RECEPTOR-MEDIATED INOSITOL PHOSPHATE METABOLISM IN RAT CEREBRAL CORTICAL SLICES

Thesis submitted to the University of Leicester for the degree of Ph.D.

by

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1987

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ACKNOWLEDGEMENTS

I would like to express my very grateful thanks to Professor S.R. Nahorski (Department of Pharmacology and Therapeutics, University of Leicester) for his excellent supervision of, and encouragement with all the work described. Many thanks also to Dr. R.F. Irvine (AFRC, Babraham) both for his invaluable contribution to the work described in Chapter 4 and for a generous supply of inositol phosphate standards. Thanks are also due to Dr. B. Potter (Department of Chemistry, University of Leicester) for N.M.R. data on Ins P_4 and to all members of the Department of Pharmacology and Therapeutics for much advice and assistance.

Especial thanks to my parents for constant support and to Miss J. Bell for her careful typing of the manuscript.

Financial support from the Medical Research Council is also gratefully acknowledged.

SUMMARY

Receptor-mediated phosphoinositide hydrolysis was studied in ^{3}H-*myo*-inositol labelled rat cerebral cortical slices. Several CNS neurotransmitter receptor agonists stimulated the hydrolysis of inositol phospholipid(s). Maximal responses to receptor stimulation showed the order, muscarinic cholinergic > adrenergic > serotonergic ≫ histaminergic. Potassium ion depolarisation and a Ca^{2+} ionophore also stimulated the accumulation of ^{3}H-inositol phosphate(s).

The metabolic sequence of muscarinic receptor-mediated phosphoinositide breakdown was examined in detail. Carbachol stimulated the sustained accumulation (> 45 min.) of ^{3}H-Ins P_1, ^{3}H-Ins P_2, ^{3}H-Ins P_3 and of a novel ^{3}H-inositol phosphate identified as Ins-1,3,4,5-P_4. Kinetic studies showed that muscarinic receptor activation results in the rapid (≪ 5 sec.) increased accumulation of ^{3}H-Ins P_2, ^{3}H-Ins P_3 and ^{3}H-Ins P_4 while the onset of ^{3}H-Ins P_1 accumulation is delayed. Using hplc, the Ins P_3 fraction was resolved into two components with the retention times of Ins-1,3,4-P_3 and Ins-1,4,5-P_3. Stimulated accumulation of Ins-1,3,4-P_3 was preceded by that of the other polyphosphates. The probable formation of Ins-1,3,4-P_3 via Ins-1,3,4,5-P_4 dephosphorylation is discussed. A phospholipid precursor for Ins-1,3,4,5-P_4 could not be identified but production of this molecule via an ATP-dependent, Ins-1,4,5-P_3 3-kinase was confirmed. Studies of the rates at which the separate ^{3}H-inositol phosphates are hydrolysed in stimulated tissue suggest considerable flux through this kinase reaction and indicate that the majority of the ^{3}H-Ins P_1 and ^{3}H-Ins P_2 accumulating in response to agonist result from ^{3}H-tris- and ^{3}H-tetrakisphosphate metabolism. Pharmacological data support these conclusions for conditions of both high and low receptor occupancy.

Lithium ions markedly affected muscarinic receptor-mediated ^{3}H-inositol phosphate metabolism, dose-dependently potentiating stimulated ^{3}H-Ins P_1 and ^{3}H-Ins P_2 accumulations while concomitantly attenuating those of ^{3}H-Ins P_3 and particularly ^{3}H-Ins P_4. The latter effects were half-maximal at 1 mM Li^{+}, exhibited a delayed onset, were not related to receptor desensitization but may be indirect consequences of Ins P_1 phosphomonoesterase inhibition. The significance of these actions is discussed in the context of the potential second messenger roles of Ins-1,4,5-P_3 and Ins-1,3,4,5-P_4.

C H A P T E R 1

INTRODUCTION

Introduction

The current study has investigated the mechanism underlying neurotransmitter receptor-mediated inositol phospholipid turnover in brain. In many other tissues the phosphoinositides are considered to be central components of a membrane transduction signalling system which serves important biological functions. The principle of transmembrane signalling is very briefly outlined in 1.1 together with means by which such processes may be related to control of neuronal function. The nature of inositol phospholipids and their specific involvement in these systems are discussed in 1.2.

1.1 General introduction

Inter-cellular communication within and between tissues or organs is achieved by chemical signals which exert both long and short term control over such cellular processes as metabolism, secretion, contractility, excitability, growth and division. The effects of these molecules on their target cell(s) are mediated via inter-action with specific receptor proteins, activation of which leads to the biological response by diverse mechanisms. A principal catagorisation of primary chemical messengers can be made on the basis of the physical location of their receptors. Those for many lipophilic molecules (e.g. steroid hormones) are present within the cell cytosol while a second diverse group of compounds including the peptide and amine hormones and neurotransmitters exert their actions by binding to receptors on the outer surface of the cell membrane.

Since the latter type of molecule does not penetrate into the cell, the information signalled by activation of the receptor must be communicated across the plasmalemma to the cell interior. A variety of membrane transduction mechanisms existing for this purpose have now been identified. Through these, the conformational change induced in the cell-surface receptor by inter-action with agonist in turn evokes either a covalent modification or change in the concentration of a secondary factor capable of altering the cell's status. In succession, a train of events is then triggered through which the particular biochemical or physiological actions expressed in response to stimulation of a given receptor are effected. Frequently the reactions involved entail modified activity of specific proteins achieved by process such as allosteric regulation or covalent changes (e.g. phosphorylation/dephosphorylation).

A vast range of cell-surface receptors and corresponding ligands are now recognised with, in many cases, separate or even single cell types expressing multiple receptor classes and sub-classes for one stimulus. In contrast, the membrane transduction mechanisms are much fewer and thus are shared in common by many types of receptor. Typical examples of these systems include situations where:

i) an ion channel forms an integral component of the cell-surface receptor such that activation of the latter allows control of plasmalemma ion fluxes and thus of ionic gradients across this membrane (e.g. the Na^+ and Cl^- channels operated by nicotinic cholinergic and $GABA_A$ receptors respectively),
ii) a single protein spans the plasma-membrane with the component exposed at the outer surface acting as the primary signal recognition site and that with access to the cytosol exhibiting protein kinase activity (e.g. the epidermal growth factor receptor),
iii) the cell-surface receptor acts via an intermediary protein(s) to regulate the ability of an enzyme, located at the cytosolic face of the plasmalemma, to catalyse the formation of a second, intracellular messenger(s) (e.g. the stimulatory and inhibitory actions of beta and $alpha_2$ adrenoceptors respectively on adenylate cyclase and the resultant effects on cAMP production). For general review of these processes see Berridge (1985).

Just as adenosine-3',5'-cyclic monophosphate (cAMP) is now recognised as the second messenger (i.e. see (iii) above) mediating the effects of numerous primary extra-cellular signals, so Ca^{2+} has long been considered to fulfill a similar role for a separate, equally varied group of ligands acting at cell-surface receptors. In resting cells, the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is around 10^{-7} M while that in the extra-cellular medium is closer to 10^{-3} M. This ionic gradient across the plasma-membrane is maintained at the expense of ATP by several processes including the Ca^{2+} pumps and exchange mechanisms present in the plasma- and various intra-cellular membranes including those of certain sub-cellular organelles (for review see Huggins and England, 1985). Agonist-receptor inter-action can disturb this closely controlled equilibrium, triggering a rise in $[Ca^{2+}]_i$ either by stimulating influx of Ca^{2+} across the cell membrane and/or by releasing Ca^{2+} from bound stores enclosed by intra-cellular membranes. Ca^{2+} from both sources is implicated in mediating the biological processes such as exocytosis and contractility modulated by a range of cell-surface receptor ligands. As with other

similar intra-cellular signals (e.g. cAMP or cGMP), the rise in $[Ca^{2+}]_i$ is believed to exert its influence by activation of a multitude of Ca^{2+} dependent proteins (including protein kinases), frequently by primary complexing with calmodulin. However, while many Ca^{2+} requiring biochemical and physiological consequences of cell-surface receptor activation have now been identified, the factor(s) coupling agonist-receptor inter-action to the increase in $[Ca^{2+}]_i$ has remained obscure. However, as the requisite Ca^{2+} can be released from intra-cellular elements, a messenger intermediate to the primary receptor stimulus and the subsequent Ca^{2+} signal has long been sought. Recent studies indicate that an appropriate second messenger is released into the cytosol when agonists acting at cell-surface receptors stimulate the hydrolysis of an inositol lipid (phosphatidylinositol-4,5-bisphosphate) located within the inner leaflet of the plasma-membrane. However, in addition to providing the signal required to mobilise intra-cellular Ca^{2+}, receptor-mediated breakdown of inositol phospholipids is also thought to produce other mediators of cellular activity (e.g. diacylglycerol) and may act as a source of precursor (e.g. arachidonic acid) for the synthesis of other molecules, subsequently released from the cell of origin to activate other cells again by inter-action with cell-surface receptors (e.g. prostanoids). Thus, the inositol phospholipids may be central components of a transmembrane signalling system capable of generating multiple intra-cellular and subsequently extra-cellular transmitter molecules. A summary of the developments culminating in this proposal is given in 1.2A.

The mechanisms by which intra-cellular signals are generated from inositol lipids in response to stimulation of cell-surface receptors are detailed in 1.2B and since the central nervous system (CNS) is the focus of the current study, emphasis is placed on the evidence for a role for the phosphoinositides in transmembrane signalling in this tissue. While many of the key features of this inositol lipid signalling system have been elucidated in non-neuronal tissues there is reason both from (a) preliminary data and (b) the existing understanding of the processes of information transfer in the CNS to suggest that important functions are served by phosphoinositide derived second messengers in brain. Before considering (a) in detail it is useful to briefly consider (b).

As the CNS is involved in both shorter and longer term processes the mechanisms for information transfer must be correspondingly flexible. This flexibility is accounted for in part by the complex structural organisation of brain but also to a significant extent by the multitude of potential interactions between the diverse mechanisms involved in electrical and chemical signalling for which this cellular framework

allows. The role of chemical messengers in trans-synaptic communication in the CNS has long been accepted and the vast majority of putative aminergic [e.g. glutamate, γ-aminobutyric acid (GABA), noradrenaline (NA), dopamine (DA), histamine (HA), 5-hydroxytryptamine (5-HT), and acetylcholine (ACh)] and peptidergic [e.g. substance P, vasopressin, neurotensin] neurotransmitters and neuromodulators now recognised to act in this capacity exert their influences by inter-action with cell-surface receptors located both pre- and post-synaptically. Thus, the actions of these molecules must be mediated via membrane transduction mechanisms such as those discussed above.

Several CNS neurotransmitters, particularly those of the amino acid type (e.g. glutamate, GABA, glycine), are believed to exert their excitatory and inhibitory actions by interaction with receptors which directly operate ion channels (e.g. mechanism (i) above). It should also be noted however, that voltage operated ion channels, responding spontaneously to the passage of an action potential and not directly under the control of receptors, also contribute notably to the electrical conductances of nervous tissue. Since both the propagation and conductance of neuronal impulses are functions of the various ionic gradients (e.g. K^+ and Na^+) across the cell membrane, modification of these potentials clearly provides one means of modulating neuronal activity by regulating the firing capacity of the cell and its ability to convey an action potential from the cell body to the terminal. As the latter property will also influence the release of chemical transmitter into the synapse the activity of subsequent neurones will also be affected. The ability of many neurotransmitters to activate feed-back inhibitory and facilitatory mechanisms regulating both their own and/or one anothers subsequent release adds a further point of control. Both actions exerted at nerve terminals and at cell-bodies involve inter-action of ligands with cell-surface receptors.

In addition to those molecules activating receptor controlled ion channels, several neurotransmitter receptor agonists have been shown to stimulate (e.g. HA, at H_2 receptors; DA at dopamine D_1 receptors; NA at beta-adrenoceptors) and inhibit (e.g. DA at dopamine D_2 receptors; ACh at muscarinic receptors and NA at $alpha_2$ adrenoceptors) the production of cAMP. As discussed in 1.2B a diverse group of cerebral receptor ligands also cause hydrolysis of inositol lipids while some of the same agonists may regulate cGMP production. The significance to cerebral function of many of the endogenous ligands exerting these actions is indicated by both the various effects observed in response to exogenous agonists and antagonists which act at the same receptor sites and by the numerous

disease-states (e.g. Parkinson's disease and senile dementia) in which abnormalities relating to the ligand/receptor systems are known to occur. Thus, by inference the intracellular messengers which mediate the actions of these stimuli must be equally significant. Precisely how these molecules regulate neuronal functions, however, remains largely obscure. Similar mechanisms to those used by identical messengers (e.g. covalent modification of specific proteins) in non-neuronal cells would obviously be expected. Therefore, potential targets would clearly include key structural or enzymic proteins associated with or involved in control of:

i) voltage and/or receptor operated ion channels.
ii) neurotransmitter receptor number.
iii) neurotransmitter release and re-uptake.
iv) the transduction and related processes transmitting signals across the cell membrane.

Examples of effects on these systems have been reported for the intracellular messengers released from inositol phospholipids and are discussed in 1.2B.

1.2 The role of inositol phospholipids in trans-membrane signalling and evidence for a function in brain

A) Historical developments and the nature of inositol phospholipids

a. Historical background:

Myo-inositol is one of nine stereoisomers of hexahydroxycyclohexane (see Parthasarathy and Eisenberg, 1986 and refs. therein). It has long been recognised as a constituent of organisms from numerous and diverse phyla (e.g. see Hawthorne, 1964; Hawthorne and Kemp, 1964) many of which require an exogenous source of myo-inositol for growth. Although other isomers of inositol occur biologically, the myo-configuration is the most common and is unique in its occurrence in combined form in the lipid fraction of various cellular membranes. Since the inositol phospholipids (phosphoinositides, see 1.2(b)) are also widely distributed, the essential nature of myo-inositol in many cells is most readily attributed to its biochemical role as a precursor for synthesis of these. In some systems, however, phosphoric acid esters of myo-inositol, derived via metabolic pathways separate from those involved in lipid synthesis, clearly have distinct, biologically important functions (e.g. phytic acid (Ins P_6) in

seed germination, see Biswas et al. 1978; Ins-1,3,4,5,6-P_5 in oxygen transport, see Bartlett, 1982 and refs. therein).

Amongst mammalian tissues, brain is one of the few shown to be capable of synthesizing myo-inositol from glucose-6-phosphate (Eisenberg, 1967). Brain was also the first animal tissue found to contain myo-inositol in lipid bound form (Folch and Woolley, 1942), although inositol was originally recognised as a lipid constituent by Anderson and Roberts (1930 a and b). Further, it was also in brain that Folch (1949 a and b) first discovered the polyphosphoinositides (see (b) below). Subsequent studies have allowed the inositol phospholipids to be resolved in to the components now recognised as PtdIns, PtdIns-4-P and PtdIns-4,5-P_2 and established many of the key features of phosphoinositide metabolism although it was not until the early 1960's that the complete structures of the brain polyphosphoinositides were conclusively defined by Ballou and co-workers (Grado and Ballou, 1961; Tomlinson and Ballou, 1961; Brockerhoff and Ballou, 1961). The early work on inositol phospholipid biochemistry has been extensively reviewed by Hawthorne (1960 and 1964) and Hawthorne and Kemp (1964) and the topics covered updated by Hawthorne and Kai (1970), Hawthorne and White (1975) and Downes and Michell (1982).

Current interest in the phosphoinositides as critical components in transmembrane signalling mechanisms also owes its origins to studies contemporary with those discussed above. As noted in many recent reviews (Michell, 1975; Berridge, 1981, 1984; Downes, 1982, 1983, 1986; Downes and Michell, 1985; Abdel-Latif, 1986), the first evidence for a receptor-mediated effect on inositol lipid metabolism was the demonstration that acetylcholine (ACh) or carbachol stimulated the incorporation of [^{32}P] ortho-phosphate into the phospholipids of pigeon pancreas or guinea-pig cerebral cortical slices (Hokin and Hokin, 1953) by activation of muscarinic receptors (Hokin and Hokin, 1954). This agonist-stimulated incorporation of radiolabel was subsequently shown to be very much more pronounced for PtdIns and phosphatidic acid (PA) than for other phospholipids (Hokin and Hokin, 1955, 1958 a and b). These original observations have recently been discussed in their historical perspective by Hokin (1985).

Since its first description, the 'PtdIns response' has been observed in a multitude of tissues and cell types exposed to a wide range of stimuli which exert their effects by interaction with cell-surface receptors and numerous theories for its function suggested (see above reviews, especially Michell, 1975). A critical step towards establishing the functional significance of the PtdIns response was to identify the reaction subject to receptor control. Although agonist-receptor

interaction could potentially promote one of several enzymic steps which would account for an increased incorporation of $^{32}P_i$ (or of ^{3}H-inositol) into phosphoinositide (see Fig. 1.2.2), the majority of evidence (for summary see Michell, 1975; Hokin, 1985) emerging from studies throughout the 1960's and early 1970's indicated the activation of a phospholipase C catalysing cleaving of the glycerol-phosphate bond of PtdIns to yield diacylglycerol (DG) and Ins-1-P so that the stimulated uptake of radiolabel into lipid is only secondary to the initial hydrolysis (see also 2.1). A phospholipase C catalysing this reaction is present in many tissues including brain although its properties are still only poorly understood (see Downes and Michell, 1985). Studies by Dawson and colleagues (Dawson et al. 1971; Jungalwala et al. 1971; Dawson and Clark, 1972) showed that hydrolysis of PtdIns by this enzyme yields two water-soluble, inositol containing products, Ins-1:2 cyclic P_1 and Ins P_1, and that the former can be degraded to the latter, non-cyclic product by a separate phosphodiesterase also present in several mammalian tissues. This evidence for the generation of an inositol cyclic phosphate as a consequence of the enzymic reaction thought to be that stimulated by receptor activation clearly presented parallels with the established cAMP signalling system and raised the possibility that Ins-1:2 cyclic P_1 might act as an intracellular messenger. Subsequent studies, however, failed to provide evidence to support this suggestion (see Michell, 1975) and although more recent work indicates that inositol cyclic phosphates may be produced as a result of receptor-mediated phosphoinositide cleavage (see Majerus et al. 1987), their significance is still unclear (see 1.2c). Thus, the means by which hydrolysis of PtdIns could be coupled to control of cellular function or activity remained obscure.

Similarly, although the association of PtdIns hydrolysis with the activation of cell-surface receptors clearly indicated a functional role for inositol lipids in the mediation of the action of hormones and neurotransmitters, the nature of this role was equally obscure. Further, a common function for phosphoinositide hydrolysis was all the more difficult to identify as the many tissues exhibiting a PtdIns response differed widely in their physiological expression to activation by the numerous ligands observed to promote PtdIns breakdown. The association of polyphosphoinositides with the plasmalemma, the divalent cation binding properties of these lipids and their particularly high content in nervous tissue had led to the early suggestion that inositol lipids might be involved in the regulation of cell membrane ion fluxes and thus in neuronal function (e.g. see Hawthorne and Kai, 1970). However, the recognition of a more general feature unifying the role of the PtdIns

response arose with the first clear proposal that hydrolysis of phosphoinositides might function as the essential link coupling receptor activation to an elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Michell, 1975). The main lines of evidence supporting this suggestion were:

i) Most cell-surface receptors stimulating PtdIns hydrolysis mediate the physiological responses evoked by their respective ligands by promoting an increase in $[Ca^{2+}]_i$ while,
ii) the cleavage of PtdIns in many tissues is not itself dependent on, and could thus precede, the increase in $[Ca^{2+}]_i$ (this point has been subject to extensive debate, see recent reviews quoted above).
iii) Agonists evoking a PtdIns response do not generally or invariably stimulate production of cAMP or cGMP respectively. Thus, the effects on inositol lipid metabolism are unlikely to be mediated by cyclic nucleotides.

Since this theory was first proposed, there have been numerous suggestions concerning the mechanism coupling receptor-activated phosphoinositide metabolism to an elevated $[Ca^{2+}]_i$ (see Berridge, 1981; Putney, 1982) and the sequence of reactions originally thought to be involved has been considerably revised. In the latter respect, the most important development concerns the lipid substrate cleaved by the receptor-stimulated phospholipase C (PLC). As discussed above, this was initially considered to be PtdIns itself. The first suggestion that polyphosphoinositides are hydrolysed in response to agonists arose from studies using crude preparations from guinea-pig brain (Durell et al. 1968, 1969) in which ACh was shown to stimulate the production of inositol bisphosphate. More recently, the studies of Abdel-Latif and colleagues (see Abdel-Latif, 1986 for review) demonstrated that stimulation of $alpha_1$ and muscarinic receptors caused a rapid loss of ^{32}P radioactivity from prelabelled PtdIns P_2 and an associated increased labelling of PtdIns and PA in rabbit iris smooth muscle (Abdel-Latif and Akhtar, 1976; Abdel-Latif et al. 1977, 1978), observations subsequently shown to correlate with an increased release of ^3H-Ins P_3 from ^3H-inositol prelabelled tissue (Akhtar and Abdel-Latif, 1980). However, since these effects appeared to require Ca^{2+} and were similarly evoked by the divalent cationophore, A23187, a role for polyphosphoinositide hydrolysis in regulation of $[Ca^{2+}]_i$ seemed unlikely at this time.

Current interest was focussed on the polyphosphoinositides by studies reported by Kirk et al. (1981) and Michell et al. (1981) which showed that

exposure of hepatocytes to vasopressin stimulated a receptor-controlled, non-Ca^{2+} mediated hydrolysis of PtdIns-4,5-P_2 which exhibited kinetics compatible with a causal role in receptor-mediated regulation of $[Ca^{2+}]_i$. These observations were subsequently confirmed in numerous cell types and soon received support from complementary studies in which the accumulation of the water-soluble, ^{3}H-inositol phosphates released in response to stimulated cleavage of ^{3}H-inositol prelabelled lipids were measured (see Berridge, 1984). This latter approach clearly demonstrated that activation of a diverse group of cell-surface receptors in a multitude of cell types stimulated the rapid, PLC catalysed hydrolysis of PtdIns-4,5-P_2 by revealing the very rapid accumulation of Ins P_3 and led Berridge (1983) to suggest that the Ins-1,4,5-P_3 released as an immediate consequence of PtdIns-4,5-P_2 breakdown acts as an intracellular second messenger by mobilising Ca^{2+} from bound stores. Evidence to support this hypothesis was first provided by Streb et al. (1983) who demonstrated that the introduction of Ins-1,4,5-P_3 into permeabilised pancreatic acinar cells stimulated the release of Ca^{2+} from an intracellular, non-mitochondrial store, an observation since repeatedly confirmed in many, more recent similar studies employing a variety of cell types (see Berridge and Irvine, 1984).

The significance of these recent developments has been considerably enhanced by the recognition that diacylglycerol (DG), the other product released together with inositol phosphate(s) by receptor-mediated hydrolysis of inositol lipid(s), also acts as a second messenger (Takai et al. 1979). DG mediates its effects via interaction with a cyclic nucleotide-independent protein kinase (C kinase) first isolated from brain by Nishizuka and co-workers (Takai et al. 1977; Inoue et al. 1977; see Nishizuka, 1984 for review). Further, since the inositol lipids from mammalian tissues are enriched in arachidonate (see 1.2b), it has been suggested (see Berridge, 1981, 1984; Downes and Michell, 1985; Abdel-Latif, 1986) that phosphoinositide cleavage may indirectly (via lipase action on DG or PA) serve to supply precursor for synthesis of the arachidonate derived signal molecules. The possibility of a link between the elevated $[Ca^{2+}]_i$, the release of arachidonate and the stimulated formation of cGMP frequently evoked by stimulation of receptors which control PtdIns P_2 hydrolysis has also been discussed by Berridge (1981, 1984) who suggests that the inositol lipids, and specifically PtdIns-4,5-P_2, are central components of a multifunctional signalling system.

It is clear from this brief chronology that studies involving brain featured prominantly in many of the original and crucial observations

establishing the occurrence, structure and function of the inositol phospholipids in mammalian tissues. More recently, however, the major advances have been made using less complex tissues of more homogenous cell type and evidence regarding the mechanism of receptor-mediated polyphosphoinositide hydrolysis in brain is considerably more limited. The object of the current study was to establish, by use of more recently developed techniques (see 2.1), whether this follows a similar reaction sequence to that currently assumed to operate in other tissues. The presently accepted scheme of events initiated by agonist-receptor interaction is discussed in more detail in 1.2B together with the available evidence deriving from previous studies in brain. Firstly, however, it is useful to very briefly note the major properties of the phosphoinositides since these are central to later discussions.

b. The structures, distribution and physical properties of the inositol phospholipids:

Since each of these subjects has previously been considered in detail (see Hawthorne and Kemp, 1964; Michell, 1975; Hawthorne and White, 1975; Downes and Michell, 1982) only those points essential to later discussions are briefly noted below. The stereochemistry of the inositol lipids and of the inositol phosphates released by their receptor-mediated hydrolysis has also been clearly described previously (see Agranoff, 1978; Parthasarthy and Eisenberg, 1986).

i) Structures: The structures of the three most widely distributed myo-inositol containing phospholipids are illustrated in Fig. 1.2.1. As shown, these are phosphoglycerides exhibiting the L- or 3-sn configuration with respect to the glycerol backbone. The phosphoric acid residue of 3-sn-phosphatidic acid is esterified via the hydroxyl group carried by the carbon atom of myo-inositol which, as a result of this substitution, becomes defined as the D-1 position. Thus, the parent lipid, PtdIns (1-(3-sn-phosphatidyl)-D-myo-inositol) and its phosphorylated derivatives, PtdIns-4-P and PtdIns-4,5-P_2, release inositol phosphates of the D-configuration (i.e. D-myo-Ins-1-P, D-myo-Ins-1,4-P_2 and D-myo-Ins-1,4,5-P_3 respectively) on cleavage of the glycerol-phosphate bond. In vitro enzymic hydrolysis of the phosphodiester bond by phospholipase(s) C (see Fig. 1.2.1) also produces D-myo-inositol 1:2 cyclic phosphate derivatives (Dawson et al. 1971; Wilson et al. 1985a), with cyclisation presumably involving mechanisms analogous to those discussed by Hawthorne (1960). The proportion of cyclic:non-cyclic inositol phosphate released appears to decrease with increasing

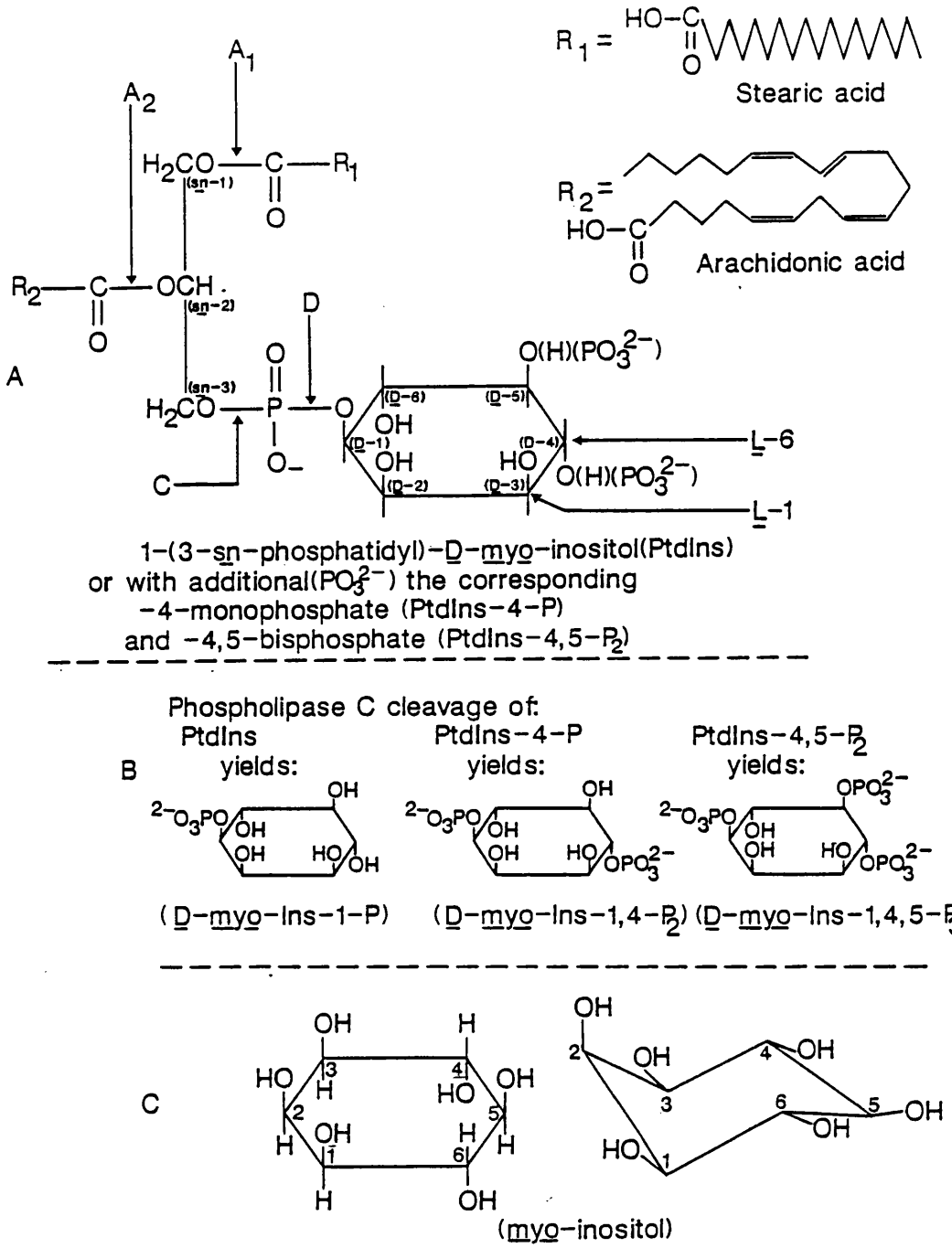


FIGURE 1.2.1 The structures of the inositol phospholipids and of the products yielded by enzymic hydrolysis of these.

Fig. A illustrates the general structure of the inositol phospholipids, specifically showing PtdIns but indicating the positions of the additional phosphate groups carried by PtdIns-4-P and PtdIns-4,5-P_2. The numbering of the inositol ring ((D-1)-(D-6)) is also shown as are the corresponding L-1 and L-6 positions. The stereochemical numbering (sn) of the glycerol backbone is illustrated as are the bonds cleaved by phospholipases A_1, A_2, C and D. The inset shows the structures of the fatty acid residues (R_1 and R_2) predominating in the phosphoinositides from animal tissues (NB. in the intact lipid, the carbonyl groups adjacent to R_1 and R_2 are contributed by the fatty acid residues indicated). Fig. B shows the inositol phosphate headgroups released on phospholipase C cleavage of each phosphoinositide. Fig. C shows the structure of unsubstituted myo-inositol, the conformation is indicated on the right (NB. the numbering of the free inositol moiety is according to Parthasarthy and Eisenberg (1986) so that the 1 position is assigned to the carbon atom closest to C-2 (bearing the axial hydroxyl group) which on substitution yields a derivative of the L-configuration).

phosphorylation of the inositol ring (Wilson et al. 1985a) and, in the particular instance of PtdIns hydrolysis, with increasing medium pH (Dawson et al. 1982). However, the diester bond of the inositol 1:2 cyclic phosphates is extremely labile to low pH (e.g. see Wilson et al. 1985a). The intact phosphoinositides are also labile to both acid and alkali. Under mildly alkaline conditions the fatty acids esterified at the glycerol sn-1 and -2 positions are preferentially cleaved. More extensive treatment with both acid and alkali also results in hydrolysis of the phosphodiester linkage via cleavage of either the glycerol-phosphate or inositol-phosphate bonds with the separate phosphoinositides yielding distinct proportions of products, the nature of which depends on the specific conditions used. In contrast, the monoester phosphate groups are more stable, although acidic conditions of varying severity can induce either migration or complete hydrolysis of these (see Hawthorne and Kemp, 1964 and refs. therein).

The other product of phospholipase C catalysed cleavage of all three phosphoinositides is sn-1,2-diacylglycerol (DG). Figure 1.2.1 indicates stearoyl and arachidonoyl fatty acid residues esterified respectively at the sn-1 and -2 positions of this moiety. This pattern is the most common for the inositol lipids derived from animal tissues and the DG released from phosphoinositides specifically in response to agonists is believed to almost invariably carry arachidonate at the 2-position, although non-receptor coupled pools of phosphoinositides may alternatively possess either oleoyl or linoleoyl residues at this position (see Berridge, 1984 for references). This enrichment of phosphoinositides with arachidonate is not, however, seen with these lipids extracted from other sources (see Michell, 1975).

ii) Distribution: The three inositol phospholipids illustrated in Fig. 1.2.1 are thought to be present in most if not all eukaryotic cells, while PtdIns, but not the polyphosphoinositides, has also been detected in a limited number of prokaryotes (see above reviews). In addition to these glycerophosphoinositides, inositol containing sphingolipids are present in both higher plants and yeast while the latter also contain inositol lipid mannosides (see Hawthorne and White, 1975; Downes and Michell, 1982 for refs.). Similar lipids have yet to be reported in animal tissues although the presence of complex structures may be indicated by recent studies (Saltiel et al. 1987). The potential occurrence of more highly phosphorylated glycerophosphoinositides than PtdIns P_2 in brain was indicated in preliminary studies by both Klenk and Hendricks (1961) and

Santiago-Calvo et al. (1963) but not subsequently confirmed (Santiago-Calvo et al. 1964), and is considered in CH.4.

In most animal tissues the phosphoinositides contribute only a minor proportion of the total cellular lipid, typical values quoted are up to about 10%. The contribution of the polyphosphoinositides (PtdIns P and PtdIns P_2) to this is thought to be minor (i.e. $\lesssim$ 10%) although in many tissues their low concentrations have frequently precluded accurate measurement; the remainder ($\sim$ 90%) is accounted for by PtdIns (e.g. see Hokin, 1985). In brain, the content of polyphosphoinositides is much greater, with PtdIns P + PtdIns P_2 representing a similar proportion of total lipid phosphorus to that accounted for by PtdIns (i.e. $\sim$ 3%, Hawthorne and Pickard, 1979). The most reliable estimates of PtdIns P and PtdIns P_2 obtained for rat brain suggest approximately 0.20 and 0.35 μmole/g fresh tissue respectively (Hawthorne, 1983) compared to a combined total of 20-40 nmol/g tissue for these lipids from rat liver (Downes and Michell, 1982). Much, though not all, brain polyphosphoinositide is associated with myelin, while the relative proportions of PtdIns P:PtdIns P_2 present in the myelin and non-myelin compartments appears to differ, with the latter exhibiting a ratio of about 2:1 and the former the reverse (see Michell, 1975). However, to what extent either the relative total brain content of PtdIns, PtdIns P and PtdIns P_2 or that exhibited by any such gross sub-fraction of tissue reflects the proportional pools of these lipids associated with a given receptor mechanism is obscure (see 3.5).

The distribution of the inositol phospholipids within and between the distinct membranes of cells has previously been discussed extensively, especially by Michell (1975) but also see Hawthorne and Pickard (1979) and Downes and Michell (1982). PtdIns is present in the highest proportion in endoplasmic reticular and associated membranes and to a lesser extent in the plasmalemma and its related structures. In contrast, PtdIns P and PtdIns P_2 are more characteristic of the latter group of membranes and it is generally thought that both the PtdIns and polyphosphoinositides associated with the plasma membrane are localised almost exclusively within the cytoplasmic face of the lipid bilayer where it is suggested that their local concentrations may reach the mM range (see above references).

iii) Physical properties: The monoester phosphate groups of PtdIns P and PtdIns P_2 render the polyphosphoinositides highly anionic at physiological pH and the negative charge carried confers unusual properties on these lipids (again see above reviews for more detailed

discussion). The most significant of these are their solubility and ion binding characteristics. Both of the polyphosphoinositides preferentially bind divalent cations and PtdIns P_2 at least exhibits moderate selectivity for Ca^{2+} although as cytosolic concentrations of Mg^{2+} are 2-3 orders of magnitude higher than those of Ca^{2+}, the physiological significance of this is unclear. The divalent cation salts of the polyphosphoinositides are soluble in non-polar solvents although in the presence of protein these ions appear to facilitate the formation of tightly bound proteo-lipid complexes thought to involve divalent cation salt bridges between protein and polyphosphoinositide. In contrast, monovalent cation polyphosphoinositide salts are more soluble in aqueous than organic solvents, dispersing in the former as spherical micelles with their hydrophobic tails and polar headgroups orientated inwards and outwards respectively. These properties necessitate the use of specialised techniques for the quantitative extraction of the polyphosphoinositides while PtdIns is generally recovered in high percentage yield from tissue by methods which are adequate for the bulk of phospholipids (for practical summary, see Simpson et al. 1987). Methods for the extraction and separation of inositol phospholipids are further discussed in CH.3 and CH.4.

B) Receptor-mediated inositol phospholipid hydrolysis in the central nervous system

It is evident from 1A(a) that as emphasis has switched over the last few years from PtdIns to the polyphosphoinositides considerable progress has been made with the recognition of two new putative second messengers (see Berridge, 1984). However, many features of this novel signalling system remain obscure and although it seems probable that Ins-1,4,5-P_3 and DG are intra-cellular signals of widespread significance, the mechanisms which control their production and metabolism are not yet fully elucidated. Further, much of the information currently available has arisen from studies on non-neural tissues and while the inferences drawn from these may apply equally to the CNS this also remains to be established (see Downes, 1986).

Figure 1.2.2 summaries data derived from many tissues to illustrate the sequence of events thought to be triggered by agonist-receptor interaction. The principal reactions involved, the enzymes catalysing these and some major alternative proposals are discussed in (a) below. Some of the more recent developments only briefly noted are considered in Chapters 3, 4 and 6. The evidence supporting the significance of a similar scheme

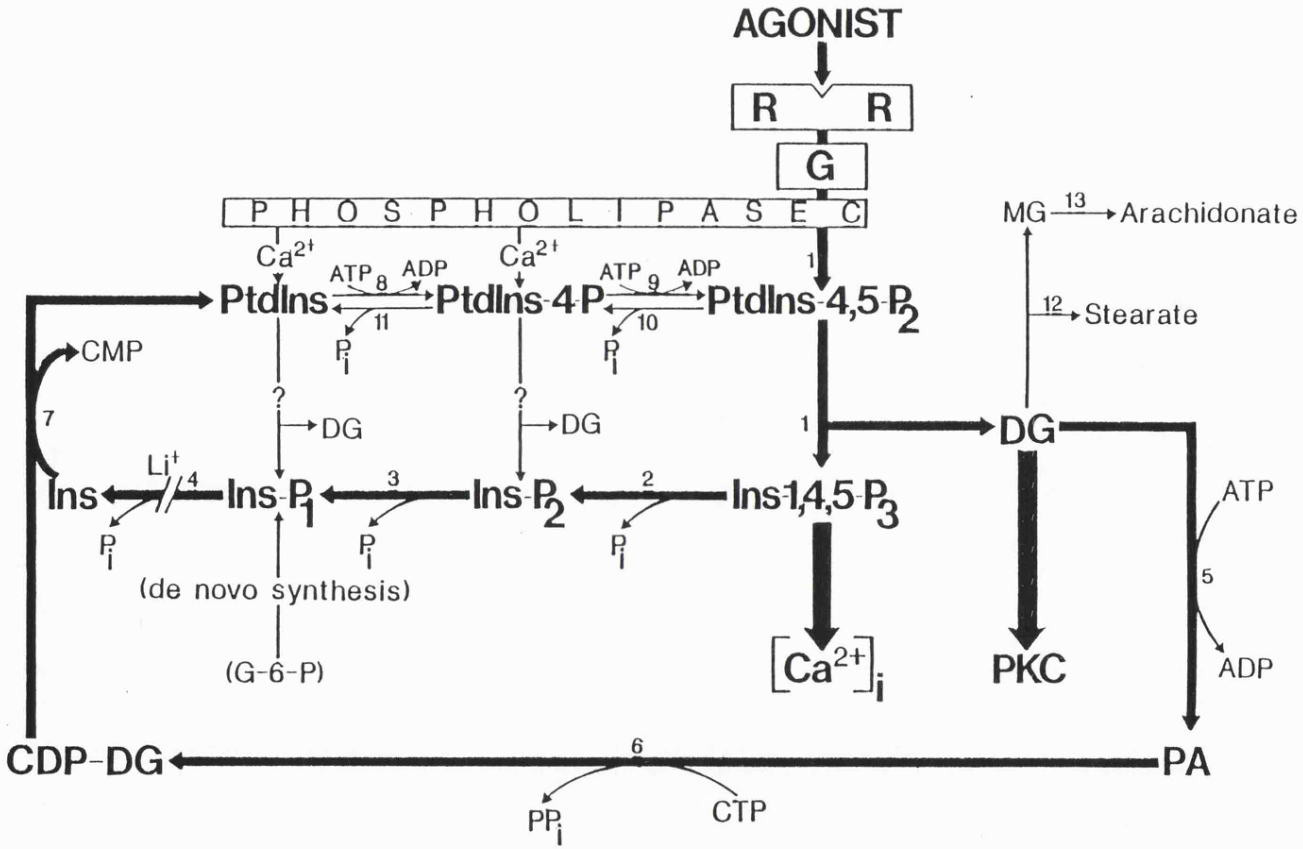


FIGURE 1.2.2 Schematic representation of the sequence of events initiated by receptor-mediated phosphoinositide hydrolysis.

Agonist-receptor interaction is shown to initiate phospholipase C catalysed hydrolysis of PtdIns P_2, potentially via intermediate involvement of a G-protein. The Ins-1,4,5-P_3 and DG produced are indicated to elevate the cytosolic free Ca^{2+} concentration and activate protein kinase C respectively. Further metabolism of these two signals is indicated via the two closed cycles shown by the bolder lines. Additional metabolic routes (e.g. deacylation of DG) are indicated by the lighter arrows. The potential hydrolysis of PtdIns and/or of PtdIns P either at the initial step and/or as a secondary consequence of the increased Ca^{2+} concentration is also indicated. Abbreviations are as in Appendix 5. Reactions 1-13 are referred to in the text as those catalysed by: (1) phospholipase C; (2),(3) and (4) inositol tris-, bis- and mono- phosphate phosphomonoesterases (phosphatases) respectively; (5) diacylglycerol (DG) kinase; (6) phosphatidate cytidylyltransferase; (7) CDP-diacylglycerol:myo-inositol phosphatidate transferase; (8) PtdIns kinase; (9) PtdIns-4-P kinase; (10) PtdIns-4,5-P_2 5-phosphomonoesterase; (11) PtdIns-4-P 4-phosphomonoesterase; (12) diacylglycerol lipase; (13) monoacylglycerol (MG) lipase.

of receptor-mediated inositol lipid hydrolysis in brain is summarised in (b), (c) and (d) below.

a. The general mechanism of agonist-stimulated phosphoinositide hydrolysis:

i) The inositol lipid substrate(s) for the receptor mechanism: It has been recognised for a number of years that the agonist-stimulated incorporation of radiolabel into inositol phospholipid(s) arises secondarily to initial phospholipase C catalysed hydrolysis of lipid (see 1.2A). The very rapid accumulation of ^{3}H-Ins-1,4,5-P_3 in ^{3}H-inositol labelled tissues and the equally rapid loss of ^{32}P prelabelled PtdIns P_2 demonstrated in a multitude of cell types (e.g. Berridge, 1983 and Michell et al. 1981) both now suggest that the latter polyphosphoinositide rather than PtdIns itself is the primary substrate for the receptor activated phospholipase C. From 1.2A the expected products of this reaction are D-myo-inositol-1,4,5-trisphosphate (Ins-1,4,5-P_3) and sn-(1-stearoyl, 2-arachidonoyl)-diacylglycerol (DG). Increased accumulation of these two molecules is detectable within seconds (or less) of exposure of tissue to appropriate ligands although production of DG has more frequently been inferred from measurement of ^{32}P PA derived via DG kinase than by direct estimation. Considerable evidence now suggests both Ins-1,4,5-P_3 and DG have second messenger functions (see Berridge and Irvine, 1984; Nishizuka, 1984; (b) below). It is important to note that of these two products it is Ins-1,4,5-P_3 which identifies the agonist labile lipid as PtdIns P_2 since DG may derive equally from other glycerophospholipids although the distinctive fatty acid composition is characteristic of the phosphoinositides.

Similarly, the most readily interpretable evidence in favour of an initial receptor-mediated hydrolysis of polyphosphoinositides rather than of PtdIns has arisen from kinetic analyses in which stimulated accumulation of Ins P_3 and Ins P_2 has been shown to precede that of Ins P_1 or inositol (e.g. Berridge, 1983; Martin, 1983; Downes and Wusteman, 1983). Most studies, however, have failed to dissociate the rates at which the two inositol polyphosphates appear on challenge with agonist (see Berridge, 1984). This may indicate the concomitant hydrolysis of both polyphosphoinositides or the rapid dephosphorylation of PtdIns P_2 derived Ins-1,4,5-P_3 to yield Ins P_2. The latter route would be consistent with the role of Ins-1,4,5-P_3 as a second messenger. This metabolic route has received support from studies in which the rates of Ins P_3 and Ins P_2 accumulations were compared in TRH-stimulated GH_3 cells maintained either at 37°C or sub-physiological temperature (Drummond et

al. 1984). Kinetic studies in carbachol-stimulated parotid gland slices (Downes and Wusteman, 1983; Irvine et al. 1985) also show that the rate of Ins-1,4,5-P_3 turnover is sufficient to account for the Ins P_2 produced. However, as most recent studies have employed radiolabelling techniques, the absolute rates of accumulation and hydrolysis of inositol phosphates in stimulated cells are yet to be determined. Thus, although PtdIns P_2 is considered as the initial substrate for the receptor mechanism, the simultaneous hydrolysis of PtdIns P cannot be excluded.

Since Ins-1,4,5-P_3 is suggested to mediate the increase in $[Ca^{2+}]_i$ through which many of the receptors controlling inositol phospholipid breakdown exert some or all their physiological effects, the receptor coupled phospholipase C activity must be independent of the agonist-triggered changes in $[Ca^{2+}]_i$. This has been considered at length in many recent reviews (e.g. see Downes and Michell, 1985 and refs. therein). Studies using crude, partially or wholly purified preparations of phosphoinositide specific phospholipase(s) C indicate that the properties exhibited by these enzymes vary depending on the physical presentation of the substrate and on the ionic environment and pH at which activity is assayed. When supplied substrate in the form of a lipid mixture resembling that of the cytosolic face of the plasma membrane, a soluble preparation from rat brain hydrolyses PtdIns P_2 at micromolar Ca^{2+} concentrations but if presented substrate in the same form and assayed in the presence of physiological intra-cellular ion concentrations shows little activity below 100 μM Ca^{2+} (Irvine et al. 1984b). Further, agents which promote an influx of Ca^{2+} across the plasma membrane stimulate hydrolysis of polyphosphoinositides both in brain (see Nahorski et al. 1986) and in certain other tissues (see Abdel-Latif, 1986) while depletion of the extra-cellular Ca^{2+} concentration variably attenuates the hydrolysis of inositol phospholipids evoked by stimulation of distinct neurotransmitter receptors in cerebral cortex (Kendall and Nahorski, 1984). The significance of all these observations is still unclear though they need not necessarily imply that receptor-mediated phosphoinositide breakdown in brain is secondary to changes in $[Ca^{2+}]_i$ for several reasons. Firstly, although the cytosolic phosphoinositide phospholipase(s) C from this tissue do show some properties characteristic of the receptor controlled activity, preferentially hydrolysing PtdIns P_2 over PtdIns (Irvine et al. 1984b), recent evidence also suggests the presence of a membrane bound enzyme capable of hydrolysing polyphosphoinositides (Van Rooijan et al. 1983). Secondly, a large number of studies now suggest that agonist-receptor interaction is coupled to phospholipase C cleavage of inositol phospholipids via a guanine nucleotide dependent regulatory

protein (see (c) below) which may act by reducing the requirement of the appropriate enzymic activity for Ca^{2+} (see Cockcroft, 1987). Since this latter mechanism is more consistent with a membrane bound than a cytosolic phospholipase C, the functional role of the latter type of activity in terms of the response to receptor stimulation is therefore uncertain. In general, it is considered that although the relevant phospholipase(s) C from many sources have an absolute requirement for Ca^{2+} (as evidenced by the aboliton of their activity in the presence of Ca^{2+} chelators), their agonist enhanced activity is not a consequence of but rather mediates (through Ins-1,4,5-P_3) receptor controlled Ca^{2+} mobilisation.

A second point related to these considerations is whether the increase in $[Ca^{2+}]_i$ triggered by Ins-1,4,5-P_3 might cause a re-direction of phospholipase C activity away from the polyphosphoinositides so that PtdIns itself is cleaved in a secondary, indirect phase of the response to receptor activation. Majerus et al. (1985) have proposed this sequence to account for the actions of thrombin on human platelets and further supportive evidence may also be inferred from recent studies in angiotensin II stimulated vascular smooth muscle cells (Griendling et al. 1986). A major potential advantage of this scheme over that discussed by Berridge (1984) is that it allows for the independent generation of the two putative second messengers, DG and Ins-1,4,5-P_3, since the former but not the latter could be equally well derived from PtdIns as from PtdIns P_2. Majerus et al. (1985) thus point out that as the cellular mass of PtdIns far exceeds that of the polyphosphoinositides, this lipid may represent the quantitatively more important source of DG. The substrate specificities of most phosphoinositide phospholipases C are not necessarily inconsistent with an initial hydrolysis of PtdIns P_2 and the subsequent cleavage of PtdIns as most of these enzymes are capable of degrading all three inositol phospholipids under appropriate conditions (see Downes and Michell, 1985). However, when assayed at micromolar $[Ca^{2+}]$ and under conditions approximating to the intra-cellular ionic environment, a crude enzyme preparation from the soluble fraction of rat brain showed marked preference for PtdIns P_2 over PtdIns if presented with a substrate in a non-bilayer form (Irvine et al. 1984b). This may indicate that a direct hydrolysis of PtdIns is unlikely although as noted above the appropriate receptor activated phospholipase C may be a membrane bound rather than a cytosolic enzyme so that while the properties of the relevant enzyme remain poorly defined a secondary hydrolysis of inositol lipids other than PtdIns P_2 may be difficult to establish in many tissues. However, kinetic studies on rat parotid gland (Downes and Wusteman, 1983; Irvine et al. 1985) are also inconsistent with a direct hydrolysis of

PtdIns and muscarinic receptor stimulation in this tissue evokes a sustained hydrolysis of PtdIns P_2. Since an equally persistent release of both Ins-1,4,5-P_3 and DG would be expected under these circumstances a secondary additional breakdown of PtdIns would seem to serve no purpose. Potentially, control of inositol lipid hydrolysis differs between tissues but in the majority of cells (including platelets) the initial receptor mediated event appears to involve hydrolysis of PtdIns P_2 rather than of other phosphoinositides.

ii) The metabolism of PtdIns P_2 derived signal molecules and re-synthesis of inositol phospholipid

As both products released by PtdIns P_2 hydrolysis are believed to act as intra-cellular second messengers, specific mechanisms for their rapid degradation would be expected. Some of the metabolic routes involved are out-lined in Fig. 1.2.2. Clearly as the PtdIns P_2 substrate for the receptor-controlled phospholipase C (PLC) is present at the inner surface of the plasmalemma, many of the enzymes catalysing the reactions indicated must be similarly located, particularly those responsible for catabolism of DG since this product is highly lipophilic. Figure 1.2.2 shows two alternative routes of DG removal. One involves sequential lipase action, initially releasing stearic acid and monoacylglycerol (MG) followed by cleavage of the latter to provide free arachidonate. The second pathway is via DG kinase and yields sn-3-phosphatidic acid (PA) which may be re-cycled into inositol phospholipid synthesis (see below). Different cells may show a preference for one route or the other (see Downes and Michell, 1985). In brain, the DG released from (poly)phosphoinositide, at least in response to muscarinic receptor stimulation, is metabolised by the second pathway (Van Rooijan et al. 1985).

Figure 1.2.2 also indicates the successive dephosphorylation of Ins P_3 through Ins P_2 to Ins P_1 and ultimately to free inositol. Both soluble and membrane bound enzymes appear to be involved in this sequence which is discussed in detail in subsequent chapters (see CH.3). An important point to note however, is that the degradation of both D- and L-myo-Ins-1-P to inositol is potently inhibited by lithium ions (Hallcher and Sherman, 1980), a feature first exploited by Berridge et al. (1982) to develop the sensitive assay for receptor-mediated phosphoinositide cleavage described in 2.1. As de novo synthesis of myo-inositol from glucose-6-phosphate also involves L-myo-Ins-1-P as an intermediate (see Partharsarthy and Eisenberg, 1986) and as brain is largely dependent on this biosynthetic route for its supply of free inositol, chronic exposure

to lithium ions may depress the capacity of the inositol phospholipid signalling system in brain (see Berridge, 1984).

The two closed cycles responsible for metabolism of both DG and inositol phosphates (see Fig. 1.2.2) merge at the level of CDP-diacylglycerol-myo-inositol:phosphatidate transferase to allow re-synthesis of PtdIns. The sub-cellular location of the transferase activity responsible for re-synthesis of the agonist-labile phosphoinositide pools is uncertain. The major site of PtdIns biosynthesis is the endoplasmic reticulum (see Michell, 1975). Specific exchange proteins exist to transport both PA from the plasma-membrane to this site and PtdIns in the reverse direction (see Berridge, 1981), but the rapid and continued hydrolysis of inositol lipid may indicate the necessity for limited PtdIns synthesis at the plasmalemma (see Downes and Michell, 1985). From PtdIns the re-synthesis of the polyphosphoinositides is completed by two ATP and Mg^{2+} requiring kinases which successively phosphorylate the D-4 and D-5 positions of the inositol ring to yield PtdIns-4-P and PtdIns-4,5-P_2. PtdIns kinase shows both soluble and membrane bound activity although in brain both this and PtdIns P kinase may be largely cytosolic enzymes, each of which is inhibited by Ca^{2+} ions (see Hawthorne, 1983). As shown in Fig. 1.2.2, 5- and 4- phosphomonoesterases also catalyse dephosphorylation of PtdIns P_2 through PtdIns P back to PtdIns. Clearly both these phosphatases and the corresponding kinases are potential targets for factors regulating receptor-mediated inositol lipid hydrolysis (see reviews quoted and Faresse, 1983).

Finally, it should be noted that recent studies (Wilson et al. 1985a) show that hydrolysis of inositol phospholipids can also yield inositol cyclic (poly)phosphates corresponding to Ins-1-P, Ins-1,4-P_2 and Ins-1,4,5-P_3 but in which a cyclic phosphodiester bond bridges the carbon atoms at the D-1 and the 2-position of the inositol ring. The metabolism of these compounds is discussed in later chapters (but see Majerus et al. 1987 for details) as is that of Ins-1,3,4-P_3, a second inositol trisphosphate accumulating in stimulated cells (Irvine et al. 1984a) (see CH.4 and CH.6).

b. Inositol phospholipid hydrolysis in brain:

i) Evidence for primary hydrolysis of PtdIns P_2: It will be noted from the previous section that many of the enzymes catalysing the cycle of reactions shown in Fig. 1.2.2 are present in brain. Despite this and the early demonstration of a phosphoinositide response in brain (see 1.2A) there is relatively little evidence that the initiating reaction in this tissue is hydrolysis specifically of PtdIns P_2. This lack of available

information derives largely from the only comparatively recent introduction of simplified assay procedures (see 2.1) yielding more readily interpretable data. Some more recent data arising from studies using these techniques are discussed in later chapters (see CH.3). The evidence from earlier studies which suggests agonist-induced hydrolysis of polyphosphoinositides is summarized below (but see Hawthorne and Pickard, 1979; Downes, 1982 for reviews).

The first indication of a receptor-mediated, phospholipase C catalysed hydrolysis of polyphosphoinositides in brain was the demonstration that ACh stimulated the accumulation of both Ins-1,4-P_2 and Ins-1-P in crude preparations from guinea-pig brain (Durell et al. 1968). An atropine-sensitive effect of cholinergic agonists on the ^{32}P labelling of PtdIns P_2 was not, however, observed in synaptosomes from the same tissue (Yagihara and Hawthorne, 1972; Schacht and Agranoff, 1972). Alternatively, Soukup et al. (1978) found that in vivo administration of both acetylcholine and noradrenaline stimulated the incorporation both of ^{3}H-inositol and ^{32}P into the polyphosphoinositides of rat brain. In contrast to these conflicting reports, numerous studies have demonstrated agonist enhanced turnover of PtdIns and PA both in vivo and in vitro (see Hawthorne and Pickard, 1979 for refs.). However, it is of interest that the latter authors have pointed out that studies measuring stimulated incorporation of ^{32}P into synaptosomal lipids have frequently revealed significantly enhanced labelling of PA but more variable data for PtdIns. This perhaps reveals the fallibility of this type of approach since factors other than (poly)phosphoinositide hydrolysis which lead to an increased supply of DG can result in an increase in both PtdIns and PA labelling, potentially yielding ambiguous data (see 2.1). Equally, the Ca^{2+} dependency of receptor-mediated inositol phospholipid hydrolysis can also be difficult to evaluate from labelling studies which measure only the secondary re-synthesis of lipid. It is, nevertheless, clear that an increased $[Ca^{2+}]_i$ can provoke breakdown of polyphosphoinositide(s) in brain as exposure of guinea-pig brain synaptosomes to A23187 stimulated loss of ^{3}H-inositol pre-labelled ^{3}H-PtdIns P_2 and an increased accumulation of ^{3}H-Ins P_2 (Griffin and Hawthorne, 1978). However, as ^{3}H-Ins P_3 was not observed in response to the ionophore in this study the predominant route of Ca^{2+}-mediated inositol lipid breakdown may be distinct from that stimulated by agonists (see CH.3).

Support for this conclusion and further evidence for a receptor-mediated, phospholipase C catalysed cleavage of polyphosphoinositides in brain has emerged from more recent studies. Van Rooijan et al. (1983) demonstrated a muscarinic receptor-mediated loss of ^{32}P pre-labelled

polyphosphoinositides from synaptosomal preparations and an increased accumulation of ^{32}P labelled inositol polyphosphates. Similarly, Berridge et al. (1983) showed that exposure of ^{3}H-inositol labelled brain slices to 1 mM carbachol rapidly stimulated the accumulation of ^{3}H-Ins P_3 and ^{3}H-Ins P_2 but not of ^{3}H-Ins P_1. However, neither of these studies extended to detailed kinetic analysis of these responses and thus while the latter in particular is strongly indicative of an initial receptor activated breakdown of polyphosphoinositide(s) confirmation of this requires further investigation. This has been the principal objective of the current studies.

ii) Cellular targets of PtdIns P_2 derived signals and possible functions in the CNS

Cleavage of PtdIns P_2 by phospholipase C yields Ins-1,4,5-P_3 and DG (see (a) above) both of which may act as intra-cellular second messengers. Ins-1,4,5-P_3 has been shown to mobilise Ca^{2+} from bound intra-cellular stores thought to be located within the endoplasmic reticulum (see Berridge and Irvine, 1984). The mechanism underlying this action of Ins-1,4,5-P_3 is uncertain (see Abdel-Latif, 1986) although as other inositol (poly)phosphates are markedly less effective or inactive in this respect, interaction of Ins-1,4,5-P_3 with a specific site (e.g. receptor-protein) is implied (Berridge and Irvine, 1984). The recent demonstrations of saturable and selective Ins-1,4,5-P_3 binding to membrane preparations from several tissues (Baukal et al. 1985; Spat et al. 1986) are consistent with this view and it is of interest that a high density of similar sites has been reported in several cerebral regions (Worley et al. 1987).

The DG released from phosphoinositides is thought to exert control over cellular function by activation of a cyclic nucleotide and Ca^{2+}-calmodulin independent protein kinase (C kinase). In the presence of DG, the threshold requirement of C kinase for Ca^{2+} is reduced such that the resting $[Ca^{2+}]_i$ becomes sufficient for its activity, and the translocation of the enzyme from the cytosol to the plasmalemma is promoted such that the phospholipid requirement of C kinase is also met, primarily by phosphatidylserine. The substrates for C kinase phosphorylation largely remain unidentified but much recent evidence concerning the cellular functions this enzyme is likely to regulate has derived from the use of synthetic diacylglycerols and the tumour promoting phorbol esters, certain of which can substitute for DG and evoke a direct activation of C kinase. These agents are resistant to metabolism and their application to cells by-passes the need for receptor activation and

can reproduce some of the actions of cell-surface receptor ligands known to stimulate inositol phospholipid hydrolysis. An important point revealed by the activity of these agents however, is that the sn-(1-stearoyl, 2-arachidonoyl)-diacylglycerol released from phosphoinositides is not a more effective activator of C kinase than many similar analogues although with respect to the latter, the enzyme does exhibit some specific requirements. The properties, potential targets and interactions of the C kinase and Ins-1,4,5-P_3 branches of the PtdIns P_2 signalling system are discussed in recent reviews (see Nishizuka, 1984; Berridge and Irvine, 1984; Downes and Michell, 1985; Abdel-Latif, 1986).

Although there is only limited evidence for agonist-stimulated cleavage of PtdIns P_2 in brain (see (i) above), both the large number of neurotransmitter receptor agonists now shown to elicit an albeit less well defined phosphoinositide response (see (c) below) and the high density of Ins-1,4,5-P_3 binding sites and of C kinase in this tissue suggest both putative second messengers may have important roles in the CNS. Demonstration of functional activity for Ins-1,4,5-P_3 has been restricted to date by its hydrophilicity and the lack of specific inhibitors of its metabolism, although Ins-1,4,5-P_3 has been shown to mobilise bound intra-cellular Ca^{2+} when introduced into permeabilised neuroblastoma cells (Gill et al. 1986). Alternatively, potential actions of DG have been easier to identify because of the availability of stable analogues capable of penetrating the cell membrane. Thus, a role for DG in regulation of the muscarinic receptor-mediated inhibition of a calcium-dependent potassium current in hippocampal neurones (Baraban et al. 1985) and of stimulated activity of voltage-sensitive Ca^{2+} channels of Aplysia bag cell neurones (De Reimer et al. 1985) has been inferred from studies with phorbol esters. Further, a very recent study (Higashida and Brown, 1986) has implicated both the PtdIns P_2 derived messengers in distinct phases of the changes in neuroblastoma cell membrane ion conductances evoked by bradykinin. There is also evidence for the involvement of DG in an auto-regulatory mechanism as in both rat hippocampal slices (Labarca et al. 1984) and astrocytoma cells (Orellana et al. 1985) phorbol esters inhibit muscarinic receptor-mediated inositol phospholipid hydrolysis, observations which may be related to the ability of protein kinase C to modulate PtdIns P kinase activity (see Van Dongen et al. 1985). A number of studies may also implicate PtdIns P_2 derived signals in control of neurotransmitter release but see Downes (1986) and (d) below. Finally, as stimulation of neuroblastoma cells by histaminergic and cholinergic agonists results in formation of cGMP via an arachidonic acid metabolite (Snider et al. 1984), it is possible that regulation of guanylate cyclase

activity could be an indirect consequence of inositol lipid hydrolysis in neuronal cells. However, a recent study shows that control of cGMP formation in this manner is not a consequence of muscarinic receptor stimulation in several cerebral regions (Kendall, 1986).

c. Neurotransmitter receptors mediating phosphoinositide hydrolysis in brain: receptor-phospholipase C coupling:

The study of receptor-mediated effects on inositol phospholipid metabolism has been greatly facilitated by the introduction of lithium ions as a tool to amplify this response (Berridge et al. 1982). As a result the technique described in 2.1 has been widely applied to establish the pharmacological character, distribution, localisation and adaptive properties of many receptor systems in the CNS (e.g. Brown et al. 1984; Daum et al. 1984; Jacobson et al. 1985; Mantyh et al. 1984; Rooney and Nahorski, 1986; Kendall et al. 1985). Thus, it now seems certain that inositol lipid breakdown is a wide-spread consequence of receptor activation in brain. A summary of the receptor sub-types identified by more recent studies is given below (see also Nahorski et al. 1986; Downes, 1986 and for earlier references Hawthorne and Pickard, 1979 and Downes, 1982).

i) Pharmacological character of receptors

Cholinergic receptors: The hydrolysis of phosphoinositides stimulated by cholinergic agonists in brain is probably a consequence exclusively of muscarinic receptor activation (Brown et al. 1984; Jacobson et al. 1985; Fisher and Bartus, 1985) although both the putative M_1 and M_2 receptor sub-types may be involved (Lazareno et al. 1985), each mediating different proportions of the total response in separate cerebral regions (Rooney and Nahorski, 1986) (see also 2.3).

Adrenergic receptors: The major sub-classes of adrenoceptors ($alpha_1$, $alpha_2$, $beta_1$ and $beta_2$) can be clearly distinguished by use of a range of selective agonists and antagonists. Noradrenaline (NA) stimulates hydrolysis of phosphoinositides in rat cerebral cortex and hippocampus by inter-action with $alpha_1$-adrenoceptors since the response is inhibited by prazosin but not by yohimbine or propranolol (Brown et al. 1984; Minneman and Johnson, 1984; Janowsky et al. 1985). In cerebral cortex there is evidence for the close coupling of $alpha_1$ receptor occupation and inositol lipid hydrolysis (Kendall et al. 1985).

Serotonergic receptors: 5-Hydroxytryptamine (5-HT) receptors are commonly classified as either 5-HT_1 or 5-HT_2 (Peroutka and Snyder, 1980). 5-HT stimulated inositol phospholipid hydrolysis in brain is best

characterised as a 5-HT_2 receptor-mediated response but see Nahorski et al. (1986).

Histaminergic receptors: Histamine (HA) is a further putative neurotransmitter which stimulates phosphoinositide hydrolysis in brain. This response is potently blocked by mepyramine but not cimetidine (Brown et al. 1984) indicating mediation via histamine H_1 receptors, with the density of which the ability of histamine to evoke inositol lipid breakdown shows a close correlation in brain (Daum et al. 1984).

Peptidergic receptors: A number of peptide transmitters have also been shown to induce inositol lipid hydrolysis in several cell lines or CNS regions; these include substance K, neurotensin, CCK-octapeptide, bradykinin, substance P and vasopressin although to date only responses to the latter two have been studied in detail (see Nahorski et al. 1986; Downes, 1986 for references).

Non-receptor stimuli: Exposure of brain slices to a variety of depolarising stimuli and agents such as divalent cationophores also appears to result in inositol lipid hydrolysis (see CH.3 and Nahorski et al. 1986).

Receptors mediating inhibition of phosphoinositide hydrolysis: By analogy with the adenylate cyclase signalling system receptors capable of mediating stimulation or inhibition of inositol phospholipid breakdown would be expected. While many agents stimulate this process in brain however, reports of inhibitory actions are limited. Baudry et al. (1986) have shown that several excitatory amino acids inhibit muscarinic receptor-mediated and depolarisation-evoked phosphoinositide hydrolysis. Similarly, Simmonds and Strange (1985) have reported a dopamine D_2-receptor mediated inhibition of TRH-stimulated phosphoinositide cleavage in bovine anterior pituitary cells. However, an equivalent effect of dopamine could not be demonstrated in GH_3 cells (J. Baird, personal communication) nor do dopamine receptor agonists or antagonists influence carbachol-stimulated or basal accumulations of inositol phosphates in rat striatal slices (Kelly, Rooney and Nahorski, unpublished). Clearly the search for inhibitory responses will be a subject for future studies.

ii) Receptor-phospholipase C coupling: Although there are several means by which this coupling could be effected, the majority of available evidence implicates a role for membrane bound, guanine nucleotide-dependent regulatory proteins (N or G-proteins) analogous to the N_s and N_i proteins mediating the actions of stimulatory and inhibitory receptors on

adenylate cyclase activity (for review of these proteins see Limbird, 1981; Gilman, 1986; for discussion of their role in control of phosphoinositide phospholipase(s) C see Downes and Michell, 1985; Abdel-Latif, 1986 and Cockcroft, 1987). In some, though by no means all instances, this G-protein appears to be closely related to N_i since pretreatment of cells with Pertussis toxin (an inactivator of N_i) abolishes both agonist-stimulated hydrolysis of PtdIns P_2 and other biochemical and physiological events thought to be a consequence of this reaction.

Evidence for the G-protein coupling of cerebral receptors to phosphoinositide phospholipase C is very limited. However, this would not generally appear to involve N_i since in vivo pretreatment with Pertussis toxin does not affect carbachol-stimulated inositol phospholipid hydrolysis in rat striatal slices (Kelly, Rooney and Nahorski, 1985) despite the fact that the toxin does have access to intra-striatal N_i (Kelly, Willcocks and Nahorski, 1987). The situation is further complicated in brain as the vast majority of phosphoinositide specific phospholipase C in this tissue is present in the cytosol while a G-protein regulated activity would be more readily envisaged for a plasma-membrane bound enzyme. However, the soluble enzyme(s) does exhibit some characteristics compatible with the receptor controlled activity since when presented with a mixed substrate containing PtdIns in ten-fold excess over PtdIns P_2 it preferentially hydrolyses the latter and exhibits substantial activity against polyphosphoinositide even at low [Ca^{2+}] (1 μM) when supplied PtdIns P_2 in a lipid mixture approximating in composition to the inner leaflet of the cell membrane (Irvine et al. 1984b). In contrast, with substrate presented in the latter form the same crude enzyme from rat brain cytosol shows little activity below 100 μM Ca^{2+} if assayed under ionic conditions reflecting those of the intracellular environment. On the basis of these observations Irvine et al. (1984b) have implied that agonist-receptor interaction could potentially alter this soluble activity by evoking a change in the presentation of the substrate to the enzyme. Alternatively, other recent studies have indicated that the relevant receptor-mediated phospholipase C activity may represent a membrane bound rather than a cytosolic enzyme (Van Rooijan et al. 1983). Further, Gonzales and Crews (1985) have shown that guanine nucleotides stimulate the release of ^{3}H-Ins P_3 from ^{3}H-inositol prelabelled cerebral cortical membranes, an observation consistent with both a plasma membrane associated phosphoinositidase C and its regulation by a G-protein(s). Clearly the membrane/cytosolic location of the receptor-sensitive phosphoinositide phospholipase C needs to be better defined in brain as does the role(s) of the soluble enzyme activities.

Alternatively, if one of the latter is responsible for receptor-mediated phosphoinositide cleavage, the results of Gonzales and Crews (1985) may indicate that a more flexible mechanism of receptor-phospholipase C coupling is required, in which event the recent proposal featuring G-proteins as programmable messengers (Rodbell, 1985) may be relevant. Finally, the means by which G-protein-phosphoinositidase C interaction leads to activation of the latter also remains to be established in brain, although it is possible that this involves a lowering of the enzyme's threshold requirement for Ca^{2+} as may be the case in other tissues and cell types (see Nahorski et al. 1986 and Cockcroft, 1987).

d. Pre- or post-synaptic and cellular location of the phosphoinositide response in brain:

As many of the earlier studies on cerebral tissue demonstrated a 'PtdIns response' in synaptosomal preparations, agonist enhanced inositol phospholipid turnover was considered to be a predominantly pre-synaptic response and thus its involvement in the regulation of neurotransmitter release was suggested (see Hawthorne and Pickard, 1979). However, although more recent work has demonstrated receptor-mediated hydrolysis of polyphosphoinositides in synaptosomes (Van Rooijan et al. 1983) both Downes (1982) and Nahorski et al. (1986) have pointed out that as these preparations are frequently contaminated with dendritic fragments (and possibly also with glial cells) the evidence for this original interpretation is equivocal and may have to be re-evaluated in favour of a post-synaptic localisation of the response as is more consistent with studies in the periphery.

Strong support for post-synaptic muscarinic and $alpha_1$ receptor-mediated responses has arisen from the use of both chemical and surgical lesioning techniques. In synaptosomes prepared from rat hippocampus, activity of choline acetyltransferase (a pre-synaptic marker) was markedly reduced by prior removal of the cholinergic innervation to this region while the enhanced turnover of PtdIns and PA mediated by muscarinic receptors in these vesicles remained unaffected (Fisher et al. 1980). Alternatively, in the same preparation, destruction of the hippocampal cell bodies by pre-treatment with ibotenate attenuated both the inositol phospholipid response and the number of muscarinic receptors (Fisher et al. 1981). Similarly, $alpha_1$ adrenoceptor-mediated accumulation of inositol phosphates exhibits denervation super-sensitivity in rat cerebral cortical slices after prior lesion of the noradrenergic imput by intraventricular injection of 6-hydroxydopamine (Kendall et al. 1985). The same receptor-mediated effect is reduced by prior kainate lesion in

slices of the rat dorsal lateral geniculate nucleus also indicating this response is a post-synaptic event (Kemp and Downes, 1986).

Although these more recent studies suggest that the former assignment of the 'PtdIns response' as a pre-synaptic event was probably largely inaccurate, both these and the earlier studies identifying responses in synaptosomes indicate that agonist-stimulated inositol lipid hydrolysis is localised in neuronal cells. Equally, the association of much cerebral polyphosphoinositide with neuronal elements suggests a similar conclusion. Receptor-mediated phosphoinositide hydrolysis in brain may however, involve additional components since muscarinic receptor activation in astrocytoma cells enhances PtdIns turnover (Cohen et al. 1983) and agonists at both these and alpha$_1$ adrenoceptors also stimulate accumulation of inositol phosphates in primary cultures of cortical astrocytes (Pearce et al. 1985). Alternatively, comparative studies on both cultured glia and neurones suggest that the phosphoinositide response is predominantly a neuronal phenomenon (Gonzales et al. 1985). Despite this, it is important to note that interpretation of the data from the current studies on muscarinic receptor-mediated inositol lipid hydrolysis may be complicated not only by the interaction of agonists with more than one sub-class of receptor (i.e. M_1 and M_2) but also by activation of responses in distinct cell types.

e. Summary:

A role for the inositol lipid signalling system in the CNS is strongly indicated by:

i) The relatively high concentrations of the inositol phospholipids in brain.
ii) The presence of the enzymes necessary to catalyse the sequence of reactions thought to be initiated by agonist-receptor interaction.
iii) The multitude of neurotransmitter receptors shown to stimulate phosphoinositide turnover.
iv) The high density of binding sites for Ins-1,4,5-P_3 and the high content of protein kinase C.
v) The demonstrated functions for PtdIns P_2 derived signals.

Although it is clear from previous sections that many aspects of this system require further investigation in brain, one particularly important point is that while the stimulated turnover of inositol phospholipids has often been demonstrated, the metabolic pathways involved have remained comparatively poorly defined. The primary objective of the current study has been to examine these in detail. The results of these investigations

should assist a preliminary evaluation of the relative extents to which DG and Ins-1,4,5-P_3 are of significance in the modulation of neuronal activity mediated by CNS neurotransmitters. Further, by providing a clearer understanding of the biochemical mechanisms underlying the actions of receptor stimuli they should assist the interpretation of, and thus enhance the value of related pharmacological studies.

CHAPTER 2

Methods for studying receptor-mediated inositol lipid hydrolysis/ inositol phosphate accumulation in cerebral cortex: practical considerations and basic characteristics of the response to muscarinic receptor stimulation

2.1 The principle of the assay for measurement of inositol phospholipid hydrolysis

The methods used in the current study to measure receptor mediated inositol lipid hydrolysis in cerebral cortical slices are described in later sections (2.2 and 2.4) and results are presented (2.3, see also 3.2 and 6.2) to support the validity of these approaches and to illustrate some of the problems associated with the interpretation of data derived from so complex and heterogenous a tissue. However, it is useful to consider firstly and very briefly, the rationale behind the experimental design employed since this is common to most of the studies described and to consider some of the advantages of this over other approaches.

An important feature of the assay used is that a direct measure of inositol lipid hydrolysis is made. Until comparatively recently, receptor-mediated effects on phosphoinositides were frequently assayed by measurement of the stimulated incorporation of radiolabel (^{32}P or ^{3}H inositol) into these lipids (see Hawthorne and Pickard, 1979; Berridge, 1981; Downes, 1982). Indeed, the first evidence for an effect of agonists on inositol lipids arose from such studies (Hokin and Hokin, 1953). However, as discussed in CH.1, one of the earliest reactions coupling receptor activation to stimulated phosphoinositide metabolism is a phospholipase C catalysed cleavage of $PtdInsP_2$. The stimulated incorporation of radiolabel into the inositol lipids is considered to reflect a compensatory re-synthesis which is therefore, only secondary to the initial hydrolysis and as such, studies measuring this can be subject to labelling artefacts which can complicate the interpretation of data (see Berridge, 1981 and references therein). These problems arise for several reasons. Firstly, and most critically, as hydrolysis of inositol lipids is now strongly implicated in the sequence of events coupling agonist-receptor interaction with a rapid increase in the intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) (Berridge, 1984; Berridge and Irvine, 1984), any effects of Ca^{2+} on the multiple stages of lipid re-synthesis will be intrinsic to the stimulated incorporation of radiolabel which is measured. As many of the enzymes involved in inositol lipid and/or phosphate metabolism may be sensitive to $[Ca^{2+}]$ (see 2.3, 3.3, 4.4), this indirect measure of lipid breakdown may give a false or misleading estimate of both the magnitude and Ca^{2+} dependency of the initial hydrolysis. Secondly, and more generally, any changes in the intracellular environment which are consequent on the products released on phosphoinositide hydrolysis, could potentially have modulatory effects on the reactions involved in the later re-synthesis of inositol lipids and would also affect measurements of label incorporation. Since receptor-mediated hydrolysis of $PtdInsP_2$ produces the two putative second

messengers, Ins-1,4,5-P_3 and DG, which influence a number of cellular processes (Berridge, 1984; Nishizuka, 1984), this is clearly an important consideration. Thirdly, if agonists were to affect concentrations of inositol lipid precursors other than by promoting hydrolysis of these lipids, then indirect effects, not relating to the primary sequence of receptor-mediated reactions, would also be monitored when measuring the incorporation of radiolabel into phosphoinositides. In addition, in the current studies (see 2.3) significant, agonist-stimulated incorporation of ^{3}H-inositol into lipids was rarely observed, a fact which may be related to the high basal incorporation of this labelled molecule through reactions not forming part of the pathway associated with the receptor mechanism (see Downes, 1982).

Alternative approaches to the study of inositol lipid hydrolysis involve the pre-labelling of tissue with radiolabel (^{32}P or ^{3}H-inositol) such that isotopic equilibrium is achieved or tissue is pulse-labelled before challenge with agonist. Such studies do allow a direct measure of lipid hydrolysis and, unlike many studies measuring stimulated incorporation of label, can be used to infer changes in the steady state concentrations of the phosphoinositides resulting from receptor activation (see Michell, 1982). However, problems with the pulse-labelling of brain slices with ^{3}H-inositol were encountered in the current study (see 2.3), while generally this approach involving lipid measurements seems relatively cumbersome compared with the assay outlined below.

In the experiments described in the present study, a direct measure of inositol lipid hydrolysis was achieved as follows. After initial preparation, tissue slices were first incubated in the presence of ^{3}H-inositol to allow uptake of this label and its subsequent incorporation into the polar headgroup of the phosphoinositides, PtdIns, PtdInsP, and PtdInsP$_2$. Following a suitable prelabelling period, receptor agonists or other stimuli were added to promote the phospholipase C catalysed cleavage of these ^{3}H-lipids. This diesteratic hydrolysis results in the release of the radiolabelled, polar headgroup (i.e. a ^{3}H-inositol phosphate) and of unlabelled diacylglycerol. Tissue samples were extracted so that these labelled products, together with excess, free ^{3}H-inositol, were obtained in a separate fraction from that containing ^{3}H-lipid. Since the three phosphoinositides are distinguished by the degree of phosphorylation of the inositol ring, the tritiated, water-soluble hydrolysis products are therefore characteristic of their lipid precursor and are identifiable, separate from other phosphates (e.g. nucleotides etc.) present in tissue extracts, by virtue of their specific labelling with ^{3}H-inositol. Thus, the presence of ^{3}H Ins-1,4,5-P_3 in extracts is indicative of ^{3}H-PtdInsP$_2$ hydrolysis. However, as this product is degraded by successive

dephosphorylation, lower inositol phosphates (Ins P_1, Ins P_2) may be derived either from Ins-1,4,5-P_3 or their corresponding lipids. Clearly, where the reaction sequence associated with the receptor mechanism is of interest, the ^{3}H-inositol phosphates must be separated from one another. This is achieved by relatively simple procedures based on the different ionic charge carried by the separate ^{3}H-inositol phosphates, a property of these hydrolysis products which is also exploited to isolate these from the excess, free ^{3}H-inositol used (see 2,4, CH.4, CH.5). As ^{3}H-inositol polyphosphates are catabolised by sequential removal of their monoester phosphate groups, each of these will yield ^{3}H-Ins P_1 and ultimately, free ^{3}H-inositol which can be re-incorporated into PtdIns. The breakdown of Ins P_1 to inositol is inhibited by lithium ions, so that in the presence of LiCl, all the ^{3}H-inositol labelled, lipid hydrolysis products are trapped at the level of ^{3}H-Ins P_1, regardless of which phosphoinositide(s) is cleaved in response to agonist. In the presence of lithium ions, ^{3}H-Ins P_1 should continue to accumulate over the entire period of stimulated phosphoinositide hydrolysis, thus the detectable response to stimuli is very much amplified. This property of lithium ions was first exploited by Berridge et al. (1982) to develop the very sensitive assay of inositol lipid hydrolysis that has frequently been used since in pharmacological studies of the receptors mediating this response.

In the current study experiments are described in which either this lithium assay has been used as a gross measure of receptor-mediated phosphoinositide hydrolysis or where the individual ^{3}H-inositol phosphates produced have been quantified separately in order to characterise the sequence of reactions involved in this response in brain. In either case, since the ^{3}H-products assayed are those arising as a result of the phospholipase C catalysed reaction (see above), a direct measure of receptor-mediated inositol lipid hydrolysis is obtained. This is not to imply that the current experimental protocol is without associated problems. Indeed, several recent observations indicate more complex pathways of inositol lipid and/or phosphate metabolism than had previously been considered. The implications of these in terms of the above methodology are discussed in later sections, as are some of the more general difficulties associated with these methods.

The basic procedures used for the preparation of and labelling of cerebral cortical slices with ^{3}H-inositol are described in 2.2. The lithium assay of ^{3}H-inositol phosphate accumulation is also detailed in 2.2 and data obtained using this is presented in 2.3 to establish some general features of stimulated phosphoinositide hydrolysis in brain slices. Methods for the extraction and separation of ^{3}H-inositol

polyphosphates are given in 2.4 (see also CH.5) and a similar protocol for the separate estimation of ^{3}H-inositol lipids detailed in 3.5. Most of these procedures are common to all the experiments described throughout the current study and thus, only specific modifications are reported in later chapters, although other methods, only used to a limited extent, are fully reported where appropriate.

2.2 Methods: Preparation of cerebral cortical slices and assay of phosphoinositide hydrolysis

In the majority of experiments these methods were identical to, or modified from, those described by Brown et al. (1984). Tissue was obtained primarily from male Sprague-Dawley rats between 200-350 g except in several later experiments where male Wistar rats of a similar weight range were used. Where comparable data were derived from both strains, no differences in the patterns of response were observed.

a. Preparation of cerebral cortical slices:

Animals were stunned by a blow to the head and decapitated. The top of the skull was cut away and the whole brain excised using a micro-spatula and immediately transferred to an ice-cooled dish. Cerebral cortex was dissected out over ice in three distinct steps. Firstly, with the hypothalamus uppermost, a vertical slice was made 1-2 mm behind the olfactory bulbs which were then dissected clear to give a clean section of frontal cortex. A second slice, approximately 2-3 mm thick, was cut vertically immediately in front of the hypothalamus. This section consists primarily of parietal cortex surrounding an inner core of the two striata. The former was trimmed from the outer edge of this section, leaving the pair of striata with the central septum intact. The remaining cortical tissue was dissected clear by turning the mid- and hind-brain section such that the hypothalamus was lowermost and applying gentle pressure to the two lobes of the cortex, from the direction of the cerebellum forwards, until these unfolded to reveal the hippocampi foremost. Hippocampi were removed and discarded. Cortical tissue was cut clear from remaining ventral connections and any striatum adhering to these sections was pinched out with forceps. The total dissection time was 3-5 min. per brain.

Total cortical tissue was pooled, transferred to a filter paper-covered plastic disc, and cross-chopped at 90° into 350 x 350 µm slices using a McIlwain tissue chopper. Chopped tissue was dispersed into separate slices by vigorous agitation in 20-25 ml modified Krebs-Henseleit buffer, pH 7.4 (Krebs composition, see Appendix 1). At this stage tissue

from two or more animals was pooled as appropriate, one cerebral cortex being sufficient for 20-25 assay tubes. Pooled tissue was transferred to 500 ml, flat-bottomed, screw-topped flasks and washed twice with 100-200 ml Krebs buffer to remove released neurotransmitters and debris (i.e. inadequately chopped tissue etc.) resulting from slice preparation. Cortical slices (primarily 0.35 x 0.35 x 2.0-4.0 mm) were resuspended in Krebs buffer at an approximate concentration of one cortex per 50 ml, incubation flasks gassed with O_2/CO_2 (95%/5%) and tissue incubated in a metabolic shaker at 37°C for 60 min. with four regular, intervening replacements of buffer and O_2/CO_2. This recovery period (to allow equilibration of tissue slices following post-mortem changes) was varied in a number of preliminary experiments (see 2.3).

b. Labelling of tissue with ^{3}H-inositol and assay of inositol lipid hydrolysis:

After 60 min. preincubation, tissue was washed twice in 100-200 ml of the above Krebs medium supplemented with 5 mM LiCl (in later experiments LiCl was omitted), then allowed to settle under gravity until uniformly packed (30-60 sec.). Aliquots of packed cortical slices (50 µl) were transferred to 6 ml capacity, flat-bottomed, polypropylene vials containing an appropriate volume of Krebs buffer (200-240 µl) such that after subsequent addition of tissue and drugs, a final volume of 300 µl was achieved.

Where it was more convenient, LiCl was omitted from the wash and incubation buffer and added at the appropriate stage to the final desired concentration as 10 µl of a 30-fold concentrated solution. Similarly, ^{3}H-inositol was normally added to the incubation medium at 1-5 µCi per tube (1 µCi unless otherwise stated) as 10 or 20 µl of an aqueous or 10-25% (v/v) ethanolic solution, depending on its source. The specific activities of the ^{3}H-inositol used varied between 10-16 Ci/mmol and hence concentrations of exogenously supplied ^{3}H-inositol were in the range 0.2-1.0 µM. For batch analysis of samples where accumulation of total ^{3}H-inositol phosphates was assayed, Krebs medium was routinely supplemented with 1 µCi ^{3}H-inositol per 300 µl.

Following the addition of cortical slices, incubation vials were gassed with O_2/CO_2 (95%/5%) and replaced in a metabolic shaker at 37°C and tissue allowed to pre-label (i.e. accumulate ^{3}H-inositol) for 30 min. before the addition of agonists. In some later experiments, this prelabelling period was extended to 60 min. to allow more convenient measurement of low concentrations of inositol polyphosphates (see CH.4). Where antagonists or cholinesterase inhibitors were to be present, these were added 15 min. prior to agonists. All drugs were added as 10 µl of a

30-fold concentrated solution in either appropriate Krebs buffer or a convenient alternative solvent as indicated.

Incubations were stopped by addition of 0.94 ml methanol:chloroform (2:1 v/v) (or in later experiments using 300 µl 1.0 M trichloroacetic acid or 7-8.3% (w/v) perchloric acid, see 2.4 and 6.1). Further 0.31 ml aliquots of each chloroform and water were then added as described by Berridge et al. (1983). Samples were vigorously vortex mixed, then centrifuged at 2-3,000 x g for 10 min. to produce a two-phase system. This comprised an upper, aqueous methanol layer (volume ~ 1.24 ml) containing the water-soluble, ^{3}H-inositol phosphates plus free ^{3}H-inositol and a lower organic phase (volume ~ 0.62 ml) containing the labelled inositol phospholipids. An insoluble tissue residue remained at the interface.

For assay of the ^{3}H-inositol phosphates, 0.75 ml of the upper phase was removed and diluted to 3 ml with distilled, deionised water and treated as below. Sample pH was approximately 7-8. For preliminary estimations of label incorporated into the inositol phospholipids, 200 µl aliquots of the lower chloroform layer were evaporated to dryness overnight at room temperature, then 5 ml of scintillant added and the radioactive content quantified by liquid scintillation counting. Counting efficiency for tritium was about 22% as determined by reference to a tritium quench correction curve constructed using ^{3}H-standards and an appropriate decay table (Packard) using the external standard channels ratio (ESCR) method.

To the diluted upper phases, 0.5 ml of a 50 % (w/v) slurry of Dowex 1 x 8 anion exchange resin (100-200 mesh) in either the formate (see below) or chloride form was added. Samples were thoroughly mixed to allow binding of the negatively charged, ^{3}H-inositol phosphates to the exchange resin which was then allowed to sediment under gravity before careful aspiration of the supernatant. The Dowex resin was washed four times with 3 ml 5 mM unlabelled inositol to remove remaining, free ^{3}H-inositol, each time with vigorous mixing and subsequent, careful aspiration of the washing medium. This procedure routinely reduced levels of unbound radiolabel to between 30 and 100 cpm/ml by the final wash. After removal of the final supernatant, resin-bound radioactivity was eluted with 0.5 ml 1.0 M ammonium formate/0.1 M formic acid or 0.5 ml 0.5 M HCl for formate and chloride Dowex resins respectively. ^{3}H-Inositol phosphates thus displaced were then quantified by liquid scintillation counting of a 0.4 ml aliquot of the eluate with 4.6 ml scintillant. Counting efficiency for tritium was of the order 17-20%, determined as above.

c. Preparation of Dowex anion exchange resins:

For the batch analysis method above and for some earlier anion exchange column separations of ^{3}H-inositol phosphates (see 2.4), two ionic forms of Dowex 1 x 8 (100-200 mesh) resin were employed, the formate and chloride forms. Resin was purchased (Appendix 3) in the chloride form and converted as required by the following procedure.

Dry resin (200-250 g) was suspended several times in 1-2 l deionised water and fine particles aspirated off with the supernatant after allowing the bulk to sediment. For the chloride form, the resin was then suspended in H_2O to give a 50% (w/v) slurry, assuming the initial weight and then stored at 4°C. The formate form was generated via an initial conversion to the hydroxide form by suspending the washed resin (Cl^- form) in approximately 5 l 1.0 M NaOH, with constant stirring for about 15 min. After sedimenting, the supernatant was aspirated and the resin washed repeatedly with several volumes of water until the supernatant pH < 9. The formate form was then generated by re-suspension of the resin in 2 l 1.0 M formic acid, again with stirring for 15 min. Finally, the Dowex was washed several more times with water until the supernatant pH > 4.2, then resuspended at 50% (w/v) in H_2O and stored at 4°C until required.

In later experiments (CH.3.5 onward), analytical grade Dowex resin in the formate form was used to achieve separation of ^{3}H-inositol phosphates. This resin (200-400 mesh) was purchased in the formate form and was prepared simply by suspending at 50% (w/v) in H_2O. Chromatographic resolution achieved with this resin was much improved compared with that found using resin prepared as above (see CH.4).

d. Recovery of inositol phosphates and expression of data:

i) Recovery of inositol phosphates: The capacity of the anion exchange resins used is about 1 m eq/g. Given this, and the volumes of resin used to bind inositol phosphates, it is unlikely that the small amounts of tissue used generate sufficient of these species to exceed the resin capacity. However, it was essential to verify this point by establishing the recovery of inositol phosphates under the specific assay conditions used.

The batch analysis method (above) was used principally to assess total ^{3}H-inositol phosphate accumulation in control and stimulated cerebral cortical slices incubated in the presence of Li^+ ions. Under these conditions, the vast bulk of total inositol phosphate accumulating is in the form of Ins P_1 (Berridge et al., 1982; Brown et al. 1984). Therefore, recovery on this method was assessed using a ^{14}C labelled Ins P_1 standard. However, it is reasonable to assume that, given the greater affinity of polyphosphates for the resin (resulting from the

greater negative charge carried), recovery of these would be, at a minimum, of a similar order to that for Ins P_1.

Ten determinations of ^{14}C Ins P_1 recovery were made in a single experiment using Dowex resin in each of the chloride and formate forms. ^{14}C Ins P_1 was applied to the resin in tissue extracted carrier as described below. Tissue incubations were performed exactly as above except that ^{3}H-inositol in the medium was replaced with an equivalent concentration of unlabelled inositol. Brain slice samples were stimulated for 45 min. with 1 mM carbachol in the presence of 5 mM LiCl in order to mimic the maximal concentrations of inositol phosphates expected to accumulate in typical experiments. Reactions were stopped as described above and 0.75 ml aliquots of upper phase diluted to 3 ml before addition of approximately 6.0 nCi ^{14}C Ins P_1 (about 114 pmol; sp.act. 55 mCi/mmol) in 10 µl aqueous solution. Methods were then followed as above and label finally quantified by counting 0.4 ml aliquots of the Dowex resin eluates. 10 µl aliquots of original ^{14}C label were counted under identical conditions to assess 100% recovery. Quench correction was made by reference to an appropriate curve constructed with ^{14}C standards using the method of external standard channels ratio.

The recoveries quoted below have been calculated on the following basis. Although 0.5 ml of a 50% (w/v) slurry of resin is added to each sample, the volume of resin remaining after the final wash (see (b) above) will exceed 0.25 ml owing to resin hydration. Therefore, calculation of recovery on the basis of dpm present in a 0.4 ml aliquot taken from a volume of 0.75 ml (resin + eluting buffer) will give an under-estimate, the magnitude of which depends on the extent to which the respective chloride and formate forms of resin swell on hydration. It has been assumed that differences between the ionic forms, in this respect, are negligible. Therefore, the estimates of recovery have been made by assuming 0.5 ml 50% (w/v) resin occupies 0.25 ml.

The maximum recovery (100%) was determined as 5.92 nCi ^{14}C Ins P_1. Recovery on formate and chloride forms of Dowex resin were respectively 5.54 ± 0.11 and 5.13 ± 0.05 nCi (mean ± SEM of 10 separate measurements). Therefore, the percentage recoveries on formate and chloride resin were 93.6 ± 1.9% and 86.7 ± 0.8%. Clearly both resins show comparable recoveries with the small, apparent differences potentially being accounted for by different relative extents of resin swelling on hydration. Further, as a small amount of resin was invariably lost during the several wash steps (see (b) above), it is probable that the true recoveries are closer to 100%. Therefore, given that the samples used were prepared so as to contain unlabelled inositol phosphates

approximating to the maximal levels expected in typical experiments, these recovery rates clearly validate the methods used.

ii) Expression of results: In the following sections the data is expressed as cpm accumulated as inositol phosphate or inositol phospholipid per 50 µl of tissue in order that, as far as is possible, values obtained at different stages can be compared. As the precise methods used (i.e. concentrations of ^{3}H-inositol used, extraction and analytical procedures) vary between chapters, direct comparisons cannot always be made. Values quoted for inositol phosphates in this chapter have been determined using similar calculations to that for the recovery of ^{14}C Ins P_1 made above, after first allowing for the fraction of upper phase assayed. Values for phosphoinositides have been similarly scaled up. Thus, typical experimental values obtained for data in this chapter were approximately 30% of those indicated. In later chapters, similar calculations have been made as appropriate. It should be noted that some variations between values for radiolabelling of both inositol lipid and phosphates are apparent between some earlier experiments and those described later. These can largely be accounted for by the use of different batches of ^{3}H-inositol. Several preliminary experiments (e.g. as in 2.3) were performed using aqueous solutions of label stored at -20°C. Thus, repeated freeze-thawing may have promoted extensive tritium exchange between the inositol and water solvent. A direct comparison between two preparations of ^{3}H-inositol, one stored at -20°C in H_2O and the second in 90% (v/v) ethanol, showed much greater label incorporation into both inositol lipid and phosphate was achieved with the latter. In later experiments the ^{3}H-inositol used was normally that obtained from a stock solution containing ethanol. Additional problems associated with contamination of the ^{3}H-inositol with unidentified ^{3}H-materials and methods used to remove these before use of the label in assays, are considered in 2.4.

Finally, where statistical analysis of data has been made, this has been achieved using either paired or unpaired Student's 't' test according to Appendix 4.

2.3 Practical considerations applying to the study of phosphoinositide hydrolysis in brain slices: basic characteristics of muscarinic receptor-mediated responses

The methods described in 2.2 form the general basis of those used throughout the current study. Although very similar procedures have previously been applied to the study of receptor-mediated phosphoinositide hydrolysis in brain slices (e.g. Brown et al. 1984), there is no guarantee that the conditions used are ideal. A number of factors may be expected to influence both the responses obtained and the interpretation of the data reported. Some of these apply generally to studies of phosphoinositide hydrolysis and others more specifically to studies using brain slices. In order (A) to verify the validity of the methods used and (B) to establish some of the basic characteristics of receptor-mediated phosphoinositide hydrolysis in brain, some of these factors are considered below (in sections (A) and (B) respectively) and are illustrated by data from preliminary experiments which form the basis for the further studies described in later chapters.

A) Practical considerations applying to study of phosphoinositide hydrolysis in brain slices

a. Suitability of brain slices for study of phosphoinositide hydrolysis:

A number of features of tissue slice preparations are specifically relevant to the characteristics of receptor-mediated responses in brain. The maintenance of a structurally intact system of cell bodies, neurones and pre- and post-synaptic receptors allows not only study of the actions of exogenous drugs but also the more physiologically relevant demonstration of effects caused by endogenous modulators released either by electrical or depolarising stimuli. However, problems do arise in correlating functional biochemical responses derived using slice preparations and receptor binding data frequently obtained using isolated membranes. An important factor here may be the maintenance of a physiological membrane potential in the former but not the latter preparation (see Nahorski et al. 1986).

As discussed by Downes (1982) a characteristic feature of stimulated phosphoinositide hydrolysis is the requirement for tissue preparations comprising whole cells to demonstrate the response, a fact related to both the differing subcellular localisation of the system components and the several energy-dependent steps of inositol lipid synthesis and headgroup cycling. Numerous studies (see Hawthorne and Pickard, 1979 and Downes, 1982) have shown responses using synaptosomes which are capable of

generating ATP though these are generally less marked than responses observed in tissue slices (Redman and Hokin, 1964). More recently inositide breakdown has been demonstrated in tissue homogenate and membrane preparations (e.g. Litosch et al. 1985), particularly in the presence of GTP or its non-hydrolysable analogues but this requires preliminary labelling of intact tissue prior to isolation of membranes.

While slices derived from cerebral tissues provide a convenient means of studying inositol lipid metabolism the data obtained are not necessarily as readily interpreted as those derived from tissues of more homogenous cell type. Thus, the cellular localisation of responses (see CH. 1) is not always clear and it is often difficult to exclude the possibility that responses to given stimulus may be mediated, at least in part, through secondary or indirect mechanisms (e.g. release of other neurotransmitters). The use of primary neuronal cultures and cell lines will no doubt provide invaluable complementary information and should prove particularly useful in allowing controlled manipulations of intracellular conditions to which tissue slices are not readily amenable. Indeed, considerable progress has already been made (see Downes, 1986). A further approach also discussed by Downes (1986) is the use of individual, vibratome cut tissue sections from localised cerebral regions (e.g. discrete brain nuclei). These preparations have the advantage of suitability to both biochemical and electrophysiological studies of receptor-mediated effects while exhibiting homogenous or, at least, largely defined cell populations.

b. Assay conditions: factors affecting the viability of tissue slices:

Clearly the metabolic status of the tissue is an important consideration when studying any biochemical response. In optimising the current assay conditions a number of factors likely to affect this were taken into account.

i) Dimensions of tissue slices: In using tissue slice preparations the slice dimensions can be a critical factor. Regardless of source, the slice size is an approximate balance between maintaining the maximum number of intact cells and the problems that arise from increasing slice dimensions. Ideal measurements vary typically between 100-500 μm depending on the cellular morphology of the tissue. In the case of brain slices, larger dimensions favour greater cellular integrity but are countered by the problems of anoxia and diffusional barriers. The latter may be particularly relevant where the early kinetics of receptor-mediated inositol lipid hydrolysis are under consideration. Preliminary

experiments in this laboratory (see Brown et al. 1984) suggested that cerebral cortical slices cross-chopped at 350 x 350 μm were handled most conveniently and produced the most consistent data in experiments designed to characterise phosphoinositide breakdown. Downes (1982) has reported identical findings with tissue from several cerebral regions and Janowsky et al. (1985) and Minneman and Johnson (1984) have both used equivalent preparations.

ii) Tissue preincubation: In the current study this refers to the post-preparational recovery period which tissue was allowed prior to labelling with ^{3}H-inositol (see 2.2). Brain tissue is known to be subject to profound post-mortem changes in the cellular concentrations of high energy metabolites, particularly creatine phosphate and adenosine triphosphate (ATP) (e.g. see Whittingham et al. 1984). Substantial degradation of inositol phospholipids is also known to occur post-mortem (Dawson and Eichberg, 1965), a situation which can only be aggravated by the massive release of transmitters (etc.) occurring during subsequent dissection and chopping of tissue. Given the multiple energy-dependent steps associated with phosphoinositide synthesis, it might be anticipated that a suitable recovery period prior to labelling of tissue with ^{3}H-inositol and subsequent agonist challenge would facilitate both more consistent lipid labelling and hence, receptor-mediated hydrolysis. Fredholm et al. (1984) have studied the immediate post-mortem changes in the concentrations of adenosine nucleotides and the restoration of these to stable levels during subsequent incubations of hippocampal slices under conditions similar to those currently used. They have correlated these changes with the electrophysiological responsiveness of these tissue slices. Their results indicate that over a 60 min. post-preparational incubation period, the energy charge of the tissue (defined as, [(ATP + ½ADP)/(ATP + ADP + AMP)] returns close to that found in vivo and that this is paralleled by improved electrophysiological responsiveness, although the absolute levels of adenosine nucleotides at the end of this period are only about 50% of those found in vivo in rat brain. Whittingham et al. (1984) have reported similar results but they suggest that although brain slices display reasonable metabolic stability over prolonged incubations (i.e. upto 8 hours), levels of energy metabolites required up to 3 hours to stabilise after slice preparation.

In a preliminary series of experiments the effects of allowing tissue a variable recovery phase prior to assay were investigated. Tissue was prepared as in 2.2 and after an initial wash in Krebs buffer was either aliquoted out into incubation medium containing ^{3}H-inositol (i.e. zero preincubation time) and treated as below or incubated in bulk in Krebs

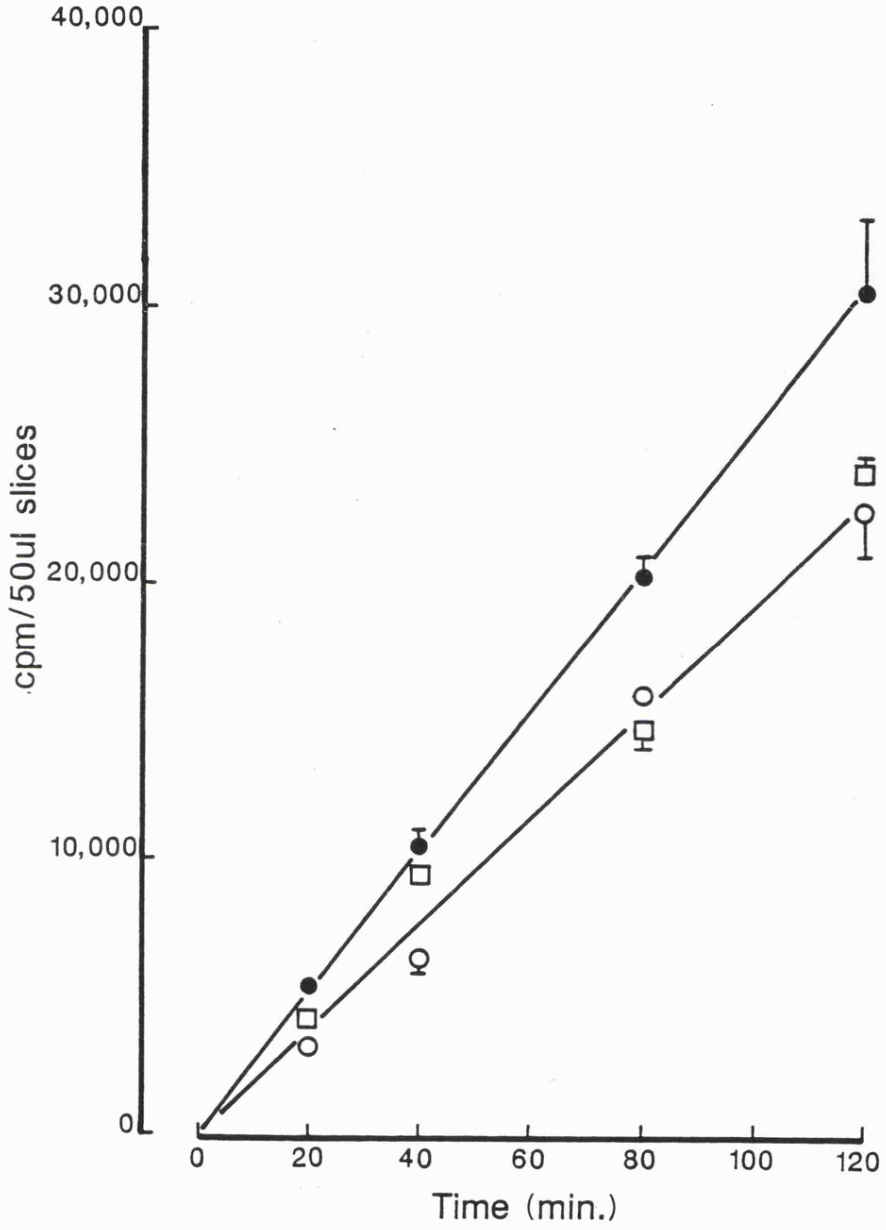


FIGURE 2.3.1 The effect of pre-incubation time on the time course of ^{3}H-inositol incorporation into brain slice phosphoinositides.

After preparation, cerebral cortical slices were allowed a recovery period (i.e. pre-incubated) of 0 min. (○), 30 min. (□) or 60 min. (●) before addition of ^{3}H-inositol (1 μCi). Tissue was then incubated for the times indicated in the presence of 5 mM LiCl before extraction of ^{3}H-inositol phospholipids according to 2.2. Results show mean ± SEM of 3 determinations made in a single experiment but similar data were obtained on two further occasions.

buffer for 30 or 60 min. as in 2.2 prior to transfer to assay tubes. In the latter case, buffer was replaced at 15 min. intervals with freshly oxygenated medium. The effects of a recovery phase thus varied between 0, 30 and 60 min. on ^{3}H-inositol incorporation into phospholipid and on agonist-induced ^{3}H-inositol lipid hydrolysis were determined in separate experiments. For the former, tissue preincubated for varying times was allowed to incorporate label under conditions as in 2.2 for up to 2 hours before reactions were stopped. After phase partitioning of solvents, 200 µl aliquots of the chloroform layer were evaporated to dryness and counted for tritium. The results of a typical experiment are shown in Figure 2.3.1. Where the effect of preincubation time on agonist-induced phosphoinositide hydrolysis were measured tissue allowed different recovery periods was allowed to incorporate ^{3}H-inositol for 30 min. prior to incubation with or without carbachol (3×10^{-4} M) or noradrenaline (NA, 1×10^{-4} M) each in the presence of 5 mM LiCl for a further 45 min. Accumulated ^{3}H-inositol phosphate was assayed as in 2.2 and corresponding lipid labelling determined as above. Figure 2.3.2 illustrates the mean results of three experiments.

From Figure 2.3.1 it is clear that the varied preincubation times had little effect on the incorporation of ^{3}H-inositol into phospholipid. Given the comments above this result is surprising but can probably be accounted for by a combination of factors. Firstly, it should be noted that while brain concentrations of polyphosphoinositides are high compared to other tissue, these two lipids (PtdInsP and PtdInsP$_2$) still only comprise a fraction of the total cerebral inositol lipid (see 3.5). As it is well established that the use of neutral solvents results in poor extraction of polyphosphoinositides (see Downes and Michell, 1982), it may be anticipated that under the current conditions labelled lipid will comprise almost exclusively ^{3}H-PtdIns (this confirmed by data in 3.5). Further, the incorporation of inositol into PtdIns is known to proceed by two mechanisms. The first is an energy-dependent route via CDP-diacylglycerol:myo inositol phosphatidate transferase (Agranoff et al. 1958). The second is a poorly understood, energy-independent, inositol headgroup exchange reaction (Paulus and Kennedy, 1960) which is stimulated by Mn^{2+} ions and cytidine nucleotides and is variably considered to occur via a reversal of the synthetic pathway (Hokin-Neaverson et al. 1978) or to be catalysed by a distinct enzyme (Takenawa et al. 1977). Both pathways occur in brain (see Dawson, 1982) and comparative studies of stimulated incorporation of $^{32}P_i$ and ^{3}H-inositol into PtdIns suggest that a substantial proportion of basal labelling with the latter results through headgroup exchange (see Downes, 1982). Thus, measurement of accumulated lipid label under the current experimental conditions will be

a poor indicator of the sensitivity of phosphoinositide labelling to the tissue's metabolic state both because incorporation into PtdIns may proceed by an energy independent mechanism and because labelled polyphosphoinositides are excluded by the extraction procedure. It is possible that as synthesis of PtdInsP and PtdInsP$_2$ require additional ATP-dependent reactions, that separate measurement of label incorporation into these would reflect the effects of varying pre-incubation times more accurately. However, such measurements may also be of limited use given the heterogenous cell population of brain slices and the inherent possibility of discrete cellular, as well as subcellular, pools of inositol lipids. Thus, total labelling even of distinct fractions of phosphoinositide will not necessarily reflect the pattern associated with the agonist-sensitive pools which are of interest here. Recent studies by Labarca *et al*. (1985) and Schoepp (1985) demonstrate this point for PtdIns showing that while ^{3}H-inositol labelling of this lipid is markedly potentiated by Mn^{2+} ions this does not result in increased accumulation of labelled Ins P$_1$ resulting from muscarinic or alpha-1 receptor stimulation. Similarly, substantial proportions of brain PtdInsP and PtdInsP$_2$ are associated with the myelin fraction (see Hawthorne and White, 1975) which would not be expected to be agonist labile. Therefore, while measurement of ^{3}H-inositol incorporation into PtdIns alone is likely to be relatively insensitive index of the effects of preincubation time on labelling of agonist-sensitive inositol lipid pools, the extraction and quantification of separate polyphosphoinositides would not necessarily be substantially preferable. Similarly, assays of inositol lipid hydrolysis by measurement of stimulated increases in ^{3}H-inositol labelling are of reduced sensitivity compared to those using $^{32}P_i$ or ^{32}P ATP, as the latter will preferentially label more rapidly turning over, agonist associated pools, while the former also results in substantial labelling of presumably larger and less metabolically active pools. For these reasons notable changes in lipid labelling are rarely seen in experiments described in the current study despite accumulations of ^{3}H-inositol phosphates approximately equating with total ^{3}H-lipid (see Figure 2.3.2). The small changes observed in lipid labelling resulting from different preincubation times illustrated in Figure 2.3.1 could be representative of more significant effects on discrete but small pools of metabolically less stable phosphoinositides which are obscured by simultaneous labelling of much larger less active pools. The data presented in Figure 2.3.2 together with later results would appear to support this view.

Comparison of Figures 2.3.2 A and B illustrates a number of the points made above. The data showing lipid labelling (again primarily ^{3}H-PtdIns) support those from Figure 2.3.1, indicating only marginal

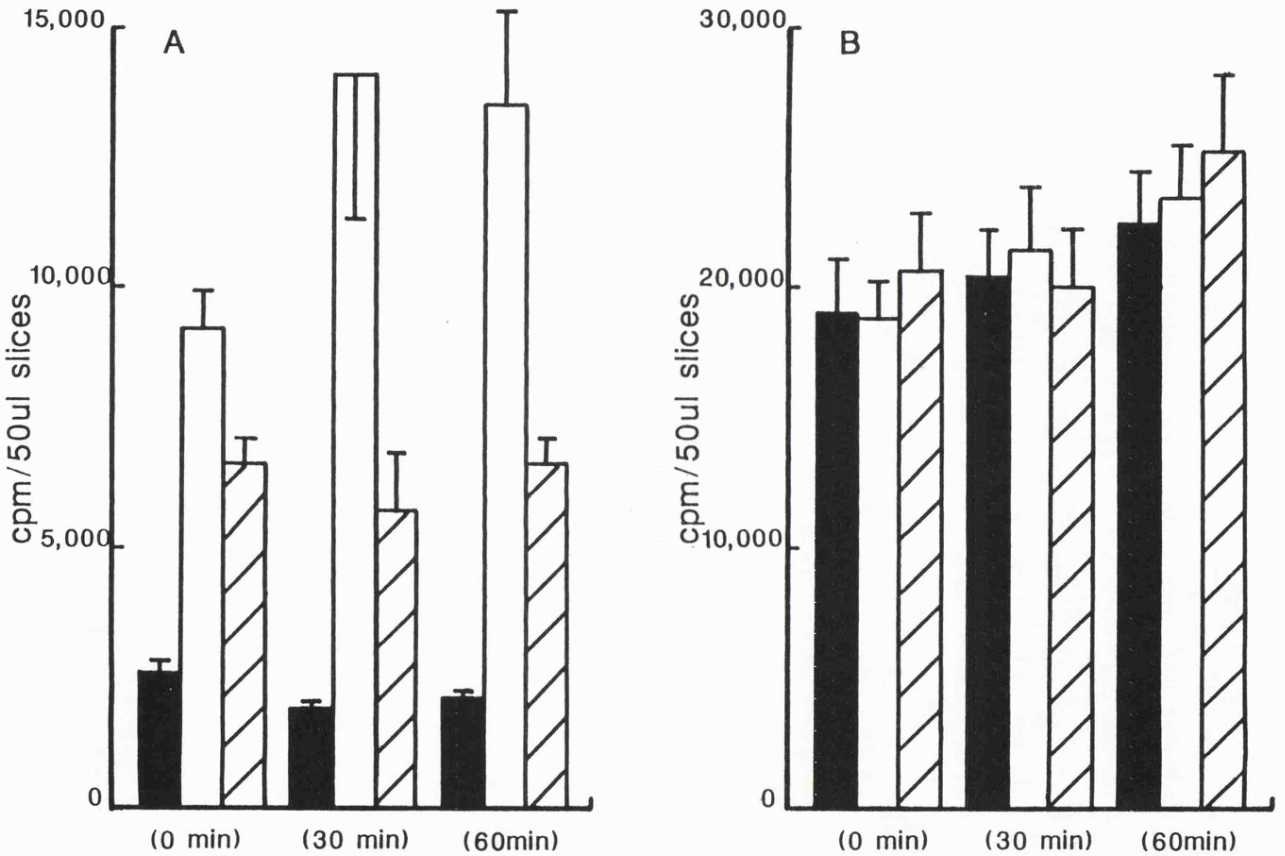


FIGURE 2.3.2 The effect of pre-incubation time on control and agonist-stimulated ^{3}H-Ins P(s) accumulations in ^{3}H-inositol labelled brain slices and corresponding effect on phosphoinositide labelling.

Tissue was labelled with ^{3}H-inositol (1 μCi) for 30 min. after being allowed a post-preparational recovery period of 0, 30 or 60 min. Brain slices were then further incubated for 45 min. in the absence (shaded bars) or presence of 0.3 mM carbachol (open bars) or 0.1 mM NA (hatched bars). 5 mM LiCl was present throughout incubations with label. ^{3}H-Ins P(s) (A) and ^{3}H-phospholipid (B) were then determined as in 2.2. Results represent the means ± SEM of at least 3 experiments performed in triplicate.

differences between tissue preincubated for 0, 30 or 60 min. Figure 2.3.1 A demonstrates that noradrenaline and carbachol respectively stimulate accumulations of inositol phosphate 2-3 and 3-7 fold over basal values. By comparison with previous work the response to NA is mediated via alpha-1 adrenoceptors (Brown et al. 1984; Minneman and Johnson, 1984) and that to carbachol through muscarinic receptors (Brown et al. 1984; Jacobson et al. 1985). In contrast to the marked accumulation of inositol phosphate induced by either agonist there is no substantial effect on the labelling of PtdIns. This indicates that potentially only a very small percentage of total ^{3}H-PtdIns (possibly within the limits of experimental error) functions as part of the receptor-mediated mechanism, which in turn implies that only pools of PtdInsP and PtdInsP$_2$ which can be derived via this specific fraction of PtdIns can be similarly associated with the receptor. Alternatively, substantially larger lipid pools may be involved whose hydrolysis is closely compensated for by constant re-synthesis. In the latter case, though not in the former, freshly synthesised receptor-coupled lipid would necessarily have to be derived from either an identical cellular pool of ^{3}H-inositol or one of very similar specific activity to that from which other ^{3}H-PtdIns is derived, otherwise marked agonist-induced effects would be anticipated. From the current data it is not possible to estimate the size of receptor associated pools of lipid though subsequent data (see (c) below) favour the earlier argument. Similarly, it is not possible to determine whether muscarinic and alpha-1 receptor responses are mediated through a common pool of lipid.

It is interesting that the response to carbachol but not that to NA shows a substantial dependence on preincubation time, with tissue preincubated for 30 min. or more yielding a fold stimulation approximately twice that obtained from tissue allowed no recovery phase. This difference is due, in part, both to a decreased basal Ins P$_1$ and markedly increased stimulated Ins P$_1$ concentrations. Whether the difference between the noradrenergic and carbachol-stimulated responses reflects a variable sensitivity at the level of the receptor or at a step down-stream is obviously not apparent. A previous study (Brown et al. 1984) has shown that muscarinic and alpha-1 receptor-mediated responses in cortex are additive, implying separate receptors may be associated with distinct phosphoinositide pools. Similarly, the greater response to carbachol in tissue given a suitable recovery phase suggests that the muscarinic receptor may have access to a greater fraction of total inositol lipid than the alpha-1 receptor. Further, since studies with rat cortex (Allison et al. 1976) have suggested that the majority of inositol monophosphate accumulating in the presence of Li^{+} ions in vivo results from acetylcholine release, it might be anticipated that immediately

following slice preparation muscarinic receptor associated lipid would undergo a more profound depletion. Thus, this system would display a greater sensitivity to preincubation time. If this explains the observed effect, then the absence of a corresponding decrease in lipid labelling would argue in favour of relatively small pools of receptor associated ^{3}H-PtdIns. Alternatively, muscarinic and alpha-1 receptors may have access to small but equivalent lipid pools with the former receptor promoting a more rapid flux to generate a larger response. A faster turnover rate would also be dependent on the metabolic status of the tissue owing to the need to replenish energy-dependent pools of $PtdInsP_2$, assuming a similar sequence of reactions as proposed for other tissues. Either explanation or potentially a combination could account for the differential sensitivities of the NA and carbachol responses to preincubation.

From these preliminary experiments it is clear that agonist-induced hydrolysis of phosphoinositides can be favourably affected to an extent by allowing tissue slices a post-preparational recovery period of between 30 and 60 min. The incorporation of ^{3}H-inositol into inositol lipids is less clearly affected by the length of the preincubation period. In the light of the present results, subsequent experiments always allowed for a 60 min. preincubation prior to assay (i.e. as in 2.2) unless otherwise stated. However, it should be pointed out that in the discussion above it is assumed from previous studies (e.g. Fredholm et al. 1984; Whittingham et al. 1984) that the effect of the preincubation period is to allow tissue concentrations of energy metabolites (ATP etc.) to stabilise or, at least, to partially recover from the low levels anticipated more immediately post-mortem. It is possible that the effects noted could be due to other factors and clearly to establish any relationship between the magnitude of agonist-induced phosphoinositide hydrolysis and tissue ATP content will require further studies in which direct measurement of both parameters is made.

iii) Tissue concentration: A third factor which might be expected to have marked effects on tissue responsiveness over relatively long term in vitro incubations is the amount of tissue per incubation volume. In turn, incubation volumes are necessarily limited by the use of reasonable levels of ^{3}H-inositol and by achieving a surface area/volume ratio consistent with adequate diffusion of O_2/CO_2 to maintain the pH of the physiological buffer used. Thus, the optimal signal to noise ratio of the assay will depend on a balance between the extent to which accumulation of ^{3}H-inositol phosphates is increased per amount of tissue and the effect of increasing tissue concentration on sample pH which could ultimately limit slice viability.

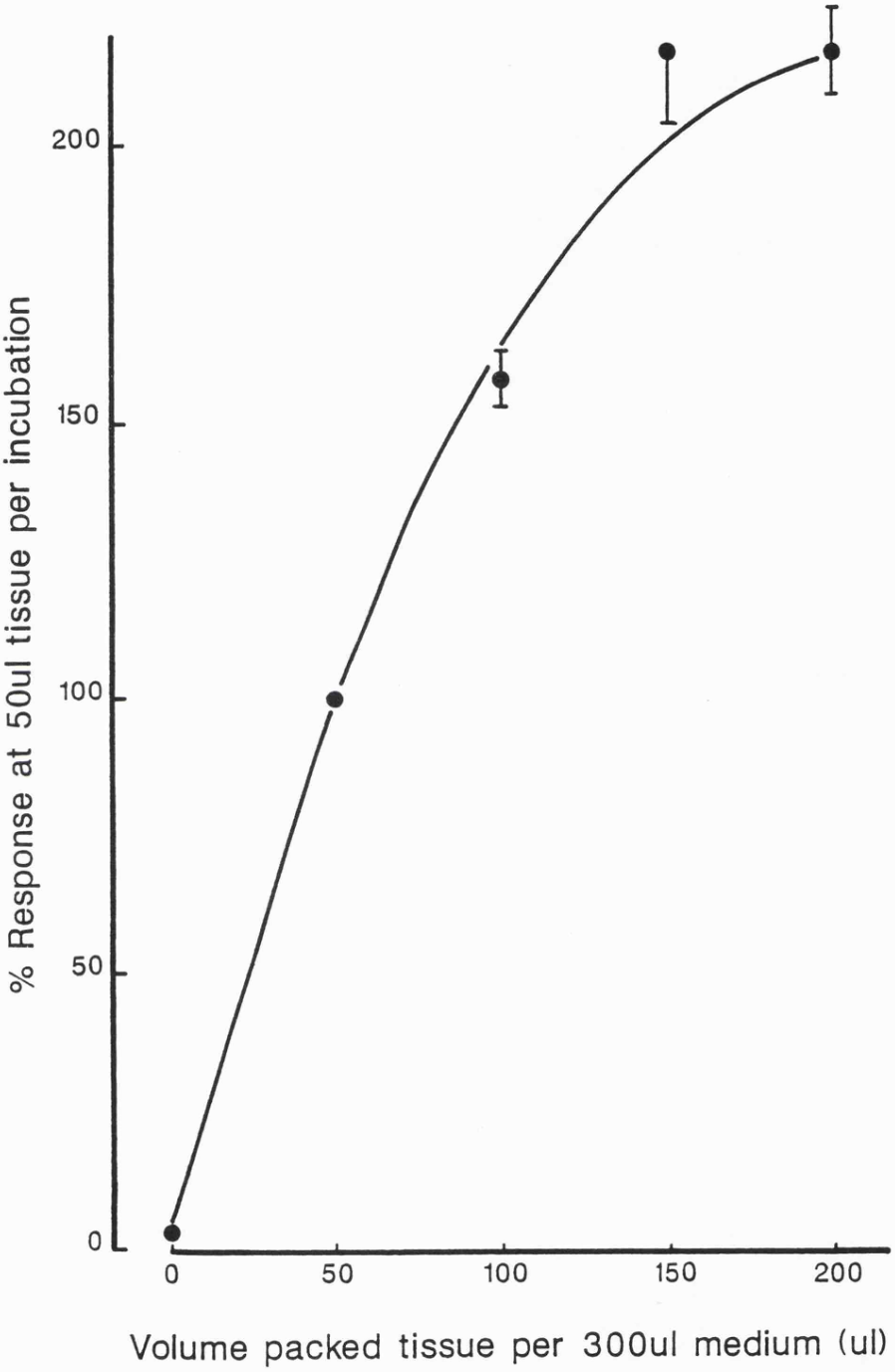


FIGURE 2.3.3 Linearity of the ^{3}H-Ins P(s) accumulation stimulated by carbachol with the density of tissue per incubation volume.

Cerebral cortical slices were prepared and pre-incubated (60 min.) as in 2.2 then 0, 50, 100, 150 or 200 µl of packed tissue transferred to assay tubes containing sufficient medium to give a final volume of 300 µl. Brain slices were then allowed to incorporate ^{3}H-inositol (1 µCi) for 30 min. prior to exposure to 1 mM carbachol for 45 min. and subsequent estimation of ^{3}H-Ins P(s) as in 2.2. 5 mM LiCl was present throughout incubations with label. Results show mean ± SEM of 3 experiments performed in triplicate and are expressed relative to the response obtained at 50 µl tissue/300 µl total volume.

In further, preliminary experiments the effect of tissue concentration on the response to agonist was investigated. Incubations were performed exactly as in 2.2 except that assays were run with either 0,50,100,150 or 200 µl of packed cortical slices. Other conditions were as in the legend to Figure 2.3.3. From the results shown in Figure 2.3.3. it is apparent that the accumulation of ^{3}H-inositol phosphate in response to carbachol only remained linear with tissue concentration up to between 50-100 µl packed slices/300 µl medium. Indeed, the doubling of concentration from 50-100 µl resulted in only a 50-60% increase in accumulated label. The plateauing of the response at above 100 µl tissue indicates that the advantage of increased amounts of tissue is negated by less tenable viability, presumably resulting from the limited buffering capacity of the medium. Thus, the results indicate that the lower end of the tissue concentration range (50-100 µl) is optimal within the limitations applied by the use of a convenient buffer volume (i.e. 300 µl). Using a similar incubation protocol, Minneman and Johnson (1984) have reported that alpha-1 receptor-mediated inositol phosphate accumulations in cerebral cortical slices show an almost identical relationship between maximum response and tissue density per incubation. In future experiments 50 µl aliquots of tissue were routinely used in a final incubation volume of 300 µl (see 2.2).

iv) Time courses of agonist-stimulated ^{3}H-inositol phosphate accumulation: A final important factor in optimising the current assay conditions was the time course of the response to agonist. Clearly this could be dependent on tissue viability but might also be expected to vary with the approach used to label tissue with ^{3}H-inositol. Different labelling methods are considered in (c) below.

A notable feature of receptor-mediated inositol phospholipid hydrolysis is the failure of the response to rapidly desensitise, unlike receptor controlled adenylate cyclase systems (e.g. Stiles et al. 1984). This has been demonstrated for several receptors coupled to inositol lipid hydrolysis in brain (e.g. histamine H_1, muscarinic, and alpha-1 adrenergic; Daum et al. 1984; Jacobson et al. 1985; Minneman and Johnson, 1984, respectively). Cerebral tissue however, is characteristically susceptible to anoxia so that over prolonged incubations, a decreased rate of accumulation of inositol phosphate in the presence of lithium ions may result not through desensitisation but more simply through decreased tissue viability. As discussed above, the latter may be an important factor with separate receptor systems displaying differential sensitivities. Further, the variably increased metabolic activity induced in the presence and absence of different agonists might therefore result

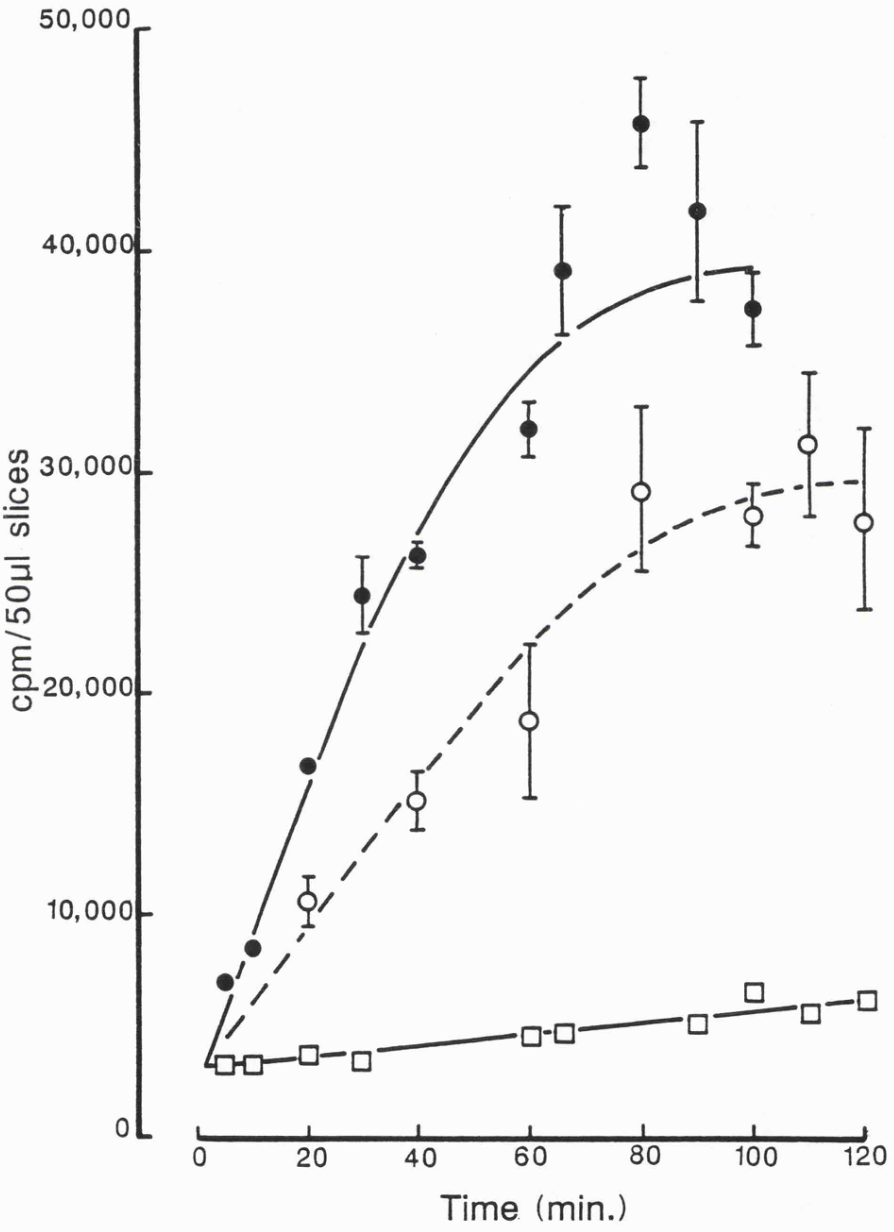


FIGURE 2.3.4 Time courses for the accumulations of ^{3}H-Ins P(s) stimulated by different doses of carbachol in ^{3}H-inositol labelled brain slices.

Cerebral cortical slices were prepared and labelled with ^{3}H-inositol (1 µCi) as described in 2.2 then further incubated in the absence (□) or presence of 0.3 mM (●) or 0.1 mM (○) carbachol for the times indicated before extraction and assay of accumulated ^{3}H-Ins P(s) as in 2.2. 5 mM LiCl was present throughout incubations with ^{3}H-inositol. Results show the mean ± SEM of 1-2 experiments run in triplicate.

in dissimilar time courses of ^{3}H-inositol phosphate accumulation (i.e. basal accumulation may be linear for a prolonged period while the maximally enhanced agonist stimulated rates may begin to decline over a comparable incubation). Assay sensitivity for a given agonist will be maximal only over a time course during which basal and stimulated rates of ^{3}H-inositol phosphate accumulation both procede linearly.

Time courses for several agonists were therefore established. Most detailed examination was afforded to the carbachol-stimulated response as the earlier results (see above) indicated that this was likely to exhibit the greatest sensitivity to decreased tissue viability. Figure 2.3.4 illustrates the time courses for ^{3}H-inositol phosphate accumulation in response to both a sub-maximal and maximally effective concentration of carbachol (Brown et al. 1984). The upper curve, showing responses to 3 x 10^{-4} M carbachol, represents the results of two experiments and indicates that stimulated values at later times (60 min. and more) were prone to greater error and demonstrated variably extensive plateauing of the response between 70 and 120 min. However, as illustrated by Figure 2.3.4 and as determined in repeated later experiments (see 3.4 and 6.4), responses to maximally effective concentrations of carbachol reliably resulted in a linear accumulation of labelled inositol phosphate over the initial 60 min. Basal accumulation, as anticipated, was linear over the entire period shown, while responses to sub-maximally effective doses of the muscarinic receptor agonist paralleled those to higher concentrations.

Figure 2.3.5 compares the time courses of responses to maximally effective concentrations of each NA (1 x 10^{-3} M, Minneman and Johnson, 1984), histamine (HA) (1 x 10^{-3} M, Daum et al. 1984) and 5-hydroxytryptamine (5-HT) (3 x 10^{-4}, Brown et al. 1984; Kendall and Nahorski, 1985a) with that to 1 mM carbachol. It is clear that the pattern of response is closely similar for all four agonists with, in each case, the time courses plateauing between 60 and 90 min. Maximal responses to carbachol, NA, 5-HT and HA, determined as the percentage of basal at the extreme of the linear portion of each curve (60 min.), were respectively 820%, 320%, 180% and 150%. This overall order of tissue responsiveness to these agonists corresponds well with that observed by Brown et al. (1984) and the maximal responses to carbachol, NA and 5-HT approximate to those reported in the references quoted above, although that to HA is somewhat smaller than that observed by Claro et al. (1986) in rat cerebral cortex.

For each of the time courses illustrated in Figure 2.3.5, lipid labelling for the corresponding points was also measured. This showed values, relative to corresponding controls, for carbachol, NA, HA and 5-HT respectively varied between 82-96%, 98-105%, 90-103% and 83-96% over the

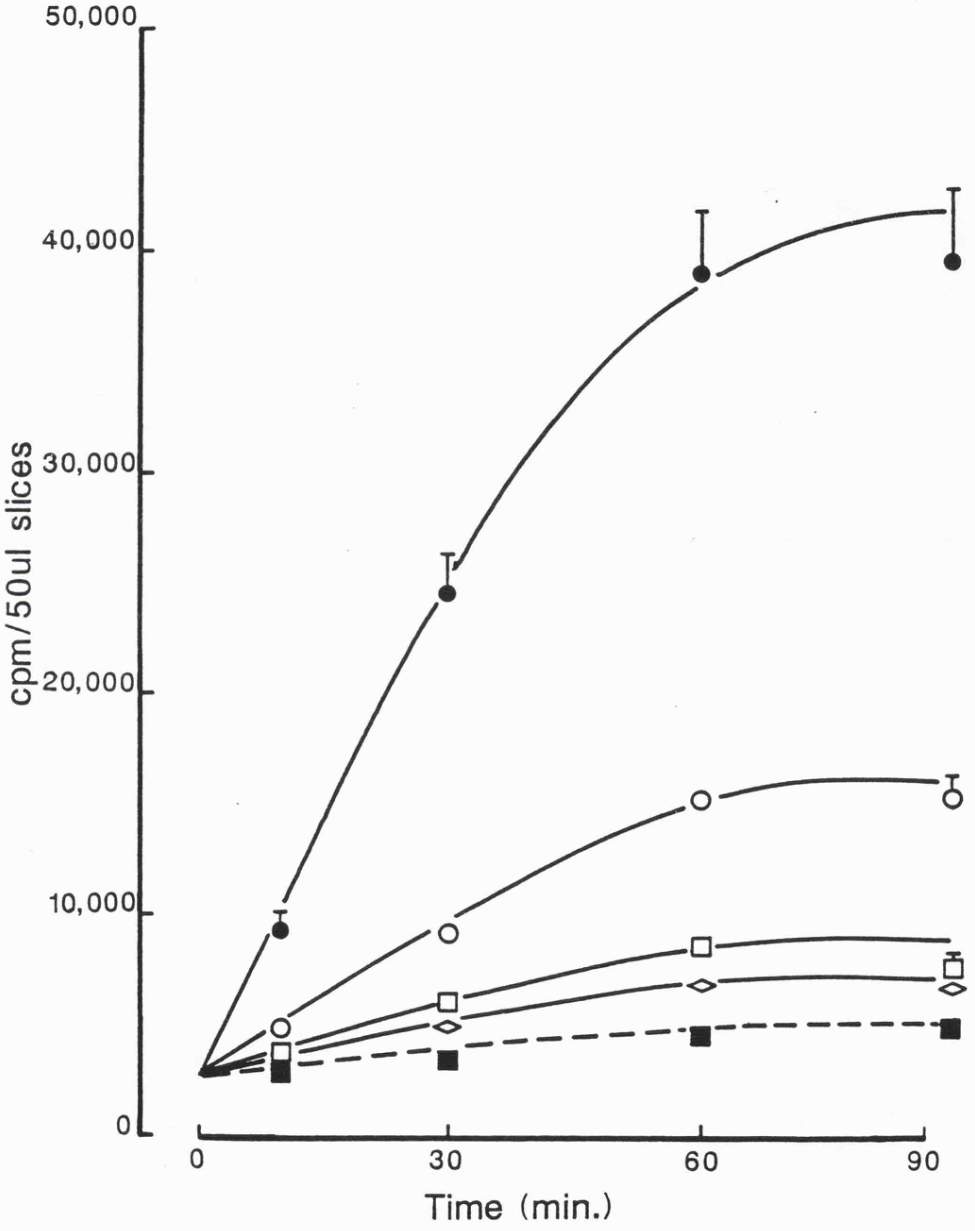


FIGURE 2.3.5 Time courses for the accumulations of ^{3}H-Ins P(s) stimulated by different neurotransmitter receptor agonists in ^{3}H-inositol labelled brain slices.

Incubations were performed as in the legend to Fig. 2.3.4. (●), (○), (□), (◇) and (■) show the responses to 0.3 mM carbachol, 1.0 mM NA, 0.3 mM 5-HT, 1.0 mM HA and control respectively. Results represent the mean ± SEM of 3 measurements made in a single experiment.

90 min. incubation period. This confirms the observations previously made, though the same comments apply.

Most importantly, these data show that responses to agonists producing inositol phosphate accumulations of widely different magnitude are all remarkably similar in respect to their time courses. Thus, the apparently very much greater increase in metabolism of inositol lipids stimulated by carbachol does not result in a less prolonged, maximally-induced accumulation of ^{3}H-inositol phosphate than that stimulated by less effective agonists. (This does not however, contradict the previous observations that the muscarinic receptor-mediated response may be more sensitive to the initial metabolic viability of the tissue). Similarly, the same holds for different doses of the same agonist which is clearly an important factor in the pharmacological characterisation of responses.

A final interesting feature of the data in Figure 2.3.5 is the response to histamine which shows a stimulated accumulation of ^{3}H-inositol phosphate only after an initial lag period such that a clear effect is not evident until 30 min. It may be pointed out that detection is limited by the magnitude of the response, while the data represents only a single experiment. However, a more detailed study (Daum et al. 1984) has reported similar time courses to HA in both guinea-pig cerebral cortical and cerebellar slices where linear accumulation of ^{3}H-Ins P_1 (in the presence of lithium ions) up to 4-5 fold basal values were apparent only between 15 and 120 min. but not at earlier times. This may indicate that responses to HA are mediated indirectly although these responses are completely antagonised by H_1 histamine receptor blockade (Daum et al. 1984; Claro et al. 1986) while the latter study shows linear accumulation of inositol phosphate over an initial 60 min. incubation with HA.

In later experiments where the batch analysis of total ^{3}H-inositol phosphates (see 2.2) was used, incubations with agonists were routinely run for 45 min. since responses to each of the agonists above (except HA) showed time courses linear up to about 60 min., while at later times responses, particularly to muscarinic receptor stimuli, were subject to greater variability. Thus, a 45 min. incubation was found to be consistent with a sensitive assay, allowing detectable accumulations of ^{3}H-inositol phosphate in response to even comparatively small stimuli while giving reliable data with more effective stimuli and falling clearly within the linear phase of both basal and stimulated responses. Nevertheless, this assay procedure does have complicating factors, largely arising through the inclusion of lithium ions (see CH.3).

c. Protocols for labelling tissue with ^{3}H-inositol:

The various factors likely to affect agonist responses considered in (b) above, are primarily practical considerations aimed at optimising the assay conditions. A further factor which is likely to be important from this point of view but which also has theoretical implications for the interpretation of data, is the approach used to label tissue with ^{3}H-inositol. While it is possible to achieve close to isotopic equilibrium labelling of polyphosphoinositides with ^{32}P within relatively short term incubations (e.g. see Weiss et al. 1982) by virtue of the rapid turnover of their monoester phosphate groups, similar labelling of all three phosphoinositides with ^{3}H-inositol or ^{3}H-glycerol requires longer periods (e.g. see Drummond et al. 1984; Drummond and Raeburn, 1984). Assay of inositol lipid hydrolysis either by measurement of stimulated incorporation of ^{32}P or by loss of ^{32}P radioactivity from prelabelled lipid is of limited value (see 2.1). Similar assay by measurement of the accumulation of ^{32}P labelled inositol phosphates is possible (Van Rooijen et al. 1983) although the lack of label specificity clearly presents problems in isolating the products distinct from other non-inositol phosphate esters which will also be labelled. The use of tissue preparations labelled with ^{3}H-inositol eliminates the latter difficulty as separate ^{3}H-inositol phosphates are isolated by relatively simple procedures (see 2.4), while measurement of a total inositol phosphate accumulation provides a crude but direct assay of lipid hydrolysis. However, as with many studies employing radioisotope labelling, the products are only quantified in terms of radiolabel and not in absolute amounts, thus problems of potentially varying specific activity arise. Despite the many recent studies of receptor-mediated inositol lipid hydrolysis, there have to date been very few reports of the chemical concentrations to which inositol phosphates accumulate in stimulated cells (for an example see Rittenhouse and Sasson, 1985). The lack of further data derives simply from the absence of convenient assays for the quantification of the very low concentrations, particularly of inositol polyphosphates, anticipated. Although the concentrations of the parent lipids can be determined by phosphate assay and an estimate of the specific activity within each phosphoinositide fraction thus calculated, this will not necessarily reflect that associated with receptor-linked pools, especially in a tissue such as brain (see (b) above), and cannot therefore be translated to the corresponding inositol phosphates.

There is therefore, no adequate means for verifying that changes in inositol phosphate or lipid labelling correspond to identical changes in mass terms. However, different labelling procedures may reflect concentration changes more faithfully than others. For example, where

inositol lipid hydrolysis has been examined in cell culture preparations, tissue is typically grown for up to several days in medium supplemented with ^{3}H-inositol, allowing labelling to approach isotopic equilibrium (e.g. Drummond et al. 1984). Clearly this is not practical with most tissue slice preparations where labelling times are restricted by tissue viability and most studies have adapted one of two basic approaches with appropriate modifications.

i) Continuous labelling/ongoing synthesis protocol: This procedure is typified by that described in 2.2 and has been employed in the majority of experiments in the current study. The essential feature of this method is that ^{3}H-label remains present throughout the incubation with agonist after allowing a prior, prelabelling period which normally varies between 30 and 60 min. (cf Brown et al. 1984 with Jacobson et al. 1985 or Daum et al. 1984) or is omitted such that agonist and ^{3}H-inositol are added simultaneously (Minneman and Johnson, 1984). The major advantage of this method is that labelled lipid degraded in response to agonist is continuously replenished from free ^{3}H-inositol (but see discussion in CH.3) so that the time scale of incubations is not restricted by a limited pool of prelabelled phospholipid. In the presence of Li^{+} ions, where degradation of ^{3}H-Ins P_1 to free ^{3}H-inositol is inhibited, accumulation of the former can proceed linearly for as long as tissue remains viable provided the receptor does not desensitise. Thus, the signal generated by low doses of agonists or by less effective stimuli (i.e. 5-HT or HA in cerebral cortex) can be maximised. This can be particularly important for pharmacological characterisation of responses. Similarly, detection of rapidly metabolised inositol polyphosphates is also facilitated by the continuous production of labelled products (see below).

The major disadvantage of this protocol over that discussed in (ii) is that it is more susceptible to the problems of changing specific activity of both free and bound inositol pools. The aim of the initial short term prelabelling period is to allow a degree of equilibration of exogenous ^{3}H-inositol with intracellular pools. In brain, free inositol concentrations are estimated at between 5 and 10 mM (Spector, 1976), presumably with this existing as multiple compartments (Margolis and Heller, 1965). Similarly, inositol lipids are associated with most membrane structures to varying degrees (see Michell, 1975). This subcellular heterogeneity of lipid bound and potentially free inositol pools also, is further complicated in brain slices by the multiple cell types present. Over the 30-60 min. period of ^{3}H-inositol labelling prior to agonist addition, it is impossible to tell whether label equilibrates equally with all cellular pools. Both the size and metabolic flux through

lipid bound and free inositol compartments will affect the extent to which label is accumulated as will any variation in the access which exogenous ^{3}H-inositol has to different pools. Agonists are not noted to promote inositol uptake into brain but the variable extent to which different agonists stimulate phosphoinositide hydrolysis and the potentially variable pool sizes of free ^{3}H-inositol and/or lipid associated with separate receptor systems could clearly result in products of very different specific activity. Thus, equating responses mediated by distinct receptors or comparing responses to different doses of a particular agonist on the basis of accumulated radiolabel may be open to misinterpretation. Equally, the time course of a response to one concentration of a specific agonist may be affected by progressive changes in the extent of labelling of precursor pools. Clearly these problems of changing specific activity are most likely to arise if tissue labelling and stimulated lipid hydrolysis are allowed to procede simultaneously. Therefore, the advantages of a continuous labelling protocol may be outweighed by the susceptibility to labelling artifacts. Data presented in later sections suggests however, that the latter may be of limited significance but that further complications may be introduced by the inclusion of Li^{+} ions in assays.

ii) Pulse labelling protocol: This procedure has been employed for study of inositol phospholipid hydrolysis both in brain (Berridge et al. 1983) and other tissue slice preparations (Downes and Wusteman, 1983). The principle difference from the method described in (i) above is that lipid labelling and hydrolysis do not proceed simultaneously. Assays involve three distinct phases. Tissue is first allowed a suitable labelling period with ^{3}H-inositol, typically 1-2 hr. Exogenous, free label is then removed by repeated washing in an appropriate buffer prior to a secondary 1-2 hr. incubation in the presence of high concentrations (1-10 mM) of cold inositol, the purpose of which is to 'chase' unbound ^{3}H-inositol from the tissue. Finally, incubations with agonist are similarly performed in the presence of 1-10 mM cold inositol so that any free ^{3}H-inositol remaining within cells, either from the initial incubation or as a result of stimulated lipid hydrolysis, is massively diluted and secondary label incorporation thus reduced to negligible levels. Assuming these procedures to be effective, initial changes in phosphoinositide and inositol phosphate labelling should reflect comparable mass changes. However, this method also has its limiting features. Firstly, since phospholipid labelling is restricted to the initial stage, ^{3}H-lipid degraded in response to agonist will be replenished from cold precursor pools so that specific activity declines.

Consequently, stimulated accumulation of ^{3}H-inositol phosphates would be expected to procede linearly only for a period which is less than the time taken for the total, initially labelled lipid pool to undergo one complete cycle. In the presence of Li^{+} ions the linear phase of the time course will be limited by the size of the labelled pool associated with receptor and the rate of hydrolysis. The latter, in turn, will be a function of agonist concentration. Thus, in establishing dose-relationships it would be necessary to ensure that time courses were linear at each concentration over the appropriate range. In the absence of lithium ions, ^{3}H-Ins P_1 accumulation would be expected to peak at a level dependent on its net rate of synthesis and degradation and then decline as ^{3}H-inositol derived from its hydrolysis became diluted with cold inositol from the 'chase' protocol and resulted in a precursor pool for lipid re-synthesis of very much reduced specific activity. Similarly, inositol polyphosphates would achieve maximal concentrations at times consistent with their sequence of synthesis and comparative rates of hydrolysis, and subsequently decline as precursor specific activity fell.

The limited ability of this labelling approach to sustain continued inositol phosphate accumulations would be expected to result in reduced sensitivity compared with assays using an ongoing synthesis protocol, particularly in pharmacological studies where agonist responses may be small. Indeed, Fisher and Bartus (1985) have noted this point in recent studies comparing responses to partial and full agonists at muscarinic receptors in guinea-pig brain. However, Claro et al. (1986) have employed pulse labelling methods to achieve pharmacological characterisation of HA-stimulated inositol lipid hydrolysis in rat cerebral cortex, a response which, as shown above, is not notably large.

Despite these potential problems, this method does have the major advantage that initial changes in lipid (and hence inositol phosphate) labelling should reflect equivalent mass changes. This is particularly important in mechanistic studies of inositol lipid hydrolysis where the point of key interest is the sequence of receptor-mediated steps. The usefulness of a pulse labelling protocol in this application is clearly exemplified by the experiments of Downes and Wusteman (1983).

As one of the principal aims of the current study was to establish that receptor-mediated inositol lipid hydrolysis in brain follows a similar reaction sequence to that proposed for other tissues, a number of experiments were performed to determine whether pulse labelling of cortical slices provides a practical approach to this problem. The methods used and the results obtained are described in the following section.

iii) Pulse labelling of cerebral cortical slices with ^{3}H-inositol: Tissue was prepared and preincubated as in 2.2. Subsequent steps were based on the methods of Berridge et al. (1983), modified to achieve, as closely as possible, equivalent incubation conditions to those described in 2.2. Tissue was incubated in bulk in Krebs buffer to give a tissue:medium ratio of 1:5. ^{3}H-Inositol was added to give a concentration of 1 µCi/300 µl of the final incubation volume. Cerebral cortical slices were incubated under these conditions at 37°C with gentle shaking for 90 min. in a sealed bottle. Medium was freshly oxygenated at 15 min. intervals. After this prelabelling period, tissue was allowed to settle and ^{3}H-medium aspirated. Slices were washed four times with 15-20 ml of Krebs buffer containing unlabelled inositol at either 1 or 10 mM and re-suspended at the original concentration and incubated as before for a further 30 or 90 min. in this medium. Washing steps were repeated and buffer replaced at 15 min. intervals. Finally, tissue was washed twice more in 15-20 ml Krebs buffer supplemented with both inositol and 5 mM LiCl, allowed to settle under gravity and 50 µl aliquots transferred to 6 ml capacity vials containing sufficient supplemented Krebs medium such that, after drug additions, a final volume of 300 µl was achieved. Vials were gassed with O_2/CO_2 (95%/5%), sealed and incubated for 10 min. at 37°C in a metabolic shaker before addition of drugs. Incubations were stopped after times illustrated in figures by addition of 0.94 ml 2:1 (v/v) methanol:chloroform. Subsequent steps were identical with previously described methods.

Initial experiments were designed simply to show agonist responses using this procedure. Figure 2.3.6 illustrates the effects of carbachol, NA and HA on both inositol phosphate and corresponding inositol lipid (essentially ^{3}H-PtdIns) labelling after a 45 min. incubation with these agonists. The relative order and magnitude of responses corresponds closely to that seen with an ongoing synthesis protocol. Absolute values (in terms of cpm accumulated) are larger than shown in Figure 2.3.2 but see 2.2(d)ii and later data. It is apparent from Figure 2.3.6 that despite the substantial accumulation of inositol phosphate, particularly in response to carbachol, there is no significant decrease in inositol lipid labelling. Over the course of several experiments this latter feature was somewhat variable but in no case did decreases in lipid radioactivity approach corresponding increases in ^{3}H-inositol phosphates stimulated by carbachol. This observation implies that of the originally labelled lipid (PtdIns using this extraction), only a small proportion may be associated with the muscarinic receptor mechanism. As responses to other agonists are substantially less marked and ^{3}H-lipid values subject to some error, the situation for other receptor systems is less clear.

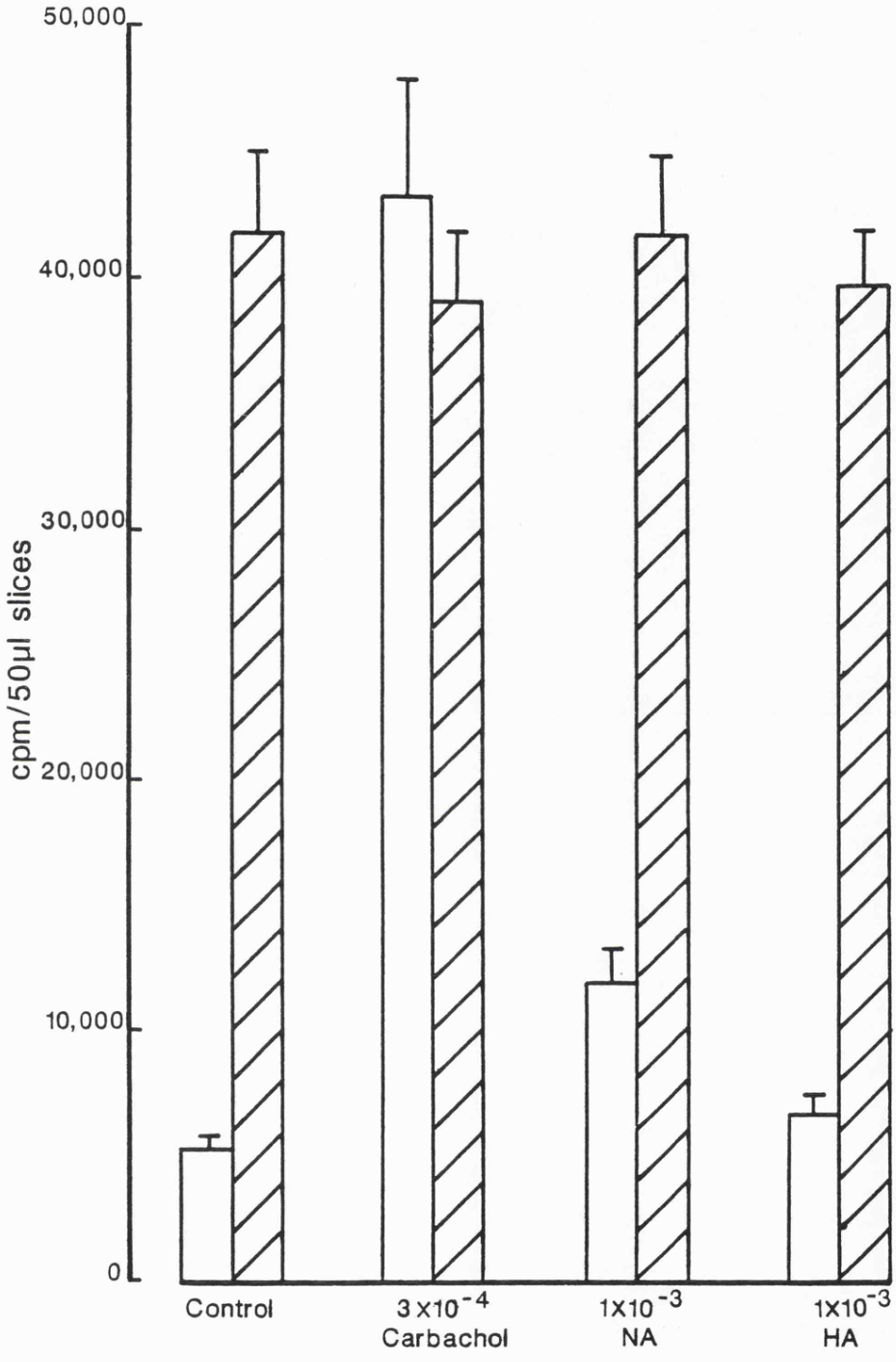


FIGURE 2.3.6 Agonist-stimulated changes in ^{3}H-Ins P(s) and ^{3}H-phospholipid labelling in cerebral cortical slices 'pulse labelled' with ^{3}H-inositol.

Brain slices were pulse labelled with ^{3}H-inositol as described in the text. 50 µl aliquots of pre-labelled tissue were then transferred to assay tubes containing medium (final vol. 300 µl) supplemented with 5 mM LiCl and 1 mM unlabelled inositol and incubated for a further 45 min. in the presence and absence of the drugs indicated. ^{3}H-Ins P(s) (open bars) and ^{3}H-inositol phospholipid (hatched bars) were then determined as in 2.2. Results represent the mean ± SEM of 6 measurements made in 2 experiments.

However, if only a limited amount of labelled phosphoinositide is available to the receptor mechanism, this cannot account for the response observed to carbachol unless this pool is replenished from ^{3}H-inositol precursor. Therefore, under the conditions described, a pool of labelled inositol may still exist despite the chase protocol with cold inositol and is thus able to maintain the agonist response for considerably longer than would be possible from the original ^{3}H-lipid precursor alone.

During the 'chase' phase with cold inositol and multiple wash steps, aliquots of medium were routinely counted for tritium in order to establish potential efflux of ^{3}H-inositol from brain slices. This indicated that for each 15 min. incubation with cold inositol (either 1 or 10 mM) approximately 0.1-0.2 μCi ^{3}H-inositol diffused out from tissue (i.e. from a total of 1.5 ml slices originally incubated with 25 μCi inositol) and that this rate could be maintained for up to at least 90 min. Such leakage of ^{3}H-material is not surprising given the likelihood of damaged cells at the cut surfaces of the slices and the 'pump leak' hypothesis for inositol uptake into brain (Spector, 1976). However, this observation does imply that despite extensive secondary incubation with cold inositol, substantial quantities of free label remain associated with cerebral slices. Since an essential feature of the pulse chase protocol is the prevention of secondary and potentially agonist-effected incorporation of label into inositol lipids, it is questionable whether this approach is of practical advantage in the current circumstances. As pointed out in section 2.3b, the incorporation of inositol into PtdIns by headgroup exchange is stimulated by Mn^{2+} ions. It therefore seemed possible that concentrations of free ^{3}H-inositol remaining within cells might be considerably reduced by including $MnCl_2$ in the incubation medium during the chase phase. The addition of Mn^{2+} ions to Krebs medium results in precipitation of insoluble salts (presumably phosphates and/or carbonates), necessitating the use of an alternative buffering system. However, as a single experiment employing $MnCl_2$ and using a Tris buffer resulted in much reduced agonist responses. This approach was not investigated further though with appropriate modification could prove useful.

As discussed above, agonist stimulation of pulse labelled tissue in the presence of Li^{+} ions would be expected to result in a linear accumulation of Ins P_1 over a period limited by the pool size of prelabelled precursor. Thus, the level at which responses plateau should reflect the relative total inositol (free and lipid bound) pools associated with distinct receptors. This assumes that the breakdown of Ins P_1 generated in response to different agonists is equally sensitive to Li^{+} inhibition, which more recent data suggests may not be true

(see Michell, 1986). However, based on this assumption several experiments were run in an attempt to obtain a relative estimate of the respective pools associated with muscarinic and alpha-1 receptors in cerebral cortex. Methods were as above using 10 mM cold inositol to chase ^{3}H-label from the tissue. Incubations with agonists were stopped after varying times up to 90 min. and samples analysed for inositol phosphate and phospholipid labelling as previously.

Figure 2.3.7 shows the results of a single experiment performed in triplicate. A second time course to carbachol showed essentially identical results although the linear phase of the response varied approximately between 20-40 and 30-50 min. over the two experiments. Corresponding lipid labelling data for the results in Figure 2.3.7 is given in Table 2.3.

Table 2.3.1 Effects of carbachol and noradrenaline on the labelling of inositol phospholipid in cerebral cortical slices pulse labelled with ^{3}H-inositol.

Time (min.)	Lipid labelling (cpm/50 µl tissue)		
	Control	Carbachol	NA
10	38,388 ± 2,493	32,610 ± 2,526	36,477 ± 1,203
30	39,423 ± 1,152	32,715 ± 2,529	34,230 ± 2,778
60	32,955 ± 1,839	25,608 ± 240	32,208 ± 1,851
90	31,470 ± 774	23,673 ± 807	30,837 ± 369

Cerebral cortical slices were pulse labelled with ^{3}H-inositol as described in the text then incubated for the times indicated with or without 3×10^{-4} M carbachol or 1×10^{-3} M NA in the presence of 5 mM LiCl. Accumulated ^{3}H-inositol phosphates (see Figure 2.3.7) and labelling of phospholipid were then determined as previously described. The results represent the mean ± SEM of three measurements made within a single experiment.

Comparison of the lipid and inositol phosphate labelling data shows no clear correlation either in absolute values or in the pattern of change over the time course. This may indicate that initial loss of ^{3}H-lipid is compensated for by resynthesis from a pool of ^{3}H-inositol such that only at later time points, when this becomes depleted, does a decrease in ^{3}H-PtdIns become apparent. However, as control values for lipid labelling show some variation across the time course without any apparent change in inositol phosphate, this interpretation is questionable. It is

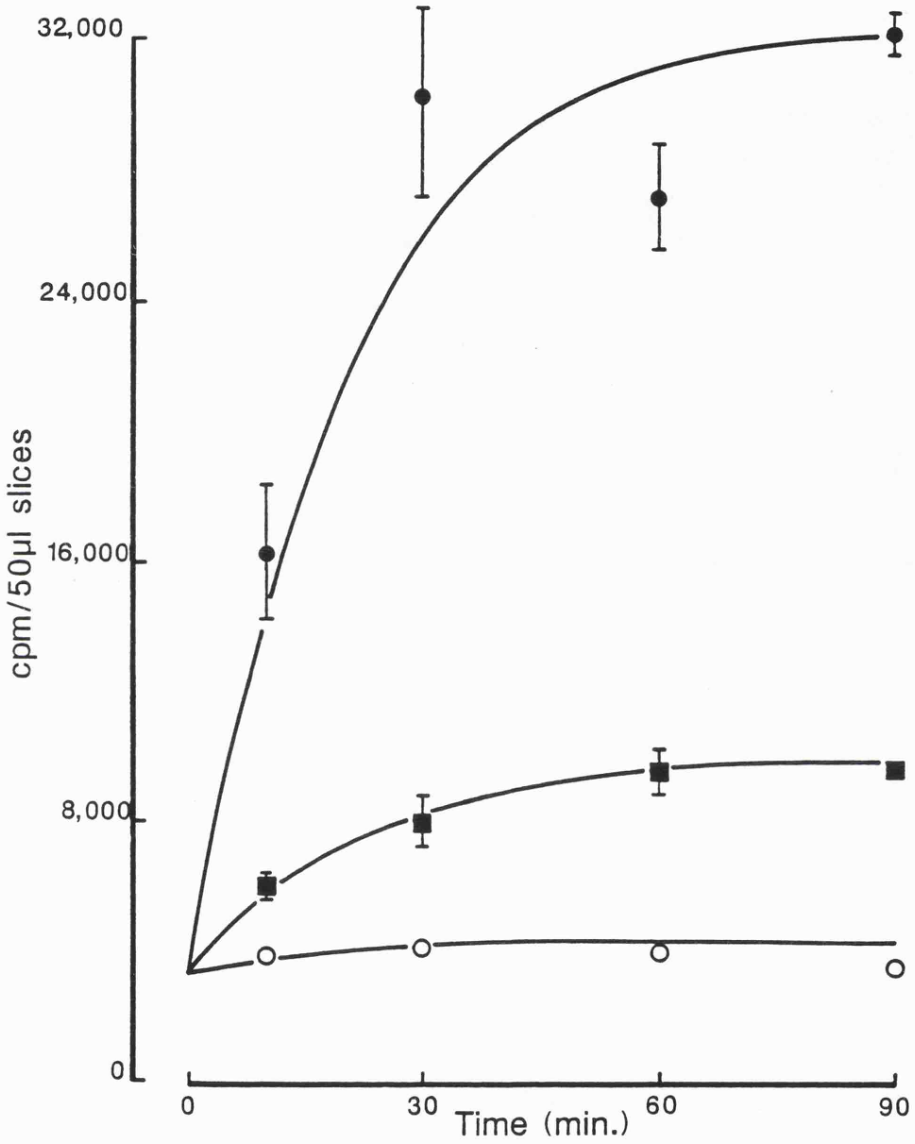


FIGURE 2.3.7 Time courses of agonist-stimulated ^{3}H-Ins P(s) accumulations in cerebral cortical slices 'pulse labelled' with ^{3}H-inositol.

Pulse labelled brain slices were incubated for the times indicated in the absence (○) or presence of 0.3 mM carbachol (●) or 1.0 mM NA (■) in medium supplemented with 5 mM LiCl and 10 mM unlabelled inositol before extraction and assay of both ^{3}H-Ins P(s) and ^{3}H-phospholipid (see also Table 2.3.1) as described in 2.2. Results represent the mean ± SEM of 3 measurements made in a single experiment.

nevertheless clear, as indicated by previous data, that a very much larger accumulation of inositol phosphate results in response to carbachol than to NA but that stimulation with either agonist produces a similar time course. From the discussion above, if the original prelabelling period allows equal equilibration of ^{3}H-inositol with the precursor pools associated with both receptor mechanisms, then this result must indicate that the total free + bound inositol available to the muscarinic receptor is several fold larger than that to the alpha-1 receptor. If the two systems shared a common pool of labelled precursor then the apparently slower rate of phosphoinositide hydrolysis induced by NA would be expected to be maintained over a longer time course. However, it must be emphasised that these deductions may be invalid if the two receptors are present on distinct cell types whose inositol uptake and intracellular distribution systems are not identical.

Comparison of these time course data with those observed with an ongoing synthesis protocol (cf Figures 2.3.5 and 2.3.7) indicates that, as predicted, the latter procedure is able to sustain a linear ^{3}H-inositol phosphate accumulation over a longer period than is a pulse-labelling protocol. From this, similar longevity of inositol polyphosphate accumulations would be anticipated using continuous labelling. A major objective of this study was to characterise the reaction sequence of inositol lipid hydrolysis in brain. Since this essentially necessitates measurement of individual inositol phosphate accumulations, an ongoing synthesis protocol appeared distinctly advantageous. Alternatively, the benefits of pulse labelling (see above) appeared questionable as the current data implied that considerable, free ^{3}H-inositol remains associated with brain slices even after prolonged 'chase' incubations. Further, even in the absence of such problems, the advantage of the pulse-chase method in allowing initial mass changes in the phosphoinositides to be inferred from labelling changes may be complicated in brain slices by the accuracy with which such changes could be assayed, given the probably small proportion of total inositol lipid label which is associated with receptor mechanisms in this tissue. For these reasons a continuous labelling approach was used in all subsequent experiments and evidence is presented (see 6.2 and 3.2) that this method is not likely to be complicated by large changes in product specific activity.

B) Basic characteristics of muscarinic receptor-mediated inositol lipid hydrolysis in rat cerebral cortex

The data discussed in previous sections essentially serve to validate the methods used to investigate phosphoinositide hydrolysis in

cerebral cortex and to highlight some of the difficulties associated with interpretation of results derived from so complex a tissue. Since it is clear from these early studies that responses to carbachol are several fold larger than to other agonists shown, attention in later chapters, where the mechanism of inositol lipid hydrolysis is considered in more detail, has focussed on that mediated via muscarinic receptors. For this reason several basic features of this response were first investigated to establish the pharmacological and physiological relevance of these later observations. These preliminary studies were brief and essentially confirm the results of more detailed reports.

a. Dose-relationship of response to muscarinic stimuli:

A requisite feature of any receptor-mediated response is that it exhibits concentration dependence over a limited range. The concentration of a particular agonist required to produce a given biochemical or physiological response is dependent not only on the drug/receptor interaction but also on the nature of steps coupling receptor activation to the response. Thus, a functional response such as phosphoinositide hydrolysis may exhibit receptor reserve such that maximal effects are apparent at corresponding low receptor occupancy if an amplification mechanism operates at an intermediary stage. The pharmacological characterisation of receptors from functional data cannot be satisfactorily achieved therefore on the basis of apparent agonist affinities alone and definition depends on the use of specific antagonists.

Previous studies of receptor-mediated phosphoinositide hydrolysis have indicated a close relationship of this response to receptor occupancy both in peripheral tissues (Jafferji and Michell, 1976) and in brain (Kendall *et al*. 1985) for certain receptor systems. Indeed, this was one line of evidence suggesting that inositol lipid breakdown is an early event subsequent to receptor activation. However, numerous reports indicate variable coupling of this response to muscarinic receptors. In rat parotid gland agonist occupation curves lie to the right of corresponding dose-response curves for phosphoinositide hydrolysis (Weiss and Putney, 1981). Comparison of data for muscarinic receptor-mediated inositol lipid hydrolysis in rat parotid gland and cerebral cortex shows that dose-response curves to full agonists in the former tissue lie to the left of those in the latter while partial agonists also give a greater percentage of the response to full agonists in parotid gland (Jacobson *et al*. 1985; Ek and Nahorski, 1986). Similarly, Fisher and Bartus (1985) have demonstrated apparently differential coupling of muscarinic receptors to phosphoinositide hydrolysis in various regions of guinea-pig brain.

Figure 2.3.8 illustrates a typical dose-response curve for carbachol-stimulated ^{3}H-inositol phosphate accumulation in the presence of 5 mM LiCl in rat cerebral cortex. It is assumed for the present purpose that measurement of Ins P_1 under these conditions is an accurate reflection of inositol lipid hydrolysis although this may not necessarily be so (see Michell, 1986; 6.3). The data presented clearly indicate a dose-related pattern characteristic of a receptor-mediated response. Maximal and half-maximal responses to carbachol were obtained at 10^{-3} M and 6×10^{-5} M respectively, representing 4.8 and 8.3 fold stimulations over basal values. The EC_{50} of 60 μM for this agonist correlates well with that observed in previous studies in rat cerebral cortex (Brown et al., 1984; Jacobson et al. 1985; Rooney and Nahorski, 1986) but is three-fold lower than that reported for guinea-pig cerebral cortex (Fisher and Bartus, 1985).

Figure 2.3.8 also demonstrates the effect of atropine, a specific muscarinic cholinergic receptor antagonist, on these responses. Consistent with earlier work (Brown et al. 1984), 10 nM atropine did not attenuate basal ^{3}H-inositol phosphate accumulation, suggesting that, under the specified conditions, little of this is accounted for by the action of endogenously released acetylcholine (ACh). In contrast, atropine produced a parallel, right-hand shift of the dose-response curve to carbachol, indicative of competitive agonist/antagonist binding at muscarinic receptors. From the shift of the parallel section of the respective curves an apparent inhibition constant (K_i) for atropine was calculated. (Calculation from the relationship: $[K_i = (A)/(K_{A2}/K_{A1}) + 1]$, where A = concentration of antagonist, and K_{A1} and K_{A2} represent the EC_{50} for agonist in the absence and presence of antagonist). This suggested a K_i of 5.5 nM which is close to the range (0.9-4.5 nM) reported by Brown et al. (1984) but suggests a slightly lower affinity of atropine than demonstrated by two more detailed studies (Jacobson et al. 1985; Rooney and Nahorski, 1986) of muscarinic receptor-mediated inositol lipid hydrolysis. However, as the current data show some variability at higher agonist concentrations, this latter difference is unlikely to be significant.

Together the results illustrated in Figure 2.3.8 are characteristic of a response mediated via muscarinic receptors. The concentration dependency of the ^{3}H-inositol phosphate accumulation by carbachol is very similar to that reported by Jacobson et al. (1985) and supports their conclusion that little receptor reserve is likely to be associated with this response in rat cerebral cortex, in contrast to rat parotid gland (see also Ek and Nahorski, 1986). The requirement for high concentrations of carbachol (1 mM, presumably reflecting high receptor occupancy) to

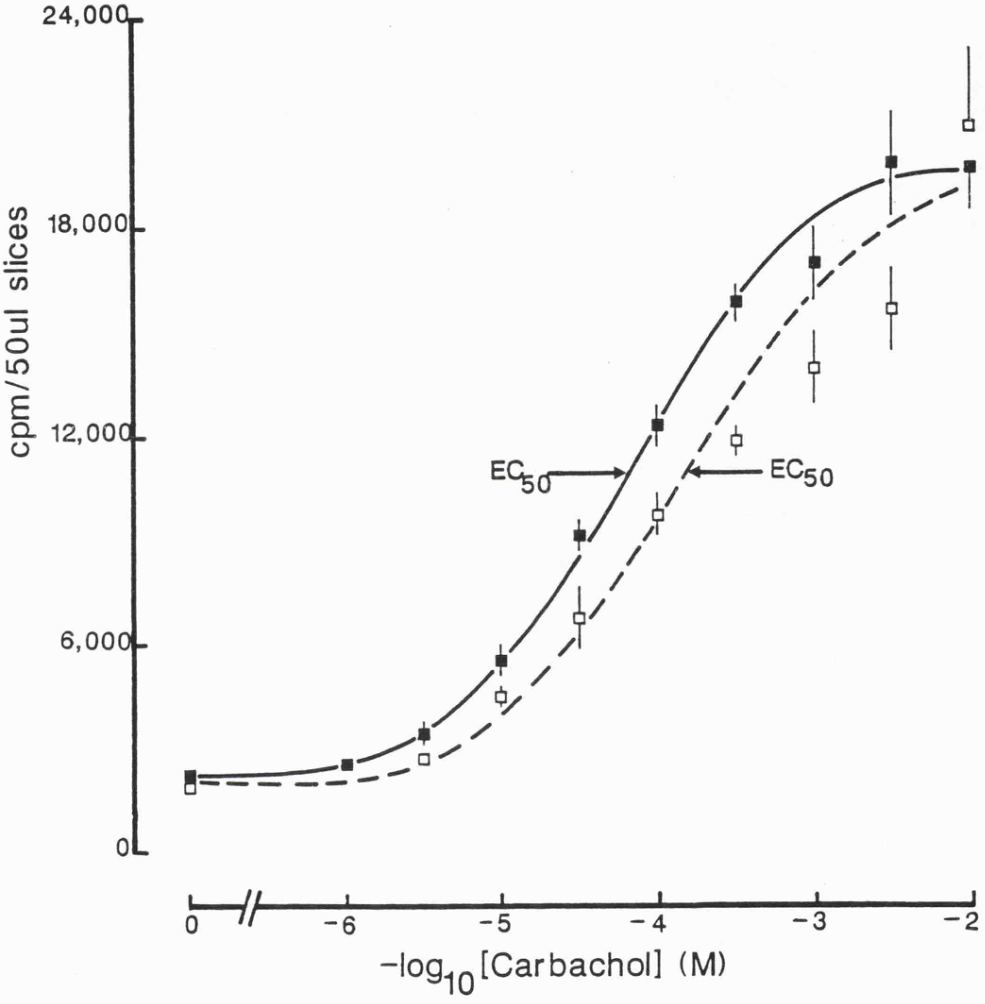


FIGURE 2.3.8 Dose-relationship for carbachol-stimulated ^{3}H-Ins P(s) accumulation in ^{3}H-inositol labelled brain slices in the absence/presence of atropine.

Cerebral cortical slices were prepared as in 2.2. After 60 min. pre-incubation tissue (50 µl) was allowed to accumulate label (1 µCi) for 30 min. then incubated for a further 45 min. with the concentrations of carbachol indicated either in the absence (■) or presence (□) of 10 nM atropine before extraction and assay of ^{3}H-Ins P(s) as in 2.2. Atropine was added 15 min. prior to agonist. 5 mM LiCl was added together with ^{3}H-inositol. Results represent the mean ± SEM of 2 experiments run in triplicate.

promote maximal accumulation of inositol polyphosphates (see 6.3), together with the time course with which these are generated (see CH.4), is also consistent with a close coupling of muscarinic receptors to phospholipase C-mediated $PtdInsP_2$ hydrolysis in brain. However, both the current and later data could be complicated via the ability of high carbachol concentrations to activate nicotinic receptors (see Bowman and Rand, 1980), stimulation of which with ACh enhances inositol lipid turnover in cultured myotubes (Adamo et al. 1985). It is therefore important to point out that later results indicate very close to 100% blockade of responses to 1 mM carbachol by 10^{-5} M atropine while Brown et al. (1984) have shown that mecamylamine, a nicotinic receptor antagonist, is ineffective against identical responses even at 10^{-4} M. It is of interest however, that Habermann and Laux (1986) have implicated a role for Na^+ channels in carbachol-stimulated inositol phosphate accumulation in vesicle preparations derived from ^{3}H-inositol pre-labelled rat cortex. In contrast, tetrodotoxin does not attenuate responses to carbachol or NA in slice preparations identical to those employed here (Rooney and Nahorski, 1986). Thus, the significance of the former observation is unclear.

A second factor potentially complicating responses to muscarinic agonists is the putative sub-classification of receptors into M_1 and M_2 based on their selective affinities for pirenzepine (Hammer and Giachetti, 1982). No attempt has been made in the current study to distinguish between these subtypes. Since both stimulation of inositol lipid hydrolysis and inhibition of adenylate cyclase are responses to muscarinic receptor activation, previous studies have suggested that these may be mediated through distinct receptor subtypes. Gil and Wolfe (1985) have proposed that in brain the former is M_1-mediated and the latter M_2 while Brown et al. (1985) infer the reverse in chick heart. In contrast, Lazareno et al. (1985) have reported that both subtypes may mediate phosphoinositide hydrolysis in rat cortex and a recent study of various cerebral regions confirms this view but suggests that the relative proportions of the response mediated via M_1 and M_2 receptors may vary according to brain area (Rooney and Nahorski, 1986).

As atropine does not distinguish between M_1 and M_2 subtypes the current data cannot be interpreted in these terms, thus at subsequent stages it must be recalled that responses could be complex. Similarly, it should be pointed out that responses to carbachol may also occur in more than one cell type. Pearce et al. (1985) have recently shown that carbachol stimulates ^{3}H-Ins P_1 accumulation in primary astrocytes labelled with ^{3}H-inositol, with an EC_{50} for this agonist of 20 μM. This response is much less marked than observed in cerebral cortical slices but

indicates a potential contribution of glial cells to the muscarinic responses reported in brain slices in later chapters. However, Gonzales et al. (1985) have recently studied muscarinic receptor-mediated inositol lipid hydrolysis in both neuronal and glial cells cultured from rat brain and, by comparison of the responses in these cells with those obtained in brain slice preparations similar to those currently used, have concluded that responses in tissue slices are primarily due to neuronal and not glial cells.

b. Responses to exogenous agonists reflect those to endogenously released transmitters:

Clearly from the data presented above and from previous studies, inositol lipid hydrolysis in brain is stimulated in a dose-dependent manner by a variety of agonists whose sites of action correspond to those of several endogenous, putative neurotransmitters (e.g. ACh, NA, HA, 5-HT). The physiological significance of these observations however, would be much enhanced if endogenously released transmitters could be shown to evoke similar responses. Several earlier studies have demonstrated increased inositol lipid turnover in brain slices and synaptosomes in response to electrical and depolarising stimuli (see Hawthorne and Pickard, 1979) although in most cases changes in lipid labelling rather than a direct measure of hydrolysis (see 2.1) have been assayed.

In many of the experiments described in subsequent chapters where the mechanism of muscarinic receptor-mediated inositol lipid hydrolysis is examined in more detail, responses have been characterised almost exclusively with carbachol as stimulus. Both ACh and carbachol are full agonists in assays of Ins P_1 accumulation in rat cortex but, although the former is marginally more potent (Jacobson et al. 1985), demonstration of maximal responses requires the presence of an anticholinesterase (Brown et al. 1984) while the latter agonist has the advantage of insensitivity to esteratic hydrolysis. Figure 2.3.9 compares the time courses for Ins P_1 accumulation stimulated by ACh in the presence of an anticholinesterase (eserine) with that resulting from the presence of physostigmine alone. The corresponding data for the response to carbachol has been superimposed from Figure 2.3.5. It is clear that both the magnitude and pattern of response to ACh and carbachol are closely similar. However, more interestingly Figure 2.3.9 shows that there is a small response in the presence of eserine alone, presumably resulting from the action of endogenous ACh leaking from tissue slices during the incubation. This response does not appear to plateau, unlike those to exogenously added agonists but this may be a consequence of the continuously increasing

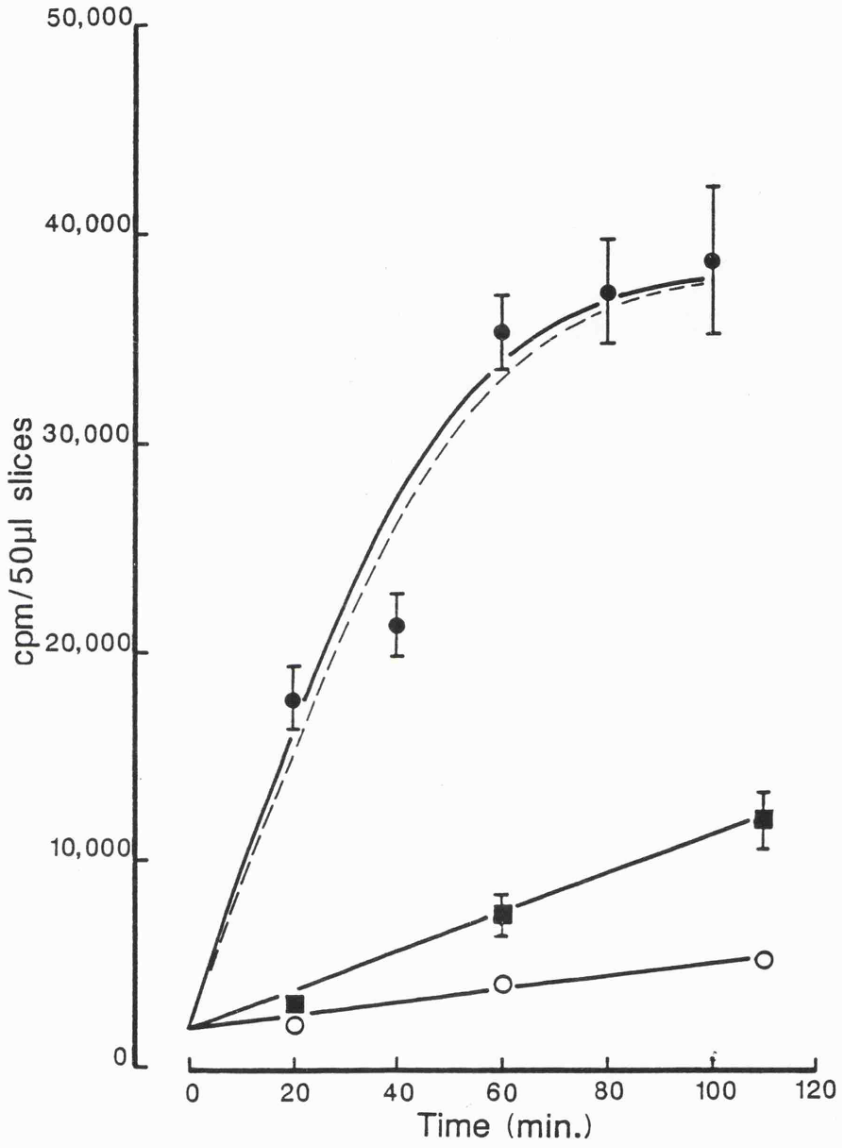


FIGURE 2.3.9 Time courses of ^{3}H-Ins P(s) accumulations stimulated by acetylcholine and physostigmine in ^{3}H-inositol labelled brain slices.

Tissue incubations were as described in 2.2. Brain slices were allowed to accumulate ^{3}H-inositol (1 µCi) for 30 min. then incubated for the times indicated in the absence (○) or presence of 50 µM physostigmine (□) or physostigmine + 0.3 mM ACh (●) prior to extraction and assay of accumulated ^{3}H-Ins P(s). 5 mM LiCl was present throughout incubations with label. Results represent the mean ± SEM of 1 experiment run in triplicate. The broken line indicates the response to 0.3 mM carbachol under identical conditions (from data in Fig. 2.3.4).

concentration of released transmitter accumulating in the incubation medium throughout the assay. A recent study from this laboratory has investigated similar responses to endogenously released ACh in more detail (Kendall and Nahorski, 1986). Depolarisation of cerebral cortical slices with either elevated potassium ion concentration (K^+) or with veratrine dose-dependently stimulated ACh release, accumulation of which was linear for up to at least 45 min. in the presence of eserine. Similar depolarisation also promoted inositol phosphate accumulation and this response was markedly potentiated in the presence of physostigmine. The enhanced response in the presence of anticholinesterase was dose-dependently blocked by atropine and suppressed by incubation in the absence of extracellular Ca^{2+}. These results clearly indicate that endogenously released ACh stimulates inositol lipid hydrolysis in a manner analogous to exogenously applied carbachol and implies that responses reported with the latter agonist in later chapters reflect those which may occur under physiological conditions. Further, using similar methods to those above, Rooney and Nahorski (1986) have been able to infer a close correlation between the regional density of cholinergic innervation in rat brain and muscarinic receptor-mediated inositol lipid hydrolysis in response to exogenous agonists.

Finally, an interesting feature of responses to depolarising concentrations of K^+ in the absence of eserine is that these are not sensitive to atropine nor to a variety of other receptor antagonists. Unlike a similar effect reported in superior cervical ganglion (Bone and Michell, 1985) this response is not potentiated by peptidase inhibitors, suggesting it cannot be attributed to an endogenously released peptide. However, this response is reduced when the $[Ca^{2+}]_e$ is reduced. Further, responses to K^+ depolarisation in cerebral cortical slices can be modulated by dihydropyridine Ca^{2+} channel agonists and antagonists (Kendall and Nahorski, 1985b), an observation recently extended to several rat brain regions for depolarisation but not agonist-induced responses (Rooney and Nahorski, 1986). Responses to K^+ are further considered in CH.3.

c. Ca^{2+} dependency of muscarinic receptor-mediated inositol lipid hydrolysis in rat cerebral cortex:

Since first demonstrated by Streb et al. (1983), Ins-1,4,5-P_3 stimulated release of Ca^{2+} ions from intracellular stores has been shown in many cell types (see Berridge and Irvine, 1984). An Ins-1,4,5-P_3 mediated release of Ca^{2+} ions has been demonstrated in cultured neuroblastoma cells (Gill et al. 1986) and it is of interest that Vicentini et al. (1985) have also been able to show a muscarinic

receptor-mediated increase in Ins-1,4,5-P_3 concentration which is unlikely to be preceded by an increased intracellular calcium ion concentration ($[Ca^{2+}]_i$) in a neurosecretory cell line. It would be anticipated, given the complexity of brain slice preparations, that similar evidence for the cause/effect relationship between inositol lipid hydrolysis and Ca^{2+} mobilisation would be difficult to obtain. This situation was further compounded in previous studies of agonist enhanced phosphoinositide turnover where agonist-stimulated incorporation of radiolabel was assayed, as these methods estimate lipid re-synthesis secondary to an initial hydrolysis and hence any associated Ca^{2+} dependency observed may simply reflect that of these secondary reactions (see 2.1). More recently, Kendall and Nahorski (1984) attempted to circumvent these latter problems using the current assay procedure as a direct assay of phosphoinositide hydrolysis to assess the calcium dependency of responses stimulated by various agonists in rat cerebral cortex. This study indicated that while responses to all agonists were abolished in the presence of EGTA, omission of Ca^{2+} from the incubation medium variably attenuated responses to NA and 5-HT and abolished that to HA but did not affect carbachol-induced Ins P_1 accumulation. The Ca^{2+} ionophore, A23187, stimulated phosphoinositide hydrolysis in a manner at least additive to NA responses and dihydropyridine Ca^{2+} channel antagonists did not inhibit receptor-mediated effects.

In the current study the Ca^{2+} dependency of muscarinic receptor-mediated responses were briefly re-evaluated. In experiments where Ca^{2+} was omitted from Krebs medium and tissue incubated in the presence of 0.5 mM EGTA, responses to carbachol were completely abolished in agreement with Kendall and Nahorski (1984) (data not shown). Figure 2.3.10 illustrates the results of further studies in which tissue was incubated throughout experiments in either normal Krebs buffer (see Appendix 1, $CaCl_2$ at 1.3 mM) or identical medium lacking added $CaCl_2$. Under the latter conditions the medium calcium ion concentration has been estimated at 10-30 μM (Kendall and Nahorski, 1984). From Figure 2.3.10 it is clear that omission of Ca^{2+} markedly attenuated responses to both ACh and carbachol. Control and physostigmine-induced inositol phosphate accumulations were also reduced in the absence of Ca^{2+} but to a lesser extent. Since control values are lower in the absence of Ca^{2+}, if the results for stimulated inositol phosphate accumulations are calculated relative to respective controls in the presence and absence of Ca^{2+} (see Figure 2.3.10B), then the attenuation of responses owing to Ca^{2+} removal, although still evident, become less marked. As the results of Kendall and Nahorski (1984) were expressed in this way, their data may not reveal effects of Ca^{2+} depletion which would be apparent in absolute terms. More

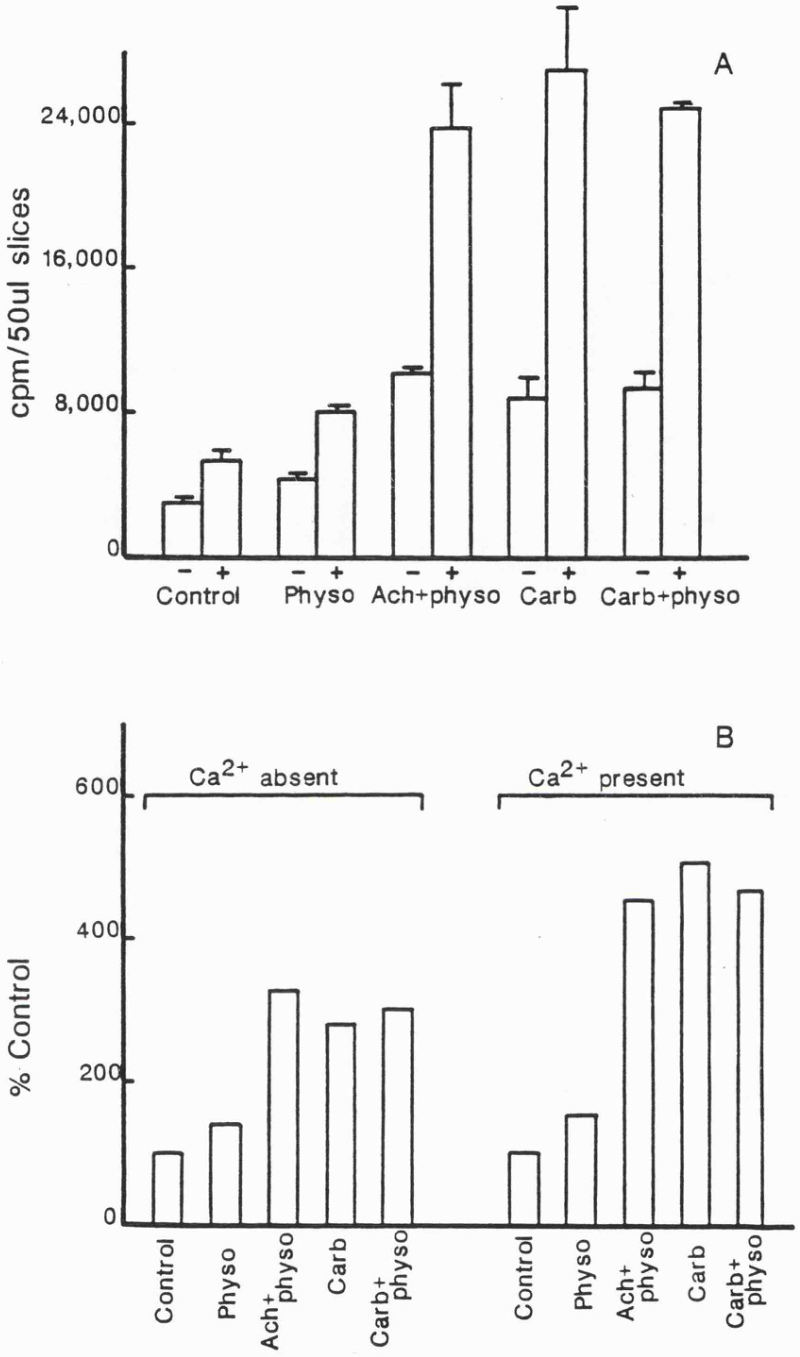


FIGURE 2.3.10 ^{3}H-Ins P(s) accumulations promoted by muscarinic receptor stimulation in the 'absence/presence' of extracellular Ca^{2+} in ^{3}H-inositol labelled brain slices.

Incubations were run as in 2.2 except that brain slices were either incubated in medium with or without the normal (1.3 mM) $CaCl_2$ content. Each set of tissue was allowed to incorporate ^{3}H-inositol for 30 min. prior to exposure to the indicated stimuli for a further 45 min. Accumulated ^{3}H-Ins P(s) was then extracted and assayed as in 2.2. 5 mM LiCl was present throughout incubations with label. Drug concentrations were: physostigmine (50 μM); ACh (0.3 mM); carbachol (0.3 mM). Results represent the means ± SEM of 1-2 experiments performed in triplicate. Fig. A shows data expressed as cpm of Ins P(s) accumulated under each condition (Ca^{2+} absent, left bar: Ca^{2+} present, right bar). Fig. B shows the same data expressed as a relative percentage of the appropriate controls.

recent results comparing the inositol lipid hydrolysis stimulated by carbachol or endogenously released ACh (Kendall and Nahorski, 1986) show that responses to both stimuli are reduced in the absence of extracellular (medium) Ca^{2+}, although the attenuation shown is not as marked as that illustrated in Figure 2.3.10. While the reduced response to endogenously released ACh in the absence of Ca^{2+} must, in part, reflect a Ca^{2+} requirement for transmitter release, the data for carbachol confirm the present results. The variable extent to which reduction in extracellular $[Ca^{2+}]$ attenuates the response to carbachol (cf Figure 2.3.10A and Kendall and Nahorski, 1986) may reflect the efficiency with which medium Ca^{2+} is depleted by simple omission of $CaCl_2$ from the Krebs buffer. Nevertheless, the reduction in muscarinic receptor-mediated responses in the 'absence' of medium Ca^{2+} (i.e. when $[Ca^{2+}]_e \sim$ 10-30 μM, see above) does imply that maintenance of maximal accumulation of inositol phosphates is dependent to an extent on $[Ca^{2+}]_e$. If this is so, then a threshold concentration in the region of 10-30 μM, below which $[Ca^{2+}]$ is variably reduced by the incubation conditions described, could account for the differences between the current and other studies. As responses are completely abolished in the presence of EGTA this may indicate a requirement of muscarinic receptor coupled phospholipase C for Ca^{2+}, although this does not necessarily imply that activity of this enzyme is dependent on an initial rise in $[Ca^{2+}]_i$ (see CH.1) since prolonged incubation in the presence of EGTA may reduce $[Ca^{2+}]_i$ below normal resting levels.

It should be noted that in the current experiments either omission of Krebs $CaCl_2$ or addition of 0.5 mM EGTA resulted in increased phosphoinositide labelling by approximately 50-75%. Kendall and Nahorski (1984) have reported similar results and suggest that as EGTA stimulates incorporation of ^{3}H-inositol but inhibits that of ^{32}P into PtdIns (Tolbert et al. 1980), Ca^{2+} depletion may inhibit the CDP-diacylglycerol: myo-inositol phosphatidate transferase reaction but stimulate inositol incorporation through headgroup exchange. If this were so, and if agonist-sensitive lipid pools are labelled primarily by the former route (as is indicated by the stimulated incorporation of ^{32}P into PtdIns in many tissues under conditions of normal $[Ca^{2+}]$, see Berridge, 1981), then the effects of Ca^{2+} removal on inositol phosphate accumulation under the current conditions, could arise from secondary changes in lipid synthesis (labelling) rather than from Ca^{2+} requirement for lipid hydrolysis. However, both routes of inositol incorporation are inhibited by Ca^{2+}, with that via CDP-diacylglycerol:myo-inositol phosphatidate transferase showing particular sensitivity (see Egawa et al. 1981) and Prpic et al. (1982) have suggested that the results of Tolbert et al. (1980) may be explained by predominant operation of the headgroup exchange enzyme in their

experiments. Thus, under conditions of low extracellular Ca^{2+}, both routes of lipid labelling would be expected to be more active than in the presence of normal Krebs calcium (i.e. 1.3 mM). If the agonist labile phosphoinositide pools are labelled by either route therefore, higher specific activity of these would be anticipated in the absence of added Ca^{2+}. Thus, as reduced ^{3}H-inositol phosphate accumulation is observed under these conditions, it seems reasonable to infer that this effect arises from a partial Ca^{2+} requirement for maximal, agonist-stimulated phosphoinositide breakdown rather than from a secondary effect whereby the reduced Ca^{2+} limits lipid re-synthesis.

2.4 Methods used for the assay of individual inositol phosphates

a. Techniques available for the resolution of inositol phosphates and the advantages of ion exchange chromatography:

i) Chromatographic methods available for the separation of inositol phosphates: Over the last 20-30 years a number of methods have been developed for the separation of inositol phosphates. Paper ionophoretic separation of inositol polyphosphates in sodium oxalate buffers has been described by Seiffert and Agranoff (1965) and Van Rooijen et al. (1983) (see CH.4), while Dawson and Clarke (1972) have used a combination of paper chromatography and ionophoresis to achieve resolution of various monophosphorylated derivatives of myo-inositol. Descending paper chromatography, principally in solvent systems described by Desjobert and Petek (1956) or Markam and Smith (1952), has been extensively used to separate inositol phosphates (see CH.4; Grado and Ballou, 1961; Tomlinson and Ballou, 1961; Brockerhoff and Ballou, 1961; Prottey et al. 1968; Irvine et al. 1984). These systems are capable of resolving a variety of isomeric Ins P_1(s), Ins P_2(s) and Ins P_3(s), although clear separation of trisphosphate isomers requires run times of 1-2 weeks (Brown and Stewart, 1966; Grado and Ballou, 1961). More recently, Emilsson and Sundler (1984) have reported a thin layer chromatographic system on polyethyleneimine using a formic acid/ammonia solvent which appears useful for the separation of certain phosphoinositols. Sherman et al. (1985) have described a gas chromatographic system with mass spectrophotometric detection for the resolution of inositol monophosphate isomers after preliminary derivatisation, while Wilson et al. (1985a) have used a similar approach to distinguish between inositol polyphosphates and their corresponding cyclic counterparts. However, as described in CH.5, high pressure liquid chromatography (hplc) is rapidly becoming the method of choice for analysis of complex mixtures of inositol phosphates.

ii) The advantages of anion exchange chromatography: Although each of the above methods is suitable for analysis of a limited number of samples, none is readily applicable to multiple batches of tissue extracts. Alternatively, the varying ionic charge carried by different inositol phosphates provides an ideal basis for their fractionation by column, anion-exchange chromatography, particularly when detection is simplified by the ease of monitoring eluates for radioactivity rather than by more cumbersome assay for phosphate. Consequently, most recent studies of inositol lipid hydrolysis have employed this approach, of which the batch analysis procedure for total inositol phosphates described in 2.2 is a further simplification. This general method has the advantage that, in addition to allowing quantification of the separate inositol phosphates accumulating in response to agonists, it may also be used to assay corresponding changes in the concentrations of the parent inositol lipids if these are first converted to their water soluble esters by relatively simple deacylation procedures (see 3.5).

Numerous variations on this principle have been developed for the separation of inositol mono and diester phosphates, most employing Dowex or equivalent anion exchange resins. Brockerhoff and Ballou (1961) and Grado and Ballou (1961) have respectively described continuous and stepwise gradient elution procedures using LiCl. Similarly, Wells and Dittmer (1965) and Lester and Steiner (1968) have used continuous gradient elution, anion exchange systems employing ammonium formate (AF) or formate/borate buffers. More recently, Bartlett (1982) has developed a system using gradient elution with both ammonium formate and hydrochloric acid (HCl) which is capable of resolving a variety of nucleoside and inositol phosphates. Cosgrove (1978) has used gradient elution with ethanolic HCl to achieve fractionation of various scyllo-inositol phosphates.

The most common system at present in use for analysis of the products of agonist-stimulated phosphoinositide hydrolysis employs a comparatively simple stepwise gradient which has the advantage over continuous gradient elution in that it is easily adapted to the simultaneous examination of tens of tissue extracts. The methods used, derive from those developed by Hawthorne and co-workers (Hubscher and Hawthorne, 1957; Hawthorne and Hubscher, 1959; Hubscher et al. 1960; Ellis et al. 1963) in which stepwise gradients of ammonium formate/disodium tetraborate and of ammonium formate/formic acid buffers are employed to resolve Ins P_1, Ins P_2 and Ins P_3 and the corresponding glycerophosphorylinositol phosphate diesters. This procedure was originally developed for comparatively large scale chromatographic separations. However, the small tissue samples commonly used to investigate receptor-mediated phosphoinositide hydrolysis

together with the very rapid turnover of inositol polyphosphates, result in very low concentrations of products. Detection of such levels of radiolabelled inositol phosphates is achieved by a modified, small scale analysis based on the system of Ellis et al. (1963) as developed by Downes and Michell (1981) and further detailed by Downes et al. (1982). This procedure, involving batch elution of short Dowex anion exchange resin columns (2-4 cm) loaded with not more than 2-3 μmol. of each phosphate ester to allow complete resolution (Hawkins et al. 1984), provides the most convenient routine means for the assay of the individual ^{3}H-inositol phosphates accumulating in response to agonist stimulation of tissue prelabelled with ^{3}H-inositol and as such has been extensively used in very many recent studies (Berridge, 1983; Downes and Wusteman, 1983; Berridge et al. 1983; Martin, 1983). Appropriately therefore, extracts from cerebral cortical slices were examined using this protocol after first establishing the validity of the separation achieved as in (b) below.

b. The extraction and separation of inositol phosphates:

i) Preliminary studies: The extent to which individual inositol phosphates accumulate varies considerably between tissues and according to stimulus and incubation conditions (e.g. see data in CH.4). For this reason it was essential to verify that the methods previously reported for the separation of inositol phosphates (see above) also remained valid under the conditions used in the current study. This is normally achieved by constructing a column elution profile, which, in terms of a stepwise gradient, means establishing the volumes of successive buffers required to displace each of the products to be resolved such that minimal cross-fraction contamination occurs and yet assay sensitivity is maximised. For routine sample analysis only a small aliquot of each total fraction can be used to quantify the product under any particular peak. Thus, as elution volumes increase, assay sensitivity falls. From the experiments described in section 2.3, it is clear that, of the agonists used, carbachol induces the greatest hydrolysis of inositol lipids as measured by the accumulation of total ^{3}H-inositol phosphate in the presence of Li^{+} ions. If this accumulation results through a sequence of reactions initiated by $PtdInsP_2$ hydrolysis and if all the agonists studied promote phosphoinositide breakdown via a common route, then it is reasonable to assume that carbachol will similarly produce the largest accumulation of Ins P_2 and Ins P_3 (see CH.3). A column separation which is valid for the analysis of extracts from carbachol-stimulated samples should therefore remain satisfactory when other stimuli are investigated. In preliminary experiments column elution profiles were established for extracts from both control and carbachol-stimulated brain slices. For

these initial studies LiCl was included in incubations since this has been reported to potentiate stimulated accumulations of not only Ins P_1 (Berridge et al. 1982) but also of Ins P_2 and Ins P_3 (Drummond et al. 1984; Thomas et al. 1984). However, see CH.3. Extraction of inositol phosphates was initially as in 2.2 but this was later modified to improve yields of polyphosphates (see (ii) below).

Samples were prepared as described in 2.2 except that aliquots from upto four tissue incubations were combined in an attempt to facilitate detection of inositol polyphosphates. Combined, neutral extracts were diluted to 4 ml with water and applied to columns of Dowex 1 x 8 (100-200 mesh, formate form) comprising 1.0 ml 50% (w/v) slurry of resin in pasteur pipettes. Column dimensions were approximately 0.4-0.6 x 2.0-3.0 cm. Sample eluate was discarded and columns successively washed with buffers as shown in Figure 2.4.1. Initially the volumes of each eluting buffer were exactly as described by Brown et al. (1984) although both the ionic strength and volumes of these buffers were varied in later experiments. Eluate was collected as 1 ml aliquots and counted for tritium with 9 ml scintillant. Under these conditions quenching did not vary significantly across the gradient.

Figure 2.4.1. shows a typical example of the elution profiles first achieved. Total bound radioactivity for the samples shown reveals a 661% stimulation by carbachol over basal levels. In each control and stimulated samples four peaks of radiolabel were apparent after an initial wash to remove free ^{3}H-inositol, as would be anticipated from data obtained with other tissues (Berridge et al. 1983; Downes and Wusteman, 1983). By analogy with previous studies (Ellis et al. 1963; Downes et al. 1982; Berridge et al. 1982) the compounds eluted respectively by buffers B,C,D and E are identified as GroPIns + Ins-1:2 cyclic P_1, Ins P_1, Ins P_2 and Ins P_3. The latter two peaks accounted for less than 10% of the resin bound radiolabel derived from carbachol-stimulated samples and showed only about a 50% increase over basal values due to agonist. The distribution of radiolabel between the first two peaks however, indicated carbachol stimulation of either or both GroPIns and Ins-1:2 cyclic P_1 in addition to the major increase in Ins P_1. Ins-1:2 cyclic P_1 is known to arise as a product of phosphodiesterase attack on PtdIns (Dawson et al. 1971) while GroPIns could result from phospholipase A_1 and A_2 attack on PtdIns or hydrolysis of lysophosphatidylinositol by either of these enzymes. An agonist-stimulated increase in either product would imply an additional, separate route of receptor-mediated phosphoinositide hydrolysis to that currently assumed (see CH.1) and would be in direct contrast to the results of Brown et al. (1984) and a more detailed study by Berridge et al. (1983). However, the pattern of ^{3}H-product elution from Dowex columns

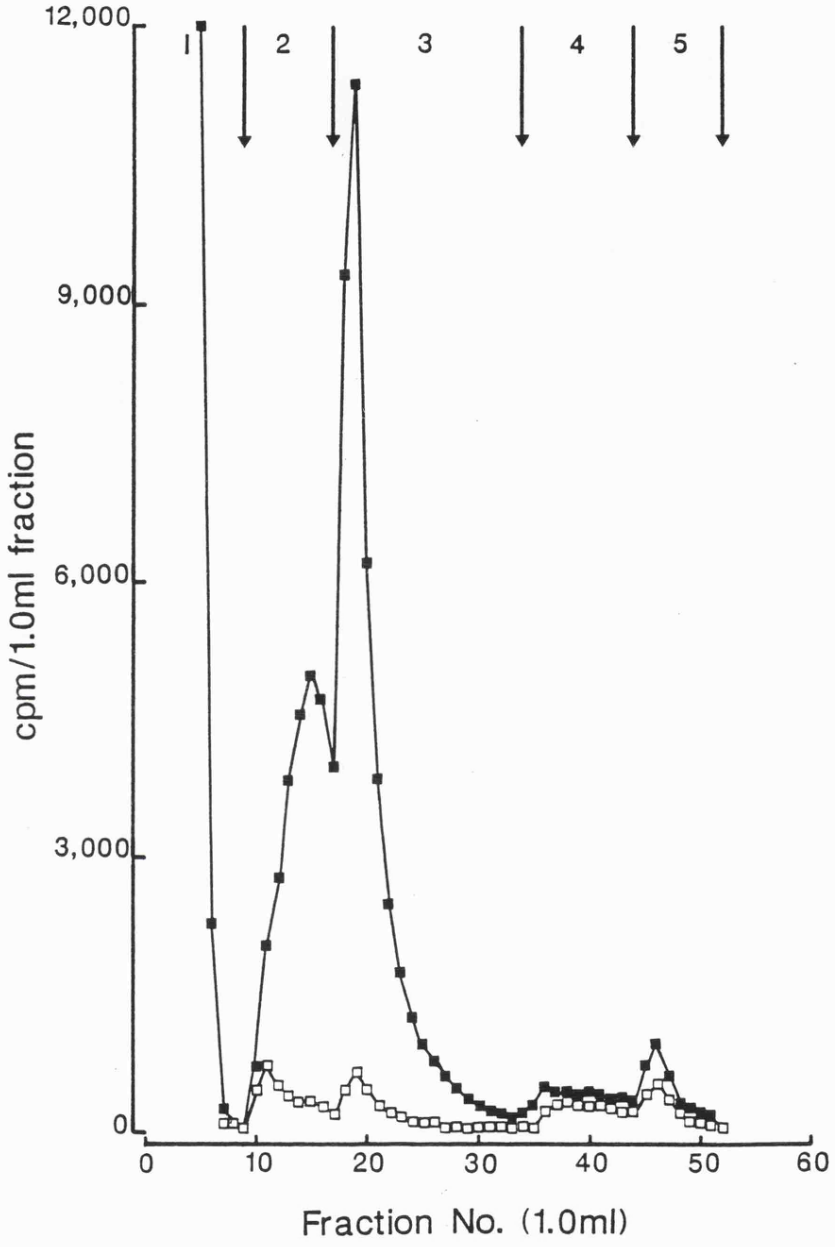


FIGURE 2.4.1 Dowex anion exchange column elution profiles of the ^{3}H products present in extracts from control and carbachol stimulated brain slices labelled with ^{3}H-inositol.

Extracts of ^{3}H-Ins P(s) were obtained under neutral conditions from ^{3}H-inositol labelled brain slices incubated for 45 min. in the presence of 5 mM LiCl and either in the presence (■) or absence (□) of 0.3 mM carbachol as described in the text and in 2.2. Pooled extracts from up to 4 tissue incubations were diluted with H_2O and a 4 ml sample applied to a 0.6 x 2.5 cm column of Dowex 1 x 8 resin (100-200 mesh, formate form). Sample was allowed to drain then the indicated volumes of buffers 1-5 applied, collected and counted for radioactivity in 1 ml aliquots to separate ^{3}H-Ins, ^{3}H-GroPIns, ^{3}H-Ins P_1, ^{3}H-Ins P_2 and ^{3}H-Ins P_3 respectively. Buffers 1-5 represent: H_2O, 0.06 M ammonium formate (AF)/0.005 M $Na_2B_4O_7$, 0.15 M AF/0.005 M $Na_2B_4O_7$, 0.3 M AF/0.1 M formic acid (FA) and 1.0 M AF/0.1 M FA.

illustrated in Figure 2.4.1 was found to be highly reproducible, using not only carbachol but also NA and HA as a stimulus. Significantly though, these latter agonists result in a much smaller accumulation of total inositol phosphate compared with that observed to carbachol (see 2.3) and on examination of extracts from brain slices stimulated with either NA or HA or carbachol the amount of radiolabel eluting in fraction B (GroPIns + Ins-1:2 cyclic P_1, Figure 2.4.1) was always proportional to the corresponding increase in Ins P_1. This suggested that a substantial amount of the radiolabel running in fraction B might in fact be Ins P_1, although as a similar effect had not been reported in previous studies (see above) using identical methodology, the reason for an earlier than expected elution of Ins P_1 was not clear. In a series of further experiments (data not shown) the elution characteristics of a ^{14}C labelled Ins P_1 standard in the same chromatographic system was examined. The standard was applied to columns either in aqueous solution both with and without upto 150 nmol. unlabelled Ins-2-P carrier or in extracts from control or 1 mM carbachol-stimulated brain slices prepared with unlabelled inositol both in the presence or absence of 5 mM LiCl (to give different accumulated Ins P_1 levels). In all cases the ^{14}C standard Ins P_1 showed radiolabel eluting in both the 0.06 M formate and 0.15 M formate fractions ((B) and (C) respectively in Figure 2.4.1). When a portion of the label eluting at 0.15 M formate was diluted in H_2O and re-applied to columns this again ran with spread between the two fractions. This problem could sometimes be overcome by using Dowex columns of increased volume but this varied between batches of resin and did not seem to suggest that the original observation was caused by column overloading since it was apparent both for ^{3}H-samples prepared with or without LiCl and for standard ^{14}C Ins P_1 run in the presence or absence of carrier Ins P_1 extracted from tissue or added exogenously as Ins-2-P. From the behaviour of the ^{14}C Ins P_1 standard it was concluded that the majority of sample extracted label eluting in both the 0.06 M and 0.15 M formate fractions (as in Figure 2.4.1) comprised Ins P_1 and that this was eluting from columns at lower ionic strength buffers owing to a lower and variable (between batches) affinity of the exchange resin for the inositol phosphates than had been anticipated from previous studies (see refs. above). Further experiments (see below) suggested a similar, though relatively less significant, phenomenon applied to Ins P_2. In additional studies, described in (ii), the ^{3}H-GroPIns present in sample extracts was eluted separate from Ins P_1 using a modified buffer as described by Emilsson and Sundler (1984) and its accumulation <u>not</u> found to be stimulated by agonists (see Figure 2.4.2). Similarly, improved chromatographic methods reported in CH.4, 5 and 6 allowed Ins-1:2 cyclic

P_1 to be resolved from both GroPIns and Ins P_1. Under these conditions no evidence for a marked effect of muscarinic receptor stimulation on the concentration of Ins-1:2 cyclic P_1 in brain slices could be found, in agreement with previous studies (Berridge et al. 1983; Brown et al. 1984).

ii) The extraction and separation of inositol polyphosphates: The chromatography of inositol phosphates thus far described was performed using samples extracted from brain slices exactly as described in 2.2. However, it is well established that inositol polyphosphates are very much more efficiently extracted under acidic than neutral conditions (e.g. Donaldson and Hill, 1986), with the former presumably preventing the binding of these highly charged molecules to insoluble proteinous material. This similarly applies to the polyphosphoinositides (see Downes and Michell, 1982; 3.5). Previous studies on numerous other tissues (e.g. rat parotid gland, Downes and Wusteman, 1983) have demonstrated agonist-stimulated accumulations of Ins P_2 and Ins P_3 many fold greater than illustrated in brain (see Figure 2.4.1; Brown et al. 1984). Thus, it seemed probable that previous measurements of inositol polyphosphate accumulations in brain represented under-estimates owing to inadequate extraction conditions, and consequently further studies were made according to modified methods.

Several different protocols have been reported for the effective extraction of inositol polyphosphates. These include the use of organic solvent mixtures acidified with HCl or of the deproteinising acids (trichloroacetic acid (TCA) and perchloric acid (PCA) (see Martin, 1983; Downes and Wusteman, 1983; Bone et al. 1984 respectively for examples of each). Of these methods that involving extraction with acidified chloroform:methanol (Martin, 1983) originally appeared the most convenient as this allows simultaneous efficient extraction of polyphosphoinositides while the other procedures require a secondary extraction of the TCA or PCA precipitated pellets with acidified solvents to achieve this (e.g. Griffin and Hawthorne, 1978; Creba et al. 1983; 3.5). However, in preliminary experiments using this preferred method it was found that the neutralised extracts (obtained by collecting upper phases analogous to those in 2.2, followed by drying in vacuum to evaporate the acid - see 6.1 for details) showed higher basal as well as higher stimulated levels of inositol phosphates when subsequently chromatographed than did samples extracted by other methods. In addition, these extracts gave much poorer chromatographic resolution of ^{3}H-inositol phosphates than did those obtained using either TCA or PCA. The problems associated with acidified organic solvent extraction of inositol polyphosphates are briefly re-considered in 6.1.

Having initially experienced difficulties with this method, a direct comparison between the efficiencies of inositol phosphate extractions achieved with neutral organic solvent (as in 2.2) and both TCA and PCA was next made. Tissue incubations were performed exactly as previously. Reactions were stopped either as in 2.2 or by addition of 300 µl of either 1.0 M TCA or PCA. Samples were left on ice for 10-20 min. then centrifuged at 3,000 x g for 20 min. to phase-partition solvent extracted samples or pellet tissue residues for acidified samples. Triplicate control and 1 mM carbachol-stimulated samples were extracted by each procedure. Aqueous phase from replicate neutral samples was combined and adjusted to 4 ml with H_2O. Aliquots (500 µl) of supernatant from TCA and PCA extracted samples were also combined as appropriate. Samples were freed of TCA by washing with 5 x 2 volumes of water saturated diethyl ether, the pH adjusted to between 6-8 with 6.25 mM $Na_2B_4O_7$ as described by Berridge et al. (1983) and sample volume finally made to 4 ml with H_2O. PCA extracted samples were stored on ice and neutralised by dropwise addition of 1.5 M KOH and 75 mM HEPES according to Bone et al. (1984), the $KClO_4$ produced being allowed to precipitate for 90 min. then removed by centrifugation. The resultant supernatant was made to 4 ml with H_2O. All samples were applied to Dowex columns and analysed for Ins P_1, Ins P_2 and Ins P_3 as in the legend to Figure 2.4.1. The results of a single experiment performed in this way are shown in Table 2.4.1.

Comparison of the stimulated values for Ins P_1 shows similar extraction under each condition, while the percentage stimulation due to carbachol estimated by neutral extraction is lower owing to a substantially higher basal value. The most apparent difference between the extraction methods is seen in the relative Ins P_2 concentrations. Both methods using acid showed comparable stimulated and control Ins P_2 values, whereas neutral extraction suggested a smaller response to carbachol and, most significantly, 3-4 fold higher basal Ins P_2 levels. As both TCA and PCA gave almost equal and relatively high yields of ^3H-Ins P_2 from stimulated samples, the high basal level of radioactivity in this fraction found with neutral organic solvent extracts may indicate that a substantial portion of 'Ins P_2' extracted under these conditions is accounted for by a distinct, but similar, ^3H-inositol labelled molecule. This is also suggested by the failure of neutral solvent extraction to give a recovery of ^3H-Ins P_2 from stimulated samples comparable with that achieved by either TCA or PCA extraction. The figures for Ins P_3 extraction by the different methods show a similar pattern to those for Ins P_2 and again the use of acid appeared to facilitate effective recovery. When organic solvents acidified with HCl were used for extraction (see above; 6.1) the results were very similar to those seen

Table 2.4.1 Comparison of methods for the extraction of ^{3}H-inositol phosphates.

Extraction	^{3}H-inositol phosphate (cpm/50 µl tissue)		
	Ins P_1	Ins P_2	Ins P_3
Neutral solvent:			
Control	2,642	896	253
1 mM carbachol	16,939	1,154	358
% stim.	641%	129%	142%
Perchloric acid:			
Control	1,790	329	134
1 mM carbachol	16,792	1,409	459
% stim.	938%	428%	343%
Trichloroacetic acid:			
Control	1,632	248	187
1 mM carbachol	15,866	1,392	521
% stim.	922%	561%	279%

Table 2.4.1 compares the extraction of ^{3}H-inositol phosphates from control and 1 mM carbachol-stimulated rat cerebral cortical slices. Tissue was prelabelled with ^{3}H-inositol (1 µCi/50 µl tissue) for 30 min. in the presence of 5 mM LiCl, then incubated with or without agonist for 45 min. before extraction and separation of the ^{3}H products as in the text. The results represent the mean values determined for triplicated samples in a single experiment but were essentially confirmed by similar studies described in 6.1.

with neutral solvents but more pronounced. A recent comparison of the same methods for extraction of inositol phosphates from control and HA-stimulated guinea-pig cerebellar slices (Donaldson and Hill, 1986) has shown almost identical results to those reported here. Hawkins *et al.* (1986) have shown that under conditions of neutral solvent extraction much of the ^{3}H-PtdInsP associated with ^{3}H-inositol labelled parotid gland slices unexpectedly partitions into the aqueous phase. Potentially an effect such as this could account for the particularly high basal but not stimulated levels of ^{3}H-inositol phosphates found here with neutral (and acidified - 6.1) organic solvents.

Clearly from this brief comparison an acid extraction protocol is preferable for inositol polyphosphates. Of the two methods described,

that employing TCA was considered the more convenient by virtue of the ease and reproducibility of freeing extracts of acid by ether extraction. In contrast, chromatograhpy of PCA extracts was occasionally complicated by either incomplete neutralisation or precipitation of $KClO_4$. Following ether removal of TCA, sample pH was routinely ≥ 4. In subsequent experiments this was adjusted to about pH 7 by addition of 5 mM $NaHCO_3$ rather than $Na_2B_4O_7$ as used above. This minor modification was made because over several experiments samples diluted in borate buffer showed delayed elution of ^{3}H-inositol on column chromatography. Commercially available ^{3}H-inositol is known to contain a number of labelled anionic contaminants which, unless removed prior to use, interfere with the assay for ^{3}H-inositol phosphates (see (iii) below). When ^{3}H-inositol from several different batches was examined by column chromatography as for inositol phosphates, the contaminating material ran largely in a fraction corresponding to Ins P_1 but also, to a lesser extent in the Ins P_2 and Ins P_3 fractions. Further, in two separate experiments when ^{3}H-inositol was applied to Dowex columns either as an aqueous solution or in borate buffer, the latter showed not only delayed elution (cpm/ml fraction 10-20 fold background radioactivity after a 50 ml wash with H_2O) but also a 3-4 fold increased level of label eluting in the Ins P_1 fraction. Borate interacts with cis 1,2, glycol groupings (see Khym, 1967 and refs. therein), thereby altering the net charge carried by a molecule containing such groups. This property led to the inclusion of borate in the solvent system described by Hawthorne and Hubscher (1958) which is adapted as a part of the stepwise gradient currently used for anion exchange column elution (see Figure 2.4.1). Potentially the effects observed here with borate may result from interaction either with ^{3}H-inositol or with contaminants contained within the label preparations used. Where reported, inclusion of borate in anion-exchange chromatographic systems has not been shown to either facilitate or interfere with the separation of inositol phosphates as might be anticipated from the varying ability of positional isomers of each inositol phosphate to complex this ion (Hubscher and Hawthorne, 1957; Lester and Steiner, 1968), although the separation of the deacylation products of several of the less polar phospholipids (i.e. GroPIns and GroPSerine) is improved in the presence of borate (Hawthorne and Hubscher, 1959). For the current purpose it was essential to ensure clean elution of all the free ^{3}H-inositol present in samples before collecting the separate ^{3}H-inositol phosphates. In this respect neutralisation of samples with $NaHCO_3$ was found to be preferable to the use of $Na_2B_4O_7$.

Having established a protocol for the acid extraction of tissue samples and a convenient means of neutralising the extracts, the

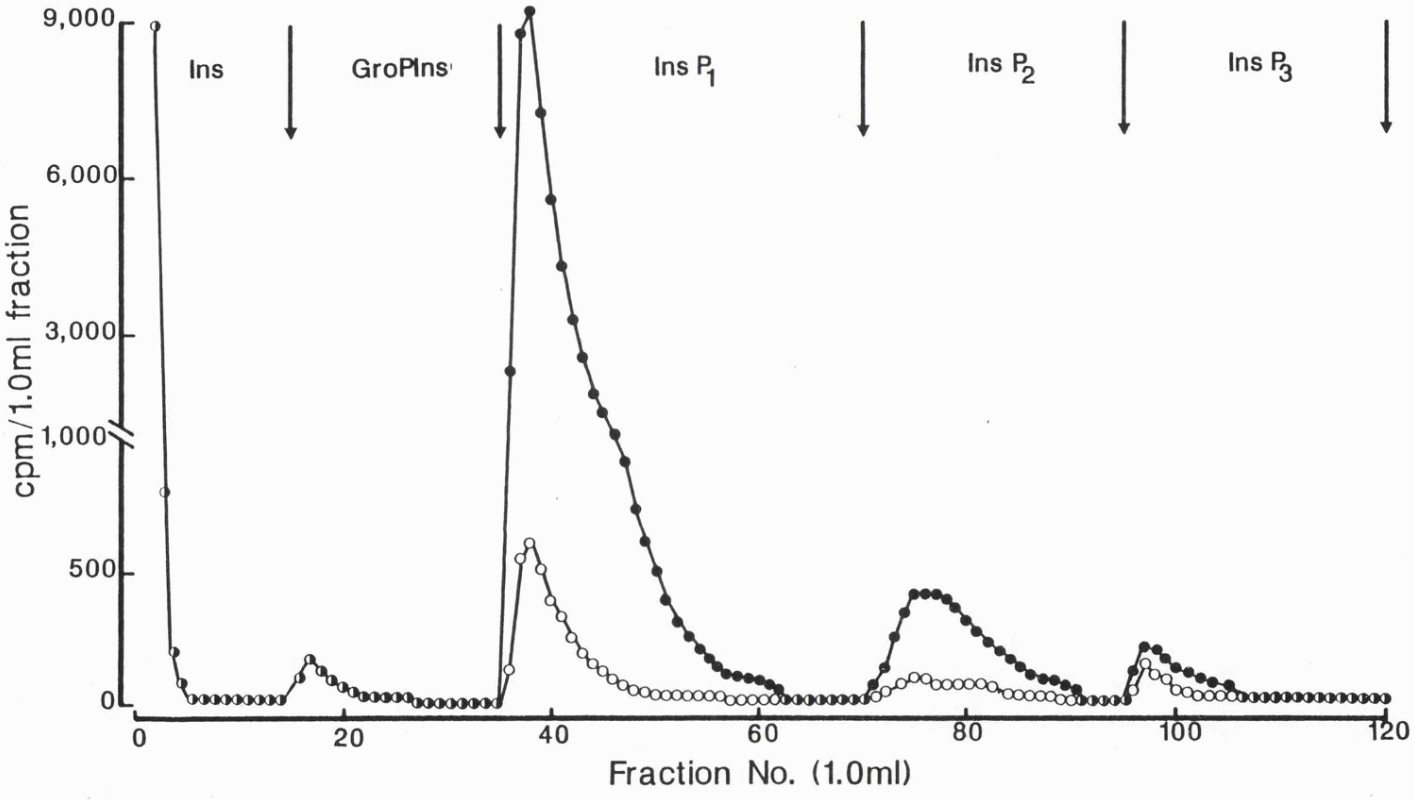


FIGURE 2.4.2 Dowex column elution profiles of ^{3}H-Ins P(s) extracted from control and carbachol stimulated brain slices labelled with ^{3}H-inositol.

Extracts of ^{3}H-Ins P(s) were obtained from TCA treated brain slices as described in the text. After removal of acid and neutralisation, samples of extracts from tissue incubated in the presence of 5 mM LiCl and either with (●) or without (○) 1 mM carbachol for 45 min. were applied to Dowex columns (see legend to Fig. 2.4.1). The fractions designated Ins, GroPIns, Ins P_1, Ins P_2 and Ins P_3 were eluted by successive application of the indicated volumes of H_2O, 0.025 M AF, 0.15 M AF/0.005 M $Na_2B_4O_7$, 0.4 M AF/0.1 M FA and 1.0 M AF/0.1 M FA respectively.

separation of inositol phosphates by anion exchange chromatography was next briefly re-examined and attempts made to estimate any cross-fraction contamination. In initial experiments a number of acid extracts from control and carbachol-stimulated brain slices were prepared as above and elution profiles for these established using the chromatograhpic system detailed in Figure 2.4.1 except that 0.2 M ammonium formate/0.1 M formic acid was used to elute Ins P_1 according to Berridge et al. (1983). The purpose of this was to determine the reproducibility of the method. Using this system poor resolution between ^{3}H-Ins P_1 and ^{3}H-Ins P_2 was repeatedly observed with stimulated but not control samples. When ^{14}C-Ins P_1 standard was run in unlabelled tissue extracted carrier this eluted cleanly at 0.2 M formate, suggesting that the poor separation of sample ^{3}H-materials might arise through elution of some Ins P_2 in the 0.2 M formate fraction. It should be noted here that an alternative anion exchange procedure using Dowex resin in the chloride form and HCl as the eluant (Griffin and Hawthorne, 1978) gave essentially the same result (data not shown).

To assess this potential cross-peak contamination more carefully, a number of ^{3}H-extracts from control and carbachol-stimulated samples were prepared and the ^{3}H-inositol phosphates fractionated using a combination of the eluants described by Ellis et al. (1963) and Emilsson and Sundler (1984) such that ^{3}H-GroPIns was eluted at 0.025 M ammonium formate, ^{3}H-Ins P_1 at 0.15 M ammonium formate/0.005 M $Na_2B_4O_7$ and ^{3}H-Ins P_2 and ^{3}H-Ins P_3 as previously (see Figure 2.4.2). Aliquots of the ^{3}H-Ins P_1 and ^{3}H-Ins P_2 fractions obtained from this primary separation were each diluted 10-fold with H_2O and re-applied to separate, fresh columns and re-eluted as shown in Figure 2.4.3. Note that re-examination of the GroPIns and Ins P_3 fractions in this way was precluded by the low levels of radioactivity present in these, but minimal cross-fraction contamination would be expected for these since Figure 2.4.2. indicates clear resolution of each from their neighbouring peaks.

Figures 2.4.2 and 2.4.3 illustrate a number of important points. Firstly, Figure 2.4.2 shows that ^{3}H-GroPIns was cleanly resolved from ^{3}H-Ins P_1 by elution according to Emilsson and Sundler (1984). This is further confirmed by Figure 2.4.3 where re-elution of the ^{3}H-Ins P_1 showed that no radiolabel was recovered in the 0.06 M formate fraction which is that expected for GroPIns (Ellis et al. 1963). Importantly, Figure 2.4.2 also shows that radiolabelling of the peak identified as GroPIns did not increase in tissue stimulated with carbachol. The identity of the 0.025 M formate fraction (Figure 2.4.2) was later confirmed as GroPIns by co-elution with an authentic standard (see Figure 5.1.1). Secondly, Figure 2.4.3A demonstrates that on re-elution, the Ins P_1 fraction isolated as in Figure 2.4.2 showed a single peak running in the 0.15 M

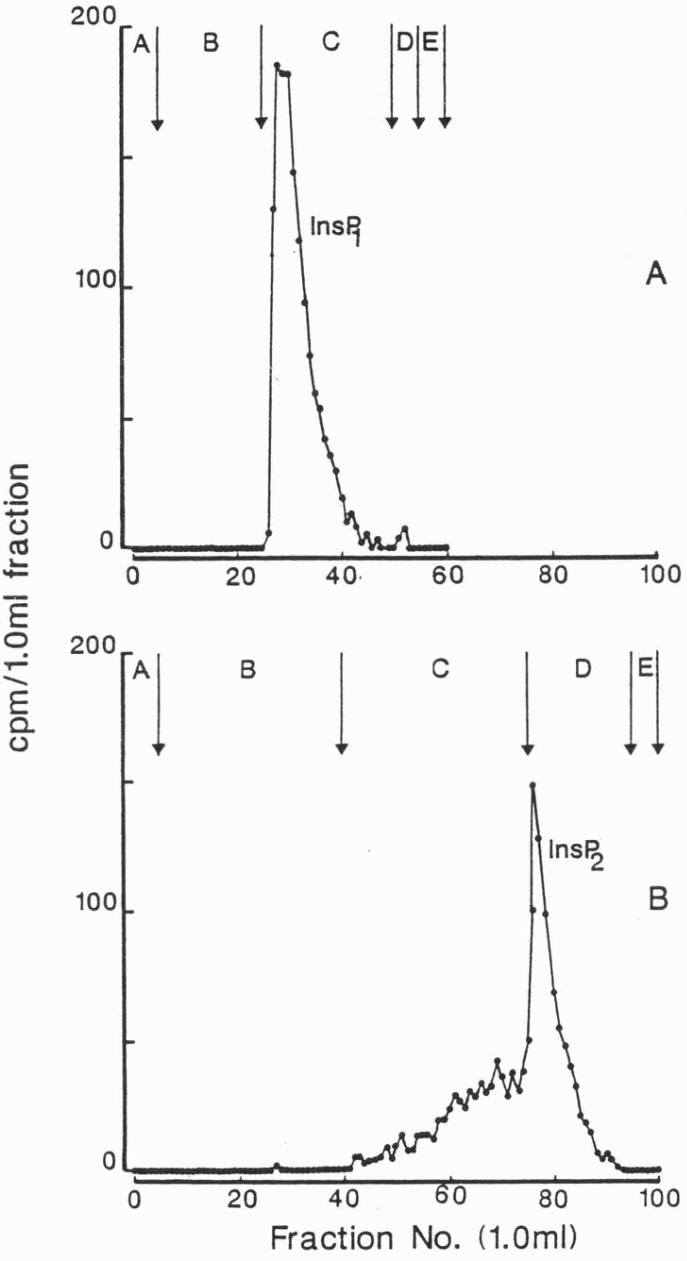


FIGURE 2.4.3 Estimation of the fraction purity achieved by Dowex chromatography.

^{3}H-Ins P_1 and ^{3}H-Ins P_2 were extracted from ^{3}H-inositol labelled brain slices stimulated with carbachol and separated as in Fig. 2.4.2. Aliquots of these fractions were diluted with H_20 and re-applied to separate fresh Dowex columns. Columns were then eluted with the volumes of A-E indicated where for: Fig. A A-E = H_20, 0.06 M AF/0.005 M $Na_2B_4O_7$, 0.15 M AF/0.005 M $Na_2B_4O_7$, 0.2 M AF/0.1 M FA and 1.0 M AF/0.1 M FA. Fig. B A-E = H_20, 0.15 M AF/0.005 M $Na_2B_4O_7$, 0.2 M AF/0.1 M FA, 0.4 M AF/0.1 M FA and 1.0 M AF/0.1 M FA.

formate fraction, with no label being eluted by higher molarity buffers. Thus, Ins P_1 isolated in this way shows no detectable contamination with other ^{3}H-inositol phosphate esters. The complete elution of Ins P_1 within this fraction is further verified by the behaviour of a ^{14}C-Ins P_1 standard (see Figure 2.4.4). Thirdly, re-elution of Ins P_2 (Figure 2.4.3B) showed no contamination with Ins P_1 nor with Ins P_3. However, approximately 50% of the re-applied ^{3}H-Ins P_2 was recovered with 0.2 M formate. From its pattern of elution this radiolabel was clearly not Ins P_1. Thus, it seems that extended elution of the resin columns used could result in elution of some Ins P_2 at 0.2 M formate and hence, this could account for the poor resolution of Ins P_1 and Ins P_2 noted above. As with the previous difficulties (see (i) above) in resolving GroPIns and Ins P_1, it is possible that the current observations were specific to the various batches of anion exchange resin used since other studies (e.g. Downes et al. 1982; Berridge et al. 1983) have not reported such problems. In later studies (see CH.4) the chromatographic separations shown here were dramatically improved by the use of analytical grade resin. However, the experiments reported in CH.3 employed the methods described here except that to avoid Ins P_1/Ins P_2 cross-contamination, the former was always eluted at 0.15 M formate as shown in Figure 2.4.2. Further, since the accumulation of ^{3}H-GroPIns in brain slice samples is small and not affected by agonists, it was eluted as a combined fraction with ^{3}H-Ins P_1 in some of the experiments described in CH.3. It should be emphasized however, that although the peaks shown on chromatograms illustrated in the current section are largely identified by analogy with previous studies (see refs. above), these identities were confirmed using the improved chromatographic techniques described in CH.4 and CH.5. Where comparable data is available, experiments performed using the methods reported here and in (iii) below gave almost identical results with those performed using the modifications reported in later sections.

iii) Summary of the methods used for the extraction and separate assay of inositol phosphate and polyphosphates: Typical experiments in which the accumulations of individual inositol phosphates were measured in rat cerebral cortex were conducted according to the following procedure which, as evidenced by the above observations, was found to be the most convenient and reliable for the extraction and subsequent chromatography of these products.

Tissue incubations, performed as in 2.2, were stopped by addition of an equal volume (300 µl) of 1.0 M TCA. Samples were left to stand on ice for 10-20 min. then vigorously vortex mixed and centrifuged at 3,000 x g for 20 min. Aliquots (500 µl) of the resultant supernatants were combined

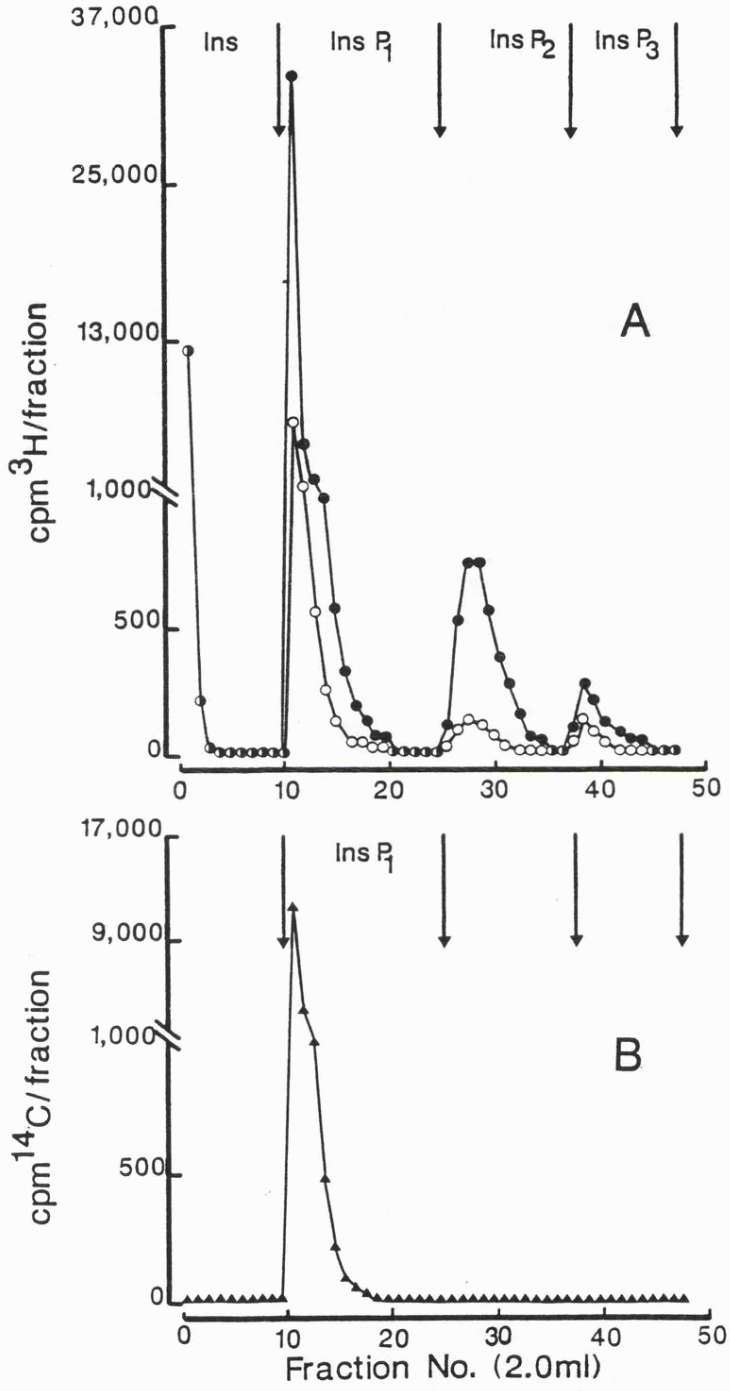


FIGURE 2.4.4 Typical elution profiles of ^{3}H-Ins P(s) achieved by Dowex chromatography.

Pooled ^{3}H-Ins P(s) extracts were prepared from TCA treated brain slices as described in the text (2.4(d)iii). Fig. A shows the elution trace for samples from tissue incubated for 45 min. in the presence of 5 mM LiCl and either with (●) or without (○) 1 mM carbachol. Fractions designated Ins, Ins P_1, Ins P_2 and Ins P_3 were eluted respectively with H_2O, 0.15 M AF/ 0.005 M $Na_2B_4O_7$, 0.4 M AF/0.1 M FA and 1.0 M AF/0.1 M FA. Fig. B shows the trace obtained with standard ^{14}C Ins P_1 run in the same system. In each case columns were as in Fig. 2.4.1.

from triplicated samples and washed with 5 x 3 ml water saturated diethyl ether. After aspirating the final ether extract, samples were made up to 5 ml with 5 mM $NaHCO_3$ and stored at 4°C. The following day 4 ml aliquots of the neutralised samples were applied to columns comprising 1 ml of a 50% (w/v) slurry of Dowex 1 x 8 resin (100-200 mesh, formate form). The eluate and an initial wash with 20 ml of water (to remove free, ^{3}H-inositol) were discarded. Columns were then successively washed with 20 ml 0.025 M ammonium formate (AF) to elute GroPIns (occasionally this step was omitted, see above), 30 ml 0.15 M AF/0.005 M $Na_2B_4O_7$ for Ins P_1, 25 ml 0.4 M AF/0.1 M formic acid (FA) for Ins P_2 and 20 ml 1.0 M AF/0.1 M FA for Ins P_3. No further label was found to elute at buffer molarities in excess of 1.0 M. Column flow rates were maintained at about 1 ml/min. by successive addition of each eluting buffer in 1 ml aliquots. Radioactivity in each total fraction was quantified by liquid scintillation counting of duplicate or triplicate 1 or 2 ml aliquots of each with 18 ml scintillant. Counting efficiency for tritium across the gradient was consistent at approximately 22%. Column recovery, as assessed with a ^{14}C-Ins P_1 standard, was close to 100%. A typical separation of ^{3}H-inositol, ^{3}H-GroPIns + ^{3}H-Ins P_1, Ins P_2 and Ins P_3 is shown in Figure 2.4.4.

As considered in (ii) above, commercially available ^{3}H-inositol contains anionic contaminants which can severely reduce assay sensitivity. In all experiments in future chapters, ^{3}H-inositol was routinely treated to minimise levels of these unidentified materials. Aliquots of stock ^{3}H-inositol (1 mCi/ml) were diluted 2-fold with water and applied to a column containing 200 µl Dowex 1 x 8 anion exchange resin (100-200 mesh, formate or chloride form). The eluate was collected and combined with a further wash of the column with a volume of H_2O sufficient to give a final dilution of label between 1-5 µCi/10 or 20 µl. Subsequent chromatography of ^{3}H-inositol as for inositol phosphates showed that contamination with anionic materials could be all but eliminated in this manner provided the label was used within several days of the clean-up procedure.

CHAPTER 3

Receptor-mediated inositol phosphate and polyphosphate accumulations in cerebral cortex and the effects of lithium

As discussed in CH.1 it is now well established that a primary reaction of receptor-mediated inositol lipid hydrolysis is a phospholipase C catalysed cleavage of $PtdInsP_2$, yielding Ins-1,4,5-P_3 and DG. Inactivation of Ins P_3 by successive dephosphorylation to inositol is thought to account for the Ins P_2 and Ins P_1 which also accumulate in stimulated cells while increased turnover of PtdIns and PtdInsP is suggested to arise from kinase activities replenishing $PtdInsP_2$. Studies using rat parotid glands pulse labelled with ^{3}H-inositol appear to confirm this cyclical metabolic sequence (Downes and Wusteman, 1983; Aub and Putney, 1984). In contrast, Majerus et al. (1985) have suggested that this scheme is not consistent with the action of thrombin on platelets and propose that while release of Ins-1,4,5-P_3 from $PtdInsP_2$ is a probable initiating reaction, the consequent rise in $[Ca^{2+}]_i$ triggered by this event promotes a direct, Ca^{2+}-dependent hydrolysis of PtdIns. The products of this secondary breakdown are Ins P_1 and DG. Therefore, this mechanism allows for the formation of the two putative second messengers, Ins-1,4,5-P_3 and DG, by distinct routes with the majority of the latter deriving from PtdIns.

Evidence concerning these mechanisms in brain is limited and although stimulated accumulation of inositol polyphosphates has been demonstrated (Durell et al. 1968; Berridge et al. 1983; Van Rooijen et al. 1983), the reaction sequences generating these have not previously been investigated in detail. In contrast, the introduction of assays employing lithium ions to trap inositol cycle intermediates at Ins P_1 (Berridge et al. 1982) has led to extensive study of phosphoinositide hydrolysis as a functional correlate for the characterisation (Brown et al. 1984; Daum et al. 1984; Minneman and Johnson, 1984; Kendall and Nahorski, 1985a) and localisation (Mantyh et al. 1984; Rooney and Nahorski, 1986) of cerebral receptors. However, such studies assume that assay of Ins P_1 under these conditions is an accurate reflection of receptor activation but since formation of this product is distal to the initial receptor-mediated step, the relationship may be complex, particularly if direct, but secondary hydrolysis of inositol phospholipids other than $PtdInsP_2$ can occur as suggested by Majerus et al. (1985). In this situation dose-response curves for Ins-1,4,5-P_3 and Ins P_1 accumulation may not be superimposable as the latter could exhibit a greater apparent receptor reserve if Ca^{2+} activated PtdIns hydrolysis represented an amplification step. In this respect, it is of interest that the dose-relationship for vasopressin-stimulated Ins P_1 accumulation lies slightly to the left of that for Ins-P_2 and Ins P_3 in superior cervical ganglia (Bone et al. 1984). However, direct PtdIns hydrolysis cannot necessarily be inferred from the existence of an apparent receptor reserve for Ins P_1 accumulation as

muscarinic receptor stimulation in rat parotid gland exhibits the latter but not the former phenomenon (Jacobson et al. 1985; Downes and Wusteman, 1983). Further, the relative positions of dose-response curves for accumulation of separate inositol phosphates might be expected to show some discrepancies as a consequence of distinct kinetic properties of the enzymes responsible for metabolism of each (see CH.6).

Despite such potential complexities, it is clear that studies of the accumulation of individual inositol phosphates are essential to an accurate understanding of the mechanism of receptor-mediated phosphoinositide hydrolysis and to less ambiguous interpretation of pharmacological data relating to assay of Ins P_1. The major apparent differences indicated by studies in rat parotid gland and in platelets (see above) suggest that the assumption of a common mechanism for all tissues and/or receptor systems may be invalid and illustrate the need to define that which operates in each specified case.

3.1 Muscarinic receptor-mediated inositol polyphosphate accumulation in rat cerebral cortex

The preliminary data presented in 2.4, in agreement with the results of Berridge et al. (1983), suggest that polyphosphoinositides may be hydrolysed as a consequence of receptor activation in brain as in many other tissues. Having established methods for the extraction and separation of inositol polyphosphates, the accumulation of these in response to a variety of stimuli was investigated in more detail in order to determine the metabolic pathways involved in receptor-mediated inositol lipid hydrolysis in cerebral cortex (see also 3.3).

As a first step toward this aim, time courses for accumulation of each inositol phosphate in response to carbachol were determined over a comparable period for which previous experiments (see 2.3) had demonstrated linear formation of ^{3}H-Ins P_1. However, while Li^+ had been included in earlier studies to inhibit Ins P_1 hydrolysis, this ion was omitted in these initial measurements of polyphosphate accumulation in order to avoid the potential complexities of differential effects of this ion on separate phosphatase steps as have been reported in other tissues (Storey et al. 1984; Drummond et al. 1984; Thomas et al. 1984). Figures 3.1.1 and 3.1.2 illustrate the mean results of three experiments performed exactly as described in 2.4.

From Figure 3.1.1 it is apparent that 1 mM carbachol stimulates the rapid accumulation of each ^{3}H-Ins P_1, ^{3}H-Ins P_2 and ^{3}H-Ins P_3 which respectively achieve 236%, 377% and 475% of appropriate control values within 5 min. of agonist addition. However, while each Ins P_2 and Ins P_3 accumulations reach maximal levels within 5 to 10 min., Ins P_1 continues

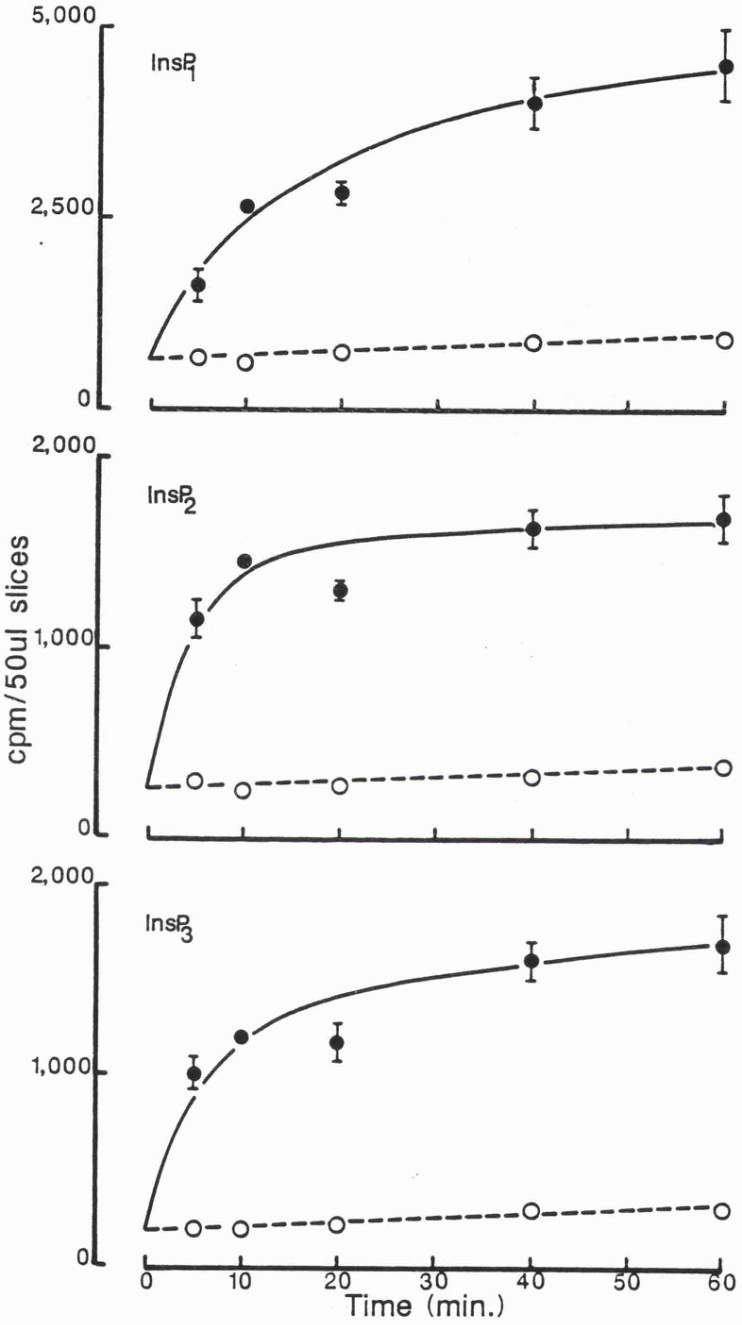


FIGURE 3.1.1 Time courses of carbachol stimulated ^{3}H-Ins P_1, ^{3}H Ins P_2 and ^{3}H-Ins P_3 accumulations in ^{3}H-inositol labelled brain slices.

Tissue was prepared, incubated and labelled as in 2.2 except that LiCl was not present. After 30 min. labelling with ^{3}H-inositol (1 μCi), 50 μl aliquots of brain slices were further incubated either in the presence (●) or absence (○) of 1 mM carbachol for the times indicated. ^{3}H-Ins P(s) were then extracted and separated as in 2.4(d)iii. Results represent the mean ± SEM of 3 experiments each run with triplicated tissue incubations. Where not shown SEM values are within the symbols.

to accumulate at a gradually diminishing rate throughout the incubation with agonist. As the accumulation of each product is determined by measurement of fraction radioactivity it is possible that the increased labelling of each inositol phosphate reflects an increase in specific activity to a small extent. This may, in part, account for the initial rapid rise in the level of each product but as both Ins P_2 and Ins P_3 accumulations rapidly plateau, it seems unlikely that major changes in the specific activity of these occurs at later times. This implies that agonist labile pools of all three phosphoinositides also achieve equilibrium with free ^{3}H-inositol over a similar period in the presence of carbachol. Given this observation, it seems probable that the more prolonged, continued increase in Ins P_1 reflects one of mass rather than specific activity since regardless of the immediate metabolic source of Ins P_1 (either Ins P_2 dephosphorylation or direct PtdIns hydrolysis) its specific activity cannot exceed that of the precursor lipid.

The addition of atropine (at a concentration 3,000-fold > IC_{50}) at a time when Ins P_2 and Ins P_3 accumulations have plateaued (see 3.2) results in a rapid decline in the labelling of all three fractions to close to, but a little above, unstimulated values. Together with the observations above, this suggests that the major proportion of the initial (first 5 min.) increase in the levels of each inositol phosphate resulting from agonist addition is also accounted for by an increase in concentration rather than specific activity because receptor blockade would not be anticipated to promote a rapid decrease in the specific activity of precursor phosphoinositide already labelled but only to prevent further secondary increases in labelling consequent on hydrolysis. The small proportion of the response not blocked by the antagonist may reflect an increase in specific activity but may also be accounted for by other factors (see 3.2). From these observations, it seems likely that the accumulations of fraction radioactivity measured, largely reflect elevated concentrations of inositide hydrolysis products and the use of continuous labelling protocol does not seem to result in progressive increases in either phosphoinositide or inositol phosphate specific activity over longer periods of stimulation. However, such changes may be significant over very short (<< 5 min.) incubations with agonists.

Comparison of Figures 3.1.1 and 3.1.2 shows that although the fold stimulation over control values is greatest for Ins P_3, over the first 5 min., the increased accumulation in cpm induced by 1 mM carbachol in each fraction is closely similar. Thus, it is not possible from the data presented to determine the sequence in which the products are formed. The substantial accumulations of each Ins P_2 and Ins P_3 nonetheless

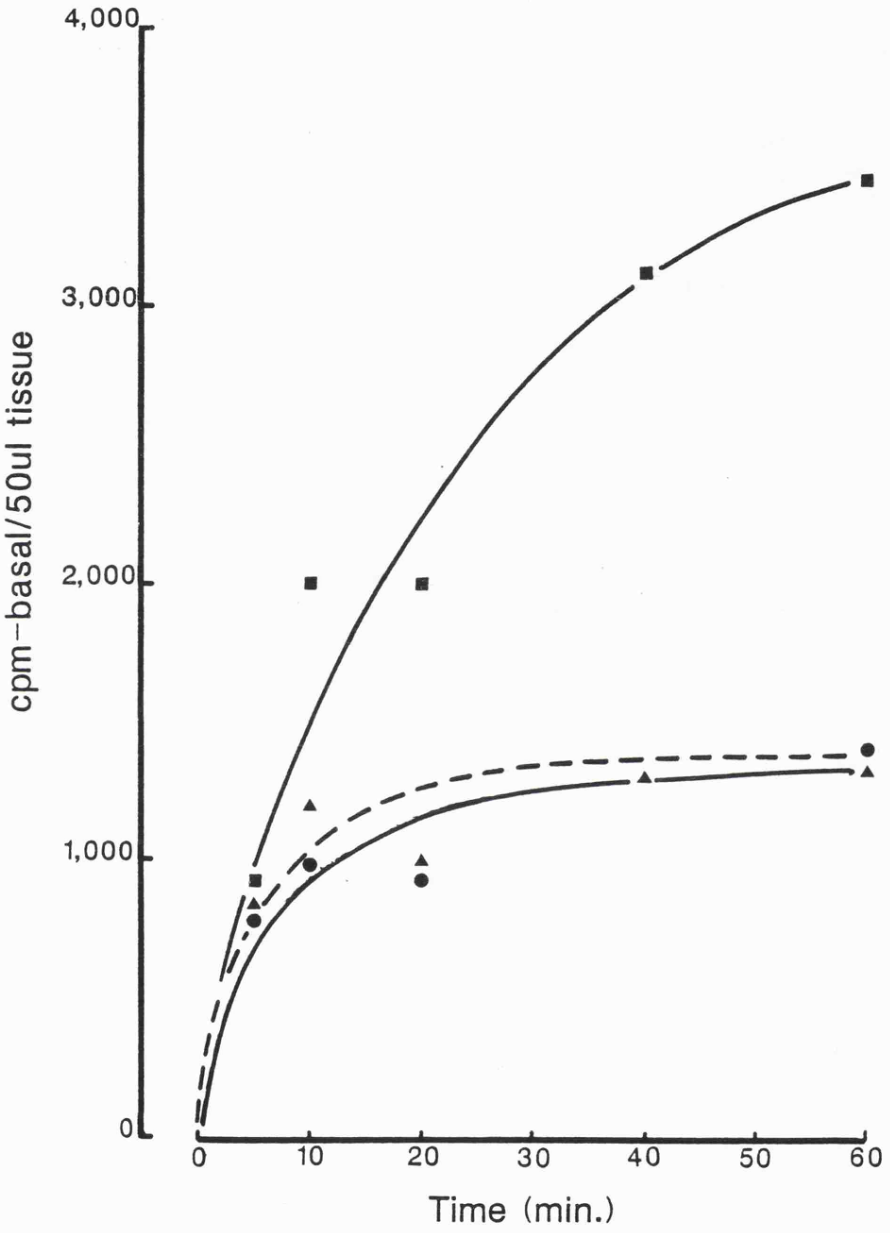


FIGURE 3.1.2 Time courses of carbachol stimulated ^{3}H-Ins P_1, ^{3}H-Ins P_2 and ^{3}H-Ins P_3 accumulations in ^{3}H-inositol labelled cerebral cortical slices.

The time course data for carbachol stimulated samples from Fig. 3.1.1 is represented after subtraction of the appropriate basal values.

demonstrates that polyphosphoinositides are substrates for a muscarinic receptor-mediated phospholipase C in rat cerebral cortex.

As the increase in product labelling is likely to represent elevated concentrations, the different extents to which the separate inositol phosphates accumulate may also be significant. From CH.2 it is clear that, in the presence of Li^+ ions, the formation of Ins P_1 continues linearly for close to 60 min. in response to carbachol, indicating a constant rate of phosphoinositide hydrolysis over this period. In the absence of Li^+ the rate of Ins P_1 accumulation begins to decline after only 10-20 min. As the rate of Ins P_1 formation would not be expected to be slower in the absence than in the presence of Li^+ ions, it seems probable that the decreased rate of accumulation results from an increased rate of Ins P_1 breakdown. The rate of hydrolysis of each inositol phosphate will be dependent on its concentration until saturation of the appropriate enzymes is approached, above which products should continue to accumulate. Since the time course for accumulation of the three inositol phosphates flattens out, it is unlikely that any of the phosphatase enzymes become saturated even with 1 mM carbachol as stimulus. The concentration at which accumulation of each product plateaus will be dependent on the rate at which its rate of breakdown accelerates towards that of its formation, and this will be a function of the K_m of each phosphatase step. As Ins P_1 accumulates to a greater extent than either Ins P_2 or Ins P_3, this implies a slower rate of turnover of the former. This is consistent with the relative K_m values estimated for Ins-1-P phosphatase from bovine brain (50-100 μM, Hallcher and Sherman, 1980) and Ins-1,4,5-P_3 5-phosphatase from human erythrocytes (25 μM, Downes *et al.* 1982) and from platelets (30 μM, Connolly *et al.* 1985) and with the comparative rates of inositol phosphate metabolism reported by Storey *et al.* (1984). Alternatively, the greater accumulation of Ins P_1 could imply its rate of formation is faster than that for Ins P_2 and Ins P_3 which would indicate a direct hydrolysis of PtdIns.

As a second messenger role has been proposed for Ins-1,4,5-P_3, a rapid rate of turnover would be anticipated, possibly exceeding that for the other inositol phosphates. From this it might be predicted that relative accumulations of products would be in the order Ins $P_1 \geqslant$ Ins $P_2 >$ Ins P_3. In this respect, the apparently equal levels of Ins P_2 and Ins P_3 in cerebral cortex are unexpected. A recent study of muscarinic receptor-mediated phosphoinositide hydrolysis in rat cerebral cortex (Jacobson *et al.* 1985) has shown similar though smaller accumulations of each Ins P_1 and Ins P_2 over the same time scale as illustrated in Figures 3.1.1 and 3.1.2. However, the corresponding increases in Ins P_3 reported were very low, with only a 30%-40% stimulation over basal values being observed by

5 min. compared to the 4-5 fold increase demonstrated in Figure 3.1.1. Since the methods used were almost identical to those employed here, it is difficult to account for the differences observed. However, as noted in 2.3, the response to muscarinic receptor stimulation can vary quite extensively in magnitude and appears to be sensitive to changes in the metabolic viability of the tissue slices so that tissue preparation and handling may be an important factor.

A much more striking difference from the current results is that observed following muscarinic receptor stimulation in rat parotid glands. 1 mM Carbachol stimulation of pulse-labelled tissue for 10 min. in the absence of Li^+ results in the largest accumulation of inositol phosphate in the Ins P_2 fraction, while Ins P_1 and Ins P_3 show approximately equal levels, about 50% of Ins P_2 (Downes and Wusteman, 1983). Despite the different labelling approach to that used here, these comparative results indicate either a distinct enzymology or route of inositol lipid and/or phosphate metabolism may be associated with muscarinic receptor stimulation in rat cerebral cortex and parotid gland.

3.2 Hydrolysis of inositol phosphates in cerebral cortex (see 6.2 also)

Downes and Wusteman (1983) have estimated the rates of hydrolysis of each inositol phosphate in rat parotid glands by first stimulating accumulation of each with carbachol, then blocking the further formation by addition of atropine and monitoring the decay of stimulated levels down to control values. Surprisingly, this did not indicate a very rapid rate of Ins P_3 hydrolysis, but showed that concentrations were still elevated above controls upto 10 min. after receptor blockade. Further, the rate of Ins P_3 breakdown did not exceed that of Ins P_2 or Ins P_1 as would be expected if each is formed in a simple sequence of dephosphorylation reactions. In order to further define the metabolic pathways associated with muscarinic receptor-mediated inositol phosphate formation in cerebral cortex a similar approach was used.

A number of preliminary experiments were first run to determine an approximate time scale over which accumulations of inositol phosphates declined. Further, more detailed studies were then made using methods exactly as described in 2.4 except that only single tissue aliquots were incubated for each time point to facilitate handling during short-term drug treatments. Since triplicate tissue samples could not be combined, the concentration of ^{3}H-inositol in the incubation medium was increased to 5 µCi/50 µl cortical slices to permit adequate detection of each inositol phosphate (see 4.1). After a 30 min. prelabelling period, samples were incubated with or without 1 mM carbachol for up to a further 30 min. prior to addition of 10 µM atropine. Reactions were stopped at intervals over

this time course as indicated in Figure 3.2.1 to ensure that the accumulation of each product had achieved an approximately maximal level and thence plateaued. This phase was chosen for the addition of the receptor antagonist as potential complications of changing specific activity which might be encountered at earlier times should be avoided in this way (see 3.1). In addition, this allows the decay of each product to be determined from maximal concentrations and thus facilitates detection and accuracy of measurement over the initial period for which the antagonist is present (i.e. when differences in the changing rates of product accumulation should be most evident). This approach does have a number of inherent problems; these are considered in detail in 6.2.

Following atropine addition, control and stimulated samples were further incubated for up to 20 min. according to Figure 3.2.1. Both control and carbachol treated samples were also run over the same period in the absence of antagonist to assess continued accumulation of inositol phosphates. The results presented in Figures 3.2.1 to 3.2.4 illustrate the means of three experiments conducted in this way.

Figure 3.2.1 allows an approximate comparison of the rates at which all three inositol phosphates decay towards control values. For the sake of clarity, controls incubated in the presence of atropine have been omitted but, in agreement with previous data (see CH.2), these were not significantly different from untreated samples. The time courses for accumulation of each inositol phosphate illustrated in Figure 3.2.1 show a very similar pattern of response to those reported above with the radiolabelling of each fraction showing an increase roughly corresponding to the greater concentration of ^{3}H-inositol included in the incubations. Stimulated concentrations of each Ins P_1, Ins P_2 and Ins P_3 were respectively 426%, 562% and 485% of control values at the time point immediately before atropine addition. After addition of the antagonist there was a rapid fall in the concentration of each labelled product such that after 10 min. in the presence of atropine, stimulated accumulations of Ins P_1, Ins P_2 and Ins P_3 were 155%, 173% and 148% of respective control values. In contrast, samples incubated in the absence of antagonist maintained a reasonably constant or slightly increased accumulation of each product. These results indicate the continuous production of each labelled inositol phosphate and demonstrate, together with previous data, that the muscarinic receptor does not desensitise to carbachol over the period of these experiments.

Figure 3.2.1 also suggests distinct initial rates at which the separate products are catabolised, since over the first 1-2 min. following atropine addition, both Ins P_3 and Ins P_2 decline nearer to control values than does Ins P_1. The data for the decay phase of each inositol phosphate

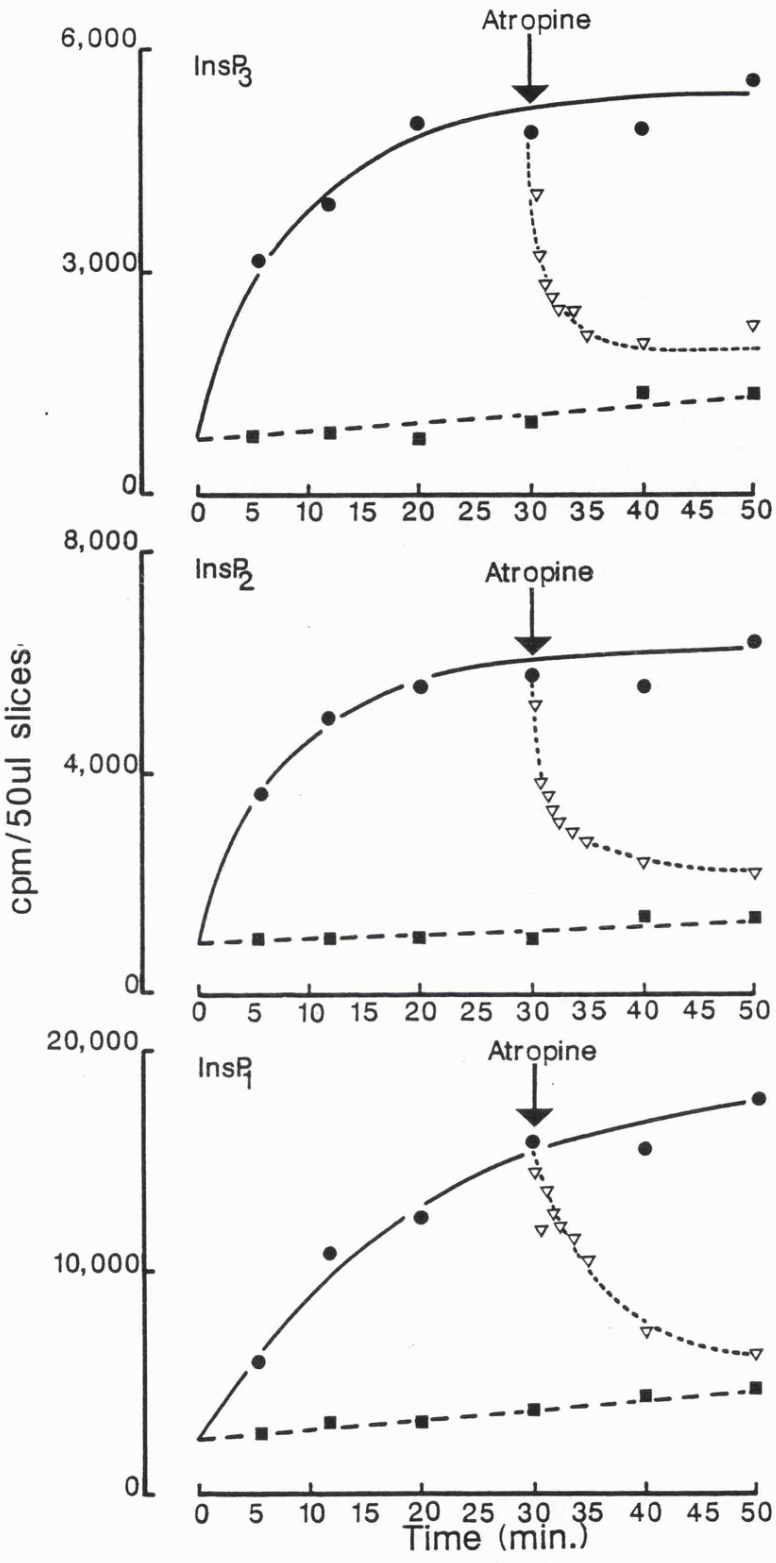


FIGURE 3.2.1 Comparison of the rates at which the ^{3}H-Ins P_1, P_2 and P_3 accumulated in response to pre-stimulation with carbachol are hydrolysed in brain slices following muscarinic receptor blockade.

^{3}H-Inositol labelled tissue was incubated in the presence (●) or absence (■) of 1 mM carbachol for the times indicated. After 30 min., 10 μM atropine or Krebs vehicle was added to both control and stimulated tissue and incubations continued for up to a further 20 min. Only the effects of atropine on stimulated samples (▽) are shown (but see text). ^{3}H-Ins P(s) were extracted and separated as in 2.4(d)iii. Results represent the means of 3 determinations made in separate experiments.

are presented in more detail in Figures 3.2.2 to 3.2.4, from which an approximate half-life ($T_{½}$) has been estimated for each product. $T_{½}$ has been determined as the time over which 50% of the total decrease due to atropine occurs. The half-life values calculated for Ins P_1, Ins P_2 and Ins P_3 are 180, 40 and 30 sec. respectively although it must be emphasised that given the associated errors these can only be approximate figures. However, there is a reasonable agreement between these estimated rates of hydrolysis and the extent to which each product accumulates. Since from both Figure 3.1.1 and Figure 3.2.1 Ins P_2 and Ins P_3 achieve constant and approximately equal concentrations in the continued presence of carbachol, there can be little difference in their rates of breakdown, an observation which is confirmed by their similar half-life values. In contrast, accumulations of Ins P_1 after 30 min. stimulation with carbachol are about 3-fold greater than those of polyphosphates which is again consistent with the apparently slower rate of hydrolysis.

A critical factor in these experiments is the time point after receptor blockade at which the separate products first show a decreased accumulation. If a sequence whereby Ins $P_3 \longrightarrow$ Ins $P_2 \longrightarrow$ Ins P_1 operates, then a decline in the concentration of Ins P_2 should only become apparent when the level of Ins P_3 falls below that at which the original rate of dephosphorylation can no longer be maintained. A similar argument applies to Ins P_1 although if receptor activation results in hydrolysis of PtdInsP and PtdIns as well as of $PtdInsP_2$, the situation would be more complex and, depending upon the events controlling breakdown of these other lipids, decreases in the concentrations of Ins P_2 and Ins P_1 might exhibit a lag period relative to Ins P_3. If, for example, a Ca^{2+}-dependent hydrolysis of PtdIns occurs as proposed by Majerus et al. (1985) and this follows from an Ins P_3-mediated increase in $[Ca^{2+}]_i$, then close to maximal accumulation of Ins P_1 could continue while concentrations of Ins P_3 and Ins P_2 declined following receptor blockade. The period over which Ins P_1 formation would persist under these circumstances would be dependent on the dose-relationship between [Ins P_3] and Ca^{2+} release and the rate at which intracellular concentrations of the latter two fall following removal of the original stimulus. In addition, secondary hydrolysis of PtdIns and/or PtdInsP should result in accumulations of Ins P_1 and/or Ins P_2 greater than can be accounted for by the flux through Ins P_3. Alternatively, if all of the Ins P_2 and Ins P_1 is formed directly by Ins-P_3 dephosphorylation, then any lag phase in the decay of the former products following receptor blockade would only be dependent on the hydrolytic rate(s) of the immediate precursor(s) and the flux through Ins-P_3 should be sufficient to account for the accumulations of all the lower phosphates.

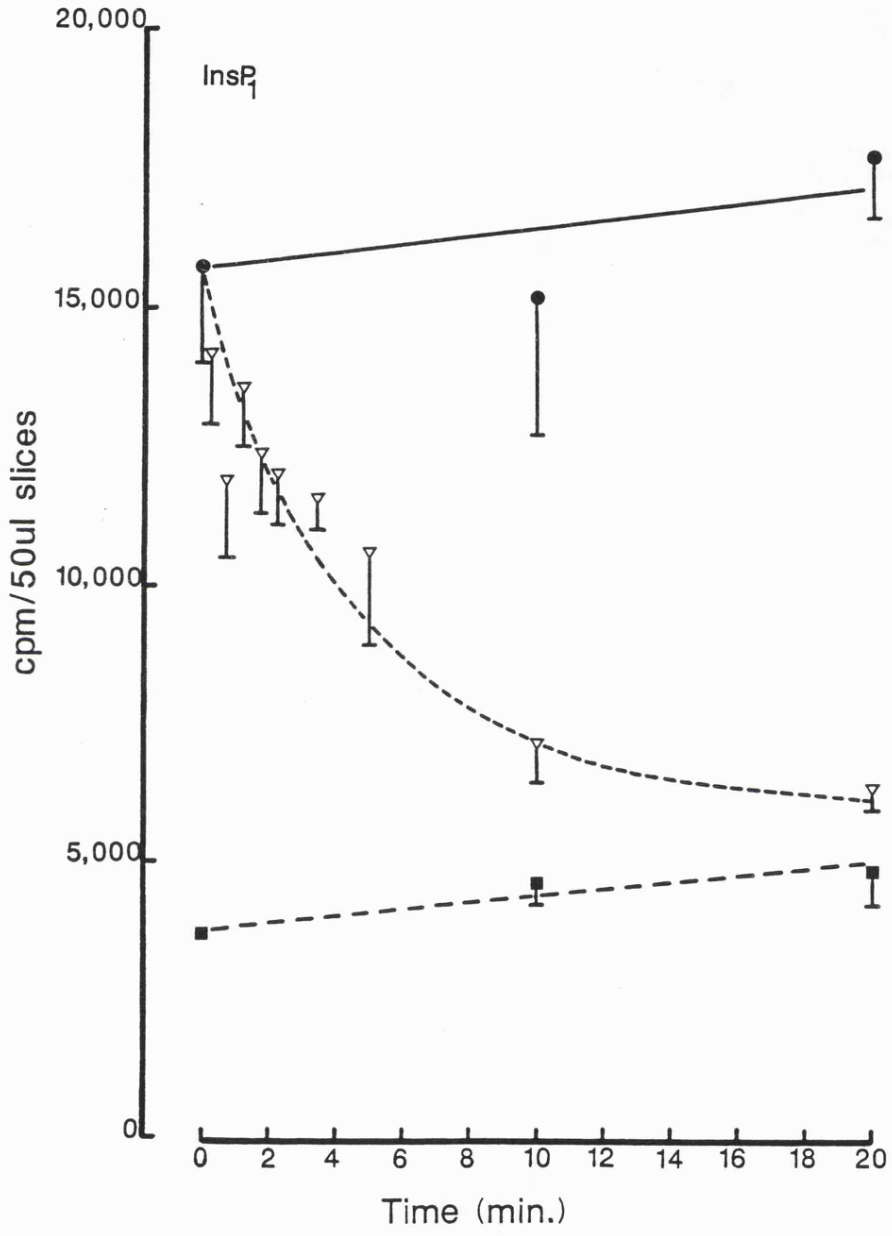


FIGURE 3.2.2 The time course of atropine induced reversal of carbachol stimulated ^{3}H-Ins P_1 accumulation.

The data from Fig. 3.2.1 for the 'decay phase' of ^{3}H-Ins P_1 induced by atropine is shown on an expanded time scale. (●) and (▽) represent carbachol stimulated samples exposed or not exposed to 10 μM atropine. (■) Indicates non-atropine treated controls. Zero time is the point of atropine addition (i.e. 30 min. on Fig. 3.2.1). Results show the mean ± SEM of 3 separate measurements.

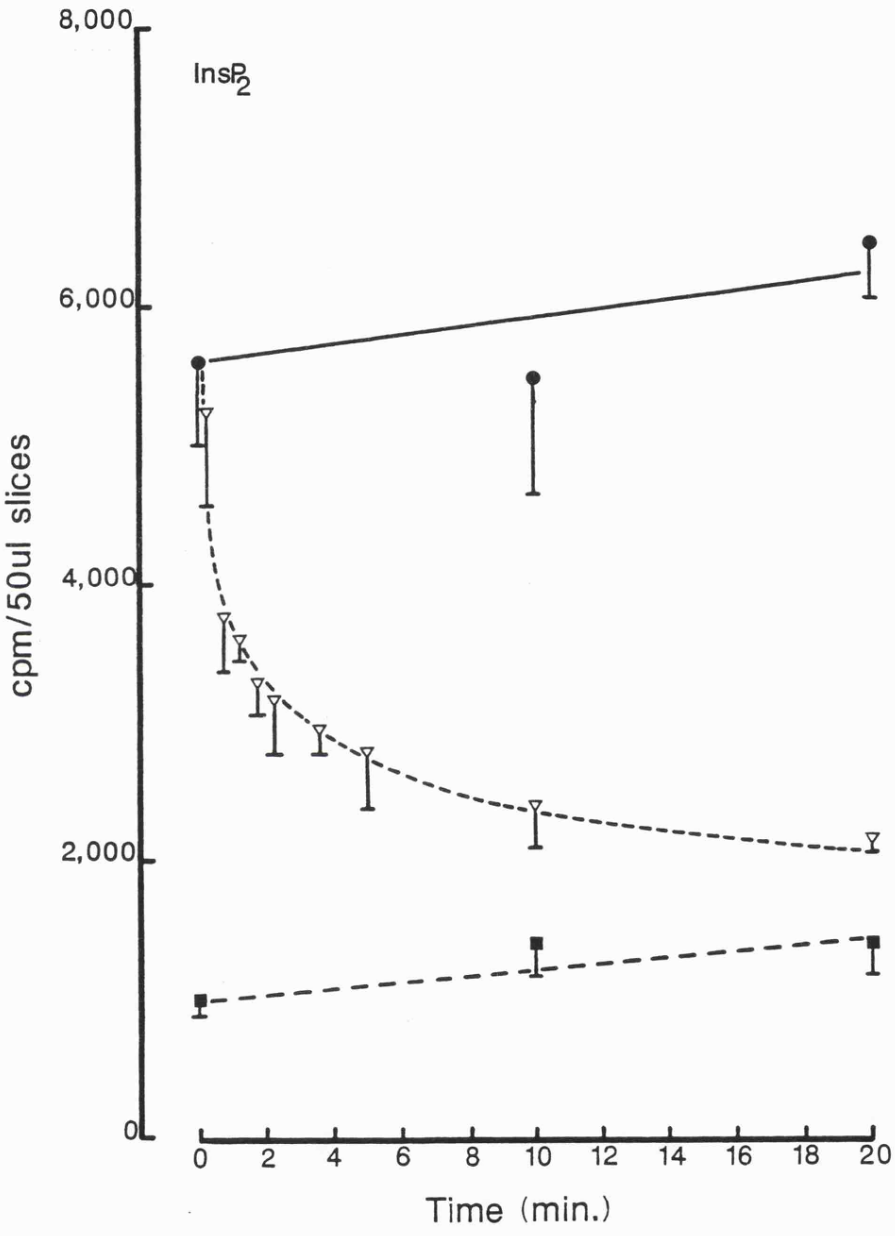


FIGURE 3.2.3 The time course of atropine-induced reversal of carbachol stimulated ^{3}H-Ins P_2 accumulation.

The data from Fig. 3.2.1 for the 'decay phase' of ^{3}H-Ins P_2 induced by atropine is shown on an expanded time scale. (●) and (▽) represent carbachol stimulated samples exposed or not exposed to 10 μM atropine. (■) Indicates non-atropine treated controls. Zero time is the point of atropine addition. Results show the mean ± SEM of 3 separate measurements.

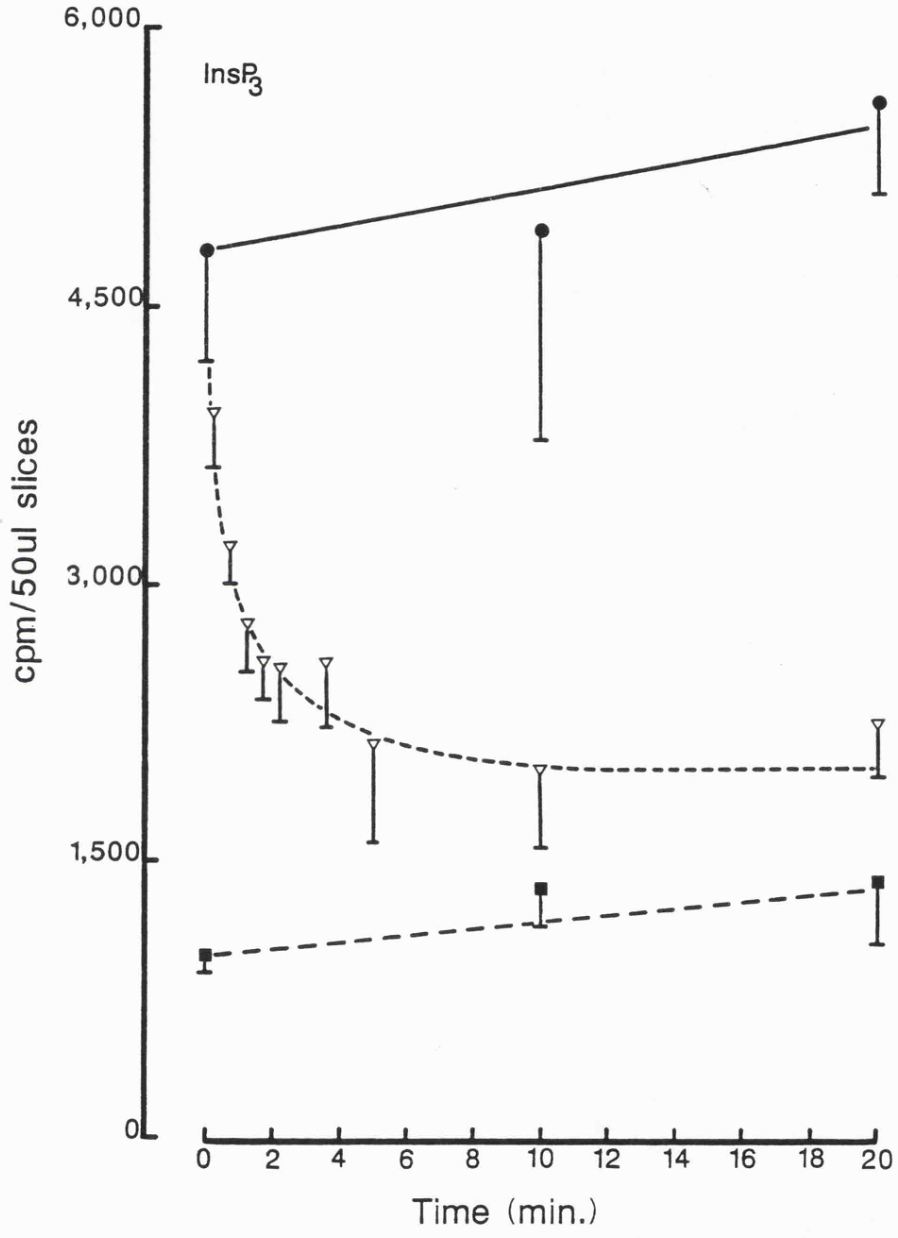


FIGURE 3.2.4 The time course of atropine-induced reversal of carbachol stimulated ^{3}H-Ins P_3 accumulation.

The data from Fig. 3.2.1 for the 'decay phase' of ^{3}H-Ins P_3 induced by atropine is shown over an expanded time scale. (●) and (▽) represent carbachol-stimulated samples exposed or not exposed to 10 μM atropine. (■) Indicates non-atropine treated controls. Zero time is the point of atropine addition (i.e. 30 min. on Fig. 3.2.1). Results represent the mean ± SEM of 3 individual measurements in separate experiments.

From the data presented in Figures 3.2.1 to 3.2.4 there is no highly significant lag period in the decline of any inositol phosphate although individual experiments showed some variation in this respect. In two out of three cases a 20-30% decrease in Ins P_3 could be detected within 15 sec. of atropine addition while only in a single experiment did a fall in the accumulation of either Ins P_1 or Ins P_2 occur in this time. This may indicate that a very short delay occurs between the time at which Ins P_3 and subsequently other products begin to decline. However, the rapid half-lives of each Ins P_2 and Ins P_3 suggest that it would be difficult to define the length of this period. For a similar reason it is also difficult to measure the initial rate of hydrolysis of Ins P_2 and Ins P_3.

If the relative radiolabelling of each fraction is representative of mass changes in concentration as suggested above, then the initial rate at which each product declines from the steady-state phase following addition of atropine should give an estimate of the flux through that inositol phosphate pool. In an attempt to determine whether accumulations of lower phosphates can be accounted for solely by dephosphorylation of Ins P_3, the T½ for this fraction has been used as a crude approximation of this initial rate. However, as the T½ is calculated from the total decrease occurring in the presence of atropine over the 20 min. period, it will lead to an underestimate of flux since at later time points the initial rate of hydrolysis declines with decreasing substrate concentration. As the accumulation of Ins P_3 approaches a constant value after about 10-20 min., it is assumed that this reflects continuing and equal rates of production and breakdown. Figure 3.2.1 shows that the concentration of Ins P_3 is about 5,200 cpm/50 µl tissue while the T½ suggests a hydrolytic rate of 1,400 cpm/0.5 min. Where the breakdown of Ins P_1 to free inositol is inhibited by Li^+ ions the total flow of label through the Ins P_2 and Ins P_3 fractions should accumulate as Ins P_1. From the data presented in CH.2 and from results shown below, typical accumulations of ^{3}H-Ins P_1 resulting from stimulation with a maximal concentration of carbachol for 45 min. are of the order 20,000-30,000 cpm/50 µl tissue in the presence of 5 mM LiCl and when cortical slices are labelled at 1 µCi ^{3}H-inositol/50 µl tissue. Comparison of the results shown in Figures 3.1.1 and 3.2.1 indicates that the 5-fold increased concentration of label used in the current experiments results in 4-5 fold more highly labelled Ins P_3 (cf 1,200 cpm and 5,200 cpm/50 µl tissue). Therefore, under conditions where less ^{3}H-inositol was used, the hydrolytic rate for Ins P_3 would be approximately 320 cpm/0.5 min. From this the total turnover through this fraction in a 45 min. period would be around 29,000 cpm. On the basis of this crude calculation it would seem that there is no need to invoke a

direct breakdown of PtdIns to account for the substantial accumulations of Ins P_1 observed in response to muscarinic receptor activation in cerebral cortex. Similarly, it may be assumed that all the Ins P_2 accumulating can also arise through Ins P_3 dephosphorylation.

However, since immediately after agonist addition the accumulation of each inositol phosphate is lower than at later times, the assumption of a constant rate of Ins P_3 hydrolysis may lead to an overestimate of the turnover of this fraction but this is likely to be offset by the underestimate introduced by using T½ as equivalent to the initial rate of breakdown. A potentially more significant complicating factor in the above calculation is the comparison of data derived in the presence and absence of Li^+ ions. Several studies have indicated that lithium may inhibit not only Ins P_1 breakdown but also that of inositol polyphosphates, albeit less potently (Drummond et al. 1984; Thomas et al. 1984; Storey et al. 1984). In the current studies, where Li^+ has been included in incubations, this has been present at 5 mM, at which concentration maximal potentiations of muscarinic, alpha-1 and 5-HT receptor-mediated accumulations of ^{3}H-Ins P_1 in cerebral cortex were reported by Brown et al. (1984). Jacobson et al. (1985) have recently shown that 10 mM Li^+ potentiates carbachol-stimulated accumulations of both Ins P_1 and Ins P_2 in cerebral cortex but does not markedly influence that of Ins P_3. From this it would be anticipated that under the current conditions, accumulation and turnover of Ins P_3 would be essentially identical in the presence and absence of Li^+. However, this was further examined in experiments described in section 3.4.

Comparison of the current data for the relative rates of inositol phosphate hydrolysis with that obtained by Downes and Wusteman (1983) indicates some discrepancies. While in cerebral cortex accumulations of both Ins P_3 and Ins P_2 show a faster initial decline towards basal levels after blockade of muscarinic receptor stimulation, the rates for all three products are approximately the same in rat parotid glands. Together with the different patterns of product accumulation this may indicate distinct enzymes or isoenzymes are responsible for metabolism of inositol phosphates in the separate tissues. However, in both parotid glands and cerebral cortex the time required for stimulated concentrations of inositol phosphates to decline completely to basal levels after atropine addition is considerable. In the present experiments, elevated levels of each product were still evident up to 20 min. following receptor blockade. As discussed in 3.1 this portion of the response (10-15% in each case) may result from an increased specific activity of the products during the initial stimulation with agonist. Alternatively, a very much slower rate of dephosphorylation may result at low inositol phosphate concentrations.

If the latter is so, then this would indicate that inositol phosphates accumulating in response to low agonist concentrations would turnover at only a fraction of the rates estimated above, assuming a simple relationship between receptor occupancy and product accumulation. A third possibility is that atropine does not completely antagonise the response to carbachol. If the response to this agonist is mediated entirely by muscarinic receptors, this is unlikely since the relative affinities of carbachol and atropine show that at 10^{-5} M the antagonist is present at about a 100-fold excess of that concentration required to exactly block the action of 1 mM carbachol. Further, in rat parotid gland, where carbachol exhibits a several fold greater potency for stimulation of Ins P_1 formation (Jacobson et al. 1985; Ek and Nahorski, 1986), the use of identical drug concentrations results in complete inhibition of the response by atropine (Downes and Wusteman, 1983). However, as noted in CH.2, carbachol is not a pure muscarinic agonist and it is possible that a portion of the response is mediated via nicotinic receptors. However, from the discussion in CH.2 this seems improbable but may be worthy of further investigation.

A final potential explanation for the second slow phase of hydrolysis is that receptor-mediated inositol lipid cleavage gives rise to multiple positional isomers of separate inositol phosphates whose rates of breakdown differ. The recent observation that muscarinic receptor stimulation in rat parotid glands results in the formation of both Ins-1,4,5-P_3 and Ins-1,3,4-P_3 (Irvine et al. 1984a) suggests that this is probable, particularly as hydrolytic rates for the former Ins P_3 are much faster than for the latter (Irvine et al. 1985). If similar Ins P_3 heterogeneity occurs in cerebral cortex it might be predicted that the large proportion (> 50%) of total radioactivity in the Ins P_3 fraction which disappears very rapidly following atropine addition is accounted for by Ins-1,4,5-P_3 while the remainder is the more stable isomer. This possibility was further investigated at a later time since resolution of these trisphosphate isomers is not achieved using the relatively simple methods discussed in 2.4 and the results of this study are reported in CH.4 and CH.6.

3.3 Inositol phosphate and polyphosphate accumulations caused by stimuli other than muscarinic receptor agonists in cerebral cortex

Although the discussion of the results in sections 3.1 and 3.2 could potentially be complicated by the accumulation of separate inositol phosphate isomers within the fractions designated Ins P_1, Ins P_2 and Ins P_3, the data presented very clearly demonstrate that polyphosphoinositide hydrolysis results from muscarinic receptor

stimulation in cerebral cortex and also indicate that the primary substrate for this mechanism is PtdIns-4,5-P_2 since Ins P_3 is both rapidly formed and hydrolysed. It was therefore of interest to determine whether other putative CNS neurotransmitters which have previously been demonstrated to enhance inositol lipid turnover showed similar patterns of response.

Data presented in CH.2 showed that NA, 5-HT and HA stimulated the accumulation of ^{3}H-Ins P_1 in the presence of Li^+ ions in cerebral cortex and previous studies have demonstrated that these responses are mediated respectively by alpha-1 adrenoceptors (Kendall et al. 1985; Minneman and Johnson, 1984), 5-HT_2 receptors (Kendall and Nahorski, 1985a; Conn and Sandersbush, 1985) and histamine H_1 receptors (Daum et al. 1984). Kendall and Nahorski (1984) have demonstrated that K^+ ion and veratrine-induced depolarisation of cerebral cortical slices results in accumulation of inositol phosphates and very recently (Kendall and Nahorski, 1985b) that this response can be modulated by dihydropyridine Ca^{2+} channel agonists and antagonists. In contrast, these latter drugs do not affect similar responses induced by addition of exogenous agonists (Brown et al. 1984; Rooney and Nahorski, 1986), which may indicate that receptor and depolarisation-mediated responses operate through distinct mechanisms. The accumulation of each Ins P_1, Ins P_2 and Ins P_3 stimulated by the various agonists above and by K^+ ions were therefore compared over a series of experiments. Methods and tissue labelling conditions were exactly as in 3.1 to allow for direct comparison with the data for muscarinic receptor stimulation.

Figure 3.3.1 illustrates the results of a single experiment in which inositol phosphate accumulations in response to each 31 mM KCl (25 mM above medium KCl) and maximally effective concentrations of NA (3 x 10^{-4} M, Brown et al. 1984) and 5-HT (3 x 10^{-4} M, Kendall and Nahorski, 1985) were measured. In the interests of clarity responses to 5-HT have been omitted though these were approximately intermediate between NA and control (i.e. almost undetectable). In further experiments responses to K^+ and NA were consistent with those shown while both 5-HT and 1 mM HA reproducibly failed to stimulate detectable, increased accumulation of any product. Since the responses to the latter agonists as determined by accumulation of total ^{3}H-inositol phosphates in the presence of lithium ions are limited (see Figure 3.3.2) perhaps this observation is not surprising. In contrast the very small response to NA is unexpected since the Ins P_1 accumulation induced by this agonist in the presence of Li^+ ions approximates to 50% of that seen with carbachol (see Figure 3.3.2).

From Figure 3.3.1 it is clear that the pattern of inositol phosphate accumulation resulting from depolarisation contrasts sharply with that

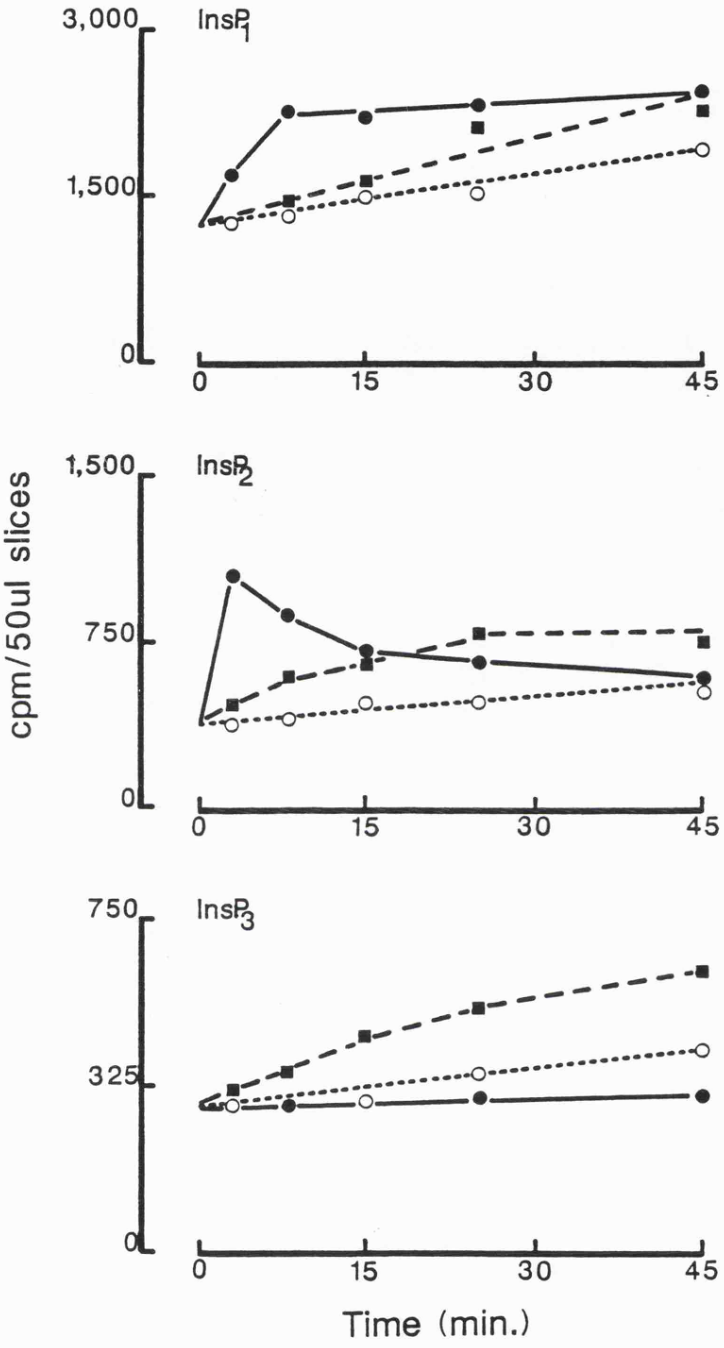


FIGURE 3.3.1 Time courses of the ^{3}H-Ins P(s) accumulations stimulated by NA and potassium ions in cerebral cortical slices labelled with ^{3}H-inositol.

Tissue incubations were performed as in 2.2 and ^{3}H-Ins P(s) extracted and separated as in 2.4. After 30 min. prior labelling with ^{3}H-inositol (1 μCi), 50 μl aliquots of brain slices were stimulated with 0.3 mM NA (■) or elevated extracellular [K^{+}] (25 mM) (●) or run as control (○) and further incubated for the times indicated. Results are from a single experiment but similar data were obtained on at least one other occasion.

induced by NA or that previously observed with carbachol. Most evident is the initial rapid accumulation of Ins P_2 which then declines over the subsequent time course. This is accompanied by an early increase in the concentration of Ins P_1 which achieves maximal levels within 10 min. and although no secondary decrease is immediately obvious, this is in part accounted for by an increased basal accumulation of Ins P_1 such that by 45 min., the percentage stimulation resulting from K^+ addition was only 129% compared to a maximum at 8 min. of 173%. Unlike responses to muscarinic or alpha-1 receptor activation there was no corresponding increase in the accumulation of Ins P_3. The rapid elevation of Ins P_2 concentration in the absence of a concomitant increase in Ins P_3 indicates that potassium-induced depolarisation may promote a phospholipase C catalysed hydrolysis of PtdInsP while the comparative rates of increased inositol phosphate accumulations and the later decrease in Ins P_2 both suggest that the Ins P_1 produced results from dephosphorylation of the former. However, as the methods used do not distinguish between different inositol phosphate isomers, the possibility of a phospholipase D-mediated cleavage of $PtdInsP_2$ to yield Ins-4,5-P_2 cannot be excluded. Although there is no evidence for such activity towards $PtdInsP_2$ in brain, a direct hydrolysis of PtdIns by a phospholipase D has been suggested for the action of fMet-Leu-Phe on human neutrophils (Cockcroft and Allan, 1984).

Under identical conditions to those currently used, Kendall and Nahorski (1986) have shown that, in the absence of a cholinesterase inhibitor, responses to K^+ depolarisation cannot be attributed to release of endogenous ACh. Equally, these responses are not blocked by tetrodotoxin nor by alpha-1, 5-HT or histamine H_1 receptor antagonists nor potentiated by peptidase inhibitors but are inhibited by depletion of extracellular $[Ca^{2+}]$. As responses to K^+ are also reversibly enhanced in the presence of dihydropyridine calcium channel agonists and antagonists, it seems probable that they may be mediated by an influx of Ca^{2+} through voltage-sensitive gates (Kendall and Nahorski, 1985b). The currently observed pattern of inositol phosphate accumulation resulting from K^+ depolarisation would seem to be consistent with an increase in $[Ca^{2+}]_i$ as several previous studies have shown very similar effects with Ca^{2+} ionophores. The addition of the calcium ionophore A23187 to ^{3}H-inositol labelled synaptosomal preparations of guinea-pig cerebral cortex resulted in decreased labelling of both PtdIns-4-P and PtdIns-4,5-P_2 but not PtdIns, with concomitant increased accumulation of Ins P_1 and Ins P_2 but not Ins P_3 (Griffin and Hawthorne, 1978). These authors concluded that the elevated intra-synaptosomal $[Ca^{2+}]$ induced by the ionophore promoted both phosphomonoesterase conversion of $PtdInsP_2$ to PtdInsP and subsequent phosphodiesterase hydrolysis of the latter. Kolesnick and

Gershengorn (1984) have also reported cleavage of PtdInsP to Ins P_2 in response to A23187 in pituitary GH_3 cells and since this did not affect thyrotropin releasing hormone (TRH) stimulation of PtdInsP_2 hydrolysis, they considered this as evidence that the latter event was not secondary to an increased $[Ca^{2+}]_i$ in these cells. Addition of A23187 to mouse peritoneal macrophages also promotes accumulation of Ins P_2 which is accompanied by a major loss of ^{3}H-inositol prelabelled PtdIns and, to a smaller extent, of polyphosphoinositides, suggesting that elevated $[Ca^{2+}]_i$ stimulates both phosphorylation of PtdIns and subsequent cleavage of PtdInsP (Emilsson and Sundler, 1984). In contrast, Akhtar and Abdel-Latif (1980) have reported increased accumulation of Ins P_3 in addition to Ins P_1 and Ins P_2 in response to calcium ionophore in rabbit iris smooth muscle. However, it now seems probable that this effect was secondary to a release of endogenous NA as it could be blocked by the alpha-1 receptor antagonist prazosin (Akhtar and Abdel-Latif, 1984 and Abdel-Latif *et al*. 1985).

As the influx of calcium induced by A23187 produces in several cell types a very similar pattern of inositol phosphate accumulation to that seen after K^+ depolarisation of cerebral cortical slices, the current data would support the view of Kendall and Nahorski (1985b) that the latter response is mediated by extracellular Ca^{2+}. The increase in $[Ca^{2+}]_i$ resulting from addition of A23187 to pituitary GH_3 cells has also been reported to increase the concentration of free arachidonic acid (Kolesnick and Gershengorn, 1984), while the same stimulus promoted accumulation of glycerophosphoinositol and lysophosphatidylinositol in macrophages (Emilsson and Sundler, 1984), presumably indicating Ca^{2+} activated phospholipase A_1 and A_2 activities. Since deacylating phospholipases, active at least towards PtdIns, are present in brain (see Dawson, 1982), a similar calcium sensitivity of these could indicate that part of the response to K^+ is mediated by release of arachidonate or its metabolites. However, this is unlikely as inhibitors of arachidonate metabolism do not affect inositol phosphate accumulations in response to K^+ in brain (Kendall, 1986).

Since Figure 3.3.1 illustrates that the time course and nature of the response to potassium are clearly distinct from that observed following agonist addition, the probable mediation of the former by extracellular calcium may suggest that the hydrolysis of PtdInsP_2 demonstrated in 3.1 for muscarinic receptor stimulation and the very much smaller response to NA shown in Figure 3.3.1 are not secondary to changes in $[Ca^{2+}]_i$. The additive responses to NA and A23187 reported by Brown *et al*. (1984) may have similar implications. However, in the current experiments, responses to K^+ were measured at 25 mM above medium concentration (i.e. a total of 31 mM K^+) as a previous study (Batty *et al*. 1985a) had demonstrated this

was maximal in stimulating total inositol phosphate accumulation in the presence of Li^+ ions. In contrast, maximal potentiation of the response to K^+ by the voltage-sensitive calcium channel agonist BAY-K-8644 is observed at a total medium potassium concentration of 12-18 mM and becomes submaximal at higher concentrations (Kendall and Nahorski, 1985b) while preliminary data (Rooney, Baird and Nahorski, unpublished) indicate that the pattern of accumulation of separate inositol phosphates at the lower potassium levels may differ from that illustrated in Figure 3.3.1. Therefore, it is possible that the similar patterns of response observed on K^+ depolarisation of brain slices and on addition of Ca^{2+} ionophore to other tissues may simply be coincidental with the concentration of K^+ ions used here, rather than a general feature of the response to latter stimulus. Thus, it may be invalid to assume that other concentrations of K^+ ions or different stimuli elevating $[Ca^{2+}]_i$ will result in increased phosphoinositide metabolism via a common pathway. Rather it might be anticipated that the varying extents and subcellular localisations of altered $[Ca^{2+}]_i$ would produce differential changes dependent on the varying sensitivity and localisation of distinct enzymes. Therefore, the observation above that depolarisation-induced responses, probably mediated by extracellular Ca^{2+}, differ from responses to receptor activation does not necessarily imply that the latter are not secondary to increased $[Ca^{2+}]_i$.

From observations in other tissues (see above) it would seem that further elucidation of the mechanism of depolarisation-induced phosphoinositide metabolism would be facilitated by measurement of both inositol lipids and phosphates since the primary effects of elevated $[Ca^{2+}]_i$ caused by ionophore appear to be on lipid interconversions in addition to diesteratic cleavage. However, in whole cells and particularly in heterogenous tissues, effects on inositol lipid labelling are likely to be complex as calcium influences many of the metabolic pathways. In brain calcium inhibits synthesis of PtdIns both through CDP-diacylglycerol phosphatidate:myo-inositol transferase and inositol headgroup exchange (see CH.2), reduces the activity of both PtdIns kinase (Kai et al. 1966) and PtdInsP kinase (Kai et al. 1968), and stimulates $PtdInsP_2$ phosphatase (Griffin and Hawthorne, 1978) and, at mM concentrations, also stimulates the soluble phosphoinositide phosphodiesterase of rat brain (Irvine et al. 1984b). Depolarisation of rat cerebral cortical slices with K^+ markedly reduces ^3H-inositol labelling of lipid, an effect partially reversed by depletion of extracellular calcium (Kendall and Nahorski, 1986) and presumably resulting from net actions of this ion on the above metabolic steps. Possibly, understanding of the physiological significance and initial

mechanism of depolarisation-induced phosphoinositide metabolism would be facilitated by studying inositol phosphate accumulations in response to stimuli over very much more rapid time courses than currently used, potentially by employing electrical stimulation as suggested by Michell (1982). Previous studies have used this stimulus (see Hawthorne and Pickard, 1979) although assay of inositide metabolism was largely by measurement of altered lipid labelling and interpretation of such data may be complex.

It would also be of future interest to determine in what way, if at all, depolarisation modifies responses to receptor activation and to establish the use of common or separate lipid pools by the two mechanisms. The former question has recently been approached by Eva and Costa (1986). These authors demonstrated a potassium ion facilitation, specifically of muscarinic receptor-mediated phosphoinositide hydrolysis in rat hippocampal slices. This did not result from an increased efficiency of agonist-receptor interaction and was not mediated by Ca^{2+} or the activation of Na^{+} channels but was suggested to arise from a facilitation by K^{+} ions of the interaction between the receptor and a GTP binding protein which other recent studies (Cockcroft and Gomperts, 1985; Litosch et al. 1985) indicate couples receptor activation to PLC hydrolysis of $PtdInsP_2$. Clearly responses to depolarising stimuli may be complex and further studies will be required to clarify the mechanisms involved.

Two further interesting features of the data presented in Figure 3.3.1 are the comparative magnitude of the inositol phosphate accumulations to NA and K^{+} and the apparent rapid desensitisation of the latter response. A number of factors may account for the second phenomenon:

i) The pool of phosphoinositide available to the depolarising stimulus may be very limited, and

ii) re-synthesis of a very small pool of lipid hydrolysed by phosphodiesterase in response to K^{+} may be inhibited by an increased $[Ca^{2+}]_i$ which may simultaneously deplete polyphosphoinositide concentrations by also promoting their breakdown via phosphomonoesterases.

iii) If, as suggested, the response to K^{+} is mediated by an influx of Ca^{2+} through voltage operated channels, rapid desensitisation of this gating mechanism could result in only a transient response. As responses to higher K^{+} concentrations show submaximal potentiation by BAY-K-8644 (Kendall and Nahorski, 1985b) this seems possible. However, as the enhancement of the K^{+} response by the calcium channel agonist is completely reversed by the antagonist PN-205-033 but the response to K^{+} alone only partially reversed, factors other than Ca^{2+} influx could be involved in the response observed here.

Secondly, as commented above, the extent of each inositol phosphate accumulation promoted by NA as shown in Figure 3.3.1 is unexpectedly small. When responses to NA and carbachol are compared in the presence of Li^+ ions, that to the former agonist is about 50% that to the latter, while in the absence of Li^+ responses to NA were barely detectable. This observation was briefly further examined by comparing responses to a variety of stimuli increasing phosphoinositide hydrolysis in cerebral cortex by measuring total ^{3}H-inositol phosphate accumulation (primarily Ins P_1) both in the presence and absence of 5 mM LiCl according to the methods described in 2.2. The results of this study are illustrated in Figure 3.3.2. Stimulation in each case was for 45 min. since this falls within the linear phase of responses at least to carbachol, NA, HA and 5-HT (see CH.2) although it must be recalled that in the absence of Li^+ ions maximum accumulation of inositol phosphates may result over a much shorter period, particularly with stimuli such as potassium. Table 3.3.1 allows further comparison of the stimulated accumulations of ^{3}H-Ins P_1 in the absence and presence of Li^+. As drugs were added to incubations in either Krebs buffer or in absolute ethanol (EtOH) distinct controls were run as indicated.

From Figure 3.3.2 it is apparent that in the presence of Li^+ ions the responses to agonists show a similar order of effectiveness to that previously observed, carbachol > NA > 5-HT $\geqslant$ HA, while responses to K^+, A23187, physostigmine and ionophore + eserine are respectively 318%, 209%, 119% and 279% of respective controls (see Table 3.3.1). The potentiation of the response to A23187 by eserine at least in the presence, though less clearly so in the absence of Li^+, indicates that part of the combined action of these drugs can be attributed to the release of endogenous ACh. It is not clear from the data whether a similar effect accounts for the action of the calcium ionophore alone but this seems unlikely since atropine does not antagonise the response to K^+ alone (Kendall and Nahorski, 1986). The responses to NA and K^+ are of similar magnitude in the presence of Li^+ while in the absence of this ion the only stimuli promoting significant Ins P_1 accumulation above relative controls are carbachol and, to a lesser extent, K^+, A23187 and ionophore + eserine. In agreement with previous observations responses to NA, 5-HT and HA were not apparent without added LiCl.

Since K^+ and A23187 show similar fold stimulations over basal values in the absence of Li^+ it is surprising that responses to the latter do not show a larger potentiation by Li^+ ions. In this respect the observation that A23187 promotes accumulation of glycerophosphoinositol (GroPIns) in macrophages (Emilsson and Sundler, 1984) is of interest since a similar action in brain could account for the relative insensitivity of this

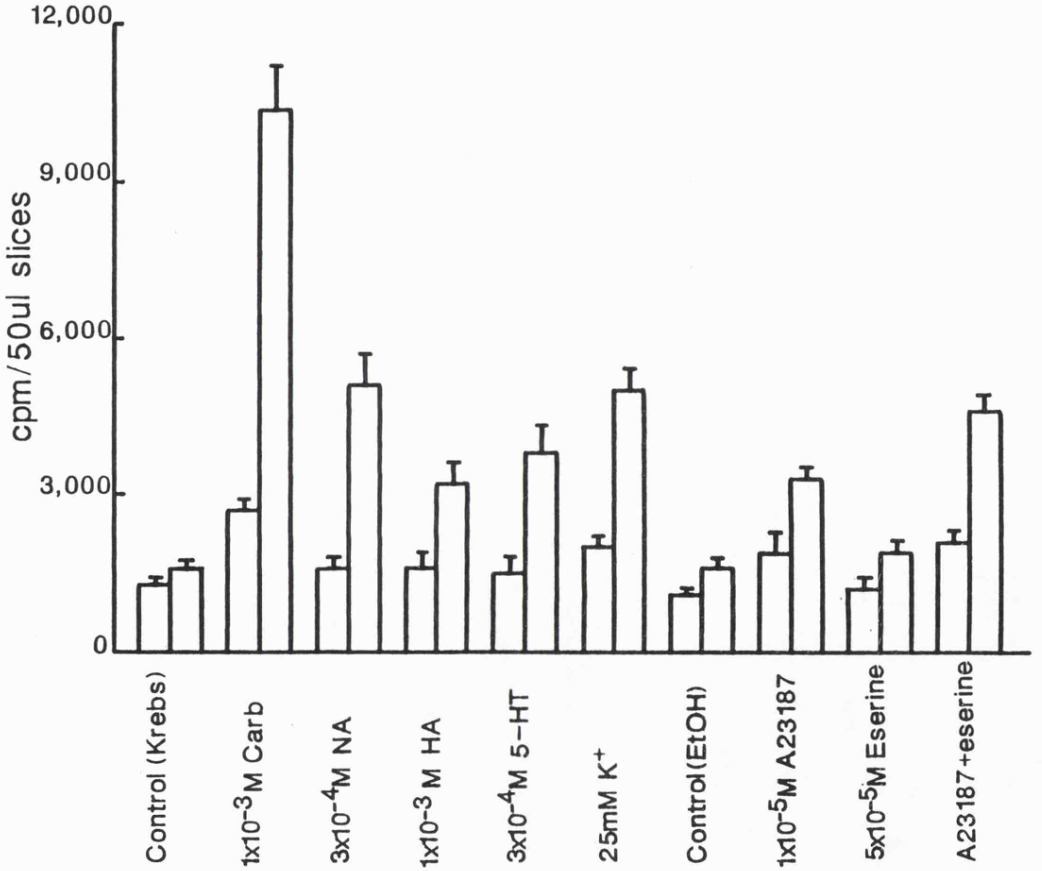


FIGURE 3.3.2 The potentiation by 5 mM LiCl of the ^{3}H-Ins P(s) accumulations promoted by different stimuli in ^{3}H-inositol labelled brain slices.

Tissue incubations, extraction and assay of ^{3}H-Ins P(s) were each performed as in 2.2. After 30 min. prior labelling with ^{3}H-inositol (1 μCi), 50 μl aliquots of brain slices were exposed to the stimuli indicated for a further 45 min. Where present (right hand of each pair of bars) 5 mM LiCl was added together with ^{3}H-inositol. Results indicate the mean ± SEM of a minimum of 3 experiments each run in triplicate.

Table 3.3.1 Comparative accumulations of ^{3}H-Ins P_1 in presence/absence 5 mM LiCl

Stimulus	% Stimulation	
	% $\frac{\text{Agonist - Li}^+}{\text{Control - Li}}$	% $\frac{\text{Agonist + Li}^+}{\text{Control + Li}^+}$
Control (Krebs)	100	100
Carbachol (1 mM)	213	674
NA (0.3 mM)	127	320
HA (1 mM)	124	210
5-HT (0.3 mM)	121	243
K^+ (25 mM)	155	318
Control (EtOH)	100	100
A23187 (10 μM)	169	209
Eserine (50 μM)	111	119
A23187 + eserine (as above)	183	279

Table 3.3.1 compares the accumulation of total ^{3}H-inositol phosphate (predominantly Ins P_1) in response to a variety of receptor and other stimuli in cerebral cortical slices. Tissue was incubated, labelled with ^{3}H-inositol and challenged with stimulus, then the ^{3}H products extracted and assayed by anion exchange chromatography as in 2.2. Results, expressed as shown (see text), represent the mean of at least three experiments performed in triplicate. Further experimental conditions are indicated in the text.

stimulus to Li^+. The assay conditions used in the current experiments would not distinguish ^{3}H-GroPIns from ^{3}H-Ins P_1, thus it is possible that a proportion of the total radiolabelled fraction accumulating in response to A23187 is accounted for by the former product. A potential alternative explanation is as discussed below.

The most notable features of the data presented in Figure 3.3.2 and Table 3.3.1 are the relative responses to NA and carbachol. The most sensitive means of establishing the potentiation of separate responses by Li^+ is by calculating the accumulation of ^{3}H-product above control values each in the presence and absence of this ion and expressing this as a relative % for each stimulus

$$\text{(i.e. } \left[\frac{\text{stimulated + Li - control + Li}}{\text{stimulated - Li - control - Li}}\right] \times 100\text{)}.$$

The respective values for carbachol and NA suggest 6.8 and 11.7 fold potentiations. However, calculation of the sensitivity of various responses to Li^+ in this way is likely to be inaccurate given the negligible responses to most stimuli, including NA, in the absence of Li^+. For this reason data has been expressed as indicated in Table 3.3.1. From this, it is nevertheless apparent that in the presence of Li^+ the response to NA is approximately 48% of that to carbachol, while Figure 3.3.2 clearly illustrates that there is no significant response to the latter agonist in the absence of Li^+. None of the other agonists used show such an evident discrepancy as NA. This unexpected effect could be accounted for if the breakdown of varying proportions of the ^3H-product accumulating in response to muscarinic and alpha-1 receptor stimulation were not sensitive to 5 mM LiCl, since this could potentially lead to a greater under-estimate of the carbachol response than of that to NA. Storey et al. (1984) have demonstrated that hydrolysis of Ins-1,4-P_2 by soluble enzymes of rat liver can proceed either by removal of the 1 or 4 monoester phosphate groups. More recently, Siess (1985) has used high pressure liquid chromatography (hplc) to show that both Ins-1-P and Ins-4-P accumulate in platelets and that the relative proportions of these and potentiations of each by Li^+ varies according to the stimulus. If distinct inositol monophosphate isomers whose hydrolyses are differentially affected by Li^+ ions are produced in separate proportions in response to NA and carbachol in cerebral cortex, then the effect noted above could be explained. This complex situation is likely to be compounded by the differential effects of Li^+ on the dephosphorylation of inositol polyphosphates which has been indicated by numerous studies (Drummond et al. 1984; Thomas et al. 1984) and by the potential for further routes of inositol phosphate metabolism which the very recent description of Ins-1,3,4-P_3 implies (Irvine et al. 1984a), and both Michell (1986) and Nahorski et al. (1986) have commented on the fallibility of assays employing LiCl.

Although Brown et al. (1984) have shown that 5 mM LiCl maximally potentiates the accumulation of total ^3H-inositol phosphates in response to various receptor agonists in cerebral cortex, in the light of the above observations this seemed worthy of more detailed re-examination, extending to the study of potential actions of Li^+ on the accumulation of separate inositol phosphates. These experiments are discussed in detail in section 3.4.

3.4 The effects of lithium on muscarinic receptor-mediated accumulation of inositol phosphates (see 6.4 also)

In experiments described in previous sections where LiCl has been included in tissue incubations, this has been present at 5 mM for reasons given above. In contrast, studies thus far of inositol polyphosphate accumulations have been conducted in the absence of this ion to avoid potential complications arising from effects of lithium on steps other than Ins P_1 dephosphorylation as are evident in hepatocytes (Thomas et al. 1984), pituitary GH_3 cells (Drummond et al. 1984) and platelets (Vickers et al. (1984). As suggested in 3.2 such effects could invalidate the calculations made comparing Ins P_1 accumulation in the presence of LiCl with flux through Ins P_3 in its absence. However, differential sensitivities of distinct inositol phosphate phosphomonoesterases to lithium could also be useful since these could potentially be exploited to further investigate the sequence and metabolic pathways involved in inositol phosphate formation. As the sensitivity to Li^+ of inositol monophosphate(s) accumulation stimulated by carbachol may, from 3.3, be of particular interest and because muscarinic receptor-mediated accumulations of polyphosphates are more amenable to study by their magnitude than are those mediated by other receptors in brain (3.3), this system was chosen for the evaluation of the effects of lithium.

In an initial series of experiments the observations of Brown et al. (1984) were extended to a detailed comparison of the dose-related effects of LiCl on each Ins P_1, Ins P_2 and Ins P_3 accumulations in the presence and absence of a maximally stimulating concentration of carbachol. Tissue incubations were performed as in 2.2 such that LiCl, where included, was added together with ^{3}H-inositol 30 min. prior to agonist. After addition of 1 mM carbachol or Krebs buffer, incubations were continued for 45 min. before termination with TCA. Subsequent sample analyses were as detailed in 2.4. The concentration-effect curves for LiCl potentiation of carbachol and control responses are illustrated in Figure 3.4.1.

In the absence of LiCl, carbachol stimulated respective accumulations of Ins P_1, Ins P_2 and Ins P_3 by 421%, 361% and 390% relative to basal values. Both these fold stimulations and the absolute accumulation of radioactivity within each fraction are consistent with those shown in Figure 3.1.1. Increasing lithium concentrations produced a dose-related potentiation of stimulated Ins P_1 accumulation with maximal and half-maximal effects at 3-5 mM and 0.5 mM, respectively, representing 6-7 and 3-4 fold enhanced responses. At concentrations of LiCl greater than 30 mM stimulated, though not control accumulations of Ins P_1 became sub-maximal. Both stimulated and control accumulations of Ins P_2 showed similar though less potent effects of LiCl, potentiation of either

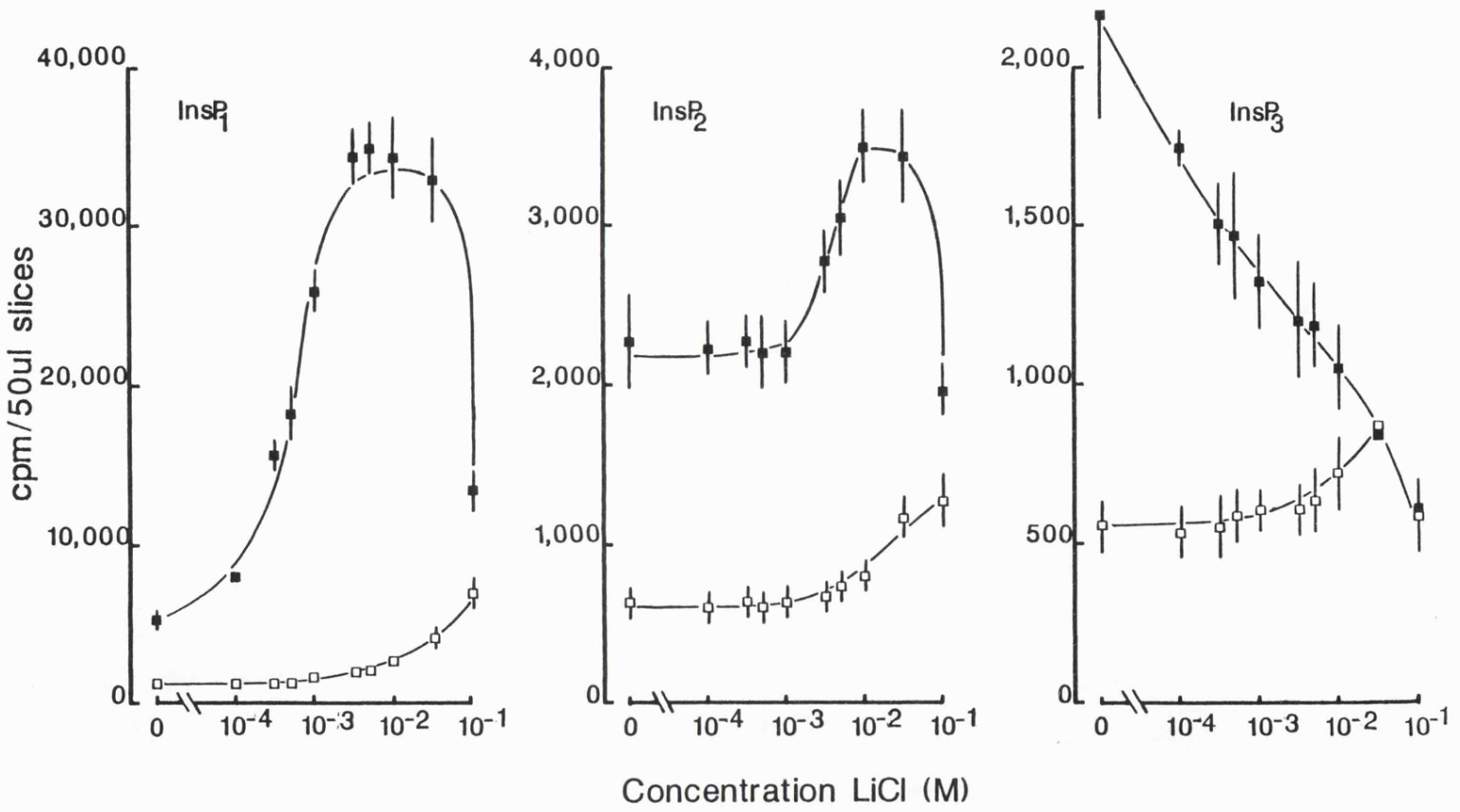


FIGURE 3.4.1 Dose-related effects of LiCl on control and carbachol-stimulated accumulations of ^{3}H-Ins P_1, ^{3}H-Ins P_2 and ^{3}H-Ins P_3 in ^{3}H-inositol labelled brain slices.

After 60 min. pre-incubation, 50 µl aliquots of tissue were incubated for 30 min. in the presence of the indicated concentrations of LiCl and 1 µCi ^{3}H-inositol. Samples were then incubated for a further 45 min. in the absence (□) or presence (■) of 1 mM carbachol before extraction and separate assay of ^{3}H-Ins P(s) as in 2.4(d)iii. Results show the mean ± SEM of 3 separate experiments.

requiring in excess of 1 mM. The maximum enhancement by Li^+ of the stimulated Ins P_2 response, occuring at 10-30 mM, was only about 1.5 fold while the EC_{50} for lithium was about 4 mM. High concentrations of lithium showed very similar effects to those seen on Ins P_1.

In marked contrast to these potentiating effects on Ins P_1 and Ins P_2, LiCl potently and dose-dependently attenuated carbachol stimulated, but not control accumulations of Ins P_3. This effect was clearly apparent at concentrations as low as 0.1-0.3 mM and half-maximal at 1 mM. Conversely, basal Ins P_3 accumulation was enhanced to a small extent between 1 and 30 mM, while higher lithium concentrations reduced both stimulated and control levels of this polyphosphate.

These discrepancies between the effects of LiCl on stimulated and control Ins P_3 accumulation suggest that the attenuation of responses to carbachol results from the combined presence of stimulus and Li^+ ions. In turn, this implies that in addition to the $[Li^+]$, the magnitude of this effect may be dependent on either the time of exposure to agonist and/or agonist dose. To investigate this possibility, time courses for the accumulation of inositol phosphates were defined in the presence of both 1 mM carbachol and 5 mM LiCl. In these experiments, to allow co-incubation of agonist and lithium over approximately the same periods, the latter was not included during the prelabelling phase with ^{3}H-inositol but was added only 5 min. before carbachol. Subsequent incubations were continued for the times indicated.

Figure 3.4.2 illustrates the effects of 5 mM LiCl on the time courses of control and stimulated accumulations of each inositol phosphate while Figure 3.4.3 shows the accumulation of each product due to carbachol above basal values to allow a simplified comparison of the current results with those obtained in the absence of lithium in 3.1. It should be pointed out that since paired comparisons for carbachol stimulation in the presence and absence of LiCl were not run, some inter-experimental variation might be suggested to account for some of the effects observed. This is unlikely because when at a later stage (see CH.6) some of the questions raised here were re-evaluated, the paired data then obtained showed a very close correlation with that presented in Figure 3.4.2.

In the presence of lithium a rapid, increased accumulation of each inositol phosphate was observed in response to carbachol although, as anticipated from Figure 3.4.1, the extent of Ins P_1 and Ins P_2 accumulations was very much greater than in the absence of this ion. However, Ins P_3 accumulation achieved a peak value by 5 min. then immediately began to decline, reaching a new steady concentration between 30 and 60 min. which from Figure 3.4.2 is approximately 60% of the initial maximum. It will be noted from comparison of Figures 3.1.1 and 3.4.2 that

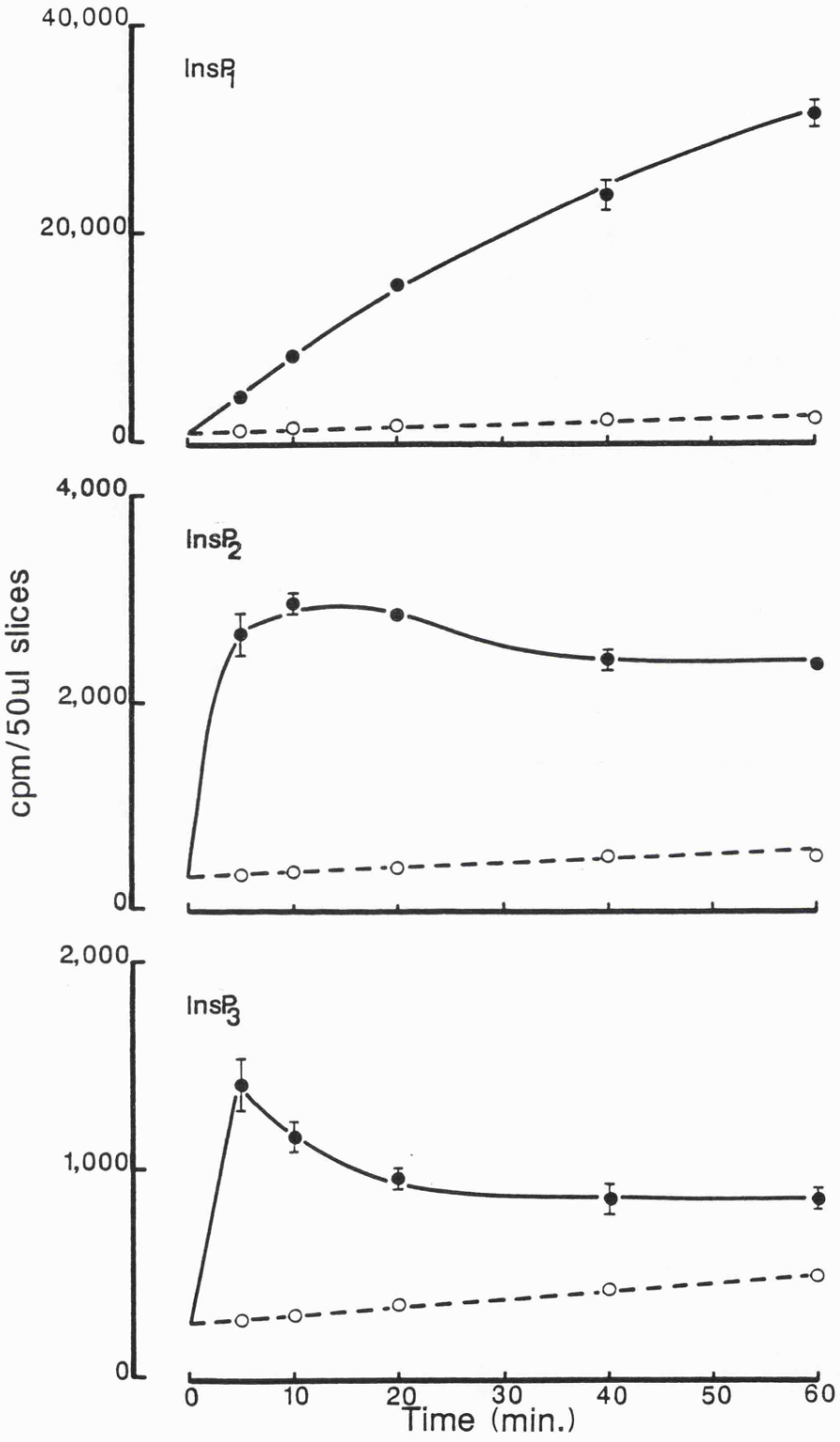


FIGURE 3.4.2 Time courses of the ^{3}H-Ins P_1, ^{3}H-Ins P_2 and ^{3}H-Ins P_3 accumulations in control and carbachol-stimulated brain slices in the presence of 5 mM LiCl.

Tissue incubations were performed as in the legend to Fig. 3.4.1 except that 5 mM LiCl was added to all samples only 5 min. prior to agonist and reactions were continued for the various times indicated. (○) and (●) show responses in the absence and presence of 1 mM carbachol. Results represent the mean ± SEM of 3 separate experiments, where not shown SEM is within the symbol.

basal accumulations of each product showed a more marked tendency to increase across the time course in the presence of LiCl. When this is taken into account for both sets of data (see Figure 3.4.3) the maximum attenuation of trisphosphate accumulation is found to be around 70%. The dose-response data illustrated in Figure 3.4.1 show a similar result at 5 mM LiCl.

Both Figures 3.4.2 and 3.4.3 show that the pattern of stimulated Ins P_3 accumulation in the presence of lithium is qualitatively paralleled by that of Ins P_2 with only a slight delay. This may imply that a proportion of Ins P_2 arises through Ins P_3 dephosphorylation although the maximum decline from peak Ins P_2 values is only 30%. In contrast, Ins P_1 accumulation appears to continue linearly at least over the first 20 min. after carbachol addition but subsequently there appears to be a small decrease in this rate, however this secondary change was not confirmed in later experiments (see CH.6).

The comparative time course data for the accumulation of the separate inositol phosphates stimulated by carbachol in the presence and absence of LiCl together with the dose-response data for this ion raise a number of interesting points relating to the mechanism of muscarinic receptor-mediated phosphoinositide metabolism. It is evident that the potent attenuating action of lithium is time-dependent and from this it would be predicted that over shorter periods of stimulation and exposure to LiCl the relationship between potentiation of responses and [LiCl] could be quite different. This possibility was also investigated in order to further clarify the time and dose-dependency of the effects observed and it is useful to compare the results of this study with those above before considering the implications of this data in more detail.

In a third series of experiments dose-response curves to LiCl in control and 1 mM carbachol-stimulated inositol phosphate accumulations were established in which lithium was added just prior to agonist and stimulation continued for only 15 min. Although a shorter period of exposure to carbachol would have been preferable for this study this was precluded by practical problems associated with handling multiple tissue samples. Figure 3.4.4 summarises the results of these experiments. From this it is clear that the dose related potentiations of both stimulated Ins P_1 and Ins P_2 accumulations by LiCl were very similar to those seen after more prolonged stimulation. Maximal and half-maximal potentiations of stimulated Ins P_1 and Ins P_2 accumulations occurred respectively at 0.25 mM and 5 mM and 1.5 and 10-30 mM, indicating a moderately higher potency on both responses than previously observed. However, stimulated Ins P_3 accumulations exhibited a biphasic dose relationship. Concentrations upto 1 mM lithium resulted in a decreased Ins P_3

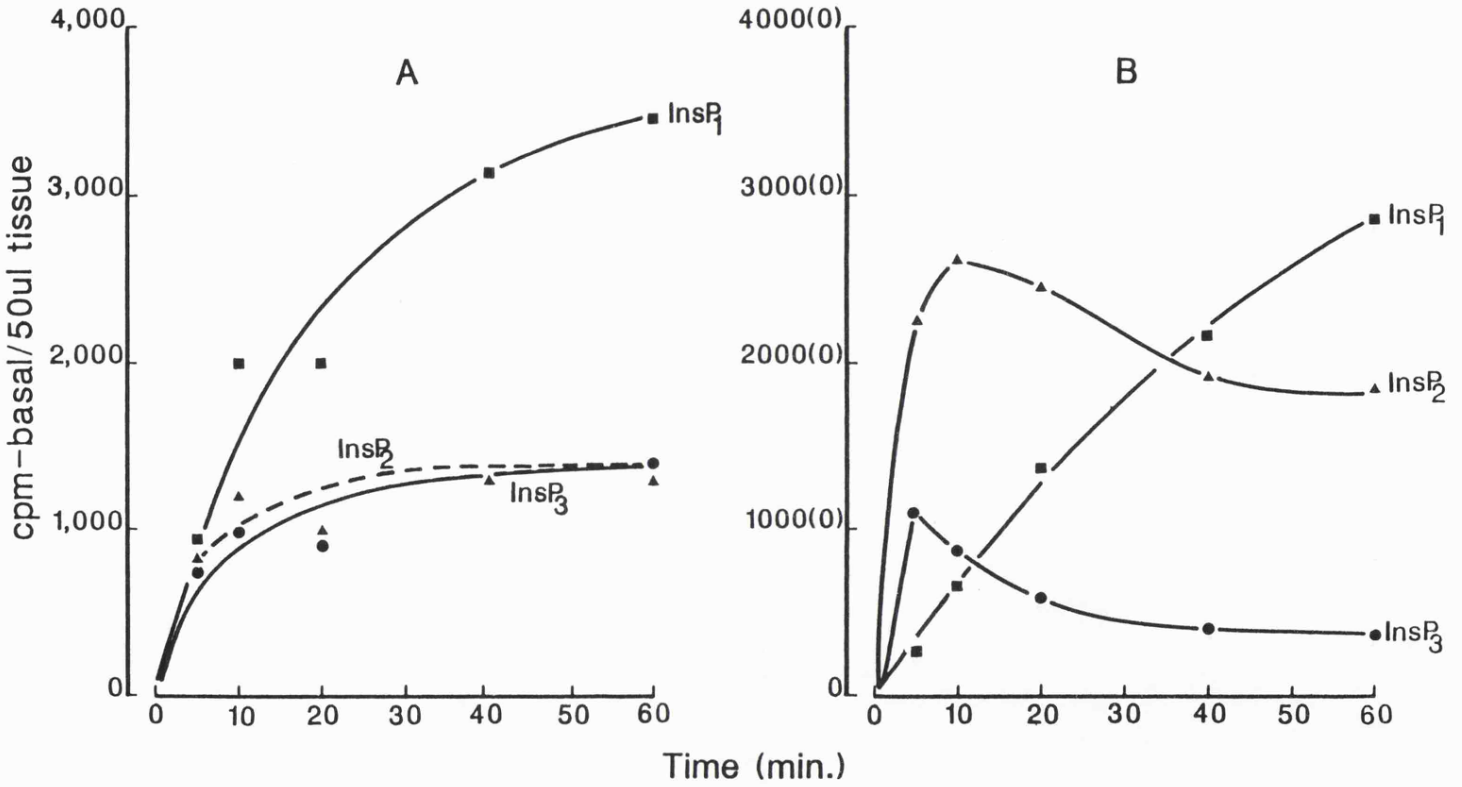


FIGURE 3.4.3 Comparison of the time courses of the ^{3}H-Ins P_1, ^{3}H-Ins P_2 and ^{3}H-Ins P_3 accumulations stimulated by carbachol in brain slices in the absence (A) and presence (B) of 5 mM LiCl.

The data from Figs. 3.1.1 and 3.4.2 has been expressed as the cpm accumulated in each inositol phosphate fraction above basal in 1 mM carbachol-stimulated tissue in order to illustrate the effects of 5 mM LiCl on the time courses of the response to agonist. For Fig. B the Ins P_1 scale = 0-40,000 while the Ins P_2/Ins P_3 scale = 0-4,000.

accumulation with a maximal attenuation of approximately 30%, while at higher lithium concentrations Ins P_3 showed a tendency to increase towards the values observed in the absence of lithium. Basal labelling of each inositol phosphate was unaffected by LiCl under these conditions except for a minor effect at the maximum concentration used.

Although the dose-response data presented in Figures 3.4.1 and 3.4.4 show some variation, both indicate differential effects of lithium on the stimulated accumulation of the separate inositol phosphates, suggesting dephosphorylation of these by distinct enzymes in cerebral cortex as suggested in hepatocytes (Thomas et al. 1984). Again both sets of data relating to the dose-dependent effects of lithium on stimulated Ins P_1 accumulation are in close agreement with similar actions reported in brain (Berridge et al. 1982; Brown et al. 1984), hepatocytes (Thomas et al. 1984) and pituitary GH_3 cells (Drummond et al. 1984) and with a detailed study of Ins P_1 phosphomonoesterase isolated from rat brain (Hallcher and Sherman, 1980). These data are also consistent with these earlier studies in identifying Ins P_1 as the major product accumulating in brain in the presence of lithium. However, there is an interesting contrast with the results of Minneman and Johnson (1984) who suggest that following alpha-1 receptor stimulation in rat cerebral cortex, the predominant inositol phosphate accumulating in the presence of lithium is Ins P_2, with a relative order of accumulation Ins P_2 > Ins P_3 > Ins P_1. These observations appear contrary to the effects of lithium in all other tissues although from Figure 3.3.1 it is apparent that stimulation of cerebral cortical slices with NA in the absence of this ion resulted in more readily detectable changes in each Ins P_2 and Ins P_3 than in Ins P_1. Since efficient extraction of inositol polyphosphates requires acidic conditions, the preferential accumulation of Ins P_2 and the use of a chloroform:methanol extraction method could account for the absence of a discernible response to NA without added lithium as noted in Figure 3.3.2. However, this seems unlikely as Minneman and Johnson (1984) have used a similar neutral extraction in their identification of the inositol metabolites accumulating in response to alpha-1 receptor stimulation and it seems probable that these latter observations may be a consequence of inadequate chromatographic procedures.

From Figures 3.4.1 and 3.4.2 it is also evident that either short or more prolonged exposure of tissue to carbachol resulted in smooth monophasic, dose-related potentiations of Ins P_1 accumulation by lithium. If, as considered in 3.3, muscarinic receptor stimulation in cerebral cortex gives rise to distinct, isomeric Ins P_1(s) the hydrolysis of which is differentially affected by lithium, this would not be anticipated. If such a phenomenon were to account for the discrepancy noted between

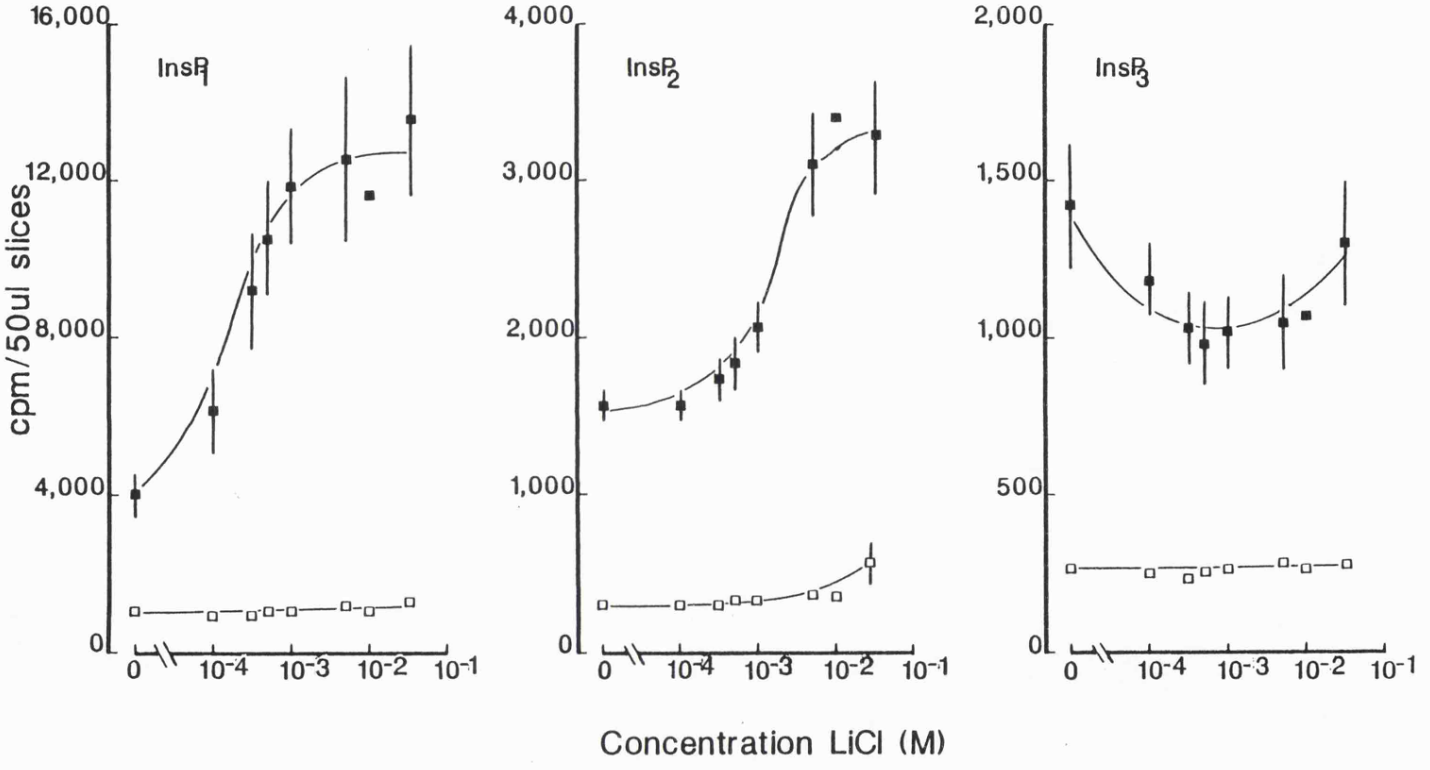


FIGURE 3.4.4 Dose-related effects of LiCl on control and carbachol-stimulated accumulations of ^{3}H-Ins P_1, ^{3}H-Ins P_2 and ^{3}H-Ins P_3 in ^{3}H-inositol labelled brain slices.

After 60 min. pre-incubation, 50 µl aliquots of tissue were incubated for 30 min. with 1 µCi ^{3}H-inositol. Samples were then incubated for a further 15 min. after the addition of the concentrations of LiCl indicated and either in the presence (■) or absence (□) of 1 mM carbachol. Ins P(s) were extracted and assayed as previously. Results represent the mean ± SEM of 3 experiments.

responses to carbachol and NA in the absence and presence of lithium, then from previous results, the response to the former agonist would be expected to be further potentiated by lithium concentrations in excess of 5 mM. Since this is clearly not so, it may be assumed either that the Ins P_1 formed in response to carbachol is of a single isomeric species (Ins-1-P) or that hydrolysis of other monophosphates is either insensitive to lithium or shows an almost identical sensitivity to that for Ins-1-P hydrolysis and that a different explanation accounts for the relative responses to NA and carbachol. Given the results of Storey et al. (1984) and Siess (1985), the former possibility seems unlikely. Further, Sherman et al. (1985) have demonstrated that acute in vivo administration of LiCl results in increased accumulation of Ins-1-P and Ins-4-P in rat brain and that these effects can be partially reversed by treatment with atropine, implying a muscarinic cholinergic component in these responses. The accumulation of Ins-4-P observed in this study was very much smaller than that of Ins-1-P which may indicate either a lesser production or a lower sensitivity of the breakdown of the former to lithium. If the latter is correct then the failure of the current dose-response data to reveal multiple components of the potentiated Ins P_1 accumulations may arise from inadequate definition of this relationship at sufficiently detailed lithium concentrations. The preliminary results suggesting apparent differential sensitivity of muscarinic and alpha-1 receptor responses to potentiation by lithium and the results of Sherman et al. (1985) clearly indicate that use of more sophisticated chromatographic techniques capable of resolving isomeric inositol phosphates may yield valuable data concerning the pathways of inositol polyphosphate metabolism in brain.

The currently observed effects of lithium on Ins P_2 and Ins P_3 accumulation are also of interest in this respect. Those on Ins P_2 are similar to effects reported in pituitary GH_3 cells (Drummond et al. 1984) and hepatocytes (Thomas et al. 1984). However, in the latter study stimulated Ins P_2 potentiation showed a higher sensitivity to lithium and was markedly biphasic with decreased Ins P_2 accumulations being apparent at in excess of 3-5 mM LiCl. The latter effect was attributed to effects of lithium on Ins P_3 phosphatase because stimulated Ins P_3 accumulation is also potentiated in these cells. These observations in hepatocytes are in striking contrast to those illustrated in Figures 3.4.1 and 3.4.3 which very clearly demonstrate that lithium suppresses stimulated Ins P_3 accumulation in brain. It should be noted however, that both previous studies in GH_3 cells and hepatocytes have used cells labelled to isotopic equilibrium with ^3H-inositol and the possibility arises that in the current study effects on Ins P_3 accumulation may be related to the labelling protocol used as discussed below. From this point of view a

pulse labelling approach may have been more useful in the present experiments, although previous attempts at such a procedure have indicated a considerable pool of free ^{3}H-inositol remains associated with brain slices even after a prolonged 'chase' with cold inositol (see CH.2). A similar problem has been noted in superior cervical ganglion (Bone et al. 1984).

Comparison of Figures 3.4.1 and 3.4.4 shows that prolonged exposure to carbachol results in a dose-dependent attenuation of Ins P_3 by lithium while shorter periods of muscarinic receptor stimulation produced a biphasic dose-relationship. In contrast, extended exposure to high lithium concentrations appeared to elevate basal Ins P_3. Together these observations suggest dual actions of lithium, with the balance between its attenuating and enhancing effects on Ins P_3 accumulation being both time and stimulation dependent. However, at all lithium concentrations (Figures 3.4.1 and 3.4.4) and at all but the earliest time point after agonist addition (Figure 3.4.3), the predominant effect of this ion appears to be a reduction of stimulated Ins P_3 accumulation. Despite the differential effects of lithium on control and stimulated Ins P_3 concentrations and the time dependency of these actions, the sustained stimulated elevation of each inositol phosphate in the presence and absence of lithium demonstrates that these phenomena are not related to muscarinic receptor desensitisation.

The time and stimulation dependency of attenuated Ins P_3 accumulation strongly suggests that this may result from a lithium-induced depletion of a receptor specific pool of a particular inositol metabolite. It has previously been argued (Batty and Nahorski, 1985) that this is unlikely to be inositol itself as free ^{3}H-inositol is present in the medium throughout. It is improbable that lithium influences the transport of ^{3}H-inositol as the labelled material when added together with this ion clearly has initial access to the phosphoinositides which can be hydrolysed by muscarinic receptor activation. In addition, stimulated Ins P_3 accumulation is reduced at lithium concentrations (0.1-0.3 mM) at which inositol can still be derived by Ins P_1 dephosphorylation. Berridge et al. (1982) have suggested that the therapeutic efficacy of lithium in the treatment of manic-depressive illness may result from the ability of this ion to lower brain concentrations of myo-inositol and thereby reduce the capacity of signalling systems involving hydrolysis of phosphoinositides. Systemic administration of lithium within the therapeutic concentration range decreases inositol levels and increases concentrations of Ins-1-P in rat brain (Alison and Stewart, 1971; Alison et al. 1976) by inhibition of Ins-1-P phosphatase (Hallcher and Sherman, 1980). Under these conditions, the major proportion of Ins-1-P

accumulating in brain is the D-enantiomer (Sherman et al. 1981) which is that suggested to derive from phosphoinositide hydrolysis, as opposed to the corresponding L-myo-Ins-1-P which arises as an intermediate in the de novo synthesis of myo-inositol from glucose-6-phosphate (Eisenberg, 1967). Both enantiomers are found in brain and hydrolysis of each is inhibited by lithium (Hallcher and Sherman, 1980). However, the recent description of an Ins-1,3,4-P_3 in rat parotid glands (Irvine et al. 1984a) suggests that, if a similar compound is present in brain (see CH.4), the metabolism of this could lead to formation of either D- or L-myo-Ins-1-P by hydrolysis of phosphoinositides but the observations of Sherman et al. (1981) may indicate that prior removal of the D-3 (L-1) phosphate is preferential.

On the basis of the relative accumulations of, and rates of hydrolysis of, D- and L-Ins-1-P, Sherman et al. (1985) suggest that in brain Ins-1-P is produced at approximately a 7-fold greater rate through phosphoinositide hydrolysis than de novo synthesis. It might be anticipated from this that in the prolonged presence of a maximally stimulating concentration of carbachol, the continued cycling of inositol lipids would be highly dependent on the availability of inositol derived from Ins P_1. Thus, although the currently observed attenuation of stimulated ^{3}H-Ins P_3 accumulation was observed at concentrations of lithium which still allow formation of ^{3}H-inositol via ^{3}H-Ins P_1 dephosphorylation, it is possible that the decreased rate of this reaction at between 0.1 and 0.3 mM lithium was sufficient to decrease lipid synthesis (labelling) to a significant extent. This would imply a limited pool of inositol which is closely associated with the receptor mechanism to account for the preferential use of lipid-derived ^{3}H-inositol over freely available, exogenously supplied label under stimulated conditions. Downes and Stone (1986) have demonstrated that prolonged muscarinic receptor stimulation in rat parotid gland in the presence of lithium results in a more marked accumulation of CDP-DG than in the absence of this ion and decreased labelling of PtdIns but not of PtdInsP or PtdInsP_2, effects which are overcome by inclusion of 10-30 mM inositol in the incubation medium. These authors concluded that although cellular inositol is depleted in the continued presence of lithium and agonist, supply of PtdInsP_2 is maintained at the expense of PtdIns. Drummond and Raeburn (1984) have reached similar conclusions with GH_3 cells. If receptor-mediated phosphoinositide hydrolysis in brain proceeds exclusively through PtdInsP_2 hydrolysis and the relationship between inositol supply and maintenance of PtdInsP_2 levels is similar to that in parotid glands and GH_3 cells, then the decreased availability of ^{3}H-inositol due to impaired ^{3}H-Ins P_1 hydrolysis should not significantly affect stimulated Ins P_3 accumulation. Alternatively, if very much less

of total brain PtdIns and PtdInsP is associated with the receptor than in these other cells, the period over which these lipids can be used to sustain the required $PtdInsP_2$ levels would be limited and sensitivity to lithium-depleted inositol concentrations would be more rapidly apparent. As discussed in CH.2 it is possible that since agonist effects on ^{3}H-inositol labelling of lipid are rarely apparent in brain (at least for PtdIns), the proportion of total cellular phosphoinositide associated with the receptor could be small although, as substantial basal labelling may occur through headgroup exchange (see CH.2), this could mask agonist-induced labelling through CDP-DG:myo-inositol transferase. It must therefore be concluded that the currently observed lithium-induced attenuation of stimulated Ins P_3 accumulation could arise through a depletion of a pool of ^{3}H-inositol available for rapid re-cycling (re-synthesis) of lipids and that this results from lithium inhibition of Ins P_1 phosphatase. However, if this accounts for the effects of lithium on stimulated Ins P_3 accumulation then the continued linear accumulation of Ins P_1 (even at times when stimulated Ins P_3 concentrations are reduced by 70%) would imply that this molecule is not only directly derived from PtdIns but also from a pool of this lipid not immediately associated with the receptor mechanism because otherwise a reduction in Ins P_1 accumulation would have to follow or coincide with that in Ins P_3. A secondary hydrolysis of PtdIns, consequent on an initial Ins-1,4,5-P_3 mediated elevation of $[Ca^{2+}]_i$, has been proposed for the action of thrombin on platelets (Majerus et al. 1985). In addition, Dixon and Hokin (1985) have demonstrated the formation of inositol-1:2 cyclic monophosphate following stimulation of mouse pancreatic minilobules with various agonists and, since this product is known to arise through phospholipase C cleavage of PtdIns (Dawson et al. 1971), have suggested that receptor activation may result in phosphodiesterase attack on this lipid. However, Wilson et al. (1985a) have demonstrated that corresponding cyclic products also arise from hydrolysis of PtdInsP and $PtdInsP_2$ in vitro. The Ins-1:2 cyclic 4,5-P_3 derived from $PtdInsP_2$ has been demonstrated to release $^{45}Ca^{2+}$ from platelets and to induce a change in membrane conductance in Limulus photoreceptors, exhibiting a considerably higher potency than Ins-1,4,5-P_3 on the latter response (Wilson et al. 1985b). Metabolism of these cyclic polyphosphates occurs initially by sequential removal of the 5 and 4 monoesterphosphates before hydrolysis of the cyclic diester bond (Connolly et al. 1986). Thus, the Ins-1:2 cyclic P_1 observed by Dixon and Hokin could arise through this route rather than directly from PtdIns. Further evidence of a direct PtdIns hydrolysis however, has very recently been presented for the action of angiotensin II on vascular smooth muscle cells (Griendling et al.

1986). Following receptor stimulation in these cells the stimulated formation of Ins P_3 and changes in polyphosphoinositide levels are transient while DG production shows an initial increase followed by a secondary, more sustained elevation which is accompanied only by a loss of PtdIns. This would appear to support the hypothesis put forward by Majerus et al. (1985). Similarly, the current data could be interpreted in this way since Ins P_1 continues to accumulate at times when stimulated Ins P_3 accumulation is reduced by up to 70%. However, data presented in 3.2 and 6.2 suggest that direct hydrolysis of PtdIns is not likely to account for a substantial proportion of the Ins P_1 accumulating in response to muscarinic receptor stimulation in brain and, as discussed below, the currently observed effects of lithium can be explained in other ways.

An alternative explanation for the action of lithium on stimulated Ins P_3 accumulation is either a direct or indirect effect of this ion on the interconversion of inositol lipids. Given the multiple cell types present in brain slices and the potentially discrete pools of phosphoinositides together with the high content of these lipids in brain, it may be difficult to detect changes affecting a limited, agonist-labile compartment (see 3.5 also). Sherman et al. (1985) have reported that in vivo administration of lithium to rats did not significantly effect concentrations of inositol lipids either in whole brain or cerebral cortex but suggested that prolonged treatment with agonists combined with high lithium concentrations might be expected to reduce these. Drummond and Raeburn (1984) have demonstrated that in GH_3 cells the combined action of TRH and lithium results in an enhanced DG accumulation over that seen with agonist alone. DG is known to activate protein kinase C (Nishizuka, 1984), an enzyme which may be involved in the modulation of PtdInsP kinase activity in rat brain (Van Dongen et al. 1985). Thus, Drummond and Raeburn (1984) have pointed out that under conditions of agonist stimulation in the presence of lithium, effects on phosphoinositide concentrations could be anticipated. Van Rooijan et al. (1985) have concluded that the majority of DG released as a consequence of muscarinic receptor-mediated phosphoinositide hydrolysis in nerve ending preparations from guinea-pig cerebral cortex is re-cycled into PtdIns. However, given the results of Downes and Stone (1986) and of Drummond and Raeburn (1984) it is possible that in the presence of lithium a different situation may prevail and secondary effects of lithium on phosphoinositide levels could be mediated through elevated DG levels. However, assuming receptor-mediated inositol lipid hydrolysis is restricted to $PtdInsP_2$, it would be difficult to account for the continued linear accumulation of Ins P_1 and the concomitant severely reduced Ins P_3 levels by an effect of lithium on

inositol lipid concentrations or hydrolysis.

A final interesting, potential explanation of the lithium attenuation of stimulated Ins P_3 accumulation is suggested by the recent observation that carbachol stimulation of parotid glands results in the production of the distinct inositol trisphosphate isomers, Ins-1,3,4- and-1,4,5-P_3 (Irvine et al. 1984a). The former appears to have a comparatively greater metabolic stability (Irvine et al. 1985). Similar heterogeneity of brain Ins P_3 could account for the currently reported effects of lithium, with this ion potentially altering the ratio of a relatively stable/unstable isomeric Ins P_3 species. The inactivation of Ins-1,4,5-P_3 as a Ca^{2+} mobilising signal is generally assumed to proceed by the specific removal of the 5-phosphate by a lithium-insensitive phosphatase (Downes et al. 1982; Connelly et al. 1985; Seyfred et al. 1984). A similar mechanism is suggested in brain by the recent identification of an Ins-1,4,5-P_3 5-phosphatase present in the particulate fraction from this tissue (Erneux et al. 1986). Since hydrolysis of the 5-phosphate by these enzymes (including that from brain) is insensitive to lithium, it seems probable that the potentiation of Ins P_3 accumulation by this ion in several tissues (Drummond et al. 1984; Thomas et al. 1984) might result from an inhibitory action on Ins-1,3,4-P_3 hydrolysis. Although the metabolic pathways involved in synthesis and degradation of this compound are not yet certain (but see CH.4 and CH.6), the formation and partial, subsequent inhibition of hydrolysis of Ins-1,3,4-P_3 in cerebral cortex could account for the biphasic dose-relationship between lithium concentration and stimulated Ins P_3 accumulation. Nevertheless, as the predominant effect of lithium on Ins P_3 is to reduce its stimulated accumulation, any inhibitory action on Ins-1,3,4-P_3 hydrolysis in brain would appear to be secondary to a more potent and significant effect of this ion elsewhere on inositol lipid/phosphate metabolism. However, if lithium were also to inhibit formation of Ins-1,3,4-P_3 in such a manner as to increase metabolism through Ins-1,4,5-P_3, the lesser stability of the latter trisphosphate (Irvine et al. 1985) could result in a more rapid turnover of total Ins P_3, resulting in diminished accumulation. Precisely how such a mechanism would influence the calculation made in 3.2 relating flux through Ins P_3 in the absence of lithium to Ins P_1 accumulation in the presence of this ion is unclear, but it is apparent from the above discussions that the inferences drawn in 3.2 may be invalid.

From the current data it is obviously not possible to conclude the accumulation of multiple Ins P_3 isomers following muscarinic receptor activation in brain although the biphasic dose-response curves to lithium over shorter periods of stimulation suggest that this is a possibility. If Ins-1,3,4-P_3 is formed in brain then a combination of a lithium

inhibition of its hydrolysis together with a second, more potent action of this ion, effectively causing Ins P_3 metabolism to be channelled through a relatively less stable isomer, could account for most of the effects observed in the current study.

Clearly the present data raise a number of questions concerning cerebral inositol metabolism, suggesting that this follows a more complex sequence of reactions than has yet been described. An additional important point to note however, is that if the attenuation of stimulated Ins P_3 accumulation induced by lithium represents a reduced flux through this fraction and if the major route of cerebral phosphoinositide hydrolysis occurs via $PtdInsP_2$ cleavage, then when receptor-mediated responses are assayed by measurement of Ins P_1 in the presence of lithium ions, this could result in an underestimate of the response. This could have severe implications for pharmacological studies since responses to different agonists or agonist concentrations could be affected to varying extents. This possibility is further considered in 6.3 and 6.4 where the effects of lithium on inositol phosphate metabolism are reconsidered. The effects of this ion on inositol lipid metabolism are briefly considered in 3.5.

3.5 Effects of lithium on ^{3}H-inositol labelling of phosphoinositides

As discussed in 3.4 it is possible that the lithium-induced attenuation of stimulated Ins P_3 accumulation arises from direct or indirect effects of this ion on interconversion of the inositol lipids associated with the receptor mechanism. If the agonist labile pools of phosphoinositides represent only a small proportion of the cellular total, such effects may be difficult to detect, particularly in a tissue of heterogenous cell type, even after separation of these lipids into individual fractions. However, in an attempt to eliminate the possibility of any gross effects of lithium on phosphoinositide labelling, comparisons of ^{3}H-inositol incorporation into each PtdIns, PtdInsP and $PtdInsP_2$ in the presence and absence of LiCl and carbachol were made after first establishing methods for the extraction and separation of these lipids.

a. Extraction and separation of phosphoinositides:

i) Extraction: In previous sections where inositol lipids have been extracted this has been achieved by phase partitioning of neutral aqueous and organic solvents to obtain an upper phase containing ^{3}H-inositol phosphates and a lower chloroform layer containing ^{3}H-inositol lipid. However, this procedure is inadequate for extraction of inositol polyphosphates and equally so for polyphosphoinositides (see below).

Methods of choice for the quantitative extraction of the latter vary according to the tissue although in the majority of cases the best results are obtained with methanol:chloroform mixtures acidified with HCl (see Hawthorne and White, 1975; Downes and Michell, 1982). In order to determine which of several reported methods would be both most effective and convenient for the present study, a brief comparison of the total ^{3}H-inositol lipid extracted by neutral and various acidified solvents was first made in preliminary experiments.

Table 3.5.1 shows the results of a single experiment comparing four separate methods where 1-4 represent:

1. extraction as described in 2.2.

2 + 3. extraction as in 2.2 except that methanol:chloroform (2:1 v/v) containing 25 mM or 500 mM HCl respectively was used.

4. extraction of TCA precipitated tissue by the method of Yagihara et al. (1973) according to Griffin and Hawthorne (1978) including three successive treatments with CH_3OH:$CHCl_3$:10 M HCl in the ratios 100:100:1.2, 100:100:1.2 and 200:100:1.2 (v/v).

Table 3.5.1 Comparative extractions of inositol phospholipid

	Extraction	Total inositol lipid (as a % of the maximum extracted)
1.	Neutral solvent (2:1 CH_3OH:$CHCl_3$)	71.1 ± 1.7%
2.	Acidified solvent (2:1 CH_3OH:$CHCl_3$ containing 25 mM HCl)	49.0 ± 1.7%
3.	Acidified solvent (2:1 CH_3OH:$CHCl_3$ containing 500 mM HCl)	100 ± 1.9%
4.	Repeated acidified solvent extraction of TCA pellet (Griffin and Hawthorne, 1978)	90.9 ± 4.1%

Table 3.5.1 compares the extraction of ^{3}H-inositol labelled phosphoinositides from ^{3}H-inositol prelabelled cerebral cortical slices by four separate methods described in the text. Results represent the mean ± SEM of three separate measurements made in a single experiment but were confirmed for methods 1-3 in two further experiments. Values are represented as a relative percentage of the maximum recovery of total ^{3}H phosphoinositide (PtdIns + PtdInsP + $PtdInsP_2$), where 100% represents 56618 cpm lipid radioactivity/50 µl tissue extracted.

Each extraction method was performed on triplicate 50 µl aliquots of cerebral cortical slices prepared and preincubated as in 2.2 and

subsequently allowed to incorporate 1 μCi of ^{3}H-inositol for 90 min. prior to extraction according to the methods above.

The results of this study clearly indicate that neutral solvent extraction yields between 20-30% lower total ^{3}H-inositol labelled lipid than the maximum achieved with either highly acidified solvent (3) or repeated acifidied solvent extraction (4). Unexpectedly, procedure (2) involving a single step extraction with only weakly acidified solvent gave about 30% less ^{3}H-lipid than neutral solvent and although Table 3.5.1 shows the results of only a single experiment very similar proportions of total ^{3}H-lipid were obtained with methods 1-3 on two further occasions. The inclusion of acid with organic solvents is generally considered to facilitate extraction of polyphosphoinositides by disruption of proteo-lipid interactions, thus it is difficult to account for the reduced total ^{3}H-lipid yield at low, but increased yield at higher, HCl concentrations. Hawkins et al. (1986) have reported that under neutral extraction conditions as in (1) (Table 3.5.1) the majority of PtdInsP partitions into the upper phase while most PtdInsP$_2$ remains associated with insoluble, interfacial protein during solvent extraction. Possibly a similar effect on one or more of the phosphoinositides occurs in the presence of weak acid although it should be noted that repeated extraction with CH_3OH:$CHCl_3$ containing HCl at 40-60 mM (4) gave an almost equivalent yield of ^{3}H-lipid to a single step extraction including 0.5 M HCl. The reason for this unusual effect was not further investigated but in subsequent experiments extractions of inositol lipids were always performed using solvents containing HCl to a minimum of 0.1 M.

From the results shown in Table 3.5.1 the most convenient and effective method for extraction of total ^{3}H-inositol lipids appears to be that described by (3). However, this has the disadvantage that a prior extraction of ^{3}H-inositol phosphates is not made and although these can be recovered from the aqueous phase following solvent partitioning, previous experiments (see 2.4) had indicated that this was less convenient than the use of either TCA or PCA. Alternatively, the method of Griffin and Hawthorne (1978) allows use of TCA for this purpose but the subsequent repeated acidified solvent extraction is time consuming for multiple samples. A more convenient method, approximating to a combination of 3 + 4, involving a single step methanol:chloroform:HCl extraction of a TCA pellet has been described by Downes and Wusteman (1983). When the extraction of total ^{3}H-inositol lipids and of separate phosphoinositides by this procedure was directly compared with that achieved with neutral solvent (see Figure 3.5.2 and below) the increased yield of lipid with this acidified solvent method was of a similar order to that achieved with procedures 3 and 4 above. Subsequent use of this method, described in

detail in (iii) below, gave reproducible yields of all three inositol lipids although whether this reflects their quantitative extraction is not certain as a proportion of inositol lipid present in some tissues is resistant to even repeated acidified solvent extraction (Michell et al. 1970). However, when the residue of tissue aliquots extracted by method (4) were dissolved in NaOH and counted for tritium, less than 200 cpm was detected, indicating an extraction efficiency of >99%. A similar efficiency would be anticipated for the method routinely used.

ii) Separation: The separation of intact inositol phospholipids is most commonly achieved by thin layer chromatography by a variety of methods extensively described by Simpson et al. (1987). Other procedures include column chromatography on DEAE cellulose (Hendrickson and Ballou, 1964) or on neomycin coated beads as detailed by Schacht (1978) and Palmer (1981), and paper and gas liquid chromatography as discussed by Hawthorne and White (1975), although of the latter two methods the former may be less reliable than others (Downes and Michell, 1982). Alternatively, if the lipids are first deacylated, the resulting water-soluble glycerophosphoinositol (phosphate) diesters can be conveniently separated into corresponding fractions by either gradient (Brockerhoff and Ballou, 1961; Wells and Dittmer, 1965) or stepwise elution of anion exchange columns (Ellis et al. 1963). This last method has recently been adapted to allow small scale analysis suitable to multiple tissue aliquots in a manner analogous to that used for the separation of inositol mono-, bis- and trisphosphates described above (Downes and Michell, 1981; Creba et al. 1983; Berridge, 1983; Hawkins et al. 1984). As similar chromatography was already in routine use for the resolution of phosphoinositide hydrolysis products this last procedure was selected for the current study.

In preliminary experiments ^{3}H-inositol labelled phosphoinositides were extracted as detailed in (iii) below, dried under N_2 and deacylated by the method of Ellis et al. (1963) exactly as described by Creba et al. (1983). However, when the resultant neutralised samples were applied to Dowex anion exchange columns a considerable portion (up to 50%) of the water-soluble radioactivity failed to adhere to the resin. Since each of the major products of mild alkaline hydrolysis of the phosphoinositides carries at least one negative charge at pH 7-8, this indicated that further breakdown of the expected glycerophosphoryl diesters had occurred. Under the conditions used such hydrolysis is not unusual (Hawthorne and White, 1975) and from a combination of the three ^{3}H-inositol labelled phosphoinositides small proportions of each labelled free inositol together with isomeric mixtures of Ins P_1, Ins P_2 and Ins P_3 would be expected from previous studies (Brockerhoff and Ballou, 1961; Ellis et al.

1963; see also Hawthorne, 1960 and Hawthorne and Kemp, 1964). However, the high proportion of uncharged tritiated material, which was presumably ^{3}H-inositol derived from PtdIns, was surprising as this might be expected to arise from strong but not mild alkaline hydrolysis and indicated that the method used might also result in considerable further hydrolysis of GroPInsP and GroPInsP$_2$ derived respectively from PtdInsP and PtdInsP$_2$. Under these circumstances the relatively simple separation of glycerophosphoryl diesters would be complicated by the presence of a variety of products such that resolution of radioactivity into gross GroPIns, GroPInsP and GroPInsP$_2$ fractions might not be representative of the original lipid distribution of radiolabel. For this reason an alternative deacylation procedure was sought. The method of Clarke and Dawson (1981) using methylamine gives rise to fewer unwanted side products but, although a simplification of this procedure has recently been introduced (Hawkins et al. 1986), this method seemed too complex in its original form for routine treatment of multiple samples. A much simpler method, derived from that of Brockerhoff (1963) which also limits further hydrolysis of lipid deacylation products, has been described by Wells and Dittmer (1965).

In further preliminary experiments a slight modification of this procedure (see iii) was directly compared with that of Creba et al. (1983). A ^{3}H-inositol lipid extract from cerebral cortical slices was divided into two equal portions and one deacylated by each method. The resultant water-soluble products were then analysed for glycerophospho-inositol (phosphate) diesters and corresponding inositol monoester phosphates by anion exchange chromatography on 0.6 x 3.0 cm columns of AG 1 x 8 Dowex resin (200-400 mesh, formate form) using a stepwise elution essentially as described by Ellis et al. (1963) except that GroPIns and GroPInsP were respectively eluted at 25 mM NH_4COOH and 250 mM NH_4COOH/ 0.1 M HCOOH. The volumes of eluants required for optimum resolution were determined by trial and error. Peaks are identified by analogy with the results of Ellis et al. (1963) and for GroPIns, GroPInsP and GroPInsP$_2$ by co-elution with standard ^{3}H-inositol labelled materials obtained by deacylation of authentic labelled lipids (Amersham and NEN). The identities of Ins P_1 and Ins P_3 were similarly confirmed at a later date (see CH.4 and 5). The results of this experiment are illustrated in Figure 3.5.1 which also shows the elution profile for a mixture of deacylation products prepared from standard ^{3}H-lipids.

Deacylation by either method resulted in >99% conversion of lipid to water-soluble radioactivity but comparison of Figure 3.5.1A and B shows that the procedure of Wells and Dittmer (1965) resulted in lesser amounts of side products than that of Creba et al. (1983). Using the latter, radiolabel running as GroPIns, GroPInsP and GroPInsP$_2$ is respectively

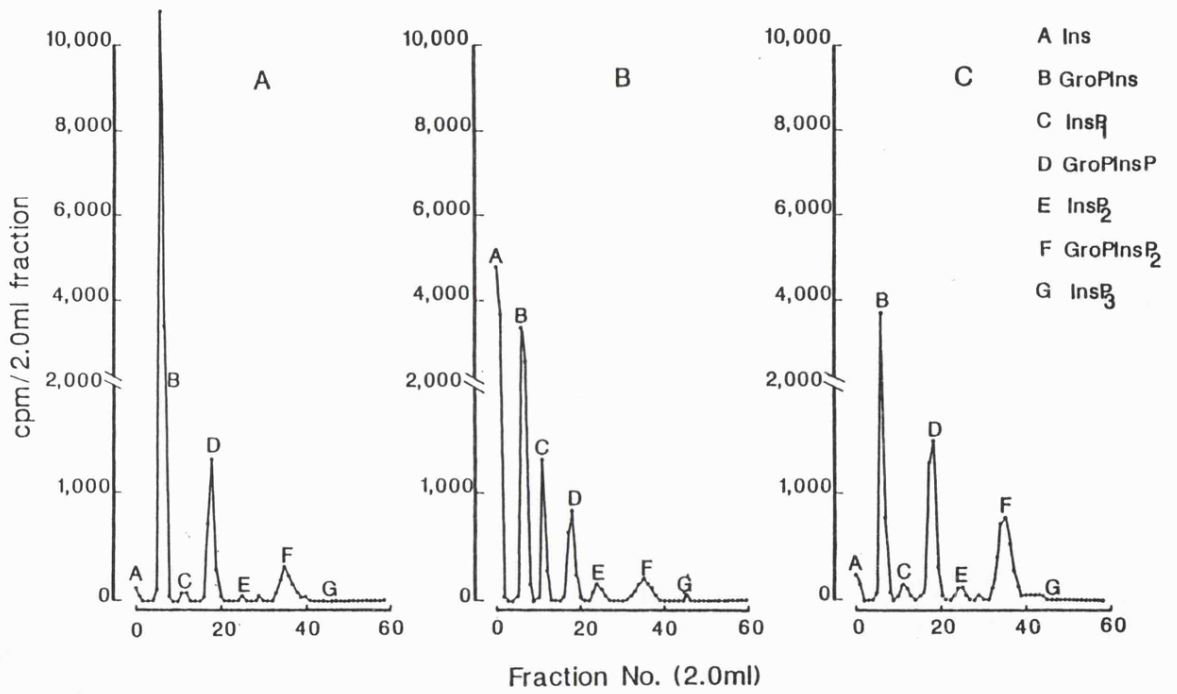


FIGURE 3.5.1 Comparison of methods for the deacylation of ^{3}H-inositol labelled phosphoinositides.

^{3}H-Inositol labelled phospholipids were extracted from brain slices (Figs. A and B) as described in the text or obtained as pure standard materials (Fig. C). Brain extracts were deacylated according to Wells and Dittmer (1965) (A) or Creba et al. (1983) (B) and a mixture of standards treated by the former procedure (C). The resultant ^{3}H-products were chromatographed essentially according to Ellis et al. (1963) on 0.6 x 3.0 cm columns of AG 1 x 8 anion exchange resin (200-400 mesh, formate form). The ^{3}H-products labelled A-G were eluted separately by washing the columns successively with H_2O (10 ml), 0.025 M AF (12 ml), 0.2 M AF (10 ml), 0.25 M AF/0.1 M FA (14 ml), 0.4 M AF/0.1 M FA (14 ml), 0.5 M AF/0.1 M FA (30 ml) and 1.0 M AF/0.1 M FA (28 ml).

32%, 76% and 71% of that achieved with the former method, while labelling of inositol, Ins P_1, Ins P_2 and Ins P_3 is increased 39, 7, 6 and 2 fold respectively. Total radioactivity recovered from columns A and B was 17574 and 16832 cpm. These results indicate that deacylation according to Creba et al. (1983) caused further hydrolysis of each glycerophosphoryl diester to a greater extent than the alternative procedure used. This is particularly notable in the formation of free ^{3}H-inositol and of ^{3}H-Ins P_1 and to a lesser extent of ^{3}H-Ins P_2. From previous studies (Brockerhoff and Ballou, 1961) of the products of strong alkaline hydrolysis of glycerophosphoinositol (phosphate) diesters, inositol would be expected to arise only from PtdIns (via GroPIns) while Ins P_1 could derive from either PtdIns or PtdInsP as Ins-1-P and Ins-2-P or Ins-4-P respectively. Similarly, Ins P_2 could arise from PtdInsP or PtdInsP_2 as Ins-1,4-P_2 and Ins-2,4-P_2 or Ins-4,5-P_2 respectively, while isomeric Ins P_3 (Ins-1,4,5-P_3/Ins-2,4,5-P_3) can derive only from PtdInsP_2. As the source of Ins P_1 and Ins P_2 is uncertain without resolution of the isomers, the lipid origin of the radioactivity in these fractions cannot be exactly determined and as the method of Creba et al. results in greater quantities of these products it is more likely to lead to inaccurate assignment of the water-soluble radioactivity to its correct original lipid precursor. Although the significance of this may be limited for PtdInsP and PtdInsP_2, the method of Wells and Dittmer (1965) seemed preferable. In subsequent experiments this was routinely used to deacylate lipid extracts as described below. However, on chromatography of the resultant products only three major fractions were normally collected to allow more rapid analysis of samples. These would correspond to GroPIns + Ins P_1, GroPInsP + Ins P_2 and GroPIns P_2 + Ins P_3. The small amounts of monoester inositol phosphates formed (see Figure 3.5.1) are unlikely to significantly alter the final total proportions of radiolabel assigned to original lipid fractions.

iii) Summary of extraction and separation methods: Based on the above observations the following methods were found to be most convenient and reliable for the extraction and deacylation of ^{3}H-inositol lipids and separation of the resulting water-soluble products.

Tissue incubations were terminated with an equal volume of 1.0 M TCA. Cortical slices were precipitated by centrifugation and inositol phosphates (where required) isolated as previously from the supernatants. The tissue pellets were washed sequentially with 1.0 ml 5% TCA containing 1 mM EDTA and 1.0 ml H_2O, according to Griffin and Hawthorne (1978). ^{3}H-lipids were extracted from the residue as described by Downes and Wusteman (1983) by addition of 0.94 ml of 2:1 (v/v) methanol:chloroform containing

100 mM HCl. Samples were allowed to stand for 10 min. at room temperature prior to partitioning of the aqueous and organic phases by addition of 0.31 ml chloroform and 0.56 ml 0.1 M HCl and subsequent centrifugation at 3,000 x g for 10 min. Upper phase was aspirated and 400 µl of the chloroform layer removed and combined from triplicated incubations to facilitate detection of polyphosphoinositides. Resultant samples were dried under a stream of N_2 and, if necessary, stored overnight at -20°C.

For deacylation, lipid extracts were re-dissolved in 1 ml of 4:1 (v/v) methanol:chloroform and 50 µl of 1.2 M NaOH in 50% methanol added. Samples were incubated for 15 min. in a water bath at 37°C prior to addition of 2 ml of each 9:1 (v/v) chloroform:methanol and water. After thorough vortex mixing, aqueous and organic phases (approximately 3 ml and 2 ml respectively, plus insoluble, interfacial residue) were separated by centrifugation at 3,000 x g for 10 min. 2.5 ml upper phase was collected for analysis of the water-soluble products and a 1.0 ml portion of the lower phase dried under N_2 and counted for tritium to assess the efficiency of deacylation which was routinely $\geqslant$99%. Upper phases were freed of NaOH by passage through 200 µl columns of a 50% (w/v) slurry of Dowex 50 (H^+ form, 50-100 mesh) and the resin washed with 1.5 ml H_2O to limit loss of products. Finally, samples were adjusted to pH 7-8 by addition of 1.0 ml 25 mM $NaHCO_3$.

Subsequent analysis of the deacylation products was by anion exchange chromatography on 0.6 x 3.0 cm columns of AG 1 x 8 (200-400 mesh, formate form). Comparisons of the separations achieved with this analytical grade resin with results obtained on Dowex resin (100-200 mesh, formate form) showed that use of the former gave very much improved resolution of peaks which were more clearly defined (without the considerable tailing observed with the other resin) and very much sharper, allowing greater assay sensitivity (cf Figures 3.5.1 and 3.5.2 with the elution profiles illustrated in CH.2). The improved chromatography afforded by use of the analytical grade resin and the application of this to the separation of various inositol phosphates is considered in more detail in CH.4. Separation of glycerophosphoinositol (phosphate) diesters on this resin was routinely achieved by elution essentially as described by Creba *et al.* (1983) and Berridge (1983). Sample (4 ml) was applied and the eluate, together with a 5-10 ml water wash (to remove free ^{3}H-inositol) was discarded, GroPIns, GroPInsP and GroPInsP$_2$ were respectively eluted with 10-20 ml 0.18 M AF/0.005 M $Na_2B_4O_7$, 10-20 ml 0.4 M AF/0.1 M FA and 10-12 ml 1.0 M AF/0.1 M FA. The upper range of volumes was occasionally required when larger quantities of ^{3}H-material were applied to columns. Radioactivity in each fraction was determined by liquid scintillation

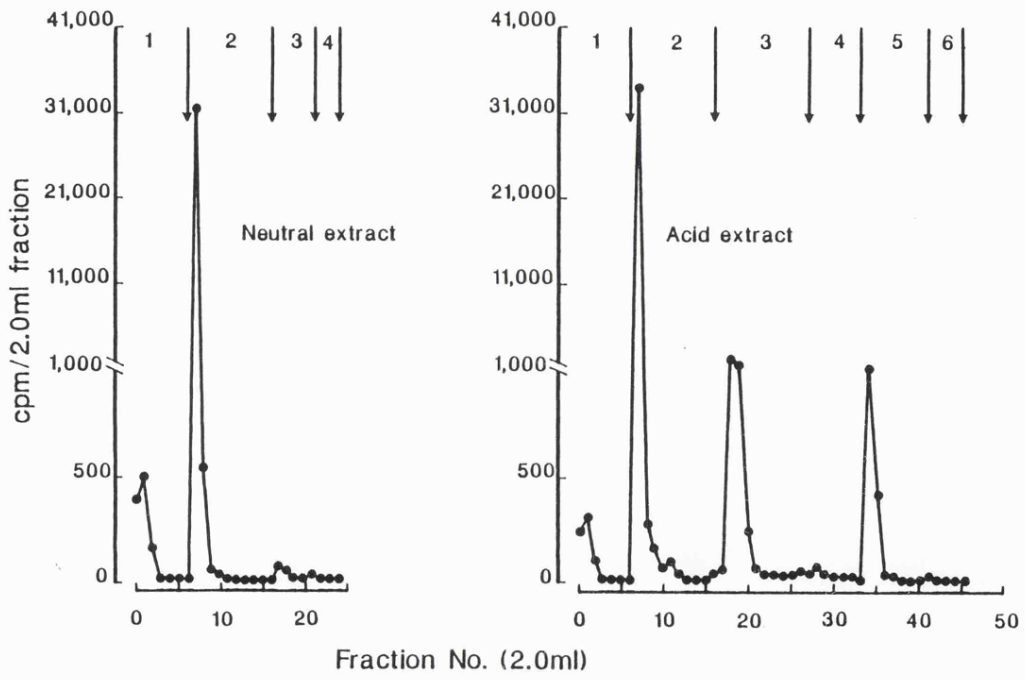


FIGURE 3.5.2 Comparison of the ^{3}H phosphoinositides extracted from ^{3}H-inositol labelled brain slices by neutral and acidified solvents.

Cerebral cortical slices were prepared as in 2.2. After 60 min. pre-incubation, 50 µl aliquots were labelled with 2.5 µCi ^{3}H-inositol for 75 min. ^{3}H-phospholipids were then extracted using neutral or acidified chloroform/methanol as in the text. Lipid extracts were pooled from triplicate incubations and a portion of each total (~50%) dried under nitrogen and deacylated. The resulting water soluble ^{3}H-products were analysed by anion exchange chromatography as in the legend to Fig. 3.5.1 except that a less detailed elution system was applied. For the neutral extract 1-4 represent: H_2O, 0.18 M AF/0.005 M $Na_2B_4O_7$, 1.0 M AF/0.1 M FA and 2.0 M AF/0.1 M FA. The major peak is ^{3}H-GroPIns derived from ^{3}H-PtdIns. For the acid extract 1-6 represent: H_2O, 0.18 M AF/0.005 M $Na_2B_4O_7$, 0.3 M AF/0.1 M FA, 0.4 M AF/0.1 M FA, 1.0 M AF/0.1 M FA and 2.0 M AF/0.1 M FA. Peaks left to right are ^{3}H-GroPIns, ^{3}H-GroPIns P and ^{3}H-GroPIns P_2 derived from ^{3}H-PtdIns, ^{3}H-PtdIns P and ^{3}H-PtdIns P_2.

counting of 2-5 ml aliquots in either the liquid or gel phase with 20 ml Hydroluma (May and Baker) or 10 ml InstaGel (Packard).

Figure 3.5.2 shows a typical example of the separation achieved and illustrates elution profiles for deacylated, ^{3}H-inositol labelled lipid samples extracted from brain slices by either neutral solvent extraction as in 2.2 or with acidified solvent by the method above. The samples shown were prepared by prelabelling rat cerebral cortical slices with 2.5 µCi ^{3}H-inositol/50 µl of tissue as in 2.2 for 75 min. prior to lipid extractions. Organic phases from triplicated samples were combined and a portion of each (~50%) deacylated and chromatographed. Neutrally extracted samples showed little radiolabel eluting later than GroPIns (<200 cpm) while samples extracted with acidified chloroform:methanol showed two peaks corresponding to GroPInsP and GroPInsP$_2$ which respectively accounted for 5000 and 2500 cpm. Total radioactivity extracted by neutral solvents represented 78% that obtained with acidification which is similar to the results reported for total ^{3}H-lipid in Table 3.5.1. The increased yield with the latter method was primarily (80-90%) a consequence of improved PtdInsP and PtdInsP$_2$ extraction with little difference (7%) with respect to PtdIns.

b. Effects of lithium on ^{3}H-inositol labelling of phosphoinositides:

Having established convenient methods for the reliable extraction and quantification of separate inositol lipids these were next applied to a brief study of the effects of lithium on lipid labelling. From section 3.4 it is evident that the effects of lithium on Ins P$_3$ accumulation are dependent both on time and the presence of stimulus. For this reason ^{3}H-inositol incorporation into cerebral cortical phosphoinositides was investigated in both control and stimulated tissue at times and under conditions approximating to those employed in 3.4 to establish the dose-response data illustrated in Figures 3.4.1 and 3.4.3.

Tissue was prepared and incubated as previously. 50 µl aliquots of brain slices were allowed to incorporate 1 µCi ^{3}H-inositol for 30 min. in the presence or absence of 5 mM LiCl prior to agonist addition. Further incubations were then for either 15 or 45 min. in either the presence or absence of 1 mM carbachol. Reactions were terminated with TCA although inositol phosphates were not quantified but see 3.4. Phospholipids were extracted and analysed for ^{3}H-inositol as in 3.5(a)iii above.

Figure 3.5.3 illustrates the results of two experiments in each of which duplicate determinations of phosphoinositide labelling were made from combined triplicate tissue incubations for each condition described. At time zero, the point of agonist addition, the percentage ratios of

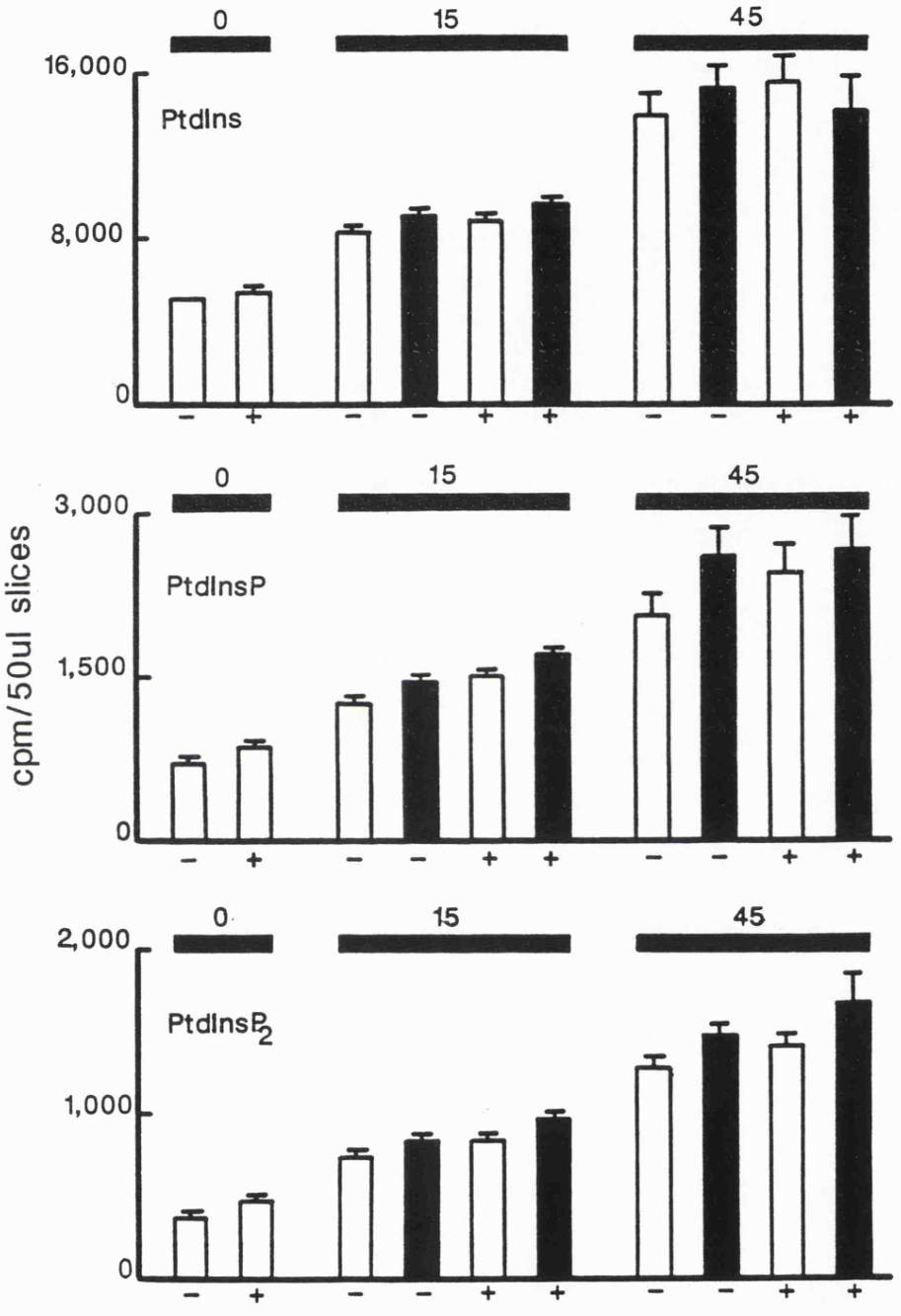


FIGURE 3.5.3 The effects of 5 mM LiCl and/or 1 mM carbachol on the labelling of brain phosphoinositides with ^{3}H-inositol.

Cerebral cortical slices, prepared as in 2.2, were labelled for 30 min. with ^{3}H-inositol (1 µCi/50 µl tissue) either (+) or (-) 5 mM LiCl. Reactions were then either stopped (0) or continued for a further 15 or 45 min. in the absence (open bars) or presence (closed bars) of 1 mM carbachol. ^{3}H-lipids were extracted and analysed as described in the text. Results represent the means ± SEM of 4 determinations, each made on duplicated samples in 2 experiments.

total ^{3}H-inositol incorporation into PtdIns, PtdInsP and PtdInsP$_2$ in the absence and presence of lithium were 82.6:11.4:6.0 and 80.2:12.8:7.0 while total labelling in the presence of lithium represented 108% that in the absence. After a further 45 min. in the absence of agonist these ratios were respectively 80.7:11.9:7.4 and 80.1:12.7:7.2 while total ^{3}H-inositol incorporation in the presence of lithium was 112% that in the absence. After 45 min. stimulation with 1 mM carbachol the percentage ratios of lipid labelling were 78.9:13.5:7.6 and 76.6:14.4:9.0 in the absence and presence of lithium while total ^{3}H-inositol incorporation in the presence of this ion was 96% that in the absence. Comparison of the total incorporation in the presence of both agonist and lithium ions with that in the absence of either agent shows only a 7% increase under the former conditions.

From these data and Figure 3.5.3 it is apparent that neither 5 mM LiCl nor carbachol stimulation, either individually or together, produce any gross changes in the labelling pattern of the inositol lipids. However, a number of small effects of varying significance can be seen. At most time points both muscarinic receptor stimulation and lithium ions separately induced small increases in the incorporation of ^{3}H-inositol into each lipid fraction. At 15 min. the combined effects of these treatments was approximately additive for each lipid although at 45 min. similar additivity was only apparent for labelling of PtdInsP$_2$. This latter action is most clearly seen from the above ratios of lipid labelling where prolonged exposure to a combination of carbachol and lithium appears to marginally increase polyphosphoinositide labelling but decrease that of PtdIns. However, it is improbable that this minor effect is causative in the action of lithium on stimulated Ins P$_3$ accumulation, firstly because of its very limited magnitude, secondly because each carbachol and lithium separately produce similar though lesser effects, and thirdly because this effect is equally apparent after both 15 and 45 min. while lithium-attenuated Ins P$_3$ accumulation is not maximal until the later time.

In contrast to the current results, Downes and Stone (1986) have recently noted that the enhanced ^{3}H-inositol labelling of PtdIns induced by carbachol in rat parotid gland is inhibited in the presence of lithium while the combined action of both agents on polyphosphoinositides is not different from that resulting with agonist alone. This implies the maintenance of polyphosphoinositides at the expense of PtdIns in the presence of both carbachol and lithium (see also 3.4). The very marked effects of muscarinic receptor stimulation and of lithium on inositol lipid labelling in this tissue are clearly not apparent in brain. Indeed from the present study it is difficult to discern clear actions of either

agent since although effects, particularly on $PtdInsP_2$ may be significant, these are very small compared to the total incorporation of ^3H-inositol into each lipid fraction. By comparison with data from other tissues, notably parotid gland, this suggests that of the total of each inositol lipid, only a small proportion is available to the receptor mechanism. If this is so then the very limited effects apparent in Figure 3.5.3 could assume much greater significance if these are specific to the receptor associated pools. It is therefore possible that if the agonist labile portions of each lipid could be more clearly defined in brain, as in parotid gland, that similar effects would be apparent in both tissues. However, the sustained maintenance of $PtdInsP_2$ levels observed in parotid gland in the continued presence of agonist and lithium ions would suggest that the combined action of these agents would be unlikely to result in a reduced stimulated Ins P_3 accumulation as is seen in cerebral cortex. In turn this could imply (a) different sites of action of lithium in the two tissues, (b) alternative routes of inositol phosphate/lipid metabolism, (c) that in brain the potentially smaller amount of PtdIns associated with the receptor is less able (in the presence of lithium) to continuously sustain the $PtdInsP_2$ pools required for continued stimulated Ins P_3 production. The latter explanation would agree with the conclusion above that in cerebral cortex pools of all three phosphoinositides, labile to muscarinic receptor stimulation, are small. This would argue against the suggestion of Majerus et al. (1985) which is that, because the cellular mass of PtdIns exceeds that of polyphosphoinositides the former could be a more significant source of receptor controlled production of DG, unless it is supposed that this is derived from a PtdIns pool separate from that immediately associated with the receptor. A direct hydrolysis of PtdIns was considered in 3.4 as a potential explanation of the means by which concomitant linear accumulation of Ins P_1 and a 60-70% reduction in Ins P_3 accumulation could be observed. In the event of direct PtdIns breakdown the effect of lithium on Ins P_3 would necessarily have to arise through an action at a step beyond the formation of this lipid and could not be attributed to inhibition of Ins P_1 phosphatase alone. The current data provides no clear evidence for an effect of lithium on the reactions between PtdIns conversion to polyphosphoinositides and Ins P_3 formation. The currently observed very limited effects of lithium on inositol lipid labelling and the associated problems of interpretation, given the heterogeneity of cellular and subcellular pools of these lipids, emphasise the value of assays for inositol phosphates where at least effects are more easily detectable and quantifiable.

As the present data implies limited receptor coupled pools of phosphoinositides it is assumed that the majority of ^{3}H-inositol incorporated represents other cellular/subcellular fractions of these lipids. The proportional labelling of PtdIns, PtdInsP and PtdInsP$_2$ under each of the above experimental conditions indicates a lesser content of polyphosphoinositides than might be anticipated in brain (see Hawthorne and White, 1975; Hawthorne and Pickard, 1979 and Hawthorne, 1983). The relative labelling of PtdInsP and PtdInsP$_2$ also shows a ratio of between 1.5-2.0:1.0 in the current experiments. Reliable estimates of these lipids from rat brain fixed rapidly either by microwave irradiation (Soukup et al. 1978) or in liquid nitrogen (Hawthorne and Kai, 1970) show a PtdInsP:PtdInsP$_2$ ratio of approximately 1:2 (i.e. the opposite of the labelling pattern above). A previous study of ^{3}H-inositol labelling of brain slices under conditions very similar to those currently used has indicated only 7-8% and 1-2% respectively of total label incorporated into phospholipid occurs as PtdInsP and PtdInsP$_2$ (Kendall and Nahorski, 1984) although a slightly different extraction procedure was used here. Alternatively, in synaptosomes prepared from guinea-pig cortex prelabelled in vivo for 2 hrs. with ^{3}H-inositol, Griffin and Hawthorne (1978) have reported almost equivalent labelling of PtdInsP and PtdInsP$_2$. It seems probable that the relative labelling of polyphosphoinositides observed in the current experiments results from a lower specific activity of PtdInsP$_2$ than of PtdInsP which would indicate that at least a portion of the polyphosphoinositides are labelled comparatively slowly. Since the receptor coupled lipid pools will turn over rapidly in the presence of agonist these would be expected to accumulate label over a comparatively short period and the results presented in 3.1 and 3.2 suggest that after several minutes in the presence of stimulus their specific activities are likely to change little. It is therefore possible that over shorter incubations with both agonist and label that changes in receptor specific pools of lipid would be more readily evident. However, it is clear from 3.4 that the effects of lithium on Ins P$_3$ accumulation require 30-40 min. to become maximal. An interesting further approach might be to pre-treat unlabelled tissue with agonist in the absence and presence of lithium for a prolonged period ($\sim$45 min.) and to then monitor the uptake of ^{3}H-inositol into the separate lipids. In this way it may be possible to magnify effects on receptor coupled lipid pools.

In conclusion, however, it is difficult to envisage from the data presented in Figure 3.5.3 that the dramatic lithium-induced reduction in stimulated Ins P$_3$ accumulation can be attributed to impaired inositol lipid interconversions. This is not only because the effects on phosphoinositide labelling are so small but also because both lithium and

carbachol individually produce similar, though lesser, effects in all three lipids to those seen with both agents together, while attenuated Ins P_3 accumulation is seen only under the latter conditions. Both the discussions above and data presented in later chapters suggest a more fruitful approach to establishing the site(s) of action of lithium on cerebral inositol metabolism will be a more detailed analysis of the effects of this ion on the pathways of inositol phosphate metabolism which the current study clearly suggests may be more complex than has previously been thought.

CHAPTER 4

Muscarinic receptor-mediated accumulation of inositol-1,3,4,5-tetrakisphosphate in rat cerebral cortex

4.1 Improved assay sensitivity for inositol phosphates indicates the presence of dual components in the trisphosphate fraction

Several previous studies (e.g. Brown et al. 1984; Jacobson et al. 1985) have indicated that stimulated accumulation of Ins P_1 in the presence of lithium is a useful functional marker of receptor activation. However, the recent evidence for potential separate routes of inositol lipid and phosphate metabolism controlled by distinct receptors in different tissues (cf Berridge, 1984 with Majerus et al. 1985 and Michell, 1986) suggests that measurement of Ins P_1 alone, under these conditions, may prove a less reliable estimate of phosphoinositide hydrolysis than originally expected and pharmacological data derived from such measurements may be open to more complex interpretations. The significance of this can only be determined by a clearer understanding of the metabolic pathways associated with each distinct receptor-mediated response which is best achieved by estimation of the separate products accumulating in the presence of different stimuli.

However, in cerebral cortex, accumulations of each inositol phosphate are small in the absence of lithium even in response to maximally effective concentrations of the most efficacious agonists. This is particularly so for inositol polyphosphates and the situation in brain is aggravated by the complex effects of lithium which may restrict use of this ion to potentiate limited responses. These problems are exemplified by the data presented in 3.3. Inositol phosphate accumulations in response to each NA, HA and 5-HT were almost undetectable in the absence of lithium and although in the presence of this ion, stimulated accumulation of Ins P_1 could be demonstrated (2.3), this was only easily quantifiable after longer periods of stimulation, under which conditions responses may be modified from those observed more immediately after receptor activation (see 3.4).

A major factor limiting assay sensitivity for small quantities of radiolabelled inositol phosphates is the volume of buffer required to elute separate fractions during chromatography (see 2.4). As noted in 3.5(a)iii the use of analytical grade (AG) resin resulted in a much improved separation of glycerophosphorylinositol (phosphate) diesters. The principle on which fractionation of these is based applies equally to the inositol monoester phosphates resulting from receptor activation and it seemed probable that the detection limit for these products would be similarly enhanced by the use of AG resin. Alternatively, assay sensitivity could potentially be improved by use of more highly labelled tissue samples.

It was considered that by achieving a higher specific activity of ^{3}H-inositol phosphates and by improving their chromatographic resolution

that detection would be sufficiently enhanced to allow more detailed analysis of both responses to less effective agonists and responses to carbachol over more rapid time courses than had previously been practical. Although data presented in earlier sections (see CH.3) had demonstrated hydrolysis of polyphosphoinositides in response to the latter agonist in cerebral cortex, these further studies should provide crucial evidence regarding the initial inositol lipid substrate(s) hydrolysed in response to receptor activation which earlier experiments had failed to reveal.

In preliminary experiments towards this goal the chromatographic characteristics of extracts from carbachol-stimulated cortical slices prepared under a variety of labelling conditions were examined using AG anion exchange resin. Initially tissue samples were incubated as in 2.2 except that ^{3}H-inositol was included in Krebs medium at either 1 or 5 µCi/50 µl cortical slices and stimulation continued for 45 min. in the presence or absence of 5 mM LiCl. The aim of this protocol was to determine whether the high radiolabelling of the resulting ^{3}H-inositol and ^{3}H-inositol phosphate fractions was likely to interfere with the subsequent small scale chromatographic separations. Analysis of samples by anion exchange chromatography was as described by Downes et al. (1982) and Berridge et al. (1983) with modifications as reported in 2.4. Elution of Ins P_1 was compared using buffers of varied ionic strength based on the separations shown in 2.4 and 3.5. Although a direct comparison of the separation achieved using AG resin and unrefined Dowex material was not made, the separation of products on the former was found to be remarkably consistent and reproducibly the volumes of eluants required for each inositol phosphate fraction were 50% or less than required using the latter resin. Increasing medium ^{3}H-inositol concentrations from 1-5 µCi resulted in approximately 4-5 fold increased radiolabelling of each inositol phosphate fraction but this did not reduce the efficiency of the separation achieved and from a later experiment (see 4.4(b)iii) cross-peak contamination is unlikely to exceed 5%. These initial results indicated that increased labelling of samples together with improved chromatography would be a practical method of improving assay sensitivity sufficiently for the desired purposes.

However, a very much more interesting observation arising from these preliminary experiments was that when extracts of carbachol-stimulated samples, labelled either according to previous methods or with higher ^{3}H-inositol concentrations, were examined on AG resin the Ins P_3 fraction showed duplicate peaks indicative of two separate components. This partial resolution was apparent with several samples analysed in a single experiment over precisely the same fractions in each elution profile. Additionally, the distribution of radiolabel between the two components

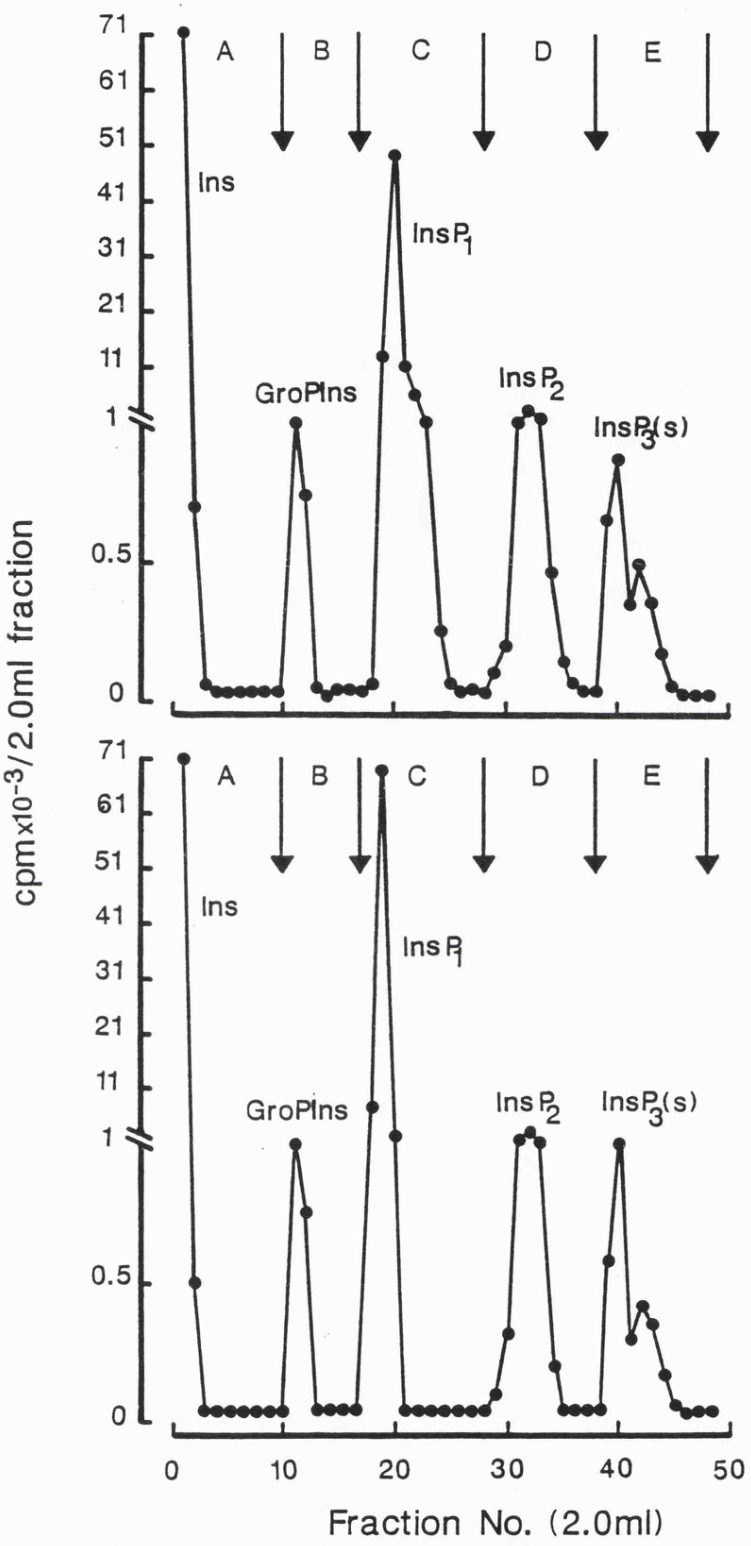


FIGURE 4.1.1 Analysis of the ^{3}H-inositol phosphate products resulting from carbachol stimulation of ^{3}H-inositol labelled brain slices using analytical grade anion exchange resin.

Extracts from 1 mM carbachol-stimulated cerebral cortical slices labelled with ^{3}H-inositol (1 µCi/50 µl tissue) were prepared as in 2.4. Incubation with agonist was for 45 min. in the presence of 5 mM LiCl. Extracts were applied to 0.6 x 3.0 cm columns of AG 1 x 8 anion exchange resin (200-400 mesh, formate form). Columns were washed successively with the solutions A-E to separate the ^{3}H-products initially identified as indicated. A-E represent: H_2O, 0.025 M AF, 0.15 M AF/0.005 M $Na_2B_4O_7$ (or 0.2 M AF, lower trace), 0.4 M AF/0.1 M FA and 1.0 M AF/0.1 M FA respectively. The elution profiles shown are typical of several run under these conditions.

under the total peak appeared to be markedly different for samples prepared in the presence or absence of lithium. Although this last observation was not entirely certain because samples incubated with and without this ion were labelled with distinct ^{3}H-inositol concentrations, the apparent effect of lithium did appear to support the chromatographic resolution of separate inositol metabolites. Typical examples of the separations observed are shown in Figure 4.1.1 (A) and (B) which each illustrate the results obtained with extracts from cerebral cortical slices labelled with 1 μCi ^{3}H-inositol and incubated for 45 min. in the presence of 1 mM carbachol and 5 mM LiCl. Attempts to resolve and identify both components in the inositol 'trisphosphate' fraction are described in the following sections.

4.2 Preliminary studies to identify the components of the 'Ins P_3' fraction

a. Resolution of the 'Ins P_3' fraction into distinct components:

Anion exchange chromatography, mainly according to Downes and Michell (1981) and Downes et al. (1982), has been extensively used for the separation of inositol phosphates in a multitude of recent studies of phosphoinositide hydrolysis. This method has previously been observed to resolve only three fractions whose labelling increases in response to agonists. Berridge et al. (1983) have suggested that in blowfly salivary gland, rat parotid gland and cerebral cortex these correspond to Ins-1,4,5-P_3, Ins-1,4-P_2 and Ins-1-P. More recent observations suggest that at least the Ins P_1 (Siess, 1985 and see Michell, 1986) and Ins P_3 fractions (Irvine et al. 1984a and 1985) isolated by this method comprise multiple isomeric species. The potential formation of Ins P_3 isomers in response to muscarinic receptor stimulation was considered as a possible explanation of the effects of lithium reported in 3.4. When dual peaks of radioactivity were observed in the Ins P_3 fraction derived from extracts of carbachol-stimulated brain slices, with an apparent selective effect of lithium on one of these (see 6.4), an obvious possibility was that this represented a partial resolution of Ins-1,4,5-P_3 and Ins-1,3,4-P_3, both of which arise from muscarinic receptor stimulation in rat parotid gland (Irvine et al. 1984a).

The first priority to allow further investigation of this was to achieve complete resolution of the two components of the peak eluted at 1.0 M formate so that each could be studied separately. In initial attempts to achieve this a remaining portion of one of the samples originally exhibiting this dual peak was re-examined. This was derived from cerebral cortical slices labelled with 5 μCi ^{3}H-inositol and

incubated with agonist for 45 min. (see 4.1). A 1-2 ml aliquot of an original 5 ml extract was applied to a 0.6 x 3 cm column AG 1 x 8 resin (200-400 mesh, formate form). Following elution with 0.4 M AF/0.1 M FA to remove free ^{3}H-inositol and displace GroPIns, Ins P_1 and Ins P_2, the column was washed with a series of buffers of gradually increasing ionic strength as shown in Figure 4.2.1. This resulted in the separate elution of two distinct peaks of radioactivity, the first at 0.6 M AF/0.1 M FA and the second at 0.9 M AF/0.1 M FA. Initially these were respectively designated Ins P_3 (a) and Ins P_3 (b) although the range of buffer molarities separating the two peaks was surprising if each represented a distinct Ins P_3 isomer. Indeed, under such crude conditions (i.e. the use of short columns and comparatively large fraction volumes) any separation of isomeric compounds would be unexpected, particularly as Irvine et al. (1984a) initially reported incomplete resolution of Ins-1,4,5-and-1,3,4-P_3 by gradient elution hplc. Several other previous studies also suggested that separation of isomeric inositol phosphates by so great a margin would be unlikely. Both Ellis et al. (1963) and Grado and Ballou (1961) using similar, though much longer columns, presumably of greater resolving power than those currently employed, have reported only partial resolution of the isomeric inositol phosphates deriving from alkaline hydrolysis of phosphoinositides. Further, Hubscher and Hawthorne (1957) and Lester and Steiner (1968) have commented that isomeric phosphoinositols should be separable in the presence of borate via the ability of this ion to complex vicinal cis hydroxyl groups and the variable availability of these groups depending the positional phosphate substitution of the inositol ring, but neither set of authors have found this rational approach successful when applied to anion exchange chromatography. Alternatively, Spector (1976) has used a similar method to separate scyllo- and myo-inositol. More recently, Cosgrove (1978) has demonstrated resolution of scyllo-inositol trisphosphate isomers by anion exchange chromatography using gradient elution with ethanolic HCl and has concluded that at a given degree of phosphorylation of the inositol ring, compounds substituted with the greatest number of adjacent equatorial phosphate groups will show longest retention by columns. However, since Ins-1,4,5-P_3 and Ins-1,3,4-P_3 are equivalent in the latter respect this provides no basis for their resolution and from the observations of the other studies quoted above, the range of buffer molarities separating (a) and (b) initially seemed contrary to separation of isomeric Ins P_3(s).

Comparison of the ionic strength of buffers required to elute each (a) and (b) with those required for lower inositol phosphates suggested that the difference in polarity between (a) and (b) was approximately equivalent to one or two units of charge. It was assumed, by analogy with

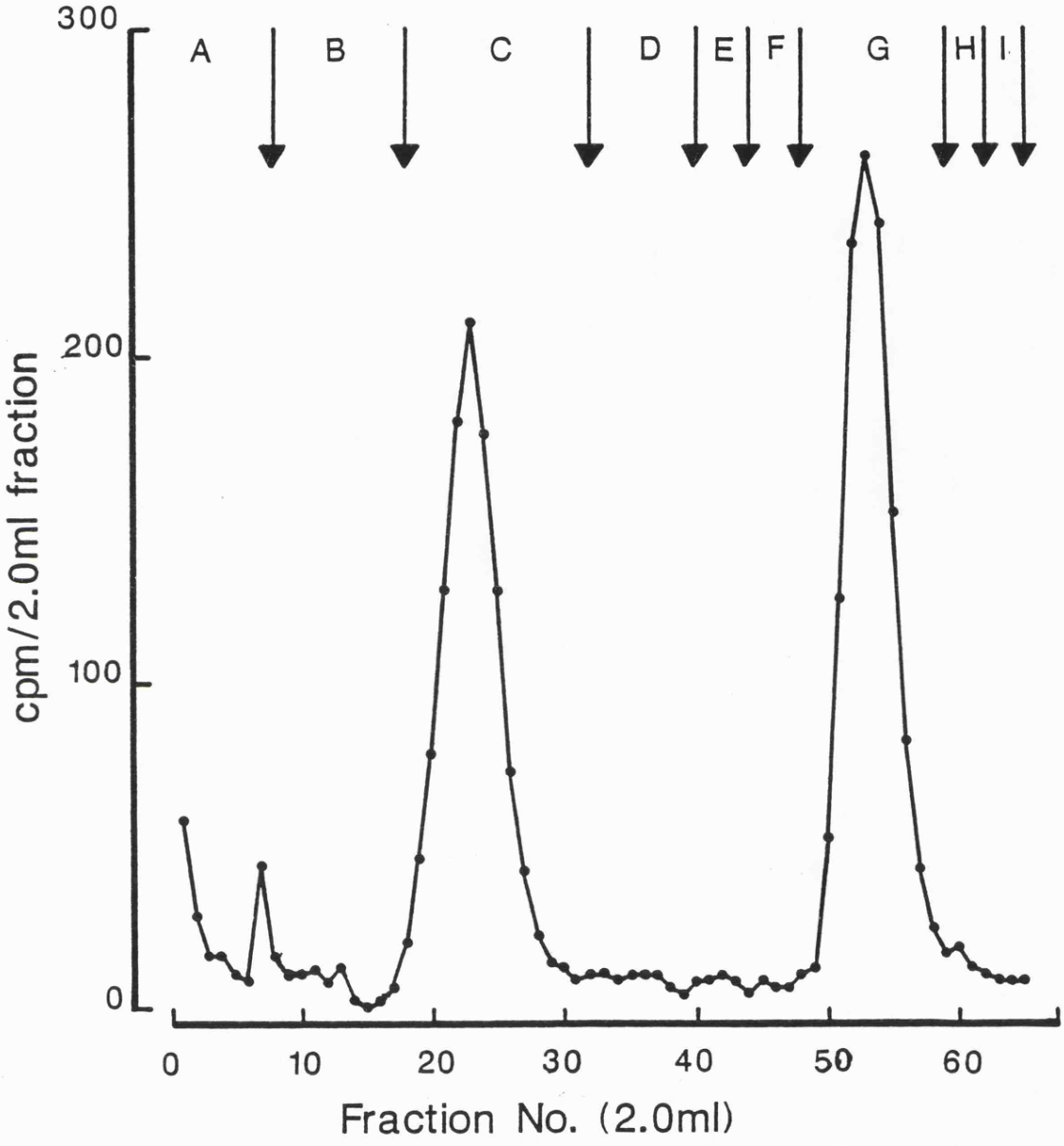


FIGURE 4.2.1 Resolution of the components of the 'Ins P_3' fraction isolated by anion exchange chromatography.

A ^{3}H-inositol phosphate extract prepared from 1 mM carbachol-stimulated, ^{3}H-inositol labelled brain slices as described in the text was analysed by anion exchange chromatography as in the legend to Fig. 4.1.1 except that after prior elution of the column with an excess (40 ml) of 0.4 M AF/0.1 M FA (to ensure complete displacement of lower ^{3}H-Ins P(s)) the column was washed with a stepwise gradient of ammonium formate (AF)/formic acid (FA) to achieve resolution of the 'Ins P_3' fraction. Buffers A-I represent: 0.4 M AF/0.1 M FA, 0.5 M AF/0.1 M FA, 0.6 M AF/0.1 M FA, 0.7 M AF/0.1 M FA, 0.75 M AF/0.1 M FA, 0.8 M AF/0.1 M FA, 0.9 M AF/0.1 M FA, 1.0 M AF/0.1 M FA and 2.0 M AF/0.1 M FA respectively. The earlier peak is 'Ins P_3 (a)' and the latter 'Ins P_3 (b)'.

previous studies where almost identical chromatographic techniques had been used (see above), that at least one of the components of the original 1.0 M formate fraction must represent Ins P_3. As no more highly phosphorylated forms of inositol than Ins P_3 had been conclusively identified in mammalian tissue it seemed probable that if only one component from (a) and (b) corresponded to a trisphosphate, the latter was the most likely candidate. This would imply that (a) represented a less highly charged molecule which was unlikely to be an Ins P_2 as previous studies clearly show these to be eluted at lower (0.4 M) molarity formate solutions (Ellis et al. 1963; Downes and Michell, 1981; Berridge et al. 1983). The only alternative compound of inositol whose mobility would approximately correspond to that of peak (a) in the elution scheme described in Figure 4.2.1 is GroPInsP$_2$. This also seemed unlikely since although small quantities of ^{3}H-GroPIns are routinely present in extracts from both control and stimulated tissues (Berridge et al. 1983), deacylation products of the parent lipids PtdInsP and PtdInsP$_2$ are not normally expected. From experiments described in 2.4 it is apparent that muscarinic receptor activation in cerebral cortex does not stimulate accumulation of ^{3}H-GroPIns. However, as the dual peaks within the Ins P_3 fraction had thus far been observed in extracts from stimulated tissue without comparison with appropriate controls, it was uncertain whether both of these responded to agonist and it was possible that low levels of each glycerophosphorylinositol phosphate diester were present in brain under both resting and stimulated conditions.

b. Further chromatography of inositol phosphates and the effect of carbachol on the components of the 'Ins P_3' fraction:

In further preliminary experiments more convenient eluting buffers for peaks (a) and (b) were first established to allow more rapid separation and easier quantification of each. Re-analysis of samples by reference to the elution characteristics of these compounds as shown in Figure 4.2.1 showed that peaks (a) and (b) were routinely cleanly resolved at 0.8 M AF/0.1 M FA and 1.0 M AF/0.1 M FA respectively. Using this system both the possible identification of Ins P_3 (a) as GroPInsP$_2$ and the effect of agonist stimulation of cerebral cortical slices on each (a) and (b) were then investigated.

i) Effects of carbachol stimulation on Ins P_3 (a) and (b): The results of a typical experiment to measure the accumulation of each inositol phosphate resulting from incubation of ^{3}H-inositol labelled tissue in the presence or absence of 1 mM carbachol under conditions exactly as previously described are illustrated in Figure 4.2.2. It is

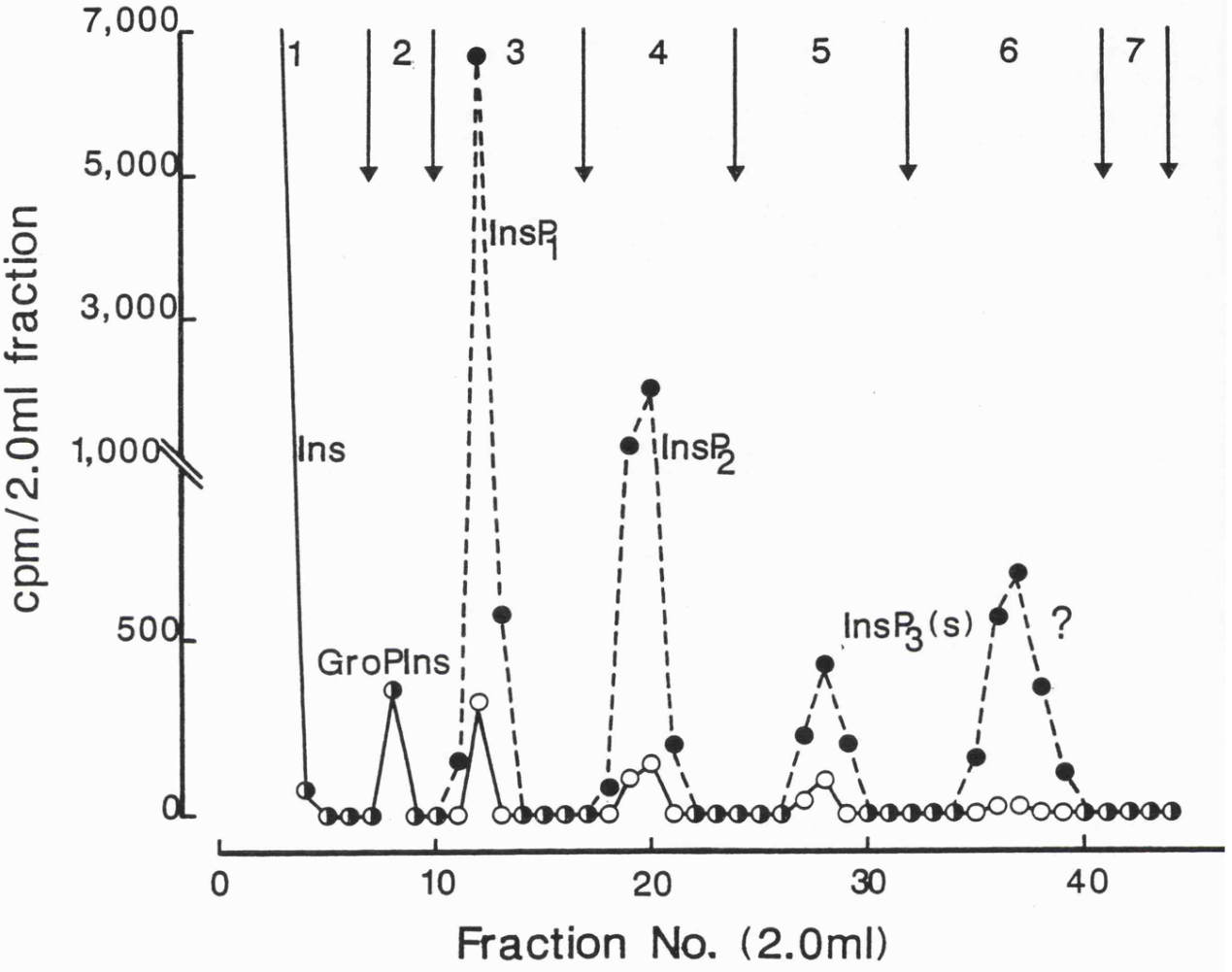


FIGURE 4.2.2 The effects of carbachol on the accumulations of '^{3}H-Ins P_3 (a)' and '^{3}H-Ins P_3 (b)' in ^{3}H-inositol labelled brain slices.

Brain slices were labelled with ^{3}H-inositol as previously then exposed to 1 mM carbachol (●------●) for 15 min. or run as control (○——○). Neutral extracts were prepared from triplicated tissue incubations as in 2.4(d)iii and analysed by anion exchange chromatography on 0.6 x 3.0 cm columns of AG 1 x 8 resin (200-400 mesh, formate form). The fractions indicated were resolved by sequential elution with the buffers 1-7 which represent: H_2O; 0.06 M AF/0.005 M $Na_2B_4O_7$; 0.2 M AF; 0.5 M AF/0.1 M FA; 0.8 M AF/ 0.1 M FA; 1.0 M AF/0.1 M FA and 2.0 M AF/0.1 M FA. The results are from a single experiment but are representative of many.

immediately evident from this that accumulation of radiolabel under each peaks Ins P_3 (a) and (b) is markedly enhanced in response to agonist. As the total of these latter two fractions corresponds to that isolated in previous sections as Ins P_3, accumulation of both products must be a consequence of muscarinic receptor occupation because atropine (10^{-5} M) effectively antagonises this response (see 3.2). Further, the rate of turnover for total 'Ins P_3' estimated in 3.2 suggests that formation and hydrolysis of both (a) and (b) must be very rapid.

The distribution of radioactivity under each of these two peaks is also of interest. In the presence of agonist the total under (b) is about twice that under (a) while in the absence of carbachol, (b) is practically undetectable. The percentage stimulation over basal is approximately 30-fold and 4-5 fold for (b) and (a) respectively. These proportions showed marked similarity to those observed for Ins-1,3,4-P_3 and Ins-1,4,5-P_3 in carbachol-stimulated rat parotid gland (Irvine et al. 1985). Therefore, although the chromatographic properties of (a) and (b) on simple anion exchange resins argued against their identities as isomeric Ins P_3(s) these results suggested that (a) might be Ins-1,4,5-P_3 and (b) Ins-1,3,4-P_3. In addition the very marked effects of carbachol on accumulation of each product was contrary to the identification of (a) as GroPInsP$_2$ since this would imply an entirely distinct mechanism of receptor-mediated PtdInsP$_2$ hydrolysis to that documented for other tissues (see Berridge, 1984) suggesting a potential combination of stimulated phospholipase A_1 and A_2 activities in addition to phospholipase C.

ii) Further chromatography of inositol phosphates: In order to eliminate the possible identification of Ins P_3 (a) as GroPInsP$_2$, ^{3}H-standards of each glycerophosphoinositol (phosphate) diester were prepared by deacylation of authentic ^{3}H-inositol labelled lipids as described in 3.5. Control and 1 mM carbachol stimulated cerebral cortical slices were extracted for both acid soluble ^{3}H-inositol labelled products and for ^{3}H-lipids and the latter also deacylated. Each standard and sample ^{3}H-materials were then examined by anion exchange chromatography for both inositol diester and monoester phosphates using methods based on those of Ellis et al. (1963) and established in 3.5.

Figure 4.2.3 compares the resulting chromatograms for the ^{3}H-monoester phosphates released in response to carbachol (A), the diester phosphates derived from the deacylation of ^{3}H-lipids from the corresponding sample (C) and the glycerophosphorylinositol (phosphates) from a mixture of authentic ^{3}H-inositol labelled lipids (B). The products from the control ^{3}H-lipid extracts were essentially identical to (C) while

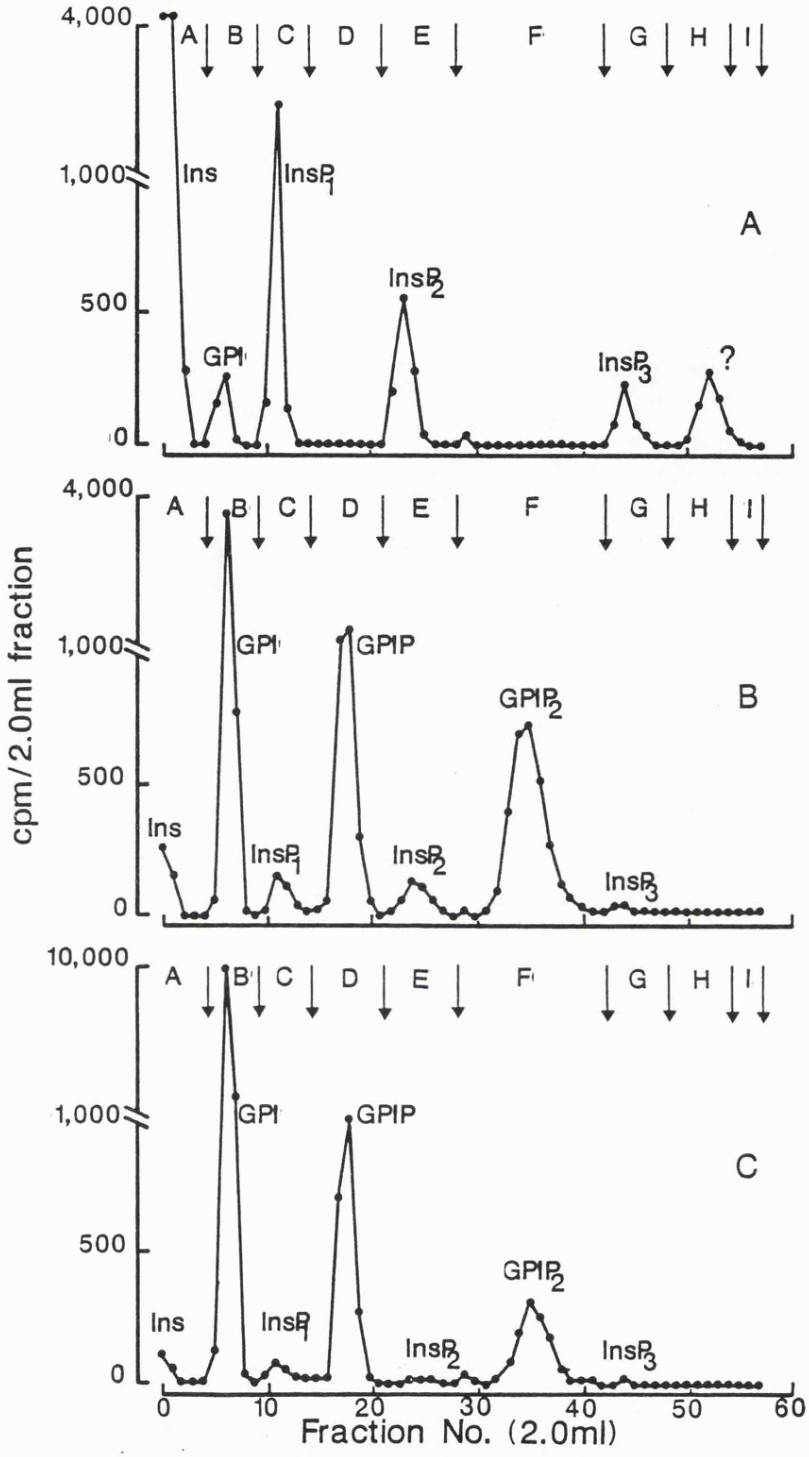


FIGURE 4.2.3 Comparative anion exchange column elution profiles of the ^{3}H-inositol phosphates derived from carbachol-stimulated brain slices and the ^{3}H-glycerophosphoryl-inositol (phosphates) derived from the ^{3}H lipids present in the same samples or from ^{3}H lipid standards.

^{3}H-Inositol phosphates (A) and ^{3}H-inositol phospholipid (C) extracts were prepared from ^{3}H-inositol labelled brain slices incubated with or without 1 mM carbachol for 15 min. Incubation conditions were as previously. The lipid extracts and a mixture of ^{3}H-inositol labelled phosphoinositides (B) were deacylated. A portion of the ^{3}H-inositol phosphate extracts and of each lipid deacylate were chromatographed as in the legend to Fig. 3.5.1 except that after eluting ^{3}H-$GPIP_2$ columns were washed as in the legend to Fig. 4.2.2. A-I represent: H_2O, 0.025 M AF, 0.2 M AF, 0.25 M AF/0.1 M FA, 0.4 M AF/0.1 M FA, 0.5 M AF/0.1 M FA, 0.8 M AF/0.1 M FA, 1.0 M AF/0.1 M FA and 2.0 M AF/0.1 M FA. Control ^{3}H-inositol phosphate extracts and ^{3}H-lipid deacylates are not shown but see text.

monoester phosphates from control tissue showed the expected reduction in labelling but the same pattern of elution profile as illustrated in (A). Each deacylated ^{3}H-lipid extract showed three major peaks of radioactivity which co-eluted exactly with those for standards of GroPIns, GroPInsP and GroPInsP$_2$, and minor, intermediate peaks, which by analogy with Brockerhoff and Ballou (1961) and Ellis et al. (1963) and from later comparison with authentic standards, are identified as ^{3}H-inositol, Ins P$_1$, Ins P$_2$ and Ins P$_3$ (see below and CH.5). As previously observed (3.5) and in accord with Ellis et al. (1963), the ^{3}H-GroPIns P$_2$ standard eluted as a comparatively broad peak at 0.5 M AF/0.1 M FA and while corresponding radiolabel was apparent after deacylation of sample ^{3}H-lipids, none of the acid soluble radioactive products whose accumulation is promoted by carbachol co-eluted at this molarity formate (see A). To displace both the Ins P$_3$ (a) and (b) higher ionic strength buffers were required. These results demonstrated unequivocally that none of the ^{3}H-products accumulating in response to carbachol corresponds to a glycerophosphorylinositol phosphate diester deriving from any of the known inositol phospholipids. Further, trace C shows no apparent lipid precursor present in extracts from stimulated tissue which could account for the formation of both Ins P$_3$ (a) and (b) but see 4.3.

The formation of small quantities of inositol monoester phosphates as side products of mild alkaline hydrolysis (deacylation) of phosphoinositides was discussed in 3.5. Each Ins P$_1$, Ins P$_2$ and Ins P$_3$ can be identified together with the major diesters resulting from degradation of the standard ^{3}H-lipids. The Ins P$_3$ produced is likely to be a mixture of Ins-1,4,5- and-2,4,5-P$_3$ with the former predominating (Brockerhoff and Ballou, 1961). Since this fraction co-eluted with the Ins P$_3$ (a) peak from carbachol-stimulated brain extracts this suggested a common identity of the latter with one or more of these trisphosphates. However, as the radioactivity within the Ins P$_3$ fraction resulting from deacylation of the standard lipid was so low this identification was only tentative and further experiments to support this conclusion are described in the following section.

c. Preparation of inositol trisphosphate standards for comparative chromatography with extracts from stimulated brain slices:

The above chromatographic evidence clearly eliminated the identification of either peaks (a) or (b) as a known diester product of inositol lipid deacylation, suggesting that both are more polar than GroPInsP$_2$ and that (a) at least might correspond to Ins-1,4,5- and/or -2,4,5-P$_3$. Further, the proportional stimulation of (a) and (b) showed marked similarity for that observed in response to carbachol for

Ins-1,4,5-P_3 and Ins-1,3,4-P_3 respectively in rat parotid gland. In view of this the albeit unlikely possibility of isomeric trisphosphate resolution being achieved on simple anion exchange columns was reconsidered, although additional studies had already shown that shallow stepwise gradients of either formate, formate/borate or formate/formic acid buffers failed to further resolve either the Ins P_1 or Ins P_2 fractions resulting from carbachol stimulation of cerebral cortical slices. Identification of either or both peaks (a) and (b) as inositol trisphosphate was initially hindered by the absence of available standard Ins-1,4,5-P_3 or Ins-1,3,4-P_3 and a defined method for resolution of these compounds. Appropriate standards either as pure compounds or as defined isomeric mixtures were therefore prepared by a variety of methods described below and their elution characteristics on anion exchange columns compared with those for the two late running peaks deriving from carbachol-stimulated brain slices in order to aid identification of the latter.

i) Preparation of standard Ins-1,4,5-P_3 and Ins-2,4,5-P_3: The preparation of Ins-(1,[^{32}P 4,5])-P_3 from ^{32}P labelled human erythrocyte ghosts has been described by Downes et al. (1982). However, a more convenient means of preparing a small quantity of ^{3}H-inositol labelled Ins-1,4,5-P_3 as a standard initially seemed to be hydrolysis of ^{3}H-authentic PtdInsP_2 as this was already available. This was attempted in two ways, firstly by enzymic hydrolysis with phospholipase C and secondly by strong alkaline hydrolysis. Until very recently the former procedure has been assumed to produce solely Ins-1,4,5-P_3. However, Wilson et al. (1985a) have shown that a purified phospholipase C from sheep seminal vesicle hydrolyses PtdInsP_2 to yield predominantly the expected Ins-1,4,5-P_3 but also a cyclic product, inositol-1:2 cyclic 4,5-trisphosphate. As inositol-1:2 cyclic monophosphate breaks down to a mixture of Ins-1-P and Ins-2-P in the presence of acid (Wilson et al. 1985a) this cyclic trisphosphate would be expected to give the corresponding Ins-1,4,5- and-2,4,5-P_3(s). Although this does not affect the results discussed below it is important to point out that the enzymic hydrolysate which is considered primarily as a marker for Ins-1,4,5-P_3 may also contain the 2,4,5 isomer as a result of the acid extraction procedure used (see below).

Phospholipase C catalysed hydrolysis of ^{3}H PtdInsP_2 was initially attempted using a commercially available B. cereus enzyme under incubation conditions which Wilson et al. (1984) have shown to be appropriate for a purified, phosphoinositide specific phosphodiesterase. However, although Sundler et al. (1978) have shown that a purified B. cereus preparation of

phospholipase C is active towards PtdIns and Drummond (1985) has found that exogenous addition of a similar bacterial enzyme to GH_3 cells results in the formation of both Ins P_3 and DG, significant hydrolysis of ^{3}H-PtdInsP_2 either in the presence or absence of 50 µM carrier PtdIns could not be detected under the current conditions. However, in identical incubations a rat brain supernatant prepared according to Irvine et al. (1979) was active towards each ^{3}H-inositol labelled PtdIns, PtdInsP and PtdInsP_2. The hydrolysis of inositol lipids by this crude preparation has been studied in detail by Irvine et al. (1984b). In the discussion below ^{3}H-Ins-1,4,5-P_3 was identified by analogy with this study as the most polar product resulting from hydrolysis of authentic ^{3}H-PtdInsP_2 when samples were analysed by anion exchange chromatography. Preparation of Ins P_3 was as described below.

A 30% (w/v) whole rat brain homogenate was prepared in 0.32 M sucrose, centrifuged at 100,000 x g for 90 min. and the resultant supernatant decanted and stored. Hydrolysis of ^{3}H-lipids using this preparation was achieved by mixing 5-10,000 cpm of phosphoinositide standard with sufficient unlabelled, carrier PtdIns to give a final concentration of 50 µM. After evaporation of lipid solvents under nitrogen, 200 µl HEPES buffer, pH 7.0 (see Appendix 1) was added, samples vortex mixed and transferred to a metabolic shaker at 37°C. A 200 µl aliquot of rat brain supernatant was added and incubations continued for 30-90 sec. prior to addition of 400 µl 1.0 M TCA. Precipitated protein was removed by centrifugation, the supernatant collected, extracted five times with two volumes of diethylether and adjusted to pH 7 with 5 mM $NaHCO_3$. Lipid hydrolysis under these conditions had the disadvantage that, as a purified enzyme preparation was not used, considerable breakdown of Ins P_3 to lower inositol phosphates occurred but sufficient Ins-1,4,5-P_3 to act as a chromatographic marker (1-2,000 cpm) could be obtained.

As a cross reference for chromatographic identification, Ins P_3 was also prepared from ^{3}H-PtdIns P_2 by alkaline hydrolysis. Briefly, several thousand cpm of lipid was dried under nitrogen, dissolved in 4 ml 2.0 M NaOH, heated to 100°C and maintained at this temperature for 30 min. Under these conditions hydrolysis of the diester bond should be complete (Hawthorne and Kemp, 1964). After cooling, the solution was freed of Na^+ ions by passage through a column of Dowex 50 (50-100 mesh, H^+ form) and finally neutralised with $NaHCO_3$. From the results of Grado and Ballou (1961) and Brockerhoff and Ballou (1961) this procedure would be expected to yield predominantly Ins P_3 (mainly Ins-1,4,5-P_3 with contaminating 2,4,5-P_3 isomer) with a smaller quantity of Ins-4,5-P_2.

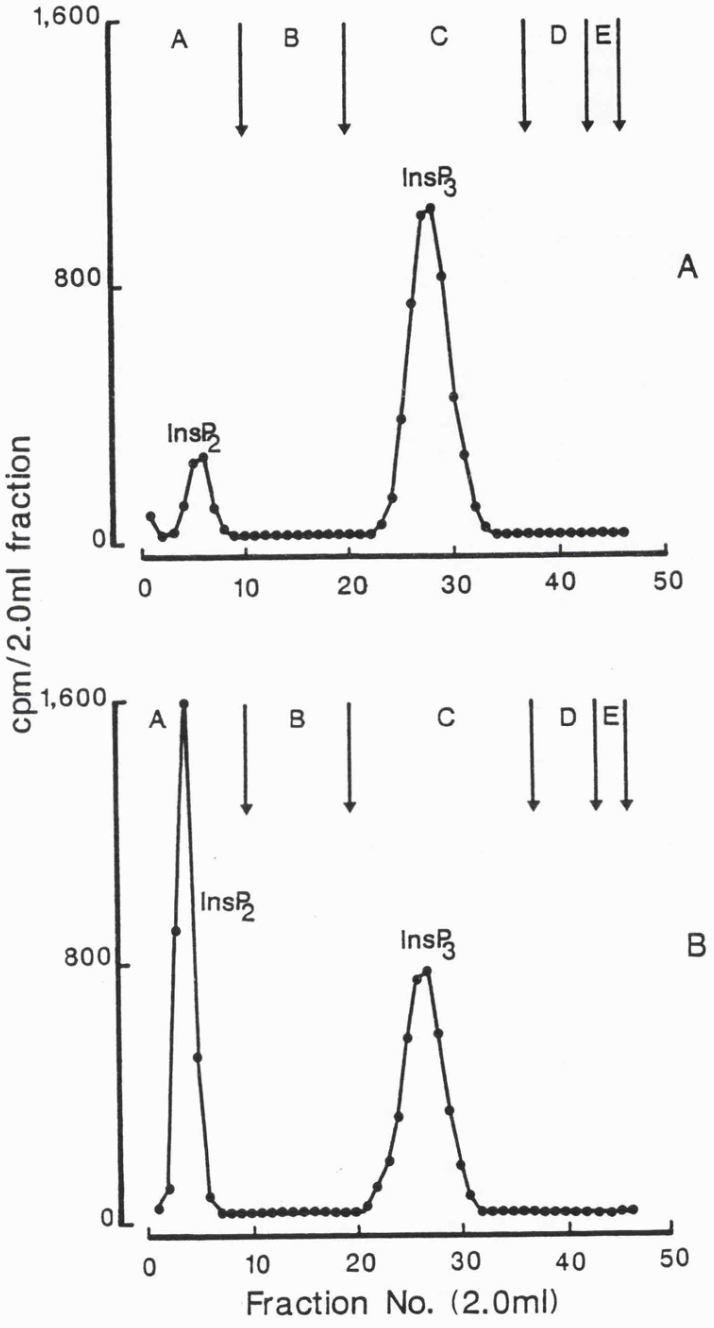


FIGURE 4.2.4 The chromatographic behaviour of different preparations of ^{3}H-Ins P_3 on anion exchange resin columns.

^{3}H-Ins P_3 (see text) was prepared from authentic, ^{3}H-inositol labelled PtdIns P_2 by either alkaline hydrolysis (A) or treatment with a crude preparation of phospholipase C from rat brain (B). Samples of each preparation were applied to separate anion exchange resin columns as previously and the columns then washed using an elution scheme based on that described in the legend to Fig. 4.2.1. A-E represent: 0.4 M AF/ 0.1 M FA; 0.5 M AF/0.1 M FA; 0.6 M AF/0.1 M FA, 0.8 M AF/0.1 M FA and 1.0 M AF/0.1 M FA. See also Fig. 4.2.5.

Samples of each the enzymic and alkaline hydrolysate of ^{3}H-PtdInsP$_2$ were examined by anion exchange chromatography using an elution scheme similar to that originally used to resolve the components of the 'Ins P$_3$' fraction described in 4.2a such that Ins P$_2$, GroPInsP$_2$ and more polar products would be separated. The results are illustrated in Figure 4.2.4. The alkaline hydrolysate contained two major products, the first eluting as expected for an Ins P$_2$ at 0.4 M AF/0.1 M FA and the second, the predominant peak, at 0.6 M AF/0.1 M FA. The enzymic hydrolysate also showed two radiolabelled fractions at the identical molarity formate buffers. The latter peak in each case is identified from above as Ins-1,4,5-P$_3$ + Ins-2,4,5-P$_3$. Neither sample exhibited radioactivity in the fraction corresponding to GroPInsP$_2$ as would be anticipated if the alkaline hydrolysis were incomplete or if intact ^{3}H-lipid remaining after enzymic breakdown was carried through the extraction procedure. Similarly, neither sample contained radioactivity which eluted at buffer molarities greater than 0.6 M AF/0.1 M FA.

These results confirmed that, within the volumes of buffers applied, Ins P$_3$ eluted in the system used only at or above 0.6 M AF/0.1 M FA and the resolution of Ins-1,4,5-P$_3$ from any Ins-2,4,5-P$_3$ present was not achieved. Comparison with the data presented in Figure 4.2.1 shows that the Ins P$_3$ prepared by either method shows very similar retention by anion exchange resin to that observed for the Ins P$_3$ (a) derived from carbachol stimulation of cerebral cortical slices which also elutes only at or above 0.6 M AF/0.1 M FA. However, although these results strongly supported the identification of 'Ins P$_3$ (a)' as an inositol trisphosphate they could not confirm its isomeric nature nor eliminate the possibility that Ins P$_3$ (b) represented Ins-1,3,4-P$_3$. This latter seemed improbable in view of the apparent co-elution of both Ins-1,4,5- and-2,4,5-P$_3$. However, in order to further investigate this possibility comparisons were made between extracts from carbachol stimulated brain slices and samples known to contain both Ins-1,4,5-P$_3$ and Ins-1,3,4-P$_3$. These experiments are described in (ii) below.

ii) The use of parotid gland extracts as a defined source of Ins-1,3,4-P$_3$: As the only defined source of Ins-1,3,4-P$_3$ and the only tissue in which accumulation of this product in proportion to Ins-1,4,5-P$_3$ had been established was rat parotid gland (Irvine *et al*. 1985) a comparative study of the relative accumulations of peaks Ins P$_3$ (a) and (b) stimulated by carbachol in this tissue and in rat cerebral cortex was next made. The previous studies which have established the identity of Ins-1,3,4-P$_3$ (Irvine *et al*. 1984a) and its metabolism relative to Ins-1,4,5-P$_3$ (Irvine *et al*. 1985) have been conducted using parotid glands

pulse labelled with ^{3}H-inositol. In contrast, the present experiments showing accumulation of Ins P_3 (a) and (b) have used a continuous labelling protocol. As pulse labelling of cerebral cortical slices is not easily achieved (2.3) initial comparisons between parotid and brain slices were made according to the current methodology. Apart from this modification parotid glands were prepared and incubated to allow as close a comparison as possible with both brain tissue and with these earlier studies. A brief description of the methods used for comparative studies is given below.

Brain slices were handled as previously allowing 30 min. prelabelling with 1 µCi ^{3}H-inositol/50 µl tissue in a volume of 300 µl before exposure to 1 mM carbachol. Rat parotid glands from two animals were excised, dissected clear of adhering tissue fragments and chopped at 150 µm intervals in three directions at 60° angles according to Hanley et al. (1980). Resulting slices were suspended by agitation in modified Krebs buffer (2.2) and washed with several volumes of the same medium before preincubation for 90 min. under identical conditions to those used for brain tissue. Labelling of parotid glands with ^{3}H-inositol was essentially as for cortical slices except that prelabelling was continued for 90 min. before agonist addition as described by Ek and Nahorski (1985). Triplicate samples of each ^{3}H-inositol labelled rat parotid gland and brain slices were incubated in the presence or absence of 1 mM carbachol for 15 min. and reactions stopped by addition of TCA. After precipitation of tissue fragments by centrifugation appropriate supernatants were combined and neutralised as previously. Aliquots of each final sample were analysed by anion exchange chromatography as described in the legend to Figure 4.2.5.

The relative accumulations of radioactivity within each inositol phosphate fraction from the two tissues are compared in Figure 4.2.5 (2) and (4). The upper traces in Figure 4.2.5 ((1) and (3)) illustrate the corresponding elution profile for an enzymic hydrolysate of authentic ^{3}H-PtdInsP_2 prepared as in (i) above to allow identification of the Ins-1,4,5-P_3 fraction. From (i) the later eluting of the labelled products in this hydrolysate can be identified as Ins-1,4,5-P_3 or as a potential mixture of isomeric Ins P_3(s). Hence Ins P_3 is marked by the proportion of the standard PtdInsP_2 hydrolysate eluting in fraction (E) (0.8 M AF/0.1 M FA). Extracts from both rat parotid gland and cerebral cortical slices show a stimulated accumulation of radiolabel under this fraction in response to carbachol. Earlier eluting peaks are identified from previous sections and by analogy with references previously quoted.

Although quantitative comparisons between the two tissues should not be drawn from Figure 4.2.5 qualitative differences are immediately

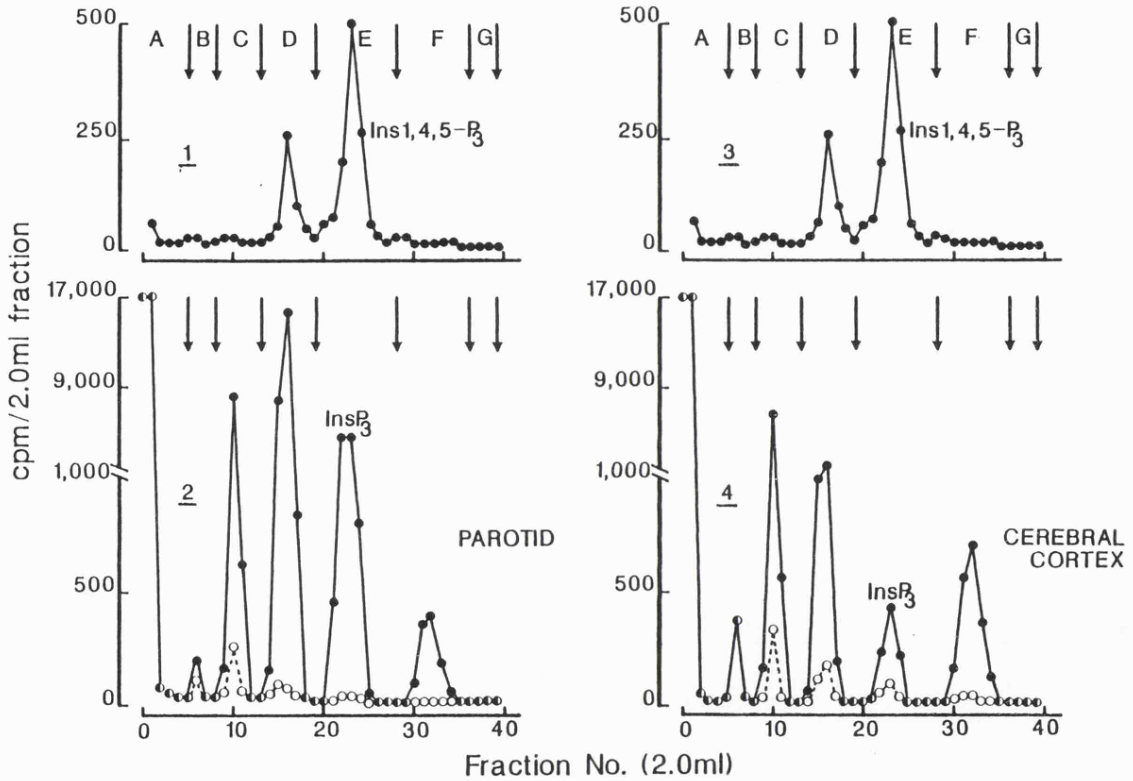


FIGURE 4.2.5 Comparative elution profiles of the ^{3}H-inositol phosphates produced in control and carbachol-stimulated rat parotid gland and cerebral cortical slices labelled with ^{3}H-inositol: identification of the Ins-1,4,5-P_3 fraction.

^{3}H-Extracts from control (O) and 1 mM carbachol-stimulated (●) rat parotid gland (panel 2) and cerebral cortex (panel 4) were prepared as in the text. Incubation of ^{3}H-inositol labelled tissues with or without agonist was for 15 min. Extracts were analysed by anion exchange chromatography as previously (see legend to Fig. 4.2.2 for buffers A-G). Panels 1 and 3 show the elution profile of an enzymic hydrolysate of ^{3}H-inositol labelled PtdIns P_2 analysed by identical methods to provide a chromatographic marker for the Ins-1,4,5-P_3 fraction. Results are from a single experiment.

apparent. In brain Ins P_1 is the major product while in parotid gland Ins P_2 predominates and Ins P_1 and Ins P_3 accumulations in this tissue are approximately equal. Relative accumulations of Ins P_1, Ins P_2 and Ins P_3 in parotid gland show a very similar pattern of response to that previously observed following muscarinic receptor activation in this tissue (Berridge et al. 1983; Downes and Wusteman, 1983), while results with brain slices are consistent with those shown in 3.1. However, most interestingly both tissues show stimulated accumulation of radioactivity under both peaks Ins P_3 (a) and Ins P_3 (b). Total ^{3}H-'Ins P_3' (a + b) in parotid gland is approximately 4-fold greater than that accumulating in cerebral cortex. Higher specific activity of parotid gland products is unlikely to account for this difference since this ratio is not consistent for each radioactive fraction. The distribution of tritium under peaks Ins P_3 (a) and (b) is markedly different in the two tissues. As from the position of the standard (upper traces), Ins-1,4,5-P_3 can be assigned as at least one component of (a) this distribution is of crucial significance. In brain, accumulation of radioactivity under (a) is about 50% that under (b) while compared to control values these fractions show 5 and 29 fold increased labelling respectively. As previously noted these relative stimulations show reasonable agreement with the data obtained by Irvine et al. (1985) in parotid gland if (a) is assumed to be Ins-1,4,5-P_3 and (b) Ins-1,3,4-P_3. However, the current data also reveals that extracts from stimulated parotid glands accumulated approximately 10 times more radiolabel in (a) than under (b) and that stimulations of these fractions were 100 and 20 fold basal values respectively. Since Irvine et al. (1985) have shown that the predominating trisphosphate accumulating in pulse labelled parotid glands after 15 min. exposure to 1 mM carbachol, in both absolute terms (i.e. cpm) and as a relative percentage of basal, is the Ins-1,3,4-P_3 isomer, the current observations raised two intriguing possibilities.

Firstly, because of the two late running peaks isolated from parotid glands the major proportion of label accumulated in the same fraction as that in which standard Ins-1,4,5-P_3 was found to elute, then (a) and (b) could not respectively be identified as Ins-1,4,5-and-1,3,4-P_3. In this situation (b) must represent a product separate from and more polar than known inositol trisphosphates. However, this assumes that the current tissue labelling conditions resulted in equivalent proportions of each Ins-1,4,5-P_3 and Ins-1,3,4-P_3 to those observed by Irvine et al. (1985). If this were not so then the second possibility was that the two peaks did represent isomeric Ins P_3(s) and that continuous and pulse labelling protocols resulted in opposite ratios of accumulated Ins-1,4,5- and 1,3,4-P_3. This latter seemed at least possible because metabolism of the

former Ins P_3 has been shown to be much more rapid than that of the latter in rat parotid glands (Irvine et al. 1985), so that if ^{3}H-Ins-1,4,5-P_3 were not continuously produced or if its specific activity declined over the 15 min. period of stimulation, then its detectable accumulation would be expected to fall relative to that of the more stable trisphosphate. In the current experiment the use of an on-going labelling protocol would be expected to eliminate such problems but the possibility remained that tissue pulse labelled with ^{3}H-inositol, as employed by Irvine et al. (1985), might be unable to sustain continued synthesis of precursor ^{3}H-PtdInsP_2 at the original specific activity over the entire period of stimulation. Such a decrease in specific activity would be dependent on the ability of labelled PtdInsP and PtdIns to buffer changes in ^{3}H-PtdInsP_2. Downes and Wusteman (1983) have shown that the total Ins P_3 accumulating in response to 1 mM carbachol in pulse labelled parotid glands remains at a constant level at least between 15 and 45 min. after addition of agonist. From the kinetics of Ins-1,4,5- and-1,3,4-P_3 hydrolysis in similar samples (Irvine et al. 1985) a considerable decline in total Ins P_3 over this period would be anticipated if sustained synthesis of ^{3}H-PtdInsP_2 could not be maintained. This indicated that the current observations would be unlikely to be accounted for by the different approach employed for labelling parotid tissue. Equally, the close qualitative agreement of the present data for the relative stimulated accumulations of each Ins P_1, Ins P_2 and Ins P_3 to those observed in pulse labelled tissue (Berridge et al. 1983; Downes and Wusteman, 1983) also supported this conclusion.

However, as it was not possible to positively eliminate the identification of (b) as Ins-1,3,4-P_3 from the current data a direct comparison of the accumulation of both Ins P_3 (a) and (b) was made in parotid glands using each a pulse and continuous labelling protocol in a further experiment. Parotid gland slices were prepared as above and split into two portions. One of these was labelled as previously and the second using a pulse labelling procedure based on that of Berridge et al. (1983) and Downes and Wusteman (1983), modified only to the extent necessary to allow as close as possible a comparison between both sets of incubation conditions.

Briefly, pulse labelled tissue was achieved by incubation of 0.6 ml gravity packed slices in a final volume of 6 ml Krebs buffer (2.2) containing ^{3}H-inositol to 0.2 μM in a water bath at 37°C. After a 90 min. prelabelling period tissue was repeatedly washed in Krebs buffer supplemented with 10 mM cold inositol then resuspended in the same medium at the original tissue/buffer concentration and incubated for a further 60 min. Following several more washes with Krebs/inositol medium, 50 μl

aliquots of tissue were transferred to 5 ml capacity, flat-bottomed vials containing sufficient Krebs buffer to give a final volume of 250 µl after addition of drugs. To allow equal total incubation times subsequent to tissue preparation, corresponding samples for continuous labelling were preincubated for 60 min. as for cortical slices then 50 µl aliquots transferred to assay vials containing Krebs medium supplemented with ^{3}H-inositol to $\sim$ 0.2 µM in a final volume of 250 µl and allowed to incorporate label for 90 min. Triplicate samples labelled according to either protocol were then incubated in the presence or absence of 1 mM carbachol for 15 min. Subsequent sample extraction and analysis were as above.

The results of this experiment are illustrated by the lower traces in Figure 4.2.6. The upper traces show the comparative elution profiles for (1) standard ^{32}P labelled Ins-1,4,5-P_3 and (3) a mixture of standard ^{32}P labelled Ins-1,4,5- and-2,4,5-P_3 in an approximate ratio of 3:1. Both standard materials were the gift of Dr. R.F. Irvine and were prepared by methods described in detail by Irvine et al. (1984a,b and 1985). The elution characteristics of these standards are consistent with those obtained with the less precisely defined isomeric mixtures of the same materials shown in Figure 4.2.4 and allow clear identification of the Ins P_3 fraction as that eluting with buffer (E) (0.8 M AF/0.1 M FA) also in agreement with earlier observations (Note: this fraction elutes as a broader band at 0.6 M formate, see Figures 4.2.1 and 4.2.4). No resolution of Ins-1,4,5- and-2,4,5-P_3 was observed.

Parotid glands labelled according to each procedure showed stimulated accumulations of ^{3}H-products co-eluting with the standard Ins P_3, although the amount of radioactivity is about 5-6 fold greater from tissue incubated under continuous labelling conditions. As the ratio of stimulated radiolabel under each Ins P_1, Ins P_2 and 'Ins P_3 (b)' is also consistently increased by 4-6 fold using continuous as opposed to pulse labelling, this difference is likely to reflect an increased specific activity rather than preferential accumulation of distinct proportions of separate products. Most importantly the relative distribution of radiolabel from stimulated samples under peaks Ins P_3 (a) and Ins P_3 (b) remains constant, with each labelling method showing a ratio a:b of 10:1.

Under pulse labelling conditions it can be assumed from Irvine et al. (1985) that the predominating labelled Ins P_3 will be the 1,3,4 isomer. Hence, because under either set of conditions the major proportion of label under peaks (a) and (b) is found in the former and because standard Ins-1,4,5-P_3 also runs with this fraction, radioactivity under (a) must represent both Ins-1,4,5-P_3 and Ins-1,3,4-P_3. That is, the separate products eluting at 0.8 and 1.0 M formate respectively cannot represent

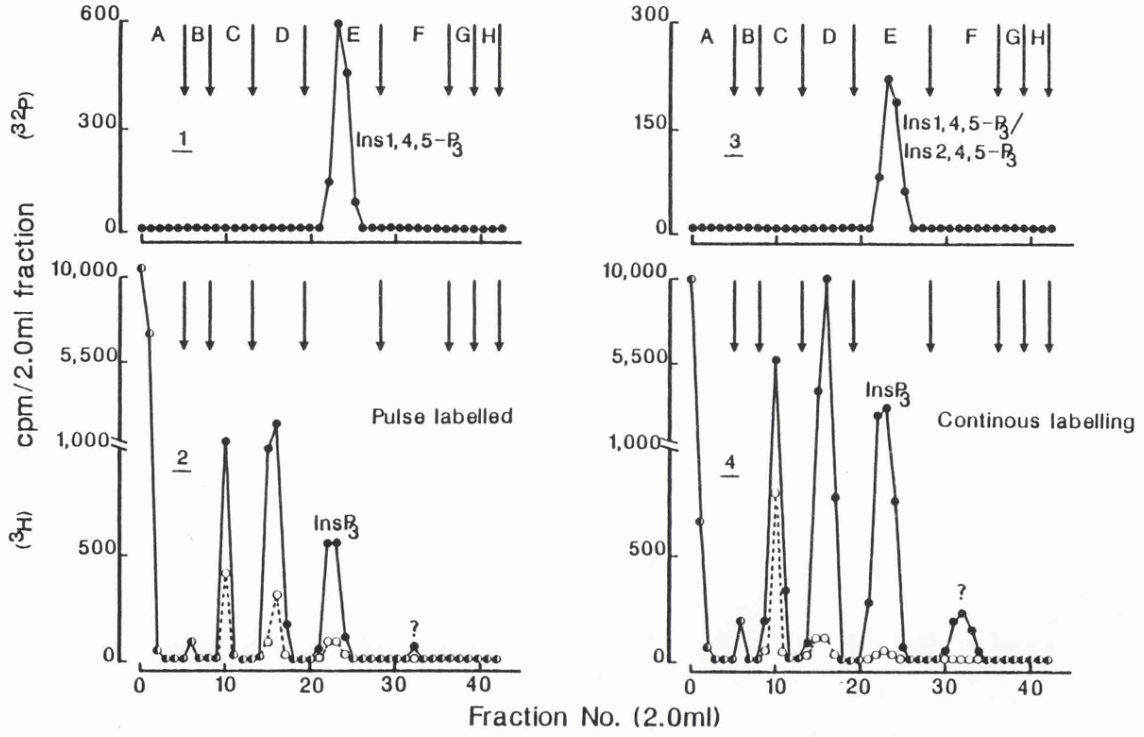


FIGURE 4.2.6 Comparative elution of profiles of the ^{3}H-inositol phosphates produced in control and carbachol-stimulated rat parotid glands labelled with ^{3}H-inositol using either a 'pulse' or 'continuous' labelling protocol: identification of the Ins-1,4,5-P_3 and Ins-2,4,5-P_3 fractions.

^{3}H-Extracts were obtained from control (○) or 1 mM carbachol-stimulated (●) rat parotid glands labelled with ^{3}H-inositol by pulse (panel 2) and continuous labelling (panel 4) methods as described in the text. Incubations ± agonist were for 15 min. Extracts were analysed as in the legends to Figs. 4.2.2 and 4.2.5. Panels 1 and 3 show the elution profiles for ^{32}P labelled standards of Ins-1,4,5-P_3 and a mixture of Ins-1,4,5-P_3 and Ins-2,4,5-P_3 respectively, analysed in an identical fashion to provide chromatographic markers for these fractions. Results are from a single experiment.

these isomeric trisphosphates. It is important to note however, that carbachol stimulation of parotid tissue does result in enhanced accumulation of label in the 1.0 M formate fraction although to a lesser relative extent than was observed in cerebral cortex and further that this response is only clearly apparent in tissue incubated with a continuous labelling protocol. This last observation suggests that, at least in parotid glands, accumulation of this ^{3}H-product is only clearly detectable when a sufficiently high specific activity is achieved. In turn this may imply a very low concentration of this product and/or a very rapid turnover.

d. Summary of data:

The data presented in this section show that carbachol stimulation of either ^{3}H-inositol labelled rat cerebral cortical or parotid gland slices results in the accumulation of a ^{3}H-product which from its chromatographic behaviour on anion exchange resin appeared distinct from:

i) the glycerophosphorylinositol phosphate diesters corresponding to the known inositol phospholipids.

ii) the inositol monoester phosphates deriving from phospho-diesteratic cleavage of the established phosphoinositides.

iii) the inositol trisphosphates known or suspected to accumulate in stimulated tissues (Ins-1,4,5-P_3, Ins-1,3,4-P_3 and Ins-2,4,5-P_3).

Further this ^{3}H-product cannot correspond to an inositol cyclic phosphate for two reasons. Firstly, any such material present in tissue samples would be hydrolysed by the acidic extraction conditions used. Secondly, the most polar cyclic phosphate anticipated would be Ins-1:2 cyclic 4,5-P_3 whose acid hydrolysis products are Ins-1,4,5-P_3 and Ins-2,4,5-P_3 both of which are eliminated by comparison with authentic standards.

Formation of this novel ^{3}H-product in cerebral cortex is probably a rapid consequence of muscarinic receptor activation because its stimulated accumulation, when determined together with that of ^{3}H-inositol trisphosphate(s) was apparent within minutes of carbachol addition (3.1) and decreased within several seconds when the response to agonist was blocked by atropine (3.2). The rapid turnover of this product in rat parotid gland is also evidenced by the labelling approach required for its detection. Similarly, the failure of previous studies to note formation of this ^{3}H-product in the latter tissue can be accounted for both by its very low level of accumulation compared to other inositol phosphates (particularly in pulse labelled tissue) and by its unexpected polarity which necessitates the use of more rigorous chromatography for its isolation from Ins P_3.

The retention of this ^{3}H-material by anion exchange resin compared to that for identified inositol trisphosphates and lower inositol phosphates suggested that its polarity differed from any of the former by a minimum of one and possibly more units of charge.

Together these observations raised the possibility that muscarinic receptor stimulation resulted in the formation of an inositol product containing four phosphate groups. From the identification by Irvine et al. (1984a) of both Ins-1,3,4- and-1,4,5-P_3 in rat parotid glands, a logical extension of this conclusion was that 'Ins P_3 (b)' might represent inositol-1,3,4,5-tetrakisphosphate (Ins-1,3,4,5-P_4). In turn the rapid turnover of this molecule, inferred from 3.2, together with this hypothetical structure suggested a plausible precursor for either or both Ins-1,4,5- and/or-1,3,4-P_3 and hence implied a potentially crucial role for the putative Ins P_4 in receptor-mediated control of $[Ca^{2+}]_i$.

However, as the current experiments provided no structural evidence to support the identification of an Ins P_4, a second possibility was that the undefined ^{3}H-product could arise through catabolism of the exogenously supplied ^{3}H-inositol such that the tritium label no longer represented an intact myo-inositol ring. Several lines of evidence initially argued against this and in favour of an Ins P_4. Firstly, the accumulation of 'Ins P_3 (b)' was apparently receptor-mediated (3.2) and by extension of the data in 3.1 and 3.2 showed kinetic characteristics very closely paralleling those for Ins P_1, Ins P_2 and Ins P_3 formation. Assuming from this that 'Ins P_3 (b)' contained label in the original myo-inositol ring, its high polarity limited the number of possible alternative identities for this product. However, the most significant support for an Ins P_4 arose from the preliminary descriptions by both Klenk and Hendricks (1961) and Santiago-Calvo et al. (1963) of phosphoinositides from human and bovine brain exhibiting inositol:phosphate ratios of 1:4. The results of the latter group seemed particularly relevant since, unlike the former report which indicated a complex inositide distinct from the established PtdIns, PtdInsP and $PtdInsP_2$, these suggested the occurrence of a conventional inositol lipid whose structure would be consistent with a phosphatidylinositol trisphosphate capable of yielding an Ins P_4 on hydrolysis by phospholipase C. In addition highly phosphorylated inositol metabolites have been identified in a variety of both animal and plant species. In the latter, metabolism of these seems particularly important during germination (see Biswas et al. 1978) while in avian and turtle red cells a role for Ins-1,3,4,5,6-P_5 in oxygen transport has been proposed (see Bartlett, 1982 for references).

Given these precedents for both highly polar inositol polyphosphates and for a potential $PtdInsP_3$, the current chromatographic data, taken in

conjunction with the results from 3.1 and 3.2 together with the identifications of Ins-1,3,4- and-1,4,5-P_3 in parotid glands (Irvine et al. 1984a), provided strong circumstantial evidence for receptor-mediated accumulation of Ins-1,3,4,5-P_4. However, clearly further studies were required to allow a conclusive identification of the putative Ins P_4. The analysis of 'Ins P_3 (b)' establishing it as Ins-1,3,4,5-P_4 and spectroscopic data supporting this conclusion are described in 4.4. The majority of these experiments were performed in collaboration with Dr. R.F. Irvine (AFRC, Babraham) as were further studies of both the kinetics of Ins P_4 formation relative to those of Ins P_3 isomers and attempts to identify an appropriate phospholipid precursor for the former molecule in cerebral cortex. However, concurrently with these studies further experiments were run assuming an Ins P_4 structure to establish (i) the relative rate of accumulation of each inositol phosphate and (ii) to attempt to obtain at least preliminary evidence for a putative PtdInsP_3. Since the results of these studies give additional support to data presented in 4.4 these are considered first in the following section.

4.3 Relative kinetics of Ins P_4 accumulation and preliminary studies to identify a PtdInsP_3 in rat cerebral cortex

Study of the accumulations of Ins-1,4,5-P_3 and Ins-1,3,4-P_3 over the first few seconds following muscarinic receptor activation in rat parotid glands has allowed Irvine et al. (1985) to establish a distinct delay in the formation of the latter product. Similar kinetics of inositol trisphosphate accumulations have also been observed in guinea-pig hepatocytes and HL 60 cells (Burgess et al. 1985). The apparent lag phase observed before stimulated accumulation of Ins-1,3,4-P_3 could be detected led to the suggestion that this product might arise through either an isomerisation of Ins-1,4,5-P_3 or by phosphodiesteratic cleavage of a PtdIns-3,4-P_2, itself produced in very limited quantities and only in response to agonist (Irvine et al. 1985). Evidence for either reaction sequence has however, been difficult to obtain.

The tentative identification in 4.2 of an Ins P_4 accumulating in carbachol-stimulated cerebral cortex and parotid gland slices and the subsequent confirmation of this as Ins-1,3,4,5-P_4 (4.4) raised a number of interesting alternative possibilities. The preliminary report of a PtdIns P_3 in brain tissue (Santiago-Calvo et al. 1963) presented a potential parent lipid precursor for this molecule and prompted the suggestion that phospholipase C catalysed cleavage of this lipid rather than of PtdInsP_2 might form the initial event in receptor-mediated phosphoinositide metabolism (Batty et al. 1985b; Nahorski et al. 1986). Formation of each Ins-1,4,5-P_3 and Ins-1,3,4-P_3 could then occur through

separate Ins-1,3,4,5-P_4 3- and 5-phosphatases respectively while the sequence of trisphosphate appearance could be dependent on the concentration and kinetics of these enzymes.

Two alternative approaches to investigate this possibility were to determine (a) the relative rates at which the putative Ins P_4 and other inositol phosphates accumulated and (b) to establish the presence or absence of a PtdInsP_3 in rat brain. Preliminary studies along each of these lines were initially made using methods previously established and the results of these are described below. Subsequent to a conclusive identification of Ins-1,3,4,5-P_4 these were re-investigated using more sophisticated techniques detailed in 4.4.

a. Relative kinetics of Ins P_4 accumulation:

The time course for polyphosphoinositide hydrolysis in response to agonists is extremely rapid (Michell et al. 1981; Berridge, 1983). However, initial rates of Ins-1,4,5-P_3 and Ins-1,3,4-P_3 accumulation can be distinguished by analysis over the first few seconds following receptor activation (Irvine et al. 1985). In order to achieve detection limits for inositol phosphates compatible with such early events, previously established methods for labelling and analysis of brain slice samples were modified as below.

Tissue was prepared as in 2.2 but after allowing a 60 min. preincubation period, 50 µl aliquots were each incubated with 5 µCi ^{3}H-inositol in a final volume of 300 µl Krebs buffer ([^{3}H-inositol]$\sim$1µM) and allowed to incorporate label for 60 min. prior to exposure to agonist. Detection of labelled products was further facilitated by combining supernatant fractions from triplicate samples after TCA extraction of tissue and on subsequent chromatography by collecting and counting individual 2 ml aliquots of each buffer thus establishing complete elution profiles for all samples. Using these methods stimulated accumulations of inositol phosphates could be reliably detected within 5 sec. of agonist addition and the baseline separation of all products under each experimental condition routinely verified.

Figure 4.3.1 illustrates the mean results of three separate experiments in which both the very rapid and later accumulation of each inositol phosphate in response to 1 mM carbachol stimulation of cerebral cortical slices was determined. Figure 4.3.2 allows comparison of the accumulation of each ^{3}H-product above basal levels over the first minute following muscarinic receptor occupation. Significant stimulated accumulation of each Ins P_4, Ins P_3 and Ins P_2 was apparent by 5 sec., the earliest time point investigated. In contrast, agonist enhanced labelling of Ins P_1 was not significant until between 20-60 sec. As considered in

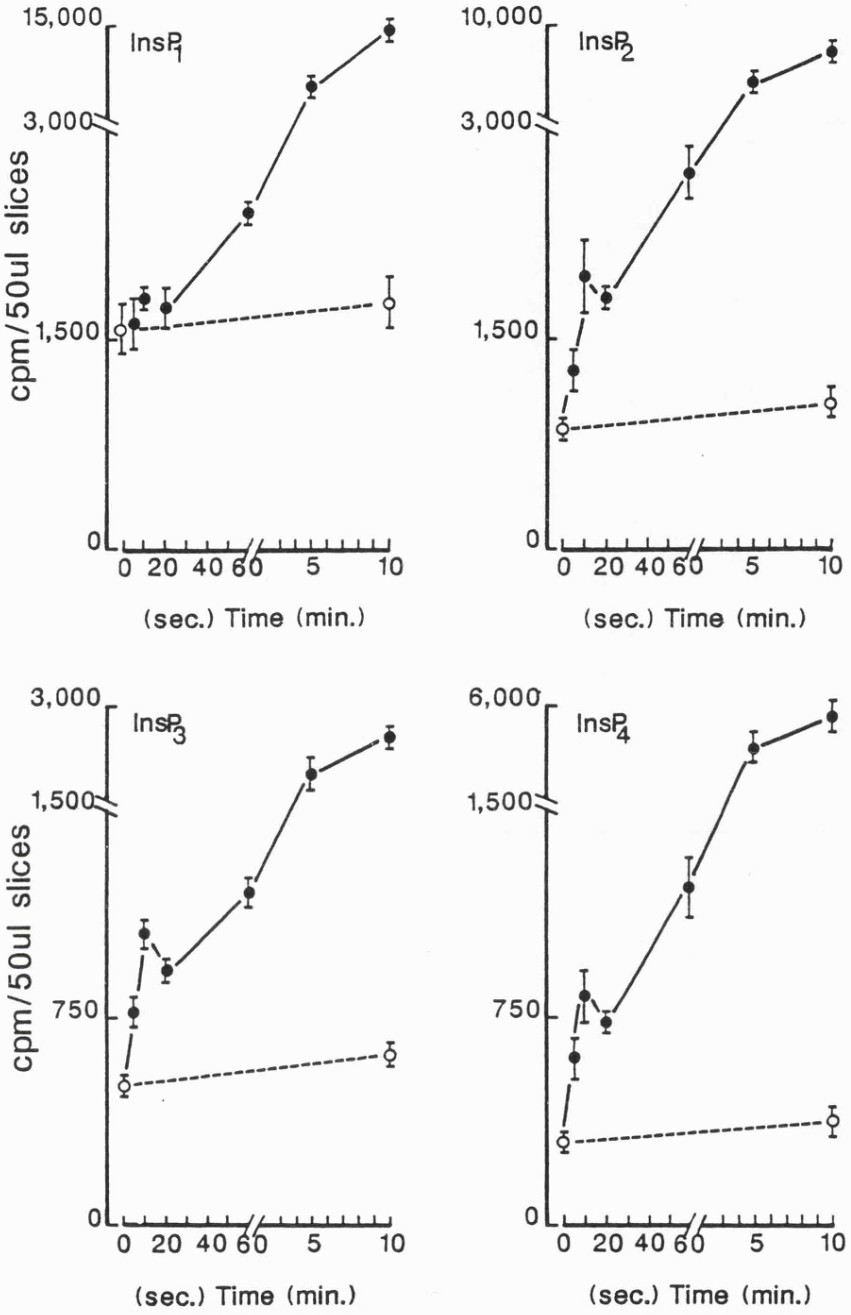


FIGURE 4.3.1 Time courses of the ^{3}H-inositol phosphate accumulations stimulated by carbachol in ^{3}H-inositol labelled brain slices.

Cerebral cortical slices were prepared and pre-incubated as in 2.2. 50 µl aliquots of packed tissue were then labelled for 60 min. with 5 µCi ^{3}H-inositol (~1 µM) in a total volume of 300 µl. Tissue was then further incubated for the times indicated either with (●) or without (○) 1 mM carbachol. ^{3}H-Ins P's were extracted, pooled from triplicated incubations and extracts neutralised as previously. Samples were analysed as in the legend to Fig. 4.2.2, a complete elution profile being constructed for each sample. Radioactivity in the Ins P_1, P_2, P_3 and P_4 fractions was calculated from the total under each peak and corrected to give a value in cpm/50 µl tissue. The results represent the mean ± SEM of 3 experiments performed in this way. Comparison of the zero time control values with the stimulated values by paired Student's 't' test indicated that carbachol significantly increased ($P \leqslant 0.05$) levels of ^{3}H-Ins P_4, P_3 and P_2 by 5 sec. Stimulated accumulation of ^{3}H-Ins P_1 was not significant by the same criteria until after 20 sec.

2.3 and 3.1 it is possible that under a continuous labelling regime changes in the radiolabelling of each inositol phosphate fraction over very rapid time courses as illustrated in Figures 4.3.1 and 4.3.2 may not necessarily reflect equal changes in concentration as increased specific activity of the precursor lipids resulting from increased flux through these is a likely immediate consequence of receptor activation. The initial rise, subsequent drop and further increase in levels of each ^{3}H-polyphosphate may be, in part, accounted for by varying specific activities. Regardless of this the increased labelling of each inositol phosphate fraction must reflect its very rapid, agonist-induced turnover. Hence, the delay before enhanced labelling of the Ins P_1 fraction is apparent compared to the immediate increases in ^{3}H-inositol polyphosphates strongly suggests that one or more polyphosphoinositides forms the immediate substrate for muscarinic receptor-mediated phospholipase C activity in brain as reported for other receptor systems in many tissues (see Berridge, 1984).

The very similar rates at which each of the inositol polyphosphates accumulate do not allow this primary lipid substrate to be identified more definitively. At all times over the first minute following carbachol stimulation, accumulation of ^{3}H-Ins P_2 exceeded that of all other products. However, as significantly increased accumulation of Ins P_1 was not evident over at least the first 20 sec. it is not clear whether the more marked increases in Ins P_2 than in Ins P_3 or Ins P_4 reflect its formation both via Ins P_3 dephosphorylation and directly from PtdInsP or merely its slower rate of hydrolysis. The evidence from 3.2 and calculations for the rate of turnover of a fraction corresponding to Ins P_3 + Ins P_4 would not contradict the latter argument. Equally, as the data does not reveal increased labelling of the putative Ins P_4 without a corresponding increase in Ins P_3 or vice versa at any time point, no evidence for a primary hydrolysis solely of PtdInsP_2 or a putative PtdInsP_3 can be inferred.

These results are entirely consistent with similar observations where the rapid kinetics of receptor-mediated Ins P_3, Ins P_2 and Ins P_1 accumulations have been studied in other tissues since the majority of these have also shown that it is difficult to dissociate rates of Ins P_2 and Ins P_3 accumulation (see Berridge, 1984). Nevertheless, the data do support the observations in 3.2 from which very rapid turnover of the Ins P_4 fraction was assumed in 4.2. However, if the putative Ins P_4 were the first product to arise from phosphoinositide hydrolysis, although it is possible that its accumulation would be closely followed by that of Ins P_3, a slightly delayed formation of Ins P_2 might be expected. In rat parotid gland a lag of 5-10 sec. is apparent for formation of Ins-1,3,4-P_3

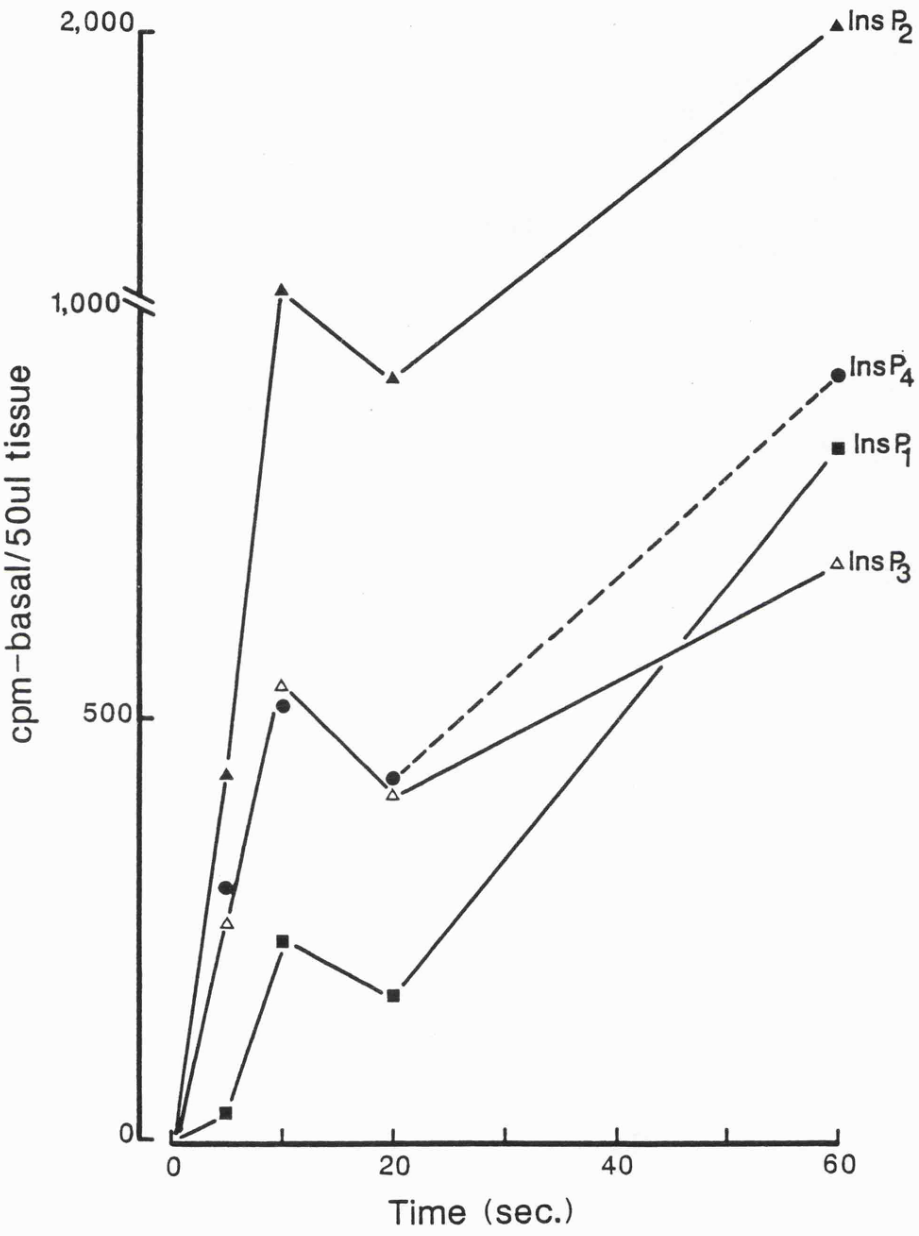


FIGURE 4.3.2 Time courses of the ^{3}H-inositol phosphate accumulations stimulated by carbachol in ^{3}H-inositol labelled brain slices.

The data presented in Fig. 4.3.1 is shown such that values for carbachol-stimulated changes in the concentrations of ^{3}H-Ins P_1, P_2, P_3 and P_4 are expressed as cpm accumulated above the respective basal levels.

but not for Ins-1,4,5-P_3 (Irvine et al. 1985) nor for Ins P_2 (Downes and Wusteman, 1983). This suggests that in this tissue, production of Ins-1,3,4-P_3 is further removed from the primary receptor promoted reaction than is Ins P_2. As with the current data showing very rapid production of Ins P_2, this would contradict an initial hydrolysis of a putative PtdInsP_3. However, in each case this could be accounted for by an extremely rapid and preferential hydrolysis of Ins P_4 through Ins-1,4,5-P_3 to Ins P_2 followed by more substantial subsequent accumulations of all products in the continued presence of high agonist concentrations. As the current experiments did not separate potential Ins P_3 isomers occurring in cerebral cortex, the significance of the rapid increase in the total Ins P_3 fraction is not clear. The apparently biphasic accumulation of each Ins P_4, Ins P_3 and Ins P_2 over the first 20-30 sec. suggested that different routes of metabolism could predominate at earlier and later time points. However, as pointed out above, initial changes in product specific activities was also a probable explanation of this biphasic pattern which was highly reproducible.

At later times the relative accumulations of inositol phosphates correspond closely with those shown in 3.1 when the previous isolation of Ins P_3 and Ins P_4 as a combined fraction is taken into account. Accumulation of Ins P_1 at 5 min. is marginally greater than that of either Ins P_2 or total Ins P_3 + P_4 while the latter two fractions are approximately equal (cf Figure 3.1.1). Also, in agreement with earlier observations, accumulations of inositol polyphosphates achieve close to maximal values earlier than does Ins P_1. The relative levels of Ins P_3 and Ins P_4 at later times are also of interest. At both 5 and 10 min. these show an approximate ratio of 1:2 (Ins P_3:Ins P_4). In the presence of lithium prolonged exposure to agonist reduced stimulated accumulation of radiolabel in the total Ins P_3 + P_4 fraction to about 30% of the initial value (3.4). In view of the fact that 60-70% of this total fraction is accounted for by Ins P_4 after 10 min. in the presence of carbachol, this suggested that the effect of lithium might be selective for this product rather than for Ins P_3. This possibility was further investigated at a later date and is considered in CH.6.

Although these preliminary experiments did not yield definitive information concerning the initial substrate for muscarinic receptor-mediated phosphoinositide hydrolysis, they did reveal significant novel data. Most importantly, evidence for the very rapid, phospholipase C catalysed breakdown of polyphosphoinositide(s) and for equally rapid accumulation of Ins P_3 was obtained for the first time in cerebral cortex. This demonstrates that although it may be difficult to prove a role for Ins-1,4,5-P_3 in control of $[Ca^{2+}]_i$ directly in this tissue at least the

rate at which Ins P_3 is produced is consistent with that in other cell types where such a role for this molecule is very strongly indicated (see Berridge and Irvine, 1984). The current data also suggest that an initial breakdown of PtdIns in brain is unlikely. If this does occur subsequent to a primary increase in Ins P_3 as suggested by Majerus et al. (1985), its significance in regard to muscarinic receptor activation in brain must be limited because:

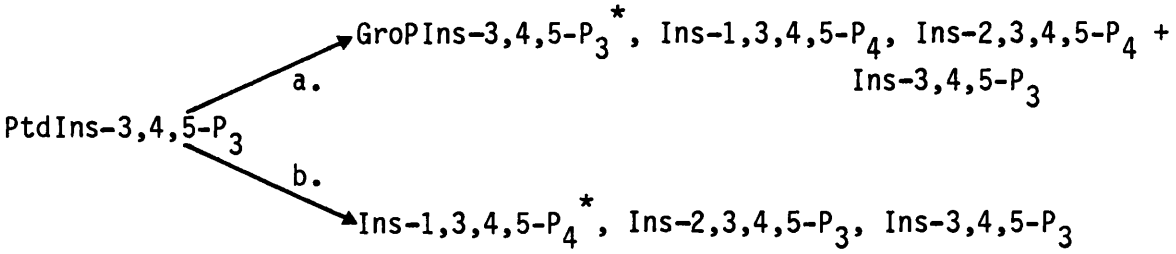
i) dephosphorylation of marked levels of ^{3}H-Ins P_2 at both early and later times is likely to account for a substantial portion of the Ins P_1 accumulating at the corresponding times and

ii) although Ins P_3 is elevated at 5 sec. a significant lag in Ins P_1 formation is clearly apparent. As from 3.2 the total Ins P_3 + P_4 fraction is very rapidly metabolised, increased accumulation of Ins P_3 will only be maintained in the continuous presence of agonist which is not representative of the situation prevailing for endogenously released ACh. Note that in the absence of physostigmine continued potassium or veratrine evoked depolarisation of cerebral cortical slices does not result in an atropine-sensitive accumulation of inositol phosphates (Kendall and Nahorski, 1986).

Finally, the current data also reveals that the novel inositol metabolite identified as Ins-1,3,4,5-P_4 (see 4.4) accumulates extremely rapidly following muscarinic receptor activation in cerebral cortex. In a single experiment, comparable with those illustrated in Figure 4.3.1, except that Ins P_4 accumulation alone was determined, carbachol stimulation of rat parotid gland resulted in production of the same ^{3}H-product again within 5 sec. While the rate at which Ins P_4 accumulated did not identify it as the primary product of stimulated inositide hydrolysis it was at least consistent with this suggestion and strongly supported the idea that this molecule is a potential precursor for Ins-1,3,4-P_3 since appearance of Ins P_4 in parotid glands showed no detectable lag phase. However, as the present experiments did not resolve potential trisphosphate isomers accumulating in cerebral cortex or parotid gland this conclusion was initially based on comparison with previous studies (Irvine et al. 1985; Burgess et al. 1985) and could not be extended to Ins-1,4,5-P_3 without further investigation. Additional studies to resolve the sequence of inositol tris- and tetrakisphosphate accumulations are described in 4.4. Initially, however, preliminary attempts were made to identify a precursor phospholipid for Ins P_4 in cerebral cortex in order to clarify the probable source of this molecule (see b. below).

b. Preliminary experiments to obtain evidence for a $PtdInsP_3$ in cerebral cortex:

Since the experiments in (a) above provided no evidence in favour of an initial preferential accumulation of Ins P_4 over other inositol polyphosphates, further preliminary studies were run to investigate the possibility that the former molecule might derive from an appropriate $PtdInsP_3$. Two alternative, crude approaches whereby evidence for such a lipid might be obtained were originally considered. These were to characterise the chromatographic properties of the products obtained from control and stimulated ^{3}H-inositol lipid extracts following either deacylation or strong alkaline hydrolysis. From previous studies (Grado and Ballou, 1961; Brockerhoff and Ballou, 1961; Ellis et al. 1963; Santiago-Calvo et al. 1963) although either method was likely to result in complex mixtures of ^{3}H-products both should yield molecules characteristic of a $PtdInsP_3$ precursor. Assuming a phosphatidylinositol-3,4,5-P_3 (PtdIns-3,4,5-P_3) the expected ^{3}H-inositol labelled products of each hydrolysis would be:



(a) = mild alkaline hydrolysis, (b) = strong alkaline hydrolysis.
*Denotes major product. Note that for deacylation by mild alkaline hydrolysis although the diester ($GroPInsP_3$) should be the sole product, previous studies using these conditions would predict further breakdown of this to yield the minor monoester products (see 3.5 and above refs.).

As standard materials were not available for comparison and because initially the only easily available chromatograhic technique was anion exchange as previously described, identification of isomeric compounds was not possible. Further, Ellis et al. (1963) have reported significant contamination of carefully prepared inositol lipid extracts with water-soluble, inositol monoester phosphates. This suggested that if the products of strong alkaline hydrolysis were examined and a product with the chromatographic properties of an Ins P_4 obtained, it might be difficult to exclude the possibility that the necessarily small quantities of this did not derive from contamination of the extract with water-soluble ^{3}H-materials arising from carbachol stimulation. Alternatively, the major product of deacylation, a $GroPInsP_3$, would be unique to a

precursor $PtdInsP_3$ and would be expected to exhibit retention by anion exchange resins intermediate between that for Ins P_3 and the putative Ins P_4. As earlier analysis (4.2) had revealed that the latter two molecules could be resolved by a wide margin and that diester and monoester phosphates from authentic inositol lipids could also be separated and identified using these methods this seemed the most rational initial approach. Although the formation of small quantities of monoester phosphates would also be produced from a $PtdInsP_3$ using the procedure reported in 3.5, this would not complicate the analysis as the only probable such molecules arising from $PtdInsP_3$ would be Ins P_3 and Ins P_4(s) which would also be separable from a $GroPInsP_3$. A more significant complicating factor is the small extent of cross-peak contamination resulting on Dowex anion exchange chromatography as discussed below.

These initial procedures were necessarily crude and could not conclusively identify a $PtdInsP_3$ but simply provide support for the existence of such by detection of a ^{3}H-inositol labelled molecule with the appropriate polarity corresponding to the expected deacylation product. However, the preliminary nature of these experiments must be emphasised since the first of these were performed prior to confirmation of the structure of the putative Ins P_4 accumulating in response to carbachol and were intended merely as a first attempt to confirm the observations reported by Santiago-Calvo et al. (1963).

As the presence of a $PtdInsP_3$ in any tissue is obviously not readily detected and as no evidence for such a molecule had been apparent amongst the deacylation products derived from ^{3}H-inositol labelled rat brain lipid extracts (see Figure 4.2.3) its concentration must be extremely low. Irvine et al. (1985) have proposed that Ins-1,3,4-P_3 might arise from phosphodiesteratic cleavage of a PtdIns-3,4-P_2 but were able to show that if such a lipid exists this must represent less than 5% of the total PtdIns-4,5-P_2 in rat parotid gland. Similarly, a PtdIns P_3 would be expected to be equivalent to only a small proportion of $PtdInsP_2$ in rat brain. To achieve suitable detection limits it was necessary to obtain a lipid extract containing appropriately highly labelled $PtdInsP_2$. In the two experiments described below ^{3}H-lipid extracts were obtained from multiple 50 µl aliquots of cerebral cortical slices incubated as in 4.3(a). Lipid extraction and deacylation were as described in 3.5 except where otherwise stated. Chromatography of products was achieved as previously described.

Figure 4.3.3 illustrates the elution profile obtained from an extract derived from tissue exposed to 1 mM carbachol for 15 min. following 60 min. prelabelling with ^{3}H-inositol. Lipid extraction was as above

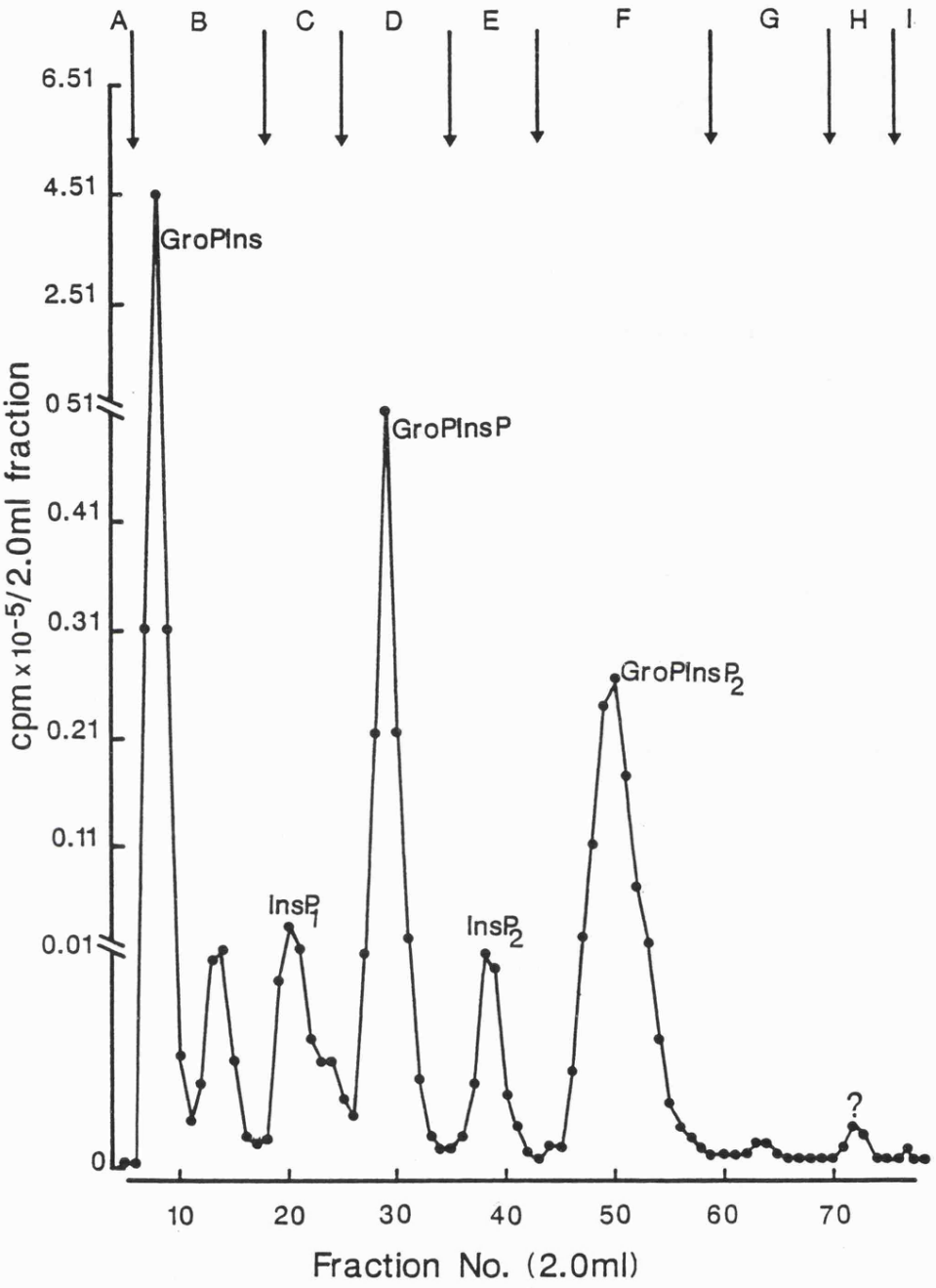


FIGURE 4.3.3 The isolation of a putative ^{3}H-GroPIns P_3 by anion exchange chromatography.

A ^{3}H-inositol labelled extract of phospholipid was obtained from carbachol-stimulated brain slices as described in the text and deacylated according to section 3.5. The neutralised deacylate was examined on a 0.6 x 3.0 cm column of AG 1 x 8 resin (200-400 mesh, formate form) eluted with the indicated volumes of buffers A-I which represent: H_2O; 0.025 M AF; 0.2 M AF; 0.25 M AF/0.1 M FA; 0.4 M AF/0.1 M FA; 0.5 M AF/0.1 M FA; 0.6 M AF/0.1 M FA; 0.8 M AF/0.1 M FA and 2.0 M AF/0.1 M FA. ^{3}H-Ins P_3 and ^{3}H-Ins P_4 extracted from carbachol-stimulated brain slices ran in fractions G and I respectively. The unidentified peak running in fraction H is discussed in the text. A similar phospholipid extract from unstimulated tissue gave qualitatively identical results.

except for a prior neutral 2:1 methanol:chloroform treatment to remove the bulk of phospholipid in order to ensure a high efficiency of deacylation under conditions as in 3.5. The labelling of the GroPIns fraction is therefore proportionally lower than shown in 3.5. The corresponding elution profile for control samples was qualitatively identical to that illustrated in Figure 4.3.3 although a more marked increased labelling of each lipid fraction due to carbachol than previously found (3.5) was noted. To provide markers for Ins P_3 and Ins P_4 a TCA extract from carbachol-stimulated tissue containing ^{3}H-inositol monoester phosphates was analysed in an identical manner. In the elution scheme illustrated in Figure 4.3.3 these ran in fraction G (0.6 M AF/0.1 M FA) and I (2 M AF/ 0.1 M FA) respectively. As anticipated from previous data this marker sample showed no radiolabel eluting in fraction H owing to prior column elution at 0.6 M formate to elute trisphosphates (see 4.2(c)i). In contrast ^{3}H-lipid deacylation products from each control and stimulated samples showed a very small peak of radiolabel running intermediate to the Ins P_3 and Ins P_4 markers. In each control and stimulated lipid extracts this was equivalent to 0.5% of the total cpm under the fraction corresponding to GroPInsP_2 while its labelling was increased about 40% in the presence of agonist as was that of GroPInsP_2.

Since the retention of this deacylation product corresponded to that expected for a GroPInsP_3 relative to Ins P_3 and Ins P_4, this initial result appeared consistent with the presence of a very low PtdInsP_3 concentration in rat brain. That this represented a small proportion of labelled PtdInsP_2 was not contrary to this suggestion since from previous studies (see above) only a fraction of each inositol lipid may be associated with the receptor mechanism and total levels of a PtdInsP_3 would necessarily have to be limited. Additionally, the possibility could not be excluded that the current result was a substantial underestimate of the putative PtdInsP_3 which by extension of the known properties of PtdInsP_2 might be difficult to extract quantitatively. When the tissue residues remaining after acidified solvent extraction were solubilised in NaOH and counted for tritium both control and stimulated samples showed greater than 10,000 cpm remained associated with these, while a similar 0.5-1% of total ^{3}H-lipid extracted always remained in the organic solvent fraction following deacylation. Either of these could account for an underestimate of a PtdInsP_3. However, the data of Santiago-Calvo *et al.* (1963) indicating the presence of a PtdInsP_3 in bovine brain, were derived from tissue homogenised in PCA and the residue extracted for lipid with neutral ethanol:chloroform (1:1) suggesting that, very much contrary to expectation, a PtdInsP_3 might not be difficult to extract. Hence, the current protocol employing an initial extraction with neutral solvent

could have been inappropriate. Nevertheless, the elution characteristics of the small unidentified fraction of radioactivity shown in Figure 4.3.3 were very close to those reported for a GroPInsP$_3$ by Santiago-Calvo *et al.* (1963).

On the basis of this result further investigation of the potential occurrence of a PtdInsP$_3$ seemed warranted. A more appropriate approach employed at a later date is discussed in 4.4. Additionally, however, this original observation was also extended at a later time using both the chromatographic technique described above and a more sophisticated hplc separation for phosphoinositols. Details of the hplc method used are described in CH.5. The results of a further experiment to characterise the unidentified fraction shown in Figure 4.3.3 are discussed below.

As the labelling of this fraction was very low a secondary chromatographic analysis of this was originally not practical. In order to allow such analysis attempts were made to obtain sufficient ^{3}H-material. The data illustrated in Figure 4.3.4 were obtained from the combined ^{3}H-lipids extracted from 60 individual 50 µl aliquots of cerebral cortical slices each incubated with 5 µCi of ^{3}H-inositol and exposed to 1 mM carbachol for 15 min. Lipid extraction was exactly as in 3.5. To achieve >99% efficiency on subsequent deacylation it was necessary to use a 2-fold greater concentration of NaOH than reported in 3.5. The resulting water-soluble products were divided into two unequal portions and one spiked with AMP, ADP and ATP and analysed by gradient elution hplc according to gradient (1), Appendix 2. The elution profile is illustrated by the lower trace in Figure 4.3.4. A larger aliquot of the deacylate was examined by Dowex anion exchange chromatography according to the elution scheme in the upper trace in Figure 4.3.4. 2 ml eluate fractions were collected and a 0.5 ml aliquot of each counted for tritium. The results shown in Figure 4.3.4 have been scaled up appropriately for the total amount of radioactivity recovered. GroPInsP$_2$ and any contaminating Ins P$_3$ were eluted from the column at 0.6 M AF/0.1 M FA (but see below). More polar products were then obtained by washing the column with 2.0 M AF/0.1 M FA and finally 3 M HCl so that detection of these would be facilitated by the sharpness of resulting peaks. The radioactivity present in the latter two fractions respectively represented 2.3 and 1.5% of that under the GroPInsP$_2$ peak. Further characterisation of the HCl fraction was not attempted since even if this originally contained any diester products considerable hydrolysis of these would be anticipated under such acidic conditions (Downes *et al.* 1982).

The peak 2.0 M formate fraction was desalted and re-examined by hplc. Briefly, this was achieved as follows. After 4-fold dilution with H_2O this fraction was re-applied to a small column comprising 200 µl 50% (w/v)

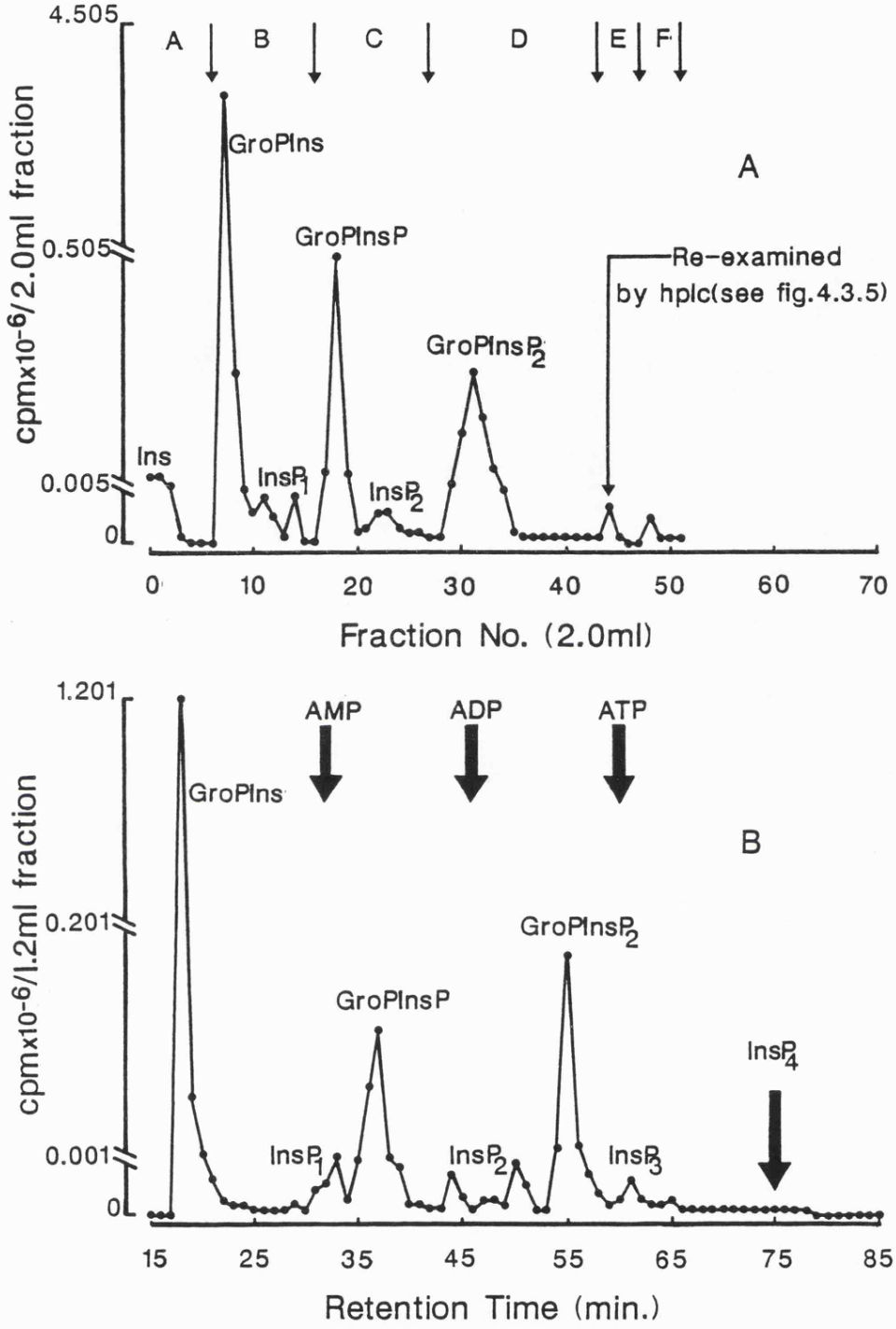


FIGURE 4.3.4 Attempts to isolate a ^{3}H-GroPIns P_3 from ^{3}H-inositol labelled brain slices using conventional and hplc anion exchange chromatography.

A bulk ^{3}H-phospholipid extract from ^{3}H-inositol labelled brain slices was prepared and deacylated as described in the text. One portion of the deacylate was examined by hplc (B) according to CH.5. A second, larger portion was analysed by Dowex anion exchange chromatography on AG 1 x 8 resin as previously (A) using an elution system where A-F represent: H_2O; 0.18 M AF/0.005 M $Na_2B_4O_7$; 0.4 M AF/0.1 M FA; 0.5 M AF/0.1 M FA; 2.0 M AF/0.1 M FA and 3.0 M HCl. Using the latter procedure 2.0 ml fractions were collected and aliquots monitored for radioactivity. Fraction E was subsequently desalted and re-examined by hplc (see Fig. 4.3.5).

slurry of Dowex 1 x 8 (200-400 mesh, Cl^- form) and the column washed with H_2O. The labelled product was re-eluted with 1 ml 2.0M LiCl and desalted according to Downes et al. (1982) except that after drying of the sample under vacuum, LiCl was removed with a single 8 ml extraction with absolute ethanol to maximise recovery which was finally between 60-75%. After evaporation of remaining solvent the sample was re-dissolved in 2 ml H_2O, spiked with ATP and analysed by hplc according to gradient (2) (Appendix 2).

The results of the primary analysis of the deacylate by each hplc and Dowex chromatography are illustrated in Figure 4.3.4. Each method revealed three major peaks of radioactivity which corresponded with those for standard GroPIns, GroPInsP and $GroPInsP_2$ prepared by deacylation of authentic ^3H-lipids. The relative proportions of label in these fractions were identical by either method. Using very similar gradient hplc Irvine et al. (1985) have shown that AMP, ADP and ATP are reasonably reliable markers for Ins P_1, Ins P_2 and Ins P_3. The close correlation between the current data and that of Irvine et al. (1985) with respect to the relative retention of identifiable nucleotide standards and glycerophosphorylinositol (phosphate) diesters suggests that the former should be equally reliable markers in the current system. The small peaks of radioactivity shown on the lower trace with retention times of 33, 45-50 and 61 min. can therefore be identified as Ins P_1, Ins P_2 and Ins P_3 respectively by comparison with the corresponding retention times for the adenosine nucleotides run as internal standards. Similar peaks corresponding to inositol monoester phosphates are also revealed by Dowex chromatography as previously noted. The hplc trace is also marked to show the expected retention time for Ins P_4.

Although both chromatographic techniques showed broadly the same pattern of products present in the deacylate, most importantly hplc revealed no product more polar than Ins P_3. In contrast, two late running peaks, albeit representing a very small percentage of total recovered radioactivity, were isolated by Dowex chromatography. The first of these is likely to correspond to that reported in the earlier experiment as having the appropriate mobility expected for a $GroPInsP_3$. When this was desalted and re-analysed by hplc it could be further resolved into two fractions which accounted for 97% of the recovered radioactivity and which exhibited the retention times relative to ATP characteristic of $GroPInsP_2$ and Ins P_3. The elution trace is shown in Figure 4.3.5 which reveals that no radiolabel was found corresponding to Ins P_4. About 3% of the recovered radiolabel (<50 cpm) ran intermediate to Ins P_3 and Ins P_4 which amounts to approximately only 0.02% of the total label originally found in the $GroPInsP_2$ fraction (see Figure 4.3.4). It must be

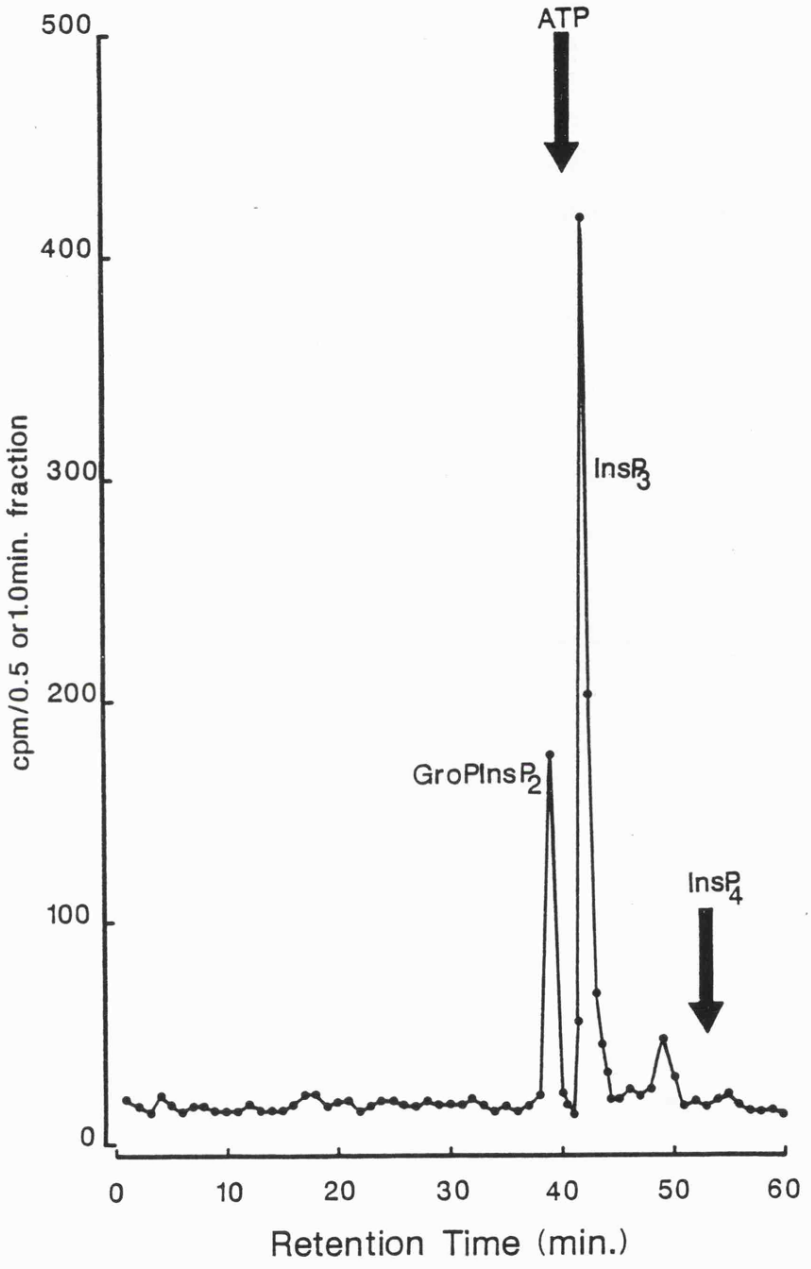


FIGURE 4.3.5 Re-examination by hplc of the putative ^{3}H-GroPIns P_3 obtained by Dowex chromatography.

Fraction E from Fig. 4.3.4 was de-salted as described in the text and examined by hplc according to CH.5 using gradient no. 2, Appendix 2. The retention time of an internal ATP marker (see CH.5) is indicated as is the expected retention time for Ins P_4. GroPIns P_2 and Ins P_3 are identified by their retention relative to ATP and by comparison with authentic standards in separate runs.

concluded from this that on Dowex chromatography a small percentage (<5%) of the peak eluted as GroPInsP$_2$ + Ins P$_3$ remains associated with the resin and may be carried forward into subsequent fractions. Data presented and reported in 4.4(b)i and iii. supports this suggestion. It is therefore very probable that at least 97% of the fraction originally observed to have characteristics appropriate to a GroPInsP$_3$ on conventional anion exchange chromatography represented GroPInsP$_2$ and/or Ins P$_3$. The nature of the remaining 3% of this fraction and of the radiolabel isolated in the current experiment at 3 M HCl (Figure 4.3.4 (A)) remains uncertain. The latter is unlikely to correspond to GroPInsP$_3$ since from the established retention for Ins P$_4$ on Dowex resin (see 4.2 and 4.4) this diester would certainly have been collected in the 2.0 M formate fraction re-examined by hplc had it been present in the original deacylate.

The labelled fraction corresponding to GroPInsP$_2$ isolated by Dowex chromatography in this experiment accounted for greater than 1.5 x 10^5 cpm. Therefore, a reliable detection limit for a putative GroPInsP$_3$ (PtdInsP$_3$) of less than 0.5% of PtdInsP$_2$ can be claimed since as little as 250-500 cpm eluting as a potential GroPInsP$_3$ on re-analysis of the 2.0 M formate fraction by hplc would have been easily detected. Given this it was concluded that the occurrence of a PtdInsP$_3$ in rat brain was unlikely unless the physico-chemical properties of this molecule were sufficiently different from those of PtdInsP$_2$ to severely invalidate the extraction or analytical methods used (see also 4.4(b)i).

Therefore, although the preliminary studies reported in this section clearly demonstrated that the product tentatively identified in 4.2 as an Ins P$_4$ accumulated very rapidly in response to muscarinic receptor activation, evidence for an appropriate lipid precursor for this molecule could not be obtained. Nonetheless, the earlier description of a PtdInsP$_3$ by Santiago-Calvo et al. (1963) and the well documented problems associated with efficient polyphosphoinositide extraction (see Downes and Michell, 1982) suggested that it would be premature to conclusively eliminate the potential occurrence of such a lipid, particularly in view of the rapid kinetics of Ins P$_4$ formation.

4.4 Identification of Ins P$_4$ and metabolism in relation to inositol trisphosphates in cerebral cortex

The experiments described in this section were performed in collaboration with Dr. R.F. Irvine (AFRC, Babraham) and methodological details of experiments performed in his laboratory are not included here as these have been fully reported elsewhere (Irvine et al. 1984a and 1985;

Batty et al. 1985b) although where appropriate the experimental rationale has been described and appropriate references given.

a. Chromatograhic, structural and spectroscopic analysis of Ins P_4:

i) Chromatographic studies: To confirm the observations made in 4.2 showing that carbachol stimulation of ^{3}H-inositol labelled rat cerebral cortical or parotid gland slices results in the accumulation of a ^{3}H-product distinct from, and more polar than, known inositol trisphosphates, a number of control and stimulated samples prepared from both tissues were initially examined by hplc. The method of Irvine et al. (1985) was originally employed for this purpose as this is the only reported means of conveniently and reliably resolving Ins-1,4,5-P_3 and Ins-1,3,4-P_3. However, extracts examined in this way revealed no labelled material eluting later than Ins P_3. Recovery of the putative Ins P_4, running in the 1.0 M formate fraction on Dowex anion exchange columns, required extension of initial hplc gradients to approximately 50% higher solvent molarity.

Figure 4.4.1 illustrates the hplc separation finally achieved between ^{3}H-Ins P_4 isolated as in (ii) from rat brain and a ^{32}P labelled standard of Ins-1,4,5-P_3 prepared from erythrocyte ghosts according to Downes et al. (1982). Routine chromatographic analysis of samples by this method is detailed in CH.5. Briefly, samples of tissue extracts were spiked with AMP, ADP and ATP and 200-300 dpm standard ^{32}P-Ins-1,4,5-P_3 and loaded onto a Partisil SAX 10 high pressure column (0.46 x 25 cm) via a 2 ml injection loop. Gradient elution to 1.7 M ammonium formate buffered to pH 3.7 with orthophosphoric acid was used to achieve resolution of labelled phosphoinositols according to Batty et al. (1985b). Eluate was monitored at 254 nM for UV absorption to detect adenosine nucleotides and either 0.25 min. (for Ins P_3 isomers) or 1.0 min. (for Ins P_4) fractions collected and counted for radioactivity. The inclusion of an internal ^{32}P Ins-1,4,5-P_3 standard in each run allowed clear identification of corresponding sample ^{3}H-material to be made. Typically Ins P_3(s) eluted between 0.75-1.0 M and Ins P_4 at 1.5 M NH_4COOH/H_3PO_4, pH 3.7, thus confirming the greater polarity of the latter molecule observed on Dowex chromatography.

Examination of extracts from both control and 1 mM carbachol-stimulated, ^{3}H-inositol labelled rat cerebral cortex or parotid gland in this system also confirmed the conclusions drawn in 4.2. Table 4.4.1 documents the relative accumulations of ^{3}H-Ins P_3 isomers and ^{3}H-Ins P_4 in each tissue following 15 min. exposure to agonist. The proportional accumulation of Ins-1,3,4-P_3 and Ins-1,4,5-P_3 in stimulated parotid gland, labelled here with an on-going synthesis protocol, shows good correlation

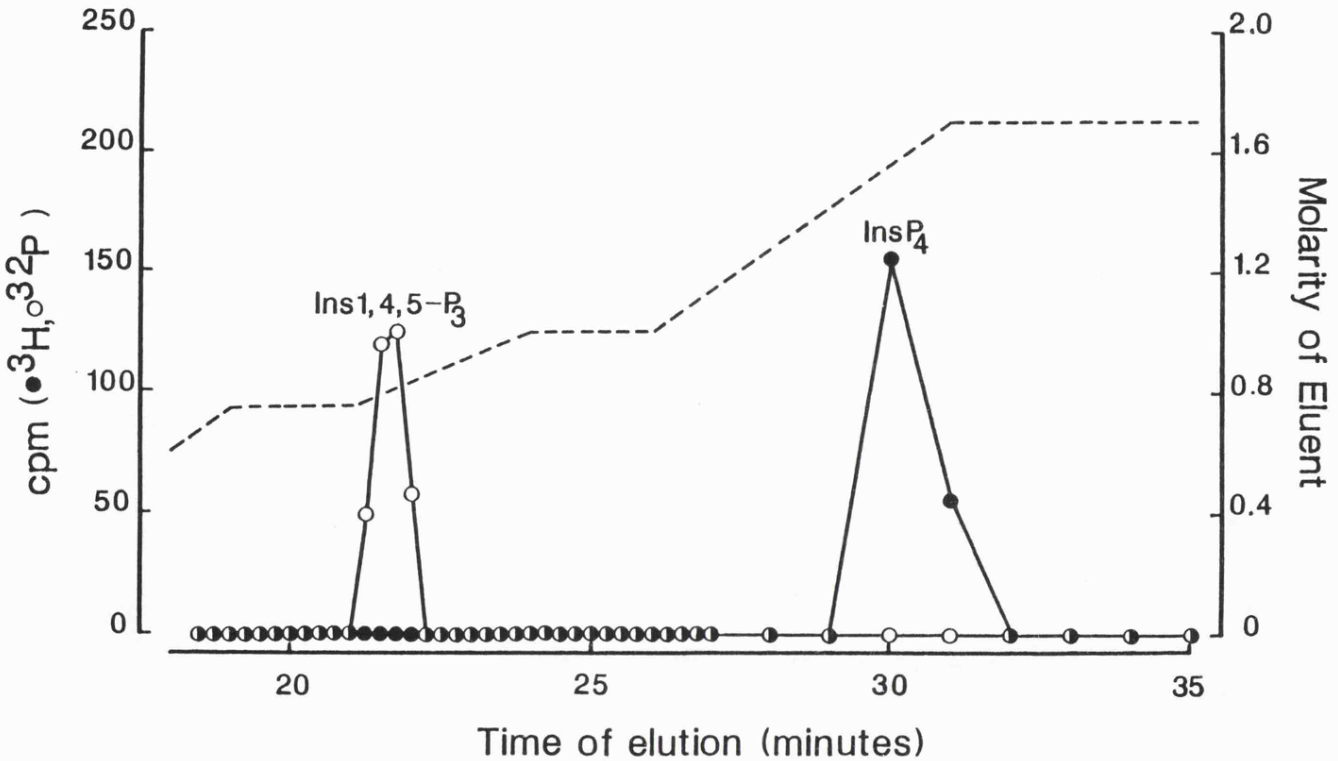


FIGURE 4.4.1 The separation of ^{32}P-Ins-1,4,5-P_3 and ^{3}H-Ins P_4 by anion exchange hplc.

^{3}H-Ins P_4 and ^{32}P-Ins-1,4,5-P_3 were obtained as detailed in the text and loaded as a 2 ml aqueous sample onto a Partisil SAX (10 μM) high performance column. The column was then subjected to gradient elution with H_2O and 1.7 M ammonium formate (pH 3.7 with H_3PO_4) as solvents and a flow rate of 1.25 ml/min. (see gradient no. 6, Appendix 2 for details). 0.20 or 1.0 min. fractions were collected as appropriate. Data is courtesy Dr. R.F. Irvine.

Table 4.4.1 Relative accumulations of ^{3}H-inositol tris- and tetrakis-phosphates in control and carbachol-stimulated rat cerebral cortical and parotid gland slices

Incubation conditions	Inositol phosphate (cpm)		
	Ins-1,3,4-P_3	Ins-1,4,5-P_3	Ins-1,3,4,5-P_4
Parotid gland (control)	10	10	not detectable
Parotid gland (1 mM carbachol)	3355	411	367
Cerebral cortex (control)	55	710	128
Cerebral cortex (1 mM carbachol)	639	2181	4874

^{3}H-Inositol labelled rat cerebral cortical and parotid gland slices were prepared as described in 4.3(a) and 4.2(c)ii (continuous labelling protocol) and subsequently incubated in the presence or absence of 1 mM carbachol for 15 min. ^{3}H-Inositol phosphates were extracted as previously described and pooled from triplicate tissue incubations. 2 ml Aliquots of each final 5 ml neutralised extract were then examined by gradient elution hplc in the presence of an internal ^{32}P Ins-1,4,5-P_3 standard as described in the text. Results are from a single experiment but are representative of further such where samples were analysed by Dowex chromatography for Ins P_4 and total Ins P_3.

with the data of Irvine et al. (1985), thus validating the assumptions made on the basis of the results shown in 4.2(c). Additionally, Table 4.4.1 shows that radiolabelled materials with the appropriate chromatographic properties of Ins-1,3,4-P_3 and Ins-1,4,5-P_3 also accumulate in response to muscarinic receptor activation in cerebral cortex although the proportion of label under the total of these two peaks and its distribution between the distinct Ins P_3 fractions is markedly different from rat parotid gland. Both this observation and the strikingly different accumulation of Ins P_4 in the two tissues is also in agreement with, and might have been predicted from the comparative data illustrated in Figures 4.2.5 and 4.2.6 (4.2).

The results obtained from these hplc analyses clearly supported the idea that the most polar product accumulating in response to carbachol carried sufficiently greater net charge than an Ins P_3 to account for a more highly phosphorylated inositol ring. To confirm this suggestion the mobility of a labelled sample of the putative Ins P_4 (prepared as in (ii)) was next compared to that for inositol tris-(Ins P_3), tetrakis- (Ins P_4), pentakis- (Ins P_5) and hexakis- (Ins P_6) phosphate standards in two further separation procedures. A mixture of standard Ins P_4 and Ins P_5 of

unknown positional isomerism was prepared by partial dephosphorylation of phytic acid (Ins P_6) according to Desjobert and Petek (1956). Cold Ins-1,4,5-P_3 and Ins (1,[4,5 ^{32}P])P_3 were prepared as described by Irvine et al. (1984a) and Downes et al. (1982) respectively. A sample of ^{3}H-Ins P_4 isolated from rat brain and containing each of these materials as internal standards was analysed by each ionophoresis in 0.1 M sodium oxalate, pH 1.5 (Seiffert and Agranoff, 1965) and by paper chromatography in propanol:ammonia:water (Desjobert and Petek, 1956; see below). In the latter system the ^{3}H-material co-migrated exactly with the internal Ins P_4 marker. On ionophoresis a partial resolution of isomeric components of the internal Ins P_4 and Ins P_5 standards was observed. The ^{3}H-inositol phosphate from rat brain co-chromatographed with the major Ins P_4 spot but also overlapped a minor spot running immediately behind this. However, when the length of ionophoretic runs was extended such that Ins P_5 and Ins P_6 migrated beyond the end of the paper and Ins P_4 isomers separated into a distinct group, the ^{3}H-material still co-chromatographed with the major Ins P_4 spot. Each paper chromatography and ionophoresis clearly separated ^{32}P-Ins-1,4,5-P_3 and the ^{3}H-brain slice sample.

In so far as it is possible to identify materials by their co-chromatography with authentic standards, both these sets of results confirmed the stimulated accumulation of an Ins P_4 in response to carbachol in cerebral cortex. However, since the positional isomerism of the standards used was necessarily undefined, it was not possible using these methods to assign the positional phosphate substitution of the inositol ring. As pointed out in 4.2 the formation of apparently the same Ins P_4 in rat parotid gland, where both Ins-1,4,5- and-1,3,4-P_3 had previously been shown to accumulate (Irvine et al. 1984a), logically suggested the possibility of the Ins-1,3,4,5-P_4 structure. Experiments to establish this are described in (ii).

ii) Structural analysis of Ins P_4 by partial dephosphorylation, periodate oxidation and reduction: A preliminary requirement of both the experiments described in (i) and those discussed here was to obtain sufficient labelled material to allow each of these analyses. Earlier time courses of Ins P_4 production in cerebral cortex (4.3a) had shown that maximal accumulations of about 20,000 dpm/50 µl tissue were achieved usually within 15 min. of agonist addition, while comparative studies with parotid gland (4.2) had indicated a much lesser accumulation in this tissue. Preparative production of ^{3}H-Ins P_4 was therefore, attempted by scaling up the procedures described in 4.3 using brain slices.

A bulk TCA extract was prepared from 40-60 separate 50 µl aliquots of cerebral cortical slices each labelled for 60 min. with 5 µCi ^{3}H-inositol

and subsequently exposed to 1 mM carbachol for 15 min. After neutralisation, the final sample (~50 ml) was applied as separate 5 ml aliquots to 0.6 x 3 cm columns AG 1 x 8 (200-400 mesh, formate form). ^{3}H-Ins P_4 was recovered from each in a single 2 ml wash with 2.0 M AF/0.1 M FA after an initial elution with 10-20 ml H_2O (for ^{3}H-inositol) and 16 ml 0.8 M AF/0.1 M FA (for total ^{3}H-GroPIns, Ins P_1, Ins P_2 and Ins P_3; see 4.4(b)ii). Recovery of Ins P_4 in this way was >95% efficient. When the ^{3}H-Ins P_4 was re-examined by Dowex chromatography radiochemical purity was also ⩾95%. Each 2.0 M formate fraction was evaporated under vacuum. Dried samples were re-dissolved in H_2O, pooled and spiked with 200 nmol unlabelled Ins-1,4,5-P_3 and de-salted by application to a Dowex column (Cl^- form) followed by re-elution with LiCl and removal of this salt, subsequent to drying, by repeated ethanol extraction (Tomlinson and Ballou, 1961; Downes et al. 1982). Recovery on de-salting was approximately 70-80% but this was very much reduced if samples were not spiked with cold Ins P_3. Using these methods up to 0.2-0.3 μCi ^{3}H-Ins P_4 could be obtained although yields were subject to some variation. Sample ^{3}H-Ins P_4 from 2-3 such preparations was used for the analyses in (i) and below.

The methods used to investigate the distribution of phosphates around the inositol ring are based on those of Grado and Ballou (1961), Tomlinson and Ballou (1961) and Irvine et al. (1984a). The logic behind these has been discussed in detail by the latter authors. Briefly, the bond between vicinal carbon atoms can be cleaved by periodate oxidation if each of these bears a hydroxyl group but not if either or both carries a phosphate substituent. Increasing phosphorylation of the inositol ring therefore limits its lability to periodate. Oxidation in this manner followed by reduction and dephosphorylation of the products will yield either an intact inositol ring or an alditol characteristic of the original phosphate substitution around this (see Figure 4.4.2). If the initial phosphate is resistant to periodate it may be rendered sensitive to oxidation by prior specific removal of one or more phosphate groups and then examined as above (see below). As the ^{3}H-Ins P_4 studied here was derived from brain tissue labelled with 2-[^{3}H]-myo-inositol, this product is assumed to retain this original ring configuration.

The methods used for periodate oxidation, for reduction of the resultant products with borohydride, for subsequent dephosphorylation of cyclic and non-cyclic polyol phosphates to corresponding polyols with alkaline phosphatase and to achieve partial Ins P_4 dephosphorylation with either alkaline phosphatase or human erythrocyte membranes, are all based on methods from the references above with modifications reported by Batty et al. (1985b). Details of chromatographic analysis and detection of

products are available from Irvine et al. (1984a) and additional sources quoted.

A sample of ^{3}H-Ins P_4 from rat brain was subjected either to the sequence of periodate oxidation, reduction and dephosphorylation or simply to extensive dephosphorylation with alkaline phosphatase alone. The resultant radiolabelled products were examined in the presence of appropriate internal standards in three systems which separate the expected cyclic and/or non-cyclic polyols. The only ^{3}H-material arising from either procedure co-migrated with authentic myo-inositol on ionophoresis in 0.1 M NaOH (Frahn and Mills, 1959) and paper chromatography in both ethyl acetate/pyridine/boric acid saturated water (12:5:4 by vol.) and propan-1-ol/ethyl acetate/water (24:13:7) (Grado and Ballou, 1961; Irvine et al. 1984a; Batty et al. 1985b).

Firstly, this result from alkaline phosphatase treatment alone supported an Ins P_4 structure, suggesting that the ^{3}H-material from rat brain comprised only a number of phosphate groups and a labelled inositol ring. Secondly, the same result following periodate oxidation demonstrated the resistance of the Ins P_4 to this treatment (this was so even after 5 days exposure to $NaIO_4$) showing that the molecule contained no vicinal hydroxyl groups. Thus, the phosphate substitution of the ring must be either as two pairs or a group of three with one single.

To distinguish between these two alternatives, samples of intact ^{3}H-Ins P_4 were subjected to partial dephosphorylation by separate methods to yield distinct inositol trisphosphates. The phosphate substitution pattern of these was then determined by chromatographic identification of the alditols resulting from each Ins P_3 after sequential periodate oxidation, reduction and complete dephosphorylation.

Partial dephosphorylation of Ins P_4 was achieved using alkaline phosphatase and a preparation of erythrocyte cell membranes. The former enzyme is largely non-specific in its hydrolysis of inositol phosphates but exhibits preferential activity towards solitary rather than vicinal phosphate groups (Tomlinson and Ballou, 1961). Thus, an Ins P_4 possessing paired phosphate groupings will be initially degraded to a complex mixture of Ins P_3(s) while that substituted to give one phosphate in isolation from the remaining three, will initially yield only a single trisphosphate. On periodate-borohydride-dephosphorylation treatment of the resultant Ins P_3(s) either a mixture of hexitols or a single pentitol will be produced respectively.

After defining conditions to allow a primary partial dephosphorylation of ^{3}H-Ins P_4 with alkaline phosphatase (Batty et al. 1985b), a bulk preparation of the Ins P_3 produced was isolated by Dowex chromatography (4.2) and subsequently degraded as above to obtain the

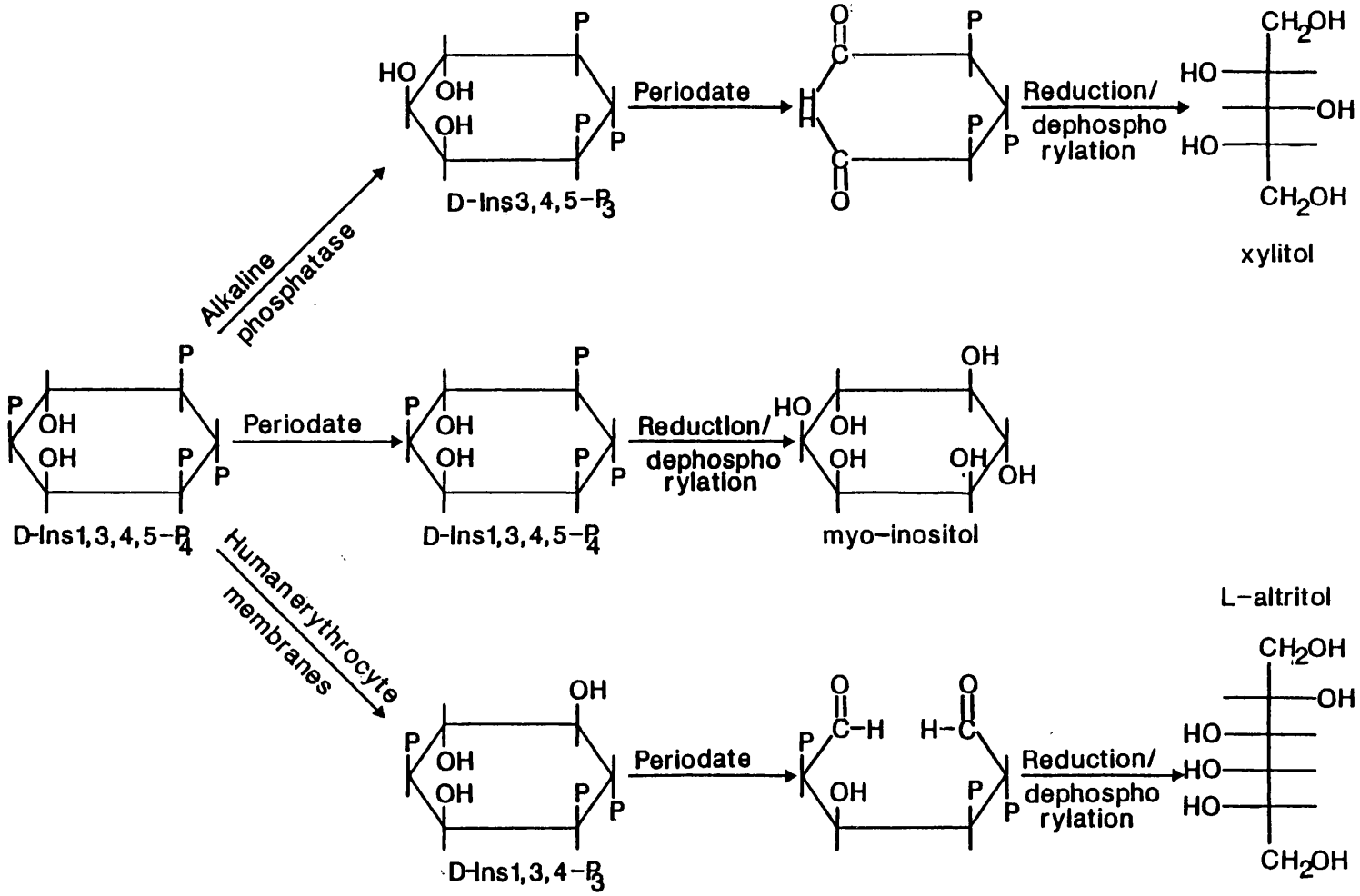


FIGURE 4.4.2 Scheme illustrating the analysis of the ^{3}H-Ins P_4 from carbachol-stimulated brain slices to establish the positions of the phosphate locants around the inositol ring.

For details of the sequences indicated see the text. Note that the original Ins P_4 and derived inositol containing structures are shown as the D-myo-inositol phosphate isomers. P represents phosphate. Data and scheme are by courtesy of Dr. R.F. Irvine.

corresponding alditol(s). These were then examined in the presence of appropriate internal standards by ionophoresis in 0.1 M NaOH (Irvine et al. 1984a) using both shorter runs to ensure no fast migrating tetritols were formed and longer runs which adequately separate most pentitols and hexitols. Only a single labelled product accounting for >90% of the original radioactivity was detected. This co-migrated with an internal xylitol standard on ionophoresis. However, as this technique does not resolve xylitol and arabitol the ^{3}H-product was also examined with appropriate standards of each of these alditols by paper chromatography in an ethyl acetate/pyridine/boric acid saturated water solvent (Grado and Ballou, 1961; Irvine et al. 1984a) to distinguish between these. This confirmed that ^{3}H-xylitol was the single labelled material produced. The only phosphate substitutions of the original ^{3}H-Ins P_4 consistent with this are D- or L-3,4,5 or 4,5,6. The latter can be eliminated because although it would also give rise to xylitol, this product would not then contain tritium as the original 2-carbon of the inositol ring and its attached hydroxyl and label bearing hydrogen would be lost on periodate oxidation. Since this confirms a D- or L-3,4,5 grouping of phosphates of the ^{3}H-Ins P_4 the resistance of the intact latter molecule to periodate also demonstrated that the remaining phosphate must occupy the D- or L-1 position.

These conclusions were further tested by a second series of partial dephosphorylation experiments using human erythrocyte cell membranes. This preparation has been shown to specifically cleave the 5-phosphate from Ins-1,4,5-P_3 (Downes et al. 1982). Similar activity towards the Ins-1,3,4,5-P_4 structure deduced above would therefore yield Ins-1,3,4-P_3. Incubation of ^{3}H-Ins P_4 in the presence of carrier unlabelled Ins-1,4,5-P_3 (from desalting) and 2 mM Mg^{2+} with human erythrocyte ghosts (2 mg protein) for 60 min. at 37°C resulted in almost quantitative (>80%) conversion to ^{3}H-Ins P_3 with less than 5% of lower inositol phosphates being formed. When a sample of the resultant ^{3}H-Ins P_3 was spiked with ^{32}P labelled Ins-1,4,5-P_3 standard and analysed by hplc as in (i), this ran relative to the standard with the retention time expected for ^{3}H-Ins-1,3,4-P_3. The same ^{3}H-Ins P_3 when subjected to periodate oxidation, reduction and dephosphorylation yield ^{3}H-altritol (>85%), indicating an original D- or L-1,3,4 or 1,2,4 phosphate substitution of the inositol ring (Irvine et al. 1984a). From the results of partial dephosphorylation with alkaline phosphatase a 3,4,5 phosphate grouping was established for the ^{3}H-Ins P_4. Thus, the ^{3}H-Ins P_3 resulting from treatment with erythrocyte membranes must necessarily be D- or L- Ins-1,3,4-P_3. The phosphate substitution of the original ^{3}H-Ins P_4 at the D- or L-1 and 4 positions is therefore confirmed.

The combined results of the chromatographic studies described in (i) and the above analyses establish the identity of the Ins P_4 accumulating in brain as D- or L- Ins-1,3,4,5-P_4 as speculatively proposed in 4.2. A very recent study revealing the probable source of this molecule in stimulated tissue (Irvine et al. 1986; see below), together with the fact that the ^{3}H-Ins P_4 studied here derived from brain slices labelled with ^{3}H myo-inositol, strongly suggests a more precise definition of this molecule as D-myo-inositol-1,3,4,5-P_4. The initial results presented in 4.2 also suggested that a very similar ^{3}H-inositol phosphate accumulates rapidly in response to muscarinic receptor activation in rat parotid gland. Downes et al. (1986) have now confirmed that this is indeed an identical Ins P_4. Several further, recent studies have extended these original observations showing that ^{3}H-inositol labelled metabolites with the chromatographic properties of ^{3}H-Ins P_4 accumulate in a variety of cell types in response to a number of stimuli (Heslop et al. 1985; Turk et al. 1986; Palmer et al. 1986; Hansen et al. 1986) and it seems probable that stimulated accumulation of this product may be a consequence common to many receptor types mediating inositol lipid hydrolysis. However, in the majority of cases Ins P_4 has been identified simply by its elution properties in hplc or Dowex chromatographic systems based on those reported above without further characterisation. Similarly, both the present more detailed analysis of Ins P_4 and that of Downes et al. (1986) are necessarily based on indirect methods involving co-chromatography of radioactivity with authentic standard materials. As a result of the studies of Irvine et al. (1986) it has very recently been possible to obtain sufficient unlabelled Ins P_4 to allow spectroscopic analysis. The preliminary results of this support the chromatographic data presented above and are described in (iii).

iii) Spectroscopic analysis of Ins-1,3,4,5-P_4: Although extraction of bulk preparations of carbachol-stimulated rat cerebral cortex yielded sufficient ^{3}H-Ins P_4 to allow each of the above studies to be performed at least in duplicate, this method obviously did not produce enough material for examination by more powerful spectroscopic techniques. Ozaki et al. (1986) have reported preliminary success in the chemical synthesis of D-myo-inositol-1,4,5-P_3 which may indicate that future studies of Ins P_4 metabolism and function (see below) may be facilitated by more ready availability of this compound synthesised by similar methods. However, for the present purpose a sample of unlabelled Ins P_4 was prepared by scaling up the procedures described in CH.6. Approximately 0.5 μmol Ins P_4 (the gift of Dr. R.F. Irvine) was examined by nuclear magnetic resonance spectroscopy (nmr) according to the legend for Figure 4.4.3

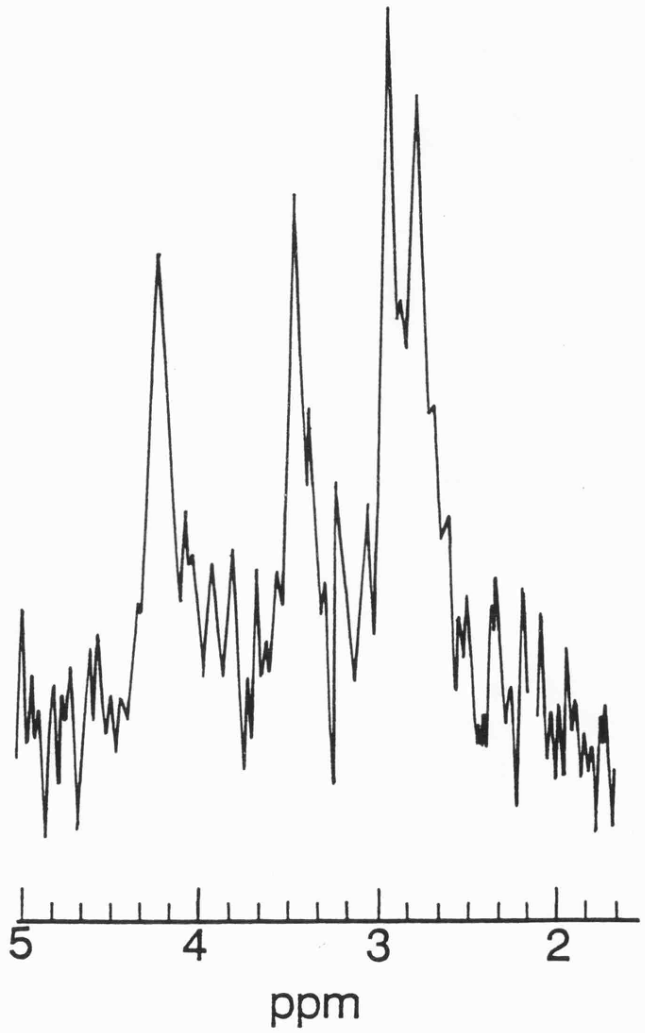


FIGURE 4.4.3 The ^{31}P NMR spectrum of inositol-1,3,4,5-tetrakisphosphate.

The spectrum was recorded at 121.5 MHz on a Bruker AM300 NMR spectrometer with broad band proton decoupling. The sample was contained in a 5 mm precision tube. Chemical shifts are given in ppm; a positive shift denotes a downfield shift from 85% H_3PO_4 as reference. The sample ($\sim$0.5 µmol) was dissolved in 100 mM triethylammonium bicarbonate buffer, pH 9.5, containing 50% D_2O. ^{31}P NMR parameters were: sweep width, 600.7 Hz; pulse width, 10 µs; aquisition time, 1.34 s; number of points, 16 K; number of reference transients, 50,000; line broadening, -1.0 Hz; gaussian broadening, 0.03 Hz; sample concentration, about 1 mM. Spectrum is courtesy of Dr. B. Potter.

which illustrates the proton decoupled ^{31}P nmr spectrum of Ins-1,3,4,5-P_4. This analysis and the interpretation of data derived from it are courtesy of Dr. B. Potter (Department of Chemistry, University of Leicester).

The spectrum presented in Figure 4.4.3 clearly shows four resonances at delta 2.74, 2.87, 3.39 and 4.14 ppm which correspond to four non-equivalent phosphate groups, indicating an assymetrical substitution of the inositol ring. Although it is not possible to define the positional phosphate distribution precisely from this data, the spectrum demonstrates unequivocally that this material is a tetrakisphosphate. Additionally, as the four phosphates are not equivalent, the possibility of symetrical substitution of the inositol ring as in Ins-1,2,3,5; 1,3,4,6 or 2,4,5,6-P_4 can be eliminated. Studies of the ^{1}H nmr spectrum for Ins P_4 have also been attempted and although this is complex and requires further analysis it also appears to suggest an unsubstituted 2 position of the inositol ring. The only substitution patterns consistent with this are: 1,3,4,5; 1,3,4,6; 1,3,5,6; 1,4,5,6 and 3,4,5,6. As 1,3,4,6 can be discounted from the data shown only 4 possibilities remain, the only one of which is entirely in accord with the results in (ii) is Ins-1,3,4,5-P_4.

To the extent which spectroscopic analysis has thus far been possible, these data confirm all the other available evidence in identifying the most polar inositol phosphate accumulating in response to carbachol in cerebral cortex as D-*myo*-inositol-1,3,4,5-tetrakisphosphate. The results presented and discussed in earlier sections establish that stimulated formation of this novel inositol metabolite is one of the most rapid biochemical responses attributable to muscarinic receptor activation in brain. The significance of these observations demonstrating receptor-mediated accumulation of an Ins P_4 in mammalian tissue for the first time is considered in the following sections.

b. Metabolism of Ins-1,3,4,5-P_4 in rat cerebral cortex:

The experiments reported in 4.3(a) demonstrated that both ^{3}H-Ins P_4 and total ^{3}H-Ins P_3 accumulate without any detectable lag following exposure of cerebral cortical slices to carbachol. Together the very rapid formation of Ins P_4 and its established pattern of phosphate substitution logically suggested it may be the immediate metabolic precursor for both inositol trisphosphates. The preliminary data suggesting a similar rate of Ins P_4 formation in parotid glands where accumulation of Ins-1,3,4-P_3 is delayed by several seconds (Irvine *et al.* 1985) and the ability of erythrocyte membranes to hydrolyse Ins P_4 specifically to this trisphosphate (4.4(a)ii) clearly supported this suggestion with respect to Ins-1,3,4-P_3. A precursor role for Ins P_4 with regard to Ins-1,4,5-P_3 was less certain as previous studies in cerebral

cortex (4.3) had not extended to isomeric resolution of Ins P_3(s) and particularly as in parotid gland no lag phase for ^{3}H-Ins-1,4,5-P_3 accumulation had been evident (Irvine et al. 1985). In order to clarify this situation, further studies were undertaken to establish the relative kinetics for ^{3}H-Ins P_4 and ^{3}H-Ins P_3(s) accumulating in response to muscarinic receptor stimulation in cerebral cortical slices (see (ii)).

However, the most definitive evidence for an initial formation of Ins-1,3,4,5-P_3 and subsequent hydrolysis of this to yield both Ins P_3(s) would be the demonstration that a parent phosphatidylinositol-3,4,5-trisphosphate (PtdInsP_3) is present in brain. Although the latter of the two studies reported in 4.3(b) contradicted the earlier experiment, the results of Santiago-Calvo et al. (1963) showed that further examination of this possibility was essential. As confirmation or denial of the occurrence of a PtdInsP_3 would clearly facilitate interpretation of the additional kinetic studies both sets of experiments were run concurrently. The examination of ^{3}H-lipid extracts for a PtdInsP_3 is described in (i) and the significance of the results obtained in relation to the kinetic studies are discussed in (ii).

i) PtdInsP_3 is not detectable in rat cerebral cortex: The methods used to establish the possible occurrence of a PtdInsP_3 were identical in approach to those described in 4.3(b) except that more sophisticated techniques were employed both to degrade ^{3}H-inositol labelled lipid extracts and for subsequent chromatographic analysis of the hydrolysis products. These methods are based on those previously suggested by Irvine et al. (1984a) for the identification of a putative PtdIns-3,4-P_2 from rat parotid glands.

Pooled ^{3}H-lipids were obtained from multiple 50 µl aliquots of cerebral cortical slices labelled with ^{3}H-inositol according to 4.3(a) by repeated extraction with acidified methanol/chloroform as described by Griffin and Hawthorne (1978) (see 3.5). Lipids were dried under nitrogen then deacylated by the method of Clarke and Dawson (1981) which has the advantage over mild alkaline hydrolysis that much smaller quantities of inositol monoester phosphates are produced as a result of secondary glycerophosphorylinositol (phosphate) diester breakdown. Each sample was then divided into two equal portions.

One of these was spiked with adenosine nucleotide markers and Ins (1,[4,5 ^{32}P])-P_3 and examined by hplc as in 4.4(a)i for any ^{3}H-products with a retention time appropriate for a ^{3}H-GroPInsP_3. Fraction collection covered that section of the gradient over which previous analyses using standard materials had shown GroPInsP_2 and more polar products, up to Ins P_4, to be recovered. GroPInsP_3 would be expected to

show a mobility intermediate between that for Ins P_3 and Ins P_4. Figure 4.4.4(A) shows a typical elution trace obtained. The retention times for the internal ^{32}P-Ins P_3 standard and that expected for Ins P_4 are marked.

The remaining half of the deacylate was further degraded by specific removal of the glycerol moiety according to the method of Brown et al. (1959). This was achieved by limited periodate oxidation followed by elimination of the resultant aldehyde with 1,1-dimethylhydrazine (Brown and Stewart, 1966; Irvine et al. 1985; see also Downes et al. 1986). This procedure effectively (i.e. >95% efficient, Irvine et al. 1985) converts glycerophosphorylinositol-4,5-P_2 to Ins-1,4,5-P_3 without inducing phosphate migration (Brown and Stewart, 1966) as is observed on strong alkaline hydrolysis which results in formation of both Ins-1,4,5-and 2,4,5-P_3 (Brockerhoff and Ballou, 1961). By analogy with this, any GroPIns-3,4,5-P_3 derived via deacylation of the putative PtdInsP_3 would be converted to Ins-1,3,4,5-P_4. Therefore, if any radiolabel ran with the expected mobility of a GroPInsP_3 prior to removal of the glycerol moiety and subsequent to this treatment ran as an Ins P_4 this would represent convincing preliminary evidence for a PtdInsP_3. After degradation in this manner, the second portion of each deacylate was also spiked with markers and ^{32}P Ins-1,4,5-P_3 standard and examined by gradient elution hplc. Figure 4.4.4(B) illustrates the elution profile obtained, again showing only the relevant section (GroPInsP_2 up to Ins P_4). Comparison of Figures 4.4.4(A) and (B) allows the increase in retention time resulting from removal of the glycerol moiety from GroPInsP_2 to yield Ins P_3 to be observed.

Each control and stimulated samples was examined using both of these procedures. The results shown are typical of those achieved with either tissue exposed to carbachol for 30 min. or that run as control. Direct analysis of deacylates revealed no detectable labelled product with a retention time greater than that observed for an internal ^{32}P Ins-1,4,5-P_3 standard (Figure 4.4.4(A)). Equally, following partial degradation of the glycerophosphorylinositol (phosphate) diesters to corresponding inositol monoester phosphates no product more polar than Ins P_3 could be observed while conversion of GroPInsP_2 to Ins-1,4,5-P_3 was almost quantitative within the same sample (Figure 4.4.4(B)).

The improved separation and definition of distinct inositol phosphate fractions permitted by the hplc system used over that which could be achieved with corresponding Dowex chromatography, and the absence of the low, but significant carry over observed with the latter method (4.3(b)), ensured that any labelled products potentially deriving from a PtdInsP_3 would have been quite clearly resolved in the above experiments. Therefore, from the extensive labelling of PtdInsP_2 (GroPInsP_2)

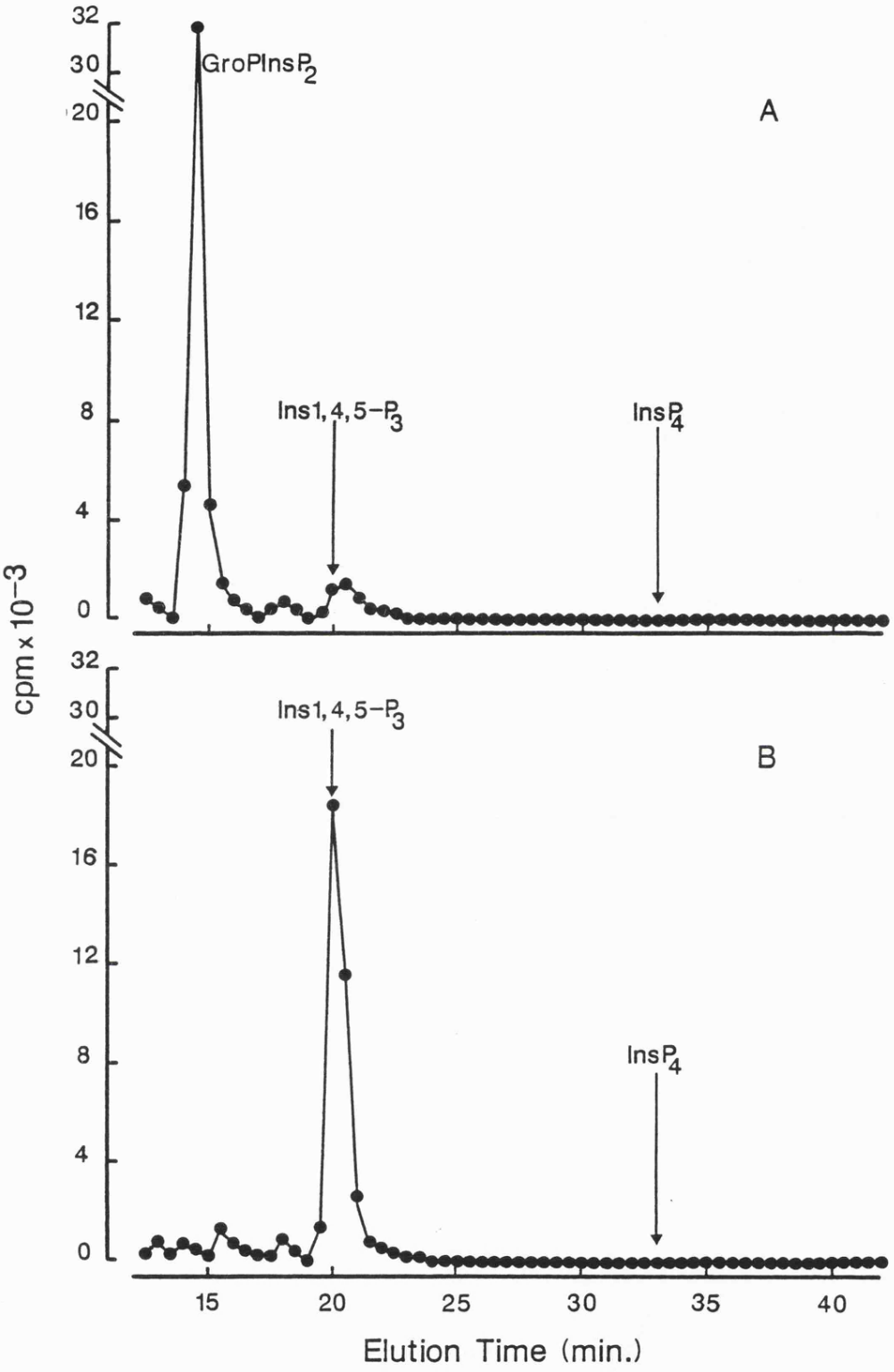


FIGURE 4.4.4 Examination of ^{3}H-inositol labelled brain phosphoinositides for a putative ^{3}H-PtdIns P_3.

Bulk extracts of ^{3}H-inositol labelled phospholipids were obtained from brain slices stimulated with 1 mM carbachol for 30 min. or run as control (not shown) as described in the text. Lipids were examined by hplc as in the legend to Fig. 4.4.1 in the presence of an internal ^{32}P Ins-1,4,5-P_3 standard after either deacylation (A) or deacylation followed by removal of the glycerol moiety (B). The elution traces show only the products derived from ^{3}H-PtdIns P_2 and the section of the gradient which would be appropriate for the products (i.e. ^{3}H-GroPIns P_3 in (A) and ^{3}H-Ins P_4 in (B)) from any ^{3}H-PtdIns P_3 present in the original lipid extract. Phospholipid extracts from unstimulated tissue gave qualitatively identical results. The data is courtesy of Dr. R.F. Irvine.

achieved by the pooling of multiple ^{3}H-lipid extracts it may be concluded that, within a detection limit of 0.5-1.0% of $PtdInsP_2$, no more highly phosphorylated inositol lipids are likely to be present in rat cerebral cortex. This confirms the more preliminary data presented in 4.3(b). However, as with these earlier results it remained difficult from the present study to exclude the possibility that the extraction procedures employed may be inadequate or inappropriate for effective recovery of a $PtdInsP_3$ (Batty et al. 1985b). The established techniques for extraction of $PtdInsP_2$ suggest that this is unlikely but as distinct salts of this lipid exhibit markedly different solubilities in polar and non-polar solvents (Downes and Michell, 1982) it seemed possible that a $PtdInsP_3$ might show similar though accentuated characteristics, and might not therefore partition into the chloroform phase during extraction. Alternatively, a $PtdInsP_3$ might be resistant to even repeat acidified solvent extraction as is a proportion of bound inositol within liver (Michell et al. 1970).

Hawkins et al. (1986) have circumvented these potential difficulties in extracting a putative $PtdInsP_3$ by collecting each the organic and aqueous phases and remaining residue after solvent extraction of tissue and by then subjecting each of these to deacylation followed by examination of all deacylates for a $GroPInsP_3$ as above. As a result of this study they have been able to convincingly demonstrate the absence of any inositol lipid more polar than $PtdInsP_2$ in rat parotid gland. Such data is not yet available for brain but the current results supported by more recent evidence lead to an identical conclusion. Attempts to reproduce the data presented by Santiago-Calvo et al. (1963) for a $PtdInsP_3$ in bovine brain which, in addition to the identification of Ins P_4, was the impetus for the present study, have suggested that their yield of product was very similar to that expected for $GroPInsP_2$ and that erroneous inositol:phosphorus ratios may have arisen owing to a partial destruction of the inositol ring during their acid hydrolysis procedure (R.F. Irvine, personal communication). Equally, examination of a Folch 'diphosphoinositide fraction' for a putative $PtdInsP_3$ by methods identical to those employed here have shown that, following deacylation and removal of glycerol moieties, less than 1% of the products formed elute in the Ins P_4 fraction on Dowex chromatography. Analysis of this small proportion by ionophoresis reveals that this is >90% Ins P_3 with no Ins P_4, demonstrating that a putative $PtdInsP_3$ would have to represent less than 0.1% of the total $PtdInsP_2$ (R.F. Irvine, personal communication). These observations confirm the conclusions drawn in 4.3(b) with respect to the fraction initially isolated as a potential $GroPInsP_3$.

The present data therefore, supported by these recent, more exhaustive studies very clearly demonstrate that a parent phosphoinositide is a highly improbable precursor of the Ins P_4 accumulating in response to receptor activation in cerebral cortex. This conclusion is further re-inforced by the definition of an alternative source of this molecule by very recent studies (Irvine et al. 1986) (see (iii) below).

ii) The relative kinetics of inositol tetrakis- and trisphosphate accumulations in cerebral cortex: The preparation of ^{3}H-inositol labelled extracts from both control and 1 mM carbachol-stimulated tissue was exactly as described in 4.3(a). To facilitate detection of inositol polyphosphates (especially Ins-1,3,4-P_3) TCA supernatant fractions from triplicate tissue incubations were pooled. After removal of acid and neutralisation, the final samples (5 ml) were split into equal portions and one half examined for each Ins P_1 through Ins P_4 by Dowex chromatography as in 4.2. The remaining sample was spiked with adenosine nucleotides and 200-300 dpm Ins(1,[4,5 ^{32}P])-P_3 standard (see above) and chromatographed for inositol trisphosphates and Ins P_4 by hplc as in 4.4(a)i.

Figure 4.4.5 shows the results of a single experiment representative of two such where samples were analysed by hplc. Corresponding data from Dowex chromatography were both qualitatively and quantitatively identical excepting that isomeric Ins P_3(s) were not resolved. The reproducibility of responses is evident from Figure 4.3.1 (4.3(a)) where the results shown, although for separate experiments, may be taken as representative of the corresponding data obtained here. Typically, a 50-100% increased accumulation of ^{3}H-Ins-1,4,5-P_3 over control values and a similar doubling of ^{3}H-Ins P_4 was observed within 5 sec. of 1 mM carbachol addition. In contrast, elevated concentrations of ^{3}H-Ins-1,3,4-P_3 were not clearly apparent until later times (10 sec. or more) although the very low labelling of this fraction made quantification over the initial period of stimulation difficult. Basal labelling of each inositol phosphate remained consistent over the time course shown (0-60 sec.) and it is interesting to note the relative levels of each. As previously observed accumulation of ^{3}H-Ins P_2 was also rapidly stimulated while ^{3}H-Ins P_1 did not differ from control values within the first 20 sec. Labelling of GroPIns was not affected by carbachol.

Comparison of these results with those derived from other tissues in which stimulated formation of separate ^{3}H-Ins P_3 isomers has been investigated shows a close agreement for their relative rates of appearance but striking differences for their respective maximal accumulations (see also Table 4.4.1). As reported for parotid glands, hepatocytes and HL 60 cells (Irvine et al. 1985 and Burgess et al. 1985

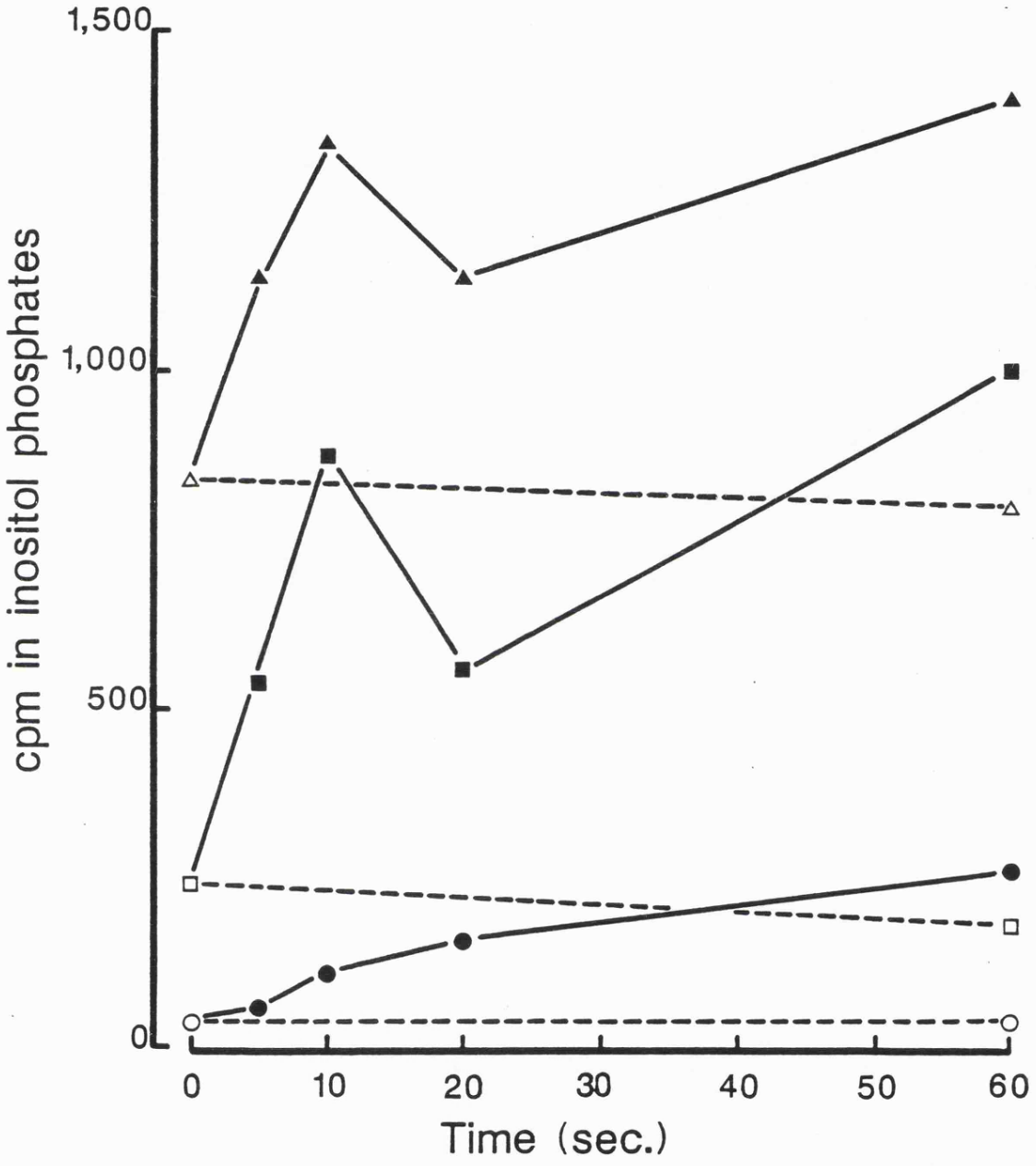


FIGURE 4.4.5 The rates of accumulation of ^{3}H-inositol tris- and tetrakis- phosphates in carbachol-stimulated brain slices.

^{3}H-Extracts were prepared from control (open symbols) and 1 mM carbachol-stimulated (closed symbols) cerebral cortical slices exactly as described in the legend to Fig. 4.3.1. Samples were analysed by hplc as in the legend to Fig. 4.4.1 (in the presence of an internal ^{32}P Ins-1,4,5-P_3 standard) to resolve ^{3}H-Ins-1,3,4-P_3 (circles), ^{3}H-Ins-1,4,5-P_3 (triangles) and ^{3}H-Ins P_4 (squares). Results are from a single experiment but similar data were obtained on one further occasion using hplc analysis and repeatedly confirmed (n = 3, see also Fig. 4.3.1) for ^{3}H-Ins P_3 and ^{3}H-Ins P_4 using Dowex chromatography.

respectively) accumulation of ^{3}H-Ins-1,4,5-P_3 was apparent without a detectable delay while increased ^{3}H-Ins-1,3,4-P_3 followed only after several seconds. These results are also consistent with those presented in 4.3(a) in demonstrating an immediate increase in total Ins P_3. Most importantly these data show for the first time both that separate, isomeric inositol trisphosphates accumulate in brain and that within the time scale resolved by these experiments Ins-1,4,5-P_3 accumulates with a time course consistent with its proposed role as a second messenger regulating $[Ca^{2+}]_i$ (see Berridge and Irvine, 1984). However, as noted in 4.3(a) the rates of accumulation of the ^{3}H-products shown do not allow absolute rates of synthesis to be compared as neither ^{3}H-inositol phosphate specific activities nor rates of hydrolysis are known. Nevertheless, given that the measurements of ^{3}H-Ins-1,4,5-P_3 accumulation represents the net balance between formation and breakdown, the enhanced labelling of this fraction observed within 5 sec. must reflect a very rapid agonist-induced turnover and if it were possible to assess the synthetic rate independently of concommitant catabolism, a several fold greater rate of Ins-1,4,5-P_3 production would be anticipated. As a very marked accumulation of Ins P_2 is also apparent within 5 sec. of carbachol addition (4.3(a)) this seems certain, assuming that receptor-mediated phosphoinositide hydrolysis is strictly limited to PtdInsP_2 (see below). The current data, together with that shown in Table 4.4.1 (4.4(a)i) also reveals that in cerebral cortex, unlike most other tissues studied (Irvine et al. 1985; Burgess et al. 1985; Hawkins et al. 1986; Turk et al. 1986; Hansen et al. 1986), the predominating Ins P_3 isomer accumulating both immediately and at later times (up to 15 min.) is Ins-1,4,5-P_3. The reason for this difference is uncertain although it seems likely to be related to differences in either prevailing routes of Ins-1,4,5-P_3 metabolism and/or factors regulating these pathways (see below). Regardless of this the results presented in Figure 4.4.5 show that Ins-1,4,5-P_3 is produced rapidly in response to receptor activation in brain as in many other tissues (Berridge, 1984) and if estimates of the initial, stimulated intracellular concentrations of this molecule made for other cells (see Berridge and Irvine, 1984) could be extrapolated to cerebral cortex a role for Ins-1,4,5-P_3 in control of $[Ca^{2+}]_i$ in brain would be strongly indicated although this may be difficult to demonstrate more directly except by use of cell culture preparations as employed in recent studies by Gill et al. (1986).

The current data also represents the first comparison of the relative rates of receptor-mediated inositol tris- and tetrakisphosphate accumulations in any tissue and confirms the results presented in 4.3(a) showing that within the time course studied, the initial rate of ^{3}H-Ins P_4

accumulation is at least as rapid as that observed for Ins-1,4,5-P_3 and exceeds that for Ins-1,3,4-P_3 by several fold. On the basis of these results a second messenger function for Ins P_4, potentially as an adjunct to Ins-1,4,5-P_3 in regulation of $[Ca^{2+}]_i$ has been suggested (Batty et al. 1985a). A number of recent studies have confirmed these results in a variety of tissues and cell types and have suggested a similar role for Ins P_4 (Heslop et al. 1985; Hansen et al. 1986; Hawkins et al. 1986). The sequence in which the separate ^{3}H-inositol tris- and tetrakisphosphates are produced in response to carbachol also supports the suggestion that Ins-1,3,4,5-P_4 is the probable metabolic precursor of Ins-1,3,4-P_3. The specificity with which the Ins-1,4,5-P_3 5-phosphatase from erythrocyte membranes hydrolyses Ins P_4 to Ins-1,3,4-P_3 (4.4(a)ii) adds further weight to this argument. Additional evidence also arises from the recent description of a 5-phosphate specific inositol trisphosphate monoesterase present in the particulate fraction from rat brain (Erneux et al. 1986). This enzyme is active towards Ins-1,4,5-P_3 but as with similar trisphosphatases from other tissues (Downes et al. 1982; Seyfred et al. 1984; Connelly et al. 1985) has a K_m for this substrate of approximately 50 μM. As the concentrations of Ins-1,4,5-P_3 required for maximal release of intracellular calcium are generally less than 10% of this (Berridge and Irvine, 1984) it is unlikely that concentrations of this Ins P_3 approach the K_m and it is probable that Ins P_4 is an alternative or co-substrate for this enzyme.

It is of interest that Erneux et al. (1986) have observed additional trisphosphatase activity of undefined specificity in the supernatant fraction from rat brain which is also active towards Ins-1,4,5-P_3. The very limited accumulations of Ins-1,3,4-P_3 (usually <30% Ins-1,4,5-P_3 in carbachol-stimulated rat cerebral cortex at both early and later times (Figure 4.4.5 and Table 4.4.1) also suggests, apparently in contrast to other tissues (Irvine et al. 1985; Burgess et al. 1985), the very rapid metabolism of this compound or alternatively a slow rate of formation relative to hydrolysis. Thus, it seems probable that rat brain contains a phosphatase of some specificity for this substrate. Burgess et al. (1985) have suggested that a similar enzyme in pancreatic acinar cells may be sensitive to inhibition by lithium ions (see also CH.6). However, as the relative accumulations of each Ins-1,3,4-P_3, Ins-1,3,4,5-P_4 and Ins P_2 are markedly different in brain and parotid glands (see 4.2; Table 4.4.1; Figure 4.4.5; Hawkins et al. 1986; Irvine et al. 1985) it seems possible that routes of inositol phosphate metabolism may be subject to separate regulatory mechanisms in the two tissues.

Although the present data provides convincing evidence that Ins P_4 represents the metabolic precursor for Ins-1,3,4-P_3 it is not equally

consistent with Ins-1,4,5-P_3 being commonly derived from this higher phosphate. For Ins P_4 to act as a precursor for both Ins P_3 isomers as suggested by both its structure and rapid formation, a 3-phosphate specific monoesterase must also be present in brain. No such activity has yet been demonstrated. However, Sherman et al. (1981) have shown that the majority of the atropine sensitive, potentiated Ins P_1 accumulation resulting in rat cerebral cortex from in vivo lithium administration is accounted for by the D-1 enantiomer. As both D- or L- ^{3}H-Ins-1,3,4,5-P_4 and ^{3}H-Ins-1,3,4-P_3 are elevated in response to muscarinic receptor stimulation, this implies hydrolysis of the phosphate esterified at the D-3 (L-1) position prior to formation of Ins P_1 because phosphatase activity toward each D- and L- myo Ins-1-P enantiomers is blocked by lithium ions (Hallcher and Sherman, 1980). If removal of the D-3 phosphate occurs at the level of Ins P_4 then this inositol phosphate could also give rise to Ins-1,4,5-P_3. A number of observations indicate that this is unlikely and suggest hydrolysis of the D-3 phosphate probably occurs at the level of Ins-1,3,4-P_3 or Ins P_2. Firstly, hydrolysis of Ins P_4 in both rat parotid gland and liver homogenates proceeds exclusively via Ins-1,3,4-P_3 (Hawkins et al. 1986; Hansen et al. 1986). Secondly, although ^{3}H-Ins P_4 accumulates very rapidly in response to muscarinic receptor activation in both cerebral cortex (see above) and rat parotid gland (Hawkins et al. 1986) this rate cannot be distinguished from that at which ^{3}H-Ins-1,4,5-P_3 and ^{3}H-Ins P_2 are also produced in both tissues (4.3(a) and above; Hawkins et al. 1986; Downes and Wusteman, 1983). If Ins P_4 were the molecule produced most immediately as a consequence of phosphoinositide hydrolysis then formation of Ins P_2, being further removed from this event, would be expected to lag behind that of Ins-1,3,4-P_3. This clearly does not occur and although the observed sequence of inositol polyphosphate accumulation could be accounted for by proposing an initial preferential 3-phosphatase breakdown of Ins P_4 to Ins-1,4,5-P_3 and subsequent rapid metabolism of this to Ins P_2 (see 4.3(a)) such reactions are not apparent in parotid gland or liver homogenates (Hawkins et al. 1986; Hansen et al. 1986). Thirdly, the failure of both the current study (4.3(b) and 4.4(b)i) and of more recent investigations (Hawkins et al. 1986) to identify a phosphatidylinositol trisphosphate indicates that Ins-1,3,4,5-P_4 must arise from a source other than a parent inositol lipid.

iii) Summary of recent developments in the study of inositol polyphosphate metabolism: The sequence of inositol polyphosphate accumulation in response to receptor activation has very recently been clarified by the description of a 3-kinase specific for Ins-1,4,5-P_3 present in a number of rat tissues including brain (Irvine et al. 1986).

Similar activity in rat parotid gland and liver has also been confirmed by Hawkins et al. (1986) and Hansen et al. (1986). The evidence presented by Irvine et al. (1986) clearly suggests that Ins-1,4,5-P_3 arising from receptor-mediated PtdInsP_2 hydrolysis can undergo either dephosphorylation to yield Ins P_2 or phosphorylation via this 3-kinase to produce Ins-1,3,4,5-P_4. Hydrolysis of the latter molecule by a 5-phosphatase then gives rise to Ins-1,3,4-P_3. As yet it is unclear which isomer(s) of Ins P_2 arise from breakdown of Ins-1,3,4-P_3 although parotid gland homogenates hydrolyse this trisphosphate to yield an Ins P_2 chromatographically distinct from the Ins-1,4-P_2 expected from Ins-1,4,5-P_3 (Hawkins et al. 1986). Hansen et al. (1986) have reported similar results with liver homogenates and have isolated two distinct Ins P_2(s) in vasopressin-stimulated hepatocytes which they identify as Ins-1,4-P_2 and Ins-1,3-P_2 although no evidence to support the latter structure was presented. It is also uncertain whether a common 5-phosphatase acts on both Ins-1,4,5-P_3 and Ins-1,3,4,5-P_4 although the enzyme(s) catalysing hydrolysis of each is/are inhibited by 2,3-bisphosphoglycerate (Tennes and Putney, 1986).

Estimates of the K_m of the 3-kinase for Ins-1,4,5-P_3 show a value of approximately 0.6 μM for the soluble enzyme present in crude supernatants from rat brain (Irvine et al. 1986; see CH.6). As this value is very close to the EC_{50} of Ins-1,4,5-P_3 required for mobilisation of intracellular calcium, the kinase is likely to compete with the calcium release mechanism for substrate. The K_m of the 3-kinase also shows that this enzyme has 75-100 fold higher affinity for Ins-1,4,5-P_3 than is exhibited by the 5-phosphatase from rat brain (Erneux et al. 1986). Therefore, unless the relative concentrations of 3-kinase and 5-phosphatase are grossly disproportionate in favour of the latter, the majority of Ins-1,4,5-P_3 would be expected to be metabolised by phosphorylation to Ins P_4 rather than by direct hydrolysis to Ins P_2. However, it is possible that changes in the intracellular environment, induced as a result of Ins-1,4,5-P_3 and/or DG release from PtdInsP_2, may regulate the activities of these enzymes such that assays performed using broken cell preparations may not reveal properties of these enzymes representative of their activities in situ unless the altered intracellular conditions promoted by agonists are carefully reproduced. In this respect the results of Biden and Wollheim (1986) are of interest since these show that increasing calcium concentrations over the physiological range 10^{-7}-10^{-5} M produces a 2-3 fold increased activity of the Ins P_3 3-kinase from RINm5F cells.

Re-examination of the current data in view of the presence of an Ins-1,4,5-P_3 kinase in rat brain shows that the kinetics of inositol

polyphosphate accumulations are in accord with the reaction sequence proposed by Irvine et al. (1986). The very rapid accumulation of each Ins P_2 and Ins P_4 and the delay in the appearance of Ins-1,3,4-P_3 can be explained by the different numbers of enzymic steps intervening between PtdInsP_2 hydrolysis and the formation of each product. As it is now clear that Ins-1,4,5-P_3 can be metabolised by two separate routes the marked accumulations of both Ins P_2 and Ins P_4 within 5 sec. of agonist addition (see Figures 4.3.1 and 4.4.5) emphasises the extraordinarily rapid rate at which PtdInsP_2 hydrolysis is promoted by receptor activation because the initial rate of accumulation of Ins-1,4,5-P_3 measured must represent at least a 2-3 fold underestimate of its rate of formation. This also suggests that the rate of Ins P_3 hydrolysis estimated in 3.2 is similarly inaccurate. Given the rate at which Ins-1,4,5-P_3 must turnover and the rapidity with which this molecule is phosphorylated to Ins P_4, it seems unnecessary to invoke breakdown of PtdInsP in response to receptor activation to account for the rapid accumulation of Ins P_2 observed in brain although this possibility cannot yet be eliminated. Although the immediate source of Ins P_2 in brain is therefore not certain, the presence of an Ins-1,4,5,-P_3 5-phosphatase (Erneux et al. 1986) and the ability of crude brain supernatants to hydrolyse low concentrations ($\sim$10-20 μM) ^{3}H-PtdInsP_2 rapidly through Ins P_3 to Ins P_2 (see 4.2(c)i) suggest that a considerable proportion of the Ins P_2 arising within 5 sec. of carbachol stimulation of cerebral cortical slices is derived directly from Ins-1,4,5-P_3. The current data also show that stimulated ^{3}H-Ins P_2 formation precedes that of Ins-1,3,4-P_3, thus eliminating the possibility that the Ins P_2 produced most immediately after receptor activation arises through dephosphorylation of this molecule. Receptor-mediated accumulation of Ins P_2 also precedes that of Ins-1,3,4-P_3 in rat parotid gland (Hawkins et al. 1986) and hepatocytes (Hansen et al. 1986) both of which tissues possess an Ins-1,4,5-P_3 and/or Ins-1,3,4,5-P_4 5-phosphatase activity. These observations in conjunction with the current data and that of Irvine et al. (1986) support the view that the Ins-1,4,5-P_3 arising from receptor-mediated PtdInsP_2 hydrolysis can be readily dephosphorylated to Ins-1,4-P_2. Since this mechanism for inactivation of the Ins-1,4,5-P_3- calcium-mobilising signal exists, this raises the intriguing question of why the second and energy-dependent route, represented by the ATP requiring 3-kinase, is also employed to catabolise an apparently substantial proportion of Ins-1,4,5-P_3. One possibility is that the Ins-1,3,4,5-P_4 and/or Ins-1,3,4-P_3 produced via this route have second messenger functions (Batty et al. 1985b; Irvine et al. 1985; Irvine et al. 1986). Hansen et al. (1986) have reported preliminary results to this effect indicating that Ins-1,3,4,5-P_4 promotes release of Ca^{2+} from

plasma membrane vesicles. As Biden and Wollheim (1986) have shown that activity of Ins-1,4,5-P_3 kinase is enhanced by increasing calcium concentrations over the range 10^{-7}-10^{-5} M it therefore seems possible that an initial Ins-1,4,5-P_3 mediated mobilisation of intracellular Ca^{2+} may serve to stimulate production of Ins-1,3,4,5-P_4 which then mediates a further rise in $[Ca^{2+}]_i$ by promoting influx of Ca^{2+} across the plasma membrane. However, clear evidence to support such a suggestion has yet to be presented.

The identification of Ins-1,3,4,5-P_4 described in this section together with the demonstration of its formation via 3-kinase action on Ins-1,4,5-P_3 (Irvine et al. 1986) have resolved the previously undefined source of Ins-1,3,4-P_3 in stimulated tissues and has introduced further potential into the intracellular signalling capacity of receptor-mediated inositol lipid hydrolysis. However, additional complexity in the metabolism of inositol phosphates is indicated by the recent observations of Wilson et al. (1985a) and Heslop et al. (1985). The first of these reports has shown that diesteratic hydrolysis of polyphosphoinositides, as with PtdIns, yields both cyclic and non-cyclic polyphosphate products. The Ins-1:2 cyclic 4,5-P_3 arising from PtdInsP_2 releases $^{45}Ca^{2+}$ from permeabilised platelets almost equipotently with Ins-1,4,5-P_3 and is several fold more potent than the latter non-cyclic trisphosphate in stimulating conductance change in photoreceptor cells (Wilson et al. 1985b). The 3-kinase described above can also phosphorylate Ins-1:2 cyclic 4,5-P_3 to yield Ins-1:2 cyclic 3,4,5-P_4 although its K_m for the cyclic trisphosphate has not been reported (Irvine et al. 1986). At present it is uncertain whether accumulation of inositol cyclic polyphosphates is a widespread occurrence in stimulated tissues but if so then the above discussion of Ins P_3(s) and Ins P_4 metabolism may well apply equally to their 1:2 cyclic derivatives. In the present study the use of acid to quench tissue incubations would have precluded detection of any Ins-1:2 cyclic 3,4,5-P_4 as this would have been hydrolysed during the extraction procedure to give predominantly Ins-1,3,4,5-P_4 with a much lesser amount of Ins-2,3,4,5-P_4. In none of the above analyses was separation of ^{3}H-Ins P_4 into isomeric fractions observed although small quantities of Ins-2,3,4,5-P_4 would not have been readily detected.

Equally, in the current experiments with cerebral slices no clear evidence could be obtained for the accumulation of inositol pentakis- and hexakis-phosphates whose presence Heslop et al. (1985) have demonstrated in blowfly salivary gland and GH_4 cells. Routine analysis of extracts from control or carbachol-stimulated brain slices by Dowex chromatography showed no radiolabel eluting later than ^{3}H-Ins P_4 even when the eluant

molarity was increased to 2.0 M formate (i.e. twice that required to displace Ins P_4). However, when a bulk preparation of ^{3}H-Ins P_4 prepared as in 4.4(a)ii was examined for higher inositol polyphosphates a small percentage of the total radiolabel was found to elute distinct from and later than ^{3}H-Ins P_4. Figure 4.4.6(A) illustrates the primary purification of ^{3}H-Ins P_4 from lower inositol phosphates as described in 4.4(a)ii showing that three fractions eluted later than Ins P_4 although the total of these comprised only 5% of the label under the Ins P_4 peak. Further identification of these was not practical owing to their low radiolabelling. However, the ^{3}H-Ins P_4 from several such initial column separations as in Figure 4.4.6(A) was pooled and re-examined by similar chromatography to assess its radiochemical purity. Figure 4.4.6(B) shows the elution profile obtained and illustrates that ^{3}H-Ins P_4 prepared as in 4.4(a)ii contains approximately 0.05% ^{3}H-Ins P_2, 1.2% ^{3}H-Ins P_3 and 3.4% of label which appears more polar than Ins P_4. The identity of the latter is unclear but in view of the small carry over of Ins P_3 into the Ins P_4 fraction evident here and the similar phenomena previously noted on Dowex chromatography (see 4.3(b) and 4.4(b)i) it is probable that at least a portion of this is residual ^{3}H-Ins P_4. The remainder and the fractions eluting separate from Ins P_4 in the primary purification may represent ^{3}H-compounds similar to the Ins P_5 and Ins P_6 reported by Heslop et al. (1985) but such identification will require further analysis. As these fractions represent such a small proportion of the total radiolabel conclusively identified as ^{3}H-Ins P_4 (4.4(a)) even in bulk preparations of the latter, derived from cerebral cortical slices exposed to maximally effective doses of carbachol, it would be difficult on more routine analysis to determine whether accumulation of these unidentified products is stimulated by agonist. Heslop et al. (1985) have suggested that the Ins P_5 and Ins P_6 demonstrated in GH_4 cells and blowfly salivary glands may derive from appropriate phosphoinositides ($PtdInsP_4$ and $PtdInsP_5$) but as no ^{3}H-inositol labelled lipids more polar than ^{3}H-$PtdInsP_2$ can be detected in brain (see above) or parotid gland (Hawkins et al. 1986) this seems improbable. Hence, it is also unlikely that Ins P_5 or Ins P_6 represent precursors for significant quantities of Ins P_4, particularly as only marginal reductions in their labelling were observed on stimulation of blowfly salivary glands with 5-HT (Heslop et al. 1985). Similarly, the level of ^{3}H-material with the chromatographic properties of an Ins P_5 in ^{3}H-inositol labelled parotid glands showed no change on exposure of tissue to carbachol while a concommitant 5-fold increased accumulation of ^{3}H-Ins P_4 was observed (Hawkins et al. 1986). Alternatively, Ins P_5 and Ins P_6 could arise through Ins P_4 by kinase activities similar to that phosphorylating Ins-1,4,5-P_3 (Irvine et al. 1986) or the inositol

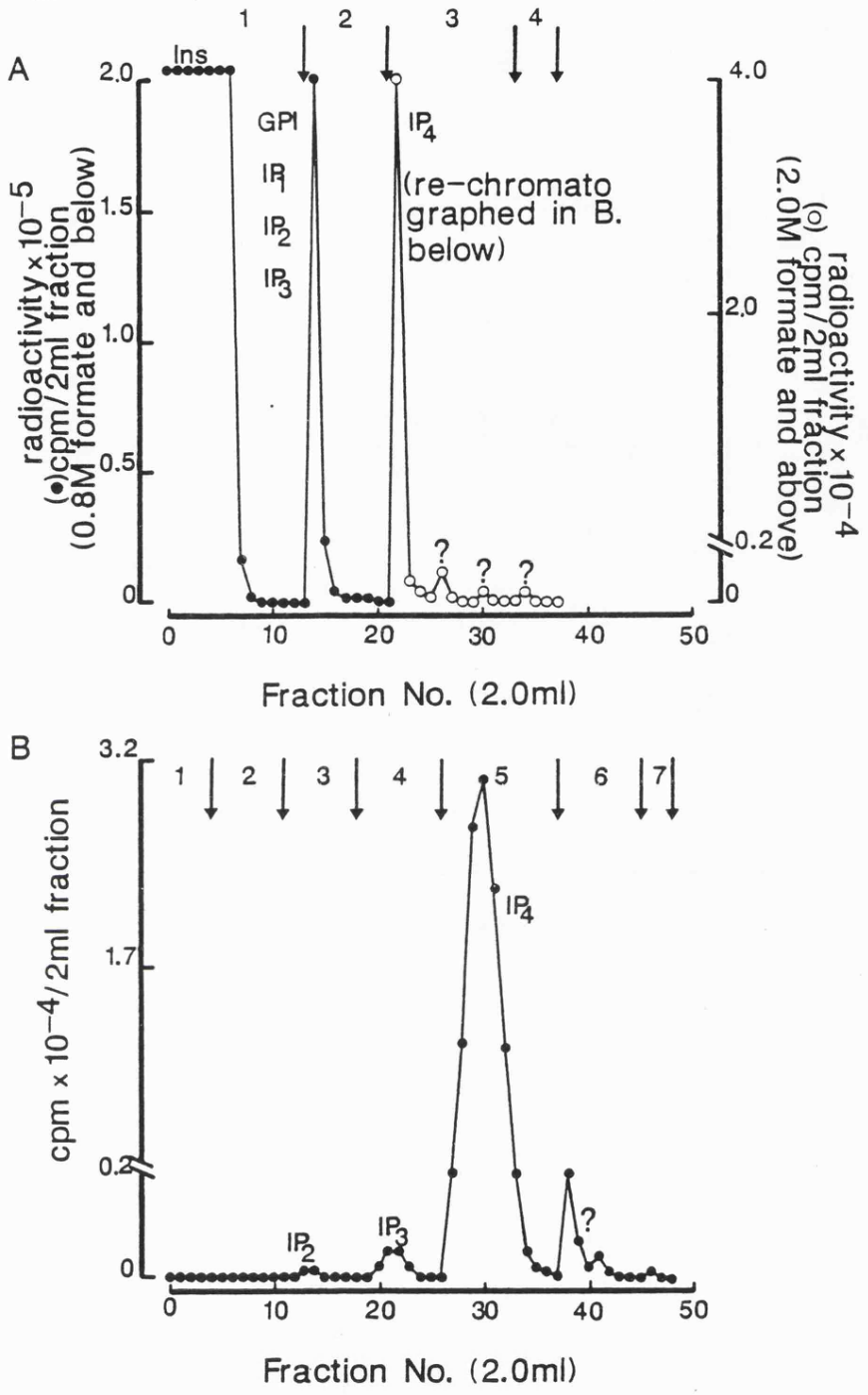


FIGURE 4.4.6 Bulk isolation of ^{3}H-Ins P_4 by anion exchange chromatography and estimation of radiochemical purity.

Fig. A shows a typical elution profile for the isolation of ^{3}H-Ins P_4 from bulk extracts of carbachol-stimulated, ^{3}H-inositol labelled brain slices prepared as described in the text. Separation of ^{3}H-Ins P_4 from lower ^{3}H-Ins P's was achieved by elution of ^{3}H-Ins with H_2O (1) and total ^{3}H-GPI + ^{3}H-IP_1 + ^{3}H-IP_2 + ^{3}H-IP_3 with 0.8 M AF/0.1 M FA (2) before displacing ^{3}H-Ins P_4 with (3) 2.0 M AF/0.1 M FA. For preparations used to determine the structure of Ins P_4 only the peak 2 M formate fractions were collected. Fig. A shows that continued elution with this buffer (3) or with 3 M HCl (4) displaces only a very small amount of further radiolabel. Fig. B shows the re-analysis of the peak ^{3}H-Ins P_4 fractions pooled from several primary separations according to (A). For (B) buffers 1-7 represent: H_2O; 0.2 M AF; 0.5 M AF/0.1 M FA; 0.8 M AF/0.1 M FA; 1.0 M AF/0.1 M FA; 2.0 M AF/0.1 M FA and 3 M HCl. Columns used for each (A) and (B) were 0.6 x 3.0 cm of AG 1 x 8 resin (200-400 mesh, formate form).

phosphate kinases known to exist in avian erythrocytes and plants (Chakrabarti and Majumber, 1978). However, liver and brain preparations which readily phosphorylate Ins-1,4,5-P_3 to Ins P_4 do not catalyse formation of higher inositol phosphates (Hansen et al. 1986; see CH.6). The significance and source of Ins P_5 and Ins P_6 in mammalian tissues therefore remains uncertain but from the data in Figure 4.4.6 indicating that at a maximum these represent only 3-4% of Ins P_4 it is unlikely that they play an important role in the rapid metabolism of inositol phosphates mediated by muscarinic receptors in cerebral cortex. The low labelling of Ins P_5 and Ins P_6 could however, reflect only a very low specific activity rather than a low concentration and their significance in relation to mammalian inositol metabolism will only become clear when their source(s) can be defined.

In contrast, the data presented in this chapter together with more recent developments discussed above clearly suggest that Ins-1,3,4,5-P_4, and/or its 1:2 cyclic derivative, is a crucial intermediate in the inositol phosphate metabolism initiated in a wide variety of tissues by a range of neurotransmitter and hormone receptors mediating PtdInsP_2 hydrolysis. Thus, although Ins P_4 is unlikely to represent the initial signal generated by phosphoinositide hydrolysis as originally suggested (4.2 and 4.3), its very rapid formation in stimulated tissues coupled with its defined source via an ATP-dependent Ins-1,4,5-P_3 3-kinase (Irvine et al. 1986) strongly indicates a second messenger function for this molecule (Batty et al. 1985b; Irvine et al. 1986), a suggestion which is beginning to receive support from currently emerging evidence (Hansen et al. 1986). The precise role, if any, played by Ins P_4 in the regulation of $[Ca^{2+}]_i$ and the means by which this is integrated with the accepted intracellular signals (Ins-1,4,5-P_3 and DG) generated by PtdInsP_2 hydrolysis remain undefined and should prove interesting areas for future research. The metabolism of Ins P_4 in cerebral cortex is further considered in CH.6.

CHAPTER 5

High pressure liquid chromatography (hplc) of inositol phosphates

5.1 Introduction

The separation of inositol phosphates into mono-, bis- and trisphosphate fractions by Dowex anion exchange chromatography has, until very recently, been considered an adequate means of analysing the products of receptor-mediated inositol lipid hydrolysis. The occurrence of the separate D- and L- enantiomers of Ins-1-P, arising respectively through cyclic metabolism of phosphoinositides and through de novo synthesis of myo-inositol from glucose-6-phosphate has long been established (see Parthasarathy and Eisenberg, 1986) but since only the D-isomer could be derived via the known routes of inositol lipid and phosphate hydrolysis separation of these was not originally considered essential to assays of phosphoinositide breakdown. The requirement for more powerful analytical methods for resolution of inositol phosphates has only become clear from a number of recent observations. The most important of these was the demonstration that the Ins P_3 accumulating in stimulated cells comprises not only the expected Ins-1,4,5-P_3 but also the Ins-1,3,4-P_3 isomer (Irvine et al. 1984a). From the discussion in CH.4 it is clear that complex isomeric mixtures of inositol phosphates may be produced in response to receptor activation and it now seems certain that not only the Ins P_3 but also the Ins P_1 (Storey et al. 1984; Sherman et al. 1985; Siess, 1985) and Ins P_2 (Hawkins et al. 1986; Hansen et al. 1986) fractions isolated by Dowex chromatography comprise multiple components. This complexity is further compounded by the occurrence of higher inositol phosphates (Batty et al. 1985b; Heslop et al. 1985) and potentially of inositol cyclic phosphates (Wilson et al. 1985a). To date no single chromatographic system has been shown to resolve all these inositol metabolites.

A number of the methods available for the separation of inositol phosphates into crude fractions were briefly described in 2.4. Several of these procedures are capable of resolving isomeric mixtures of these compounds. For example, descending paper chromatography using solvent systems as described in 4.4(a)ii has been successfully applied to the separation of various isomers of each inositol mono-, bis- and trisphosphates although with increasing levels of phosphorylation resolution becomes poorer so that for Ins P_3(s) clear separation requires 10-14 days (Grado and Ballou, 1961; Brown and Stewart, 1966). Alternatively, ionophoresis will resolve isomeric mixtures of some higher inositol phosphates (see 4.4(a)i). Both electrophoresis and ion exchange chromatography allow separation of inositol phosphates on the basis of molecular charge. The latter procedure has the advantage that tens of samples can be handled simultaneously. The separation of inositol phosphates by stepwise gradient elution from Dowex anion exchange columns

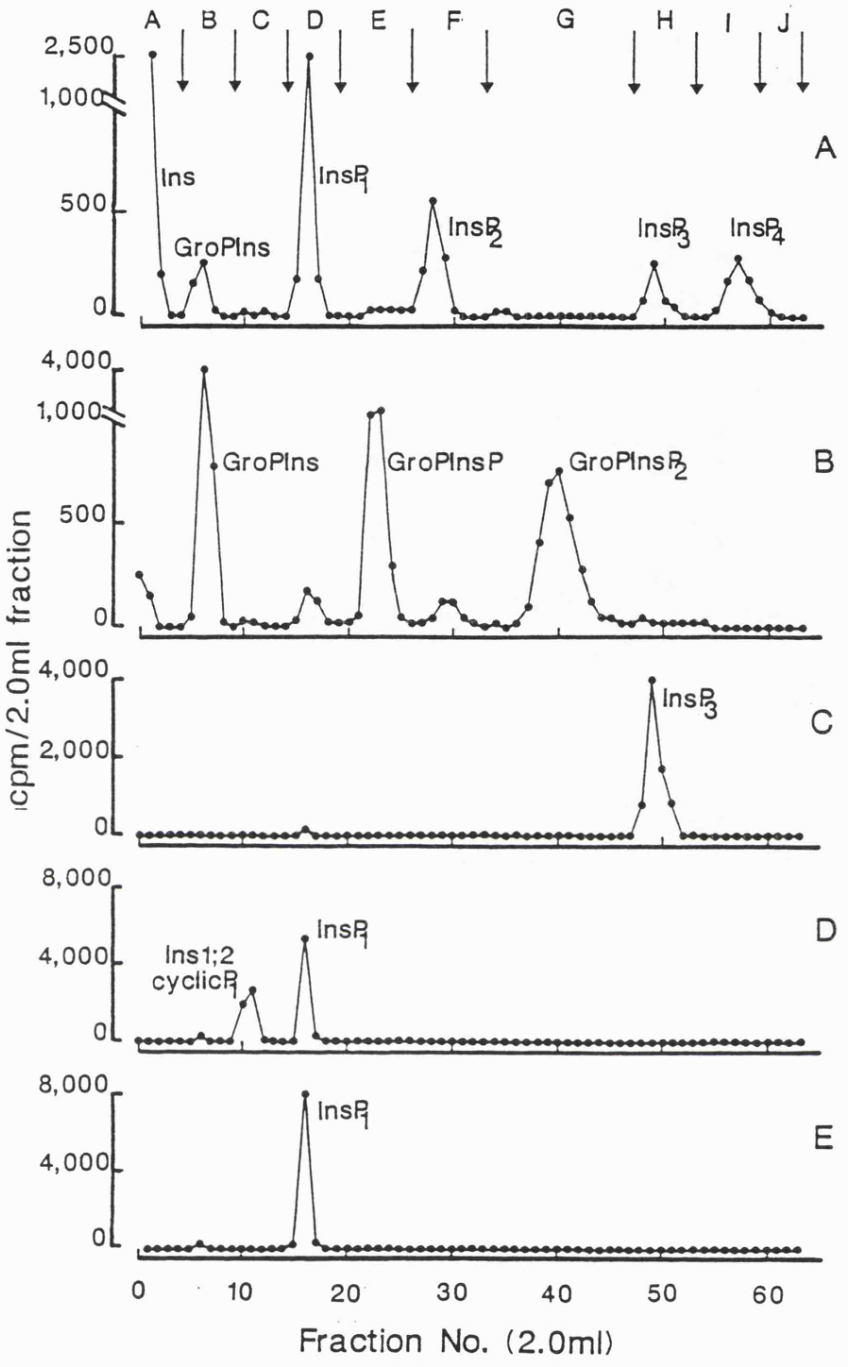


FIGURE 5.1.1 The inositol mono- and diester- phosphates separated by conventional anion exchange chromatography.

Panels A-E show separations of mixtures of sample (A) and standard (B-E) ^{3}H-inositol labelled phosphates.

A: an extract of ^{3}H-Ins P's from carbachol-stimulated brain slices.
B: a mixture of glycerophosphorylinositol (phosphates) from authentic ^{3}H-phosphoinositides.
C: authenic ^{3}H-Ins-1,4,5-P_3 (see Figs. 4.2.5 and 4.2.6 for other Ins P_3(s)).
D: a mixture of ^{3}H-Ins-1',2-cyclic P_1 and ^{3}H-Ins P_1 prepared by the action of a crude phospholipase C preparation on authentic ^{3}H-PtdIns.
E: the mixture from D after treatment with acid. A ^{14}C-Ins P_1 standard (D-Ins-3-P) also ran with the ^{3}H-Ins P_1 prepared as in D.

Separations were achieved on AG 1 x 8 resin columns as previously. Buffers A-J which gave optimal resolution were respectively: H_2O; 0.025 M AF; 0.06 M AF/0.005 M $Na_2B_4O_7$; 0.2 M AF; 0.25 M AF/0.05 M FA; 0.4 M AF/0.1 M FA; 0.5 M AF/0.1 M FA; 0.8 M AF/0.1 M FA; 1.0 M AF/0.1 M FA; 2.0 M AF/0.1 M FA. The latter was used to ensure no label eluted later than ^{3}H-Ins P_4.

has been described in detail in 2.4, 3.5 and 4.2 and a summary of the inositol monoester and diester phosphates easily resolved in this way is presented in Figure 5.1.1. However, although this method is convenient, the use of short columns (2-4 cm) and of stepped gradients restricts the separation of compounds to those which carry markedly different molecular charge. Isomeric inositol phosphates possess almost identical charge and separation of these must rely upon more subtle molecular features such as slight variations in pk_a and in charge distribution induced by substitution of the inositol ring at different positions. Cosgrove (1978) has suggested that such differences will be of magnified significance at low pH when the overall charge carried will be small and on this basis has demonstrated the resolution of various scyllo-inositol tris-, tetrakis- and pentakisphosphates by conventional gradient elution anion exchange chromatography using HCl/NaCl or ethanolic HCl solvents. However, inositol cyclic phosphates and glycerophosphorylinositol phosphates are unlikely to be stable to chromatography at such low pH and these methods have not been shown to adequately resolve inositol mono- and bis-phosphates. Additionally, the use of continuous gradient elution of conventional ion exchange columns is hardly a suitable approach for the analysis of multiple tissue extracts. Equally paper chromatography and ionophoresis are not practical for this purpose. Alternatively, hplc provides a relatively convenient means of separating inositol phosphates and by virtue of the high density of sites capable of interacting with sample molecules, high performance columns should be able to exploit the finer details of molecular structure to give much improved resolution over that achieved by Dowex column chromatography.

5.2 HPLC methods available for the separation of inositol phosphates and associated problems

The application of hplc to the separation of inositol phosphates is still in its early stages and although the technique has been applied with a variety of aims to different groups of phosphoinositols over the last 5-6 years, the available methods are limited and none yet developed has been shown to resolve all the products potentially arising from receptor-mediated phosphoinositide hydrolysis. Most of the systems described to date achieve separation on the same principle as employed for Dowex anion exchange chromatography. The hplc separations available for inositol phosphates and the basic methods involved have recently been summarised by Simpson et al. (1987). The systems used in CH.4 and CH.6 are all simple modifications of that originally described by Irvine et al. (1985) and details of the development of these are briefly described in 5.3 below.

Although this method has a number of associated problems it is the only system in which separation of Ins-1,3,4-P_3 and Ins-1,4,5-P_3 has been characterised, hence its selection for the current study (see 4.4(a)i). In addition to allowing relatively convenient quantification of distinct Ins P_3(s) this method also has the advantage over other hplc separations that a greater range of inositol metabolites can be resolved. In its original form (Irvine et al. 1985) this gradient elution anion exchange separation was shown to resolve each Ins P_1, Ins P_2 and Ins P_3 both from one another and from each corresponding glycerophosphorylinositol (phosphate) diester. Recent modifications of the buffer molarity and gradient used have extended this range to include Ins P_4 (Batty et al. 1985b), Ins P_5 and Ins P_6 (Heslop et al. 1985). As the retention of these compounds increases with increasing molecular charge it would be anticipated from the ability of this system to resolve isomeric Ins P_3(s) that separation of any potential Ins P_4 and Ins P_5 isomers would also be possible using this method. Whether the same system can be expected to give complete resolution of isomeric Ins P_1(s) and Ins P_2(s) is uncertain but the recent results of Hawkins et al. (1986) and Hansen et al. (1986) suggest that at least some of the latter may also be resolved. Alternative methods which have been demonstrated to resolve a variety of Ins P_1 isomers have used similar, though not identical, anion exchange columns and very similar eluting buffers (Hallcher and Sherman, 1980; Hokin-Neaveson and Sadeghian, 1984; Binder et al. 1985; Siess, 1985). This may indicate that by employing weaker molarity buffers and/or shallower gradients the system used for Ins P_3 isomers may also be applicable to lower inositol phosphates. These possibilities have not been investigated in the current study where hplc has been used almost exclusively for the separation of Ins P_3(s) and as a convenient means for investigating the potential occurrence of a PtdInsP_3 in rat brain (see 4.3(b) and 4.4(b)i).

Other than the separations described below and those for Ins P_1(s) (see refs. above) few further hplc methods have been specifically developed for resolution of isomeric inositol phosphates although Wilson et al. (1985a and b) have described a procedure for the separation of mono-, bis- and trisphosphates from their corresponding 1:2 cyclic derivatives using anion exchange columns eluted with neutral ammonium formate. However, as the retention times for glycerophosphorylinositol phosphate diesters and for isomeric inositol monoester phosphates in this system have not been reported this seems of limited application. In the present study, the potential occurrence of inositol cyclic phosphates within tissue samples has not been examined and the use of acid to quench tissue incubations would have prevented detection of these species (see 4.4(b)iii). However, it would be expected from the above discussion that

the isomeric inositol phosphates produced as a result of acid hydrolysis of the 1:2 cyclic phosphate bond would show some resolution in the hplc system used to separate Ins P_3 isomers. In this respect it is of interest to note that a shoulder, trailing behind the Ins-1,4,5-P_3 peak was observed with some samples analysed and may represent Ins-2,4,5-P_3 which would be expected to elute at approximately this position (R.F. Irvine, personal communication). However, in no instance was this completely resolved and was only equivalent to a fraction of the radioactivity under either the Ins-1,4,5- or-1,3,4-P_3 peaks. As no extraction procedure effective for both cyclic and non-cyclic inositol polyphosphates from tissues has been described until very recently (Ishii et al. 1986) it was not possible to further examine the potential accumulation of the former in cerebral cortex by analysis of samples according to Wilson et al. (1985a and b).

Although the method of Irvine et al. (1985) appears the most useful yet described for high resolution of inositol phosphates it does have a number of associated problems. These arise primarily from eluants required to displace particularly higher inositol phosphates (Ins P_3 and above) from the strong anion exchange (SAX) columns used. The more polar compounds are displaced from the column using high molarity ammonium formate buffered to pH 3.7 with orthophosphoric acid. The latter is required to allow optimal resolution of isomeric Ins P_3(s) (Irvine et al. 1985). In the current study preliminary attempts were made to replace phosphate with other anions (e.g. Cl^- or citrate) but this resulted in either poorer peak definition or high basal UV absorption which interfered with detection of nucleotide markers (see 5.3(c)). The combination of high concentrations of ammonium formate and of phosphate in the eluant buffer produces a number of problems. Firstly, unless eluate fractions are desalted, the high background of inorganic phosphate precludes detection of unlabelled inositol phosphates and thus these cannot be easily used to characterise separations. Secondly, the high molarity salts required at the upper end of the gradients reduce counting efficiency for radiolabelled inositol phosphates extracted from ^{3}H-inositol label tissue. This is particularly significant for inositol polyphosphates whose accumulation in some tissues or in response to certain agonists are already low (see 4.1). A related problem arises because resolution of isomeric Ins P_3(s) is limited and these can only be quantified separately by frequent collection (0.2-0.5 min.) of small aliquots of column eluate. This requirement for analysis of such small fractions together with the low accumulation of inositol polyphosphates and the associated low counting efficiency restricts the usefulness of an on-line radioactivity monitor equipped with a low volume flow through

cell. A consequence of this is that large numbers of individual fractions must be collected and each counted for radioactivity which to an extent limits the automation of sample injection which would otherwise facilitate more convenient and rapid handling of samples. To a large extent these difficulties limit the numbers of samples which can be analysed and clearly, unless resolution of inositol phosphates to the level of isomeric compounds is essential, the use of Dowex chromatography is preferable.

A new method for the separation and measurement of inositol phosphates by hplc using on-line detection has recently been reported (Meek and Nicoletti, 1986; Meek, 1986). This should avoid some of the above difficulties and has the advantage that both cold and radiolabelled compounds can be estimated. However, both the detection limit for the former and the resolution of isomeric compounds suggest that as yet this method is inadequate for assay of inositol phosphates derived from small tissue aliquots but with further refinement may prove a useful alternative to current procedures. At present the method of Irvine et al. (1985) represents the only viable means of measuring separate Ins P_3(s) and is thus the only means of measuring changes in the levels of the putative second messenger, Ins-1,4,5-P_3. The resolution of inositol phosphates achieved using this method and the development of the gradients used in CH.4 and CH.6 are detailed in 5.3.

5.3 Methods used for analysis of inositol phosphates by hplc

The methods described in this section represent a general description of those used for analysis of extracts from ^{3}H-inositol labelled cerebral cortex by hplc. However, although the basic procedure remained constant, the gradients used for analysis of samples were varied either to achieve improved chromatographic resolution for a specific purpose or to compensate for the variability observed between different columns and/or accuracy of high pressure pumping systems used. The general method is therefore described in (a) below while examples of the varied gradients employed and the means by which these were established are briefly described in (c). The separations of inositol phosphates shown in Figures 5.3.1 to 5.3.3 show the retention times for sample and standard materials determined using methods referred to in 4.3(b) while those shown in Figure 5.3.4 (A) and (B) are representative of methods used most routinely in CH.6.

a. Basic procedure for analysis of ^{3}H-inositol phosphates by gradient elution hplc:

The preparation of tissue extracts for analysis by hplc was identical to that previously described for Dowex anion exchange chromatography except that samples were routinely spiked with a mixture of nucleotide markers (see below) and/or an internal Ins(1,[4,5 ^{32}P])-P_3 standard. Nucleotide markers included either AMP, ADP and ATP or these together with corresponding guanosine nucleotides, with approximately 50 nmoles of each being added per 2 ml sample extract. Where ^{32}P Ins-1,4,5-P_3 was included this was present at 200-600 dpm per sample and was quantified separate from ^{3}H-inositol labelled products extracted from brain by dual isotope counting. Measurement of ^{32}P radioactivity alone allowed the spill over for this label into the tritium channel to be estimated at less than 3% even under conditions of maximum quenching. All standard or marker compounds were mixed with ^{3}H-inositol labelled samples prior to injection on to the hplc column. Recovery of labelled standards on chromatography was close to 100%.

Separation of inositol phosphates was achieved using a Partisil (10 μM) strong anion exchange (SAX) high pressure column, 0.46 x 25 cm. Gradients for column elution were generated using either an ACS dual high pressure pump system programmed via an Apple computer according to the Profiler gradient system (Drew Scientific) or using LKB 2150 high pressure pumps and 2152 gradient controller. The column eluants were H_2O (pump A) and either 0.28 M ammonium formate (AF) or 1.7 M AF buffered to pH 3.7 with orthophosphoric acid (pump B). Gradients are expressed as the percentage composition of the eluant contributed from pump B at a given time and are listed in Appendix 2. All buffers for column elution were passed through 0.2 μm cellulose acetate filters and de-gassed by bubbling with helium prior to use.

Samples in a final 2 ml volume were applied to the column via a standard six port hplc injection valve equipped with a 2 ml capacity loop. Subsequent to injection, sample was flushed on to the column and the elution begun. For all gradients a preliminary wash at 0% (i.e. with H_2O) was run for 10-15 min. to ensure that the high levels of ^{3}H-inositol present in samples did not interfere with the chromatographic analysis. Column eluate was passed directly through a variable wavelength ultraviolet (UV) spectromonitor and thence either to waste (for collection of free ^{3}H-inositol) or the fraction collector. Eluate absorption was monitored at 254 nm to allow detection of nucleotide markers and 1 min. fractions (1.2 ml) collected across the gradient except over the range of buffer molarity at which Ins P_3(s) eluted. To achieve separate measurement of Ins-1,3,4-P_3 and Ins-1,4,5-P_3 either 0.2, 0.3 or 0.5 min.

fractions were collected, this frequency depending on the resolution achieved with different columns. Each fraction was counted for radioactivity after addition of four volumes of 50% methanol (1.0-5.0 ml) and between 4-15 ml scintillant depending on fraction volume. Counting efficiency for tritium varied considerably across the gradient but was not significantly different for the distinct trisphosphates. The identification of sample ^{3}H-inositol labelled products is described in (b).

b. Identification of ^{3}H-inositol labelled products present in brain slice extracts and preparation of standards:

Initially extracts from control or stimulated brain slices were examined for ^{3}H-inositol phosphates up to Ins P_4 using gradients modified from 4.4(a)i. Following the identification of Ins P_5 and Ins P_6 in GH_4 cells and blowfly salivary gland the gradient of extended buffer molarity described by Heslop et al. (1985) was also used. This showed no peaks of radioactivity eluting later than ^{3}H-Ins P_4 although from 4.4(b)iii any ^{3}H-Ins P_5 or ^{3}H-Ins P_6 present in brain slice extracts could have been difficult to detect given their very low labelling in combination with the poor counting efficiency for tritium resulting from use of such high molarity buffer. Subsequent analyses were restricted to Ins P_4 and less polar inositol phosphates.

The peaks marked on each trace illustrated are identified either by co-elution with an internal ^{32}P standard (Ins-1,4,5-P_3 only) or by co-elution with authentic radiolabelled materials in successive runs, and any variability in relative retention times constantly monitored by the routine inclusion of the nucleotide markers (see below). Standards of ^{3}H-GroPIns, ^{3}H-GroPInsP and ^{3}H-GroPInsP$_2$ were prepared by deacylation of authentic ^{3}H-inositol labelled lipids (see 3.5). Standard Ins(1,[4,5-^{32}P])-P_3 was obtained as in CH.4 or was the gift of New England Nuclear. Ins-1,4,5-P_3 was also identified by its comparative retention time in separate runs with authentic ^{3}H-Ins-1,4,5-P_3, either purchased from, or the gift of, Amersham International. Ins P_1 was identified by co-elution with ^{14}C-labelled D-myo-inositol-3-phosphate (Amersham) and by its retention time relative to a mixture of isomeric inositol phosphates prepared by enzymic hydrolysis of ^{3}H-inositol labelled PtdIns (see below). Using the gradients illustrated no resolution of isomeric Ins P_1(s) was observed. Ins-1:2 cyclic monophosphate was also prepared from PtdIns. This was achieved using a soluble preparation of phospholipase C from rat brain as described in 4.2(c)i except that incubations were stopped by addition of 1.25 ml methanol:chloroform (2:1 v/v) and subsequently of 0.41 ml each chloroform and H_2O. After centrifugation to partition organic and

aqueous phases the upper layer containing the water-soluble inositol phosphates was collected and stored. The products of ^{3}H-inositol labelled PtdIns hydrolysis under these conditions are identified as Ins-1:2 cyclic-P_1 and Ins P_1 on the basis of previous studies (Dawson et al. 1971; Irvine et al. 1979). Ins-1:2 cyclic P_1 and Ins P_1 could be separated either by hplc (Figure 5.3.1) or by Dowex chromatography, in the latter case using buffers adapted from those described by Ellis et al. (1963) and Emilsson and Sundler (1984) (Figure 5.1.1). In either system Ins-1:2 cyclic P_1 was the earlier eluting product and could be clearly resolved from authentic ^{14}C-labelled Ins P_1 which co-eluted exactly with the second product resulting from ^{3}H-PtdIns hydrolysis. Figure 5.1.1 shows the separation of Ins-1:2 cyclic P_1 and Ins P_1 by Dowex chromatography and illustrates that after treatment of a mixture of these products with 0.5 M TCA at 0°C for 20 min. and subsequent removal of the acid, all radioactivity was recovered in the Ins P_1 fraction as a result of the hydrolysis of the cyclic 1:2 phosphate bond. Acid extracts from brain slices showed a peak of radiolabel which co-eluted exactly with Ins P_1 prepared from ^{3}H-PtdIns and with authentic ^{14}C-Ins P_1 both on hplc and Dowex chromatography.

Ins P_4 is identified in the elution profiles shown on the basis of results described in CH.4. Ins-1,3,4-P_3 was identified in two ways. Firstly, by its retention time relative to a ^{32}P labelled Ins-1,4,5-P_3 which was characteristic of that expected for Ins-1,3,4-P_3 (Irvine et al. 1985). Secondly, when extracts from carbachol-stimulated parotid gland were prepared as in 4.2(c), the predominant trisphosphate ran exactly as anticipated for Ins-1,3,4-P_3 while a much smaller peak corresponding to Ins-1,4,5-P_3 ran immediately behind.

The peak shown as Ins P_2 on the illustrated elution profiles is identified on the basis of its retention intermediate to that of ^{3}H-GroPInsP and GroPInsP_2. In addition, when the latter standards were prepared from the corresponding lipids small peaks of radioactivity could be detected as minor products, some of which can be identified as Ins P_2(s) (see 3.5 and 4.2). On the hplc gradient shown these exhibited retention times approximately corresponding to the peak labelled Ins P_2 in extracts from cerebral cortex thus supporting this latter identification. No attempt was made to resolve isomeric Ins P_2(s) or Ins P_1(s).

For each gradient a series of standard and sample materials prepared as above was run to characterise the separations shown and this process repeated whenever a fresh column was used.

c. Modification and verification of gradients:

The gradient elution method originally reported by Irvine et al. (1985) allowed separation of Ins-1,3,4- and-1,4,5-P_3 and of each lower

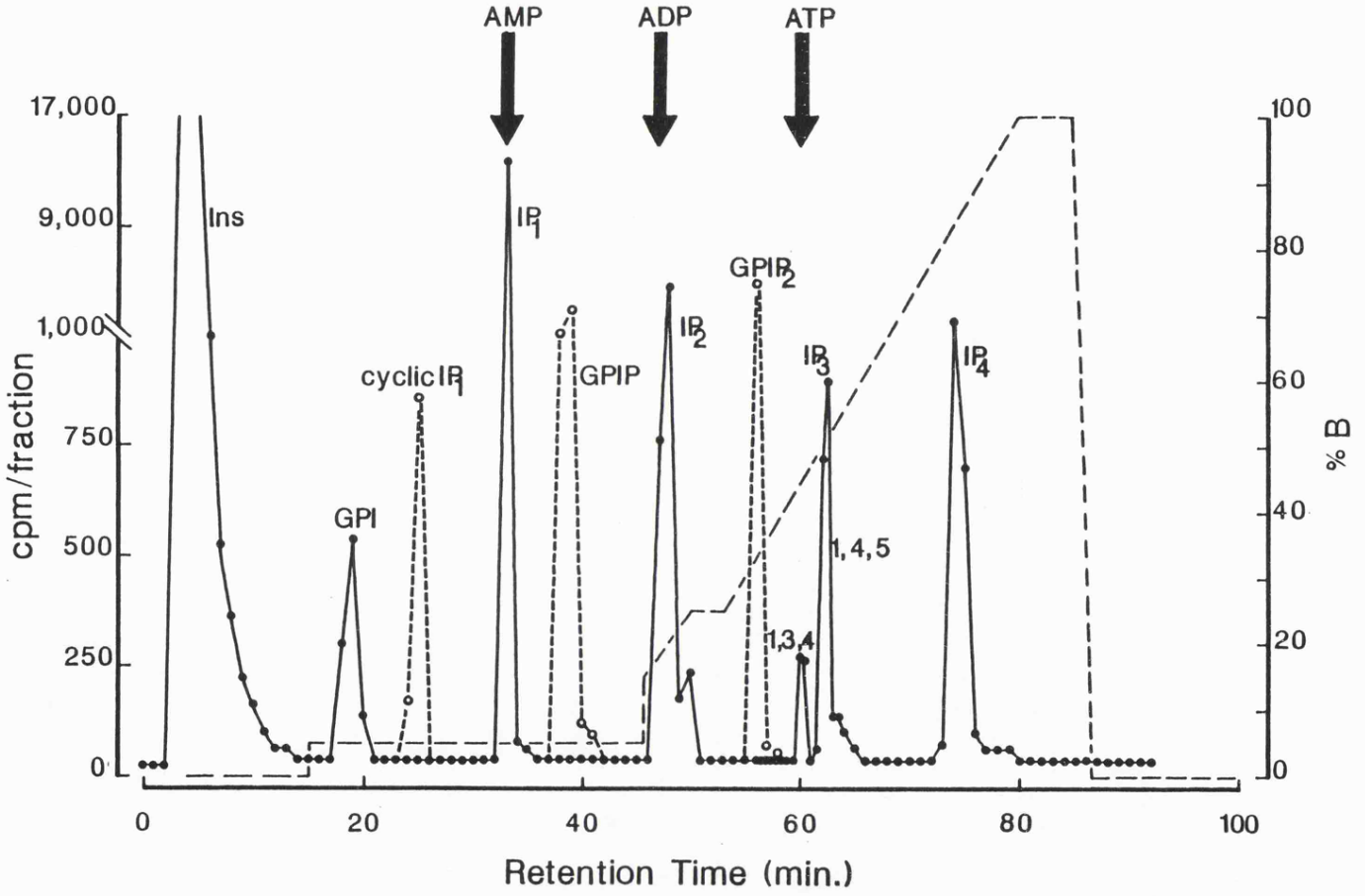


FIGURE 5.3.1 The separation of ^{3}H-inositol labelled inositol phosphates by anion exchange hplc.

The figure illustrates the separation by an hplc gradient modified from 4.4 and used to examine brain phosphoinositides for a PtdIns P_3 in section 4.3 (i.e. gradient no. 1, Appendix 2). The continuous line shows the ^{3}H-Ins P's present in an extract from carbachol-stimulated brain slices. The elution times of the other indicated Ins P's were determined in separate runs. The adenosine nucleotides were employed as internal markers in all hplc analyses (see text). The gradient used is superimposed as % B, where B = 1.7 M NH_4COOH, pH 3.7, with H_3PO_4.

inositol phosphate. This method was subsequently modified to allow elution of Ins P_4 (see 4.4(a)i). The illustrated separations were all achieved using gradients based on these two methods. However, the difference in the molecular charge carried by GroPIns upto Ins P_4 is large. This means that less highly phosphorylated compounds elute at only a very low percentage of the maximum solvent molarity required for the most polar inositol phosphates. For this reason if a single eluant is used across the entire gradient, the resolution between lower inositol phosphates tends to be limited unless the pumps used to generate the gradient are highly accurate at low output volumes. In the current experiments adequate resolution between GroPIns and Ins P_1 was difficult to achieve using a buffer which at maximum molarity (100% gradient) would displace Ins P_4. Similarly, using the linear gradient originally reported by Irvine et al. (1985) resolution between inositol monoester phosphates and glycerophosphorylinositol phosphates was also limited. Therefore, for the purpose of the studies reported in 4.3(b), in which clear separation of inositol mono-and diester phosphates was essential, a modified gradient was developed to allow better resolution of all the likely tritiated products arising from deacylation of ^{3}H-inositol labelled brain lipids (see Figure 5.3.1). This was found to be best achieved using two separate eluants, one for weakly polar inositol phosphates and a second for more highly charged molecules. Previous studies using Dowex chromatography had shown that GroPIns could be resolved not only from Ins P_1 but also from Ins-1:2 cyclic P_1 (see Figure 5.1.1.) by elution at 0.025 M ammonium formate (AF). Initial attempts were therefore made to improve Ins P_1 and GroPIns resolution on the same basis but using hplc. Further preliminary studies had also shown that elution of hplc columns at 5% of a 1.7 M AF solution, pH 3.7, H_3PO_4 would displace Ins P_1 as a sharp peak. The ability of various dilute ammonium formate solutions to displace GroPIns as a narrow band from columns was therefore tested when these were also run at 5% of the maximum. Isocratic elution of SAX hplc columns at 5% of a 0.28 M ammonium formate solution for approximately 10 min. was found to effectively resolve GroPIns and Ins-1:2 cyclic P_1 and if similar elution was then continued using 1.7 M AF (pH 3.7) H_3PO_4, Ins P_1 was eluted separately and sharply. Continued isocratic elution also displaced GroPInsP and gave better resolution from Ins P_1 than could be achieved by simply shallowing the gradient described by Irvine et al. (1985). However, to maintain a sharp Ins P_2 peak the gradient had then to be stepped as shown in Figure 5.3.1. Introducing a further short period of isocratic elution after displacing Ins P_2 also improved the separation between this inositol phosphate and GroPInsP_2. Clear resolution of Ins P_3(s) and Ins P_4 was not impaired by this procedure. In this way a

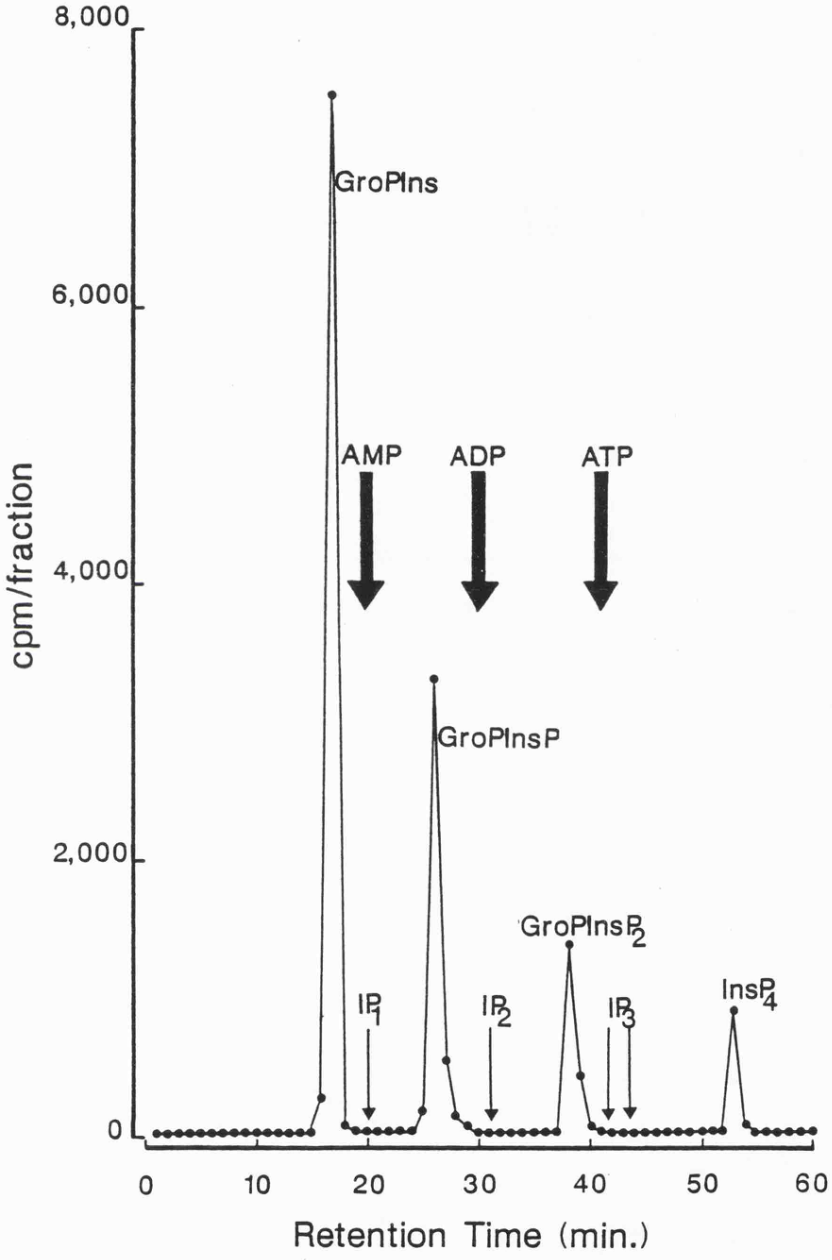


FIGURE 5.3.2 The resolution of ^{3}H-glycerophosphorylinositol phosphates by anion exchange hplc.

The figure illustrates the separation of standard glycerophosphoryl-inositol phosphates using an hplc gradient modified from that shown in Fig. 5.3.1 and used for the re-analysis of the putative ^{3}H-GroPIns P_3 fraction isolated by Dowex chromatography in 4.3 (see Fig. 4.3.5 and gradient no. 2, Appendix 2). The retention times for Ins P_1-Ins P_4 and AMP, ADP and ATP are also indicated (for GroPIns P_3 see also Fig. 6.5.3).

very distinct separation of the lower inositol phosphates was achieved and the results shown in Figure 5.3.1 for sample and standard materials serve to validate the identification of peaks made in Figure 4.3.4 (4.3(b)).

Although the separation of inositol phosphates was very effective using this method the run time per sample was considerable and this gradient was also modified to give that illustrated in Figure 5.3.2 which is representative of that used for re-analysis of the putative 'GroPInsP$_3$' fraction in 4.3(b). Figure 5.3.2 shows the retention times for each glycerophosphorylinositol (phosphate) and for Ins P$_4$, hence any GroPInsP$_3$ present in samples (see 4.3(b) and Figures 4.3.4 and 6.5.3) would have been expected to show a retention time around 45-50 min. A further slight modification of the same gradient used with a separate column is illustrated in Figure 5.3.3 and shows the very limited resolution achieved between GroPIns and Ins P$_1$ using this method with a single eluant across the gradient (1.7 M AF, pH 3.7, H_3PO_4) compared to that obtained in Figure 5.3.1 using dual, weak and strong eluants.

Over the course of a number of hplc analyses columns were periodically replaced and between separate columns the retention times for inositol phosphates varied slightly. For this reason and to allow for any variability in the accuracy of the gradient resulting from pump error, samples were routinely spiked with nucleotide markers and/or ^{32}P Ins-1,4,5-P$_3$. Irvine et al. (1985) have suggested the use of adenosine nucleotides as approximate markers for inositol phosphates in the currently employed system. In the present study where gradients were tailored to either particular requirements (see above) or altered to allow for limitations inherent to the equipment used (i.e. the inability of pumps to achieve accurate low output volume), these markers alone or together with GMP, GDP and GTP were routinely included. In most instances the retention times measured between AMP-GMP, ADP-GDP and ATP-GTP were found to bracket those found respectively for Ins P$_1$, Ins P$_2$ and Ins P$_3$. ATP eluted either just before or co-eluted with Ins-1,3,4-P$_3$ and GTP just after Ins-1,4,5-P$_3$ (see Figure 5.3.4). The spiking of samples with both ATP and GTP was found to be particularly useful when establishing ideal gradients for the resolution of inositol trisphosphates on new columns since gradients most effectively resolving the nucleotides were similarly efficient for Ins P$_3$(s). As the nucleotides could be detected by the on-line UV monitor it was therefore convenient to run trial gradients with these first. The separation of Ins-1,3,4-P$_3$ and Ins-1,4,5-P$_3$ achieved on different columns of the same type varied to the extent that resolution of these required collection of fractions from 0.5 min. down to 0.2 min. apart and occasionally the angle of the gradient had to be modified appropriately. Figure 5.3.4 (A) and (B) show typical examples of the

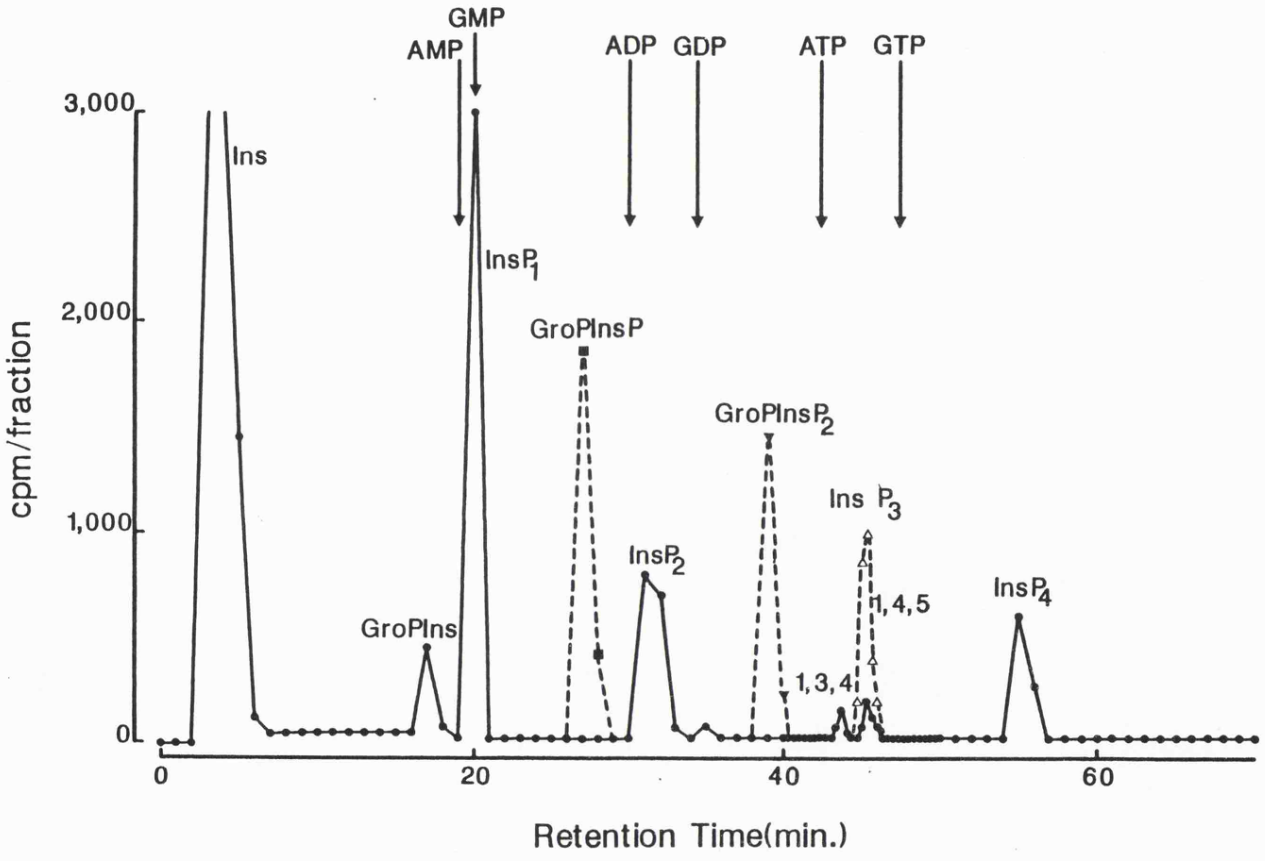


FIGURE 5.3.3 The separation of ^{3}H-inositol phosphates by gradient elution hplc.

The continuous line indicates the ^{3}H-inositol phosphates present in extracts from carbachol-stimulated brain slices and separated by gradient no. 3, Appendix 2. The retention times for ^{3}H-GroPIns P and ^{3}H-GroPIns P_2 are also indicated as are those of the adenosine and guanosine nucleotides.

separation achieved between Ins P_3(s) and Ins P_4 using gradients slightly shallowed over the Ins P_3 region as compared to that reported in 4.4(a)i. Details of all gradients are given in Appendix 2.

The routine spiking of samples with nucleotides not only allowed the potential usefulness of a gradient for the separation of particular inositol phosphates to be gauged but also allowed any major error or variation in the pumped gradient to be noted since the relative retention times for the set of nucleotides determined from the UV trace obtained with every sample would change dramatically should error occur. When samples were not also spiked with an internal ^{32}P Ins-1,4,5-P_3 standard this allowed the consistency between runs to be verified. However, between columns obtained from different sources slight variation in the exact co-elution of ATP and Ins-1,3,4-P_3 was observed contrary to previous reports (Irvine et al. 1985; Burgess et al. 1985), suggesting that the nucleotides cannot be taken as internal standards for inositol phosphates but only as approximate markers for these and as a measure of the reproducibility of the method.

In addition to the use of nucleotides for the latter purpose the accuracy of the gradients used was also determined by a more direct method. This was achieved by pumping a gradient from 0-100% of a 0.5% acetone solution and monitoring the absorption of the eluate at 254 nm. Over the range 0-100% of the gradient the change in absorbance is linear so that a visible estimate of the pumping accuracy could be obtained. Using this method and a flow rate of 1.2 ml/min. the system tested was found to be accurate only at above 2-5% of the gradient maximum which would account for the difficulty experienced in obtaining clear resolution of GroPIns and Ins P_1 reported above. However, since the major purpose for which hplc was used was separation of Ins-1,3,4- and-1,4,5-P_3 and because where higher resolution of the least polar inositol phosphates was required the procedure described above using the dual eluant system was used, this inaccuracy does not significantly affect the data presented.

Using these methods extracts from control and stimulated brain slices were examined and the stimulated accumulations of Ins P_1, Ins P_2, Ins-1,3,4- and-1,4,5-P_3 and of Ins P_4 confirmed. All samples were found to contain GroPIns and occasionally much smaller levels of ^3H-products co-eluting with GroPInsP and GroPInsP_2 although none of these was affected by muscarinic receptor stimulation. The data presented in most of the figures shown is representative of that derived from brain slices stimulated for 10-15 min. with 1 mM carbachol and although elution profiles for appropriate controls are not illustrated, the stimulation due to agonist can be seen from data presented in CH.3, 4 and 6.

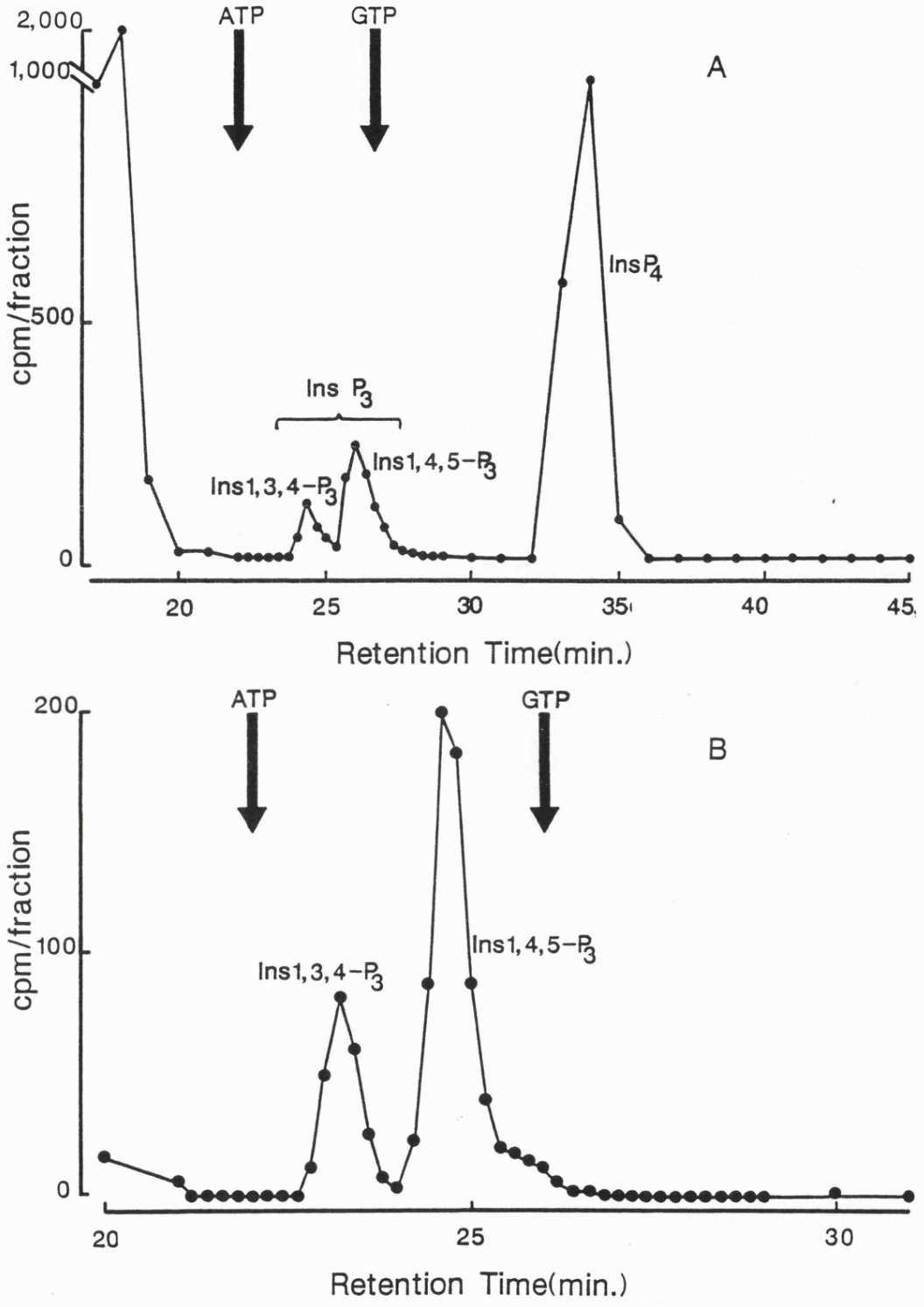


FIGURE 5.3.4 The separation of ^{3}H-inositol tris- and tetrakisphosphates by gradient elution hplc.

Fig. A shows the separation of ^{3}H-Ins-1,3,4-P_3, ^{3}H-Ins-1,4,5-P_3 and ^{3}H-Ins-1,3,4,5-P_4 achieved using gradient no. 4 (Appendix 2). Fig. B illustrates the resolution achieved between Ins-1,3,4-P_3 and Ins-1,4,5-P_3 using a slightly shallower gradient (no. 5, Appendix 2). Samples in both Figs. A and B were extracted from carbachol-stimulated brain slices labelled with ^{3}H-inositol. The relative retention of ATP and GTP is also indicated.

In the present study no attempt has been made to resolve any potential isomeric Ins P_1(s) and Ins P_2(s) whose accumulation is stimulated in response to receptor activation although as noted in 5.2 it may be possible to achieve separation of some of these using modifications of the above methods. Thus, for the present purpose the major advantage of hplc over Dowex chromatography was the ability of the former to allow separate measurement of Ins-1,3,4- and Ins-1,4,5-P_3. Other than these all the inositol phosphates shown to be resolved by hplc can be adequately separated by Dowex chromatograhy as shown in Figure 5.1.1.

CHAPTER 6

The metabolism of inositol phosphates in rat cerebral cortex

Together with the identification of Ins-1,3,4-P_3 (Irvine et al. 1984a) and the subsequent description of an Ins-1,4,5-P_3 3-kinase (Irvine et al. 1986), the data presented in CH.4 served to help establish the existence of a novel pathway of inositol phosphate metabolism, all the components of which are present in brain. Whether this represents primarily an alternative means of terminating the mobilisation of calcium triggered by Ins-1,4,5-P_3 or a mechanism for generating additional signal molecules remains to be established. The discussion in preceding sections raised the possibility that metabolism of Ins-1,4,5-P_3 through Ins P_4 could be important in both these respects. Any role for either Ins-1,3,4,5-P_4 or Ins-1,3,4-P_3 in regulation of $[Ca^{2+}]_i$ or other intracellular functions will only become clear when appropriate studies of the effects of these compounds can be made under defined conditions and/or when specific inhibitors of their synthesis or catabolism become available. Since the metabolism of neither can yet be selectively impaired, evidence for functional activity of these molecules is most likely to arise from studies analogous to those defining a role for Ins-1,4,5-P_3 in control of $[Ca^{2+}]_i$ (e.g. Streb et al. 1983), whereby these inositol metabolites are introduced into suitable isolated cells. Clearly brain slice preparations are not readily amenable to such experiments and support for any action of Ins-1,3,4,5-P_4 or Ins-1,3,4-P_3 in this tissue will most probably arise from a better understanding of the factors affecting metabolism of these compounds.

A number of the conclusions previously drawn with respect to Ins P_4 metabolism in brain were based on results shown in CH.3 in addition to the more specific data presented in CH.4. However, in these earlier experiments Ins P_4 was not determined separate from trisphosphates and both its rapid turnover and its production as a result of muscarinic receptor stimulation were inferred from measurement of a fraction now known to comprise Ins-1,4,5-P_3, Ins-1,3,4-P_3 and Ins-1,3,4,5-P_4. Although this should not invalidate the assumptions that metabolism of each is rapid and is initiated by a receptor mediated step, it is apparent that more accurate qualification of these statements in terms of specific rates of turnover and pharmacological characteristics can only be achieved by measurement of each separate product. Similarly, the previously described effects of lithium on 'Ins P_3' accumulation may also require more precise definition, particularly as preliminary data (4.1) suggested that these may be more specific to Ins P_4. Further, while a number of possible routes of inositol phosphate metabolism and sites where lithium could potentially interfere with these were considered in 3.4, the more recent developments described in 4.4(b)iii considerably extended this scope.

The experiments reported in this section primarily represent re-evaluations of some of the observations made in earlier chapters which have become essential in view of the now established dual routes of Ins-1,4,5-P_3 metabolism. These studies were directed towards either clarifying or confirming the points raised above such that the comparative rates of inositol phosphate metabolism, the pharmacological characteristics of inositol polyphosphate accumulation and the potential sites of action of lithium were re-investigated. Some of these studies are as yet incomplete but the preliminary results are presented as these either support fresh interpretations of previous observations or form the basis for future studies. Initially, however, the efficiency of several different extraction procedures for inositol phosphates were briefly examined to confirm that the method previously established for less polar compounds is also effective for Ins P_4. The results of this are considered in 6.1 and subsequent studies performed using a modified extraction (detailed here) are described in the following sections.

6.1 Comparison of methods for the extraction of inositol phosphates

The method used in previous sections to achieve effective extraction of inositol phosphates involves use of trichloroacetic acid (TCA) to quench tissue incubations and was described in detail in 2.4. For extraction of inositol polyphosphates this was found to be equally efficient to an alternative procedure using perchloric acid (PCA) and to be preferable to use of either neutral or acidified mixtures of chloroform:methanol, both of which resulted in higher basal but not stimulated values for Ins P_2 and Ins P_3. This latter effect was also noted in the current comparison and is discussed below. Bone et al. (1984) have verified the effectiveness of a PCA extraction of inositol polyphosphates by adding exogenous ^{32}P labelled standards of these to tissue samples and monitoring recovery of these on subsequent sample processing. Measurements of this type should give a reasonable estimate of the efficacy of a particular method but these conditions do not exactly correspond to those for the extraction of endogenous materials, for which the only guarantee of effective recovery using any one particular method is that alternative approaches give the same result. As both PCA and TCA give approximately equivalent yields of ^3H-inositol phosphates from both control and stimulated brain slices, use of the latter procedure would be expected to be as reliable for Ins P_1, Ins P_2 and Ins P_3 as the PCA method reported by Bone et al. (1984). Although there was no reason to expect that the current procedure would prove any less effective for Ins P_4, it seemed desirable to confirm this by brief comparison with other methods.

Since a simple method for the chromatographic resolution of Ins-1:2 cyclic P_1 from other inositol phosphate fractions was also established in CH.5 this brief study was extended to include a preliminary examination of neutral extracts for this product as discussed below.

The different extraction methods used for the purpose of this comparison were essentially those described in 2.4 except for the modifications noted and are summarised as (a)-(e) below. Each was performed on triplicate 50 µl aliquots of cerebral cortical slices according to the legend to Table 6.1.1. Extracts of inositol phosphates from triplicated samples were pooled, neutralised where appropriate, then analysed by anion exchange chromatography (see below). The results presented are for a single experiment but are broadly consistent with previous studies reported in 2.4. The conditions for extraction of ^{3}H-inositol phosphates and neutralisation of extracts were:

(a) As in 2.4 using 1.0 M TCA.

(b) As in 2.4 except that after TCA addition, tissue fragments were more thoroughly disrupted by brief sonication of samples rather than by vortex mixing.

(c) As in 2.2 using neutral chloroform:methanol (1:2 v/v) with subsequent additional H_2O and chloroform.

(d) Incubations were stopped by addition of 0.94 ml methanol: chloroform (2:1 v/v) acidified to 0.1 M with HCl. A two-phase system was generated by adding 0.31 ml of each $CHCl_3$ and 0.1 M HCl and phases partitioned by centrifugation at 3,000 x g for 10 min. The upper aqueous layer containing ^{3}H-inositol phosphates was dried under vacuum to evaporate the acid then re-dissolved in H_2O.

(e) Reactions were terminated by addition of 300 µl 7% (w/v) PCA and samples allowed to stand on ice for 10-20 min. Tissue fragments were spun down by centrifugation at 3,000 x g for 20 min. and 500 µl of the resultant supernatants collected. These were neutralised using a procedure based on that described by Khym (1975) as modified by Sharps and McCarl (1982) and Downes et al. (1986). To each 500 µl supernatant 125 µl 10 mM EDTA was added followed by 500 µl of a 1:1 (v/v) mixture of freon (1,1,2-trichlorotrifluroethane) and tri-n-octylamine. Samples were thoroughly vortex mixed and the resultant three phases produced partitioned by brief centrifugation (5 min.) in a microfuge. The upper aqueous phase contains the ^{3}H-inositol phosphates, the middle phase tri-n-octylamine perchlorate and the lower phase unreacted amine in the water immiscible freon solvent (Downes et

al. 1986). A 500 µl portion of the upper phase (pH~ 4-5) was collected, pooled from triplicate samples and adjusted to pH 6-7 by addition of 3.5 ml 5 mM $NaHCO_3$.

The extracts obtained by each method were examined by Dowex anion exchange chromatography, largely according to 4.2 but including an additional eluant intermediate to that used for ^{3}H-GroPIns and ^{3}H-Ins P_1 to allow separate measurement of any Ins-1:2 cyclic P_1 as demonstrated in CH.5. Although this would not be present in acidified extracts (see 4.4(b)iii), the 1:2 cyclic bond should be preserved on neutral chloroform: methanol treatment. Dixon and Hokin (1985) have suggested that stimulated production of Ins-1:2 cyclic P_1 in tissue exposed to agonist would constitute good evidence for direct hydrolysis of PtdIns. This is not necessarily so since the 1:2 cyclic derivatives of each Ins-1,4-P_2 and Ins-1,4,5-P_3, shown to result from PtdIns P and PtdIns P_2 hydrolysis by purified phospholipase C preparations (Wilson et al. 1985a), would also result in formation of Ins-1:2 cyclic P_1 as they are degraded initially by removal of the 5 and 4 monoester phosphates (Connolley et al. 1986). Nevertheless, the demonstration of a stimulated accumulation of Ins-1:2 cyclic P_1 would at least be indicative of either of these additional routes of inositol lipid/phosphate metabolism and could provide a useful basis for subsequent studies. As any cyclic phosphate present in the tissues would not survive the acid extractions these served as controls for the neutral solvent extraction protocol.

The chromatographic analysis of samples was also modified to the extent that after elution of ^{3}H-Ins P_4 at 1.0 M formate, columns were washed either at twice this molarity buffer (extractions (a)-(d)) or at intermediate eluant concentrations up to 2.0 M formate (extraction (a)) so that any inositol phosphates more polar than Ins P_4 would also be displaced. The results are presented in Table 6.1.1 while Figure 6.1.1 allows a qualitative comparison of samples extracted by methods (a), (c) and (d).

The data presented in Table 6.1.1 allow a quantitative comparison to be made of the extraction achieved for each inositol phosphate under each set of conditions. This confirms most of the conclusions drawn and discussed in 2.4. Extraction with either TCA or PCA gave very similar yields of each GroPIns, Ins P_1, Ins P_2, Ins P_3 and Ins P_4 and although the latter method appears marginally more effective for Ins P_3 and Ins P_4, showing approximately 25% more radioactivity in these stimulated fractions, further experiments would be required to confirm this. Similarly, little advantage is gained on TCA extraction when tissue is subjected to sonication which, by more thoroughly disrupting the structure

Table 6.1.1 Comparative extractions of inositol phosphates.

Extraction	^{3}H-Inositol phosphate (cpm 50 µl tissue)						
	GroPIns	*(Ins-1:2 cyc.P)	Ins P_1	Ins P_2	Ins P_3	Ins P_4	Higher phosphates
(a) TCA							
Control:	3272	208	4098	2132	1260	346	23
Stim:	2829	329	25205	10819	3194	3452	54
(b) TCA							
Control:	3665	231	5469	2295	1590	372	32
Stim:	3040	626	25730	11145	3478	3433	69
(c) Neutral solvent							
Control:	5623	848	3691	9018	944	136	34
Stim:	6096	1632	19844	12554	838	142	92
(d) Acid solvent							
Control:	5292	1281	14172	7616	4397	496	43
Stim:	5423	1521	33666	16688	7235	2223	59
(e) PCA							
Control:	3057	196	4645	1480	1273	224	25
Stim:	3432	217	29988	10823	3990	4341	44

Cerebral cortical slices were labelled with ^{3}H-inositol (5 µCi/50 µl tissue) for 60 min. as previously described then incubated for 15 min. in the presence or absence of 1 mM carbachol. Reactions were stopped as detailed in the text and appropriate triplicate extracts of ^{3}H-inositol phosphates combined and neutralised. Aliquots of each sample were analysed for ^{3}H-inositol phosphates by anion exchange chromatography as in Figure 5.1.1. Results represent a single experiment and are expressed as radioactivity within each inositol phosphate fraction per 50 µl of tissue.
* See text.

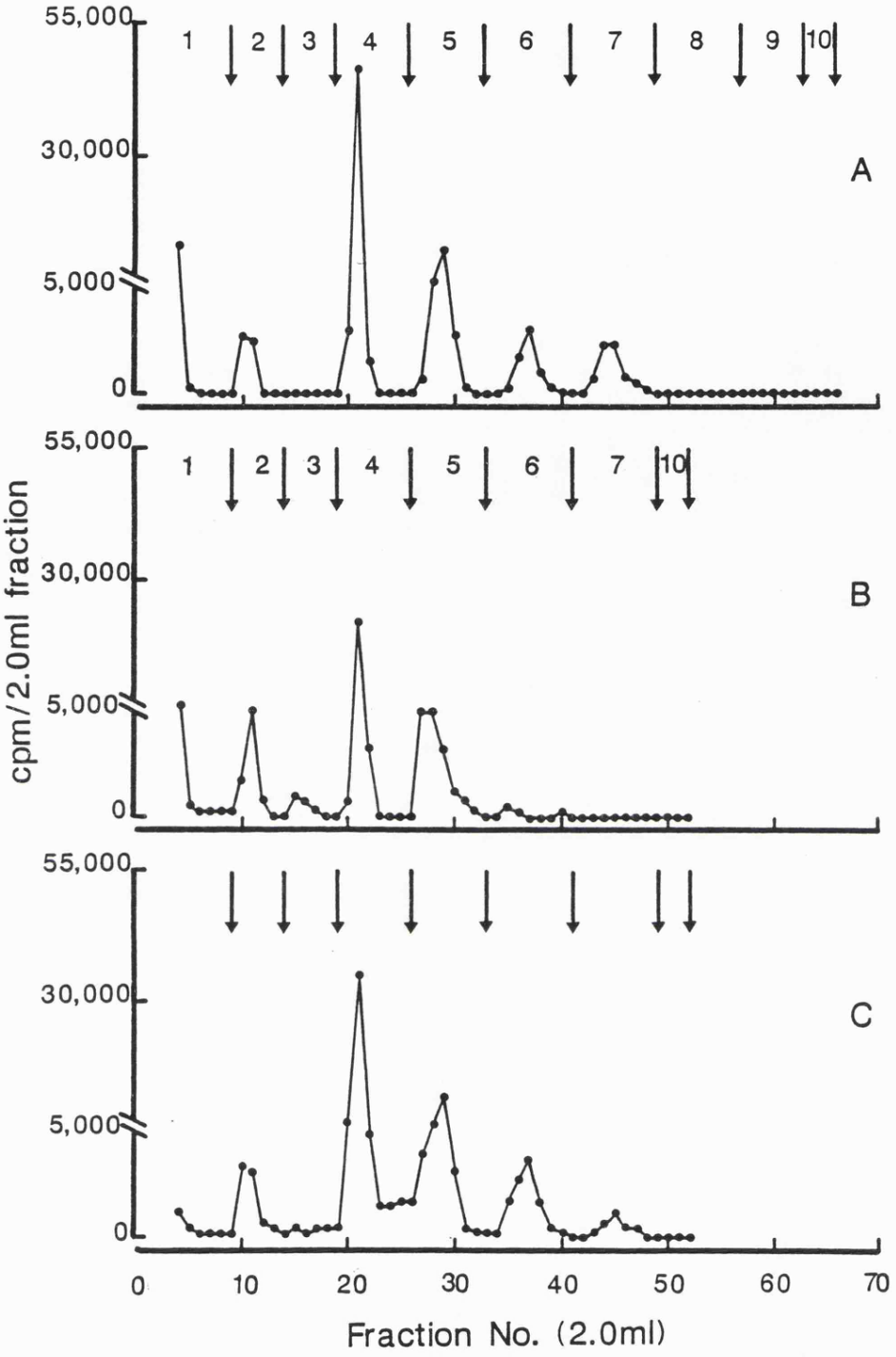


FIGURE 6.1.1 Dowex anion exchange column elution profiles of ^{3}H-inositol phosphates extracted from 1 mM carbachol-stimulated brain slices by different methods.

Cerebral cortical slices were labelled with ^{3}H-inositol as in 4.3 and exposed to 1 mM carbachol for 15 min. Reactions were stopped with 1.0 M TCA (A), neutral solvent (B) or acidified solvent (C) and samples neutralised as in text before application to 0.6 x 3.0 cm columns AG 1 x 8 resin (200-400 mesh, formate form). Columns were eluted with the indicated volumes of buffers 1-10 (A) or 1-7 followed by 10 (B + C). Buffers 1-10 represent: H_2O (Ins); 0.025 M AF (GroPIns); 0.06 M AF/0.005 M $Na_2B_4O_7$ (Ins 1:2 cyclic P_1); 0.2 M AF (Ins P_1); 0.5 M AF/0.1 M FA (Ins P_2); 0.8 M AF/0.1 M FA (Ins P_3); 1.0 M AF/0.1 M FA (Ins P_4); 1.2 M AF/0.1 M FA; 1.5 M AF/0.1 M FA and 2.0 M AF/0.1 M FA (for any Ins P(s) more polar than Ins P_4).

of tissue slices, might have been expected to give an improved yield over that obtained with the standard method previously used whereby tissue fragments are only agitated by vortex mixing. It should be pointed out however, that with each TCA and PCA extractions the ratio of stimulated Ins P_4 to Ins P_3 is lower than normally found in other experiments where radiolabelling of Ins P_4 (after 15 min. exposure to carbachol) was more typically 1.5-2.0 fold that of Ins P_3. However, as each PCA and TCA extraction protocols gave similar results and as higher yields of ^{3}H-Ins P_4 have previously been obtained using trichloroacetic acid, this discrepancy cannot be accounted for by ineffective extraction. Variation in tissue viability is a more probable explanation of the low yield of ^{3}H-Ins P_4 since any inadequacy in tissue preparation or incubation will be most notably reflected in the accumulation of this product whose synthesis is reliant on a further ATP-dependent step than is that of any other inositol phosphate measured.

Each of the figures presented in Table 6.1.1 has been adjusted to allow for background radioactivity. Despite this, a small amount of label extracted by each method eluted later than Ins P_4. However, in no case did this exceed 2% of the maximum stimulated level of accumulated ^{3}H-Ins P_4 and for all samples accounted for a very much smaller fraction of the total radiolabel recovered. This is unlikely to indicate the presence of more polar inositol phosphates (e.g. Ins P_5 or Ins P_6) because neutral solvent extraction yielded less than 5% of the ^{3}H-Ins P_4 extracted from stimulated samples with either TCA or PCA and yet gave the highest radiolabelling of the post Ins P_4 fraction. On the basis of this observation and the previously noted cross peak contamination seen on Dowex chromatography, it is probable that radioactivity eluting as 'higher inositol phosphates' can be accounted for either by carry over from earlier eluted fractions and/or by marginal error in correcting for background radioactivity.

With each extraction procedure used, a small proportion of the total radioactivity recovered ran under the 0.06 M formate peak. Table 6.1.1 shows that the effect of carbachol on this was somewhat variable. To an extent radiolabel eluting in this fraction, previously shown to correspond to that for Ins-1:2 cyclic P_1 (see CH.5), is also likely to represent contaminant. This seems probable because every acid extraction as well as the neutral solvent method revealed label in this fraction. The nature of this contaminant is uncertain but is unlikely to represent cross peak contamination from either GroPIns or Ins P_1 because previous experiments using highly radiolabelled standards of each had shown essentially complete containment of each within the 0.025 M and 0.200 M formate fractions respectively. More probably the label eluting at 0.06 M formate

represents contaminant within the original ^{3}H-inositol. Although this was routinely minimised as described in 2.4, a small amount of anionic material could usually be detected within 'cleaned' samples of ^{3}H-inositol on examination by Dowex chromatography. When present this tended to elute either before or together with Ins P_1 and, to a lesser extent, with Ins P_2 but was not normally sufficient to significantly affect the assay sensitivity. In the present situation even very low levels (< 0.02%) of contaminant could account for the label eluting in the Ins-1:2 cyclic P_1 fraction evident in acid extracted samples. As radioactivity eluting at 0.06 M formate is present in each extract it is difficult to determine to what extent, if any, label extracted with neutral solvent and running in this fraction can be identified as Ins-1:2 cyclic P_1. It is of interest to note however, that samples extracted with either neutral or acidified organic solvents showed higher labelling of this fraction than where deproteinising acids were employed.

Samples extracted by both protocols involving use of organic solvents also exhibited a number of further unusual characteristics which are evident from Table 6.1.1 and Figure 6.1.1. Firstly, extraction with neutral solvent resulted in a very much higher basal labelling of the Ins P_2 fraction. This was 4-6 fold greater than observed with either TCA or PCA and since the latter both showed effective yield of Ins P_2 from stimulated tissue, this strongly suggested that, as noted in 2.4, a substantial portion of the 'Ins P_2' extracted in the presence of organic solvent may be accounted for by a distinct but similar ^{3}H-inositol labelled material. The elution profile for a neutral solvent extract from stimulated tissue is shown in Figure 6.1.1 (B). This shows that the Ins P_2 peaks running in TCA and neutral solvent extracts do not exactly co-elute. It is important to note that where standards of inositol phosphates have been applied to columns in H_2O, the elution profiles for these correspond exactly with those obtained with TCA or PCA extracted samples (data not shown). It is also of interest to note from Figure 6.1.1 and Table 6.1.1 that while neutral solvent and TCA gave almost equivalent yields of Ins P_2 from stimulated samples the former extracted practically no Ins P_3. Although some difference between the ability of neutral solvent to extract Ins P_2 and Ins P_3 would be anticipated this would not be expected to be so dramatic. This again suggests that a large proportion of neutrally extracted 'Ins P_2' represents a separate ^{3}H-inositol labelled material. As Hawkins _et al_. (1986) have reported that much of the PtdIns P extracted with neutral solvent partitions into the aqueous phase, it seems probable that the current observation could be a consequence of this. However, a similar phenomenon was also found here when samples were extracted with acidified

organic solvent, a procedure which is normally considered to give effective extraction of polyphosphoinositides, with these partitioning into the chloroform phase (Downes and Michell, 1982). Using an acidified solvent extraction, the basal labelling of each Ins P_1, Ins P_2 and Ins P_3 was between 2-4 fold higher than observed with either TCA or PCA. This latter finding is confirmed not only by the results reported in 2.4 but also by a recent comparative study of methods for extraction of inositol phosphates (Donaldson and Hill, 1986). The stimulated level of Ins P_3 and Ins P_2 extracted with acidified solvent shows approximately the same increase over basal as that achieved with PCA suggesting that, although the former method is effective for recovery of inositol polyphosphates, it results in simultaneous extraction of other ^{3}H-materials which from Figure 6.1.1 approximately co-elute with inositol phosphates on Dowex chromatography. However, Table 6.1.1 also shows that the yield of ^{3}H-Ins P_4 obtained with acidified solvent was only 50-65% of the stimulated level achieved with either TCA or PCA. An elution profile for an acidified solvent extract from stimulated tissue is illustrated in Figure 6.1.1 (C). As with the neutral solvent extract, the Ins P_2 peak does not exactly co-elute with the corresponding peak in TCA extracts while there is very poor Ins P_1/Ins P_2 resolution. The reason for the latter feature is unclear but could be accounted for by the presence of a substantial quantity of a labelled product of intermediate polarity to Ins P_1 and Ins P_2 or the presence of some unlabelled material interfering with the chromatographic separation. However, the latter would not explain the very high basal levels of inositol phosphates evident from Table 6.1.1 and, as pointed out above, the results with both neutral and acidified solvents may arise from unusual partitioning of ^{3}H-inositol lipids under the extraction conditions used. If this is so then studies using neutral solvent to extract ^{3}H-inositol phosphates (e.g. CH.2; Brown et al. 1984; Jacobson et al. 1985; Fisher and Bartus, 1985) would be likely to underestimate the magnitude of agonist-induced responses while studies using acidified solvent to extract ^{3}H-inositol lipids (e.g. CH.3.5; Downes and Wusteman, 1983) could similarly underestimate the levels of these. Clearly further experiments will be required to determine more conclusively the cause of the unusual effects noted here with organic solvent extractions.

It is evident from the comparisons made that an acid extraction procedure is essential for the effective recovery particularly of inositol polyphosphates and clearly of the procedures compared, those using TCA or PCA are preferable both because of the better yields of Ins P_4 obtained and because subsequent chromatographic analysis of these is neither impaired nor complicated as when organic solvent is used. For the latter

reason it is difficult to determine precisely the relative efficiency of solvent extraction methods. A more detailed chromatographic analysis of the ^{3}H-products extracted with neutral or acidified solvents, possibly using either hplc as in CH.5 or conventional anion exchange as in 3.5, may help to identify the problems associated with these methods. One major advantage of neutral solvent extraction however, is that it should preserve any acid labile components (such as Ins-1:2 cyclic P_1) in their original form. Unfortunately, the current data with regard to Ins-1:2-cyclic P_1 is ambiguous owing to the presence of label in this fraction even in acid treated samples. However, even if all the radioactivity from neutral extracts eluting within the 0.06 M formate fraction is assumed to represent Ins-1:2 cyclic P_1 it is clear that carbachol has only a limited effect on its accumulation, promoting only a 2-fold increase compared to a 5-600% increase in ^{3}H-Ins P_1 determined for the corresponding sample. This small effect of agonist could simply reflect the very rapid hydrolysis of Ins-1:2 cyclic P_1 but from the relative accumulation of this molecule and that of Ins P_3 (TCA or PCA extracts) this would imply either a more rapid hydrolysis of the cyclic product, which seems unlikely particularly as the Ins P_3 is predominantly the Ins-1,4,5-P_3 isomer, or that the Ins-1:2 cyclic P_1 is both slowly produced and rapidly removed. The latter possibility is more difficult to exclude and again it is clear that further studies involving higher purity ^{3}H-inositol and preferably several means of analysing extracts for Ins-1:2 cyclic P_1 (i.e. ionophoresis or paper chromatography - Dawson and Clarke, 1972 - in addition to Dowex chromatography) will be necessary before the presence or absence of this phosphate can be confirmed. A previous study involving rigorous analysis of neutral brain extracts has found no evidence for the production of Ins-1:2 cyclic P_1 on carbachol stimulation (Berridge *et al.* 1983). However, small quantities of this product in ^{3}H-inositol labelled mouse pancreatic mini lobules have been detected but only represent a very small fraction of labelled Ins P_1 (i.e.$\sim$ 1%) (Dixon and Hokin, 1985). Possibly such low levels of Ins-1:2 cyclic P_1 suggest that its production either by phospholipase C catalysed PtdIns hydrolysis or dephosphorylation of Ins-1:2 cyclic 4-P_2 and Ins-1:2 cyclic 4,5-P_3 represent very minor routes of inositol lipid/phosphate metabolism. If the production of Ins-1:2 cyclic P_1 may be taken as a measure of direct PtdIns hydrolysis (but see above) then the current estimate of its accumulation, which is very probably an overestimate owing to fraction contamination, supports the earlier conclusions (3.2 and 4.3a) and those reported in 6.2 that metabolism of PtdIns by this route is of limited significance to muscarinic receptor-mediated inositol lipid hydrolysis in brain.

In conclusion the results of this brief comparison of extraction methods for inositol phosphates emphasise the advantages of using deproteinising acids for non-cyclic products up to and including Ins P_4, thus confirming the data in 2.4. However, in contrast to the earlier study, the most convenient procedure from a practical point of view was found to be that using PCA since the method for neutralising these samples, recently introduced by Downes et al. (1986), was considerably more rapid than repeated ether extraction of TCA. Khym (1975) has reported the efficiency of this neutralisation procedure for both aqueous PCA and TCA tissue extracts, but as both acids gave equivalent extraction of ^{3}H-inositol phosphates the procedure established by Downes et al. (1986) using PCA as described above was adopted for routine use in the experiments reported in subsequent sections.

6.2 The hydrolysis of inositol phosphates in cerebral cortex

The results presented in Chapters 3 and 4 clearly established that activation of muscarinic receptors in cerebral cortex is accompanied by a very rapid hydrolysis of PtdIns-4,5-P_2, leading to an increased accumulation of Ins-1,4,5-P_3. On the basis of the rates at which both Ins P_2 and Ins-1,3,4,5-P_4 are also produced on exposure of tissue to agonist, the turnover of Ins-1,4,5-P_3 appears to be consistent with its role as a putative second messenger. Alternatively, the relative time courses for Ins-1,3,4-P_3 and Ins P_1 accumulation imply less rapid rates of metabolism for the immediate precursors of these molecules. The more direct estimates of the comparative rates at which the separate inositol phosphates are hydrolysed also support similar conclusions (see 3.2) and, to the extent which measurements have thus far been possible, are consistent with the role of PtdIns P_2 as the sole substrate for receptor-mediated phospholipase C activity in brain. However, as previously noted, these observations were complicated both by the earlier measurement of an 'Ins P_3' fraction of which Ins-1,4,5-P_3 only constituted a limited proportion and by the false assumption that turnover of this fraction is not influenced by lithium ions.

In order to clarify this situation and to obtain a preliminary measure of the relative extents to which Ins-1,4,5-P_3 is metabolised through the two alternative pathways represented by phosphatase and kinase activities in brain, a more detailed analysis was made of the rates at which each separate inositol phosphate is degraded. This was achieved using the experimental approach described in 3.2 whereby ^{3}H-inositol labelled cerebral cortical slices were first stimulated with carbachol until accumulations of inositol phosphates had reached new steady state

values, then the time courses of decline from these down to control levels monitored when excess atropine was added to displace agonist from the muscarinic receptors. This protocol has a number of practical and theoretical limitations which are discussed below.

The conditions for incubation of brain tissue were exactly as described in 3.2 except that labelling with ^{3}H-inositol was continued for 60 min. prior to agonist addition. ^{3}H-Inositol labelled cerebral cortical slices were then incubated in the presence or absence of 1 mM carbachol for 30 min., by which time accumulations of each inositol phosphate plateau at maximal levels (see 3.1 and 4.3(b)). Both control and stimulated sample incubations were then either stopped or continued for up to 15 min. after addition of 10^{-5} M atropine or appropriate Krebs vehicle. Reactions were terminated by addition of 300 µl 7% (w/v) PCA and neutralised extracts of ^{3}H-inositol phosphates from pooled, triplicated samples obtained as in 6.1. Final samples (5 ml) were divided into equal portions and one half analysed for Ins P_1 through Ins P_4 by Dowex chromatography as in 4.2 except that Ins P_4 was eluted using 6 ml 2 M ammonium formate. The remainder was spiked with nucleotide markers and Ins (1,[4,5 ^{32}P])-P_3 and analysed for ^{3}H-Ins-1,3,4-and-1,4,5-P_3 by hplc according to gradient 4 (see Appendix 2) as described in CH.5. In this way the decay of all inositol phosphates from steady state, carbachol-stimulated levels down to control values were determined over a 15 min. period subsequent to atropine blockade of the initial response.

The combined results of three or four experiments are presented in Figures 6.2.1 to 6.2.5. In each case only the data for stimulated samples, incubated in the presence or absence of atropine, is shown for the sake of simplicity. This data has been calculated by subtracting the zero time (point of atropine addition) basal value from each stimulated value and expressing the remainder as a percentage of the zero time stimulated value above basal. Thus the control value for each inositol phosphate is represented by the abscissa. Calculation by this method was essential since appropriate controls could not be run for each time point but is valid within a small error since control values did not change markedly over the period illustrated and, as previously noted, were unaffected by atropine. Expressing data in this way substantially reduced the inter-experimental variation unavoidably associated with the protocol used. The mean values representing 100% for Ins P_1, Ins P_2, Ins P_3 and Ins P_4 were 21,876 ± 2,935, 9,327 ± 1,716, 2,522 ± 549 and 6,747 ± 856 cpm/50 µl tissue respectively. The mean percentage stimulations due to carbachol at zero time were 605 ± 88%, 1,060 ± 105%, 399 ± 16% and 3,234 ± 330% respectively for Ins P_1, Ins P_2, Ins P_3 and Ins P_4. The corresponding percentage stimulations for Ins-1,3,4-P_3 and Ins-1,4,5-P_3

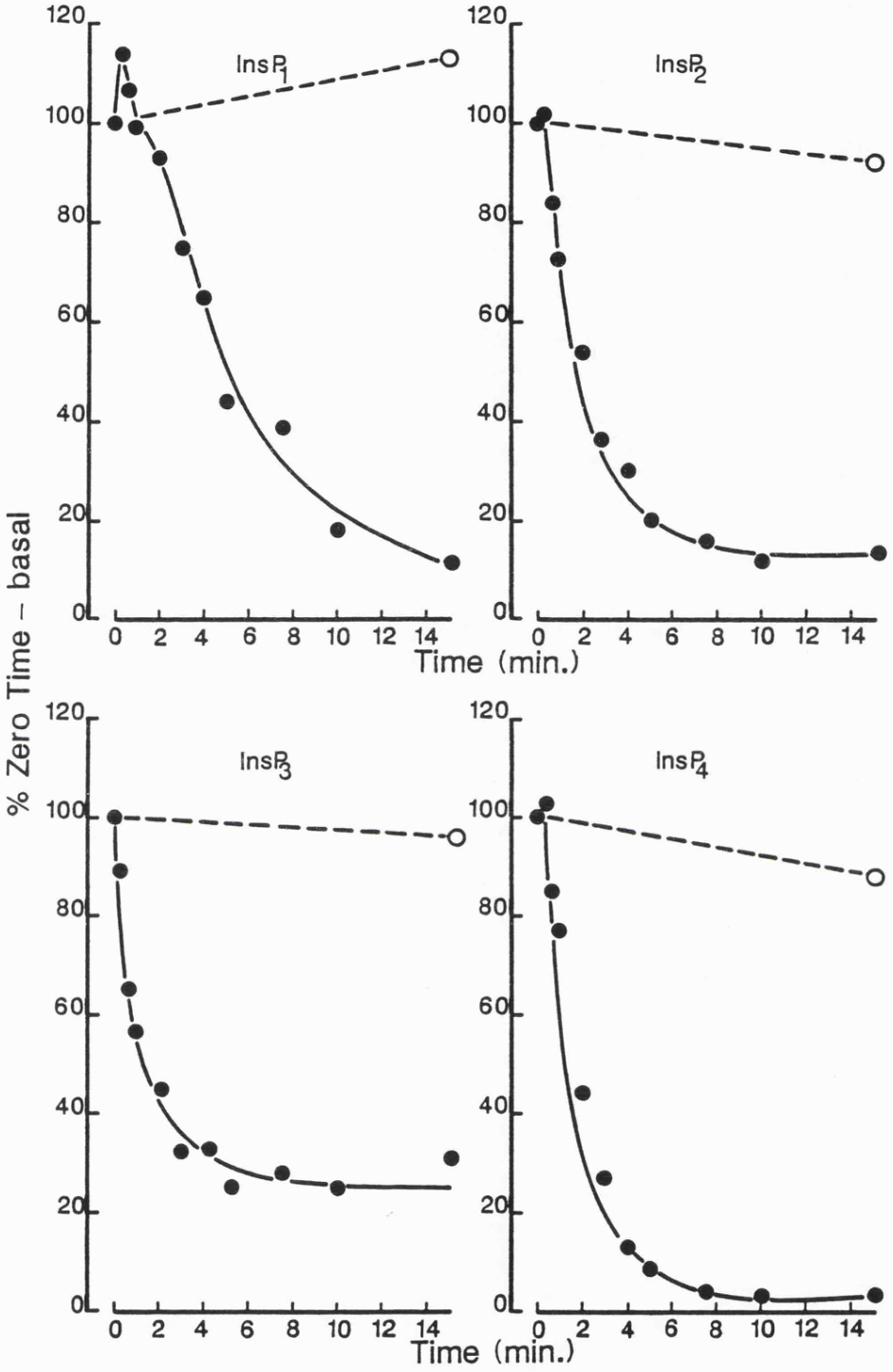


FIGURE 6.2.1 Comparison of the rates of hydrolysis of ^{3}H-inositol phosphates from carbachol-stimulated steady-state accumulations in cerebral cortical slices following muscarinic receptor blockade.

Brain slices were labelled with ^{3}H-inositol as in 4.3 and stimulated with 1 mM carbachol for 30 min. or run as control. Samples were then further incubated for the varying times shown after addition of 10^{-5} M atropine (●) or Krebs vehicle (○) at the point indicated as zero time (note that values for stimulated samples only are shown, control values are represented by the abscissa - see text). Incubations were stopped with PCA, neutral extracts prepared from pooled, triplicated tissue incubations and samples analysed for Ins P_1 through Ins P_4 as previously. Results are expressed as described in the text and represent the mean values from four experiments.

were 471 ± 98% and 211 ± 15% although relative basal values for the latter trisphosphate were typically 3-4 fold greater than for the former. Estimates of radioactivity (cpm) in the total $InsP_3$ fraction isolated by Dowex chromatography and hplc differed to an extent, owing to the lower counting efficiency for tritium obtained with the hplc eluates. Where rates are calculated below these have been determined on the basis of the 100% values reported above measured using Dowex chromatography. When allowing for separate Ins P_3 isomers, the 100% value has been broken down into proportions for Ins-1,4,5-and-1,3,4-P_3 as indicated by hplc analyses.

Figure 6.2.1 allows comparison of the effect of atropine addition on the accumulation of label under each major inositol phosphate fraction. The discontinuous lines show that in the absence of atropine, stimulated accumulations of all inositol phosphates were maintained at constant levels over the period studied, thus confirming that each had achieved a new steady state value. In contrast, the addition of antagonist resulted in a dramatically decreased accumulation of each inositol phosphate. It is evident from Figure 6.2.1 however, that the initial rates at which the separate inositol phosphate fractions declined differed considerably. The most immediate atropine-induced effect was a fall in the concentration of ^{3}H-Ins P_3 which was the only fraction affected within 20 sec. of receptor blockade, the earliest time monitored. A slight lag was apparent before either Ins P_2 or Ins P_4 accumulations began to decrease while levels of Ins P_1 changed little over the first 1-2 min. following removal of the stimulus. However, although the latter prolonged delay is likely to be a genuine effect since it is confirmed by 3-4 separate measurements at different time points, the shorter lag phase for Ins P_2 and Ins P_4 must be considered of lesser significance as it is dependent on only one time interval. Nevertheless, when the Ins P_3 fraction was resolved into its isomeric components the pattern of immediate change was consistent with that shown in Figure 6.2.1. The relative rates of Ins-1,3,4-P_3 and Ins-1,4,5-P_3 decay toward control values are compared in Figure 6.2.2. This shows that, as for total Ins P_3, there was a rapid decrease in the stimulated accumulation of Ins-1,4,5-P_3 while changes in the level of Ins-1,3,4-P_3 did not become evident until 40 sec., when concentrations of both Ins P_2 and Ins P_4 had also begun to fall. Each of these observations supports an initial receptor-mediated hydrolysis of PtdIns P_2 and the subsequent formation of other inositol phosphates either via phosphorylation or dephosphorylation of the Ins-1,4,5-P_3 produced as a consequence of this primary event. This metabolic sequence is also consistent with the relative half-lives ($T_{½}$) which can be estimated for each inositol phosphate from the data presented in Figures 6.2.1 and 6.2.2. If these are calculated as the time over which 50% of the maximal

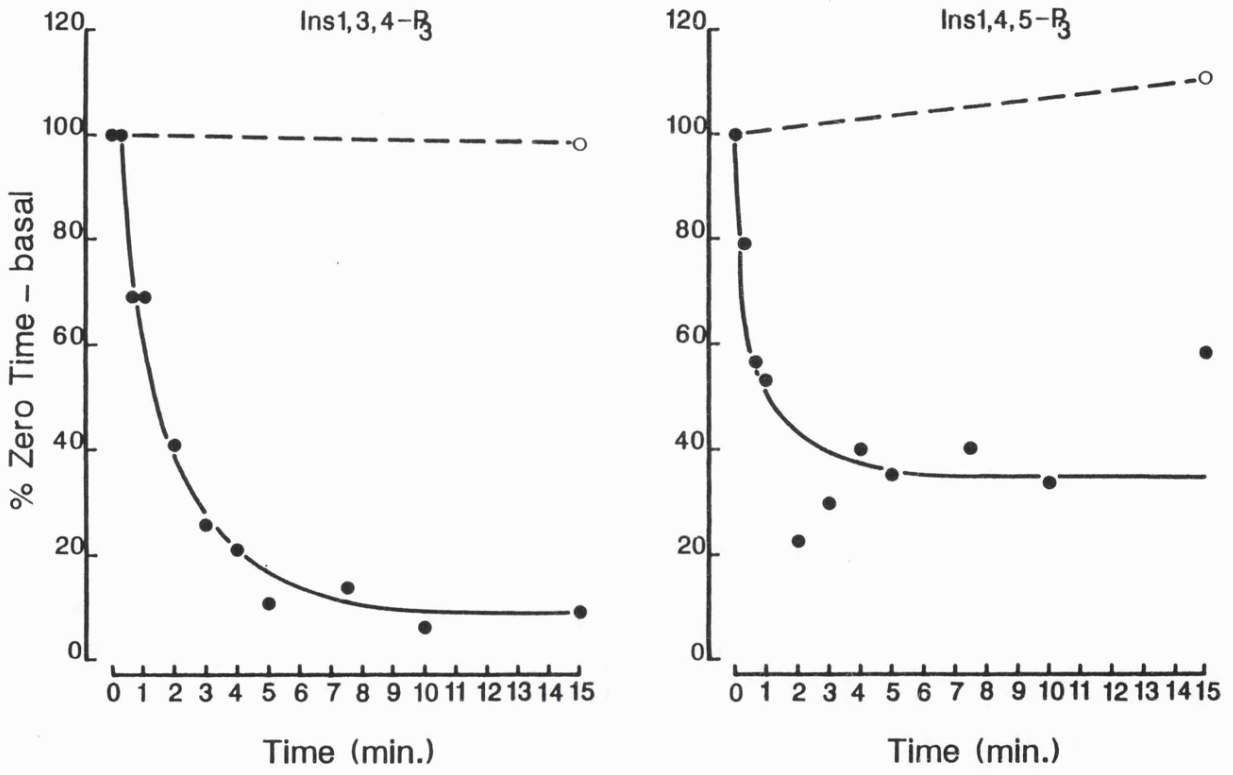


FIGURE 6.2.2 Comparison of the rates at which carbachol-stimulated accumulations of ^{3}H-Ins-1,3,4-P_3 and ^{3}H-Ins-1,4,5-P_3 decay towards control values in brain slices following muscarinic receptor blockade.

The experimental protocol was as in the legend to Fig. 6.2.1 except that aliquots of the identical samples were analysed by gradient elution hplc for inositol trisphosphates as in CH.5 (see Fig. 5.3.4). Results are expressed as detailed in the text and represent the means of 2-3 experiments.

attenuation of the response by atropine occurs, the respective T½(s) for Ins P_1, Ins P_2, Ins P_3 and Ins P_4 are 267, 93, 40 and 67 sec. and those for Ins-1,3,4-P_3 and Ins-1,4,5-P_3 73 and 27 sec. Given the error arising from inter-experimental variation and the problems associated with this experimental approach (see below), these values are in reasonable agreement with those obtained in CH.3. The T½(s) for each Ins P_1, Ins P_2 and Ins P_4 also correlate well with the relative maximal accumulations of these inositol phosphates since the ratios for each parameter are almost parallel (i.e. T½ Ins P_1:Ins P_2:Ins P_4 = 4.0:1.4:1.0; max. accumulation 3.2:1.4:1.0). The relative half-lives of total Ins P_3 or of the separate isomeric components of this fraction fit this pattern less well. However, it must be pointed out that the estimates of half-time made can only be approximate both for reasons considered below and because a certain degree of error is associated with the graphical analysis made. The latter point is emphasised by Figures 6.2.3 to 6.2.5 where the above data has been expressed on a logarithmic time scale. From these figures the T½ values for Ins P_1, Ins P_2, Ins P_3 and Ins P_4 have been determined as 320, 110, 88 and 90 sec. and those for Ins-1,3,4-P_3 and Ins-1,4,5-P_3 as 95 and 52 sec. respectively. It is clear from this that there is some discrepancy between these figures and those above which illustrates the approximate nature of any value quoted. Equally, it is apparent that the most significant error is associated with the T½ for Ins-1,4,5-P_3. The significance of this and potential explanations are considered below.

One of the essential purposes of the current experiments was to allow an estimate of the extents to which Ins-1,4,5-P_3 is metabolised directly via kinase and phosphatase activities. Heslop *et al.* (1985) have recently measured the relative stimulated accumulations of ^{3}H-Ins P_4 in blowfly salivary gland and GH_4 cells and have commented that the proportionally larger accumulation found in the former tissue or in rat cerebral cortex (Batty *et al.* 1985b) as opposed to that in GH_4 cells may simply be a consequence of the labelling approaches employed. Typically, labelling of cerebral cortex or salivary glands with ^{3}H-inositol is short term (1-4 hrs.) while that of most cell lines is allowed to continue over a period of days. In the latter situation close to isotopic equilibrium should be achieved such that changes in inositol phosphate labelling should reflect corresponding mass changes while over shorter labelling periods this is not necessarily so (see 2.3). Thus, in cerebral cortex or salivary gland the relatively larger increases in ^{3}H-Ins P_4 could, in part, be accounted for by changes in specific activity. If this were so the current data could not be interpreted in terms of mass flux through distinct metabolic pools. It is therefore of crucial significance that the results presented in Figures 6.2.1 to 6.2.5 clearly indicate that the

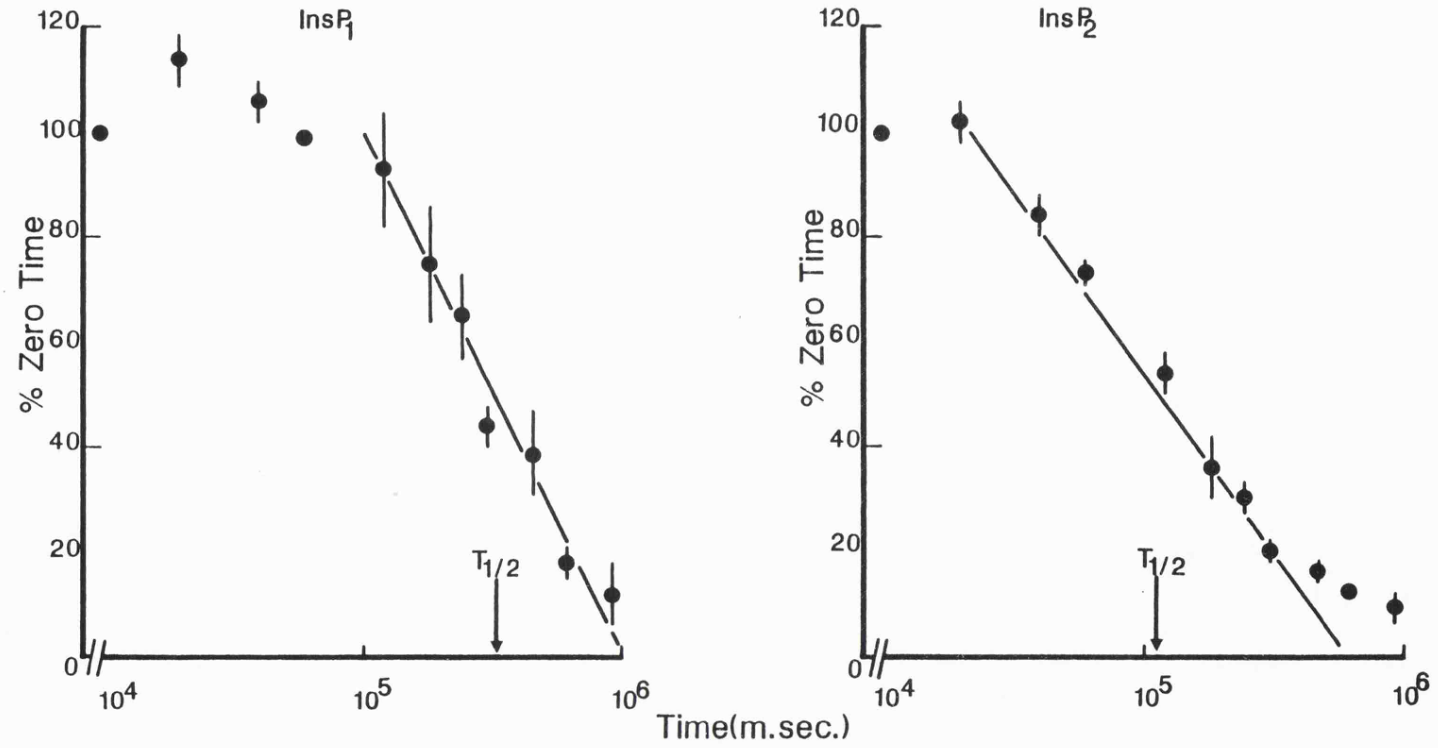


FIGURE 6.2.3 Comparison of the rates of hydrolysis of ^{3}H-Ins P_1 and ^{3}H-Ins P_2 from carbachol-stimulated steady-state accumulated levels in brain slices following muscarinic receptor blockade.

The relevant data from Fig. 6.2.1 is plotted using a logarithmic time scale. Results represent the mean ± SEM of four experiments and are expressed as described in the text. NB. Only data for the atropine-induced reversal of carbachol-stimulated inositol phosphate accumulations are shown.

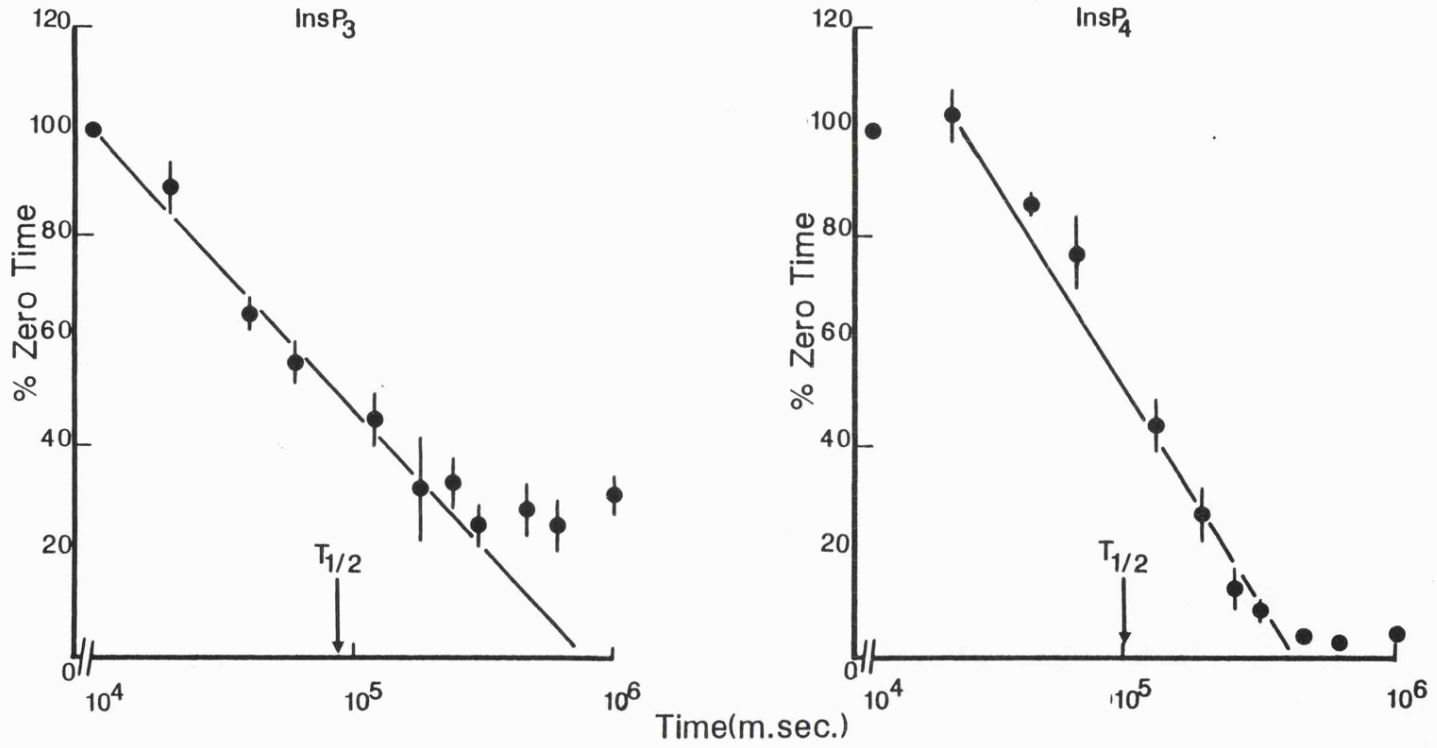


FIGURE 6.2.4 Comparison of the rates of hydrolysis of ^{3}H-Ins P_3 and ^{3}H-Ins P_4 from carbachol-stimulated steady-state concentrations in brain slices following muscarinic receptor blockade.

The relevant data from Fig. 6.2.1 has been re-plotted on a logarithmic time scale. Results represent the mean ± SEM of four experiments. NB. Only data applying to the atropine-induced reversal of the response to agonist are shown.

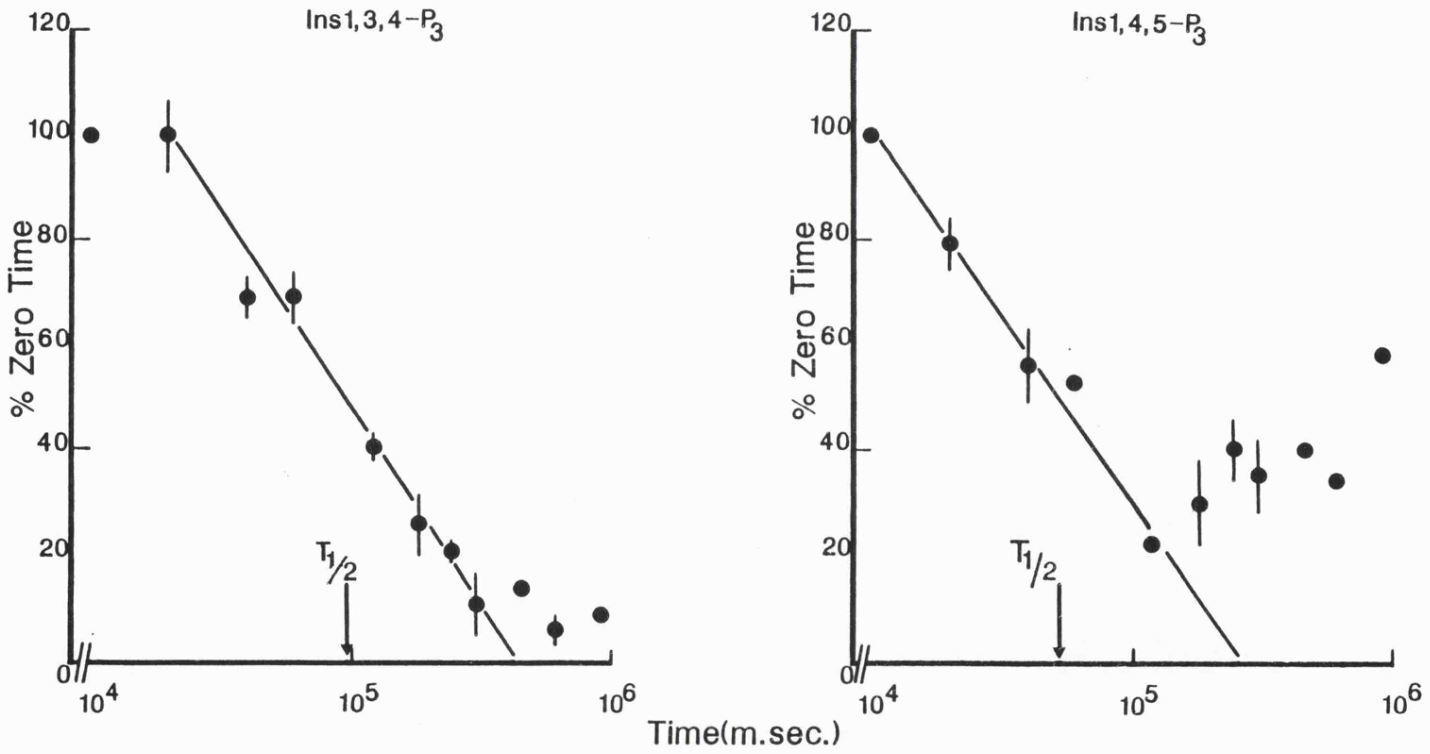


FIGURE 6.2.5 Comparative rates of hydrolysis of ^{3}H-Ins-1,3,4-P_3 and ^{3}H-Ins-1,4,5-P_3 from carbachol-stimulated steady-state concentrations in brain slices following muscarinic receptor blockade.

The data from Fig. 6.2.2 has been re-plotted on a logarithmic time scale. Results represent the mean ± SEM of three experiments (or 2 experiments where SEM not indicated). NB. Only results applying to the atropine-induced reversal of the carbachol response are shown.

increased radiolabelling of the Ins P_4 fraction promoted by carbachol is very efficiently reversed (~97%) by atropine suggesting that any change in specific activity must be minimal (see 3.2). The same experimental approach has allowed Hawkins et al. (1986) to draw an identical conclusion with respect to muscarinic receptor-mediated ^{3}H-Ins P_4 accumulation in ^{3}H-inositol labelled parotid gland. The present data also indicates an 85-90% reversal by atropine of the stimulated labelling of each the Ins P_1, Ins P_2 and Ins-1,3,4-P_3 fractions, suggesting that the effects of carbachol on these, as for Ins P_4, essentially reflect increases in concentration and not specific activity. However, both Figures 6.2.2 and 6.2.5 show that although the stimulated accumulation of Ins-1,4,5-P_3 is reduced by 60-70% within 2 min. of atropine addition, this is not further attenuated even 15 min. subsequent to receptor blockade. The significance of this observation is unclear but is unlikely to represent an increased specific activity of ^{3}H-Ins-1,4,5-P_3 as this would have to be between 3-10 fold greater than is apparent for any metabolite derived from this precursor. Equally, as the effect is restricted to Ins-1,4,5-P_3 and for reasons previously stated (see 3.2), a stimulatory action of carbachol on nicotinic receptors is improbable. One potential explanation is that the Ins-1,4,5-P_3 fraction contains more than one component of which a proportion is more slowly metabolised. However, the only source of a further isomeric Ins P_3 would be either phosphorylation of an Ins P_2, for which there is no evidence in brain, or via hydrolysis of Ins-1,3,4,5-P_4 other than by 5-phosphatase attack. Given the apparently exclusive breakdown of Ins P_4 to Ins-1,3,4-P_3 in parotid gland (Hawkins et al. 1986) and liver (Hansen et al. 1986) homogenates and the exact co-elution of sample ^{3}H-material with an internal ^{32}P Ins-1,4,5-P_3 standard on the current hplc analysis, this seems unlikely unless the Ins-1,4,5-P_3 3-kinase activity in brain is reversible. However, a more probable explanation is that owing to the relatively small accumulation of Ins-1,4,5-P_3 above basal values compared to that for other inositol phosphates, this particular response is especially difficult to quantify. For this reason any error associated with the measurements of inositol phosphates is that much more significant for Ins-1,4,5-P_3 and probably accounts for the failure of the decay curve for this molecule to return closer to basal, although the above possibilities cannot be eliminated. Despite this, the clearly evident and highly efficient atropine-induced reversal of the stimulated accumulations of at least four of the five inositol phosphate fractions measured, show that it is reasonable to conclude that the changes in radiolabelling determined reflect almost equivalent changes in concentration and, from the presumed metabolic pathways involved, that this observation can be extended to include

Ins-1,4,5-P_3. In turn this suggests that estimates of the flux through certain of the inositol phosphate fractions should be accurate within the limits of error associated with the half-lives and maximal accumulations reported above.

The decline in accumulation of each inositol phosphate was measured from an initial steady state. As, by definition, under these conditions the rates of formation and breakdown for any given product are equal, the flux through each Ins P_1 and Ins P_2 must be equivalent to or greater than that through Ins-1,4,5-P_3 depending on whether receptor-mediated inositol lipid hydrolysis is restricted to PtdIns P_2 or also includes PtdIns and/or PtdIns P. In contrast, assuming its sole production via 3-kinase activity (see CH.4), turnover of the Ins P_4 fraction must be equal to that through Ins-1,4,5-P_3 or less than this by an amount dependent on the extent to which the latter undergoes direct dephosphorylation. Further, assuming hydrolysis exclusively by 5-phosphatase activity, flux through Ins P_4 should be equal to that through Ins-1,3,4-P_3. The turnover of each fraction should be estimable from the corresponding maximal accumulation and $T\frac{1}{2}$ (but see below). Calculations have been made as in 3.2 using the 100% figures quoted above and the half-lives from Figure 6.2.1 and 6.2.2. On this basis flux through Ins P_1, Ins P_2, Ins-1,3,4-P_3, Ins-1,4,5-P_3 and Ins P_4 is estimated at 2,212, 2,588, 448, 947, and 2,930 cpm/min/50 µl tissue. Figure 6.2.6 represents these fluxes schematically. Clearly these values disagree with the above predictions but it is likely that these discrepancies can be accounted for by a number of (a) flaws inherent to the experimental rationale and (b) technical difficulties associated with the protocol used, both of which are likely to invalidate some of the above estimates more than others.

a. <u>Problems associated with the experimental design:</u>

These arise because in making the above calculations of flux three assumptions are made.

i) That on addition of antagonist the response to agonist is instantly terminated (see (iii) and b(i)).

ii) That on effective removal of the stimulus, the changing concentrations of each inositol phosphate are not either directly or indirectly dependent on one another. The validity of this assumption is uncertain since the kinetics of none of the enzymes involved in brain have been thoroughly investigated, nor have factors affecting these. Therefore, it is possible that some steps are subject to regulation either by inositol phosphates themselves or by other mediators whose concentrations are altered as a consequence of inositol lipid and/or

phosphate metabolism. In the latter respect changes in intracellular calcium and diacylglycerol concentrations could be important, particularly the former which appears to modulate activity of the Ins-1,4,5-P_3 3-kinase in RINm5F cells (Biden and Wollheim, 1986). Any such effects in brain are an unknown quantity at present and cannot be taken into account.

iii) This is related to (i) and is the assumption that immediately the antagonist occupies the receptor no further inositol phosphate is generated. In theory this is only strictly valid for the reaction most closely coupled to the receptor because those inositol phosphates which arise through more distal reactions will initially continue to be produced, albeit at decreasing rates, from the existing pools of their immediate precursors. Thus, hydrolytic rates are not measured entirely independent of concomitant synthesis for products other than the first in the metabolic sequence. The currently measured $T_{½}$ values for other products will not therefore be the true half times for hydrolysis from the steady state accumulated concentrations but these values plus additional undefined times dependent on the sum total of accumulated precursor(s) and the net rate for breakdown of these. This will lead to the greatest relative inaccuracy of an estimated $T_{½}$ when the pool(s) of precursor(s) is considerably larger than the steady state level of its breakdown product whose half-life is being assessed (i.e. as for Ins-1,3,4-P_3). Any inaccuracy in the measured $T_{½}$ is obviously translated to the estimate of flux. The significance of this to the above calculated fluxes can be gauged approximately.

In the present instance, assuming the route of inositol lipid/ phosphate metabolism outlined in 4.4(b)iii (see Figure 6.2.6), the only product whose hydrolysis could be expected to be measured independently of any continued synthesis is that for Ins-1,4,5-P_3 (but see (b)). However, as the maximal accumulation of Ins-1,4,5-P_3 is 4-5 fold lower than that of Ins P_4 and as its rate of metabolism is rapid, 3-kinase activity is likely to continue at its original rate for only a very limited period so that the estimate of $T_{½}$ for Ins P_4 should not be substantially invalidated by (iii) above. In contrast, the relative steady state concentrations of Ins-1,3,4-P_3 and Ins P_4 are such that the former may continue to be produced at a gradually declining rate for a considerably longer period, so that its <u>apparent</u> rate of hydrolysis could be an underestimate by several (5-6) fold. Similar arguments apply to Ins P_2 and Ins P_1 which are unlikely to be replenished from existing Ins-1,4,5-P_3 for an extended time after atropine addition but may be so, in the case of the former, from the pre-formed Ins P_4 pool and, in the latter case, both from this and the Ins P_2 pool. However, as both the Ins P_1 and Ins P_2 maximal

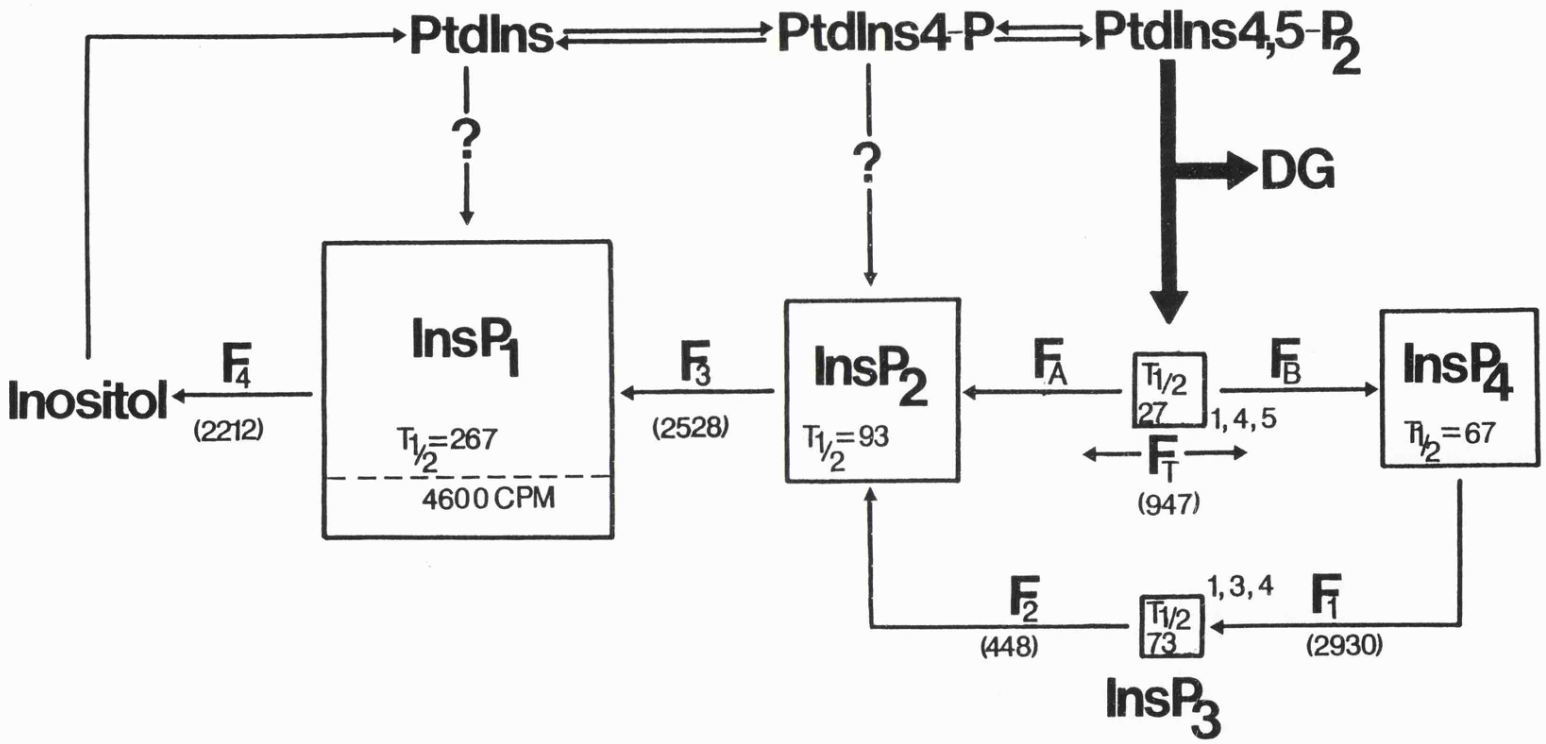


FIGURE 6.2.6 Schematic representation of the fluxes calculated through the separate ^{3}H-inositol phosphate fractions at steady-state in 1 mM carbachol-stimulated brain slices.

The data presented in Figs. 6.2.1-6.2.5 are represented diagrammatically. The box sizes for each inositol phosphate indicate the proportional maximal accumulation of radiolabel in the appropriate fraction (expressed as cpm of inositol phosphate/50 µl cerebral cortical slices - the area partitioned by the broken line for ^{3}H-Ins P_1 gives the scale). $T_{\frac{1}{2}}$ values represent the half-lives (in seconds) of each inositol phosphate, calculated from Figs. 6.2.1 and 6.2.2. F represents the flux through an indicated reaction and the corresponding values in brackets show the turnover in terms of cpm of inositol phosphate metabolised/50 µl tissue/min. For Ins-1,4,5-P_3, F_T is the total flux (F_A + F_B) through this fraction. Precursor phospholipid pool sizes are not indicated for previously stated reasons (see text and 3.5).

accumulations are large relative to those of their precursors, their apparent hydrolytic rates are unlikely to be greater than 1-2 fold underestimates. This could quite easily account for the small disparity between the estimated flux through Ins P_4 as compared with that through Ins P_1 or Ins P_2. Similarly, the greater discrepancy between the apparent fluxes through Ins P_4 and Ins-1,3,4-P_3 could be explained in this way. However, a corollary of the latter conclusion is that if flux through Ins P_4 and Ins-1,3,4-P_3 is equal, then the half-time for the latter to decline from its steady state concentration must be 6-7 fold less than currently determined which suggests a $T_{½}$ of approximately 11 seconds. Both the original estimate and this revised value suggest a considerably more rapid metabolism of Ins-1,3,4-P_3 in brain than was previously suggested in carbachol-stimulated rat parotid gland (Irvine et al. 1985). As the accumulation of Ins P_4 is small relative to that of Ins-1,3,4-P_3 in parotid gland (see 4.4(a)i; Hawkins et al. 1986) this difference between the two tissues is not likely to result from an over-estimate of half-time for the trisphosphate in parotid similar to that originally made here, but rather suggests separate enzymes or regulatory mechanisms operate in the distinct systems. However, it remains to be established whether hydrolysis of Ins-1,3,4,5-P_4 occurs exclusively via removal of the 5-phosphate in brain as seems to occur in other tissues (Hawkins et al. 1986; Hansen et al. 1986) and if this is not so then the above conclusions do not necessarily hold.

A second point that emerges from this re-evaluation of the fluxes determined above is that those through Ins P_1 and Ins P_2 are likely to be underestimates by about 1-2 fold at most. This does, however, assume that muscarinic receptor-mediated hydrolysis of phosphoinositides in brain is restricted to PtdIns P_2. This latter is difficult to verify from the current data since neither the potential isomeric Ins P_1(s) or Ins P_2(s) arising via all possible pathways of inositol lipid/phosphate metabolism could be measured separately. Despite this, as only a small proportion of each total inositol lipid fraction appears to be directly associated with the muscarinic receptor in brain (see CH.2), the prelabelled pools of PtdIns and PtdIns P present at the time of atropine addition will only correspond to a relatively small percentage of the total label in the Ins P_1 and Ins P_2 pools respectively and as such will not represent substantial amounts of potential precursors which could otherwise invalidate the estimates of flux by the mechanism described in (iii) above. The closest estimates of turnover through Ins P_1 and Ins P_2 which can be made therefore are 2,212-4,424 and 2,588-5,176 cpm/min/50 µl tissue under steady state conditions. As the flux through Ins P_4 is about 2,930 cpm/min/50 µl tissue this suggests that a minimum of 66% and 57% of

that through Ins P_1 and Ins P_2 can be accounted for by hydrolysis of Ins P_4 through Ins-1,3,4-P_3. As a considerable portion of Ins P_2 probably also arises through direct dephosphorylation of Ins-1,4,5-P_3, this suggests that a very substantial fraction of accumulated Ins P_1 and Ins P_2 must arise from higher inositol phosphates rather than directly from PtdIns and PtdIns P. A more precise definition of these proportions must await the availability of specific phosphatase inhibitors.

b. Technical limitations of the assay procedure:

The most surprising of the above calculated rates for turnover of inositol phosphates was that for Ins-1,4,5-P_3 which suggested a flux of only 947 cpm/min/50 µl tissue. Although the disparity between this and the corresponding figures for Ins P_1 and Ins P_2 could be accounted for by invoking a direct hydrolysis of PtdIns and/or PtdIns P (but see above) this would not explain the 3-fold greater flux through Ins P_4. Since no inositol phosphates more polar than Ins P_4 can be easily detected in brain and because the available evidence suggests that this molecule is very unlikely to arise through cleavage of a PtdIns P_3 (see 4.3(b) and 4.4(b)), it is reasonable to assume its production solely through Ins-1,4,5-P_3 kinase. To allow for this, either the flux through Ins P_4 must be an over-estimate or that through Ins-1,4,5-P_3 an underestimate, assuming the kinase reaction is irreversible. For a variety of reasons the error is most likely to be associated with the $T_{½}$ for Ins-1,4,5-P_3.

i) All the above estimates of half-life assume blockade of muscarinic receptors is instantaneous on atropine addition. This may not be strictly accurate as under the current protocol drugs are introduced in a low volume (10 µl) so that a finite time must elapse before equal diffusion throughout the medium (300 µl) and tissue slices is achieved. Within the time scale of the present experiments where earliest sampling is 20 sec. after atropine addition, this very limited delay is unlikely to be significant. However, if the true $T_{½}$ for Ins-1,4,5-P_3 is considerably less than estimated then diffusional difficulties could assume more importance in experiments aimed at improving the current time resolution. Although even in the latter situation this problem would not be expected to be limiting, it would affect the estimate of $T_{½}$ for Ins-1,4,5-P_3 most, both because it assumes greatest significance for the compound most rapidly metabolised and because for inositol phosphates produced later in the metabolic sequence the factors discussed in (a) are proportionally much more important.

ii) As the metabolism of Ins-1,4,5-P_3 is much more rapid than that of other inositol phosphates (from the apparent $T_{½}$ values above) any

technical limitation of the assay affects an estimate of its half-life by the greatest relative extent. Two particular factors apply here. Firstly, the very limited maximal accumulation of Ins-1,4,5-P_3 in brain which is secondly aggravated by the very low counting efficiency for tritium achieved with hplc eluates. Although this applies equally to both trisphosphates, the basal values for these are such that the percentage increase in Ins-1,4,5-P_3 is only about half that for Ins-1,3,4-P_3 so that changes in the concentration of the former are measured only over a very narrow range and any associated error becomes more significant. This applies particularly when decreases in maximal accumulation rather than vice versa must be accurately measured (see iv).

iii) In contrast to (ii) both the maximal accumulations (6-7,000 cpm/50 µl tissue) and the percentage stimulation over basal values (~3,000%) for Ins P_4 are large and consequently much easier to quantify, especially as the hydrolytic rate is also slower. This applies equally to Ins P_1 and Ins P_2 and, not disregarding the discussion in (a), suggests that at least the apparent hydrolytic rates for these can be estimated that much more accurately.

iv) A final point which applies to all the inositol phosphates but from (ii) is most relevant to Ins-1,4,5-P_3, is that the design of the assay is necessarily such that measurements of decreases from steady state concentrations are made. Although this allows easy quantifications of maximal accumulations, the small changes occurring at early times after antagonist addition are often difficult to measure against the background of any inter or intra experimental variation. For this reason and to eliminate any problems arising from (i), the first time monitored was 20 sec. after atropine addition while subsequent time intervals were large compared even to the estimated $T_{½}$ for Ins-1,4,5-P_3. As a result Figure 6.2.2 shows that the critical phase of the decay curve for Ins-1,4,5-P_3 is only marked by 3-4 separate points compared to up to 6-7 points for all other inositol phosphates, again emphasising that the latter are likely to be more reliable.

In addition to these factors it must be recalled that all the estimates of flux are derived using $T_{½}$ as a measure of inositol phosphate metabolism while, as noted in 3.2, this will be an underestimate of the initial rate at which compounds will be hydrolysed under steady state conditions. Owing to (i)-(iv) above this again applies most significantly to Ins-1,4,5-P_3. Despite all these problems however, the total flux which can be estimated from the current experiments for a fraction corresponding to that previously defined as 'Ins P_3' does show reasonable agreement with that determined in 3.2 (cf 4,325 cpm/min/50 µl slices from above and

2,800 cpm/min/50 µl tissue from 3.2) suggesting that within the inherent limitations these studies are consistent.

It is clear that with the exception of a(ii), all the various factors discussed in (a) and (b) above combine to indicate that of the fluxes estimated, that through Ins P_4 is probably the most reliable. As the turnover of the Ins P_4 fraction is 3-fold that apparent for Ins-1,4,5-P_3 this suggests the T½ estimated for the latter will be an underestimate by at least this extent. However, as a proportion of Ins-1,4,5-P_3 must also be catabolised by direct dephosphorylation to account for the rapid production of Ins P_2 without concomitant formation of Ins-1,3,4-P_3 (4.3(a) and 4.4(b)ii), the half-time of 27 sec. for Ins-1,4,5-P_3 must be an over-estimate by a factor of greater than 3-fold, perhaps indicating a value of 5-10 sec. or less. In the absence of a better measure of the flux through Ins-1,4,5-P_3 it is impossible to determine the relative extents to which this is metabolised by the kinase and phosphatase pathways. Nevertheless, the current data strongly indicates a considerable flux through Ins P_4 during stimulation and from the rates calculated even if the half-life for Ins-1,4,5-P_3 is as short as 5 sec. between 60-70% of this product is likely to undergo initial phosphorylation, while if its T½ is greater than 5 sec. this value could be substantially larger. Such a conclusion is consistent with the relative affinities of Ins-1,4,5-P_3 3-kinase (Irvine et al. 1986) and 5-phosphatase (Erneaux et al. 1986) for this substrate in brain. However, it is of interest to note that Storey et al. (1984) have estimated the half-life of Ins-1,4,5-P_3 in intact hepatocytes at about 4 sec., assuming catabolism exclusively via the phosphatase. A similarly active enzyme in brain, together with the capacity of the 3-kinase, might indicate an even faster rate of Ins-1,4,5-P_3 turnover than predicted above and although this would suggest a lesser proportion of the total metabolism through Ins P_4, it would also underline the extent to which production of lower inositol phosphates can be accounted for solely by receptor-mediated PtdIns P_2 hydrolysis.

In future a more accurate definition of the relative significance of kinase and phosphatase activities to Ins-1,4,5-P_3 metabolism may be achieved by establishing the proportions of Ins P_2 arising directly from the separate Ins P_3 isomers. At present the identities of any isomeric Ins P_2(s) produced by these different routes are unclear (see 4.4(b)iii) but if the respective products are mutually exclusive to their precursors it should be possible to determine the extent to which Ins-1,4,5-P_3 is directly dephosphorylated using a procedure similar to that employed here. However, as the expected product of the latter reaction (Ins-1,4-P_2) is identical to that which could arise from diesteratic cleavage of PtdIns P, it might still be difficult to unequivocally identify PtdIns P_2 as the

primary and only substrate for receptor-mediated phospholipase C activity. Precise definition of the sequences of inositol phospholipid and phosphate metabolism will require either use of specific inhibitors or alternative experimental approaches. One latter such possibility is the use of crude or purified enzyme preparations to determine the routes and rates of inositol phosphate metabolism. Where breakdown of Ins-1,3,4,5-P_3 has been studied in crude homogenates from either liver (Hansen et al. 1986) or parotid gland (Hawkins et al. 1986) this seems to occur with similar rapidity to that currently observed, although a direct comparison is not possible, and in both tissues results in formation of an Ins P_2 distinct from Ins-1,4-P_2. Although the pattern of metabolism observed in such experiments may not be representative of that produced by an agonist since it may be extremely difficult to accurately reproduce the intracellular conditions prevailing in a stimulated cell, such studies should allow many of the factors regulating inositol phosphate hydrolysis to be recognised. In turn this should permit a more reliable analysis of data derived from the current type of study.

Summary

In conclusion, although errors inherent to the experimental design preclude definitive determination of the relative extents to which Ins-1,4,5-P_3 is metabolised by kinase and phosphatase activities, the present results suggest a best estimate in excess of 50% is likely to be accounted for by the former pathway. Regardless of its proportional significance, it is therefore clear that flux through Ins P_4 during muscarinic receptor stimulation in cerebral cortex is considerable. Hawkins et al. (1986) have reported a similar conclusion with respect to the same system in rat parotid gland. In addition while it is difficult to exclude the possibility that hydrolysis of PtdIns and/or of PtdIns P occurs as either a direct or indirect consequence of muscarinic receptor activation, the present data suggests that a high percentage, if not all, of the total Ins P_1 and Ins P_2 accumulating in brain can be accounted for by dephosphorylation of higher inositol phosphates. This implies that receptor-mediated inositol lipid hydrolysis in this tissue is substantially restricted to PtdIns P_2. The half-lives determined for separate inositol phosphates are obviously not precise but, from corrections made on the bases discussed above, indicate that hydrolysis of Ins-1,4,5-P_3 in particular is extremely rapid ($T_{½} \sim$ 5-10 sec.) as would be anticipated from its proposed second messenger role (Berridge and Irvine, 1984). In view of previous studies (Irvine et al. 1985) the rate at which Ins-1,3,4-P_3 can be estimated to decline from its accumulated level in brain is perhaps more surprising, particularly as this is more rapid than

that for Ins P_4 which may also have a second messenger function (Hansen et al. 1986). This is not to imply that on an equimolar basis breakdown of Ins-1,3,4-P_3 is faster than for Ins P_4 since the revised half-times for these fractions are relative to their grossly different maximal accumulations and represent estimates of the times taken for a 50% decrease from their respective steady state concentrations. The large accumulation of Ins P_4 suggests its initial rate of synthesis exceeds that for its hydrolysis by several fold, but as a steady state is reached the latter rate must increase at later times. To allow for this, the breakdown of Ins-1,3,4-P_3 must similarly accelerate but this appears to occur over a very much smaller concentration range than observed for Ins P_4. This may suggest that metabolism of Ins-1,3,4-P_3 is subject to some form of control. Certainly comparison of both the relative accumulations and apparent rates of Ins-1,3,4-P_3 hydrolysis in carbachol-stimulated rat cerebral cortex and parotid gland indicate marked differences between the systems associated with muscarinic receptors in the two tissues which also exhibit reciprocal steady state accumulations and appropriately different half-lives for Ins P_4. It may be of future interest to determine whether these differences have functional significance. The half-life determined for Ins P_4 in cerebral cortex (60-70 sec.) is approximately 4-fold greater than that estimated in rat parotid gland (Hawkins et al. 1986). At present the relevance of this observation in relation to any potential second messenger role of Ins P_4 is unclear although the considerable flux through this fraction in both tissues is consistent with some function, particularly if catabolism of Ins-1,4,5-P_3 through 5-phosphatase activity is as efficient as implied by Storey et al. (1984).

However, it should be emphasised that all the above discussion relates to metabolism of inositol phosphates under conditions where continued presence of agonist allows a steady state to be achieved. This is not necessarily representative of the physiological situation where transient release of endogenous acetylcholine, itself rapidly metabolised, may result in very short term exposure of muscarinic receptors to agonist. Given this and the other associated limitations, the current data must be regarded critically. Nevertheless, the present results do provide a useful basis for future study and give valuable confirmation of the validity of the labelling protocol used since they indicate changes in radioactivity can be essentially interpreted in terms of concentration. In addition to this, the complete reversal by atropine of carbachol-stimulated Ins P_4 accumulation demonstrates that this response is mediated by muscarinic receptors in brain as originally suggested (4.2) but not previously confirmed. The dose-relationship between agonist concentration and inositol phosphate accumulation is further investigated in 6.3.

6.3 The concentration dependency of carbachol stimulated inositol polyphosphate accumulation in cerebral cortex

The proportional significance of 3-kinase and 5-phosphatase activities to metabolism of Ins-1,4,5-P_3 was considered in the previous section where it was suggested that substantial flux occurs through both these pathways under the steady state conditions resulting from stimulation of cerebral cortical slices with a maximally-effective concentration of carbachol. However, there is no guarantee that these conclusions hold at lower agonist concentrations which may be more representative of those to which receptors are exposed *in vivo*. In the above experiments and when inositol polyphosphate accumulations have been studied in earlier chapters, the stimulus was 1 mM carbachol. Although this muscarinic receptor agonist has been shown to elicit maximal phosphoinositide hydrolysis at 1 mM when the measure of this response is Ins P_1 accumulation in the presence of lithium (see CH.2; Brown *et al.* 1984; Jacobson *et al.* 1985; Rooney and Nahorski, 1986), an identical dose-relationship has not been demonstrated in brain by measuring the accumulation of inositol polyphosphates. The stimulated accumulation of Ins P_1 in the presence of lithium has previously been assumed to faithfully reflect receptor activation (see refs. above) but several recent developments in the study of inositol lipid hydrolysis suggest that this might not necessarily be so. For example:

a. If, as a consequence of an Ins-1,4,5-P_3 mediated rise in $[Ca^{2+}]_i$, hydrolysis of phosphoinositides other than PtdIns P_2 accounts for a substantial proportion of the accumulated Ins P_1 and/or Ins P_2 (see Majerus *et al.* 1985), a corresponding fraction of the total response might be expected to exhibit greater receptor reserve (i.e. agonist concentration-response curves would lie to the left).

b. If breakdown of some of the Ins P_1 produced is insensitive to lithium (see Michell, 1986) this could also affect dose-response data (see (d) also).

c. In brain lithium has complex effects on Ins P_3 metabolism (Batty and Nahorski, 1985) in addition to inhibiting hydrolysis of Ins P_1 and Ins P_2. The significance of these effects may vary either with agonist and lithium concentration and/or with the length of tissue exposure to both agents. Potentially this could result in distorted dose-response curves to agonists.

d. The initial product of receptor-mediated inositol lipid hydrolysis is Ins-1,4,5-P_3 (Irvine *et al.* 1986) which can be metabolised by two routes. If, at different agonist concentrations, the relative proportional flux through these alters, anomalous dose-response data might again be

expected, particularly if these distinct pathways ultimately yield different proportions of isomeric Ins P_1(s) (see (b)).

e. The kinetic properties of the enzymes involved in inositol phosphate metabolism might be expected to result in an agonist displaying higher potency in promoting accumulation of one product than in stimulating that of another (see discussion below).

Despite these potential complications, Bone et al. (1984) have shown that in superior cervical ganglia the dose-relationships between oxytocin concentration and accumulation of each Ins P_1, Ins P_2 and Ins P_3 are essentially identical in the presence of lithium, although under the same conditions the potency of vasopressin in stimulating accumulation of these products appears marginally higher on Ins P_1 > Ins P_2 > Ins P_3. In brain, although dose-related stimulated accumulations of Ins P_1 are demonstable in response to carbachol, NA, 5-HT and HA in the presence of lithium, when maximally effective concentrations of the same agonists are employed in the absence of this ion marked responses are only evident for muscarinic receptor stimulation (see 3.3). From this it might be expected that even this latter response would be difficult to quantify at sub-maximal agonist concentrations, particularly when polyphosphate accumulations are the measure of receptor activation. To test this possibility and investigate the significance of some of the above complicating factors in brain, the dose-relationships between carbachol concentration and accumulation of each inositol phosphate were examined both in the presence and absence of lithium.

The results of this study are illustrated in Figure 6.3.1 and the experimental protocol used described in the accompanying legend. It should be noted that stimulation with agonist was limited to 10 min. in order to avoid potential complications arising from the inclusion of 5 mM LiCl in some assays (see 3.4 and 6.4). To minimise inter-experimental variation the results of individual experiments for separate inositol phosphates were first expressed as relative percentages of the response occurring at 10^{-2} M carbachol in the presence of lithium and the means of three separate measurements then calculated on this basis. Results for Ins P_1 accumulations are not shown as high background radioactivity, presumably indicating unusually high contamination of the free ^{3}H-inositol used (see 2.4), severely restricted assay sensitivity in these experiments specifically for this fraction. The 100% values for Ins P_2, Ins P_3 and Ins P_4 accumulations respectively represent 18,743 ± 5,281, 2,170 ± 557 and 3,508 ± 927 cpm/50 µl tissue. Within the limits of the associated error, maximal and half-maximal stimulated accumulations of each inositol phosphate, both in the presence and absence of lithium, occurred at 10^{-3} M

and 10^{-4} M carbachol respectively. In the presence and absence of lithium the maximal percentage stimulations of Ins P_2, Ins P_3 and Ins P_4 accumulations over basal were 1,250% and 775%, 313% and 357% and 5,800% and 2,500%. The substantial discrepancy between the fold stimulations for Ins P_4 arises from a slightly higher basal value in the presence of lithium but as control ^{3}H-Ins P_4 levels are typically very low this cannot be considered as highly significant, particularly as lithium did not affect basal Ins P_4 levels over four further experiments (see 6.4). In contrast, the increased basal Ins P_2 and Ins P_3 accumulations in the presence of lithium were found to be very reproducible phenomena and are reflected in the potentiated stimulated responses. Maximal stimulated Ins P_2, Ins P_3 and Ins P_4 accumulations in the presence of lithium were 323%, 129% and 114% respectively of those in the absence. The limited potentiation of Ins P_3 accumulation by lithium is consistent with data presented in 6.4 where this and other effects of lithium are considered in more detail.

The most important point illustrated by Figure 6.3.1 is that carbachol-stimulated accumulations of each Ins P_2, Ins P_3 and Ins P_4 show very similar, if not identical concentration dependency. Although 5 mM lithium markedly potentiated the accumulation of Ins P_2 and to a lesser extent of Ins P_3 this did not significantly influence the position of the dose-response curve to agonist. It should be noted however, that at this concentration of lithium and over this period of exposure of tissue to both lithium and carbachol, the attenuating actions of this ion, previously noted on total Ins P_3 + Ins P_4 accumulation (see 3.4), would not have developed. Further data presented in 6.4 indicate that prolonged incubations in the presence of 5 mM LiCl and 1 mM carbachol result in remarkably different patterns of inositol tris- and tetrakisphosphate accumulations from those seen more immediately following receptor activation. The dose-response data for carbachol-stimulated Ins P_1 accumulation shown in 2.3 were generated under conditions where these latter actions of lithium are maximal. Despite this, comparison of this data with that currently reported shows a close correlation between the dose-relationship for carbachol stimulation of Ins P_1 accumulation and that for each Ins P_2, Ins P_3 and Ins P_4 accumulation. Both sets of data show maximal responses to carbachol at 1 mM while the former indicates an EC_{50} for this agonist of 60-70 μM and the latter a corresponding value of 100 μM. This slight variation in potency is unlikely to represent a significant difference between the efficacy of carbachol in promoting inositol mono- and poly-phosphate accumulations as both measurements are subject to some error and both fall within the range of values determined

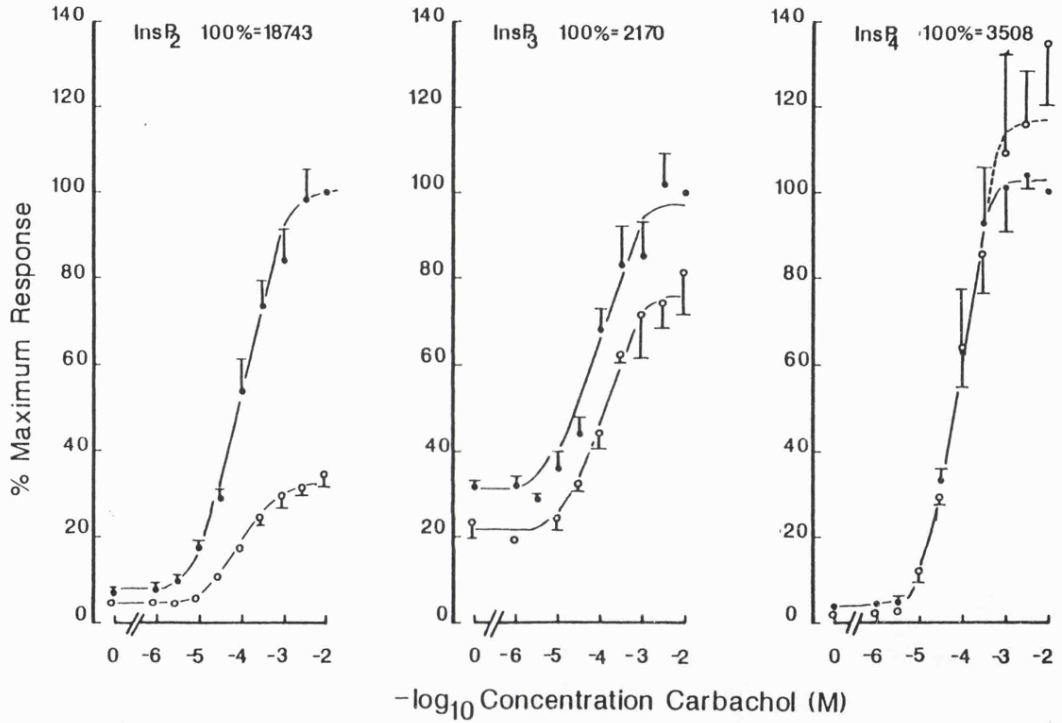


FIGURE 6.3.1 Dose-relationship for carbachol-stimulated accumulation of ^{3}H-inositol polyphosphates in cerebral cortex: the influence of lithium ions.

Brain slices were prepared and pre-incubated as previously, then 50 µl aliquots labelled with 5 µCi ^{3}H-inositol for 60 min. before addition of the indicated concentrations of carbachol. Incubations were continued for a further 10 min. then stopped by addition of PCA and neutralised extracts prepared from pooled, triplicated tissue samples as in 6.1 before analysis by anion exchange chromatography as previously. Closed circles indicate the presence and open symbols the absence of 5 mM LiCl. Where present LiCl was added 5 min. prior to agonist. Results represent the means ± SEM of 3 experiments and are expressed as detailed in the text. The 100% values in terms of cpm inositol polyphosphate/50 µl tissue were as indicated.

by previous studies in rat cerebral cortex (Brown et al. 1984; Jacobson et al. 1985; Rooney and Nahorski, 1986). Together with results from 3.4 and 6.4 the current data, and that from 2.3, indicate that although under certain conditions lithium appears to severely distort the pattern of receptor-mediated inositol phosphate accumulation, the use of this ion to potentiate responses in order to allow their more ready pharmacological characterisation does not lead to invalid dose-response curves. This point is emphasised by the close correlation between dose-response data where Ins P_3 in the absence, or Ins P_1 accumulation in the presence of lithium is the measure of receptor activation. In turn, as the potency for carbachol in promoting accumulation of Ins P_1 and Ins P_3 is very similar, this may indicate that the majority of Ins P_1 derives from polyphosphate dephosphorylation rather than through a calcium dependent hydrolysis of PtdIns (see (a) above). This conclusion supports those reached in 6.2 although, as Ins P_1 accumulation was not established and Ins P_3 isomers not resolved in the current experiments, an exact coincidence of the dose-response curves for carbachol-stimulated Ins-1,4,5-P_3 and Ins P_1 accumulations cannot necessarily be inferred from Figure 6.3.1. The effects of lithium on inositol phosphate accumulations are further considered in 6.4 but although this study further clarifies the attenuating actions of this ion on stimulated Ins P_3 and Ins P_4 accumulations, it remains difficult to resolve the apparent paradox whereby the dose-relationship between carbachol concentration and Ins P_3 accumulation determined over short periods of stimulation in the absence of lithium corresponds so closely with that for Ins P_1 accumulation determined under conditions where Ins P_3 and Ins P_4 accumulations are so markedly altered by the presence of this ion. As lithium has not been demonstrated to reduce stimulated Ins P_3 accumulation in tissues other than brain (Batty and Nahorski, 1985; see 3.4 and 6.4) it is difficult to compare the current observations with other studies although it is notable that where dose-relationships between agonist concentration and stimulated accumulations of separate inositol phosphates have been examined, these have shown a similar close agreement in the dose-dependency of Ins P_1, Ins P_2 and Ins P_3 accumulations to that observed here (Rubin, 1984; Bone et al. 1984).

In addition to the above, the current results also raise other interesting points. The essentially identical dose-dependency of stimulated Ins P_2 and Ins P_4 accumulations suggest that at each agonist concentration the relative proportions of Ins-1,4,5-P_3 metabolised via the 5-phosphatase and 3-kinase enzymes remains constant. Although this is not necessarily so since the extent to which the accumulated Ins P_2 is derived directly from Ins-1,4,5-P_3 and Ins-1,3,4-P_3 is not clear, this observation

does indicate that the conclusions reached in 6.2 are likely to apply equally at lower and higher agonist concentrations, in turn suggesting that substantial flux through Ins P_4 might be expected under physiological conditions. However, in view of the relative affinities of Ins-1,4,5-P_3 3-kinase and 5-phosphatase for their substrate the closely corresponding dose-response curves for carbachol stimulation of Ins P_2 and Ins P_4 accumulation is perhaps surprising. In brain the K_m of the former enzyme for Ins-1,4,5-P_3 is about 0.6 μM (Irvine et al. 1986; see 6.5) while that of the latter is about 50 μM (Erneux et al. 1986), suggesting that the 3-kinase will achieve saturation at considerably lower substrate concentrations than the 5-phosphatase. From 6.2 the changes in the radiolabelling of each inositol phosphate fraction in the current experiments is assumed to reflect an almost equivalent change in product concentration. Therefore, as the dose-response curves for carbachol-stimulated accumulation of each Ins P_2, Ins P_3 and Ins P_4 are virtually superimposable, this implies that both Ins P_2 and Ins P_4 accumulations increase in proportion to the rise in Ins P_3 concentration. Such a situation would only be expected at Ins-1,4,5-P_3 concentrations at or below the K_m of the 3-kinase since as this enzyme approaches saturation further increases in substrate concentration will not result in proportionally equivalent increased Ins P_4 accumulation. If the 3-kinase became saturated over the range of increased substrate concentrations occurring over the dose-response curve to carbachol, this agonist would be expected to display a higher potency in promoting Ins P_4 than either Ins P_3 or Ins P_2 accumulation. Although Ins-1,4,5-P_3 and Ins-1,3,4-P_3 were not resolved in the current experiments this is not likely to affect the argument both because the major portion of Ins P_3 in brain is accounted for by Ins-1,4,5-P_3 and because even in the presence of lithium, when the accumulation of Ins-1,3,4-P_3 may be enhanced (see 6.4), this does not result in an altered potency for carbachol stimulation of Ins P_3 accumulation. If the current data can be taken to indicate that the 3-kinase does not approach saturation this implies maximally stimulated Ins-1,4,5-P_3 concentrations are unlikely to considerably exceed 0.6 μM in brain. Nevertheless, such concentrations would be consistent with those found to be effective in mobilising intracellular calcium in numerous cell types (see Berridge and Irvine, 1984). However, as the fold stimulations of Ins-1,4,5-P_3 over basal levels in response to carbachol are small in brain (2-3 fold, see 6.2) this would indicate that control concentrations of this molecule are around 0.2 μM, at which concentration Ins-1,4,5-P_3 3-kinase would also be expected to be active. As the specific activity of ^{3}H-inositol phosphates has been suggested to change little on receptor activation (see 6.2), this, together with the relative half-lives of

Ins-1,4,5-P_3 and Ins P_4, would suggest a curious imbalance in the basal levels of these two molecules with the former being typically 4-5 fold higher than the latter (see 4.4). This may indicate that the activity of Ins-1,4,5-P_3 3-kinase and/or of other enzymes involved in inositol tris- and tetrakisphosphate metabolism may be controlled by other factors in addition to substrate concentration. The very recent demonstration that [Ca^{2+}] can modulate Ins-1,4,5-P_3 3-kinase activity in RINm5F cells (Biden and Wollheim, 1986) supports this suggestion. If similar regulatory mechanisms operate in brain then the current data do not necessarily restrict maximal Ins-1,4,5-P_3 concentrations to those over which the 3-kinase would be expected to display first order kinetics, since the properties of this enzyme in an intact stimulated cell may be subtly different from those found under less physiological conditions. However, as complex mechanisms may operate to regulate metabolism of inositol phosphates, it might be expected that these would be of varying significance at the different concentrations of products accumulating in response to varying doses of a given agonist so that the concentration-effect curves for separate inositol phosphate accumulations would exhibit distinct characteristics. Clearly this does not appear to be so and, as with the apparently paradoxical effects of lithium (see above and 6.4), is a phenomenon which requires further investigation.

Finally, the dose-dependency for carbachol-stimulated Ins P_2, Ins P_3 and Ins P_4 accumulations shows a close correlation with the corresponding data previously reported for Ins P_1 and thus supports the conclusions drawn in 2.3. However, the current data also show that responses to muscarinic receptor stimulation can be quite clearly detected and quantified even at low agonist receptor occupancy and in the absence of lithium. This provides a very marked contrast with other receptor systems coupled to phosphoinositide hydrolysis in brain. For example, in the presence of lithium, stimulation of alpha-1 receptors with a maximally effective concentration of NA produces a ^{3}H-Ins P_1 accumulation which corresponds to about 50% of that resulting from muscarinic receptor stimulation with 1 mM carbachol under the same conditions (see 3.3; Brown et al. 1984). Assuming that the specific activities of the ^{3}H-products are equal, it would be anticipated from this that a half maximally effective concentration of carbachol and a maximally effective dose of NA would produce equal accumulations of, at least, Ins P_1 and possibly also of polyphosphates in the absence of lithium. However, while the present results show that responses to carbachol are apparent even at concentrations well below the EC_{50} of this agonist, earlier experiments demonstrated that responses to maximal NA concentrations were barely detectable. Although the current data were generated using assay

conditions allowing much greater sensitivity (see 4.1) than those previously employed, this observation is consistent with those in 3.3 where muscarinic and alpha-1 receptor-mediated responses were more directly compared. On the basis of these earlier experiments it was suggested (3.3) that muscarinic receptor-mediated responses might exhibit a lower sensitivity to lithium, potentially as a consequence of an associated, proportionally greater production of an Ins P_1 whose hydrolysis is not inhibited by this ion. It is of interest to note that a similar explanation could, in part, account for the apparently contrary effects of lithium on stimulated Ins P_1 and Ins P_4 accumulations reported in 6.4.

In conclusion, the current data demonstrate that the dose-response curves for carbachol-stimulated Ins P_2, Ins P_3 and Ins P_4 accumulations are essentially superimposable. By comparison of these with earlier results (2.3 and 3.4) it is inferred that although prolonged muscarinic receptor stimulation in the presence of lithium may alter the pattern of response obtained at the level of Ins P_3/Ins P_4, this phenomenon does not appear to invalidate the assumption that measurement of stimulated Ins P_1 accumulation in the presence of this ion is a faithful reflection of reactions coupled more closely to the receptor. In addition the close agreement between the dose-response curves for carbachol-stimulated accumulations of each separate inositol phosphate provides dual support for the conclusions drawn in 6.2. Firstly, this result implies that a calcium dependent hydrolysis of PtdIns is less likely to account for the majority of accumulated Ins P_1 and Ins P_2 in brain than is polyphosphate dephosphorylation, assuming that the former would produce an apparent receptor reserve. Secondly, the same data suggest that the relative proportions of flux through Ins-1,4,5-P_3 5-phosphatase and 3-kinase previously estimated at 1 mM carbachol are likely to be equally valid at lower agonist concentrations. Finally, these results together with the apparent kinetic properties of Ins-1,4,5-P_3 3-kinase (Irvine _et al_. 1986; 6.5), may suggest that carbachol stimulation of muscarinic receptors in brain produces a sufficiently elevated Ins-1,4,5-P_3 concentration for this in turn to evoke a rise in $[Ca^{2+}]_i$. However, confirmation of this last suggestion will clearly require considerable further study while the significance of each of the above observations will only be established by more precise definition of both the proportions of the separate isomeric inositol phosphates accumulating in brain and the dose-relationships between agonist concentration and each of these responses.

6.4 The effects of lithium on inositol tris- and tetrakisphosphate accumulations

The data presented in 3.4 demonstrated differential effects of lithium on carbachol-stimulated accumulations of separate inositol phosphates, showing that this ion dose-dependently potentiated Ins P_1 and Ins P_2 accumulations while simultaneously reducing that of 'Ins P_3' by up to 70%. The magnitude of the latter effect varied with the length of exposure of tissue to lithium and agonist such that on short term stimulation the attenuated 'Ins P_3' accumulation was partially reversed at higher lithium concentrations while both these and lower doses resulted in diminished 'Ins P_3' levels over prolonged incubations with carbachol. A number of potential explanations which might account for some of these observations were also considered in 3.4. Briefly, these were:

a. That via inhibition of Ins P_1 phosphomonoesterase lithium might restrict the availability of free inositol for re-synthesis and cycling of receptor associated phosphoinositides.
b. That lithium may directly or indirectly affect the inter-conversion or hydrolysis of phosphoinositides.
c. That, again acting either directly or indirectly, lithium may promote metabolism through a comparatively less stable component of the 'Ins P_3' fraction such that only accumulation of product in this fraction and not flux through it was reduced.

Of these possibilities (a) seemed unlikely to account for all the observed effects of lithium because in this situation a reduction in the rate of Ins P_1 accumulation would be expected either to follow or coincide with the diminished, stimulated Ins P_3 accumulation regardless of whether the former is derived via polyphosphate dephosphorylation or direct PtdIns hydrolysis since a limiting supply of inositol for lipid synthesis should affect each receptor associated phosphoinositide fraction and not PtdIns P_2 specifically. However, stimulated Ins P_1 accumulation continues almost linearly in the presence of lithium for up to 30-40 min. after a reduced Ins P_3 concentration first becomes evident. Further, the observed dual effects of higher lithium concentrations suggest at least two distinct sites of action for this ion, one resulting in potentiated and one attenuated 'Ins P_3' accumulation. The former may reflect an inhibitory action of lithium on Ins-1,3,4-P_3 hydrolysis (see 3.4 and 4.4(b)ii).

As with (a) above, (b) also seemed an unlikely cause of the effects of lithium since no clear evidence for this could be found although such an action could be difficult to detect in brain (see 3.5). Alternatively, option (c) appeared more probable especially as the results presented in

CH.4 demonstrated that not only does the fraction originally isolated as 'Ins P_3' contain both Ins-1,3,4-and-1,4,5-P_3 but also Ins P_4. Further, preliminary data reported in 4.1 suggested a selective effect of lithium on stimulated Ins P_4 accumulation. For these reasons the effects of lithium on control and carbachol-stimulated inositol phosphate accumulations were re-investigated in order to determine more specifically the major site(s) at which this ion affects inositol lipid/phosphate metabolism.

In an initial series of experiments the time courses for inositol phosphate accumulations in the presence and absence of both 1 mM carbachol and 5 mM LiCl were examined under similar incubation and labelling conditions to those originally employed in CH.3 so that the current and earlier results are comparable. The experimental protocol was as described in the legend to Figure 6.4.1. Extraction and chromatography of ^{3}H-inositol phosphates were exactly as detailed in 6.1 and 6.2 respectively. Figure 6.4.1 allows comparison of the time courses of carbachol-stimulated accumulation of each Ins P_1 and Ins P_2 in the presence and absence of lithium while Figure 6.4.2 illustrates the corresponding data for Ins P_3 and Ins P_4.

As previously observed, lithium markedly potentiated stimulated Ins P_1 and Ins P_2 accumulations such that after 45 min. these were respectively 780% and 217% of the concentrations measured in the absence of this ion. In the presence of lithium Ins P_1 accumulation in response to carbachol was linear over the entire time course while Ins P_2 rapidly achieved a maximal level which then was maintained over the duration of the experiment, suggesting that the hydrolysis of Ins P_2 is less effectively inhibited at 5 mM LiCl than is that of Ins P_1. This result is consistent with the dose-response data presented in 3.4. In the absence of lithium the pattern of stimulated Ins P_2 accumulation was essentially the same as in the presence of this ion while accumulation of Ins P_1 continued linearly only for 10-15 min. before achieving a new steady state level as reported in 3.1. Basal accumulations of each Ins P_1 and Ins P_2 were also potentiated by lithium such that by 45 min. values found in the presence of this ion were respectively 333% and 225% those in the absence. Maximal stimulated accumulations of each Ins P_1 and Ins P_2, occurring at 45 min., were respectively 1425% and 608%, and 833% and 863% in the presence and absence of lithium. Each of these results is approximately comparable with the corresponding data reported in 3.1 and 3.4.

In contrast, the currently defined effects of lithium on carbachol-stimulated Ins P_3 accumulation differ markedly from those reported in 3.4. Consistent with this previous study, muscarinic receptor stimulation resulted in a rapid accumulation of Ins P_3 above control levels with close

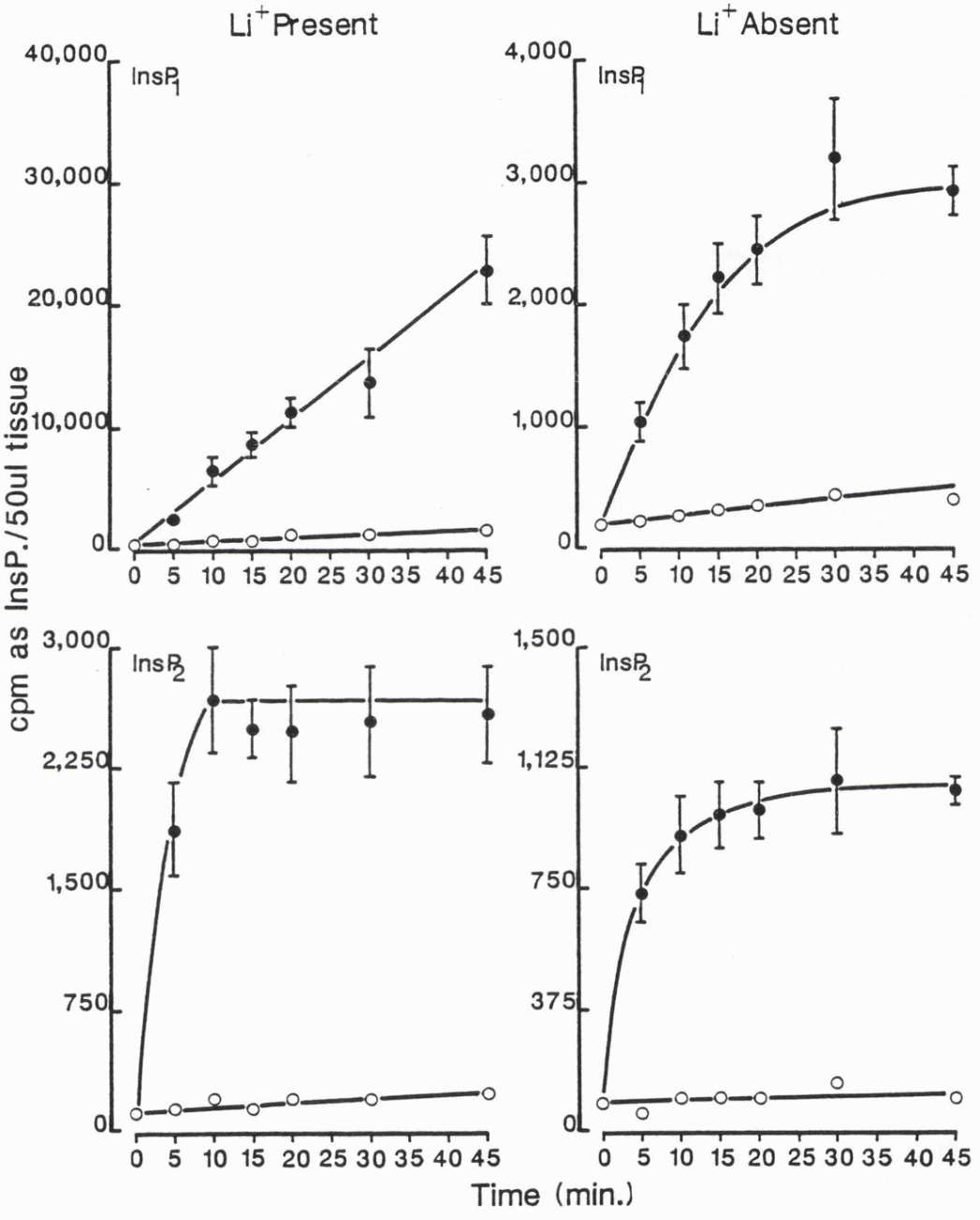


FIGURE 6.4.1 The effects of 5 mM LiCl on the time courses of control and carbachol-stimulated accumulations of ^{3}H-Ins P_1 and ^{3}H-Ins P_2 in brain slices.

Cerebral cortical slices were prepared and pre-incubated as in 2.2 then 50 µl aliquots labelled with 1 µCi ^{3}H-inositol for 30 min. in the presence or absence of 5 mM LiCl. Following the subsequent addition of 1 mM carbachol (closed symbols) or Krebs vehicle (open symbols), incubations were continued for the times indicated before extraction, neutralisation and analysis of ^{3}H-Ins P(s) from pooled triplicated samples as previously. Results represent the mean ± SEM of 3 experiments. Where not indicated SEM was within the symbol.

to maximal concentrations being achieved within 5 min. of agonist addition. These respectively represented 488% and 509% of basal values in the presence and absence of lithium. However, while earlier experiments clearly demonstrated that in the presence of lithium more prolonged exposure to carbachol resulted in a subsequent reduction in 'Ins P_3' accumulation, a similar effect here was not so obviously apparent. After 45 min. in the presence of lithium and agonist, Ins P_3 accumulation remained at about 80% of the maximal value observed at 10 min. The significance of this limited secondary decline in stimulated Ins P_3 accumulation in the presence of lithium is difficult to assess in view of the inter-experimental variation associated particularly with the corresponding data obtained in the absence of this ion. However, it should be pointed out that as these initial experiments employed labelling conditions equivalent to those used in 3.4 (to allow appropriate comparisons) and yet Ins P_3 and Ins P_4 were resolved, the assay sensitivity for Ins P_3 was reduced by more than 2-fold compared with earlier experiments where these were estimated as a combined fraction. For this reason even the maximally stimulated accumulations of Ins P_3 currently determined were extremely low and are therefore potentially subject to greater error than the data shown in Table 6.4.1. Thus, although a highly significant, lithium-induced attenuation of Ins P_3 accumulation was not detected here, the small but apparently reduced accumulation observed after more prolonged exposure to agonist is likely to represent a genuine effect (see below).

Despite this it is clear that a substantial proportion of the earlier defined effect of lithium on 'Ins P_3' accumulation can be attributed more specifically to an effect on Ins P_4 since the stimulated accumulation of this product was severely attenuated in the presence of lithium. As determined for total Ins P_3 + Ins P_4 this effect only became apparent after prolonged exposure to both lithium and agonist so that more immediately, carbachol resulted in approximately equivalent 20-50 fold increased accumulations of Ins P_4 above basal both in the presence and absence of lithium. While this maximal stimulated concentration, achieved in either case within 5-10 min. was maintained across the time course in the absence of lithium, in the presence of this ion stimulated accumulations began to decline between 10-15 min., reaching a new steady state only after 30-45 min. At this later time carbachol-stimulated Ins P_4 accumulation in the presence of lithium was reduced to 33% that in the absence. Basal Ins P_4 accumulation was not influenced by lithium within the limits of detection.

The time dependency and magnitude of the lithium-induced reduction in stimulated Ins P_4 accumulation closely paralleled the corresponding effect

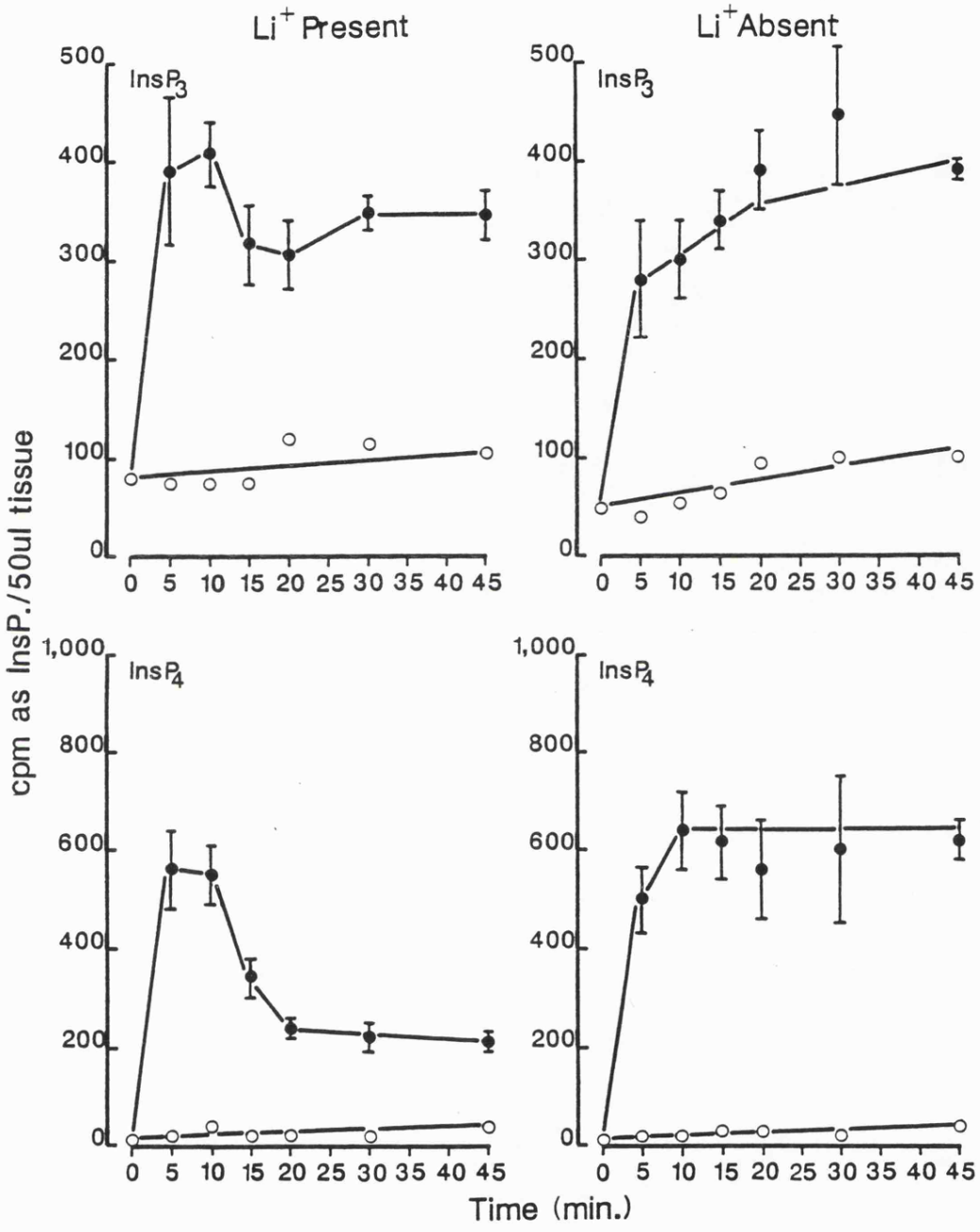


FIGURE 6.4.2 The effects of 5 mM LiCl on the time courses of control and carbachol-stimulated accumulations of ^{3}H-Ins P_3 and ^{3}H-Ins P_4 in brain slices.

The experimental protocol was as in the legend to Fig. 6.4.1 and the accumulations of ^{3}H-Ins P_3 and ^{3}H-Ins P_4 shown correspond to the same samples as in Fig. 6.4.1. Results represent the mean ± SEM of 3 experiments.

previously noted on the 'Ins P_3' fraction. Thus, it appeared that the earlier results (3.4) could largely be accounted for by a preferential reduction of Ins P_4 rather than of Ins-1,4,5-P_3 as originally assumed. However, Burgess et al. (1985) have suggested that lithium may inhibit the hydrolysis of Ins-1,3,4-P_3 in pancreatic acinar cells, a conclusion supported by the lithium potentiation of stimulated Ins P_3 accumulation in hepatocytes (Thomas et al. 1984) and GH_3 cells (Drummond et al. 1984) and the apparent insensitivity of Ins-1,4,5-P_3 5-phosphatase to lithium (Downes et al. 1982; Seyfred et al. 1984; Connelly et al. 1985; Erneux et al. 1986). Thus, it is possible that a lithium potentiation of Ins-1,3,4-P_3 accumulation could offset a decreased accumulation of Ins-1,4,5-P_3 when these two are estimated together as in the present study. Clearly in order to establish the probable cause of the very marked combined effects of lithium and carbachol on Ins P_4 accumulation the two trisphosphates must be measured separately. For this reason it is useful to examine the data in Table 6.4.1 before considering the implications of the above results in more detail.

Table 6.4.1 shows the results of an experiment in which the effects of 5 mM LiCl on each control and 1 mM carbachol-stimulated accumulations of Ins-1,3,4-P_3, Ins-1,4,5-P_3 and Ins P_4 were examined under conditions where the attenuating actions of this ion are maximal. The results are representative of a single experiment but are supported by a second in which Ins P_3 isomers were not resolved but where assay sensitivity for total Ins P_3 was increased 5-fold over that achieved above by heavier labelling of samples with ^{3}H-inositol as in 4.3(a).

Table 6.4.1 reveals that the most apparent effect of lithium was a reduction in the stimulated accumulation of Ins P_4 which was decreased by about 70% compared with that measured in the absence of this ion. In addition Table 6.4.1 clearly demonstrates that lithium also attenuated the stimulated accumulations of each Ins-1,3,4-and-1,4,5-P_3 by 30-40% thus confirming the similar, though less obvious effect illustrated in Figure 6.4.2. However, while the basal levels of neither Ins-1,4,5-P_3 nor Ins-1,3,4,5-P_4 were notably affected by lithium, that of Ins-1,3,4-P_3 was increased by approximately 2-fold. Since both control and carbachol-stimulated concentrations of ^{3}H Ins-1,3,4-P_3 in brain are typically small the significance of this observation is difficult to assess from a single experiment. Nevertheless, this would be consistent with a lithium inhibition of Ins-1,3,4-P_3 hydrolysis as suggested by Burgess et al. (1985). Further, as Ins-1,3,4-P_3 is thought to arise exclusively via 5-phosphatase attack on Ins-1,3,4,5-P_4 (Batty et al. 1985b; Hawkins et al. 1986; Hansen et al. 1986; Irvine et al. 1986) it would be anticipated, under conditions of prolonged exposure to both lithium and agonist, that

Table 6.4.1 Effects of 5 mM LiCl on control and 1 mM carbachol-stimulated accumulations of ^{3}H-inositol tris- and tetrakisphosphates.

Incubation	Inositol phosphate (cpm)					
	Ins-1,3,4-P_3		Ins-1,4,5-P_3		Ins-1,3,4,5-P_4	
	- Li^+	+ Li^+	- Li^+	+ Li^+	- Li^+	+ Li^+
1. Control	20	51	465	444	71	89
2. 1 mM Carbachol	328	221	1030	664	1377	365
3. % Stimulation	1640%	433%	222%	150%	1939%	410%
4. % Stimulated $+Li^+/_{-Li^+}$	67%		64%		27%	
5. % $\left[\frac{[\text{Stim/control}]\ +Li^+}{[\text{Stim/control}]\ -Li^+}\right]$	26%		68%		21%	

Cerebral cortical slices were labelled with ^{3}H-inositol (2 µCi/50 µl tissue) for 60 min. either in the presence or absence of 5 mM LiCl then further incubated for 45 min. after addition of 1 mM carbachol or Krebs medium. Samples were extracted and appropriate triplicates combined as previously, and subsequently analysed by hplc for Ins P_3 and Ins P_4. Results represent a single experiment.

any reduction in stimulated Ins P_4 accumulation would be reflected by a corresponding decrease in Ins-1,3,4-P_3. Table 6.4.1 shows that stimulated Ins-1,3,4-P_3 is only reduced by about 30% in the presence of lithium while that of Ins P_4 is diminished by 70%. This result also suggests that lithium may significantly inhibit hydrolysis of Ins-1,3,4-P_3. If such an effect is taken into account by expressing the data as shown in (5) Table 6.4.1 it can be seen that the percentage reduction induced by lithium in the fold carbachol stimulation of each Ins-1,3,4-P_3 and Ins P_4 is approximately equal at about 70-80% while that for their ultimate, common precursor (Ins-1,4,5-P_3) is only decreased by 30-40%.

Although it is clear that further studies will be required to confirm these preliminary observations, the present data strongly suggest that lithium markedly alters muscarinic receptor-mediated inositol tris- and tetrakisphosphate metabolism in cerebral cortex. However, as discussed below, it is difficult to determine from these results the mechanism by which lithium induces a reduction in stimulated Ins P_4 accumulation. Despite this, the data do clarify some of the points previously raised (see 3.4). Firstly, the data presented in 3.4 clearly demonstrated dual effects of higher (> 1 mM) lithium concentrations on stimulated 'Ins P_3' accumulation with the balance between the potentiating and attenuating actions of these being dependent on the time of exposure to agonist. From above it seems probable that at 5 mM, LiCl partially inhibits Ins-1,3,4-P_3 hydrolysis which would account for the biphasic dose-relationship between 'Ins P_3' accumulation and [Li^+] observed after 15 min. stimulation with carbachol. However, at this time lower doses of lithium reduced stimulated 'Ins P_3' accumulation, an effect common to all lithium concentrations after more prolonged exposure to agonist. At 5 mM LiCl this attenuating action takes between 10-15 min. to develop. The current data confirms an identical time dependency for the lithium-induced reduction in stimulated Ins P_4 accumulation. On the basis of this lag phase it was previously suggested (3.4) that the attenuating effect of lithium might arise through depletion of a receptor associated pool of an inositol metabolite. An alternative possibility is that this effect is indirectly mediated via a secondary factor whose accumulation is promoted in the presence of lithium and agonist. Both options are further considered below but it is important to note that a delayed decreased accumulation of 'Ins P_3' or Ins P_4 has thus far only been demonstrated at 5 mM LiCl. At lower concentrations the reduced 'Ins P_3' accumulation apparent after 15 min. exposure to carbachol and lithium may be more pronounced than at 5 mM such that at these doses there may be no lag phase. The significance of this delay will only become clear when time courses both to separate tris- and tetrakisphosphates have been

determined over a range of lithium concentrations, particularly since higher doses may inhibit the hydrolysis of Ins-1,3,4-P_3 and may therefore indirectly also affect breakdown of Ins P_4 such that, initially, any attenuating effects of high lighium concentrations on the latter are masked.

However, if the lag phase before a reduced stimulated accumulation of Ins P_4 becomes apparent is a genuine effect, the attenuating actions of lithium may result indirectly as a consequence of this ion either depleting a pool of an inositol metabolite or promoting accumulation of some product presumably arising from stimulated inositol lipid breakdown. The former possibility was considered in 3.4 where it was suggested that the effects of lithium on 'Ins P_3' accumulation were unlikely to result from inhibition of Ins P_1 phosphatase limiting availability of inositol for lipid re-synthesis. The reasons for this being, that ultimately the rate and maximal accumulation of Ins P_1 and Ins P_2 respectively would be subject to the same reductions observed with Ins P_3 unless these lower phosphates are derived from pools of phosphoinositides distinct from those immediately associated with the receptor. Further, in rat parotid gland where the combined action of carbachol and lithium does appear to reduce availability of inositol for PtdIns synthesis, stimulated accumulation of Ins P_3 is unlikely to be attenuated by lithium as concentrations of PtdIns P and PtdIns P_2 are maintained even in the presence of both agonist and this ion (Downes and Stone, 1986). Clearly the combination of lithium and carbachol produces different effects in brain although it is difficult to assess the significance of effects on phosphoinositide levels in this tissue (see 3.5). However, if the attenuating action of lithium was exclusive to Ins P_4, as largely appeared to be so from Figures 6.4.1 and 6.4.2, this would imply that the effect of this ion could not be attributed to either impaired lipid synthesis or hydrolysis. However, as Table 6.4.1 suggests that the stimulated accumulation of Ins-1,4,5-P_3 is also reduced in the presence of lithium, neither possibility can be excluded. Nevertheless, the attenuating actions of lithium seem unlikely to be direct consequences of Ins P_1 phosphatase inhibition but may be mediated by a secondary factor, the accumulation of which is promoted in the presence of lithium and agonist.

This raises several possibilities since the concentrations of each Ins P_1 and Ins P_2 are markedly increased in the presence of both agents and thus either could activate a negative feed-back mechanism. However, both seem unlikely mediators of the effects of lithium as attenuated 'Ins P_3' accumulation is apparent at lithium concentrations (0.1-0.3 mM, see 3.4) which do not affect Ins P_2 accumulation and which are well below the EC_{50} for this ion on stimulated Ins P_1 accumulation. Alternatively,

the effects of lithium could be mediated through an elevated concentration of diacylglycerol (DG) or a metabolite of this molecule. Stimulation of GH_3 cells with TRH in the presence of lithium results in a greater accumulation of DG than does exposure to agonist alone (Drummond and Raeburn, 1984) while muscarinic receptor stimulation in rat parotid gland in the presence of this ion results in potentiated CMP-PA and phosphatidic acid (PA) accumulations over those caused by exposure to carbachol alone (Downes and Stone, 1986). The immediate metabolic fate of the DG arising from phosphoinositide hydrolysis in brain is uncertain but as this molecule is a known activator of C kinase (Nishizuka, 1984) and as this enzyme may function as part of a feed-back mechanism modulating PtdIns P_2 synthesis (Van Dongen et al. 1985), an effect of DG on inositol lipid/phosphate metabolism might be anticipated. This view is further supported by the recent observations that phorbol esters reduce muscarinic receptor-mediated inositol phosphate accumulation in hippocampal slices (Labarca et al. 1984) and astrocytoma cells (Orellana et al. 1986) and increase the rate of Ins P_3 dephosphorylation in human platelets (Molina y Vedia and Lapetina, 1986). However, the potentiated accumulation of CMP-PA and of PA in carbachol-stimulated parotid gland appears to result from the lithium inhibition of Ins P_1 phosphatase reducing the availability of free inositol for PtdIns synthesis (Downes and Stone, 1986). A similar action in brain is far from clear and, as the pools of receptor associated phosphoinositides may be small in this tissue (see CH.2 and 3.5), might be expected to reduce the maximal accumulations of inositol phosphates independently of any secondary action of DG. Thus, although it is possible that the attenuating actions of lithium are mediated through a secondary factor, the nature of this is unknown.

Equally, it is difficult to determine the step(s) at which the attenuating actions of lithium are exerted. The marked reduction in stimulated Ins P_4 accumulation may indicate either suppression of 3-kinase activity or activation of Ins-1,3,4,5-P_4 5-phosphatase. The latter seems less likely, firstly because in combination with a lithium inhibition of Ins-1,3,4-P_3 hydrolysis this might be expected to promote accumulation of this trisphosphate and secondly because the same 5-phosphatase may hydrolyse both Ins-1,4,5-P_3 and Ins P_4, in which event equivalent reductions in the stimulated concentration of each product might be anticipated in the presence of lithium. However, an effect of lithium on 5-phosphatase activity cannot be excluded. Alternatively, the reduced stimulated accumulation of Ins-1,4,5-P_3 itself may indicate that the attenuating action of lithium is exerted at a step more proximal to that mediated by the receptor than the reaction catalysed by the 3-kinase. If this is so it suggests that the 30% reduction observed in stimulated

Ins-1,4,5-P_3 concentration is sufficient to cause a 70% decrease in stimulated Ins P_4 accumulation, assuming that the latter effect is a direct consequence of the former which is not necessarily so. From the data in 6.3 it was suggested that over the range of the dose-response curves to carbachol, the Ins-1,4,5-P_3 5-phosphatase and 3-kinase may exhibit first order kinetics. Under these conditions a 30% reduction in substrate concentration should reduce turnover through the two routes by the same relative proportion. However, in the presence of lithium there appears to be a disproportionate effect on stimulated Ins P_4 accumulation since while this is reduced by 70% there is little or no change in the accumulations of Ins P_1 and Ins P_2. In the latter respect the data presented in 3.4 indicated that the rate of, and maximal accumulation of, Ins P_1 and Ins P_2 respectively did fall by about 30-40% following the lithium-induced attenuation of 'Ins P_3', which would be consistent with the extent by which Ins-1,4,5-P_3 was currently found to decrease. Whether this is a genuine effect or not, the present results would suggest that in the presence of lithium, metabolism of Ins-1,4,5-P_3 via 3-kinase action becomes of proportionally lesser significance. In turn as such an effect is not readily attributed solely to the limited reduction in Ins-1,4,5-P_3 concentration, this may imply that other factors are involved in the regulation of this enzyme and that acting either directly or indirectly, lithium interferes with this regulatory mechanism. Alternatively, it is possible that the reduced stimulated accumulation of Ins P_4 is not reflected in the levels of Ins P_1 and Ins P_2 for other reasons e.g.

i) Breakdown of the Ins P_1 ultimately derived from Ins P_4 is not sensitive to lithium such that this component of the muscarinic receptor-mediated response is not fully estimated in assays measuring only Ins P_1 accumulation either in the presence or absence of lithium.

ii) A substantial proportion of the stimulated Ins P_1 and/or Ins P_2 accumulation in brain arises from sources other than through polyphosphate dephosphorylation. From 6.2 this is unlikely in the absence of lithium but is a possibility which cannot be excluded.

Clearly further studies will be essential to establish both the step(s) at which the attenuating actions of lithium on stimulated Ins-1,4,5-P_3 and Ins P_4 accumulations are exerted and the nature of any secondary factor mediating these effects. Possibly a better understanding of the complex effects of lithium in brain could be achieved by a detailed investigation of both the lithium and agonist dose-dependency of these and of the ways in which these relationships vary with the length of tissue exposure to both agents. Although the current results, even together with

those in 3.4, do not represent a sufficiently comprehensive study in this respect they do allow some interesting comparisons with other tissues. Firstly, the current data show that lithium potentiates both stimulated Ins P_1 and Ins P_2 and possibly Ins-1,3,4-P_3 accumulations in brain suggesting that this system has a number of features common to many tissues where similar effects of this ion have been observed (Berridge et al. 1982; Thomas et al. 1984; Rubin, 1984; Drummond et al. 1984; Burgess et al. 1985). Secondly, despite this, the attenuating action of lithium on stimulated Ins-1,4,5-P_3 and Ins P_4 accumulations also suggest that the metabolism of inositol lipids/phosphates in brain exhibits marked differences from other tissues where the currently available data indicates that corresponding effects are unlikely (Thomas et al. 1984; Drummond et al. 1984; Burgess et al. 1985; Downes and Stone, 1986). However, whether or not the effects of lithium observed here are unique to brain is difficult to determine as many previous studies have not resolved inositol tris- and tetrakisphosphates. Similarly, if these effects are specific to brain, it is not clear whether they reflect major differences between this and other tissues in the pathways associated with receptor-mediated inositol lipid and/or phosphate metabolism or more subtle differences in the mechanisms regulating these. Comparison of data from rat brain and parotid gland suggest that the latter is perhaps more probable since in both tissues the principal sequence of receptor initiated reactions appear comparable (see CH.4 and cf Batty et al. 1985b and Hawkins et al. 1986) while both the relative proportions of resultant products accumulating (see CH.4 and 6.2) and the effects of lithium in the two tissues (cf present data and Downes and Stone, 1986) show certain marked differences. It is therefore possible that a better understanding of the factors regulating inositol lipid and phosphate metabolism will in turn clarify the mechanism(s) by which lithium exerts its complex effects on this system in brain. Until these mechanisms are established it is difficult to assess how these are likely to influence the interpretation of data from pharmacological studies of phosphoinositide hydrolysis where lithium is frequently employed at relatively high concentrations (5-20 mM, see CH.2) over prolonged periods of stimulation to potentiate limited responses. Nevertheless, the current data show that when lithium is used under such conditions to study muscarinic receptor-mediated inositol lipid hydrolysis in brain the response observed at the level of Ins P_3 and Ins P_4 is grossly distorted from that in the absence of this ion. It may be of future interest to determine whether this also applies equally to other similar receptor-mediated responses in brain.

As a role for Ins-1,4,5-P_3 in control of $[Ca^{2+}]_i$ has been proposed (Berridge and Irvine, 1984) and as Ins-1,3,4-P_3 and/or Ins-1,3,4,5-P_4 may

also act as intracellular signals (Irvine et al. 1985; Batty et al. 1985b; Hansen et al. 1986) it might also be of interest to determine whether the effects of lithium on the stimulated accumulations of these molecules is translated to cellular function. In this respect it is notable that although lithium potentiates stimulated Ins P_3 accumulation in pancreatic acini (Rubin, 1984) and hepatocytes (Thomas et al. 1984) it does not affect amylase secretion or phosphorylase activation respectively in these cells. However, stimulation of adrenal glomerulosa cells with angiotensin II in the presence of lithium results in a lower production of aldosterone than does exposure to agonist in the absence of this ion (Balla et al. 1984). This effect of lithium appears restricted to stimuli promoting hydrolysis of inositol lipids and was suggested to arise via an inhibition of Ins P_1 phosphatase resulting in a limited supply of inositol for continued phosphoinositide cycling although it was not effectively reversed in the presence of high (10 mM) exogenous inositol concentrations (Balla et al. 1984). In rat parotid gland muscarinic receptor stimulation in the presence of lithium reduces PtdIns levels below those in response to agonist alone as hydrolysis of Ins P_1 to free inositol is impaired and yet despite this, in stimulated tissue, polyphosphoinositides are maintained at the same concentrations both in the presence and absence of lithium (Downes and Stone, 1986). If a similar situation prevails in glomerulosa cells, the effects noted by Balla et al. (1984) could result from lithium attenuated accumulations of Ins P_3 and/or Ins P_4 analogous to those currently reported. However, accumulation of Ins P_3 in response to angiotensin II in adrenal glomerulosa cells is potentiated in the presence of lithium (Enyedi et al. 1985) although as neither potential isomeric Ins P_3(s) nor Ins P_4 were resolved in this study, the significance of this is not clear. Thus, it is possible that a lithium potentiated accumulation of Ins-1,3,4-P_3 in these cells masks a decreased accumulation of Ins-1,4,5-P_3 and/or Ins P_4 and that this latter effect results in a diminution of the calcium signal which is promoted by the agonist and required for fully effective aldosterone production.

Finally, in summary the current results suggest that lithium exerts multiple effects on inositol metabolism in brain. This ion potentiates accumulation of Ins P_1 and Ins P_2 and possibly also inhibits hydrolysis of Ins-1,3,4-P_3. At lower concentrations (< 1 mM) lithium reduces the stimulated accumulation of total Ins P_3 and Ins P_4, an effect also seen at higher doses but only apparent after more prolonged periods of stimulation (30-45 min.). At 5 mM, lithium severely attenuates carbachol stimulated Ins P_4 accumulation and reduces that of Ins-1,4,5-P_3 to a lesser extent, while simultaneously potentiating accumulation of both Ins P_1 and Ins P_2. These actions are consistent with the inhibition of inositol mono-, bis-

and-1,3,4-trisphosphate phosphatases to varying degrees by lithium but are unlikely to be completely accounted for in this way. The attenuating effects of lithium suggest either direct or indirect action(s) exerted at further undefined site(s). These latter effects also suggest that either the routes of inositol lipid and/or phosphate metabolism or the regulatory mechanisms controlling these are more complex than is yet evident in brain. Since lithium so markedly alters the metabolism of inositol tris- and tetrakisphosphates in brain it might be expected that the data deriving from studies where Ins P_1 accumulation in the presence of this ion is the sole measure of receptor activation would be substantially invalid. However, dose-response curves to carbachol determined using such methods correspond well with those determined for polyphosphate accumulation in either the presence or absence of lithium (see 6.3). The significance of this observation and therefore of the attenuating actions of lithium to the interpretation of such pharmacological data is unclear and this apparent anomaly requires further investigation. As Ins-1,4,5-P_3 and/or Ins P_4 may have second messenger functions the reduction in their stimulated concentrations in the presence of lithium may lead to impaired cellular responses. Again further studies will be essential both to establish the nature of any cause and effect relationship between the effects of lithium on inositol metabolism and altered cellular activity and to determine the significance of any such relationship in terms of the therapeutic efficacy of lithium. The current data indicate each of these may be interesting areas for future research.

6.5 Preliminary studies on the Ins-1,4,5-P_3 3-kinase from rat brain

It is clear from the discussion in previous sections that the metabolism of Ins-1,4,5-P_3 in brain can proceed initially either by dephosphorylation to Ins P_2 or by kinase activity to yield Ins P_4. As Ins-1,4,5-P_3, and possibly Ins-1,3,4,5-P_4 and/or metabolites of this, may act as intracellular messengers, it is also evident that the enzymes catalysing these reactions may be pivotal to the control of cellular function and as such may represent key sites for regulation and for integration of the inositol lipid signalling system with other such mechanisms.

Since its recent description (Irvine *et al.* 1986), Ins-1,4,5-P_3 3-kinase activity has been confirmed in a variety of tissues and cells (Hawkins *et al.* 1986; Hansen *et al.* 1986; Stewart *et al.* 1986) although there is conflicting evidence regarding the sensitivity of the enzyme to calcium ion concentration. In contrast to Irvine *et al.* (1986) both Biden and Wollheim (1986) and Rossier *et al.* (1986) have demonstrated enhanced

activity of this enzyme as $[Ca^{2+}]$ is increased over the physiological range. Detailed data is not yet available for the 3-kinase from brain but indirect evidence (Baird and Nahorski, 1986) may indicate that $[Ca^{2+}]$ can also modulate the activity of this enzyme in cerebral cortical slices. In addition the results presented in the preceding section and those from 3.4 may suggest that complex mechanisms regulate inositol polyphosphate metabolism in brain, potentially involving a number of factors and sites of action.

Clearly any modulation of the activity of the enzymes involved in inositol tris- and tetrakisphosphate metabolism could complicate many of the earlier discussions. Thus, a better understanding of such regulatory mechanisms is essential to the accurate interpretation of biochemical and pharmacological data and should provide valuable information regarding the relative significance of the alternative routes of Ins-1,4,5-P_3 metabolism. By implication, this may in turn indicate possible functions of Ins-1,3,4-P_3 and/or of Ins P_4.

For these reasons, studies were undertaken to determine appropriate conditions for the assay of Ins-1,4,5-P_3 3-kinase and then, using these, to establish some of the basic characteristics of this enzyme from brain. It should be pointed out that these represent only preliminary studies which must have limited significance as no attempt was made to purify this enzyme activity or to assay this under conditions representative of the intracellular environment prevailing within stimulated cells. However, the results are briefly described below since these essentially confirm those of Irvine et al. (1986) and, within the above limitations, lend support to the previous conclusions regarding the capacity and rapidity of this pathway of Ins-1,4,5-P_3 metabolism in brain. Further, the experiments described should also provide a useful basis for future more detailed studies aimed at assessing the effects of a variety of factors (e.g. calcium, lithium, DG) on Ins-1,4,5-P_3 3-kinase activity.

a. Methods for the preparation and assay of Ins-1,4,5-P_3 3-kinase:

In all experiments these methods were based on those of Irvine et al. (1986).

i) Enzyme preparation: Whole rat brains were minced with scissors, suspended in 20-25 ml 0.15 M sucrose and homogenised by several strokes using a motor-driven glass-teflon homogeniser. The homogenate was adjusted to give a final 20% (w/v) tissue concentration by addition of further sucrose solution and centrifuged at 100,000 x g for 90 min. The resultant supernatant was decanted, divided into multiple aliquots and stored at either 4°C or -40°C. In some experiments this crude preparation

was used immediately but in the majority of cases only after up to several weeks frozen storage. Several cycles of freeze-thawing did not appear to appreciably affect enzyme activity.

ii) Enzyme assay: Assays of the 3-kinase were performed using the crude supernatant fraction isolated above although in many experiments this was diluted up to 1,000-fold in either 0.15 M sucrose or an identical solution containing bovine serum albumin (BSA) at a final assay concentration of 0.5-1.25% (w/v). Where a 'non-enzyme' control was required a supernatant preparation which had been heated to, and maintained at, 100°C for 30 min. was used. Enzyme incubations were carried out in a final volume of 200 µl. This included 140 µl tris-maleate buffer, pH 7.5 (final concentration 50 mM) containing adenosine triphosphate (ATP) and $MgCl_2$ in a molar ratio of 1:2. The final concentration of ATP was varied in some later experiments but except where otherwise stated was 10 mM. The remaining incubation volume comprised 10 µl ^{3}H-Ins-1,4,5-P_3 (concentrations as indicated in figures and legends) and 50 µl enzyme. Where other factors were present (e.g. 2,3-bisphosphoglycerate) these were included in the buffer to give the final concentrations indicated. Incubations were performed at 37°C in a metabolic shaker for the times shown and were started by addition of either enzyme or substrate. Reactions were terminated by addition of 200 µl 7% (w/v) PCA followed by 50 µl 5% (w/v) BSA. Precipitated protein was sedimented by brief centrifugation and 400 µl supernatant collected and mixed with 250 µl 10 mM EDTA. To the resultant solution 400 µl of a 1:1 (v/v) mixture of 1,1,2-trichlorotrifluoroethane:tri-n-octylamine was added. After vortex mixing and centrifugation a neutral extract of ^{3}H-inositol phosphates was prepared from the upper phase as in 6.1.

iii) Analysis of products and expression of results: Final neutralised assay extracts were analysed for ^{3}H-products either by Dowex or hplc anion exchange chromatography as described in CH.4 and 5 respectively. In later experiments, having established, under defined conditions (see below), that the major reaction product was Ins P_4, the activity of the kinase was determined more simply by separating a combined fraction of lower inositol phosphates from ^{3}H-Ins P_4 by Dowex chromatography, and quantifying only the latter.

In some experiments, to achieve the required substrate concentrations, ^{3}H-Ins-1,4,5-P_3 (1 Ci/mmol) was mixed with unlabelled material such that substrate and product specific activities within and between experiments varied considerably. Therefore, where appropriate,

results have been expressed either as the percentage conversion of the original ^{3}H-substrate or in terms of pmol product obtained.

b. Results and discussion:

The primary aims of these preliminary studies were two-fold. Firstly, to confirm the presence of an active Ins-1,4,5-P_3 kinase in brain and secondly, to establish conditions suitable for defining the basic kinetic properties of this enzyme. For the latter purpose it was essential to demonstrate that the crude enzyme preparation and incubation protocol used did not result in substantial hydrolysis of either the initial substrate or immediate reaction product and then to modify the conditions such that substrate phosphorylation proceeded linearly over an appropriate and convenient time course. The demonstration of 3-kinase activity in the brain supernatant fraction used and characterisation of the reaction products are considered in (i) below while further studies are described in (ii).

i) The presence of a soluble Ins-1,4,5-P_3 3-kinase in rat brain: The concentrations of Ins-1,4,5-P_3 required to promote maximal release of membrane bound Ca^{2+} ions are typically between 0.5-1.0 μM and are suggested to be within the range of intracellular [Ins-1,4,5-P_3] achieved within stimulated cells (see Berridge and Irvine, 1984). In those tissues where agonist-mediated increases in the accumulation of separate Ins P_3 isomers have been determined, the initial rapid changes ($\lesssim$ 10 sec.) in Ins-1,4,5-P_3 suggest less than 5-10 fold increases over basal levels (Burgess et al. 1985; Batty et al. 1985b; Heslop et al. 1985; Hansen et al. 1986). This implies that under resting conditions the concentration of Ins-1,4,5-P_3 is likely to be of the order of 50-100 nM or higher. In preliminary experiments to demonstrate the presence of Ins-1,4,5-P_3 3-kinase activity in the soluble fraction from rat brain a range of substrate concentrations which would bracket those expected under both stimulated and control conditions in intact cells were employed.

Figure 6.5.1 (A) and (B) demonstrate the phosphorylation of Ins-1,4,5-P_3 to Ins P_4 by the supernatant fraction prepared from whole rat brain as in (a) but diluted 10-fold to give a 2% (w/v) supernatant before inclusion in the assay. Figure 6.5.1 (A) shows the elution profiles obtained on Dowex chromatography for samples of ^{3}H-Ins-1,4,5-P_3 incubated (right) or not incubated (left) with the enzyme. The untreated sample shows a single peak at 0.8 M formate as anticipated for a pure standard of Ins P_3, while that incubated with enzyme shows three distinct peaks at 0.4, 0.8 and 1.0 M formate, respectively corresponding to ^{3}H-Ins P_1 + ^{3}H-Ins P_2, ^{3}H-Ins P_3 and ^{3}H-Ins P_4. It is important to note that despite

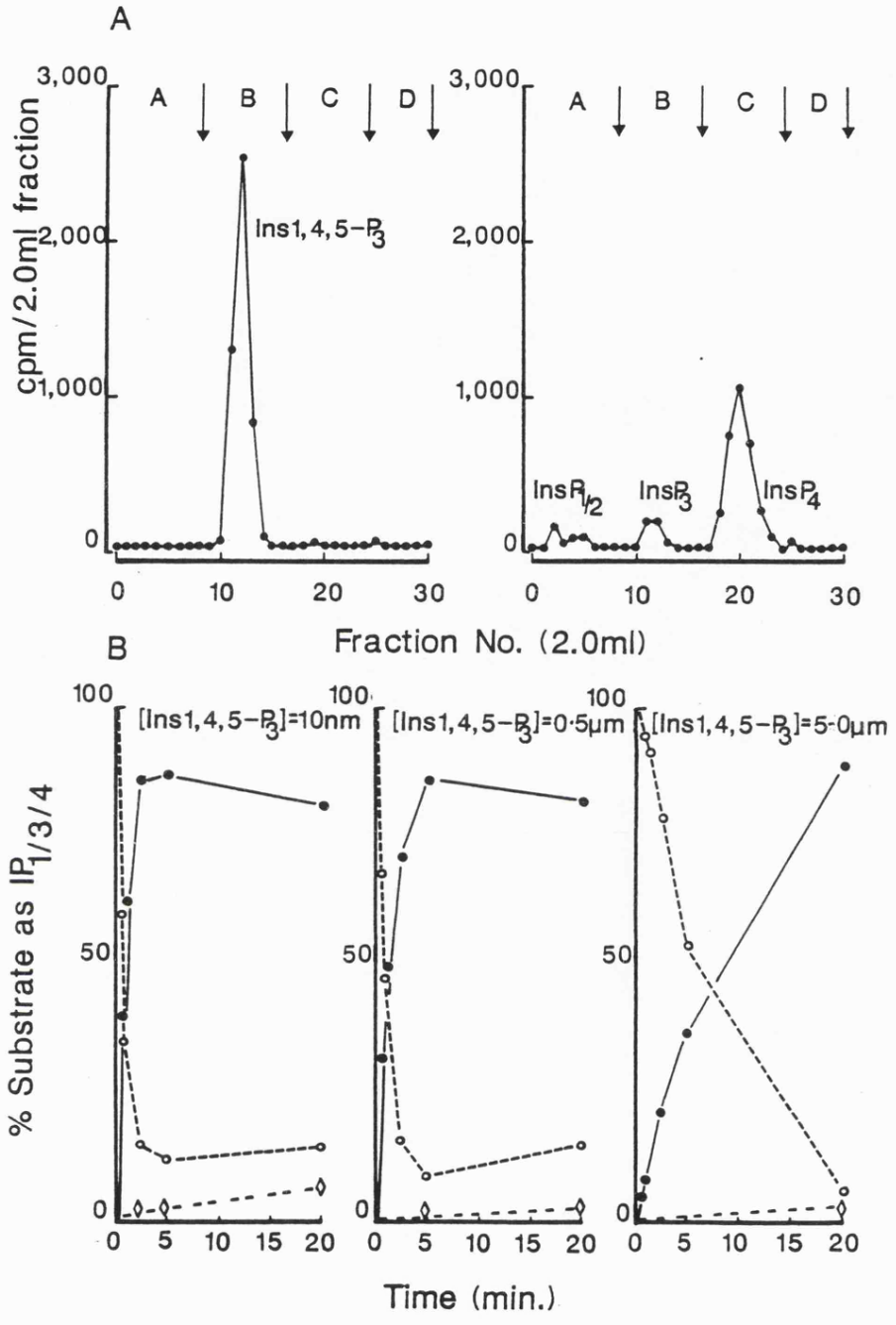


FIGURE 6.5.1 The conversion of ^{3}H-Ins-1,4,5-P_3 to ^{3}H-Ins P_4 by a soluble kinase from rat brain.

A whole rat brain supernatant (20% w/v) was prepared as in the text and diluted 10-fold with 0.15 M sucrose. 50 µl of this preparation was incubated at 37°C in Tris-maleate buffer (50 mM, pH 7.5) containing 10 mM ATP and 20 mM $MgCl_2$ and ^{3}H-Ins-1,4,5-P_3 at the indicated concentrations in a final volume of 200 µl. Reaction products were extracted as in the text. Fig. A shows the elution profiles from anion exchange columns for a ^{3}H-Ins-1,4,5-P_3 standard before (left) and after (right) incubation under these conditions for 5 min. Buffers A-D represent: 0.4 M AF/ 0.1 M FA; 0.8 M AF/0.1 M FA; 1.0 M AF/0.1 M FA and 2.0 M AF/0.1 M FA. Fig. B shows the time course for ^{3}H-Ins-1,4,5-P_3 (○), conversion to ^{3}H-Ins P_4 (●) and ^{3}H-Ins P_1 (◇) under similar conditions using the indicated substrate concentrations. ^{3}H-products were separated as in CH.4. Results are from a single experiment but were essentially reproduced on several further occasions.

elution of columns with formate concentrations up to 2.0 M no radioactivity exhibiting greater polarity than ^{3}H-Ins P_4 was recovered. This suggests that while the soluble fraction from rat brain possesses Ins P_3 kinase activity, it is unable to rapidly catalyse formation of significant quantities of either Ins P_5 or Ins P_6. A similar observation has been reported with homogenate preparations from both rat liver and brain (Hansen et al. 1986). Although the incubation period used in the current experiment was limited to 5 min. essentially identical results were obtained even after up to 30 min. (not shown).

Figure 6.5.1 (B) illustrates the time courses both for the rate of Ins-1,4,5-P_3 removal from the incubation medium and that for Ins P_4 accumulation under conditions identical to those in Figure 6.5.1 (A), except that the substrate concentration was varied between 10 and 5,000 nM. Figure 6.5.1 (B) also shows the rate of ^{3}H-Ins P_1 accumulation. A similar proportion of ^{3}H-Ins P_2 and a small, but detectable, amount of ^{3}H-inositol were also found in these incubations but this data has been omitted for clarity. It is clear from Figure 6.5.1 (B) that the time courses for the decline in ^{3}H-Ins-1,4,5-P_3 concentration and for accumulation of ^{3}H-Ins P_4 are practically mirror images of one another at each substrate concentration, suggesting the direct formation of the latter from the former. It is also evident that while accumulation of ^{3}H-Ins P_4 was apparent within 30 sec. of enzyme addition, accumulation of lower inositol phosphates was extremely limited even after 10-20 min. This may suggest formation of the latter through Ins P_4 dephosphorylation or simply a very slow rate of either Ins-1,4,5-P_3 and/or Ins P_4 breakdown. In either case it appears that under the conditions used there is little phosphatase activity capable of degrading either the substrate or major product, Ins P_4. However, although a substantial proportion of brain Ins-1,4,5-P_3 5-phosphatase is associated with the particulate fraction (Erneux et al. 1986) this observation is unlikely to reflect the complete absence of trisphosphatase activity in the preparation used since whole rat brain supernatants are known to hydrolyse Ins-1,4,5-P_3 (Erneux et al. 1986; 4.2; see below). Possibly such activity is largely inhibited at high ATP concentrations.

Finally, Figure 6.5.1 (B) illustrates two further interesting points. Firstly and most significant, is the rate at which Ins P_4 accumulates. At both lower substrate concentrations the maximal conversion (Ins P_3 to Ins P_4) of 85-90% is complete within 2.5 min., while even at higher concentrations there is close to 50% conversion within 5 min. Since these assays included the enzyme at a concentration approximately 200-fold lower than would be anticipated in whole brain, the rate of Ins-1,4,5-P_3 metabolism through this kinase reaction would suggest a half-life of this

molecule in stimulated tissue of less than 5 sec., assuming steady state concentrations in the range 0.5-5.0 μM, linearity of reaction rate with enzyme concentration, and a homogenous distribution of the enzyme in brain. Although, as previously, such estimates could be invalidated by any regulatory mechanisms not apparent under the current conditions, this very approximate value corresponds closely with that determined in 6.2. In view of the fact that the current estimate is based on conditions where trisphosphatase activity is negligible, this emphasises the capacity of brain to effectively remove the Ins-1,4,5-P_3 signal. The second important point illustrated by Figure 6.5.1 (B) is the active conversion of Ins P_3 to Ins P_4 even at concentrations (10 nM) predicted to be 5-10 fold lower than those expected under basal conditions in whole tissue (see above). This is of interest since, as noted in 6.3, control levels of ^{3}H-Ins-1,4,5-P_3 in brain are typically 4-5 fold higher than those of ^{3}H-Ins P_4 despite the probable more rapid turnover of the former, at least in the presence of agonist. Thus, these observations may indicate that factors other than Ins-1,4,5-P_3 concentration regulate or trigger the 3-kinase activity in intact cells.

Comparison of the current data with that so far available from other studies shows a number of differences. As would be anticipated from the identical conditions used, the present results correlate well with those of Irvine et al. (1986), by analogy with which study it is assumed that the Ins P_4 found here is the Ins-1,3,4,5-P_4 isomer which also accumulates in carbachol-stimulated brain and parotid gland slices (Batty et al. 1985b; Downes et al. 1986). However, the present data also contrast sharply with the results of Hawkins et al. (1986) and Hansen et al. (1986) since, although these studies indicate the presence of Ins-1,4,5-P_3 kinase in rat parotid gland and liver respectively, they also demonstrate a considerable extent of either substrate and/or product hydrolysis, with the former report indicating that only 15% of substrate Ins-1,4,5-P_3 accumulates as Ins P_4 when parotid gland homogenates are incubated with ATP and Mg^{2+} ions. These discrepancies may arise in part from the use of different tissues possessing different proportions of the relevant enzymes, but seem more likely to relate to preparational differences since both Hansen et al. (1986) and Hawkins et al. (1986) have used whole tissue homogenates rather than a supernatant as employed here. As a large proportion of brain Ins-1,4,5-P_3/Ins-1,3,4,5-P_4 5-phosphatase may be membrane bound (Erneux et al. 1986) it is possible that the results in Figure 6.5.1 (B) are less representative of the extents to which Ins-1,4,5-P_3 undergoes hydrolysis and phosphorylation than might have been observed with an homogenate. Alternatively, the exclusion of phosphatase activity to a significant extent suggests that the supernatant preparation

is a more convenient means of establishing the basic characteristics of the enzyme activity of primary interest to the current study.

Although the data presented in Figure 6.5.1 clearly demonstrate that the soluble fraction from rat brain contains enzymic activity capable of increasing the polarity of Ins-1,4,5-P_3 by an extent consistent with the phosphorylation of this substrate to an Ins P_4, they do not show that the product is specifically Ins-1,3,4,5-P_4. Equally, these results give no indication of the substrate specificity of this reaction. Both these points have previously been established under identical conditions (Irvine et al. 1986) but brief attempts were made to confirm these observations by comparatively simple methods, although it is emphasised that these would not be adequate in the absence of earlier work (Irvine et al. 1986).

Firstly, that the increase in Ins-1,4,5-P_3 polarity on anion exchange chromatography is the result of a phosphorylation specifically at the D-3 position of the inositol ring was confirmed by reaction of Ins-1,4,5-P_3 with the rat brain supernatant and ATP as described above, except that incubations were allowed to continue for 30 min. in an attempt to maximise any secondary breakdown of the Ins P_4 produced. The products were then analysed for Ins P_3(s) and Ins P_4 by hplc and compared with those resulting from an identical incubation with heat inactivated brain supernatant and with the products accumulating in response to carbachol stimulation of intact cerebral cortical slices. The results are shown in Figure 6.5.2. In this figure (A) demonstrates that pretreatment of the enzyme preparation by boiling prevented conversion of Ins-1,4,5-P_3 to Ins P_4 (hydrolysis to lower inositol phosphates was also prevented). In contrast the incubation of Ins-1,4,5-P_3 as in the legend to Figure 6.5.1 resulted in almost quantitative loss of substrate (B), with approximately 75% of the initial radioactivity being recovered in a fraction which co-eluted exactly with authentic Ins-1,3,4,5-P_3 extracted from stimulated brain slices (C). In the presence of active kinase a further 7% of the starting material was also recovered in a fraction with the precise chromatographic retention expected for Ins-1,3,4-P_3. These results strongly suggest that the initial and predominant reaction by which Ins-1,4,5-P_3 is metabolised under the conditions used involves phosphorylation at the D-3 position of the inositol ring to yield Ins-1,3,4,5-P_4. The production of a small, but significant, quantity of Ins-1,3,4-P_3 also indicates hydrolysis of this product by 5-phosphatase activity. The remaining 16-18% of the initial substrate not accounted for was assumed to have been degraded to lower inositol phosphates either directly or via Ins P_4 and Ins-1,3,4-P_3.

It is of interest to note here that as lithium ions markedly attenuate carbachol-stimulated Ins P_4 accumulation in cerebral cortical

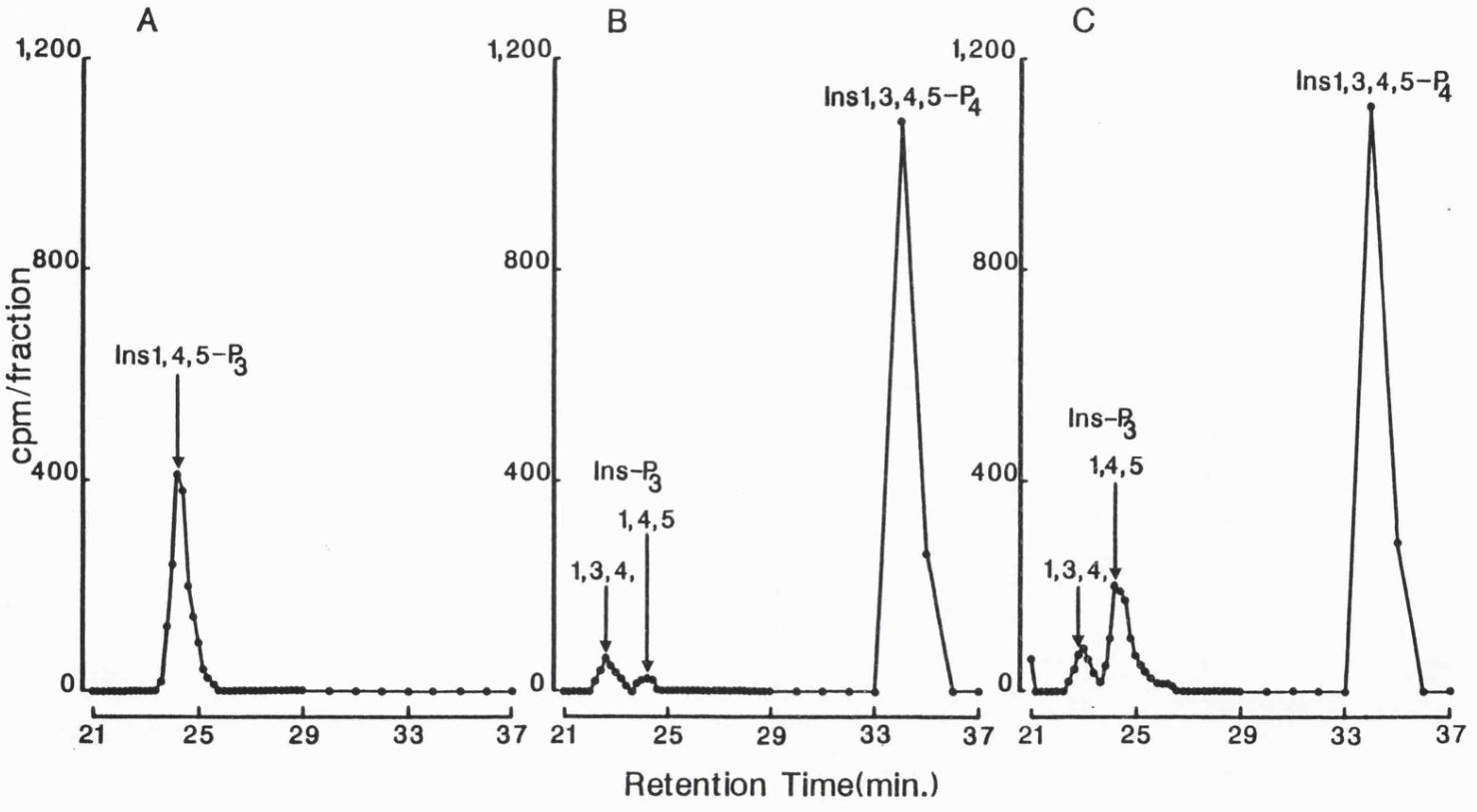


FIGURE 6.5.2 The inositol tris- and tetrakis- phosphates produced by incubation of ^{3}H-Ins-1,4,5-P_3 with a crude kinase preparation from rat brain.

Incubation of ^{3}H-Ins-1,4,5-P_3 (1.0 μM) with a whole rat brain supernatant was as in the legend to Fig. 6.5.1 except that an enzyme preparation either pre-treated by heating to 100°C (A) or not treated in this way (B) was used. After 30 min. at 37°C incubations were stopped and neutral extracts prepared and analysed for ^{3}H-Ins P_3 and ^{3}H-Ins P_4 by hplc as in CH.5. An extract from carbachol-stimulated ^{3}H-inositol labelled brain slices was similarly analysed (panel C).

slices, an effect of these ions on the conversion of Ins-1,4,5-P_3 to Ins P_4 might be anticipated. In the above experiment a parallel incubation was run in which 5 mM LiCl was included with the active enzyme. Under these conditions there was no clear effect of lithium on Ins P_4 accumulation nor on that of Ins-1,3,4-P_3, indicating that the previously observed effects of this ion (see 6.4) are unlikely to reflect a direct action on the 3-kinase. However, such an effect of lithium cannot be completely excluded on this simple basis since, as the conversion of Ins P_3 to Ins P_4 is essentially complete within 5 min. in the absence of this ion, a limited effect of lithium on either the K_m or V_{max} of the kinase might not have been clearly apparent over the 30 min. incubation used and is a possibility which requires further study.

Secondly, the substrate specificity of the demonstrated 3-kinase activity was briefly investigated by providing the crude brain supernatant with an alternative substrate. GroPIns P_2 was selected for this purpose for two reasons. Firstly, this molecule exhibits a phosphate substitution around the inositol ring with the D-1,4,5 configuration and thus, unless the enzyme exhibits high specificity, would be expected to be phosphorylated at a comparable rate to Ins-1,4,5-P_3. Secondly, if the kinase were able to utilise GroPIns P_2 as a substrate with a comparable or greater efficiency to that which it displays for Ins-1,4,5-P_3, this could indicate that PtdIns P_2 might also be a substrate for the enzyme. This in turn would suggest that a PtdIns P_3 could be formed in brain and provide an alternative source of Ins P_4. Figure 6.5.3 illustrates the results of a single experiment in which ^{3}H-GroPIns P_2 was either incubated with active (B) or heat inactivated enzyme (C) or left untreated (A) and the reaction products analysed by hplc. The enzyme concentration used was 10-fold higher than that employed in Figures 6.5.1 and 6.5.2. The ^{3}H-substrate was prepared by deacylation of a bulk lipid extract of ^{3}H-inositol labelled brain slices, from which ^{3}H-GroPIns P_2 was isolated by Dowex chromatography as in 3.5. The substrate concentration was not defined but from previous measurements of the PtdIns P_2 content of brain (see Hawthorne and White, 1975; Downes and Michell, 1982) was estimated at 10-30 μM. Figure 6.5.3 (A) demonstrates that the substrate was not significantly contaminated with ^{3}H-Ins P_3 while (B) shows that incubation of ^{3}H-GroPIns P_2 in the presence of enzyme, ATP and Mg^{2+} resulted in the production of a molecule chromatographically distinct from the established inositol mono- and diester phosphates. This ^{3}H-product accounted for 20% (i.e. 0.4-1.2 nmol) of the original ^{3}H-substrate and exhibited the chromatographic retention anticipated for a GroPIns P_3. Further evidence to support this structure was not sought, although analysis of the products of alkaline hydrolysis of this material would at least be

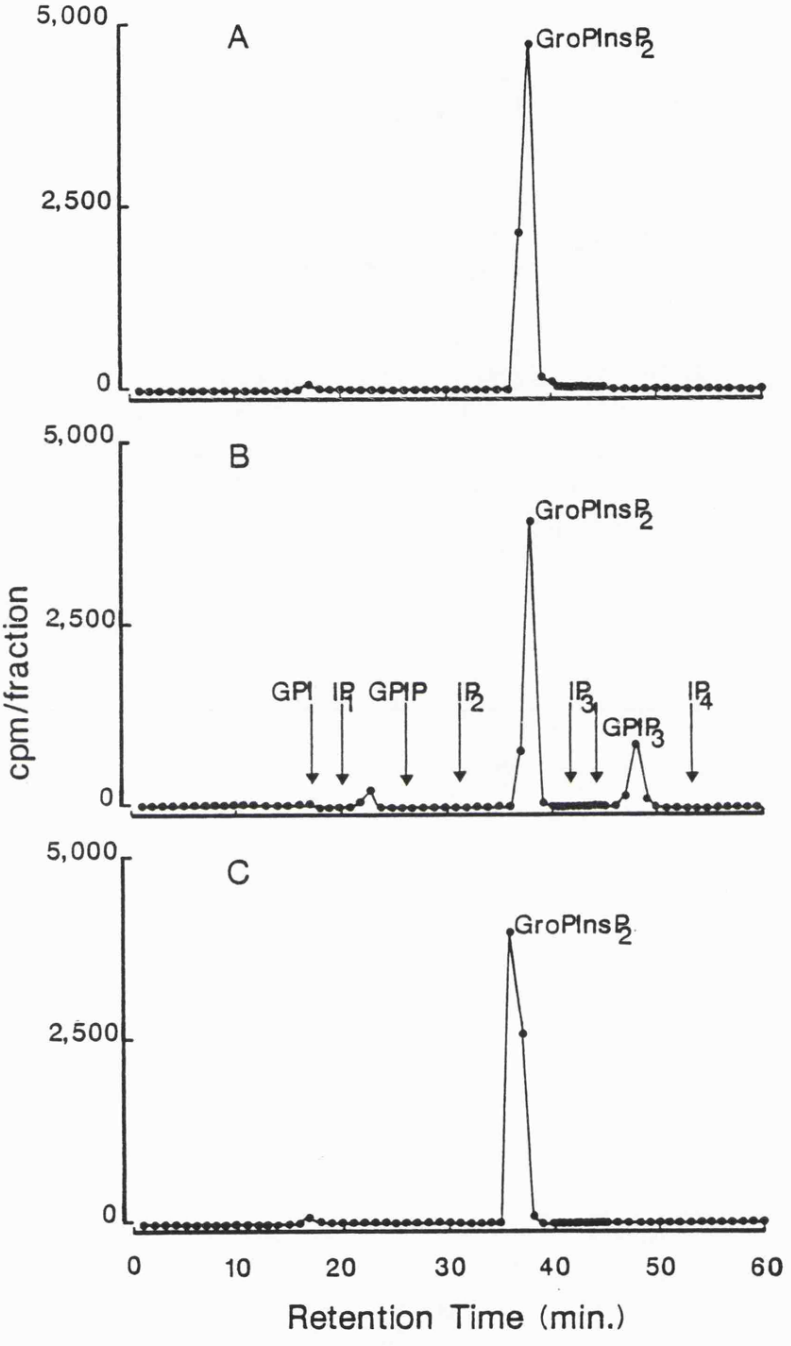


FIGURE 6.5.3 The activity of Ins-1,4,5-P_3 3-kinase on ^{3}H-GroPIns-4,5-P_2. ^{3}H-GroPIns P_2 was prepared by deacylation of ^{3}H-PtdIns P_2 extracted from ^{3}H-inositol labelled brain slices. Fig. A shows the purity of this substrate as determined by hplc according to Fig. 5.3.2. An estimated 2-6 nmol (10-30 μM - see text) ^{3}H-GroPIns P2 was incubated for 20 min. at 37°C with 50 μl of a 20% (w/v) whole rat brain supernatant in a final volume of 200 μl 50 mM tris-maleate buffer containing 10 mM ATP and 20 mM Mg^{2+}. Reaction products were extracted into PCA as previously. Figs. B and C respectively show hplc traces of the products resulting from incubation with active and heat inactivated enzyme. Results are from a single experiment.

expected to demonstrate that phosphorylation had occurred at the inositol and not the glycerol moiety. It is of interest that Hansen et al. (1986) have also reported the phosphorylation of GroPIns P_2 to a presumed GroPIns-3,4,5-P_3 by a similar enzyme preparation. The data presented in Figure 6.5.3 indicate that the kinase activity present in the soluble fraction from rat brain is able to utilise GroPIns P_2 as a substrate. However, assuming that the same enzyme catalyses phosphorylation of GroPIns P_2 and Ins-1,4,5-P_3, the data also show that the former is phosphorylated at less than 10% of the rate at which Ins-1,4,5-P_3 is used. Extracts from control or stimulated ^{3}H-inositol labelled brain slices contain negligible levels of ^{3}H-GroPIns P_2 but significant levels of ^{3}H-Ins-1,4,5-P_3 (see CH.4). Thus, it seems reasonable to assume that Ins 1,4,5-P_3 represents a more probable substrate for the kinase activity demonstrated. However, the intracellular concentration of Ins-1,4,5-P_3 in stimulated cells is likely to be in the micromolar range while it is possible that local concentrations of PtdIns P_2 within the plasma membrane may be closer to the millimolar range (see Downes and Michell, 1982). In view of the ability of the kinase to utilise GroPIns P_2 as a substrate, it may be of interest to determine any activity of this enzyme toward PtdIns P_2, although previous studies (see 4.4) would predict that this is likely to be negligible.

Taken together with the more detailed studies of Irvine et al. (1986) these results confirm the presence of an active and selective Ins-1,4,5-P_3 3-kinase in rat brain. However, although GroPIns P_2 provides a comparatively poor substrate for this enzyme it is clear that the kinase does not exhibit absolute specificity and on the basis of the current results it is difficult to exclude the possibility that at high concentrations other, related inositol compounds (e.g. Ins-1:2 cyclic 4,5-P_3, Ins-1,3,4-P_3, PtdIns P_2) would not also undergo phosphorylation. Despite this, as the enzyme appears to introduce a phosphate specifically at the D-3 position of the inositol ring, Ins-1,3,4-P_3 seems unlikely to represent an alternative substrate for the enzyme. Additionally, as Ins-1,4-P_2 is not phosphorylated by the soluble brain preparation (Irvine et al. 1986), it is possible that activity of this enzyme is restricted to trisphosphates or inositol phosphates bearing a vicinal phosphate pair at the 4 and 5 positions. The present data also suggest that substitution of the D-1 monoester phosphate with a glycerol moiety, as in GroPIns P_2 or PtdIns P_2, severely limits activity of the kinase. This is particularly significant as Ishii et al. (1986) have shown that a considerable proportion of the Ins P_3 accumulating in thrombin stimulated platelets is in the form of Ins-1:2 cyclic 4,5-P_3. Although this cyclic ester is a substrate for Ins-1,4,5-P_3 3-kinase (Irvine et al. 1986) it seems

possible, from the present data, that the cyclisation of the D-1 phosphate would render this a less suitable substrate such that, unless the intracellular concentration of Ins-1:2 cyclic 4,5-P_3 in stimulated cells markedly exceeds that of Ins-1,4,5-P_3, little Ins-1:2 cyclic 3,4,5-P_4 would be expected to accumulate. Clearly more detailed structure activity profiles for the enzyme will be essential to confirm these conclusions but it is of interest to note that the data so far available suggest the kinase exhibits very similar structural requirements for its substrate to those displayed by the Ins-1,4,5-P_3 'receptor' mediating release of intracellular Ca^{2+} (cf Berridge and Irvine, 1984 with Irvine et al. 1986 and the current results).

ii) Basic kinetic properties of Ins-1,4,5-P_3 3-kinase: Having established the presence of an active and relatively specific Ins-1,4,5-P_3 3-kinase in the soluble rat brain preparation used, further experiments were next conducted to define the capacity of this enzyme and its affinity for its substrates. In order to measure these parameters it was necessary to first modify the incubation conditions used such that linear rates of reaction could be achieved over a suitable time scale. From Figure 6.5.1 it is clear that the use of a 10-fold dilution of the initial preparation (a 20% w/v supernatant) resulted in metabolism of about 80-90% of the substrate (at concentrations between 10 and 500 nM) when incubations were run for only 2-3 min. Therefore, reaction rates were initially compared over a series of enzyme dilutions using low substrate concentrations (5-10 nM). Over several experiments it was found that when the original preparation was diluted up to 100-fold (i.e. 0.2% w/v supernatant), reaction rates were still linear for less than 5 min. However, further dilution resulted in disproportionately decreased or, occasionally, complete loss of activity which was prevented by inclusion of BSA in the assay. This suggested that at high dilutions the kinase was exposed to presumably non-specific protease activity, against which it was protected either in the presence of BSA or when brain supernatants were of sufficiently high endogenous protein concentration. These points are illustrated by Figure 6.5.4 (A) and (B). The lower figure (B) shows the time course of Ins P_4 accumulation as a result of kinase action on Ins-1,4,5-P_3 when the substrate was incubated with brain supernatants of between 100-500 fold dilutions, prepared in the presence and absence of 2% (w/v) BSA (final concentration 0.5%). Clearly as the dilution increased, a greater proportion of the enzyme activity was prevented from loss in the presence of BSA. Note that at 500-fold dilution (+ BSA) of the enzyme reaction kinetics were linear over about 15 min. This linear phase could be extended by dilution to 1,000-fold as routinely used in later

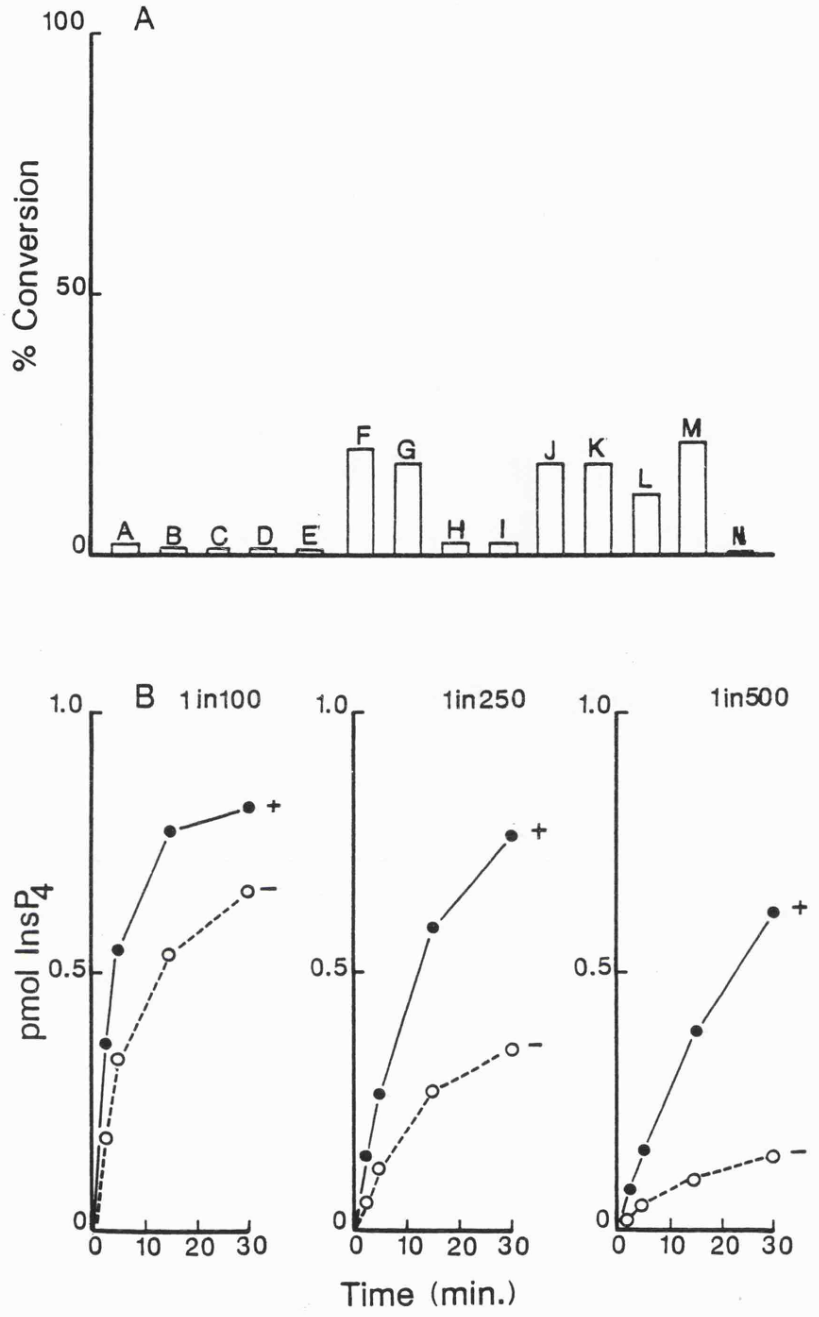


FIGURE 6.5.4 The activity of Ins-1,4,5-P_3 kinase at various dilutions and protection of activity against loss at high dilution.

Fig. A illustrates the protective effect of BSA but not of PMSF (or EtOH vehicle) on the ability of a high dilution of Ins-1,4,5-P_3 kinase to catalyse the formation of ^{3}H-Ins P_4. Incubations were run as previously except that the stock enzyme preparation (a 20% w/v brain supernatant) was diluted 1000-fold before inclusion in the assay. The concentration of ^{3}H-Ins-1,4,5-P_3 was 5 nM and the incubation time 15 min. at 37°C. A-N represent incubation of: enzyme alone; BSA (0.5% w/v) alone; BSA (1.25% (w/v) alone; EtOH (0.5%) alone; PMSF (0.5 mM) alone; enzyme + 0.5% BSA; enzyme + 1.25% BSA; enzyme + 0.5% EtOH; enzyme + 0.5 mM PMSF; enzyme + 0.5% BSA + EtOH; enzyme + 1.25% BSA + EtOH; enzyme + 0.5% BSA + 0.5 mM PMSF; enzyme + 1.25% BSA + 0.5 mM PMSF; control (using heat inactivated enzyme alone). Fig. B shows the time course of ^{3}H-Ins-1,4,5-P_3 (5 nM) conversion to ^{3}H-Ins P_4 by various dilutions of a 20% (w/v) whole rat brain supernatant incubated either with (●) or without (○) 0.5% BSA in the assay medium. Results are from a single experiment.

experiments. Figure 6.5.4 (A) compares the effectiveness of different BSA concentrations and of a protease inhibitor (PMSF - see legend) in protecting the enzyme activity. PMSF was diluted in absolute ethanol (EtOH) and hence the corresponding controls. It is evident from Figure 6.5.4 (A) that at 1,000-fold dilution (i.e. a 0.02% w/v supernatant) there was negligible kinase activity without added BSA. Incubation of ^{3}H-Ins-1,4,5-P_3 with BSA alone resulted in no Ins P_4 production, suggesting that the effect of this protein when included with brain supernatants is due to an ability to protect endogenous kinase activity in the latter. Increasing the BSA concentration from 2% to 5% (0.5-1.25% w/v final) did not further enhance the conversion of Ins-1,4,5-P_3 to Ins P_4 by the soluble brain preparation. Neither PMSF nor ethanol vehicle produced an effect equivalent to BSA and did not potentiate or negate the effect of BSA when co-incubated with this protein and enzyme. This suggests that if the effect of BSA is to protect the kinase from protease activity, the latter is unlikely to involve hydrolysis at serine residues, which reaction is typically inhibited by PMSF.

The linearity of the reaction kinetics achieved under the conditions quoted in the legend to Figure 6.5.4 (A) is further illustrated by Figure 6.5.5 where the time courses of Ins P_4 accumulation were compared over a range of substrate concentrations using a 0.02% w/v whole rat brain supernatant diluted in sucrose containing 2% w/v BSA. Clearly under these conditions the reaction kinetics were linear even at the lowest substrate concentrations for up to 15-20 min., with only 20-40% of the substrate being consumed in each case over this period. Initial rates of reaction at 5, 50 and 500 nM Ins-1,4,5-P_3 were 0.021, 0.230 and 1.000 pmol/min. respectively, suggesting deviation from first order kinetics only at concentrations approaching the K_m of the kinase (see below) for this substrate.

Having established conditions under which convenient reaction kinetics could be achieved, further studies were then made to define the K_m of Ins-1,4,5-P_3 3-kinase for each ATP and Ins P_3 using methods exactly as described in the legend to Figure 6.5.5. Initial studies were performed using concentration ranges of ATP and Ins-1,4,5-P_3 from 0.5-10 mM and 0.1-5.0 μM respectively. However, unusual reaction rates were found when low ATP concentrations were employed, particularly in conjunction with higher levels of Ins-1,4,5-P_3, such that as concentrations of the latter substrate approached the maximum used, the rates of Ins P_4 production declined from those seen at sub-micromolar substrate concentrations. Although other factors could account for this anomaly the most probable explanation was that at low ATP and high

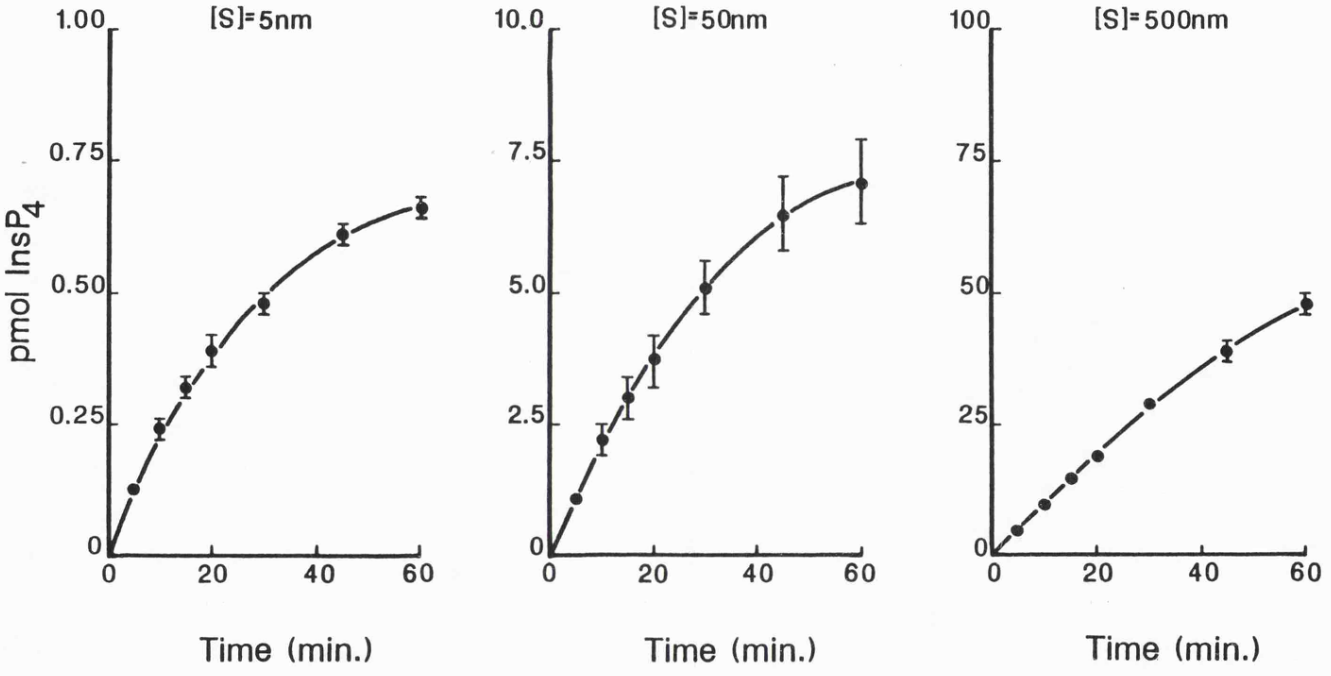


FIGURE 6.5.5 The conversion of ^{3}H-Ins-1,4,5-P_3 to ^{3}H-Ins P_4 by a crude kinase preparation: linearity of the reaction with time at varying substrate concentrations.

^{3}H-Ins-1,4,5-P_3 at 5, 50 or 500 nM was incubated at 37°C for the indicated times with 50 µl of enzyme in a final volume of 200 µl medium containing (final concentrations) 50 mM tris-maleate (pH 7.5), 10 mM ATP and 20 mM $MgCl_2$. The enzyme consisted of a 20% (w/v) whole rat brain supernatant diluted 1000-fold in 0.15 M sucrose containing 2% (w/v) BSA. Reactions were stopped by addition of PCA and ^{3}H-Ins P_4 separated from lower ^{3}H-inositol phosphates by anion exchange chromatography as previously. The results represent the mean ± SEM of 3 experiments except where SEM is included within the symbols shown.

Ins-1,4,5-P_3 there was significant loss of either the inositol phosphate substrate or product owing to phosphatase activity endogenous to the crude preparation used. Since both Ins-1,4,5-P_3 and Ins-1,3,4,5-P_4 are assumed to be catabolised primarily by hydrolysis of the 5-phosphate group, attempts were made to eliminate this problem by the inclusion of 2,3-bis-phosphoglycerate, an inhibitor of the Ins-1,4,5-P_3 5-phosphomonoesterase associated with human erythrocyte membranes (Downes et al. 1982). The addition of 5 mM 2,3-bisphosphoglycerate to incubations appeared to largely eliminate the above difficulties. However, it is not certain whether or not the appropriate phosphatase activities are entirely blocked at this concentration as, when using high ($\sim$5 μM) Ins-1,4,5-P_3 concentrations the dilution of stock ^{3}H-substrate with unlabelled material gave very low specific activities which, together with the high enzyme dilutions, resulted in accumulation of only very limited quantities of ^{3}H-Ins P_4, such that any secondary hydrolysis of this product would have been difficult to detect. Nevertheless, as little or no ^{3}H-Ins P_2, ^{3}H-Ins P_1 or ^{3}H-inositol was present after incubation of enzyme with 0.5 mM ATP and 5 μM ^{3}H-Ins-1,4,5-P_3 in the presence of 5 mM 2,3-bisphosphoglycerate, there is likely to be minimal loss of the original substrate under these conditions.

In a final set of experiments the original incubation conditions used for study of Ins-1,4,5-P_3 3-kinase were thus modified to those described in the legend to Figure 6.5.6. This shows the results of two experiments in which the kinase activity was assayed at either a fixed level of ATP (10 mM) while the Ins-1,4,5-P_3 concentration was varied (A), or the level of Ins-1,4,5-P_3 maintained at 5 μM while the ATP concentration was varied (B), in order to allow an estimate of the K_m for both substrates. In each experiment Mg^{2+} was present at twice the molar ATP concentration. Each Figure 6.5.6 (A) and (B) shows the effect of increasing substrate concentration on the rate of reaction, defined as pmol Ins P_4 produced per min. The inset in each figure shows a standard, double reciprocal plot (Lineweaver-Burke) of the same data, from which the appropriate K_m and V_{max} values have been estimated as shown. Both Figures 6.5.6 (A) and (B) show that phosphorylation of Ins-1,4,5-P_3 by the soluble fraction from rat brain displayed normal Michaelis-Menten kinetics with a K_m of approximately 0.7 μM and 1.3 mM for Ins-1,4,5-P_3 and ATP respectively. The former value agrees well with that determined by Irvine et al. (1986) (0.6 μM) and supports the discussions in previous sections. The K_m of Ins-1,4,5-P_3 3-kinase for ATP has not previously been reported but the value estimated falls well within the concentration ranges of this nucleotide found in vivo (typically 2 to 2.5 mM in rat brain, Bachelard and McIlwain, 1985). However, it might be anticipated from this K_m that,

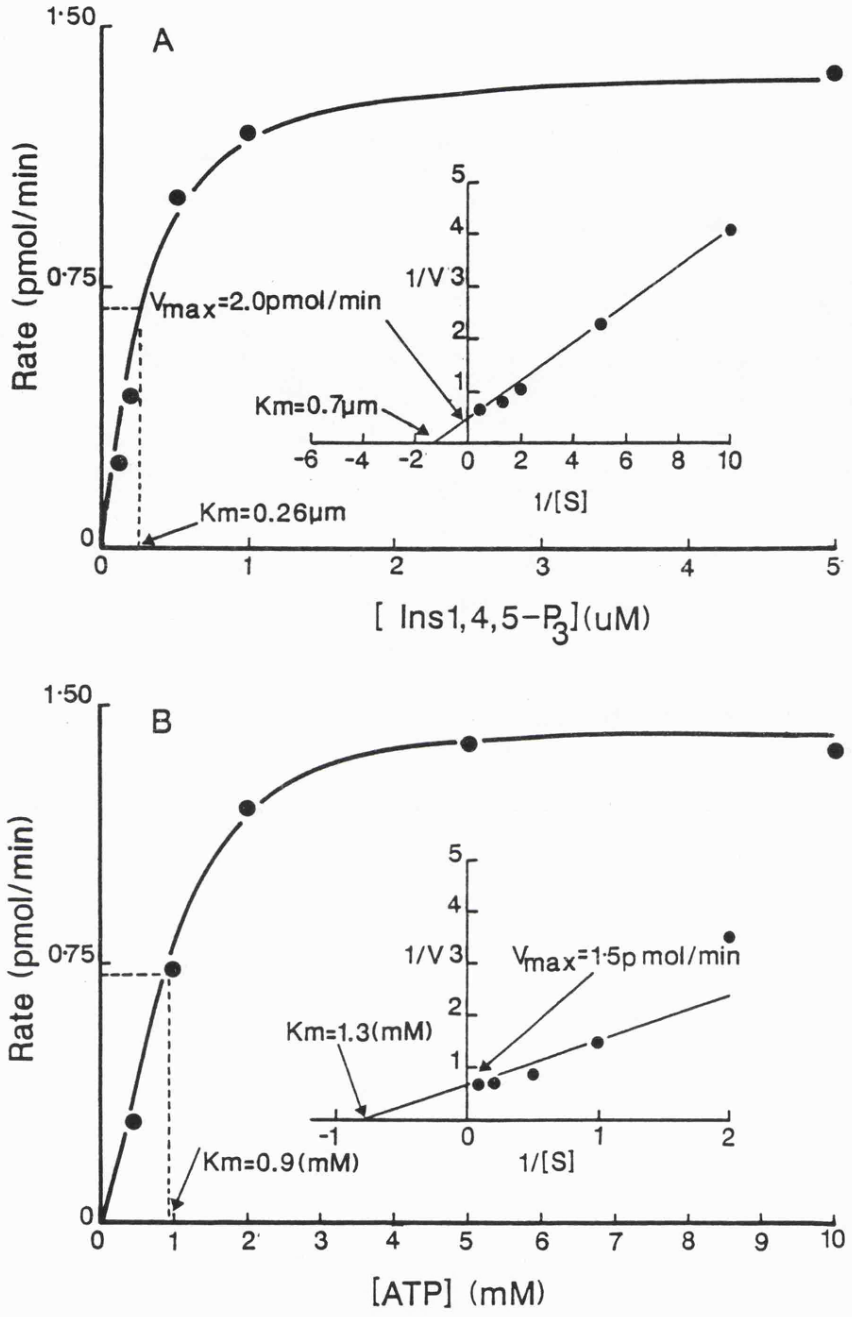


FIGURE 6.5.6 Determination of the K_m of Ins-1,4,5-P_3 3-kinase for its substrates.

Fig. A shows the estimation of the K_m for Ins-1,4,5-P_3. ^{3}H-Ins-1,4,5-P_3 at concentrations between 0.1-5.0 µM was incubated at 37°C for 15 min. with 50 µl of enzyme in a final volume of 200 µl medium containing 50 mM tris-maleate (pH 7.5), 10 mM ATP, 20 mM $MgCl_2$ and 5 mM 2,3-bisphosphoglycerate. Product ^{3}H-Ins P_4 was quantified as previously. Fig. B shows the estimation of the K_m for ATP. ^{3}H-Ins-1,4,5-P_3 (5 µM) was incubated as above except that the medium ATP concentration was varied over the range 0.5-10.0 mM with Mg^{2+} at twice the molar ATP content. Product ^{3}H-Ins P_4 was determined as above. Enzyme in each case consisted of a 20% (w/v) whole rat brain supernatant diluted 1000-fold in 0.15 M sucrose containing 2% (w/v) BSA. The inset in each Fig. A and B shows a double reciprocal plot of the same data. Each set of results is from a single experiment.

in a tissue particularly sensitive to anoxia such as brain, the magnitude of agonist-induced accumulations of Ins P_4 would be highly dependent on methods of tissue handling. In this respect the data presented in CH.2 is of interest since this demonstrated that responses (total ^{3}H-inositol phosphate accumulation) to carbachol in cerebral cortex appear more dependent on allowing tissue a post-preparational recovery phase than are responses to NA. Assuming that the effect of preincubation is to restore cellular ATP levels to close to in vivo concentrations (Fredholm et al. 1984) and that ATP-dependent steps are involved in responses to both agonists (via the requirement for PtdIns P_2 synthesis), it is possible that the greater sensitivity of muscarinic receptor-mediated responses to impaired tissue viability reflects a proportionally greater dependence on Ins-1,4,5-P_3 metabolism through 3-kinase activity than is associated with alpha-1 receptor-mediated responses.

Both Figures 6.5.6 (A) and (B) indicate a maximum reaction velocity of around 1.5-2.0 pmol Ins P_4 produced per min. Although the protein content of the enzyme preparation used was not determined, this value translates to approximately 1.8-2.5 nmol Ins-1,4,5-P_3 phosphorylated/min/mg protein assuming 8-10% (w/w) of whole rat brain wet weight constitutes protein. Clearly this is only a very approximate value but it nevertheless is in close agreement with the figure very recently estimated by Hansen et al. (1986) for the specific activity of the same enzyme activity in homogenates from rat brain cortex and between 2 and 3-fold greater than determined for a similar preparation from rat liver by the same authors. The good correlation between the present data obtained with a supernatant fraction from whole rat brain with that determined using a cerebral cortical homogenate (Hansen et al. 1986) suggests that the enzyme is predominantly cytosolic, as concluded by Irvine et al. (1986), and that its activity may be roughly homogenously distributed in brain.

In conclusion although the present results do not provide any evidence regarding the possible regulatory mechanisms affecting Ins-1,4,5-P_3 3-kinase activity, they clearly confirm the presence of this enzyme in brain and, under the assay conditions employed, reveal basic characteristics of this enzyme consistent with the inferences drawn with respect to Ins-1,4,5-P_3 metabolism via Ins P_4 in previous sections. As the capacity of brain to hydrolyse Ins-1,4,5-P_3 directly to Ins P_2 has not yet been clearly defined, it is not possible to predict from the data presented which of the kinase or phosphatase activities is likely to represent the predominant metabolic route. However, it is of interest that Irvine et al. (1986) have suggested (on the basis of their data for rat brain 3-kinase and that of Storey et al. (1984) for the Ins-1,4,5-P_3 5-phosphatse from rat liver) that hydrolysis and phosphorylation of

Ins-1,4,5-P_3 may proceed at approximately similar rates. The current data show close agreement with that of Irvine et al. (1986) with respect to the K_m of the 3-kinase for its inositol phosphate substrate, but suggest an estimate of the rat brain content of this enzyme several fold higher than determined by these authors but in agreement with that reported by Hansen et al. (1986). Together with the approximately 100-fold higher affinity of the brain kinase (K_m 0.6-0.7 μM, see above) than 5-phosphatase ($K_m \sim$ 50 μM, Erneux et al. 1986) for Ins-1,4,5-P_3, this perhaps suggests that metabolism for this putative second messenger proceeds primarily via Ins P_4 and Ins-1,3,4-P_3 in brain. Clearly accurate evaluation of the preferred pathway of Ins-1,4,5-P_3 metabolism within stimulated cells will require further studies involving use of purified enzymes, assayed under more physiologically relevant conditions than employed here. However, despite its associated limitations, the present data demonstrate properties of the soluble Ins-1,4,5-P_3 3-kinase which support the results reported in 6.2 and conclusions in 6.3, suggesting that considerable and rapid flux through the inositol tris/tetrakisphosphate pathway is a probable, physiologically significant consequence of muscarinic receptor activation in rat cerebral cortex. This in turn may imply a functional role for Ins P_4 and/or Ins-1,3,4-P_3, although again it is apparent that extensive additional studies will be required to support this suggestion. It is probable that a clearer understanding of the significance of Ins-1,4,5-P_3 metabolism via 3-kinase action will arise from more detailed investigation of factors modulating this activity, for which the currently reported observations should provide a useful basis.

CHAPTER 7

Concluding Discussion

The current study has employed recently introduced techniques which allow the hydrolysis of inositol phospholipids to be assayed directly, in order to characterise this response to receptor activation in cerebral cortex. Numerous previous studies have shown that stimulation of various neurotransmitter receptors promotes the enhanced turnover of phosphoinositide(s) in brain (see Hawthorne and Pickard, 1979; Downes, 1982 and 1986; Nahorski et al. 1986). Consistent with these earlier observations, data presented in CH.2 and 3 demonstrate that exposure of ^{3}H-inositol labelled cerebral cortical slices to acetylcholine (or carbachol), noradrenaline, 5-hydroxytryptamine or histamine results in an increased accumulation of ^{3}H-inositol phosphate(s) and that each of these responses is potentiated by lithium ions. Further data in CH.2 show that the response to cholinergic agonists is mediated via muscarinic receptors, also in agreement with earlier studies (Brown et al. 1984; Jacobson et al. 1985).

Examination of the Ca^{2+} dependency of carbachol-stimulated ^{3}H-inositol phosphate(s) accumulation showed that this response is abolished in the presence of EGTA and is substantially ($\sim$40-50%) attenuated when the extra-cellular [Ca^{2+}] is reduced from 1.3 mM to approximately 10-30 μM (see CH.2). The former observation implies that the mechanism coupling muscarinic receptor activation to phosphoinositide cleavage in brain has an absolute requirement for Ca^{2+}, while the latter suggests a partial dependency on $[Ca^{2+}]_e$. Although interpretation of these data could potentially be complicated by the experimental protocol employed, in which the effects of Ca^{2+} depletion were studied over periods of prolonged (45 min.) receptor stimulation, the latter observation is consistent with the ability of the divalent cationophore, A23187 (Kendall and Nahorski, 1984), and of K^{+} ion depolarisation to promote inositol phosphate accumulation in cerebral cortex (Kendall and Nahorski, 1985b; Rooney and Nahorski, 1986). However, these observations do not necessarily imply that receptor-mediated phosphoinositide cleavage in brain is <u>secondary</u> to an initial increase in $[Ca^{2+}]_i$ since the magnitude, pattern and time course of the inositol phosphate response to carbachol all differ markedly from that promoted by agents thought to act by elevating $[Ca^{2+}]_i$ directly (see CH.3 and Baird and Nahorski, 1986) while responses to other agonists (e.g. NA) are additive with those to the Ca^{2+} ionophore (Kendall and Nahorski, 1984). At present the significance of the attenuated muscarinic receptor-mediated response at reduced $[Ca^{2+}]_e$ and of the effects observed to A23187 and K^{+} ion depolarisation is uncertain. Clearly, further studies are required in which the response to receptor stimulation in brain slices is evaluated over both much more

rapid time courses and more extensive and carefully controlled extracellular Ca^{2+} concentrations and in which the effects of these conditions are determined on each of the separate ^{3}H-InsP(s) resulting from phosphoinositide hydrolysis. Additionally, it should prove of future value to more thoroughly characterise the products of phosphoinositide hydrolysis resulting from exposure of brain slices to elevated $[K^{+}]$ and Ca^{2+} ionophores. The current data (CH.3) indicate that the metabolic sequence promoted by the former stimulus may be distinct from that initiated by muscarinic receptor activation since depolarisation with K^{+} ions promoted enhanced ^{3}H-Ins P_1 and ^{3}H-Ins P_2 accumulation without a detectable increase in ^{3}H-Ins P_3 in ^{3}H-inositol labelled tissue. A more recent study (Baird and Nahorski, 1986) has also demonstrated a proportionally greater Ins P_2 accumulation in brain in response to K^{+} ions than observed to carbachol but also showed significant Ins P_3 production to both receptor and non-receptor stimuli. Further studies on the influence of depolarisation and/or of Ca^{2+} ionophores on receptor-mediated inositol phospholipid/phosphate metabolism may, therefore, yield valuable information concerning the modulatory influence of Ca^{2+} on these processes.

The major objective of the present study was to determine whether receptor-mediated inositol lipid hydrolysis in brain follows a similar metabolic route to that proposed for other tissues (see CH.1). Having briefly examined the responses to several stimuli, therefore, that to muscarinic receptor activation was selected for the purpose of this study since this was much larger than observed for any of the other agents noted above. In agreement with Berridge et al. (1983), 1 mM carbachol stimulation of ^{3}H-inositol labelled cerebral cortical slices resulted in a several fold increased accumulation of ^{3}H-Ins P_1, ^{3}H-Ins P_2 and ^{3}H-Ins P_3 which remained elevated for up to 45-60 min. after agonist addition but which rapidly returned close to basal levels on addition of an excess of atropine. This effect of antagonist demonstrates a continuing turnover of each ^{3}H-inositol phosphate in the presence of agonist, indicating that the muscarinic receptor does not desensitize to carbachol over the time course of these experiments. The effective reversal of the stimulated levels of each ^{3}H-inositol phosphate (> 80% in each case) by atropine also strongly suggests that the agonist-increased accumulations of these ^{3}H-products closely reflect their increased chemical concentrations. Further, on addition of atropine to tissue slices previously stimulated to close to steady state accumulated levels of each ^{3}H-inositol phosphate, the relative rates of ^{3}H-product decline toward basal levels showed ^{3}H-Ins P_3 decayed faster than ^{3}H-Ins P_2 faster than ^{3}H-Ins P_1, as would be

consistent with a sequential route of product formation. However, although these studies clearly demonstrated the presence in brain of a muscarinic receptor-mediated phospholipase C active towards PtdIns P_2, their more precise interpretation in terms of a specific phosphoinositide substrate(s) for the receptor mechanism was complicated by the potential occurrence of separate Ins P_3 isomers as described in rat parotid gland (Irvine et al. 1984a) and subsequently in many other tissues (see Abdel-Latif, 1986).

A more rigorous analysis of the products of muscarinic receptor-mediated phosphoinositide cleavage in brain (using methods described in CH.4) showed that the ^{3}H-fraction initially designated as ^{3}H-Ins P_3 could be resolved into three components, two inositol trisphosphates with the chromatographic properties of Ins-1,3,4-P_3 and Ins-1,4,5-P_3 and a novel inositol tetrakisphosphate identified as Ins-1,3,4,5-P_4 (Batty et al. 1985b). Kinetic studies (CH.4 and 6) demonstrated that ^{3}H-Ins-1,4,5-P_3, ^{3}H-Ins-1,3,4,5-P_4 and ^{3}H-Ins P_2 accumulate without a detectable delay (i.e. $\ll$ 5 sec.) in ^{3}H-inositol labelled cerebral cortical slices exposed to 1 mM carbachol and that agonist enhanced formation of ^{3}H-Ins-1,3,4-P_3 and ^{3}H-Ins P_1 follow this initial response. Subsequent similar studies in many tissues and cell types (e.g. Hawkins et al. 1986; Hansen et al. 1986) have confirmed this sequence of events in response to a wide variety of cell-surface receptor agonists previously shown to stimulate inositol phospholipid cleavage. Detailed examination of brain phosphoinositides labelled with ^{3}H-inositol failed to detect an appropriate phospholipid precursor for Ins-1,3,4,5-P_4 (CH.4), a result confirmed in rat parotid gland (Hawkins et al. 1986). Similarly, the presence of the higher inositol phosphates (^{3}H-Ins P_5 and ^{3}H-Ins P_6), which have been detected in a variety of cell types after prolonged ^{3}H-inositol labelling (e.g. Heslop et al. 1985; Hawkins et al. 1986), are not present in significant quantities in cerebral cortical slices (i.e. these represent $<$ 5% of stimulated ^{3}H-Ins P_4 concentrations), at least following short term (1-2 hr.) labelling. Thus, these are unlikely to be precursors of receptor-mediated ^{3}H-Ins P_4 accumulation in brain. In contrast, the Ins-1,4,5-P_3 3-kinase, recently demonstrated in several tissues (Irvine et al. 1986), is present in brain (see 6.5) and rapidly catalyses the ATP-dependent formation of Ins P_4.

In the absence of demonstrated alternative metabolic routes, the data from the current study therefore suggests that an early event coupled to muscarinic receptor activation in cerebral cortex is the phospholipase C catalysed cleavage of PtdIns-4,5-P_2, resulting in the very rapid accumulation of Ins-1,4,5-P_3 and DG. The rapidity of this response is

illustrated by the observation that not only concentrations of ^{3}H-Ins-1,4,5-P_3 but also those of ^{3}H-Ins-1,3,4,5-P_4 increase by about 100% within 5 seconds of agonist addition. Since the ^{3}H-Ins P_4 is most probably derived via Ins-1,4,5-P_3 kinase, the initial cleavage of PtdIns P_2 must occur within a fraction of this time. The kinetics of PtdIns P_2 hydrolysis may be further emphasised by the rate of ^{3}H-Ins P_2 accumulation following receptor stimulation since this may also arise from Ins-1,4,5-P_3 via a specific 5-phosphatase. Although a direct breakdown of PtdIns-4-P may also account for the formation of ^{3}H-Ins P_2 in stimulated brain slices, the results presented in CH.6 indicate that, following prolonged receptor stimulation, metabolism of higher polyphosphates (^{3}H-Ins P_3 and ^{3}H-Ins P_4), ultimately derived from ^{3}H-PtdIns P_2, is the more significant source of ^{3}H-Ins P_2.

The kinetic data from this study, now confirmed in many tissues, also suggest dephosphorylation of ^{3}H-Ins-1,3,4,5-P_4 accounts for the delayed accumulation of ^{3}H-Ins-1,3,4-P_3 frequently observed on receptor stimulation. However, it should be noted that although this sequence of reactions (see CH.4) is now widely accepted, the current study has <u>not</u> demonstrated that metabolism of Ins-1,3,4,5-P_4 occurs <u>exclusively</u> via 5-phosphatase activity in brain. Indeed, the results presented in CH.6 showing the disparity between the maximal steady state accumulated levels of ^{3}H-Ins-1,3,4-P_3 and ^{3}H-Ins-1,3,4,5-P_4 in carbachol-stimulated brain slices, together with the measured rates of hydrolysis of these species, must indicate either metabolism of ^{3}H-Ins P_4 by additional, alternative routes or a very much more rapid turnover of ^{3}H-Ins-1,3,4-P_3 than is apparent in other tissues (e.g. rat parotid gland - Irvine <u>et al</u>. 1985). It should also be emphasised that, to date, the proportion of ^{3}H-inositol trisphosphate designated as ^{3}H-Ins-1,3,4-P_3 in brain slice extracts has been identified solely by its retention time on hplc. Further studies are therefore required in which the route(s) and product(s) of Ins-1,3,4,5-P_4 dephosphorylation in brain are more thoroughly characterised. Ideally these should include cautious comparison between metabolism in intact stimulated tissue with that by broken cell preparations particularly since recent observations may indicate differences between these, at least with respect to Ins-1,3,4-P_3 metabolism (cf Shears <u>et al</u>. 1987 and Irvine <u>et al</u>. 1987). These studies should assist interpretation of results such as those presented in 6.2.

At present, assuming production and degradation of Ins-1,3,4,5-P_4 solely via 3-kinase and 5-phosphatase activities respectively, the latter data strongly suggest that ^{3}H-inositol tris- and tetrakisphosphate dephosphorylation accounts for a large proportion of the lower ^{3}H-inositol

phosphates which accumulate in brain slices following muscarinic receptor stimulation (see 6.2). This in turn implies that PtdIns-4,5-P_2 is the prime, and potentially the exclusive, substrate for the receptor activated phospholipase C in rat cerebral cortex. This conclusion contrasts sharply with that of Ackermann et al. (1987) who suggest that muscarinic receptor-mediated Ins P_1 accumulation in rat brain results predominantly from PtdIns rather than polyphosphoinositide hydrolysis. This proposal is based on comparisons of inositol phosphate accumulations in vivo and the properties of isolated inositol phosphate phosphatases in vitro and studies of the effects of lithium ions on both these parameters. However, unless the relevant enzymes are assayed under conditions strictly appropriate to those in the stimulated cell, the first two situations are not necessarily comparable. Additionally, data from CH.3 and 6 show that lithium ions distort the pattern of inositol phosphate accumulation stimulated by muscarinic receptor agonists not only at the level of Ins P_1 and Ins P_2 but also at the level of Ins P_3 and Ins P_4. Such effects may therefore influence the interpretation of data derived from lithium treated brain tissue. In contrast, the current study has attempted to determine the flux through the separate inositol phosphate fractions in intact, stimulated cells. Although, as noted in 6.2, these measurements may be complicated by a variety of factors, they clearly show a sustained and continuous hydrolysis of PtdIns P_2 sufficient to promote substantial Ins P_1 and Ins P_2 accumulation in the continued presence of agonist. Under these conditions, release of both Ins-1,4,5-P_3 and of DG will be persistent such that the functional significance of a direct hydrolysis of PtdIns or PtdIns P is uncertain. Therefore, while the data presented here cannot eliminate the possibility of secondary cleavage of inositol phospholipids other than PtdIns P_2, they indicate that this is likely to be limited. Again, further studies of the isomeric products of inositol polyphosphate metabolism may help to clarify this situation since separate measurement of these may allow the predominating routes of inositol phospholipid and phosphate hydrolysis to be better defined.

The current study also suggests that the use of lithium ions may help to further elucidate these pathways and to identify potential regulatory mechanisms. Data presented in CH.3 and CH.6 show that lithium ions dose-dependently potentiate muscarinic receptor-mediated ^{3}H-Ins P_1 and ^{3}H-Ins P_2 accumulation yet concomitantly suppress that of ^{3}H-Ins P_3/ ^{3}H-Ins P_4 in ^{3}H-inositol labelled brain slices. The latter action is largely due to an effect on ^{3}H-Ins P_4 accumulation which may be most readily attributed to an indirect inhibition of Ins-1,4,5-P_3 3-kinase by this ion, although a precise mechanism cannot yet be inferred. While

similar effects of lithium to those observed here on Ins P_1 and Ins P_2 have previously been reported both in brain (e.g. Hallcher and Sherman, 1980; Berridge et al. 1982; Jacobsen et al. 1985) and in many other tissues (e.g. Drummond et al. 1984; Thomas et al. 1984), metabolism of Ins-1,4,5-P_3 and Ins-1,3,4,5-P_4, where studied, has been found to be unaffected by lithium ions, at least after short-term (5 min.) stimulation (Hansen et al. 1986). More extensive investigation will, however, obviously be required in order to establish whether the inhibitory actions of lithium ions observed here on metabolism of higher inositol polyphosphates are restricted to the CNS and studies of effects in distinct cerebral regions may also prove of future value. Berridge (1984) has discussed the possibility that the therapeutic actions of Li^+ may be related to the ability of this ion to inhibit Ins P_1 dephosphorylation, thus leading to a selective reduction in CNS concentrations of free myo-inositol and a consequent down-regulation of the phosphoinositide signalling system in this tissue (see also Drummond, 1987). At present it is difficult to assess the significance of the current observations in terms of this potential mode of action for lithium but the data shown may indicate a more complex situation since, whether acting directly or indirectly, Li^+ ions appear to exert multiple effects on cerebral inositol metabolism. The better characterisation of these effects may help to both identify the routes of metabolism and functions of phosphoinositide derived messenger molecules in brain and consequently to clarify the basis of lithium's therapeutic efficacy. For these reasons it may be of interest to more accurately define the dose-dependency of the attenuating actions of lithium on Ins P_4 accumulation and to investigate the agonist dose- and time-dependency of this effect and to establish any agonist specificity, particularly since preliminary data indicate selectivity for muscarinic over alpha-1 adrenergic receptor-mediated responses (Kendall and Nahorski, 1986). Investigation of the actions of lithium ions on the metabolism of the DG moiety derived from agonist stimulated phosphoinositide cleavage may also be valuable in this respect (see CH.3 and CH.6).

The results presented here also demonstrate that, in the absence of lithium ions, both the initial rate of accumulation and subsequent rate of turnover of Ins-1,3,4,5-P_4 stimulated by muscarinic receptor activation are very rapid in brain. This may indicate that Ins-1,3,4,5-P_4 and/or the Ins-1,3,4-P_3 derived from it has a second messenger function(s). This point is emphasised by the extent to which Ins-1,4,5-P_3 appears to be metabolised via an ATP-dependent mechanism in brain, despite the existence of an alternative, non-energy dependent route (5-phosphatase) for the

efficient inactivation of this primary signal. Further, the widespread significance of the former metabolic pathway is demonstrated by a large number of recent studies which show that activation of a diverse group of cell surface receptors coupled to phosphoinositide cleavage leads to the formation of both Ins P_4 and Ins-1,3,4-P_3 in many tissues (Batty et al. 1985; Heslop et al. 1985; Irvine et al. 1986; Hawkins et al. 1986; Hansen et al. 1986; Turk et al. 1986; Palmer et al. 1986; Biden and Wollheim, 1986). A clear function of equally wide significance has, however, yet to be defined for either molecule. It is of interest, nonetheless, that while Ins-1,3,4,5-P_4 is inactive in releasing Ca^{2+} from bound, intracellular stores, Ins-1,3,4-P_3 is effective in this respect, although several fold less so than Ins-1,4,5-P_3 (Irvine et al. 1986b). In contrast, Irvine and Moor (1986) have presented evidence that Ins-1,3,4,5-P_4 but not Ins-1,3,4-P_3 may be involved in the regulation of Ca^{2+} flux across the plasma membrane. Since this action of Ins-1,3,4,5-P_4 may be dependent on the prior release of Ca^{2+} from the Ins-1,4,5-P_3 sensitive intracellular store (Irvine and Moor, 1986), it is also significant that recent studies indicate the potential activation of Ins-1,4,5-P_3 3-kinase by changes in $[Ca^{2+}]_i$ over the physiological range (Biden and Wollheim, 1986; Zilberman et al. 1987). These observations raise the possibility that the initial release of Ca^{2+} triggered by Ins-1,4,5-P_3 may serve to potentiate the formation of Ins-1,3,4,5-P_4 and subsequently, thereby mediate translocation of Ca^{2+} across the cell membrane. In view of these potential inter-actions, it is of further interest to note that K^+ depolarisation of cerebral cortical slices not only promotes phosphoinositide hydrolysis in its own right (Kendall and Nahorski, 1985b) but also potentiates the response evoked by carbachol, particularly with respect to Ins P_4 accumulation (Baird and Nahorski, 1986), while the response to agonist alone is attenuated by a reduction in $[Ca^{2+}]_e$ (see CH.2). The significance of these observations in terms of the role(s) of phosphoinositide derived second messengers in cellular function in the CNS will, however, only be revealed by future investigations.

In conclusion, the current study has revealed several novel aspects of receptor-mediated inositol phospholipid metabolism in brain. Most importantly, by separate measurement of the different products accumulating in response to muscarinic receptor activation, the initial phospholipase C catalysed cleavage specifically of polyphosphoinositides has been clearly demonstrated for the first time in the CNS. Consistent with its putative second messenger role, the metabolism of Ins-1,4,5-P_3 has also been shown to occur rapidly in brain and to produce two further

inositol compounds, Ins-1,3,4-P_3 and Ins-1,3,4,5-P_4, not previously identified in this tissue. Taken together, the results relating to muscarinic receptor-mediated phosphoinositide cleavage, although open to alternative interpretation, are most consistent with a sequence involving a rapid and sustained (in the continued presence of agonist) hydrolysis predominantly of PtdIns-4,5-P_2 to yield Ins-1,4,5-P_3 and DG. The metabolic fate of the latter cannot be inferred from the data presented but the former appears to be rapidly degraded sequentially through Ins P_2 and Ins P_1 to free inositol or to be equally rapidly converted via a 3-kinase to Ins-1,3,4,5-P_4, with substantial flux resulting at least through the second route under conditions of both high and low receptor occupancy (see 6.2 and 6.3). Degradation of Ins-1,3,4,5-P_4 in turn most probably occurs via initial removal of the 5-phosphate group although if this is the sole pathway it seems likely that distinct enzymes or regulatory mechanisms control tris- and tetrakisphosphate metabolism in brain than operate, for example, in rat parotid gland (see CH.6).

Although extensive further investigation is clearly required to fully elucidate the mechanism of receptor-mediated phosphoinositide hydrolysis in brain, the current studies have, by establishing some of the probable metabolic sequences involved, provided preliminary evidence to show that the initial response to agonists has many features common to those reported for this system in less complex tissues. These observations together with the novel data relating to inositol tris- and tetrakis-phosphate metabolism and the influence of therapeutically relevant Li^+ ion concentrations on this process should provide interesting bases for future work both in the CNS and in peripheral tissues.

APPENDICES

A1: Assay Buffers

a. Krebs-Henseleit (Krebs): The buffer was prepared freshly for each experiment and gassed with O_2:CO_2 (95%:5%) for approximately 20 min. prior to use to give a pH$\sim$ 7.4. The composition was:

	(g/l)	(mM)
NaCl	6.94	118.6
KCl	0.35	4.7
$MgSO_4$, 7 H_2O	0.29	1.2
$CaCl_2$, 2 H_2O	0.19	1.3
KH_2 PO_4	0.16	1.2
$NaHCO_3$	2.10	25.0
Glucose	2.10	11.7

b. Incubation medium for brain phosphoinositide phosphodiesterase (see 4.2): The buffer had the following composition (final assay concentrations in mM) and was adjusted to pH 7.0.

	(mM)
HEPES	25.00 mM
NaCl	50.00 mM
$CaCl_2$	0.20 mM
deoxycholate	0.50 mg/ml

c. Incubation medium for brain Ins-1,4,5-P_3 3-kinase: The buffer had the following composition (final assay concentrations in mM) and was adjusted to pH 7.5.

	(mM)
Tris-maleate	50.0 mM
ATP	10.0 mM (but see text)
$MgCl_2$, 6 H_2O	20.0 mM (but see text)
2,3-bisphosphoglycerate	5.0 mM (where present)

Note: Where high enzyme dilutions were used 0.5% w/v (final concentration) BSA was included in the assay medium to prevent loss of activity - see text.

A2: Gradients for hplc analysis of inositol phosphates:

All gradients except No. 1 are for a two solvent system comprising A: H_2O and B: 1.7 M NH_4COOH, pH 3.7, with H_3PO_4 and are expressed as the percentage contributed by B at each time point. For gradient No. 1 elution from 12.1 to 22.0 min. was at 5% B, where B = 0.28 M NH_4COOH followed by elution with 1.7 M NH_4COOH/H_3PO_4 as above. Flow rates were 1.2 ml/min. except for No. 6 (1.25 ml/min.).

1		2		3	
T	%B	T	%B	T	%B
0.0	0	0.0	0	0.0	0
12.0	0	12.0	0	12.0	0
12.1	5	12.1	2	12.1	2
22.0	5	16.0	2	16.0	2
22.1	5	16.1	5	16.1	5
46.0	5	24.0	5	24.0	5
46.1	15	47.0	59	47.0	59
50.0	25	52.0	100	52.0	100
53.0	25	55.0	100	60.0	100
83.0	100	56.0	0	61.0	0
88.0	100				
90.0	0				

4		5		6	
T	%B	T	%B	T	%B
0.0	0	0.0	0	0.0	0
10.0	0	10.0	0	5.0	0
15.0	44	17.0	44	10.0	44
17.0	44	25.0	59	12.0	44
23.0	59	35.0	100	18.0	59
33.0	100	40.0	100	23.0	59
40.0	100	41.0	0	33.0	100
41.0	0			39.0	100
				41.0	0

A3: Drugs and chemicals etc:

All radiochemicals were from either New England Nuclear or Amersham International. Other chemicals were obtained from Fisons, Aldrich, BDH or Sigma.

Dowex anion exchange resin was obtained from Sigma; analytical grade resin was from Bio-Rad.

Drugs were from Sigma.

Enzyme preparations (where purchased) were from Sigma or Boehringer Mannheim.

High performance columns for hplc were from HPLC Technology or Technicol.

Liquid scintillation cocktails were from Fisons or May and Baker.

A4: Statistical analysis of data:

Where statistical significance is indicated in the text or figure legends this has been determined by Student's 't' test for either paired or unpaired data using a computer program whereby 't' values are calculated according to the formulae below. Groups of data have been considered significantly different if $P < 0.05$.

Comparison of paired data was according to:

$$'t' = \frac{\frac{Ed}{n} \times \sqrt{n-1}}{\sqrt{\frac{Ed^2}{n} - \left|\frac{Ed}{n}\right|^2}} \qquad \text{with } n - 1 \text{ degrees of freedom}$$

where E denotes 'the sum of'
d = the difference between each pair
n = total number of pairs

Comparison of unpaired data was according to:

$$'t' = \frac{\frac{Ex_2}{n_2} - \frac{Ex_1}{n_1}}{\sqrt{\left(Ex_1^2 - \frac{(Ex_1)^2}{n_1} + Ex_2^2 - \frac{(Ex_2)^2}{n_2}\right)\left(\frac{n_1 + n_2}{n_1 n_2}\right)}{n_1 + n_2 - 2}}$$

with $n_1 + n_2 - 2$ degrees of freedom

where Ex_1 and Ex_2 denotes the sum of values in groups 1 and 2
n_1 and n_2 = the number of sample values in groups 1 and 2

A5: Abbreviations:

Note: Ins P_1, Ins P_2, Ins P_3, Ins P_4, Ins P_5 and Ins P_6 refer to myo-inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphates respectively. Where appropriate the positions of the phosphate locants around the inositol ring are given to define a specific isomer (i.e. Ins-1-P, Ins-1,4-P_2, Ins-1,4,5-P_3). Enantiomeric compounds are ascribed as D- or L- only where essential. The abbreviations InsP or InsP(s) refer to a total inositol phosphate(s) fraction of imprecisely defined composition. The abbreviations PtdIns (or PI), PtdIns P (or PIP) and PtdIns P_2 (or PIP_2) refer specifically to phosphatidylinositol and its -4-monophosphate and -4,5-bisphosphate derivatives respectively. Similarly, GroPIns or (GPI), GroPIns P (or GPIP) and GroPIns P_2 (or $GPIP_2$) refer specifically to the deacylation products of these lipids. Where the potential occurrence of isomeric forms of inositol phospholipids is discussed these are designated as appropriate (i.e. PtdIns-3,4-P_2). Unless otherwise stated, 'inositol' refers to myo-inositol

Acetylcholine: ACh
Adenosine-3',5'-cyclic monophosphate: cAMP
Adenosine-5'-diphosphate: ADP
Adenosine-5'-monophosphate: AMP
Adenosine-5'-triphosphate: ATP
Ammonium formate: AF
Bovine serum albumin: BSA
Central nervous system: CNS
Cholecystokinin: CCK
Cytidine-5'-diphosphate: CDP
Cytidine-5'-monophosphate: CMP
Cytidine-5'-triphosphate: CTP
Diacylglycerol: DG
Diethylaminoethyl: DEAE
Dopamine: DA
Ethanol: EtOH
Ethylenediaminotetraacetic acid: EDTA
Ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N', tetraacetic acid: EGTA
Extra-cellular free calcium ion (Ca^{2+}) concentration: $[Ca^{2+}]_e$
External standard channels ratio: ESCR
Formic acid: FA

Formylmethionyl-leucylphenylalanine: fMet-Leu-Phe

γ-(gamma)-aminobutyric acid: GABA

Glycerophosphorylinositol: GroPIns or GPI

Glycerophosphorylinositol-4-monophosphate: GroPIns-4-P, GroPIns P or GPIP

Glycerophosphorylinositol-4,5-bisphosphate: GroPIns-4,5-P_2, GroPIns P_2 or GPIP_2

Glycerophosphorylinositol-3,4,5-trisphosphate: GroPIns-3,4,5-P_3 or GroPIns P_3

Glycerophosphorylserine: GroPSer

Guanine nucleotide binding protein: G-protein

Guanosine-3',5'-cyclic monophosphate: cGMP

Guanosine-5'-diphosphate: GDP

Guanosine-5'-monophosphate: GMP

Guanosine-5'-triphosphate: GTP

Half-life (half-time): $T_{½}$

High performance liquid chromatography: hplc

Histamine: HA

N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid: HEPES

5-hydroxytryptamine: 5-HT

Inorganic phosphate: P_i

Inositol (myo-inositol): Ins

Inositol bisphosphate: Ins P_2

Inositol-1:2-cyclic-4-bisphosphate: Ins-1:2 cyclic 4-P_2

Inositol-1:2-cyclic monophosphate: Ins-1:2 cyclic P_1

Inositol-1:2-cyclic-4,5-trisphosphate: Ins-1:2 cyclic 4,5-P_3

Inositol hexakisphosphate (phytic acid): Ins P_6

Inositol monophosphate: Ins P_1

Inositol pentakisphosphate: Ins P_5

Inositol phosphate(s) - as a total fraction of undefined composition: Ins P or Ins P(s)

Inositol tetrakisphosphate: Ins P_4

Inositol trisphosphate: Ins P_3

Intracellular free calcium ion (Ca^{2+}) concentration: $[Ca^{2+}]_i$

Monoacylglycerol: MG

Noradrenaline: NA

Nuclear magnetic resonance: NMR

Perchloric acid: PCA

Phenylmethylsulphonylfluoride: PMSF

3-sn-phosphatidic acid: PA

Phosphatidylinositol or 1-(3-sn-phosphatidyl)-D-myo-inositol: PtdIns or PI

Phosphatidylinositol-4,5-bisphosphate or
1-(3-sn-phosphatidyl)-D-myo-inositol-4,5-bisphosphate:
PtdIns-4,5-P_2, PtdIns P_2 or PIP_2
Phosphatidylinositol-4-monophosphate or
1-(3-sn-phosphatidyl)-D-myo-inositol-4-monophosphate:
PtdIns-4-P, PtdIns P or PIP
Phosphatidylinositol-3,4,5-trisphosphate or
1-(3-sn-phosphatidyl)-D-myo-inositol-3,4,5-trisphosphate:
PtdIns-3,4,5-P_3 or PtdIns P_3
Phospholipase C: PLC
Phytic acid: Ins P_6
Thyrotropin releasing hormone: TRH
Trichloroacetic acid: TCA

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SUMMARY:

Receptor-mediated phosphoinositide hydrolysis was studied in ^{3}H-*myo*-inositol labelled rat cerebral cortical slices. Several CNS neurotransmitter receptor agonists stimulated the hydrolysis of inositol phospholipid(s). Maximal responses to receptor stimulation showed the order, muscarinic cholinergic > adrenergic > serotonergic > histaminergic. Potassium ion depolarisation and a Ca^{2+} ionophore also stimulated the accumulation of ^{3}H-inositol phosphate(s).

The metabolic sequence of muscarinic receptor-mediated phosphoinositide breakdown was examined in detail. Carbachol stimulated the sustained accumulation (> 45 min.) of ^{3}H-Ins P_1, ^{3}H-Ins P_2, ^{3}H-Ins P_3 and of a novel ^{3}H-inositol phosphate identified as Ins-1,3,4,5-P_4. Kinetic studies showed that muscarinic receptor activation results in the rapid (< 5 sec.) increased accumulation of ^{3}H-Ins P_2, ^{3}H-Ins P_3 and ^{3}H-Ins P_4 while the onset of ^{3}H-Ins P_1 accumulation is delayed. Using hplc, the Ins P_3 fraction was resolved into two components with the retention times of Ins-1,3,4-P_3 and Ins-1,4,5-P_3. Stimulated accumulation of Ins-1,3,4-P_3 was preceded by that of the other polyphosphates. The probable formation of Ins-1,3,4-P_3 via Ins-1,3,4,5-P_4 dephosphorylation is discussed. A phospholipid precursor for Ins-1,3,4,5-P_4 could not be identified but production of this molecule via an ATP-dependent, Ins-1,4,5-P_3 3-kinase was confirmed. Studies of the rates at which the separate ^{3}H-inositol phosphates are hydrolysed in stimulated tissue suggest considerable flux through this kinase reaction and indicate that the majority of the ^{3}H-Ins P_1 and ^{3}H-Ins P_2 accumulating in response to agonist result from ^{3}H-tris- and ^{3}H-tetrakisphosphate metabolism. Pharmacological data support these conclusions for conditions of both high and low receptor occupancy.

Lithium ions markedly affected muscarinic receptor-mediated ^{3}H-inositol phosphate metabolism, dose-dependently potentiating stimulated ^{3}H-Ins P_1 and ^{3}H-Ins P_2 accumulations while concomitantly attenuating those of ^{3}H-Ins P_3 and particularly ^{3}H-Ins P_4. The latter effects were half-maximal at 1 mM Li^{+}, exhibited a delayed onset, were not related to receptor desensitization but may be indirect consequences of Ins P_1 phosphomonoesterase inhibition. The significance of these actions is discussed in the context of the potential second messenger roles of Ins-1,4,5-P_3 and Ins-1,3,4,5-P_4.