DIFFERENT-SIZED RAT MESENTERIC ARTERIES

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ABSTRACT

PURINERGIC CONTROL OF SMOOTH MUSCLE TONE IN DIFFERENT-SIZED RAT MESENTERIC ARTERIES

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It is known that different-sized blood vessels perform different functions within the circulation. Analysis of the literature suggests that there are diameter-dependent differences in the characteristics of neuronal regulation of arterial smooth muscle tone. The experiments described in this thesis were designed to investigate this by systematically studying the neuronal control of arterial tone in three sizes of artery using the rat mesentery as a model vascular bed. The role of purinergic transmission was of particular interest. My experiments show that α,β-meATP and suramin are far less potent in large arteries than in smaller vessels. Contractions evoked by brief trains of nerve stimulation are mainly purinergic in small and medium-sized arteries but almost entirely adrenergic in large arteries. Investigation of the sources of calcium for contraction revealed that blockade of L-type calcium channels has very little effect on agonist or nerve-evoked contractions. Inhibition of calcium-induced calcium release also caused hardly any reduction of contractile responses. Despite the pharmacological differences observed in whole tissue experiments, immunohistochemical analysis showed no substantial differences in the expression pattern of P2X receptor isoforms in the three sizes of artery. Electrophysiological experiments indicate that P2X current densities are broadly similar in all arteries, and in contrast to contraction studies, currents were always suramin sensitive. In addition, all post-junctional responses to P2X receptor agonists are abolished in vas deferens from $P2X_1$-deficient mice. The two main conclusions that can be drawn from my data are i) purinergic transmission is more important in sympathetic nerve-mediated control of arterial tone in small arteries which are crucial in blood pressure regulation ii) almost all the calcium required for smooth muscle contraction is provided by calcium entry through the P2X receptor channel. This may have important implications for the treatment of hypertension.

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1. INTRODUCTION

1.1 AIMS OF THESIS

Neuronal control of peripheral vascular smooth muscle tone is achieved through the action of the sympathetic nervous system. ATP and noradrenaline are co-transmitters released from sympathetic nerve terminals and together mediate most vasoconstrictor effects of nerve stimulation. Their relative contribution to the overall response has however been seen to vary greatly, depending on experimental conditions and on the tissue used. One of the aims of this thesis was to investigate the properties of sympathetic transmission in arteries and to determine whether the relative importance of noradrenaline and ATP varies with arterial diameter. The rat mesenteric bed was the model system used and three sizes of artery were compared throughout all studies.

The essential cellular event triggering smooth muscle contraction is an increase in the cytosolic calcium concentration. There are three means by which calcium levels in vascular smooth muscle cells can rise in response to sympathetic nerve stimulation. ATP-mediated activation of P2X receptors allows influx of extracellular calcium into the cytosol. This activation also depolarizes the smooth muscle cell, which can lead to the opening of voltage-dependent calcium channels. In addition, noradrenaline activates α_1-adrenoceptors causing calcium release from intracellular stores. There are conflicting reports as to the relative importance of these pathways, again with an implied diameter dependence. Studying different-sized vessels from one arterial bed would also provide evidence for possible differences in the sources of increased intracellular calcium depending on arterial diameter.

The studies described in this thesis were thus designed to systematically address two key questions. Firstly, what are the relative roles of ATP and noradrenaline in mediating sympathetic vasoconstriction and does purinergic transmission become more important as arteries become smaller? Several approaches were used: in addition to characterising neurogenic contractions, the properties of P2X and P2Y receptor-mediated control of vascular smooth muscle tone were analyzed in different-sized arteries. Experiments were conducted both in whole tissue and at the single cell level. To complement the results of these functional studies, potential diameter-

dependent differences in expression of both P2X and P2Y receptor subtypes were determined using molecular and immunohistochemical techniques. In a further investigation of the role of different P2 receptor subtypes, the effect of deletion of the $P2X_1$ gene on purinergic contractile responses was studied.

The second question addressed by these experiments is: what contribution do the above mentioned pathways make towards increased cytosolic calcium associated with sympathetic nerve-evoked arterial contraction and do their relative roles change with vessel diameter? One aspect of the latter question which is of particular interest, is the contribution that voltage-dependent calcium channels make toward calcium influx associated with P2X receptor-mediated vasoconstriction. In addition, any potential contribution from intracellular calcium stores was assessed.

The following introduction is split into two parts. The first is designed to give the reader a general overview of several topics which will set the scene for the second, more specific part of this chapter. It will discuss several important milestones in the history of purines research and will introduce the receptors that mediate nucleotide signaling. The second part relates more directly to the subject of this thesis. It will outline the role of the sympathetic nervous system and nucleotide receptors in the regulation of arterial smooth muscle tone.

1.2 PURINERGIC SIGNALING AND P2 RECEPTORS

In order to give a general introduction to the field of P2 receptor signaling, I will give an historical overview, quoting several key papers that established an extracellular signaling role for ATP, culminating in the co-transmission hypothesis. In addition, I will describe the classification of P2 receptors leading through to the advances that were made with the availability of molecular data. An in depth account of the properties of P2 receptors will be given, paying particular attention to P2X receptors.

1.2.1 HISTORICAL PERSPECTIVE OF NUCLEOTIDE SIGNALING

Today, the study of the extracellular effects of ATP and other nucleotides and their cell surface receptors is a large research field in its own right. Initially, there was however substantial opposition to the notion that ATP could act as a signaling molecule outside the cell. This was mainly because much research had emphasized its role as the molecule fueling all energy-requiring processes inside the cell. In the following three subsections I will review the evidence from several key papers which a) recognized the extracellular action of purine compounds, b) lead to the proposal that ATP could act as a neurotransmitter and c) resulted in the hypothesis that ATP is not only released from nerves, but co-released with other transmitters.

1.2.1.1 Early History

ATP was isolated by two groups in 1929 (Lohmann; Fiske & Subbarrow), although adenine and adenosine had been identified many years previously. Around the same time Drury & Szent-Györgyi published a landmark paper recognizing the profound extracellular effects exerted by purine compounds (Drury & Szent-Györgyi, 1929); this could be described as the dawn of purine receptor research. They found that tissue extracts of sheep heart, kidney, brain and spleen significantly slowed the mammalian heart. The compound isolated from these extracts was AMP. Intravenous injection of AMP mimicked the negative chronotropic effect of the tissue extracts, as did injection of adenosine. Other effects of intravenous injection were impaired atrio-ventricular conduction, a general drop in blood pressure, general arterial dilatation and inhibition of intestinal movement. Similar findings were made in several other studies e.g. von Euler & Gaddum (1931) who also identified adenosine and an unknown related compound (presumably AMP) as the active substances isolated from tissue extracts. These early experiments made the link between crude tissue extracts and individual compounds thus identifying the active substance in each extract. As a result, subsequent studies were able to target their research more precisely by testing the effects of specific adenine compounds.

Despite the obvious extracellular effects of ATP and related compounds, much of the later research conducted into ATP focused on its intracellular functions. This was largely due to work

by Lipmann (1941) who proposed that ATP was the universal intracellular energy-providing molecule. Some groups nevertheless continued studying the compound's extracellular actions. Numerous studies were conducted, describing the biological actions of adenine compounds. Drury (1936) determined that the negative chronotropic effect of ATP was focused on the sino-atrial node, while Dubois-Ferière (1945) found a substantial blood pressure lowering effect of ATP in the rabbit. Green & Stoner (1950) summarized their own work on ATP and wound shock drawing together research from the previous 20 years in an important book entitled 'Biological Actions of Adenine Nucleotides'. They investigated the cardiovascular effects of adenine nucleotides, both on the isolated heart and after intravenous injection in the whole animal (or in man). Again, a general depressor effect was observed: negatively chronotropic and ionotropic, a drop in systemic blood pressure and general vasodilatation. They observed that the activity of adenine compounds increased with the length of the phosphate chain i.e. adenosine least active - ATP most active. Interestingly, the vasodilatory effect was not universal: vasoconstriction was seen in pulmonary and renal arteries. This was an early indication of what is now known to be the potent vasoconstrictor effect of ATP. All of these early experiments relied on the classical approach of using whole organs and biological assays to determine the activity of the compounds under investigation. In modern science, these techniques have been largely superceded by biochemical, pharmacological or physiological assays using isolated tissue or single cells.

The fact that ATP had such profound extracellular effects in these experiments suggested that it might have similar effects *in vivo*. A mechanism by which it is released from the cytosol into the extracellular space was therefore required. In an effort to identify the source of released ATP, research attention also turned to the nervous system. Very significant work in this field was carried out by Holton and Holton. In preliminary studies they found that electrical stimulation of sensory nerves in the rabbit ear resulted in vasodilatation and the appearance of ATP or a related substance in the venous effluent (Holton & Holton, 1953). It was also seen that injection of ATP mimicked the dilatory effect of nerve stimulation, which lead to the suggestion that ATP was liberated upon stimulation of the auricular nerve. The presence of ATP was detected with a luminescence assay and it was proven to be released from nerves and not from blood. When the auricular nerve had degenerated after section, electrical stimulation produced no increase in ATP

in the venous effluent (Holton, 1959). These experiments provided the first clear indication that ATP might play a role as transmitter that is released from nerve terminals.

1.2.1.2 ATP as a transmitter

It had therefore been established that ATP both had substantial physiological effects, and could be released from nerves. This suggested the existence of a junction or synapse where liberated ATP could exert its effects in target tissues. Around the same time, unexpected observations were made in autonomic neuroeffector transmission. Classically, it was believed that noradrenaline acting through α-adrenoceptors and acetylcholine acting through muscarinic receptors mediated all effects of the autonomic nervous system. However, some responses were seen which could not be accounted for by the two transmitters; these were described as non-adrenergic non-cholinergic (NANC). Important studies that made the link between ATP and NANC are described below. Two preparations that were frequently used at the time were the guinea-pig vas deferens and taenia coli.

Burnstock and Holman used intracellular recording techniques to study membrane potential changes associated with sympathetic nerve-mediated stimulation of vas deferens smooth muscle cells. Excitatory junction potentials (EJPs) evoked by transmural stimulation of the guinea-pig vas deferens were abolished by guanethidine or bretylium, which selectively block sympathetic nerves. However, α-adrenoceptor antagonists such as yohimbine and phentolamine had no effect (Burnstock & Holman, 1964). This suggested that a sympathetic transmitter other than noradrenaline was mediating the observed responses. One of the explanations proposed at the time was anatomical (limited access of drugs to receptors), and later work suggested the presence of a novel adrenoceptor that was resistant to commonly used antagonists (Hirst & Neild, 1980). However, it soon became apparent that this was an example of NANC transmission in the vas deferens, i.e. these antagonist-resistant responses were being mediated by ATP.

Other studies focused on the sympathetic innervation of the gastrointestinal tract. When excitatory responses of the mammalian stomach were blocked, transmural electrical stimulation

was found to evoke inhibitory responses that were resistant to adrenoceptor antagonists. Responses evoked by stimulation of perivascular nerves were however not resistant to such drugs (Paton & Vane, 1963). This suggested that a distinct network of neurons was present in the wall of the stomach, a theory supported by further evidence from studies on the guinea-pig taenia-coli. When contractile responses were blocked with atropine, two patterns of inhibitory response were observed. Stimulation of perivascular nerves at low frequencies caused only a slight relaxation of the tissue whereas the same stimulation applied transmuraly caused a substantial response. In addition, adrenergic neuron blockers abolished responses to perivascular stimulation but hardly affected those to transmural stimulation (Burnstock *et al*, 1966). These results seemed to confirm the presence of intramural nerves mediating responses of smooth muscle that were neither cholinergic nor adrenergic.

A possible candidate for the unidentified transmitter substance was ATP, as indicated by research in the preceding decades (see above). Burnstock *et al* (1970) studied the compounds released from guinea-pig stomach and Auerbach's plexus obtained from turkey gizzard upon stimulation of non-adrenergic inhibitory nerves. Adenosine and inosine, breakdown products of ATP, were obtained from stomach preparations while ATP, ADP and AMP were isolated from Auerbach's plexus. When exogenous ATP was added to the perfusate of the stomach vasculature, it was broken down to adenosine, inosine and adenine. Exogenous application of ATP mimicked the inhibitory effect of nerve stimulation in the taenia coli of various species. In addition, quinidine, a compound that had been shown to antagonise ATP-mediated responses, inhibited responses to both ATP and nerve stimulation.

Further evidence for NANC transmission was produced during the late 1960s and by 1970 such transmission had been shown to be present not just in the gastrointestinal tract, but also the cardiovascular and urinogenital systems. This experimental data was drawn together in a review by Burnstock where he first proposed the concept of purinergic nerves (Burnstock, 1972). In using the criteria for a neurotransmitter as put forward by Eccles (1964) he outlined the evidence in favour of ATP as the transmitter in NANC nerves:

(i) *Transmitters must be stored and synthesised in the nerve terminal.* This was demonstrated by Su *et al* (1971) and more recently by e.g. von Kügelgen & Starke (1991a)

(ii) *Transmitters must be released from nerve and effect of nerve stimulation must be mimicked by application of exogenous transmitter.* As shown by Burnstock *et al* (1970) and e.g. Ramme *et al* (1987); von Kügelegen *et al* (1994).

(iii) *A mechanism for transmitter inactivation must be present.* For example, Burnstock *et al*, 1970 and e.g Zimmermann (1999).

(iv) *Drugs altering nerve-mediated responses must have same effect on responses to exogenously applied transmitter.* As demonstrated by Satchell *et al* (1972) and more recently by e.g McLaren *et al* (1994)

As Burnstock himself pointed out, more experiments were required to confirm that ATP was the transmitter in these nerves (the recent examples show this has now been done). Nevertheless, this was the first step in establishing a role for ATP as a neurotransmitter.

1.2.1.3 Co-transmission Hypothesis

Classically it was believed that noradrenaline was the only transmitter released by sympathetic nerves. This was based largely on what has become known as Dale's Principle (1934), which states that a given nerve can only synthesize and release one transmitter. The research outlined below however showed that this was not the case. It has changed our understanding of sympathetic transmission (and neurotransmission in general) and may lead to the development of new therapeutic targets. With respect to this thesis, the fact that more than one transmitter mediates vasoconstriction may provide a new means of manipulating neuronal control of arterial tone. This may eventually result in new anti-hypertensive treatments.

Dale's Principle was one of the main reasons for the initial reluctance in accepting Burnstock's proposal that sympathetic nerves secrete both noradrenaline and ATP. An important early indication that co-release of noradrenaline and ATP from secretory cells may indeed be possible came from work on adrenal chromaffin cells. Cell fractionation studies had shown that the majority of the catecholamines in chromaffin cells are stored within membrane bound vesicles

(Blaschko & Welch, 1953). Further analysis of these chromaffin granules revealed that they existed in both light and heavy fractions of centrifugation. 'Heavy' granules were rich in catecholamines and adenine nucleotides while 'light' ones contained a lower concentration of noradrenaline and no nucleotides (Hillarp, 1960). The fact that these granules are involved in catecholamine secretion was strongly suggested by Douglas & Poisner (1966). They showed that substantial amounts of catecholamines and adenosine compounds (mostly AMP and adenosine, some ADP and ATP) appeared in the venous effluent upon stimulation of chromaffin cells by either acetylcholine or activation of the splanchnic nerve. The efflux of AMP and noradrenaline followed the same time course and the ratio of total adenine nucleotides to catecholamines in the effluent remained constant throughout the stimulation period. The ratio of total adenine nucleotides and noradrenaline in the effluent was also similar to that previously reported in chromaffin granules of the same species (Hillarp & Thieme, 1959). These results were interpreted to show that catecholamines (and ATP) are released from heavy chromaffin granules.

As sympathetic nerves innervate arterial smooth muscle and mediate neuronal control of arterial tone, it is of significance that chromaffin cells and post-ganglionic sympathetic neurons are developmentally similar (both originate from neural crest cells). This raised the possibility of similar co-storage and co-release in both cell types. Indeed, noradrenaline storage vesicles similar to those in chromaffin cells were seen in sympathetic nerves (von Euler & Lishajko, 1962) and these were later shown to also contain ATP (von Euler et al, 1963). Although it was not alluded to at the time, direct evidence for noradrenaline and ATP co-transmission in a physiological context came from elegant experiments conducted by Su and co-workers. They demonstrated that adenosine labeled with tritium was taken up by the isolated guinea-pig taenia coli and converted largely into [^{3}H]ATP. Electrical stimulation of sympathetic nerves supplying the tissue, induced a reduction in smooth muscle tone, which was accompanied by a substantial increase in tritium overflow. Both effects were blocked by incubation with guanethidine and were therefore caused by activation of sympathetic nerves (Su *et al*, 1971). Langer & Pinto (1976) studied contractions of the cat nictitating membrane smooth muscle. After near total noradrenaline depletion with reserpine, substantial responses to nerve stimulation remained. Residual contractions were unaffected by adrenoceptor antagonists and were shown not to be mediated by acetylcholine, while application of exogenous ATP and ADP evoked contractions in

both reserpine-treated and control tissues. These data lead the authors to propose that ATP or ADP may be mediating the non-adrenergic component of the response.

Both the above studies tie in nicely with previously mentioned work by Burnstock & Holman (1964). These experiments focused on electrical changes in the post-junctional membrane of tissues innervated by sympathetic nerves. Experiments on guinea-pig vas deferens showed that excitatory junction potentials (EJPs) were inhibited by drugs blocking sympathetic transmission (e.g. guanethidine) but were resistant to α-adrenoceptor antagonists (e.g. phentolamine mad yohimbine). This shows that electrical changes of the post-junctional membrane, which underlie smooth muscle contraction were also not mediated by noradrenaline. Co-transmission was therefore supported by evidence of i) ATP release from sympathetic nerves, ii) sympathetic nerve-mediated but noradrenaline-independent responses of smooth muscle and iii) sympathetic nerve-mediated but noradrenaline-independent EJPs. The arguments in favour of co-transmission were discussed by Burnstock in his key review entitled "Do some nerve cells release more than one transmitter" (1976). In this paper he suggested that ATP is co-released with noradrenaline from sympathetic and acetylcholine from parasympathetic nerves, theories which have since been confirmed. Today it is clear that co-transmission is not exceptional but a common feature of the nervous system, for example, several combinations of transmitters and neuropeptides can be released from autonomic nerves (for review see Lundberg, 1996).

1.2.2 P2 RECEPTORS

As interest in the field of nucleotide research grew, ATP and related compounds were shown to have a wide range of effects in various tissues. This is well illustrated by the vasculature, where nucleotides can produce vasoconstrictive, vasodilatory and proliferative effects. A crucial step in the understanding of these physiological effects is the identification of the receptors which mediate them. In the next section I will describe the initial classification of P2 nucleotide receptors and the limitations of this early system. This will be followed by an account of the dramatic effect that molecular cloning has had on our understanding of P2 receptor classification and function. Both P2X and P2Y receptors will be discussed although the focus will be on P2X receptors.

1.2.2.1 Initial Identification and classification of P2 Receptors

Purine receptors can be divided into P1 and P2 subtypes based on the initial proposal by Burnstock (1978). This distinction could unambiguously be made thanks to antagonists such as and xanthine and its derivatives (e.g. theophylline), which are selective for P1 receptors. In addition, P1 receptors were said to be potently activated by adenosine and AMP while ATP and ADP were agonists at P2 receptors. A further distinction was that P1 receptors modified cellular cAMP levels while P2 receptors did not (today it is clear that the latter point is incorrect as some P2Y receptors can modify adenylate cyclase activity; see Table 2). Since then, molecular studies have confirmed this division. The nomenclature has also been changed, and P1 receptors have been subdivided in to A_1, A_2 and A_3 receptors for adenosine; for a review of the current nomenclature see Ralevic & Burnstock (1998).

Further studies on P2 receptors soon revealed that more than one receptor was involved in mediating the observed responses. An important finding concerning functional differences between P2 receptor types was that the time course of responses differed depending on the agonist used. Fedan *et al* (1982a) found that β,γ-meATP evoked transient contractions in guinea-pig vas deferens which were monophasic, regardless of agonist concentration. This resulted in a concentration response curve that was also monophasic (i.e. only one P2 receptor was activated throughout). In the case of ATP or adenosine tetraphosphate however, raising agonist concentration produced a response that contained both a transient and a sustained component. This resulted in a biphasic concentration response curve. The two types of response were a strong indication of different signal transduction pathways and therefore different receptors. These results were extended by experiments using the early P2 receptor antagonist aminoproprionyl ATP ($ANAPP_3$). For agonists producing biphasic responses, $ANAPP_3$ inhibited the transient but not the maintained contraction and converted the concentration response curve to monophasic (Fedan *et al*, 1982b). $ANAPP_3$ therefore appeared to selectively block responses at one of the receptors. Although the interpretation given at the time centered on differences in agonist hydrolysis, these results show the clear distinction between 2 different P2 receptors.

Many studies attempted to distinguish between P2 receptors using agonist potencies. For example one investigation compared responses in the guinea-pig bladder with those in the taenia coli (Burnstock *et al*, 1983). 2MeSATP was found to be 200 times more potent than ATP in causing relaxation of the taenia coli but equipotent with ATP in contracting the bladder. This was interpreted as an indication of two different P2 receptors, 2MeSATP being a potent agonist at the receptor in the taenia, but a weak agonist at the P2 receptor in the bladder. Another explanation could however also have been differential agonist breakdown in the two tissues. More conclusive evidence for the presence of two distinct P2 receptors came from experiments in one tissue. Kennedy *et al* (1985) found that both α,β-meATP and ATP caused contraction of the rat femoral artery but ATP was much less potent. When the smooth muscle tone of the vessel was raised with noradrenaline, ATP caused relaxation while α,β-meATP caused further contraction. Removal of the endothelium abolished the relaxant effect of ATP. This suggested that ATP and 2MeSATP were acting through two distinct receptors. Further confirmation of this result came from raised tone experiments using 2MeSATP. In the resting rabbit portal vein this compound had no effect but in a raised tone preparation it produced a relaxation at a higher potency than ATP (Kennedy & Burnstock, 1985). These data and an extensive review of the literature lead Burnstock & Kennedy (1985) to the proposal of two subtypes of P2 receptor. P_{2x} receptors mediate a rapidly desensitising contraction of vascular and bladder smooth muscle and have an agonist rank order potency of α,β-meATP = β,γ-meATP > 2MeSATP ≥ ATP. P_{2y} receptors on the other hand, cause sustained relaxation of the same smooth muscles and have the following order of agonist potency: 2-MeSATP ≥ ATP > α,β-meATP = β,γ-meATP. In addition, P_{2x} receptors were desensitised by α,β-meATP whereas P_{2y} receptors were not. Following on from this proposal, numerous studies were conducted in vascular tissue confirming this pharmacologically and functionally based distinction between P_{2x} and P_{2y} receptors.

At this point, based on findings in whole tissue, clear functional and apparently clear pharmacological differences between P2 receptor subtypes were established. The pharmacological differences however relied to a large part on differences in agonist potency, which, as will be explained later, is not an ideal basis for receptor classification. The difference in time courses was less ambiguous and suggested distinct signal transduction pathways for the two receptors. More detailed explanations for these functional differences could however be

obtained by studying responses at the single cell level. Electrophysiology studies by Benham & Tsien (1987) showed that ATP directly gated a non-selective inward cation current in dissociated vascular smooth muscle cells that activated and inactivated rapidly and reversed around 0 mV. Similar recordings were made in rat sensory neurones (Krishtal *et al*, 1988) and bullfrog cardiac cells (Friel & Bean, 1988). These results indicated that the P2 receptor activated by ATP in these cells was a ligand-gated ion channel, i.e. a P2X receptor.

Friel & Bean however also observed another response to ATP application in bullfrog cardiac cells. In this case a slowly developing and maintained outward current was evoked, which reversed around –90 mV (Friel & Bean, 1988). This response was clearly very different to the one observed by Benham & Tsien. It was concluded that a hyperpolarising potassium conductance was being activated as a result of a second messenger induced release of calcium from intracellular stores. This was supported by biochemical studies in other tissues, which determined that activation of P_{2y} receptors induced the production of inositol trisphosphate (Brock *et al*, 1988) and activation of phospholipase C (Carter *et al*, 1988). In these experiments, ATP was therefore activating a G-protein coupled P2Y receptor, rather than an ionotropic P2X receptor.

As the effects of ATP were studied in more diverse tissues it became clear that not all effects could be accommodated by the two pharmacological profiles described above. Shortly after the initial classification into P_{2x} and P_{2y}, Gorden published a review confirming and extending the subdivision of P2 receptors to include P_{2t} and P_{2z} receptors (Gorden, 1986). The P_{2t} receptor was expressed on platelets and reported to mediate ADP-induced platelet aggregation, an effect inhibited by ATP (Cusack *et al*, 1985). Evidence was presented that this receptor was coupled to adenylate cyclase and intracellular calcium mobilisation (Sage *et al*, 1990). In contrast, activation of the P_{2z} receptor lead to the permeabilization of the cell, allowing influx of otherwise membrane impermeant dyes such as Lucifer Yellow or Fura-2. It was identified on several types of macrophage and other immune cells (Greenberg *et al*, 1988; El-Moatassim & Dubyak, 1992).

In addition, UTP was also found to evoke functional responses in certain cell types. Two frequently qouted reviews surveyed early experiments on the effects of UTP attempting to

specify the receptors involved (Seifert & Schulz, 1989; O'Conner *et al*, 1991). At this stage it was still unclear whether UTP and ATP were acting at the same site and how many receptors were mediating observed responses. O'Conner proposed the existence of a distinct P_{2u} receptor, which was activated by both ATP and UTP. This was confirmed in a later study by Motte *et al* (1993) which demonstrated that, in addition to the 2MeSATP-sensitive P_{2y} receptor, there was another receptor that was sensitive to both ATP and UTP.

Using this system of P2 receptor classification, there were thus five main subtypes with the following agonist profiles (Dubyak & El-Moatassim, 1993):

P_{2x} (ion channel):L–β,γ-meATP ≥ α,β-meATP = ATP = ADP > AMP

P_{2y} (GPCR): 2MeSATP ≥ ADPβS > ATP = ADP >> α,β-meATP = β,γ-meATP = UTP

P_{2u} (GPCR): UTP ≥ ATP = ATPγS > ADP > 2MeSATP > α,β-meATP = β,γ-meATP

P_{2t} (GPCR or ion channel): 2MeSADP > ADP

P_{2z} (pore forming ion channel): BzATP >ATP = ATPγS > > > ADP

Like all previous classification systems this one used responses in native tissues or cells relying heavily on agonist potencies to distinguish between receptor subtypes. As a result, the list of subtypes remained incomplete and the pharmacological profiles were not entirely accurate. In hindsight this approach was less than ideal; however the lack of selective antagonists, which would be more useful, made it the only option available. There are several problems associated with classifying receptors based on agonist sensitivity. Firstly, the functional responses that were assayed are not a direct reflection of agonist potency. For example, several cellular events separate the activation of a receptor on a smooth muscle cell and the initiation of cellular contraction. Differences in the excitation contraction-coupling pathway may result in apparent differences in agonist potency. Secondly, the compounds used may not have been pure. All purinergic agonists are closely related molecules and samples of any one compound are frequently contaminated by small amounts of similar compounds. This may have lead to unwanted activation of further purinergic receptors, which are sensitive to these contaminants. Finally, many ligands are subject to metabolic degradation. As a result, one cannot be sure

whether the compound applied is producing the observed response or one of its degradation products.

As increasingly diverse properties of P2 receptors were reported, it became evident that this classification system was no longer satisfactory. What was famously described as an 'apparent random walk through the alphabet' clearly needed to be replaced by a unified and systematic nomenclature that could easily accommodate the discovery of new receptor subtypes. In 1994 Abbracchio & Burnstock proposed a new classification system based on a comprehensive review of the literature with the goal of identifying pharmacologically and functionally similar P2 receptors. Although they took the important step of grouping all P2 receptors into two classes, ion channel and G-protein coupled receptor, further subdivision was still based on agonist profiles. The proposed scheme therefore required some modification. This is exemplified by their subdivision of P2X receptors into four isoforms, three of which are now known to be the same smooth muscle $P2X_1$ receptor. This once more demonstrates the shortcomings of using agonist potencies to classify receptors.

A definitive system of classifying P2X and P2Y receptors can only be created by characterising the proteins at the molecular level. With the help of nucleotide sequence data one can unequivocally group receptors into structurally similar subtypes and distinguish between individual isoforms. In the following two sections I will describe the dramatic advances that have been made in our knowledge of P2 receptor structure and function resulting from the availability of molecular data.

1.2.2.2 P2X receptors

P2X receptors were first cloned simultaneously by two independent groups: $P2X_1$ by Valera *et al* (1994) and $P2X_2$ by Brake *et al* (1994). Due to the lack of similarity with other ligand-gated ion channels, a homology-based approach was unsuccessful. Both groups therefore used expression cloning to obtain the sequence of the chosen receptor. The channels were isolated using a cDNA library constructed from tissue known to display robust excitatory responses to ATP: vas deferens for $P2X_1$ (e.g. Sneddon & Westfall, 1984) and rat pheochromocytoma PC12 cells for

$P2X_2$ (e.g. Nakazawa *et al*, 1990). When expressed heterologously, both cloned receptors displayed properties characteristic of the tissue from which they were isolated. These are for example, rapidly inactivating responses and α,β-meATP sensitivity for $P2X_1$ receptors and sustained responses and α,β-meATP insensitivity for $P2X_2$ receptors. Within two years, five further P2X isoforms had been cloned using strategies based on homology with the first two channels. Key structural and functional properties of the P2X receptor family are described below.

1.2.2.2.1 *Structure*

P2X receptors form a class of ligand-gated ion channel with unique structural characteristics. Seven isoforms have been identified, $P2X_1$ – $P2X_7$, which are between 379 and 595 amino acids in length and share between 26 and 47 % sequence identity. All isoforms share a similar overall structure, the only major difference being the size of the intracellular C-terminal tail. The sequences contain several fully conserved amino acids throughout the polypeptide chain e.g. cysteine, lysine and argenine. I will now outline our current knowledge regarding the structural characteristics of P2X receptors that govern some of its pharmacological and functional properties.

Membrane topology

The initial determination of the primary sequence of the P2X protein (Valera *et al*, 1994; Brake *et al*, 1994) indicated that these receptors represented a new and structurally distinct family of ligand-gated ion channels (North, 1996). Unlike the two other classes of ligand-gated ion channel (the nicotinic superfamily and the excitatory amino acid superfamily), P2X receptors have a predicted structure of two membrane spanning regions linked by a large extracellular loop (Fig. 1) . The only other ion channels with a similar membrane topology are subunits of certain sodium channels (Canessa *et al*, 1994; Linguelia *et al*, 1995), certain degenerins of C. elegans (Huang & Chalfie, 1994) and inward rectifier potassium channels (Doupnik *et al*, 1995); none of these channels are however ligand-gated. Despite these implied structural similarities, P2X

receptors do not share significant homology with any other class of ion channel. Figure 1 shows a schematic representation of the P2X subunit structure.

Valera *et al* (1994) and Brake *et al* (1994) proposed this structure based on the following features:

- hydrophobicity plots of the amino acid sequence showed only two regions long enough to span the membrane as an α-helix.
- the large hydrophilic domain linking the transmembrane sequences contains five possible consensus sites for N-linked glycosylation, suggesting it is on the extracellular side of the membrane. The fact that the previously solubilized $P2X_1$ receptor had a higher molecular mass than predicted from the amino acid sequence suggests these sites are used *in vivo* (compare Bo *et al*, 1992 and Valera *et al*, 1994).
- the lack of an N-terminal signal sequence suggests it (and hence the C-terminal) resides in the cytoplasm.
- numerous proline and serine residues in the C-terminal make this region a candidate for phosphorylation, and therefore located intracellularly.

The primary sequences of all remaining P2X isoforms predict a very similar structure (Collo *et al*, 1996; Surprenant *et al*, 1996). Strong support for this proposed membrane topology comes from work by Newbolt *et al* (1998) on $P2X_2$ receptors. Mutagenisis studies introducing artificial consensus sequences for N-linked glycosylation confirmed the extracellular location of the loop. Sites engineered immediately following TM1 (first transmembrane domain) and preceding TM2 (second transmembrane domain) were both glycosylated. In contrast, a site introduced in the N-terminal and presumably intracellular region of the protein was not modified. These results define the boundaries of the extracellular loop (before the peptide chain enters the membrane) and confirm the cytoplasmic location of the N-terminus. In a further elegant experiment two cDNAs were concatenated and expressed in oocytes to form functional channels (Newbolt *et al*, 1998). As each subunit was shown to contribute to the currents recorded, this data provides direct proof that both N- and C-termini are located on the same side of the membrane (i.e. the cytoplasmic side; Fig. 1).

Initially, it was believed that a short hydrophobic segment of protein immediately upstream of the second transmembrane domain, formed a re-entrant loop embedded in the cell membrane (Brake *et al*, 1994). Recent work by Schmalzing and colleagues however contradicts this theory. They found that an asparagine residue in close proximity to the putative loop is efficiently glycosylated with complex carbohydrate groups (Rettinger *et al*, 2000a). This would not be possible if adjacent segments of the protein were contained within the cell membrane as the required enzymes would be unable to gain access to target amino acid residues. This study also confirmed earlier work by Torres *et al* (1998) on recombinant $P2X_2$ receptor channels, showing the essential role of glycosylation in the formation of functional channels. In each case, elimination of all consensus sequences for N-linked glycosylation prevented cell surface expression of functional P2X receptors.

Ion permeation

An important aspect of understanding and manipulating channel function is to identify which parts of the protein are involved in forming the ion-conducting pore. There is evidence to suggest the second transmembrane domain of a structurally similar channel (Lingueglia *et al*, 1995) is involved in forming the ion-conducting pore. This domain of the $P2X_2$ receptor was therefore subjected to the substituted cysteine accessibility method in order to determine which residues contribute to the channel pore (Rassendren *et al*, 1997). The results indicated that three residues (I328, N333 and T336) in the N-terminal portion of TM2 contribute to the pore, presumably lying in the outer vestibule. Two further residues located well within TM2 (L338 and D349) are believed to lie on either side of the narrowest portion of the channel pore i.e. the channel gate. A study by Egan *et al* (1998) used a similar approach. Although this group's results are not identical to those of Rassendren and colleagues, it is of interest to note that they suggested residue G342 forms part of the channel gate: this amino acid lies precisely within the region delineated as the channel pore by Rassendren *et al*. Both studies were also in agreement that the secondary structure of TM2 is unlikely to be a simple α-helix. More recent work also implicates amino acids in the first transmembrane segment of the $P2X_2$ receptor. The same technique as above was used to determine which residues lying within and either side of TM1 are exposed to the aqueous medium. Evidence from this work suggests that a valine residue at position 48 on

the extracellular face of the transmembrane segment is also involved in the channel gating process (Jiang *et al*, 2001). It may therefore be that amino acids in both transmembrane domains co-operate to control channel opening and closing.

Ligand-binding sites

The rational design of subunit selective P2X receptor agonists would be greatly assisted if the precise location and configuration of ligand binding sites were known. With respect to antagonists a limited amount of information is available. During the initial identification of the suramin insensitive $P2X_4$ receptor, it was noticed that a lysine residue in the extracellular loop, which was conserved in $P2X_1$ and $P2X_2$ receptors, was replaced by a glutamate in $P2X_4$ subunits (Buell *et al*, 1996). Mutagenisis of this residue to lysine changed the receptor into the suramin-sensitive phenotype, indicating a crucial role for the amino acid at this position. A portion of the extracellular loop was later also implicated in the reduced PPADS sensitivity of the rat $P2X_4$ receptor as compared to the human protein (Garcia-Guzman *et al*, 1997).

With respect to the ATP binding site, more detailed analysis has been undertaken. Many ATP-binding proteins contain a conserved amino acid sequence known as a Walker motif. A crucial part of this sequence is the positively charged lysine residue, which is thought to interact with one of the negative charges on the ATP molecule. Positively charged lysine and arginine residues are also believed to be important in ATP binding proteins lacking a Walker motif (e.g. P2 receptors). Two independent studies on $P2X_1$ (Ennion *et al*, 2000) and $P2X_2$ (Jiang *et al*, 2000) receptors examined the effect on ATP-evoked responses of mutating conserved lysine and arginine residues located in the extracellular loop. This part of the protein is thought to contain the ATP binding site. Despite differing in detailed results (a possible reflection of subtype specific differences in receptor structure) the studies agree that positively charged residues in close proximity to the transmembrane domains appear to be most important in agonist binding; a putative ATP-binding site is thought to form near the opening of the channel pore (Fig. 1).

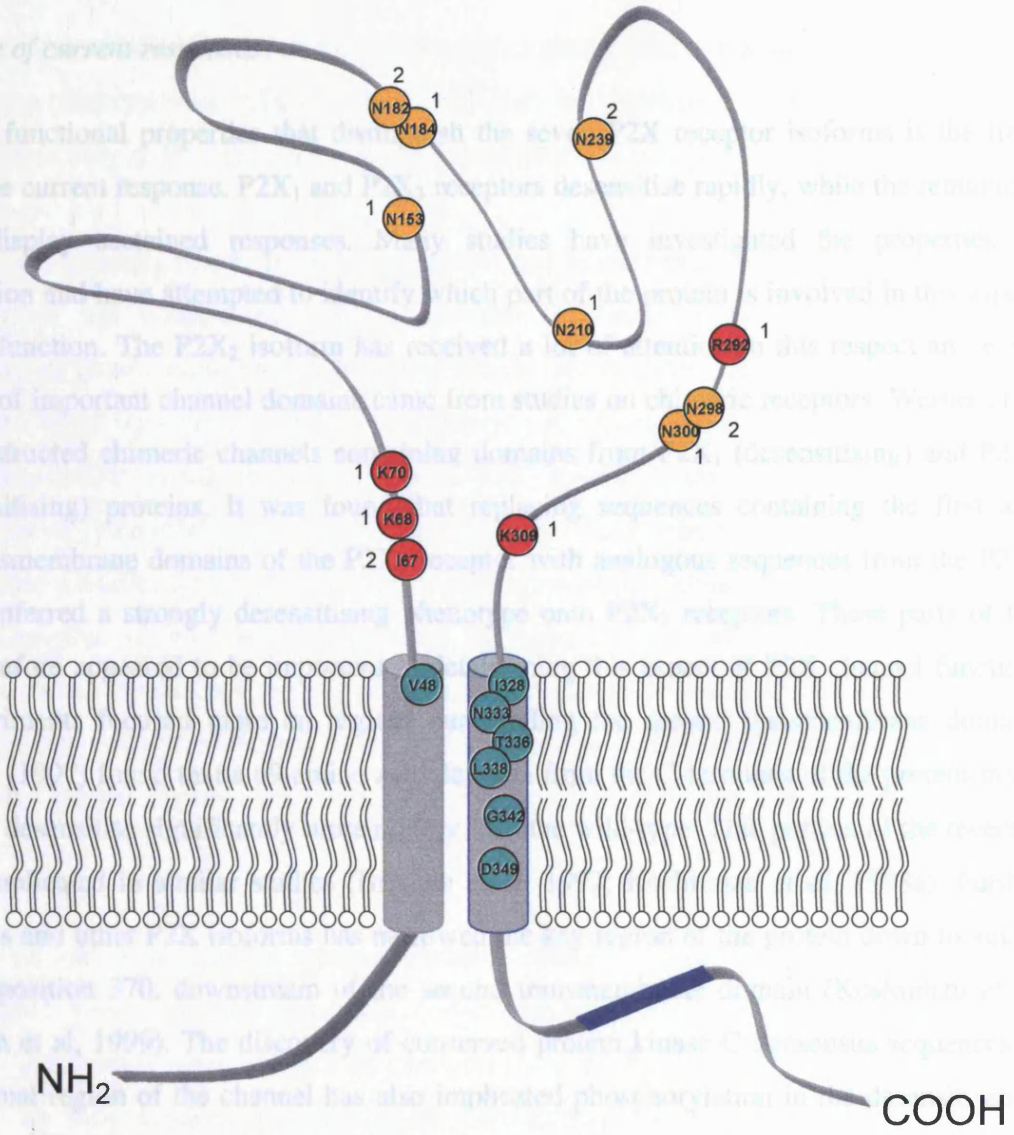


Figure 1 Structure of a P2X receptor subunit with several key amino acid residues. The diagram shows a schematic representation of the characteristic P2X receptor membrane topology: two transmembrane domains linked by a large extracellular loop. The positions of certain amino acids are given, which have been shown to be important in structural and functional studies; the numbers indicate in which P2X isoform each residue contributes to channel function. The role of amino acids is given by the colour: yellow indicates glycosylation sites and red denotes residues involved in ligand binding. Residues in green are thought to form part of the channel pore in $P2X_2$ receptors. The portion of the intracellular C-terminal highlighted in blue has been implicated in $P2X_2$ receptor desensitisation. The whole C-terminal including the second transmembrane domain are involved in $P2X_2$ subunit interactions.

Time course of current responses

One of the functional properties that distinguish the seven P2X receptor isoforms is the time course of the current response. $P2X_1$ and $P2X_3$ receptors desensitise rapidly, while the remaining receptors display sustained responses. Many studies have investigated the properties of desensitisation and have attempted to identify which part of the protein is involved in this aspect of channel function. The $P2X_2$ isoform has received a lot of attention in this respect and early indications of important channel domains came from studies on chimeric receptors. Werner *et al* (1996) constructed chimeric channels containing domains from $P2X_1$ (desensitising) and $P2X_2$ (non-desensitising) proteins. It was found that replacing sequences containing the first and second transmembrane domains of the $P2X_2$ receptor with analogous sequences from the $P2X_1$ channel, conferred a strongly desensitising phenotype onto $P2X_2$ receptors. These parts of the protein therefore appeared to be important in determining this aspect of P2X channel function. Later experiments focused more on regions surrounding the second transmembrane domain. Simon *et al* (1997) found that a 69 amino acid deletion from the C-terminus of the protein made the channel desensitise significantly more rapidly than the wild-type. This portion of the receptor was also implicated in similar studies (Brändle *et al*, 1997; Koshimizu *et al*, 1998a). Further work on this and other P2X isoforms has narrowed the key region of the protein down to amino acids near position 370, downstream of the second transmembrane domain (Koshimizu *et al*, 1999; Smith et al, 1999). The discovery of conserved protein kinase C consensus sequences in the N-terminal region of the channel has also implicated phosphorylation in the desensitisation process (Boue-Grabot *et al*, 2000). Other theories regarding the regulation of desensitisation involve the actin cytoskeleton in the case of $P2X_1$ (Parker, 1998) and the phosphorylation state of key N-terminal residues as controlled by calcium-dependent calcineurine in $P2X_3$ receptors (King *et al*, 1997).

Subunit assembly

A further area of interest is how P2X receptor subunits form functional channels. All known ligand-gated ion channels are oligomeric proteins consisting of multiple subunits arranged around a central aqueous pore. Nicotinic acetylcholine receptors contain five homologous

subunits and other channels, such as ionotropic glutamate receptors, contain four subunits. It is therefore very probable that P2X receptors will also be multimeric proteins. The fact that heteromeric assembly of P2X subunits is possible supports this viewpoint.

It is known that subunits can form both homomeric and heteromeric receptors but it is unclear which parts of the channel protein are involved in inter-subunit interactions. Greater understanding of these interactions may reveal which factors determine heteromeric assembly and may also shed light on channel stoichiometry. A recent study examined the effect of targeted sequence deletions on the assembly of rat $P2X_2$ and $P2X_3$ subunits (Torres *et al*, 1999a). Deletion of either intracellular N- or C-terminal portions of the protein did not affect assembly, which confirms a similar finding for the C-terminal region of the human $P2X_2$ channel (Lynch *et al*, 1999). Deletion of the C-terminal portion including the second transmembrane domain however prevented interactions. The general protein domains involved in subunit interactions have therefore been described. However, these data do not give detailed information at the amino acid level, and further experiments will be required to pinpoint which residues are crucial for subunit assembly.

Other channels that share the P2X receptor's unusual subunit structure (e.g. amiloride-sensitive sodium channel, inward rectifier potassium channel) are believed to assemble as tetramers, and initially it is was thought possible that P2X channels share this stoichiometry. This configuration is supported by early experiments studying the heterologously expressed extracellular domain (ECD) of the $P2X_2$ receptor (Kim *et al*, 1997). Several molecular techniques were used to demonstrate that the ECD assembled into a stable tetramer, and specific binding of ATP indicated that the protein had folded correctly. More recent studies on intact $P2X_1$ and $P2X_3$ receptors however disagree with these findings. Chemical cross-linking using specially synthesised PPADS dimers and analysis of purified receptor protein with blue native PAGE suggested both receptors from stable trimers (Nicke *et al*, 1998). The authors however note that a trimer bundle of α-helices would form a pore far smaller than that predicted by previous functional studies (Evans *et al*, 1996). A proposed explanation is that the pore region is formed by a different, non-helical secondary structure, as had already been suggested in earlier studies (Rassendren *et al*, 1997). A trimeric stoichiometry was also predicted by Stoop *et al* (1999). A

recent study in rat $P2X_7$ receptors has concluded that this channel could exist in different stoichiometries depending on the site of expression: monomers in brain astrocytes / glial cells and multimeric complexes with perhaps as many as six subunits in bone marrow and peritoneal macrophages (Kim *et al*, 2001).

1.2.2.2.2 *Properties and Distribution*

The cloning of P2X receptors allowed each isoform to be studied individually using heterologous expression systems. This analysis has clearly delineated the properties of each P2X receptor isoform and shows that P2X receptors can be distinguished based on a range of pharmacological and functional properties. The most important characteristics of each isoform are given in Table 1 and are summarized below. P2X receptors can be broadly classed into two groups: $P2X_1$ and $P2X_3$ receptors are rapidly desensitising and methylene-substituted ATP derivative sensitive, while the remaining isoforms are slowly desensitising and methylene-substituted ATP derivative insensitive. Another general feature distinguishing different subunits is antagonist sensitivity: all isoforms except $P2X_4$ and $P2X_6$ (in the rat) are sensitive to the P2 receptor antagonists suramin and PPADS. $P2X_7$ receptors are unusual in that they are substantially more sensitive to an ATP derivative (BzATP) than the parent compound. This isoform also becomes permeable to low molecular weight molecules upon prolonged exposure to agonist. Initially, this property was thought to be unique to the $P2X_7$ isoform, but recently $P2X_2$ (Virginio *et al*, 1999) and $P2X_4$ (Khakh *et al*, 1999) receptors have been shown to behave in the same way. With respect to distribution, certain clear trends can be seen. $P2X_1$ receptors are principally found in vascular and other smooth muscles, while $P2X_{2\text{-}6}$ receptors have mainly been characterised in neuronal tissue. For example, $P2X_2$ is found in sympathetic ganglia; $P2X_3$ only in sensory neurons; $P2X_4$ and $P2X_6$ are widespread throughout the brain; $P2X_5$ is found in certain sensory ganglia. $P2X_7$ receptors on the other hand are mainly found in immune cells.

The $P2X_1$ channel is 399 amino acids in length and was originally cloned from rat vas deferens (Valera *et al*, 1994). Vas deferens had previously been shown to respond strongly to ATP in whole tissue studies (e.g. Dunn & Blakeley, 1988), while data from isolated myocytes showed that ATP evoked currents with a rapid onset characteristic of direct gating of an ion channel (e.g.

Data determined at cloning	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
Rat tissue cloned from	Vas deferens (Valera *et al*, 1994)	PC 12 cells (Brake *et al*, 1994)	DRG neurons (Lewis *et al*, 1995; Chen *et al*, 1995	SCG neurons (Buell *et al*, 1996) Hippocampus (Bo *et al*, 1995)	Coeliac ganglion (Collo *et al*, 1996) Heart (Garcia-Guzman *et al*, 1996	SCG neurons (Collo *et al*, 1996) Brain (Soto *et al*, 1996)	Brain (Surprenant *et al*, 1996)
Predicted number of a.a.	399	472	397	388	417	379	595
Agonist rank order potency	2MeSATP ≥ ATP > α,β-meATP >> ADP	ATP ≥ 2MeSATP ≥ ATPγS not α,β-meATP	ATP ≥ α,β-meATP ≥ ATPγS >> ADP	ATP = 2MeSATP > ATPγS >> ADP not α,β-meATP	ATP ≥ 2MeSATP ≥ ATPγS >> ADP not α,β-meATP	ATP ≥ 2MeSATP ≥ ATPγS >> ADP not α,β-meATP	BzATP >> ATP > 2MeSATP > ATPγS >> ADP not α,β-meATP
Desensitisation	rapid	slow	Rapid	slow	slow	slow	slow
Antagonists	Suramin, PPADS	Suramin, PPADS	Suramin, PPADS	Suramin, PPADS insensitive	Suramin, PPADS	Suramin, PPADS insensitive	PPADS

Table 1a Data on P2X receptor structure and properties obtained from initial cloning studies.

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
Single channel conductance (Evans, 1996)	~ 18 pS	~ 21 pS	Channel openings too rapid	~ 9 pS	n.d.	n.d.	n.d.
Distribution	Smooth muscle (Vulchanova *et al*, 1996; Mulryan *et al*, 2000), platelets (Mahaut-Smith *et al*, 2000)	Mainly neuonal tissue e.g. SCG (Zhong *et al*, 2000) dorsal horn (Vulchanova *et al*, 1996)	Only sensory neurons (e.g Lewis *et al*, 1995; Chen *et al*, 1995; Virginio *et al*, 1998b)	Mainly neuronal tissue e.g throughout brain, ganglia and spinal cord (Collo *et al*, 1996)	Mainly dorsal root and trigeminal ganglia (Collo *et al*, 1996)	Widely overlapping with $P2X_4$	Mainly immune cells and CNS (Surprenant *et al*, 1996; Collo *et al*, 1997)
Special characteristics	TNP-ATP potent antagonist (Virginio *et al*, 1998a) Only L-β,γ-meATP potent agonist (Trezise *et al*, 1995)	Lower pH increase potency of agonists Inhibited by raised extracellular calcium (Evans *et al*, 1996) Prolonged agonist application induces pore formation (Virginio *et al*, 1999)	TNP-ATP potent antagonist (Virginio *et al*, 1998a) Both stereoisomers of β,γ-meATP agonist (Trezise *et al*, 1995)	Human receptor more sensitive to PPADS (Garcia-Guzman *et al*, 1997) Prolonged agonist application induces pore formation (Khakh *et al*, 1999)	Very small currents from recombinant receptors (Collo *et al*, 1996)	Do not readily form homomers (Torres *et al*, 1999; King *et al*, 2000)	Bz ATP more potent than ATP Inhibited by raised extracellular divalent cations (Virginio *et al*, 1997) Prolonged agonist application induces pore formation (Surprenant *et al*, 1996)

Table 1b Summary of data relating to function and distribution of P2X receptor isoforms obtained from later studies. N.d. indicates not determined.

Nakazawa & Matsuki, 1987). The properties of the cloned channel correspond very closely to those of P2X receptor-mediated responses in several types of native smooth muscle i.e. vas deferens, arterial and bladder (Khakh *et al*, 1995; Evans & Kennedy, 1994; Vial & Evans, 2000). As this thesis focuses on vascular smooth muscle, the $P2X_1$ receptor is of greatest interest and will be discussed in greatest detail. A detailed account of its properties follows, drawing particular attention to the similarities between the cloned $P2X_1$ receptor and native smooth muscle.

Pharmacology

Many studies have characterised vascular P2X receptors, and all reveal a similar pattern of results. Experiments have been conducted in rat pulmonary arteries (Liu *et al*, 1989), the rabbit ear artery (von Kügelgen & Starke, 1991b), rat mesenteric arteries (Juul *et al*, 1993), the rat tail artery (Evans & Kennedy, 1994), rabbit coronary arteries (Corr & Burnstock, 1994), guinea-pig submucosal arterioles (Galligan *et al*, 1995), as well as many other vascular preparations. Some key pharmacological and functional properties of native and recombinant $P2X_1$ receptors are compared in Table 2.

	Recombinant $P2X_1$ receptor	**Native Smooth Muscle P2X receptor**
Nature of response	Non-selective cation current	Non-selective cation current
Time course of response	Transient, rapidly desensitising at high agonist concentrations [1]	Transient, rapidly desensitising at high agonist concentrations [2]
ATP sensitivity (EC_{50})	~ 0.4 μM [3]	0.6 μM [2] (c)
2MeSATP sensitivity (EC_{50})	~ 0.3 μM [3]	0.4 μM [2] (c)
α,β-meATP sensitivity (EC_{50})	~ 1 μM [3] 2.2 μM [1]	0.3 μM [4] (t); ~ 1 μM [5] (t); ~ 2 μM [6] (t) 1.2 μM [2] (c)
L-β,γ-meATP sensitivity (EC_{50})	1.9 μM [1]	4.1 μM [4] (t) 10.5 μM [2] (c)
Suramin sensitivity	pIC_{50} = 6.1 [7] pIC_{50} = ~ 6 [1]	pK_B = 5.3 [8] (t); pA_2 = 5.6 [9] (t); pA_2 = 5.5 [4] (t) pIC_{50} = 5.4 [2] (c)
PPADS sensitivity	pIC_{50} = 5.8 [7] pIC_{50} = ~ 6 [1]	pK_B = 6.6 [8] (t); pK_B = 6.3 [4] (t) pIC_{50} = 7.1 [2] (c)

Table 2 Comparison of several key functional properties showing the similarity between recombinant $P2X_1$ receptors and P2X receptors of native smooth muscle. (c) indicates single cell and (t) whole tissue experiments. [1] Evans *et al*, 1995; [2] Lewis & Evans, 2000a; [3] Valera *et al*, 1994; [4] Galligan *et al*, 1995; [5] Lewis *et al*, 1998; [6] Khakh *et al*, 1995; [7] Bianchi *et al*, 1999; [8] Khakh *et al*, 1994; [9] McLaren *et al*, 1994.

It is clear that the properties of the native smooth muscle P2X receptor closely parallel those of recombinant $P2X_1$ receptors. Both evoke rapidly desensitising responses and both show similar sensitivity to ATP and several of its derivatives. The P2 receptor antagonists suramin and PPADS have similar potencies at both receptors. The smooth muscle P2X receptor is potently activated by methylene-substituted ATP derivatives, a property shared only by $P2X_1$ and $P2X_3$ subunits. However, $P2X_1$ receptors are unique in their sensitivity to the L-isomer of β,γ-meATP, which distinguishes them from the very similar $P2X_3$ isoform. The fact that L-β,γ-meATP is an agonist in vascular smooth muscle therefore strongly supports the theory that $P2X_1$ receptors are the dominant P2X isoform in this tissue. α,β-meATP and β,γ-meATP are also useful pharmacological tools when studying $P2X_1$-mediated responses in whole tissue experiments as they are resistant to metabolic degradation (e.g. Juul *et al*, 1993; Trezise *et al*, 1995; von Kügelgen *et al*, 1995).

In addition, $P2X_1$ receptors are characterised by several other pharmacological properties. They are activated by diadenosine polyphosphates with between four and six phosphate groups, both in whole tissues and isolated cells (Ralevic *et al*, 1995; Lewis *et al*, 2000a; Steinmetz *et al*, 2000a); these are naturally occurring compounds that are released during platelet activation. BzATP has also been reported to be a potent agonist at recombinant $P2X_1$ receptors (Bianchi *et al*, 1999). With respect to antagonists, there have been reports of compounds that may selectively block $P2X_1$ receptors with greater potency than other isoforms, e.g. Ip_5I (King et al, 1999), NF023 (Ziyal *et al*, 1997; Soto *et al*, 1999) and NF279 (Rettinger *et al*, 2000b). Certain members of the MRS series of compounds have also been described as selective antagonists for $P2X_1$ receptors, e.g. MRS-2220 (Jacobson *et al*, 1998) and MRS-2179 (Brown *et al*, 2000). Trinitrophenol-substituted adenine nucleotide derivatives (TNP-ATP, TNP-ADP and TNP-AMP) have been reported to potently antagonize the $P2X_1$ receptor, although they are also active at $P2X_3$ and $P2X_{2/3}$ channels (Virginio et al, 1998a). Susceptibility to metabolic breakdown however suggests these compounds may be of little use in whole tissue studies (Lewis *et al*, 1998).

The crucial importance of the $P2X_1$ isoform in vas deferens smooth muscle, which is very similar to vascular tissue, has recently been further underlined by studies in $P2X_1$-deficient mice

(Mulryan *et al*, 2000). ATP failed to produce any response in vas deferens from $P2X_1$-null animals, either in whole tissue experiments (see Chapter 7 of this thesis) or in isolated smooth muscle cells. In addition, no EJPs were recorded in response to electrical stimulation. These data give direct evidence the $P2X_1$ receptor is the P2X isoform that mediates the depolarising and vasoconstrictive effect of ATP in the vas deferens. Similar findings in the bladder have also confirmed the central role of the $P2X_1$ receptor in this type of smooth muscle (Vial & Evans, 2000).

Ion permeation

$P2X_1$ channels gate a non-selective inward cation current that is carried mainly by sodium and have a single channel conductance of ~ 19 pS (Valera *et al*, 1994; Evans, 1996). Several years before the receptor had been cloned, Benham & Tsien (1987) identified such an ATP-gated current in single smooth muscle cells of the rabbit ear artery. They estimated the channel was roughly 3.3:1 selective for calcium over sodium. Slightly higher figures have subsequently been obtained by other groups (e.g. Valera et al, 1994; Evans *et al*, 1996), which is probably a reflection of different experimental approaches. These values lead to the conclusion that approximately 6 % of the current is carried by calcium (Benham & Tsien, 1987), a value in line with estimates made by the measurement of changes in intracellular calcium levels (Pacaud *et al*, 1994). As with other P2X isoforms, the reversal potential for the channel has been found to be close to 0 mV using physiological internal and external ion concentrations (Valera *et al*, 1994, Lewis & Evans, 2000a); this confirms that the receptor is a non-selective cation channel.

Distribution

Immunohistochemical and *in-situ* hybridization studies have shown that $P2X_1$ receptors are expressed at high levels in the smooth muscle layer of these tissues: artery (Nori *et al*, 1997; Chan *et al*, 1998; Lewis *et al*, 2000a), bladder (Vulchanova *et al*, 1996) and vas deferens (Mulryan *et al*, 2000). Despite the previously mentioned dominance of the $P2X_1$ isoform, expression of $P2X_2$ (Nori et al 1998), $P2X_4$ (Soto *et al*, 1996; Lewis & Evans, 2000a) and $P2X_5$

(Lewis & Evans, 2000a) receptors has also been detected in vascular smooth muscle. In addition, northern analysis suggests that $P2X_7$ may also be expressed in smooth muscle of human saphenous vein (Cario-Toumaniantz *et al*, 1998). The functional significance of these other isoforms is as yet unclear. Although $P2X_1$ receptors are principally found in smooth muscle, they are also expressed in platelets (Sun *et al*, 1998, Mahaut-Smith *et al*, 2000) as well as sensory ganglia, sympathetic ganglia and spinal cord (Collo *et al*, 1996). Important sites of expression of the remaining P2X receptors are given in Table 1.

1.2.2.2.3 *Heteromultimers / splice variants*

At the outset, research into P2X receptors focused on characterising the properties of individual subunits. A single isoform was expressed in a heterologous system therefore giving rise to homomeric receptors. It is however known that other ion channels can form heteromeric assemblies. Nicotinic acetylcholine, GABA and ionotropic glutamate receptors consist of multiple subunits, some of which exist in more than one isoform. As a result, many different subunit combinations are possible, creating receptors with a range of functional properties. Immunohistochemical and in-situ hybridization studies show that the distribution of P2X isoforms overlaps in many tissues. If more than one isoform is expressed in a cell, heteropolymerisation of different subunits into one channel may therefore also occur in P2X receptors.

Table 1 shows that homomeric P2X receptors consisting of one type of subunit have distinct pharmacological properties. Incorporating more than one type of subunit into the channel to form a heteromeric receptor can however modify these properties, resulting in a further diversification of channel phenotypes. Moreover, native tissues may have properties that cannot be accounted for by homomeric channels. In this case, knowledge of heteromeric receptors can be useful in assigning the P2X phenotype of a native tissue to a recombinant receptor. Splice variants are another means of modifying the properties of P2X subunits.

A recent study has identified 11 possible pairwise combinations of P2X subunits based on biochemical analysis (Torres *et al*, 1999b); unfortunately the functional properties of these receptors were not characterised. Four functional heteromers have however been identified:

(i) The $P2X_{2/3}$ was the first heteromeric channel discovered. Rat nodose neurons were found to be α,β-meATP sensitive and produce sustained responses, a phenotype that could not be accounted for by any known homomeric P2X receptor. When combinations of $P2X_1$ to $P2X_4$ subunits were expressed, one channel was found which very closely reproduced the unusual phenotype, a $P2X_{2/3}$ receptor (Lewis *et al*, 1995). This receptor shared properties of both contributing subunits to form a channel with a novel functional profile characteristic of responses in no-dose neurons: the non-desensitising responses of $P2X_2$ subunits and the α,β-meATP sensitivity of $P2X_3$ subunits. Direct proof of heteromeric assembly was provided by experiments where both proteins were co-immunoprecipitated with antibodies to one of the two subunits (Radford *et al*, 1997).

(ii) A further example of heteromeric assembly is the $P2X_{4/6}$ receptor (Le *et al*, 1998). $P2X_4$ subunits are known to form functional channels while functional expression of homomeric $P2X_6$ receptors has proven very difficult (Torres *et al*, 1999b). Curiously, $P2X_{4/6}$ receptors have very different properties to $P2X_4$ channels: they are α,β-meATP and suramin sensitive. The very extensively overlapping distribution of the two subunits (Collo et al, 1996) and the inability to express functional $P2X_6$ channels lead the authors to suggest that this may be a major native P2X receptor in the CNS.

(iii) Functional $P2X_{1/5}$ receptors have been expressed in heterologous systems by several groups (Haines *et al,* 1999; Le *et al,* 1999; Surprenant *et al*, 2000) These studies found the new receptor to share properties of each subunit: large but biphasic currents; sensitivity to α,β-meATP and sensitivity to TNP-ATP. One difference in the results of the studies is the inhibitory effect of TNP-ATP, although this could be attributed to different antagonist application protocols. Both early papers point out that areas of overlapping expression of the two subunits in the dorsal horn of the spinal cord (Collo *et al*, 1996) could be sites where native $P2X_{1/5}$ channel are found. Surprenant *et al* (2000) proposed the sympathetic neuroeffector junction in guinea-pig submucosal arterioles as a site for native $P2X_{1/5}$ function.

(iv) $P2X_2$ and $P2X_6$ subunits can also co-assemble to form functional channels (King *et al*, 2000). Although in this receptor, the $P2X_2$ phenotype is more dominant than in $P2X_{4/6}$ receptors, some differences to the $P2X_2$ phenotype were found: agonist potencies were lower, currents were biphasic in acidic conditions and suramin antagonism showed an altered pH dependence.

Structural and functional diversity can also be achieved by alternative splicing. The existence of P2X receptor splice variants was first demonstrated for the $P2X_2$ isoform. Three variants were isolated of which only one formed functional channels. This channel showed slightly lower antagonist sensitivity and substantially faster desensitisation than the wild-type receptor. *In situ* hybridization suggests that this, and even the non-functional splice variants are expressed throughout rat neuronal tissue (Brändle *et al*, 1997; Simon *et al*, 1997). Other studies on the same receptor reached similar conclusions (Koshimizu *et al*, 1998b). Splice variants have also been reported for $P2X_4$ receptors (Carpenter *et al*, 1999) and $P2X_1$ receptors (Ohkubo *et al*, 2000).

1.2.2.3 P2Y Receptors

P2Y receptors have been shown to play a substantial role in the regulation of vascular tone. Unlike P2X receptors however, their activation can result in both vasoconstriction and vasodilatation. To date six P2Y receptor isoforms have been identified of which $P2Y_{1,2,6}$ and possibly $P2Y_4$ are involved in the regulation of vascular tone. In the next section, the most important properties of the four vascular receptors will be described. A more detailed account, including the remaining isoforms and all references is given in Table 3. The most potent agonists for these receptors are 2MeSADP for $P2Y_1$, UTP or ATP for $P2Y_2$ and $P2Y_4$ and UDP for $P2Y_6$. Suramin is an antagonist at all rat isoforms except $P2Y_4$, while $P2Y_1$ receptors are also sensitive to PPADS. Activation of all isoforms leads to a PLC-mediated release of calcium from intracellular stores, although $P2Y_1$ receptors can also modify adenylate cyclase activity.

P2Y receptors are widely expressed in the vasculature and can mediate both contraction and relaxation. Arterial smooth muscle cells have been shown to express $P2Y_2$ (Miyagi et al, 1996)

	$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$	$P2Y_{11}$	$P2Y_{12}$
Tissue originally cloned from	Chick embryo (Webb *et al*, 1993)	Mouse neuroblastoma cell line, NG108-15 (Lustig *et al*, 1993)	Human genomic DNA (Communi *et al*, 1995)	Rat aortic smooth muscle (Chang *et al*, 1995)	Human placenta (Communi *et al*, 1997)	Rat platelets (Hollopeter *et al*, 2001)
Predicted no. of a.a.	362	373	365	328	365	342 (human)
Agonist rank order potency	2MeSADP = 2MeSATP > ADP > ATP (Tokuyama *et al*, 1995) also Ap_4A (Pintor *et al*, 1996)	UTP = ATP ≥ Ap_4A (Lazarowski *et al*, 1995; Nicholas *et al*, 1996)	UTP = ATP = Ap4A = ITP (Bogdanov *et al*, 1998) UTP more potent than ATP at human receptor	UDP >> UTP ≥ ATP > ADP (Nicholas *et al*, 1996)	ATPgS = BzATP > ATP > ADPβS > 2MeSATP (Communi *et al*, 1999)	2MeSADP > ADP (human) (Hollopeter *et al*, 2001)
Antagonists	Suramin, reactive blue 2 (Boyer *et al*, 1994) PPADS (Schachter *et al*, 1996) MRS-2179 (human) (Camaioni *et al*, 1998)	Suramin (Charlton *et al*, 1996) not PPADS	Reactive blue 2 (Communi *et al*, 1996) not suramin, not PPADS	Reactive Blue 2 more than suramin (Chang *et al*, 1995)	Suramin more than reactive blue 2 Not PPADS (Communi *et al*, 1999)	2MeSAMP, C1330-7 (human) (Hollopeter *et al*, 2001)
Distribution (non-vascular tissue)	Heart, brain, skeletal muscle, spleen, lung, liver, kidney (Tokuyama *et al*, 1995) also platelets (Fabre *et al*, 1999)	Heart, brain, testis, osteoblasts, (Lustig *et al*, 1993; Bowler *et al*, 1995) Pulmonary epithelia (Cressman *et al*, 1999)	Placenta (Communi *et al*, 1995), lung (Nicholas *et al*, 1996)	Stomach, lung, GI tract, aorta (Chang *et al*, 1995) thymus, brain (Communi *et al*, 1996)	Spleen, HL-60 cells (Communi *et al*, 1997)	Platelets, brain (human) (Hollopeter *et al*, 2001)
Coupling	Activation of PLC via $G_{q/11}$ also inhibtion of AC via $G_{i/o}$	Activation of PLC via $G_{q/11}$ and $G_{i/o}$	Activation of PLC via $G_{q/11}$ and $G_{i/o}$	Activation of PLC via $G_{q/11}$	Activation of PLC via $G_{q/11}$ also activation of AC	Inhibition of AC via $G_{i/o}$

Table 3 Summary of the most important data on the function and distribution of P2Y receptor isoforms.

and $P2Y_6$ receptors, which the latter were originally cloned from (Chang *et al*, 1995). $P2Y_4$ receptors are not generally found in this tissue, although low level expression of the protein has been reported (Malmsjö *et al*, 2000a). Smooth muscle cells can express all four isoforms when maintained in culture (Erlinge *et al*, 1998) but under these conditions, they are thought to display a proliferative phenotype found in disease states. The two isoforms most widely associated with the endothelium are $P2Y_1$ and $P2Y_2$ as demonstrated by Pirotton *et al* (1996) in bovine aortic endothelial cells, although another study found $P2Y_{1,2,4}$ and $P2Y_6$ isoforms on cultured human umbilical vein endothelial cells (Jin *et al*, 1998).

The properties and distribution of all mammalian P2Y receptor isoforms are summarised in Table 3. The missing numbers in the sequence represent receptors, which have been identified in non-mammalian species or have not yet been shown to be functional proteins. P_{2y2} and P_{2y5} were cloned from chick DNA and P_{2y8} from Xenopus laevis. The P_{2y7} protein is identical to the leukotriene B4 receptor. $P2Y_{10}$ and $P2Y_{11}$ were cloned from human DNA but appear not to be functional receptors.

1.3 P2 RECEPTORS AND THE REGULATION OF ARTERIAL TONE

The remainder of the introduction focuses more closely on the subject matter of this thesis: the purinergic control of arterial tone. One of the most important means of regulating arterial smooth muscle tone is through the action of the sympathetic nervous system. There follows a description of the mechanism of sympathetic transmission and the importance of ATP in the neuronal control of vascular tone. Nucleotides altering vascular smooth muscle function can however also be released from non-neuronal sources. I will therefore give a general outline of the effects that extracellular nucleotides can have on arterial function and which P2 receptors are involved. In addition, the importance of calcium in smooth muscle contraction will be discussed.

1.3.1 OVERALL BLOOD PRESSURE REGULATION

The circulation ensures that the metabolic demand of all tissues in the body is met, i.e. the necessary supply of oxygen and nutrients is provided while at the same time unwanted waste products are removed. In order for this process to be efficient, arterial pressure within the system must be maintained at the required level. The two factors that determine arterial pressure are cardiac output and peripheral resistance. A key factor affecting cardiac output is blood volume, while a key factor affecting peripheral resistance is vascular smooth muscle tone. Within the vasculature, it is the smallest arteries and arterioles which pose the greatest resistance to blood flow, and they are therefore most important determining overall systemic blood pressure (Mulvany, 1996). Factors affecting smooth muscle tone in these vessels will have the greatest effect on arterial pressure. Short-term regulation of blood pressure is achieved through the sympathetic nervous system, which constantly adjusts arterial smooth muscle tone. This is described in detail in section 1.3.2. Long-term regulation of blood pressure on the other hand is mediated by hormones which prinicpally control blood volume. Hormones of the renin-angiotensin system play a key role: angiotensin II and antidieuretic hormone (ADH). Overall blood pressure regulation is complex and involves several interacting systems; a detailed account of these processes is beyond the scope of this discussion.

1.3.2 SYMPATHETIC TRANSMISSION

From section 1.2.1.3 it is clear that ATP is released from sympathetic nerves; one of the aims of this thesis was to characterise the role that ATP plays in sympathetic vasoconstriction in different sized arteries. In the next subsections I will illustrate the structure of arteries and the interactions sympathetic nerves form with underlying smooth muscle cells. In addition, the properties of sympathetic transmission and recent evidence proving co-transmission of noradrenaline and ATP will be described.

1.3.2.1 Structure and Innervation of Arteries

Structure

A cross-sectional view of an arterial wall reveals three distinct structural layers or 'tunics'. The tunica intima is the innermost layer facing the vessel lumen. A layer of endothelial cells cover the inner surface of the artery which rest on a thin basal lamina. This is followed by the subendothelium, a layer of connective tissue which becomes thicker as arterial diameter increases. In medium and large arteries a prominent elastic lamina consisting of elastin, separates the tunica intima from the tunica media. This membrane has numerous openings allowing diffusion of material into the medial layer of the vessel; arterioles have no elastic lamina. The tunica media is the thickest layer of the artery wall. It contains layers of helically arranged smooth muscle cells. The larger the artery, the more layers of muscle cells; the smallest arteries have a single layer while large muscular vessels can have up to forty layers. Arterial smooth muscle cells are characterised by gap junctions forming intercellular connections. These couple neighbouring cells into a functional syncitium, which allows waves of electrical excitation to spread throughout clusters of smooth muscle cells. As a result contractile responses initiated at the outer layer of smooth muscle are propagated through many layers of cells, spreading toward smooth muscle near the lumen of the vessel. Interspersed between smooth muscle cells are elastic and collagen fibers as well as variable numbers of elastic laminae, again depending on vessel size. The outermost layer of an artery is the tunica adventitia, which in large muscular arteries, can be separated from the tunica media by a further elastic lamina. It contains large numbers of elastic and collagen fibers, as well as connective and adipose tissue which become continuous with the surrounding, non-vascular tissue. Nerve cells, such as sympathetic axons are found along the medio-adventitial border. The tunica adventitia becomes very thin in arterioles.

Innervation

Sympathetic neurons innervate arteries by forming a highly branched network of fibres that surround the vessel. They are located at the medio-adventitial border beneath the exterior surface of the artery. Although most axons occur in bundles, small vessels can be innervated by

individual neurons (Klemm *et al*, 1993). Distal neurons in the terminal region of the nerve run close to the smooth muscle layer (1 – 2 μm away) and are characterised by large numbers of swellings called varicosities, which are the sites of transmitter storage and release. These structures contain numerous small and large synaptic vesicles as well as mitochondria (Luff & McLachlan, 1989). The size of varicosities varies depending on the tissue, but they are roughly 1 to 3 μm in diameter e.g. in axons innervating guinea-pig submucosal arterioles (Luff et al, 1988). Sympathetic varicosities contain small granular and large dense-cored vesicles, both of which contain ATP and noradrenaline. Comparing data from several vessels shows that the size of the contact area between the varicosity and the smooth muscle cell and the number of synaptic vesicles are proportional to the size of a varicosity (Luff et al, 1995).

Sympathetic innervation density i.e. the density of junctions capable of transmission, has been investigated by electron microscopy using serial section studies. Not all varicosities form neuroeffector junctions although the proportion is higher than previously believed: for example up to 70 % of varicosities in sympathetic axons innervating guinea-pig submucosal arterioles (Luff & McLachlan, 1989). This number can however vary considerably depending on the species. It is thought that the density of junctions increases as arterial diameter decreases, suggesting a greater role for neuronal control in small arteries (Luff & McLachlan, 1989). In vessels with numerous junctions, varicosities are found in close apposition to the smooth muscle membrane (Cowen, 1984), 70 – 100 nm in the case of guinea-pig submucosal arterioles (Luff *et al*, 1987). The large numbers of small synaptic vesicles found in such junctional varicosities can be grouped into clusters, which are located close to the prejunctional membrane; these clusters have been termed 'prejunctional membrane specialisations' (PMSs). Unlike synapses or neuromuscular junctions, such PMSs are only found in a proportion of neuroeffector junctions; estimates range from 15 – 20 % (Luff *et al*, 1991). Physiological studies have shown that the probability of transmitter release from a given varicosity is quite low (between 1% and 10%; Brock & Cunnane, 1987; see Section 1.6.2), and it is possible that the presence of PMSs in varicosities correlates with a greater chance of transmitter release. Special structural features can also be found at the post-junctional membrane. Recent work by Bennett and colleagues investigated the spatial distribution of P2X receptors on the surface of smooth muscle cells. In both rat mesenteric artery (Hansen *et al*, 1999) and urinary bladder (Dutton *et al*, 1999) they

show that $P2X_1$ receptors form clusters that are juxtaposed to sympathetic nerve varicosities. The proximity of post-junctional receptors lends great support to the concept that varicosities are the site of transmitter release.

These structural and morphological differences in the innervation of arteries (i.e. density of varicosities forming neuroeffector junctions, proportion of neuroeffector junctions containing PMSs, distance between pre- and post-junctional membranes) may contribute to the differing functional properties seen in different arteries. For example, the postjunctional effect of a transmitter may depend on its ability to diffuse across the junctional cleft. The size of the cleft might therefore affect the relative contributions of noradrenaline and ATP to the observed response.

1.3.2.2 Mechanism of Sympathetic Transmission

Arterial tone is under the dual control of hormonal and neuronal regulation, neuronal control being achieved through the activity of the sympathetic nervous system. The sympathetic nervous system controls arterial tone on a heart beat to heart beat basis in response to signals received from peripheral blood pressure sensors. These are called baroreceptors and are located in the carotid sinuses and the aortic arch. Increased stretching of the aortic and carotid artery wall caused by a rise in blood pressure activates the baroreceptors, which send signals to the nucleus tractis solitarius (NTS) located in a brain stem region called the medulla. The NTS plays a crucial role in the integration of sensory (e.g. baroreceptor and chemoreceptor) inputs regulating cardiovascular function (Ralevic, 2000). Efferent signals from the NTS travel along the spinal cord to the preganglionic sympathetic neurons, which project to the prevertebral and paravertebral ganglia. These ganglia are formed by the cell bodies of postganglionic sympathetic neurons whose axons spread into the periphery, innervating all resistance arteries.

The electrical events occurring at the sympathetic neuroeffector junction were studied in the guinea-pig vas deferens in early experiments conducted by Burnstock and Holman in the 1960s. Electrical stimulation of the nerve innervating the vas deferens produced a depolarisation of the post-junctional membrane termed an excitatory junction potential or EJP (Burnstock & Holman,

1961). However, small depolarisations occurring at random are also recorded in the absence of electrical stimulation. These are described as spontaneous EJPs or sEJPs (Burnstock & Holman, 1962). By analogy with the neuromuscular junction, it was concluded that sEJPs result from the random release of single quanta of transmitter which activate postjunctional receptors. EJPs are therefore composed of an integral number of quanta, each of which has the same amplitude as a sEJP. Large numbers of quanta released simultaneously produce the large depolarisation that is the EJP. ATP and noradrenaline in the nerve terminal are stored in vesicles. The fact that reserpine (which blocks uptake of noradrenaline into vesicles) abolishes noradrenaline release, confirms that only noradrenaline stored in vesicles is released during neurotransmission.

Postganglionic sympathetic nerve terminals contain large and small vesicles, both of which are believed to be involved in transmitter release. ATP and noradrenaline are thought to be stored in both types of vesicle (Fried, 1980). Experiments on the properties of sympathetic transmission have revealed that a vesicle is released in response to every 10 to 100 stimuli; transmitter release is therefore intermittent (Blakeley & Cunnane, 1979). This intermittence does not arise from the failure of the action potentials to propagate into the varicosity but because every action potential has only a low probability of evoking transmitter release (Brock & Cunnane, 1987; Astrand & Stjärne, 1989). Experiments that studied single varicosities in isolation confirmed the intermittent nature of transmitter release and also determined that the probability of transmitter release may vary between varicosities (Lavidis & Bennett, 1992).

Regulation

An important means by which transmitter release is regulated is by the transmitters themselves. Both ATP and noradrenaline can modulate their own and each other's release by activating receptors expressed on the pre-junctional nerve terminal rather than the post-junctional smooth muscle cell. In the case of arteries, differences in this modulation, for example by variations in the expression of such pre-junctional autoreceptors, may cause differences in the contractile response to nerve stimulation.

Adenosine, formed by the breakdown of ATP, is one of the earliest observed modulators of sympathetic transmission. In the vas deferens, adenosine receptor agonists such as 2-chloroadensoine were seen to potently inhibit EJPs, i.e. ATP release (Sneddon *et al*, 1984). Inhibition of transmitter release is also mediated by prejunctional α_2-adrenoceptors. The α_2-selective adrenoceptor antagonist yohimbine has been shown to potentiate both adrenergic and purinergic postjunctional responses in the guinea-pig saphenous and ileocolic artery (Fujioka & Cheung, 1987; MacDonald *et al*, 1992). Recent work in the rat tail artery however suggests that yohimbine increased the efflux of tritiated noradrenaline but not that of ATP (Msghina *et al*, 1999). Pre-junctional β-adrenoceptors have also been widely reported to facilitate the release of noradrenaline from sympathetic nerve terminals (e.g. Brock et al, 1997). It is however not clear whether these receptors decrease (Goncalves et al, 1996) or increase (Brock et al, 1997) the release of ATP.

An indication of ATP-mediated prejunctional modulation in arteries came from experiments showing a substantial reduction of noradrenaline overflow caused by ATP and ATPγS (von Kügelgen *et al*, 1989). This suggested a role for prejunctional P2Y receptors, which was confirmed by the later demonstration that the effect was pertussis toxin-sensitive (von Kügelgen *et al*, 1993). Further evidence supporting a potentiating effect of prejunctional P2Y receptors on transmitter release came from work by Ren & Burnstock (1997). Other reports suggest the presence of facilitatory pre-junctional P2X receptors, which increase the release of noradrenaline (Boehm, 1999).

The curious agonist and antagonist profile of prejunctional inhibition of nodrenaline release in the rat tail artery, lead Westfall and co-workers to classify the relevant receptors as novel P_3 receptors. β,γ-meATP and 2-chloro-adenosine appear to be agonists while α,β-meATP antagonised the effect of these compounds (Shinozuka et al, 1990). Futher studies demonstrated facilitation of noardrenaline release with a similar pharmacological profile in the rabbit saphenous artery (Todorov *et al*, 1994) and the rabbit ear artery (Ishii *et al*, 1995). This theory has however not received widespread support and it is still unclear whether this class of receptor actually exists.

1.3.2.3 Co-transmission in Sympathetic Nerves

In section 1.2.1.3 I outlined the historical evidence which lead to the proposal that ATP and noradrenaline are co-released from sympathetic nerves. In the subsequent years much evidence accumulated supporting this proposal. Several key papers are discussed below leading up to recent experiments which directly prove co-transmission.

A large part of the work investigating co-transmission focused on sympathetic control of smooth muscle tone in vas deferens. Experiments using electrical stimulation of nerves to evoke contractions of the rat, mouse and guinea-pig vas deferens revealed a biphasic response: a fast initial transient contraction followed by a slow tonic one. Treatment with α-adrenoceptor antagonists such as phentolamine or prazosin, or noradrenaline depletion with reserpine blocked the second phase of contraction but did not affect the initial phase (McGrath, 1978). It therefore appeared that noradrenaline was mediating the sustained portion of contraction but another, unidentified transmitter produced the transient response. In the same year, Westfall and co-workers used radio-labeled adenosine in an attempt to prove the release of ATP from sympathetic nerves innervating the guinea-pig vas deferens. Tissues were incubated with [^{3}H]noradrenaline and with [^{3}H]adenosine which was taken up and converted to [^{3}H]ATP. Conditions were chosen that blocked vas deferens contraction, thus reducing the possibility of ATP release from smooth muscle cells. With this approach, they demonstrated release of adrenaline and ATP into the effluent upon nerve stimulation, which was blocked by TTX (Westfall *et al*, 1978). The authors suggested that ATP was being released with noradrenaline from sympathetic nerves, which would also account for the biphasic contractions to nerve stimulation previously observed. Similar results were obtained by Muramatsu *et al* (1981) studying sympathetic transmission in the dog basilar artery. This group also showed release of tritiated noradrenaline and ATP upon nerve stimulation after the tissue had been incubated with radiolabeled precursors of these compounds. Contractions in response to nerve stimulation were attenuated by TTX or after sympathetic denervation but not by pre-treatment with reserpine. The interpretation of these data was that ATP and noradrenaline were both being released from the same sympathetic nerve terminals.

The γ-receptor hypothesis

One of the most prominent challenges to the co-transmission hypothesis came from Hirst and Neild. They proposed an alternative explanation for the inability of adrenoceptor antagonists to block sympathetic nerve induced depolarisation of the post-junctional membrane. Using focal iontophoretic application (rapid pressure ejection) of noradrenaline they showed two types of response: a local vasoconstriction that was blocked by phentolamine and a membrane depolarisation that was not abolished in the presence of phentolamine. They concluded that a novel type of adrenoceptor (γ-adrenoceptor) insensitive to available antagonists, was mediating the changes in membrane potential which explained the persistence of EJPs after adrenoceptor blockade (Hirst & Neild, 1980). However, evidence was presented during the following years that disproved the theory. Some important experiments are summarised below.

A strong indication that sympathetic nerve-mediated depolarisation of the post-junctional membrane is not mediated by noradrenaline came from studies with $ANAPP_3$, a selective P2 receptor antagonist. Electrically evoked EJPs in vas deferens were reduced by $ANAPP_3$ but not by prazosin (Sneddon *et al*, 1982), suggesting a role for ATP in the observed response. The fact that EJPs were not abolished probably reflects the unsatisfactory antagonistic potency of $ANAPP_3$. Direct evidence that ATP was responsible for the rapid depolarisation of the postjunctional membrane came from studies using iontophoretic application of ATP. Evoked and spontaneous excitatory junction potentials (EJPs and sEJPs) recorded from the guinea-pig vas deferens were abolished by desensitisation of post-junctional P2X receptors with α,β-meATP. Iontophoretic application of ATP mimicked EJPs but noradrenaline did not. Application of α,β-meATP abolished electrical responses to ATP but not those to noradrenaline (Sneddon & Burnstock, 1984a). These results further supported the hypothesis that ATP, co-released from sympathetic nerves with noradrenaline, mediated EJPs evoked by nerve stimulation. Similar experiments in the rabbit mesenteric artery (von Kügelgen & Starke, 1985) and the rat tail artery (Sneddon & Burnstock, 1984b) lead to the same conclusions. Allcorn *et al* (1986) also studied EJPs in rodent vas deferens. Again, α,β-meATP abolished spontaneous and evoked electrical events in vas deferens smooth muscle cells. Pre-treatment with 6-hydroxdopamine, which

destroys sympathetic nerves, however also abolished EJPs. This result strongly supported the concept that ATP was also being released from sympathetic nerves.

Direct evidence in favour of ATP mediating EJPs came from later work by Evans & Surprenant (1992). These experiments were carried out in the same tissue used by Hirst & Neild and showed that suramin abolished EJPs while adrenoceptor blockade had no inhibitory effect and even enhanced responses. Studies in $P2X_1$ receptor-deficient mice showed that EJPs are not evoked in the absence functional channels (Mulryan *et al*, 2000). This proves that ATP and not noradrenaline mediates sympathetic nerve-evoked EJPs in these smooth muscle cells.

In an attempt to further strengthen the argument for co-transmission, other studies looked directly at the release of transmitters from nerves rather than their post-junctional effects. Many attempts were made to demonstrate the release of ATP along with noradrenaline from sympathetic nerves by measuring transmitter efflux in response to nerve stimulation (for review see Sneddon *et al*, 1996; von Kügelgen *et al*, 1998). The main difficulty in interpreting these data is however identifying the source of ATP. In whole tissue experiments there are several cell types in addition to neuronal cells that can release ATP such as smooth muscle and endothelial cells. This concern was addressed by von Kügelgen and colleagues by using cultured sympathetic neurons. Electrical stimulation of neurons evoked noradrenaline and ATP overflow with a similar time course. The release of both transmitters was reduced by a similar degree in the presence of TTX or ω-conotoxin and by removal of extracellular calcium (von Kügelgen *et al*, 1994). Although a small number of non-neuronal cells were present in culture, possible release of ATP from these cells was shown to be minimal. These results therefore represent direct proof of co-release of ATP and noradrenaline from sympathetic nerves.

Thanks to these experiments the concept of ATP and noradrenaline co-transmission is now accepted. One aspect of this process that however remains unclear is whether the two transmitters are released from the same or from different synaptic vesicles. Initially, it was believed that noradrenaline and ATP are stored in the same vesicles in sympathetic nerve terminals suggesting that the two transmitters are always released in a constant ratio. This assumption was based mainly on the analogy with adrenal chromaffin cells where this was seen

to be the case (Douglas & Poisner, 1966). There are indeed current studies which support this view (e.g. Brock & Cunnane, 1999). Recent research into co-transmission has however revealed that this may not always be the case. In the guinea-pig vas deferens it was seen that activation of pre-junctional β-adrenoceptors has opposite effects on the release of sympathetic co-transmitters: noradrenaline release is enhanced while ATP release is reduced (Goncalves *et al*, 1996). Another study of transmission in the guinea-pig vas deferens, found that the two transmitters were released from sympathetic nerves with different time courses and in differing ratios throughout the period of nerve stimulation. These discrepancies were not due to differential rates of transmitter clearance or additional release from other cell types. The conclusion drawn was that there are two pools of vesicles containing different ratios of ATP and noradrenaline (Todorov *et al*, 1996). Later work by the same group suggests that sympathetic nerves can alter the amount of noradrenaline 'recruited' into synaptic vesicles as a result of frequency-dependent autoinhibition of noradrenaline release by prejunctional α_2-adrenoceptors (Todorov *et al*, 1999). A similar role for α_2-adrenoceptor-mediated modulation of noradrenaline release was found by Msghina *et al* (1999).

1.3.3 NUCLEOTIDE-MEDIATED CONTROL OF ARTERIAL TONE

The experiments presented in this thesis investigating arterial P2 receptors had several aims: the characterisation of P2X receptor-mediated contractions; the comparison of the relative roles played by noradrenaline and ATP in sympathetic transmission and the characterisation of P2Y receptors mediating vasoconstriction. All these experiments were conducted with a view to comparing results in arteries of different diameters. In the following section I will outline the current knowledge concerning each of these aspects of purinergic control of arterial tone. I will also briefly describe the sources of extracellular nucleotides in the cardiovascular system and the role that endothelium plays in controlling vascular tone.

1.3.3.2 P2 Receptor-Mediated Smooth Muscle Contraction

Relative roles of noradrenaline and ATP in sympathetic vasoconstriction

Many contraction studies conducted in the 1980s demonstrated sympathetic co-transmission in a number of different arteries (Sneddon & Burnstock, 1984b; von Kügelgen & Starke, 1985; Ishikawa, 1985). Subsequent work extended these findings by attempting to determine the relative role of each transmitter in mediating the observed response. This was generally done by electrically stimulating sections of artery and using selective antagonists to block each component of contraction. Data produced from this research was far more variable than that characterising the pharmacology of P2X-mediated contractions. A very important early observation was that the proportion of adrenergic and purinergic transmission is strongly dependent on the parameters of stimulation. This was first described by Kennedy *et al* (1986) in the rabbit central ear artery. They showed that the relative purinergic component of contractions in response to both a one-second train of stimulation and continual stimulation was greater when the frequency of stimulation was reduced. Later work also focused on effects of train length. Sjöblom-Widfeldt *et al* (1990) compared single stimuli with continuous trains and found that responses to the former were largely purinergic while the latter were almost entirely adrenergic. Contractions to stimulation at two different frequencies and train lengths were investigated by Evans & Cunnane (1992). This study clearly showed that reducing not just the frequency but also the duration of stimulation substantially increased the purinergic component of the response: ATP-mediated contraction dominated the response to short trains of stimulation. Recent studies on the *in vivo* pattern of sympathetic nerve activity suggest that individual neurons fire in intermittent high-frequency bursts (Johnson & Gilbey, 1996). This contrasts with parameters of electrical stimulation used in many studies, which produce long trains of continuous activity. It may therefore be that during the brief trains of firing seen *in vivo*, ATP is the principal transmitter mediating sympathetic vasoconstriction.

The relative contribution of the two transmitters has however also been seen to vary greatly from one artery to another. For example, the rabbit saphenous artery has been shown to have a large purinergic component at several different parameters of stimulation (Burnstock & Warland, 1987). Similar results were also obtained in the rabbit hepatic artery (Brizzolara & Burnstock,

1990) and rabbit splenic artery (Ren & Burnstock, 1997). In contrast, the rat tail artery has only a small purinergic component of contraction (e.g. Bao & Stjärne, 1993). Other arteries where only a small purinergic response has been demonstrated include canine auricular artery (Haniuda *et al*, 1997) and guinea-pig cutaneous arteries (Morris, 1999). The relative roles of ATP and noradrenaline therefore also depend on the tissue studied.

For practical reasons, most experiments are conducted in fairly large vessels. Although these arteries are easy to dissect and experiment on, they are not the most important vessels in the physiological regulation of blood pressure. Studies have shown that small arteries and arterioles contribute over 70% of resistance to blood flow in the rat mesentery (Mulvany, 1996). Altering the tone of these vessels will have the greatest effect on blood pressure. Resistance vessels must therefore be studied in order to obtain an accurate picture of blood pressure regulation *in vivo*. Certain groups have indeed focused on investigating smaller arteries. Ramme *et al* (1987) characterised responses in relatively small branches of the rabbit jejunal artery. They found that responses to a low frequency train of stimulation were entirely purinergic, the first time this had been demonstrated in any artery. A study following on from this focused on arterioles of the guinea-pig submucosa. In this case, even comparatively long, high frequency stimulation evoked responses that were again entirely purinergic (Evans & Surprenant, 1992). On the basis of these data, it could be speculated that while adrenergic and purinergic components combine to mediate transmission in larger arteries, purinergic transmission dominates in small arteries. To my knowledge, no study has yet systematically investigated the effect of arterial diameter on the relative adrenergic and purinergic components of contraction. The experiments in this thesis were therefore designed to address this question.

P2Y receptor-mediated control of smooth muscle tone

Arterial smooth muscle tone is also under the control of P2Y receptors, which unlike P2X receptors, can mediate both vasoconstriction and vasodilation. Experiments in this thesis focus on their vasoconstrictor actions. Vascular P2Y receptors were initially identified through their endothelium-dependent vasodilator action (Kennedy *et al*, 1985; Hopwood & Burnstock, 1987) which we now know is mediated by endothelial $P2Y_1$ and $P2Y_2$ receptors (Pirotton *et al*, 1996;

Ralevic & Burnstock, 1996). However P2Y receptors expressed on arterial smooth muscle can also mediate vasoconstriction, independent of P2X receptors. An early observation of this was made by Saiag *et al* (1990). They found that ATP and UTP evoked contractions of rat tail and femoral arteries after removal of the endothelium. Responses to ATP were transient and abolished by removal of extracellular calcium, while those to UTP were sustained and hardly reduced in calcium-free solution. ATP was therefore evoking desensitising P2X receptor-mediated contractions, which are crucially dependent on influx of extracellular calcium. Responses to UTP however persisted in the absence of extracellular calcium, indicating calcium release from internal stores mediated by a G-protein coupled P2Y receptor. It was concluded that vascular smooth muscle expresses vasoconstrictor P2X and 'pyrimidinoceptors' ($P2X_1$ and $P2Y_2$).

In the same year, von Kügelgen and Starke presented evidence for two receptors mediating vasoconstriction in rabbit basilar artery, both distinct from P2X receptors. One interpretation of this result could be expression of both $P2Y_2$ and $P2Y_4$ or $P2Y_6$ receptors (von Kügelgen & Starke, 1990). The fact that UTP evokes contractions independent of P2X receptors is shown by cross-desensitisation experiments (e.g Miyagi *et al*, 1996); desensitisation of P2X receptors with α,β-meATP has no effect on UTP-evoked responses confirming that the agonist is not acting through P2X receptors.

Subsequent research has confirmed the existence of contraction-mediating P2Y receptors in many different vascular preparations, e.g. rat tail artery (McLaren *et al*, 1998a), rat mesenteric artery (Juul *et al*, 1993), rat aorta (Lopez *et al*, 1998), rat vas deferens (Bültmann *et al*, 1999a) and human coronary arteries (Malmsjö *et al*, 2000a). Although it is generally believed that the $P2Y_2$ isoform is expressed on vascular smooth muscle there is evidence that disagrees with this theory. This is mainly based on suramin sensitivity. In the rat, $P2Y_2$ receptors are suramin sensitive and some groups have reported uridine nucleotide – induced contractions (i.e. mediated by P2Y receptors) that are not blocked by suramin. Hartley *et al* (1998) observed UDP-mediated responses in rat pulmonary arteries that were relatively insensitive to suramin; it was concluded that this tissue expressed functional $P2Y_6$ receptors. Suramin-resistant contractions to UTP were seen in canine coronary arteries, suggesting a role for $P2Y_4$ receptors (Matsumoto *et al*, 1997).

As for P2X receptors, there are no studies that have investigated possible differences in the properties of P2Y receptors throughout the vascular tree. Experiments are described in Chapter 3 which have characterised the pharmacological properties of UTP-sensitive P2Y receptors in different-sized arteries of the same vascular bed.

1.3.3.3 Role of the Endothelium

The endothelium plays an important role in vascular function. On a purely anatomical level, it acts as a barrier preventing the interaction of blood constituents with the smooth muscle layer of the vessel. In addition, it controls vascular tone by releasing vasodilators e.g. prostacyclin, NO and EDHF, and it inhibits platelet activation also through prostacyclin and NO. Endothelial cells are believed to express $P2Y_1$ and $P2Y_2$ receptors. In each case, receptor activation stimulates phospholipase C resulting in increased intracellular levels of calcium, which leads to the calcium/calmodulin-dependent activation of endothelial constitutive nitric oxide synthase (ecNOS) (for review see Boarder & Hourani, 1998). NO then diffuses into the smooth muscle layer where it causes cells to relax resulting in vasodilatation. More recently $P2Y_2$ receptors have also been implicated in UTP-induced but NO-independent vasorelaxation (Malmsjö *et al*, 1998). This suggested a role for endothelium-derived hyperpolarising factor (EDHF), which was confirmed by latter work (Malmsjö *et al*, 1999a). Very recently, evidence of endothelial P2X receptor expression has also been reported. $P2X_1$ receptors were found on rat mesenteric artery endothelial cells (Hansen *et al*, 1999), $P2X_2$ receptors on the endothelium of small rat cerebral arteries (Loesch & Burnstock, 1999) and $P2X_4$ receptors on cultured endothelial cells from several human blood vessels (Yamamoto *et al*, 2000). The functional role of these channels is not yet clear.

1.3.3.4 Non-neuronal Sources of Extracellular Nucleotides and Nucleotide Breakdown

Extracellular nucleotides that have an effect on the cardiovascular system can be released from several sources as reviewed by Dubyak & El-Moatassim (1993). All cells contain high concentrations of cytoplasmic ATP, and an obvious source of extracellular ATP is therefore cell

lysis, for example resulting from injury. By its nature this process is sudden and unregulated. Interestingly, many cell types involved in dealing with tissue damage express P2 receptors: platelets, endothelial and smooth muscle cells as well as several immune cells.

A further means is by controlled exocytotic release. For my purposes I will concentrate on cells of the cardiovascular system although it also occurs in immune cells. Platelet dense granules contain very high concentrations of ATP and ADP and lower concentrations of UTP. Release of these nucleotides during platelet activation and degranulation raises their local concentration to roughly 50μM with significant consequences for vascular function. Other compounds released upon platelet activation are diadenosine polyphosphates. These are naturally occurring agonists at P2 receptors and will therefore contribute to the modulation of smooth muscle tone *in vivo*. Nucleotides can also be released from endothelial cells. Arterial tissue has been shown to release ATP in response to α_1-adrenoceptor activation, an effect nearly abolished by removal of the endothelium (Seeda *et al*, 1990). Shear stress caused by changes in blood flow can also induce nucleotide release from endothelial cells (Ralevic *et al*, 1992), and both ATP and UTP can be released from smooth muscle cells. Release from sympathetic nerves plays a vital role in the regulation of smooth muscle tone (see section 1.3.3.2).

Nucleotide breakdown

The potent actions of extracellular nucleotides described in the preceding sections require a system to ensure that their 'resting' concentration is kept low. Equally, after release into the extracellular space they must be rapidly inactivated once the desired signal had been produced. Both processes are achieved by a group of enzymes found at the exterior surface of the cell membrane, which degrade nucleoside triphosphates into the diphosphate, mononphosphate and finally the nucleoside. This field has recently been reviewed (Zimmermann, 1999; Zimmermann & Braun, 1999). Several families of enzymes are capable of degrading nucleotides: the alkaline phosphatases, ecto-5'-nucelotidase, ecto-NTPases and the ecto-phosphodiesterase/nucleotide pyrophosphatases (PDNP). One interesting feature of the ecto-ATPases is their structural

similarity to P2X receptors, i.e. two transmembrane domains linked by a large extracellular loop (Zimmermann & Braun, 1999).

1.3.4 CALCIUM AND SMOOTH MUSCLE CONTRACTION

Upon P2X receptor activation, calcium can enter the smooth muscle cell either through the P2X channel or through voltage-dependent calcium channels, which are opened by the concomitant depolarisation of the cell. In addition, this rise in cytosolic calcium can trigger calcium release from intracellular stores. It is unclear which of these processes leading to increased intracellular calcium levels dominates in P2X receptor-mediated control of arterial smooth muscle tone. One aim of this thesis was to address this question. The role and function of voltage dependent calcium channels and the process of calcium-induced calcium release will now be discussed. Initially, I will outline the cellular events that are triggered by calcium which lead to contraction of the smooth muscle cell.

1.3.4.1 Excitation-contraction coupling

The crucial step in contraction of smooth muscle is a rise in the cytosolic calcium concentration. The most important components of the contractile machinery are myosin, actin, calmodulin, calponin, myosin light chain kinase and myosin light chain phosphatase. An increase in the level of calcium inside the smooth muscle cell causes calmodulin to bind four calcium ions and activate myosin light chain kinase (MLCK). Myosin is one of the contractile proteins. It consists of two heavy chains and two pairs of light chains. The heavy chains each have a globular head domain and a long tail domain. Each pair of light chains is associated with the head region of the heavy chain. MLCK phophorylates a specific serine residue in one pair of light chains which results in a conformational change in the region linking the head and tail domains of the heavy chains. The head of the peptide chain is made more mobile by this modification enabling a Mg^{2+}-ATPase enzyme in the head region of the myosin chain to be activated. Actin, the other contractile protein, then binds to myosin activating the Mg^{2+}-ATPase, which catalyses the formation of cross-bridges between the two contractile proteins. The cyclic formation of these

cross-bridges generates force resulting in contraction of the smooth muscle cell. This process will continue in the presence of raised cytosolic calcium. Once calcium levels drop, MLCK is inactivated by the dissociation of calmodulin and myosin is dephosphorylated by myosin light chain phosphatase. Cross-bridge formation stops and the smooth muscle cell relaxes.

1.3.4.2 Sources of Calcium

1.3.4.2.1 *Calcium Channels*

Calcium is one of the most important intracellular signaling entities, affecting a wide range of cellular processes. Its concentration in the cytosol is therefore precisely controlled. In excitable cells, calcium is the key regulatory link between excitation and initiation of the cellular response (secretion of neurotransmitters in neurons and contraction in muscle). Voltage-dependent calcium channels (VDCC) enable a cell to very rapidly increase the cytoplasmic calcium concentration in response to membrane depolarisation by allowing calcium influx from the extracellular space.

The great majority of voltage-dependent calcium entry in smooth muscle cells is mediated by L-type channels, although there is also evidence of T-type channel expression (for review see Gollasch & Nelson, 1997). The properties of these channels will now be briefly outlined. These two channel types can be distinguished by clear differences in their functional properties. Firstly, T-type channels activate at negative potentials of –60 to –50 mV, while L-type channels activate at more positive potentials of -45 to –35 mV. Secondly, T-type channels inactivate roughly ten times more quickly than L-type channels producing responses that are much more transient than L-type channel currents. In addition T-type channel currents in smooth muscle cells are generally significantly smaller than L-type currents. With respect to channel blockers, L-type channels are unique in their sensitivity to the dihydropyridine (e.g. nifedipine), benzothiazapine (e.g. diltiazem) and pheylalkamine (e.g. verapamil) classes of calcium channel antagonists. T-type channels on the other hand are far more sensitive to Ni^{2+} than L-type. Cd^{2+} non-selectively blocks all VDCC. Although T-type currents are difficult to record due to their small size, they can be detected by their different potential of activation and their resistance to dihydropyridines.

An important means of L-type channel regulation is phosphorylation of the channel protein. Increased levels of cAMP and cGMP have been shown to inhibit L-type calcium channels in vascular smooth muscle cells (Ousterhout & Sperelakis, 1987; Xiong *et al*, 1994). Vasodilators such as NO or prostacycline are produced by activation of endothelial $P2Y_1$ and $P2Y_2$ receptors and cause increased production of cGMP and cAMP, respectively. These dilators may therefore mediate their effects through phosphorylation and inhibition of L-type channels. The resulting decrease in calcium influx would lead to reduced levels of intracellular calcium and vasodilatation. Phosphorylation is also mediated by Protein Kinase C (PKC). This enzyme is activated by calcium and diacyl glycerol; the latter is released together with IP_3 after activation of many G-protein-coupled receptors. PKC has been shown to phosphorylate L-type channels resulting in a potentiation of calcium currents in dissociated smooth muscle cells (Loirand *et al*, 1990). In addition, calmodulin has been shown to mediate both calcium-dependent facilitation and inactivation of L-type calcium channels (Zühlke *et al*, 1999). Calcium can therefore regulate its own influx by modulating the activity of the channel through which it enters the cell.

As P2X receptor activation depolarises vascular smooth muscle cells, VDCCs are considered to play a significant role in the purinergic component of sympathetic vasoconstriction. There are indeed studies, which show that L-type calcium channels are important in mediating calcium influx in response to P2X receptor activation. In experiments in the rat tail artery, nifedipine substantially reduced contractions in response to nerve stimulation (Surprenant et al, 1983). Both α,β-meATP-evoked and nerve-evoked purinergic contractions were potently inhibited by nifedipine in dog mesenteric arteries (Omote *et al*, 1989). Blockade of L-type channels had a similar effect on the purinergic component of neurogenic responses in the rabbit ileocolic artery (Bulloch *et al*, 1991) and substantially inhibited contractions to exogenous α,β-meATP in renal arterioles of the rat (Inscho *et al*, 1995). One study in very small arteries of the guinea-pig submucosa however produced different results. In these experiments, responses to exogenous α,β-meATP were completely resistant to nifedipine, indicating that L-type channels are not required for calcium influx in this tissue (Galligan *et al*, 1995). One interpretation of this result is that the size of the artery is again a crucial factor. Does the requirement for L-type channels decrease with decreasing arterial diameter? Once more however, systematic studies investigating

the diameter-dependence of voltage-gated calcium influx are lacking. Experiments are described in this thesis that were designed to investigate this question.

1.3.4.2.2 *Calcium-induced calcium release*

Calcium-induced calcium release (CICR) is an important further means of regulating the cytosolic calcium level. Calcium that either enters the cell through calcium permeable channels or is released from IP_3 sensitive stores, can trigger the release of further calcium from intracellular stores. In the case of smooth muscle cells, this might produce sufficient amplification of the calcium response to trigger contraction. The release of calcium from the sarcoplasmic reticulum is mediated by ryanodine receptors which are activated by a rise in the cyoplasmic concentration of calcium and possibly also by cADP-ribose (Kannan *et al*, 1996). All three isoforms of the ryanodine receptor have been identified in vascular smooth muscle (Neylon *et al*, 1995).

Calcium-induced calcium release has been shown to be involved in ATP-mediated contraction of vascular smooth muscle. In certain experiments, ATP acting at P2X receptors produced a transient depolarisation and rise in intracellular calcium in rat portal vein smooth muscle cells. The rise in calcium was shown to be independent of VDCC or IP_3 sensitive calcium stores, while caffeine or ryanodine substantially decreased the response. It was concluded that the ATP-gated calcium entry represented only a small part of the calcium response, the rest being mediated by CICR (Pacaud *et al*, 1994). They did however acknowledge the difficulty in dissociating calcium influx from calcium release due to their mutual interdependence. This had however previously been achieved for depolarisation-induced responses. By replacing extracellular calcium with strontium they were able to demonstrate that the calcium release from intracellular stores occurred at a slight delay to the initial strontium influx (Gregoire *et al*, 1993). This suggests that calcium release is being triggered by the slightly earlier influx of strontium. Later work in human vascular smooth muscle demonstrated that the P2X receptor mediated rise in cytosolic calcium was partly due to calcium influx and partly due to CICR (Loirand & Pacaud, 1995).

Evidence therefore exists for the involvement of CICR in ATP-mediated smooth muscle contraction. In order to further characterise the sources of increased intracellular calcium for P2X receptor-induced arterial contraction, I have studied the role of this process in my experiments. It was again of interest to determine whether the results suggested any dependence on arterial diameter.

1.4 SUMMARY

The previous sections have given an overview of several aspects of purinergic signaling, which are pertinent to the subject matter of this thesis. The following chapters describing the experiments I have conducted on the role of purinergic transmission in the control of arterial smooth muscle tone are divided as follows:

Chapter 2 – Methods
Chapter 3 – Agonist-evoked Contractions
Chapter 4 – Neurogenic Contractions and Sources of Calcium
Chapter 5 – Immunohistochemistry and Molecular Biology
Chapter 6 – Electrophysiology
Chapter 7 – Transgenic Mouse Studies
Chapter 8 – General Discussion

2. METHODS

2.1 RAT MESENTERIC ARTERY DISSECTION

Male rats (200 g - 300 g) were killed either by cervical dislocation or CO_2 asphyxiation, both followed by femoral exsanguination. The viscera were exposed and a 5 cm portion of ileum with attached mesenteric arcade was removed. The tissue was pinned out in a Sylgard (184 silicone elastomer, Dow Corning, USA) lined dissection dish containing the appropriate physiological solution (see below), stretching out the arcade away from the gut wall to clearly expose all blood vessels (Figure 2). Arteries were identified by their structural rigidity and appearance: because of their thicker walls, arteries are far less compliant than veins. They also have characteristic longitudinal striations which are visible when illuminated with light at an oblique angle. Vein walls, on the other hand, are fragile and uniform in appearance.

Initially all coarse fat was removed to expose the blood vessel, which was covered with a thin layer of adipose and connective tissue. Using fine forceps, the connective tissue was pulled away from the artery until just a thin membrane was visible. This was then cut along the wall of the artery with fine irridectomy scissors leaving the vessel with a clearly defined edge, virtually free of any excess connective tissue. For all experiments described three different sizes of mesenteric artery were used. Large arteries correspond to the main branch of the mesenteric artery and medium-sized arteries correspond to second or third order branches. Small vessels are equivalent to fifth or sixth order arteries and are the last branch of the mesenteric arcade before the vessels enter the gut wall (Figure 2).

2.2 ARTERIAL CONTRACTION STUDIES

Functional properties of rat mesenteric arteries were characterised by studying contractile responses of freshly dissected artery segments. These vessels retain their contractility for many hours when kept under physiological conditions. If the artery is dissected very carefully and superfused with oxygenated Ringer's solution, sympathetic nerves innervating the blood

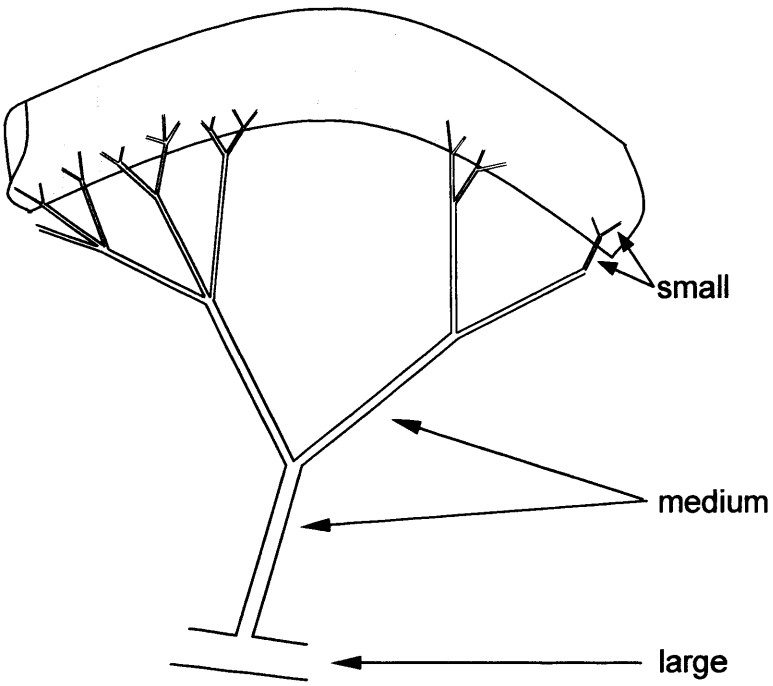


Figure 2 Schematic diagram of rat mesenteric arcade. A section of ileum with attached blood vessels. Veins as well as fat and connective tissue are not shown. Large arteries correspond to the superior mesenteric artery or first order branch. Medium arteries are second or third order and small arteries are fifth or sixth order branches.

vessels remain viable thus allowing the investigation of neurogenic contractions. Arteries ranging in outside diameter from 50 μm to 750 μm were studied in these experiments. Because of this great range of sizes, two different methodologies had to be used: Diamtrak video imaging microscopy for small arteries and wire myography for medium and large arteries.

2.2.1 SOLUTIONS AND DRUGS

In experiments where contractions were evoked by application of exogenous agonists, tissues were superfused with a minimal physiological saline solution (PSS), which was sufficient to keep arteries viable for at least 10 hours (composition in mM: NaCl 150, HEPES 10, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, pH to 7.3 with NaOH). When contractions were evoked by electrical stimulation of periarterial nerves, tissues were superfused with oxygenated Ringer's solution as this was found that to maintain reproducible nerve-evoked responses for much longer than PSS. Ringer's solution had the following composition in mM: NaCl 120, glucose 11, $NaHCO_3$ 22, KCl 5, NaH_2PO_4 1, $CaCl_2$ 2.5 and was gassed with 95% O_2/5% CO_2 to maintain a pH of 7.4. Magnesium was omitted from the solution as this has previously been found to enhance contractile responses of arteries to nerve stimulation (Evans & Cunnane, 1992).

The majority of drugs were made up in deionised water as concentrated stock solutions, stored as frozen aliquots and diluted in PSS to the final bath concentration. Stock solutions of nifedipine and cyclopiazonic acid were made up in ethanol and DMSO respectively and were stored as frozen aliquots. Stock solutions of phenylephrine were made on the day of use and discarded after the experiment. Due to its low water solubility, caffeine was made up directly in PSS at the required concentration. Stock solutions of tetrodotoxin and $CdCl_2$ were stored at 4°C.

2.2.2 DIAMTRAK

Diamtrak video imaging microscopy was used for the smallest arteries, which could not be studied by any other method because of their size. In this system, isotonic changes in vessel

diameter are measured by tracking movements of the arterial wall from an image of the vessel on a video monitor.

Small arteries were pinned out in a Sylgard-lined organ bath (2 ml volume) and stretched to 120 – 150 % of their resting length in order to simulate the intraluminal pressure produced by blood flow *in vivo*. It was found that this improved the contractility of vessels and helped their resting diameter return to baseline after agonist application. The organ bath was superfused with physiological solution (1.5 ml/min) pre-warmed to 32 – 35°C by being passed through a heating jacket. The bath temperature was monitored with a temperature probe.

The organ bath was placed on the stage of an inverted microscope (Nikon TMS) and the image recorded with a black and white video camera (JVC TK-S350). The output of the camera was fed into a PC equipped with a frame grabber card and translated into a digital signal. The image of the artery was then displayed on a video monitor and analysed on line with Diamtrak® software which allows continual measurement of vessel diameter (Figure 3). The programme relies on the contrast between the dark image of the artery and the light background to track movements of the outer edge of the vessel wall. Clearly defined edges are therefore required and care was taken to remove all connective tissue from the artery to produce a clean section for programme to track. The data output was digitised and stored to disc. It was also converted back to an analogue signal, which was fed into a chart recorder to provide a real-time display of vessel diameter. The sampling frequency was 10 Hz and changes in diameter of 0.7 μm could be resolved.

The preparation was equilibrated in flowing solution for at least 30 min prior to agonist application. Vessels that gradually relaxed during this time yielded reliable and reproducible results. A small proportion of arteries (5 - 10 % of preparations) were obviously damaged during dissection as they spontaneously constricted rather than relaxed. It was found that these vessels did not generate good quality data and they were therefore discarded.

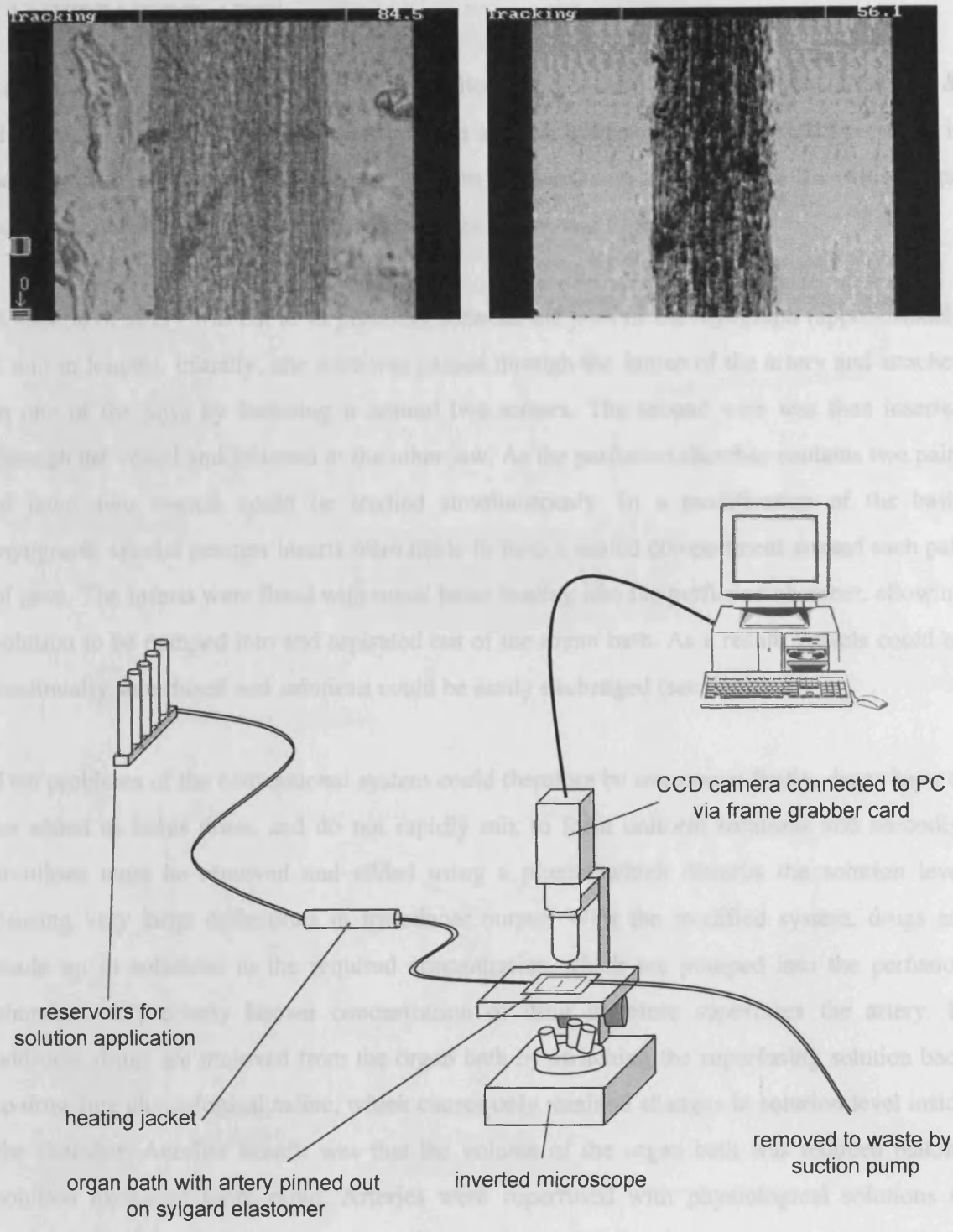


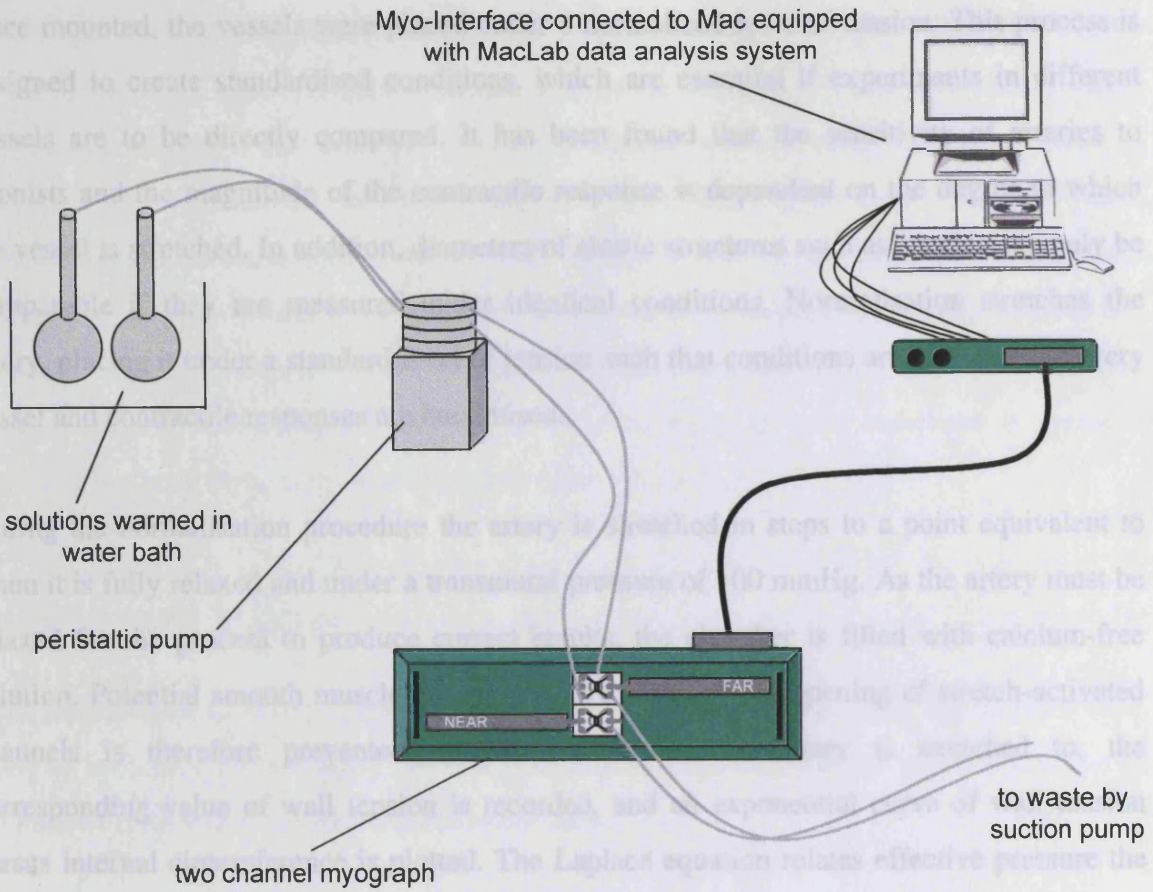
Figure 3 Scheme of experimental setup for Diamtrak studies. Solutions flow by gravity from raised reservoirs to the organ bath via a heating jacket; at the same time solution is pumped from the organ bath. Solution is therefore continually flowing allowing easy application of agonists and antagonists. An image of the artery is recorded by the cctv camera and fed into a PC where it is analyzed with Diamtrak software. Sample images of a relaxed (left) and constricted artery (right) are shown at the top of the figure.

2.2.3 WIRE MYOGRAPHY

Large and medium-sized arteries were studied using a Mulvany myograph (Mulvany & Halpern, 1977), which measures the force that a vessel generates as it contracts. The vessel is held in a fixed position, connected to both a micrometer and a force transducer and contractions are measured as isometric changes in vascular tone.

A section of artery was cut to fit precisely between the jaws of the myograph (approximately 2 mm in length). Initially, one wire was passed through the lumen of the artery and attached to one of the jaws by fastening it around two screws. The second wire was then inserted through the vessel and fastened to the other jaw. As the perfusion chamber contains two pairs of jaws, two vessels could be studied simultaneously. In a modification of the basic myograph, special perspex inserts were made to form a sealed compartment around each pair of jaws. The inserts were fitted with metal tubes leading into the perfusion chamber, allowing solution to be pumped into and aspirated out of the organ bath. As a result, vessels could be continually superfused and solutions could be easily exchanged (see Figure 4.)

Two problems of the conventional system could therefore be overcome: firstly, drugs have to be added as bolus doses and do not rapidly mix to form uniform solutions and secondly, solutions must be removed and added using a pipette which disturbs the solution level causing very large deflections in transducer output. With the modified system, drugs are made up in solutions to the required concentration which are pumped into the perfusion chamber. A precisely known concentration of drug therefore superfuses the artery. In addition, drugs are removed from the organ bath by switching the superfusing solution back to drug-free physiological saline, which causes only minimal changes in solution level inside the chamber. Another benefit was that the volume of the organ bath was reduced making solution exchange more rapid. Arteries were superfused with physiological solutions (2 ml/min) warmed to 37°C in a water bath and pumped to the myograph chamber by a Minipuls peristaltic pump. The chamber itself was also heated to 37°C and the temperature was maintained with a thermostatic probe; solutions were removed by a vacuum pump (Fig. 2).



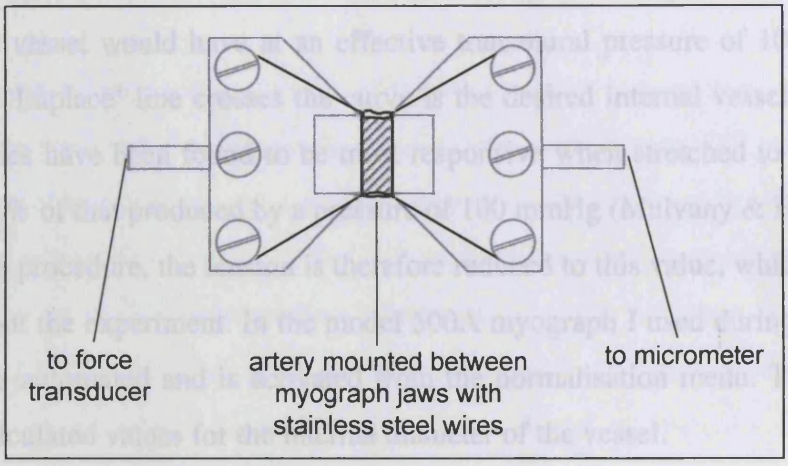


Figure 4 Scheme of myograph experimental set-up. Solutions containing drugs are warmed in a water bath and pumped to the myograph chamber by a peristaltic pump. A suction pump continually removes solution so arteries are exposed to constantly flowing physiological saline. Data are collected by the Myo-Interface and sent to MacLab data analysis system. Traces of artery tension are displayed and stored on a Macintosh computer. Inset shows detail of one channel in the myograph recording chamber.

Once mounted, the vessels were placed under a normalized level of tension. This process is designed to create standardised conditions, which are essential if experiments in different vessels are to be directly compared. It has been found that the sensitivity of arteries to agonists and the magnitude of the contractile response is dependent on the degree to which the vessel is stretched. In addition, diameters of elastic structures such as arteries can only be comparable if they are measured under identical conditions. Normalisation stretches the artery, placing it under a standard level of tension such that conditions are the same for every vessel and contractile responses are maximised.

During the normalisation procedure the artery is stretched in steps to a point equivalent to when it is fully relaxed and under a transmural pressure of 100 mmHg. As the artery must be relaxed for the process to produce correct results, the chamber is filled with calcium-free solution. Potential smooth muscle contraction as a result of the opening of stretch-activated channels is therefore prevented. For each diameter the artery is stretched to, the corresponding value of wall tension is recorded, and an exponential curve of wall tension versus internal circumference is plotted. The Laplace equation relates effective pressure the artery would be under *in vivo* to the wall tension and internal circumference determined from the normalisation procedure. This relation is then used to plot a straight line representing the wall tension the vessel would have at an effective transmural pressure of 100 mmHg. The point where the 'Laplace' line crosses the curve is the desired internal vessel diameter. Rat mesenteric arteries have been found to be most responsive when stretched to a wall tension equivalent to 90 % of that produced by a pressure of 100 mmHg (Mulvany & Halpern, 1977). At the end of the procedure, the tension is therefore reduced to this value, which the vessel is held at throughout the experiment. In the model 500A myograph I used during my PhD, this whole process is automated and is activated from the normalisation menu. The programme also provides calculated values for the internal diameter of the vessel.

2.2.4 Validation of techniques

In order to mount an artery in the myograph, two stainless steel wires, each with a diameter of 40 μm must be inserted through the vessel lumen. Medium and large arteries had internal diameters of well over 80 μm and could be studied using myography. Small vessels however

had an approximate mean internal diameter of 60 μm, making them too small to be mounted in the myograph. These vessels therefore had to be investigated using Diamtrak video imaging microscopy. The fact that two experimental systems were employed, made it important to establish that this difference in methodologies had no bearing on results generated. Medium-sized arteries were small enough to also be studied with the Diamtrak system if a very low power microscope objective was used. Conducting experiments on the same type of artery allowed the direct comparison of results obtained with the two different systems. Concentration-response curves for selected agonists were compared in medium vessels using both myography and Diamtrak video microscopy.

2.2.5 DRUG APPLICATION

Both for experiments using exogenous agonists and nerve stimulation, drug applications were made by switching the perfusing solution from normal physiological solution to a solution of the desired drug at the required final concentration.

2.2.5.1 Agonists

In each case, agonists were applied until a maximal response was reached, at which point application was stopped and the drug washed out. One exception was UTP, which had to be used in very high concentrations. As a result, applications had to be as brief as possible to minimise agonist usage. For 1 and 3 mM UTP applications were stopped after 3 minutes; readings of tension were then compared with those recorded for lower concentrations at the same time point. At the start of all experiments, arteries were challenged with an application of 60 mM KCl or 10 μM α,β-meATP, which was found to improve the subsequent contractility of the vessel. Preliminary studies had shown that some vessels became more responsive after repeated agonist applications. These studies also gave an indication of the probable maximal concentration of each agonist. In experiments constructing concentration response relationships, a high concentration of agonist was therefore first applied until responses were reproducible before the full range of applications was made. About 40% of preparations became more responsive after this treatment (80% when phenylephrine was the

agonist). If not taken into consideration, this increase in responsiveness may have yielded inaccurate concentration response curves.

2.2.5.2 Antagonists

In experiments testing the effect of antagonists, reproducible control responses to the agonist were always obtained prior to antagonist application. Tissues were then superfused with the antagonist alone to allow equilibration with the tissue and the receptor, after which both antagonist and agonist were applied concomitantly. Because of different sites of action, and different rates of equilibration, the duration of pre-superfusion varied depending on the antagonist used: 10 minutes for $CdCl_2$, 15 minutes for nifedipine and prazosin; 30 minutes for suramin and PPADS. As nifedipine and PPADS are photosensitive, solutions of these drugs were stored in darkness until used. During use, bottles were protected from light with aluminium foil and lights illuminating the organ bath were switched off.

2.2.5.3 Depletion of Calcium Stores

Intracellular calcium stores were depleted with 30 μM cyclopiazonic acid (CPA). After reproducible responses to the chosen agonist were obtained, CPA was applied in the absence of extracellular calcium for 30 minutes. Calcium was removed from the PSS to increase the rate at which calcium was extruded from the sarcoplasmic reticulum leading to better store depletion than in normal calcium solution. Contractile responses to α,β-meATP however rely on extracellular calcium. Ninety seconds prior to agonist application, calcium was therefore restored to the physiological solution. After 90 sec in normal calcium solution, the agonist was applied in the continued presence of calcium and CPA. The difference between responses in the absence and in the presence of CPA corresponded to the component of contraction mediated by calcium release from intracellular stores. To verify that calcium stores were fully depleted using this protocol, the effect of CPA was first tested on contractions evoked by 10 mM caffeine, which are known to be caused by the release of calcium from intracellular stores.

2.2.6 NERVE STIMULATION

For myograph experiments, medium and large arteries were stimulated with two platinum electrodes mounted in the plastic jaws either side of the vessel. In Diamtrak studies, the electrode was a blunt glass microelectrode filled with Ringer's solution into which a silver wire had been inserted. It was positioned in close proximity to the artery about 1 mm from the diameter-recording site. A silver chloride earth pellet was placed in the bath to complete the circuit. For both systems, the electrodes were connected to an Applegarth Electronics stimulator (Oxford, U.K.) through which electrical pulses were delivered. The electrical stimulation parameters were chosen to reflect the fact that sympathetic neurones fire in intermittent bursts rather than sustained trains (Johnson & Gilbey, 1994). Arteries were stimulated for 1 second at a frequency of 10 Hz i.e. 10 pulses at 10 Hz to replicate one such burst of activity. A short pulse width (0.2 – 0.3 ms) was chosen to avoid direct stimulation of the muscle tissue. In addition, low voltages were used (10 – 40 V) to give large responses that were clearly visible above background noise but were still nerve-evoked. Stimulation parameters were optimized for every preparation by testing the effect of tetrodotoxin (TTX, 0.3 μM) on contractions and modifying parameters accordingly. When responses were abolished in the presence of TTX, they were presumed to be neurogenic in origin.

Antagonists were applied until a minimum reproducible response to nerve stimulation in the presence of the drug was obtained. In experiments using α,β-meATP to desensitise the P2X receptor, arterial diameter/tone was allowed to return toward baseline in the continued presence of α,β-meATP before the effect on nerve-evoked contractions was measured.

2.3 IMMUNOHISTOCHEMISTRY

Immunohistochemistry was used to identify which P2X receptor isoforms are expressed in the three sizes of mesenteric artery. Antibodies raised against all seven isoforms were used. Four of the seven antibodies ($P2X_{1, 2, 4}$ and $P2X_7$) are commercially available from Alamone laboratories (Israel). Anti-$P2X_5$ and $P2X_6$ antibodies were obtained from Roche Bioscience (Ca, USA) and anti-$P2X_3$ was a gift from Lucy Vulchanova (University of Minnesota). Each antibody was raised against a C-terminal sequence of the rat P2X receptor. The epitope, supplier and dilution factor of each antibody are detailed in Table 4.

Antibody	Epitope	Dilution	Supplier
$P2X_1$	DPVATSSTLGLQENMRTS (residues 382-399)	1:200	Alomone Laboratories
$P2X_2$	SQQDSTSTDPKGLAQL (residues 457-472)	1:200	Alomone Laboratories
$P2X_3$	VEKQSTDSGAYSIGH (residues 383-397)	1:5000	Lucy Vulchanova
$P2X_4$	KKYKYVEDYEQGLSGEMNQ (residues 370-388)	1:200	Alomone Laboratories
$P2X_5$	RENAIVNVKQSQILH (residues 437-451)	1:1000	Roche Bioscience
$P2X_6$	EAGFYWRTKYEEARA (residues 357-371)	1:1000	Roche Bioscience
$P2X_7$	KIRKEFPKTQGQYSGFKYPY (residues 576-595)	1:200	Alomone Laboratories

Table 4 Amino acid residues and location within the protein sequence of epiotpes for all P2X receptor antibodies. The dilution used for immunohistochemical staining and the supplier of the antibody are also given.

Sections of artery were dissected in phosphate buffered saline (PBS, composition in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.5, pH to 7.4 with NaOH) and placed in a small plastic tube containing Tissue-Tek (Miles Inc., Elkhart, In) embedding fluid. Care was taken to ensure that the vessel was in the correct orientation for transverse sectioning i.e. floating vertically in the fluid. Before the vessel drifted out of position, the tube was snap frozen by placing it in a mixture of hexane and dry ice. Once the Tissue-Tek was frozen, tissue samples were stored at -20°C for at least 24 hours before sectioning.

In order to avoid the loss of artery sections during the incubation procedure, glass slides were 'subbed' to ensure that the tissue adhered properly. The procedure was as follows: Slides are placed in steel racks, soaked overnight in 5 % Decon and then washed under hot running water for 30 minutes. They were then washed five times in milli-Q H_2O for five minutes and dried in an oven for 30 minutes. The slides were then placed successively in the following tanks for one minute while agitating: tank 1. subbing solution (2 % 3-aminopropyltriethoxysilane in acetone); tank 2. acetone; tank 3. acetone; tank 4. milli-Q H_2O; tank 5. milli-Q H_2O. After subbing, the slides were dried in an oven and ready for use.

Sections of artery were cut to a thickness of 12 μm using a Cryostat and two to three sections were mounted on one slide. After sections had dried they were fixed by submerging in a 2% paraformaldehyde solution (composition: 2 g paraformaldehyde, in 40 ml 0.2 M Na_2HPO_4, 10 ml 0.2 M NaH_2PO_4 and 50 ml deionised H_2O) for 10 minutes and then washed in PBS twice for 15 minutes. Excess buffer was blotted off and 100 μl of 0.5% Triton-X in blocking solution (10% donkey serum and in PBS) was carefully pipetted onto the sections. The blocking solution helps to reduce non-specific immunoreactivity and Triton-X permeabilizes the smooth muscle cell membrane allowing access of the antibody to the whole receptor molecule. After 30 minutes the slides were washed twice in PBS for 15 minutes and again dried of excess solution. They were then incubated overnight at 4°C with primary anti-P2X antibody diluted in 10 % donkey serum in PBS (100 μl per slide).

The following morning, the slides were washed six times in PBS for 10 minutes. After blotting off excess buffer, 100 μl of the secondary antibody (FITC-conjugated anti-rabbit raised in donkey, Jackson Immunoresearch, 1:100 dilution in 10% donkey serum in PBS) was pipetted onto sections and tissues were incubated at room temperature for a further two

hours. Slides were then washed twice for 15 minutes in PBS. Excess fluid was again removed from the slides, 15µl of Citifluor mounting fluid was applied and sections were sealed with a cover slip. Up to three controls were used to check for non-specific binding; in each case the protocol up to application of the primary antibody was identical:

(i) where available, a blocking peptide was incubated with its corresponding primary antibody for one hour prior to the incubation. This peptide corresponds to the epitope to which the antibody is raised and should abolish all immunoreactivity with the tissue. Using a blocking peptide will show whether there is non-specific binding of the primary antibody to a site that does not correspond to the epitope. A blocking peptide was available for anti-$P2X_{1,2,4,5}$ and $P2X_7$ antibodies and was used at a concentration equivalent to the antibody.

(ii) control slides were first incubated with blocking solution rather than primary antibody and then with secondary antibody to check for non-specific binding of the latter.

(iii) a final control slide to check for tissue autofluorescence was incubated with blocking solution replacing both primary and secondary antibody incubations.

Tissue sections were visualized under an epifluorescence microscope (Nikon labphot 2 with EFD3 fluorescence filter attachment), images were recorded with a COHU CCD camera and captured using Scionimage software on an Apple Power Macintosh. If the fluorescence intensity was too great, neutral density filters were used to reduce brightness. The same filters were used on all slides from one experiment. Images were stored as tif files and optimized using Corel Photoshop software.

2.4 MOLECULAR BIOLOGY

To complement the immunohistochemical studies, patterns of P2X and P2Y receptor isoform expression were also studied using RT-PCR. RNA was extracted from each of the three types of artery and reverse transcribed in to cDNA. PCR reactions were then performed using primers that are specific for each P2X and P2Y receptor isoform. As no antibodies are available for P2Y receptors, PCR is the only means available for studying their subtype distribution.

2.4.1 RNA EXTRACTION

Total RNA was isolated from small, medium and large mesenteric arteries for reverse transcription as described by Chomczynski & Sacchi (1987). RNAse-free conditions had to be used throughout the RNA extraction procedure. Dissection instruments were washed with ethanol, treated with RNAse-away and wrapped in aluminium foil until use. If solutions could be autoclaved, they were treated with diethyl pyrocarbonate (DEPC) to destroy any enzymes present. 1 ml of DEPC per litre was added to the solution which was shaken vigorously to form an even suspension and then autoclaved. Dissection dishes were lined with agarose made up in DEPC-treated PBS and all tissues were dissected in DEPC-treated PBS.

After dissection, the artery was placed in a round-bottomed 1.5 ml eppendorf tube containing 484.4 µl denaturing solution (4M guanidium thiocyanate, 25mM sodium citrate pH7, made up with DEPC-treated H_2O) + 3.6 µl beta-mercaptoethanol (final concentration 105 mM) and put on ice. The tissue was then homogenised using a Polytron homogeniser until it was visibly broken up. Arteries frequently remained intact even after prolonged homogenisation. This was a particular problem with small and medium arteries as they were not large enough to always be drawn into the blade of the Polytron. As an additional disruption step, the tissue suspension was therefore spun through a Qiagen shredder column at 13,000 rpm for 5 minutes.

Once tissue disruption was complete, 8.3 µl of 30% sarkosyl detergent (BDH) was added to the solution, which was then briefly vortexed. The volume at this point was made up to 500 µl with denaturing solution. To the tissue suspension, 33 µl of 3M sodium acetate (pH 4.5 made up in DEPC-treated H_2O) and 600 µl of 5 : 1, Phenol : SEVAQ (25 : 1, chloroform : isoamylalcohol) were added. The mixture was vortexed, placed on ice for 10 minutes and centrifuged at 13,000 rpm and 4°C for 20 minutes. The aqueous layer was then very carefully removed from the top avoiding the interface and placed in a new tube. 1 ml of cold absolute ethanol was added to the RNA solution, which was then precipitated at -20°C overnight. The solution was then centrifuged at 13,000 rpm and 4°C for 30 minutes to pellet the precipitated RNA. In order to facilitate visualisation the tubes were orientated in the centrifuge with the hinge facing away from the centre. The pellet would therefore be expected to form along the

hinge side of the tube. After spinning, as much supernatant as possible was removed by pipette and the pellet washed with cold 70 % ethanol (made up in DEPC-treated H_2O). Tubes were centrifuged at 13,000 rpm for 2 - 3 minutes and washed twice more as above. The pellet was then dried for 2 - 3 minutes in an oven at 60°C (tubes left open) and resuspended in 22 µl of DEPC-treated H_2O by vortexing and trituration.

As an alternative, commercially available RNA extraction kits were also tried (RNeasy Kit by Qiagen and Total Quick RNA kit by Talent). In each case the procedure was performed as described in the instructions, which yielded a solution of RNA equivalent to the final step of the isolation method described above.

2.5.2 cDNA SYNTHESIS

After RNA had been isolated from the tissue it was reverse transcribed into cDNA for subsequent PCR analysis. Thin-walled PCR eppendorf tubes were used for cDNA synthesis reactions. Each 22 µl sample of RNA was split into two equal halves, one reaction to include reverse transcriptase and one without reverse transcriptase. The volume of cDNA synthesis reactions was 20 µl for each tube and the components were added in the following order: 1 µl of oligo-dT primers (0.5µg/µl solution containing equal quantities of TTTTTTA, TTTTTTG and TTTTTTC; the extra terminal base aids annealing of the primers) was added to 11 µl RNA and the mixture heated in a thermocycler to 70°C for 10 minutes. The tubes were then snap cooled by placing directly on ice. After a brief centrifugation the following was added:

4 µl of first strand buffer (5 x stock)

2 µl DTT (0.1M)

2 µl dNTPs (each at 5mM)

1 µl of 'RNAase-out' RNAse inhibtor

The mixture was briefly centrifuged and placed in a water bath at 42°C for 2 minutes. 1 µl (200 units) of Superscript reverse transcriptase was then added to 'reaction' tubes and 1 µl of DEPC-treated H_2O added to 'control' tubes. The tubes were incubated at 42°C for 60 minutes, then heated to 70°C for 15 minutes in a thermocycler and finally place directly on ice. The cDNA solutions were stored at -20°C for future use.

The enzyme-free tubes served as a control for contamination with genomic DNA. If contaminating genomic DNA were present, the subsequent PCR reaction would yield a product even though no cDNA had been synthesised from RNA. If on the other hand, there was no contaminating DNA, the reaction lacking reverse transcriptase would contain no DNA template and should therefore produce no product after PCR. A control tube was included for every sample of cDNA that was reverse-transcribed.

2.4.3 PCR OF ARTERIAL cDNA

Primers specific for each P2X and P2Y receptor were used to detect which isoform is expressed in each size of mesenteric artery. The sequences of forward and reverse primer for each receptor isoform are given in Table 5. Each reaction volume was 25 µl and thin-walled PCR tubes were used throughout. The primers were added to the tube first, followed by a mix of the remaining constituents. Using a master mix made pipetting more accurate as adding very small volumes accurately to each individual tube (e.g. 0.25 µl of enzyme) is very difficult. It also ensured that all components were added to all tubes. A reaction using actin primers would therefore have been performed as follows:

1 µl each of forward and reverse actin primers (each containing 25 pmol/µl of primer) was added to each tube. The forward and reverse primers for all P2X receptors are already mixed and only 1 µl of total primer solution is required. As a result 19 µl of H_2O is added to the mix below for PCR using P2X primers. Enough mix containing the remaining components was then made up and divided between all reaction tubes. The quantities per tube were:

0.5 µl cDNA

0.75 µl $MgCl_2$ (to give a final concentration of 1.5mM)

2.5 µl of NH_4 Taq buffer (10 x stock)

1 µl dNTPs (5mM for each)

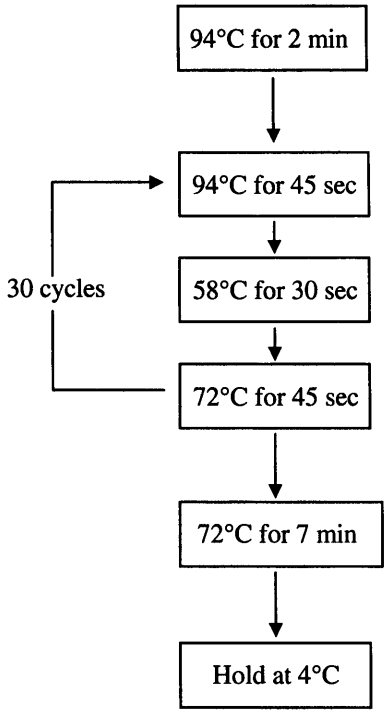
18 µl H_2O

0.25 µl Taq polymerase

Gene		Primer sequences (5' to 3')	Product size (bp)
$P2X_1$	forward reverse	CCTTGGCTATGTGGTGCGAGAGTC AGGCAGGATGTGGAGCAATAAGAG	382
$P2X_2$	forward reverse	CATCTTCAGGCTGGGTTTCATTG AGGGTCACAGGCCATCTACTTG	477
$P2X_3$	forward reverse	CCCCATTTTGCCCCATCTTGA ACTCGCTGCCGTTCTCCATCTTAT	252
$P2X_4$	forward reverse	GTGGGACTGCAACCTGGATAGAGC CTGAGCGGGGTGGAAATGTAACTT	424
$P2X_5$	forward reverse	CAACCGCCTGGACAACAAACACA CTGAGCAGGCCCCACCGAGAT	628
$P2X_6$	forward reverse	GACTGGAGAGGGGGTTGGGGTAAT AGGCAGGTGCTTCAGAATAGGTTG	311
$P2X_7$	forward reverse	GCAACTCTGGCGGCTTCATCC AGGCACAGAGGCGGCTTTTAGT	557
$P2Y_1$	forward reverse	TGGTGGCCATCTCCCCTATTCTCTT ATCTCGTGCCTTCACAAACTC	595
$P2Y_2$	forward reverse	TTCCACGTCACCCGCACCCTCTTATTACT CGAGTTCCCCAACTCACACATACAAATGATTG	539
$P2Y_4$	forward reverse	CTTCTCTGCCTGGGTGTTTGGTTGGTAGTA TCCCCCGTGAAGAGATAGAGCACTGGA	474
$P2Y_6$	forward reverse	GCCAGTTATGGAGCGGGACAATGG AGGAACAGGATGCTGCCGTGTAGGTTG	352

Table 5 Sequences of forward and reverse primers for PCR reactions detecting $P2X_1$ – $P2X_7$ receptors and $P2Y_{1,2,4}$ and $P2Y_6$ receptors. The size of the expected product amplified from the reaction is given in the right-hand column.

Each quantity was multiplied by the total number of reactions to produce the mix. 24 µl of mix is then added to each tube containing primers, briefly centrifuged and place in the thermocycler. The thermocycler settings for reactions using actin as well as all P2X primers were as follows:



The thermocycler settings for P2Y primers were as above except for the second (annealing) stage of the repeated cycle. In this case the temperature used was 55°C for $P2Y_1$ primers, 65°C for $P2Y_2$ and $P2Y_4$ primers and 66°C for $P2Y_6$ primers.

Actin primers were used to check for successful RNA extraction and the absence of any contaminating genomic DNA, as this is a ubiquitous and abundant mRNA. If results with these primers were satisfactory, all P2X and P2Y primers were subsequently tested. RNA extracted from brain was used as a positive control for the expression of all P2 receptor isoforms; a sample of this tissue will contain transcripts for all P2X and P2Y receptors.

2.4.4 VISUALISATION OF PCR PRODUCTS

TAE buffer (in 1 l H_2O to make 50 x stock solution: 242 g Trizma base, 57.1 ml glacial acetic acid, 37.2 g $Na_2EDTA.2H_2O$ pH to 8.5 with HCl/NaOH) was the running buffer used for electrophoresis and was used to make agarose gels. All subsequent steps were performed using 1 x TAE buffer. All PCR reactions were run on 1.5 % agarose gels containing 50 μg/ml ethidium bromide. Prior to loading the samples, 4 μl of loading dye (sucrose and dye) was added to each sample to track progress of band migration. A mixture of known molecular weight markers was placed in the first well of each gel to check for the size of PCR products. The gels were run at 150 V for 15 - 25 minutes and bands were visualised under a UV transilluminator. A picture of the gel was taken using a digital camera connected to a PC and gel images were captured using Kodak 1D Image Analysis Software.

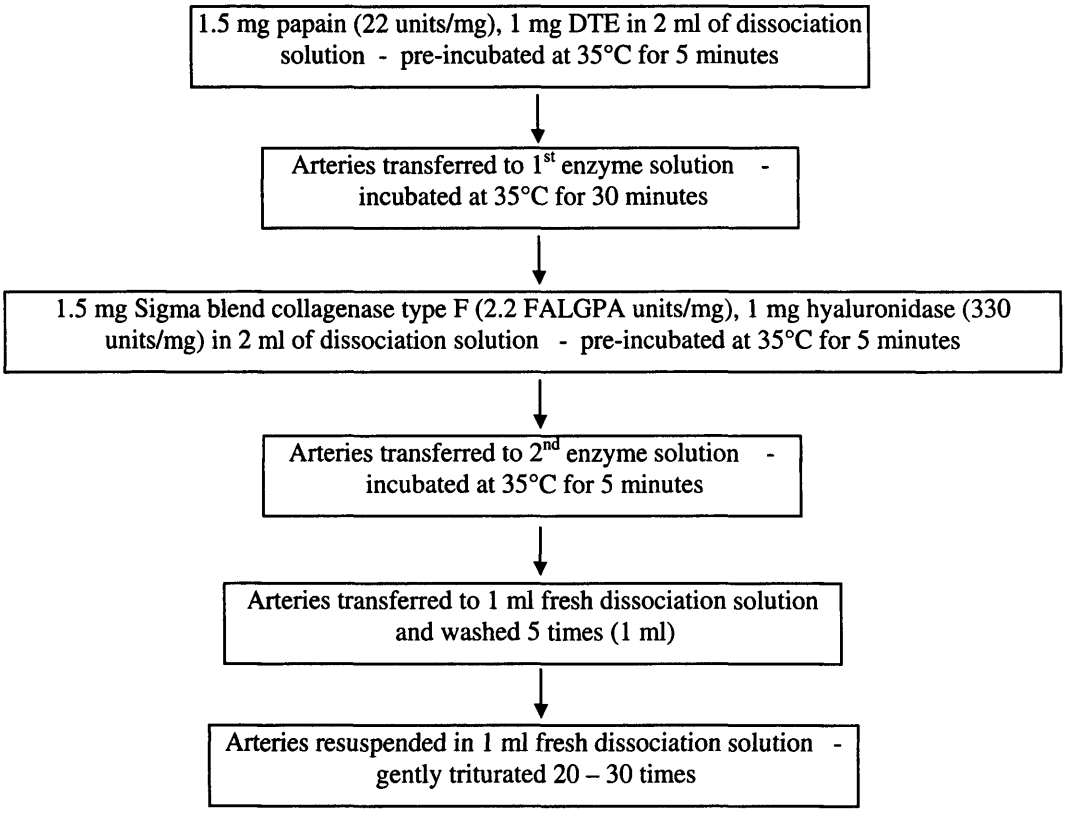
2.5 ELECTROPHYSIOLOGICAL CHARACTERISATION OF P2X RECEPTORS AND CALCIUM CHANNELS

In addition to investigating functional responses of whole artery segments, currents recorded from single smooth muscle cells were used to characterise the P2X receptors expressed in mesenteric arteries. It was of particular interest to compare results obtained in these experiments with those from contraction studies.

2.5.1 SMOOTH MUSCLE CELL DISSOCIATION

Arteries were dissected as described under section 1.1 using nominally calcium-free dissociation solution of the following composition: (in mM) NaCl 137, KCl 5.4, HEPES 10, $MgCl_2$ 1, Na_2HPO_4 0.44, NaH_2PO_4 0.4, $NaHCO_3$ 4.2, pH to 7.4 with NaOH. This solution was used to ensure that the smooth muscle cells remained elongated and did not contract during the dissociation process. Medium-sized vessels were cut open longitudinally to expose the lumen and then cut into three segments of roughly equal length. Large vessels were opened longitudinally and then cut into transverse strips approximately 1 mm wide. It was

not possible to further manipulate small arteries and they were left intact. The following protocol of enzymatic digestion was then used:



The suspension of dissociated cells was then plated onto 13 mm glass cover slips (~ 100 µl on each) and left to adhere for 2 - 3 hours at 4°C. Cells were stored at 4°C and used within 8 hours. All arteries were subjected to the same enzymatic digestion using the same concentration of enzymes, although the duration of incubation varied with the size of the vessel and the freshness of the enzymes. The above protocol was used for medium-sized arteries; for small arteries the first and second incubations were 32 and 15 minutes respectively, while for large vessels they were 30 and 12 minutes.

2.5.2 CURRENT RECORDINGS

All electrophysiology experiments were performed on cells dissociated from all three sizes of artery and were conducted at room temperature (20 - 24°C). Cells were superfused with

physiological solutions at 2 ml/min from a raised reservoir (gravity feed) and drugs were applied using a rapid U-tube application system (Fenwick *et al*, 1982; Evans & Kennedy, 1994). Antagonists were also applied by bath superfusion. Both the perfusion and drug application systems were connected to 5-way manifolds to allow simple exchange of solutions for superfusion/application. Micro-electrodes were pulled using an automated two-step electrode puller (Narishige, Japan) and fire polished. When filled with internal solution they had a resistance of 2 - 6 MΩ. Recordings were made in the whole cell patch clamp configuration with an Axopatch 200B amplifier using standard techniques. Data were collected and analysed using pClamp 7 software (both Axon Instruments, USA). Cells were maintained at holding potentials which were corrected for liquid junction potentials.

2.5.2.1 Solutions

Both the superfusing external solution and internal pipette solutions used depended on the experimental protocol: for P2X receptor current recordings the external bath solution was PSS (in mM: NaCl 150, KCl 5, HEPES 10, $CaCl_2$ 2.5, $MgCl_2$ 1, pH to 7.3 with NaOH) and the internal pipette solution was $I_{K\text{-gluconate}}$ (in mM: 140, NaCl 5, EGTA 9, HEPES 10, pH to 7.3 with KOH). For calcium current recordings the bath solution was E_{barium}, (in mM: NaCl 130, KCl 5.4, Glucose 10, HEPES 10, $BaCl_2$ 10, EGTA 0.1, pH to 7.3 with NaOH) and the internal pipette solution was I_{CsCl} (in mM: CsCl 150, HEPES 10, EGTA 10, TEA 10, phosphocreatine 8.8, $MgCl_2$ 2.1, Na_3GTP 0.3, $CaCl_2$ 0.2, pH to 7.3 with CsOH). When recording potassium currents, the solutions used were PSS and $I_{K\text{-gluconate}}$.

2.5.2.2 P2X receptor currents and suramin inhibition

The U-tube application system was triggered with pClamp software and agonist applications were 500 ms in duration. Cells were at a holding potential of –60 mV. Because of run-down of currents in response to P2X receptor activation, the first recording made in a cell was always the largest and taken to be the cell's 'true response'

2.5.2.3 Calcium currents

These experiments were conducted replacing calcium with barium as the charge carrier as this is known to have several beneficial effects on currents: amplitudes are larger, they do not inactivate as quickly and there is less run-down of responses . The external perfusing solution was E_{barium} and the internal pipette solution was based on CsCl (I_{CsCl}) as intracellular caesium blocks potassium currents. It was necessary to block potassium currents because they have amplitudes of up to 1 nA and would mask much smaller calcium currents. Phosphocreatine and GTP were included in the internal solution to allow the synthesis of ATP in a regenerative cycle, which helps reduce run-down of currents. The pipette solution was allowed to dialyse with the cell cytosol for 2 - 3 minutes before recordings were started. For current-voltage relationships, cells were maintained at a holding potential of -60 mV and subjected to 10 depolarising steps to command potentials 10 mV more positive than the previous (i.e. -60 mV to -50mV, -60mV to -40mV, -60mV to -30mV etc.) up to the final command potential of +40 mV. Depolarizations were 50 ms in duration. After recording calcium currents, the superfusing solution was switched to E_{barium} containing 1mM $CdCl_2$ and after 5 minutes another set of recordings was made. Currents in the presence of $CdCl_2$ were later subtracted from currents recorded in the absence of the blocker in order to remove any leak current from the response and obtain the pure calcium current.

In addition, step depolarizations to two command potentials were performed, which had been shown by the I-V relationship to produce the largest calcium currents. These were also repeated in the presence of 1μM nifedipine (presuperfusion as above) to detect any non-L-type calcium currents. A final recording in the presence of 1 mM cadmium was made to subtract any leak currents from both the calcium current and the nifedipine-resistant current.

2.5.2.4 Potassium currents

The external perfusing solution was PSS and the internal pipette solution was $I_{Kgluconate}$. Cells were held at a holding potential of -60 mV and then hyperpolarized to -120 mV. From there 12 step depolarizations to command potentials 20 mV more positive than the previous were

made (i.e. -100 mV to -80mV, -100mV to -60mV, -100mV to -40mV) up to the final command potential of +120 mV.

2.6 TRANSGENIC MOUSE STUDIES

2.6.1 CONTRACTION STUDIES

In order to assess the effect of deleting the $P2X_1$ gene on mouse physiology, it was essential to conduct functional studies in tissues where the receptor would normally be expressed at high levels. The vas deferens was chosen because $P2X_1$-deficient male mice were infertile. As the vas deferens is involved in reproduction and expresses high levels of $P2X_1$ protein, this was an appropriate choice of tissue. Moreover, it is robust and reliably produces good experimental data. The function of the vas deferens was tested by studying its contractile behaviour.

Contraction experiments on mouse vas deferens were conducted in a traditional organ bath set-up. Male mice were killed by cervical dislocation and vasa deferentia carefully dissected. A piece of surgical suture silk was then firmly tied to each end of the tissue and a loop tied in each thread. With one loop attached to a steel holder and the other to a force transducer, the vessel was submerged in 15 ml organ bath containing Ringer's solution and placed under an initial tension of 1 g. Four organ baths were used simultaneously. Each organ bath was connected to a 60 ml reservoir of solution, which was used to wash the tissue; both chambers were gassed with 95% O_2/5% CO_2. Agonists and antagonists were added in the form of a concentrated stock solution (in deionised H_2O) to give the desired concentration when diluted in the organ bath. Continual bubbling of the solution in the organ bath chamber ensured rapid mixing of added drugs. After drug application, tissues were repeatedly washed with the solution in the reservoir. Neurogenic contractions were evoked through two silver electrodes positioned either side of the tissue, which were connected to a Grass stimulator. Reproducible responses were obtained when vas deferens were stimulated every 6 minutes. The stimulation parameters were again designed to stimulate sympathetic nerves rather than muscle tissue. A pulse width of 0.3 ms was used throughout all nerve-evoked contractions. For the majority of experiments 60 pulses at 20 Hz and 40 V was used; the parameters used when comparing the

effects of shorter trains of stimulation were 10 pulses at 20 Hz and 20 V, and 5 pulses at 10 Hz and 20 V.

2.6.2 MOUSE TAIL GENOTYPING

To confirm the genotype of mice used in pharmacological studies, PCR reactions were conducted on samples of tail tissue. Primers specific for the mouse $P2X_1$ gene and the neomycin resistance gene were used to determine whether animals were wild-type, heterozygote or knock-out.

2.6.2.1 DNA extraction

Mouse tail samples 5 mm in length were placed in a 1.5 ml eppendorf tube containing 0.4 ml of tail buffer of the following composition: 10 mM Tris-HCl (pH8), 50 mM NaCl, 50 mM EDTA and 0.5 % SDS to which 0.5 mg/ml proteinase K (Sigma P-0390) had been freshly added. The mixture was incubated overnight at 55 – 60°C. After incubation, 0.1 ml 5M NaCl was added and the digest vortexed for 5 - 10 seconds. 0.5 ml of chloroform was then added, the tube gently mixed for 5 - 10 seconds and spun in a centrifuge at 13.000 rpm for 5 minutes. The top layer of solution was then carefully transferred to a new tube taking care not to disturb the interface. 1 ml of cold absolute ethanol was added to precipitate the DNA and the tube mixed well. The excess ethanol was then removed by pipette taking care not to lose any DNA. 1 ml of 70 % ethanol was added to wash the DNA, centrifuged at 13.000 rpm for one minute and then removed again by pipette. After another wash, as much ethanol as possible was removed from the tube and the DNA left to air dry for a few minutes. The DNA was then resuspended overnight in 100 µl of sterile H_2O in a 0.5 ml eppendorf tube. It was diluted 1/10 for use in PCR reactions.

2.6.2.2 PCR

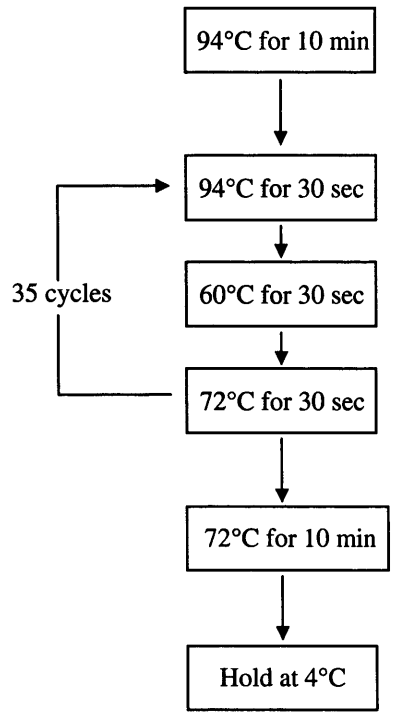
For these reactions, 'Ready To Go' PCR beads from Amersham Pharmacia were used. In order to reduce consumption, the beads were divided and used for two rather than one reaction. The components for each reaction were: 0.5 µl of forward and 0.5 µl of reverse

primers for the $P2X_1$ (20 pmoles of each) and neo^R (20 pmoles of each) gene respectively (2 µl in total), 1 µl DNA, 9.5 µl sterile H_2O (containing ½ PCR bead).
Primer sequences (5' to 3'):

Wild-type $P2X_1$ gene
Forward AACCCAGATCCCACCAACGAAC
Reverse TCACCCAATGACGTAGACCAGAAC

Neo^R gene in knock-out construct
Forward GGTTCTCCGGCCGCTTGGGTGGAG
Reverse GCGCGCCTTGAGCCTGGCGAACAG

A mix of all components except the DNA was made to reduce the number of pipetting steps and ensure every tube contained all constituents. For four reactions the following mix was made: 2 PCR beads were dissolved in 38 µl of H_2O to which 2 µl of each forward and reverse primer ($P2X_1$ and neo^R) was added. The 46 µl mix was divided into four tubes containing 11.5 µl, and 1 µl of template DNA was added to each to give a total reaction volume of 12.5 µl. The following protocol was used for the PCR reaction:



PCR products were loaded on an agarose gel as described in section 1.5.4 and visualised on a U.V. transilluminator. Images were captured using Kodak 1D Image Analysis Software.

2.7 DATA ANALYSIS

2.7.1 GENERAL METHODS

Data are described as mean ± s.e.m. throughout, with n = number of observations. These parameters were calculated using Microcal Origin 5.0 software. The effects of antagonists or potentiators are expressed as a percentage of the control response in the absence of the compound. Differences between means were tested using a Student's t-test, either two sample two-tailed or paired, as appropriate. A p value of < 0.05 was considered to be statistically significant. Statistical tests were performed using Microsoft Excel 97.

2.7.2 CONCENTRATION-RESPONSE RELATIONSHIPS

Concentration-response relationships were analysed using the Hill equation. The Hill equation provides a means of analysing the binding interaction between receptor and its ligand. In addition to describing the potency of a ligand at a receptor, it is also used to estimate the number of ligand molecules that bind to a receptor in order to produce the observed response. I have however mainly used the Hill equation to compare agonist potencies.

Concentration-response data were fitted by the least squares method with the following form of the Hill equation using Microcal Origin software:

$$\text{response} = \alpha[A]^H / ([A]^H + [A_{50}]^H)$$

where α is the asymptote, H is the Hill co-efficient, [A] is the concentration of agonist, and A_{50} is the concentration of agonist producing 50% of the maximal response (EC_{50}). The parameter pA_{50} is equivalent to $-\log EC_{50}$.

The affinity of a competitive antagonist for a receptor can be determined by using Schild analysis to calculate its pA_2 value (Arunlankshana & Schild, 1959). This approach was used to compare the potency of suramin in the three sizes of mesenteric artery. A number of assumptions must be made when using Schild analysis to calculate antagonist potency: the level of response is directly proportional to the number of activated (occupied) receptors; the agonist acts at only one receptor subtype; both agonist and antagonist bind competitively and reversibly; the antagonist causes parallel right-ward shifts of the concentration-response curve with no decrease in the maximal response. In order to perform a Schild regression, full concentration response curves must be constructed in the absence and in the presence of at least three different concentrations of antagonist. This was done for medium-sized arteries. Because of time constraints, full concentration response curves were not constructed in small and large arteries. In these vessels, data from two concentrations of agonist were used to estimate the pA_2 value for suramin.

2.7.3 Electrophysiology

Data for kinetic parameters of P2X receptor currents were calculated using the Clampfit mode of pClamp 7 software. The decay constant τ was determined from a mono-exponential curve fitted to the current trace using the same software.

3 AGONIST - EVOKED CONTRACTIONS

3.1 INTRODUCTION AND AIMS

The circulation is divided into a series of vascular beds that control the blood supply to different organs and tissues. Within the arterial side of each bed, vessels branch from a large conduit artery down to small resistance arteries and arterioles. Conduit vessels are responsible for the bulk supply of blood to an organ while resistance arteries are located deep within the tissue, optimising blood flow and regulating blood pressure. Because of these differing roles, it is conceivable that the type and density of receptors and channels expressed controlling vascular function may differ depending on the size of the vessel.

Such diameter dependent heterogeneity of expression has indeed been demonstrated for several ion channels, e.g. potassium (Quayle *et al*, 1996) and calcium channels (Bowles *et al*, 1997), and there is evidence to suggest that this may also be true of P2 receptors (e.g. Steinmetz *et al*, 2000b). One of the aims of the work described in this chapter was to determine whether there are differences in the properties of P2X and P2Y receptors between large and small arteries of the same vascular tree. A pharmacological approach based on agonist and antagonist sensitivities was used to characterise and compare the functional properties of P2X and P2Y receptors in small, medium and large rat mesenteric arteries. Using subtype selective agonists and antagonists also provided data on which P2X and P2Y receptor isoforms are involved in mediating contractile responses. This work has been published (see Appendix 1).

3.2 CONTRACTIONS EVOKED BY α,β-meATP

Contractions of rat mesenteric arteries in response to P2X receptor activation were characterised by studying concentration response relationships to the metabolically stable ATP analogue α,β-meATP. Previous studies have shown that ATP is subject to enzymatic degradation in whole tissue experiments (Evans & Kennedy, 1994; Khakh *et al*, 1995; Zimmermann & Braun, 1999). This problem was avoided by using α,β-meATP, which is largely resistant to breakdown.

α,β-meATP evoked concentration-dependent contractions in all sizes of artery. At low concentrations (≤0.3 μM for small and medium; ≤10 μM for large) responses were somewhat sustained with vessel diameter/tone only gradually returning toward baseline during agonist application (Fig. 5). At higher concentrations (1 and 3 μM for small and medium; 30 and 100 μM) the rapid desensitisation of the P2X receptor became more apparent. Contractions were more transient in nature, reaching a maximum and then declining back toward baseline in the continued presence of the agonist. This effect was pronounced approaching maximal concentrations of α,β-meATP (≥10 μM for small and medium; ≥300 mM for large). Arteries constricted very rapidly, reaching a peak response within ~ 5 sec and then quickly relaxed during agonist application, producing a very transient trace of diameter or tone. To overcome P2X receptor desensitisation, applications of P2X agonists were separated by 30 minutes. Preliminary studies had shown that this interval gives reproducible responses to P2X receptor agonists.

Although the time course of responses was very similar in all arteries, there was a substantial difference in the sensitivity to α,β-meATP. Medium arteries were ~50 times and small arteries ~200 times more sensitive to the agonist than large vessels (Table 6).

	EC_{50}	pA_{50}	Hill slope	n
Small	0.4 μM	6.4 ± 0.1	1.5 ± 0.2	5
Medium	2.5 μM	5.6 ± 0.2	1.2 ± 0.2	4
Large	107 μM	4.0 ± 0.1	0.9 ± 0.1	4

Table 6. Summary of data on potency of α,β-meATP in mesenteric arteries. The difference in pA_{50} values is significant between small and medium ($p<0.005$), small and large ($p<0.001$) and medium and large arteries ($p<0.005$).

To demonstrate that the sensitivity of an artery was the same regardless of which experimental system was used, medium-sized arteries were studied using both Diamtrak and myography. The results determined with Diamtrak are essentially the same as those obtained with the myograph (EC_{50} = 2.3 μM, pA_{50} = 5.7 ± 0.1 and Hill slope = 1.2 ± 0.1; n = 4).

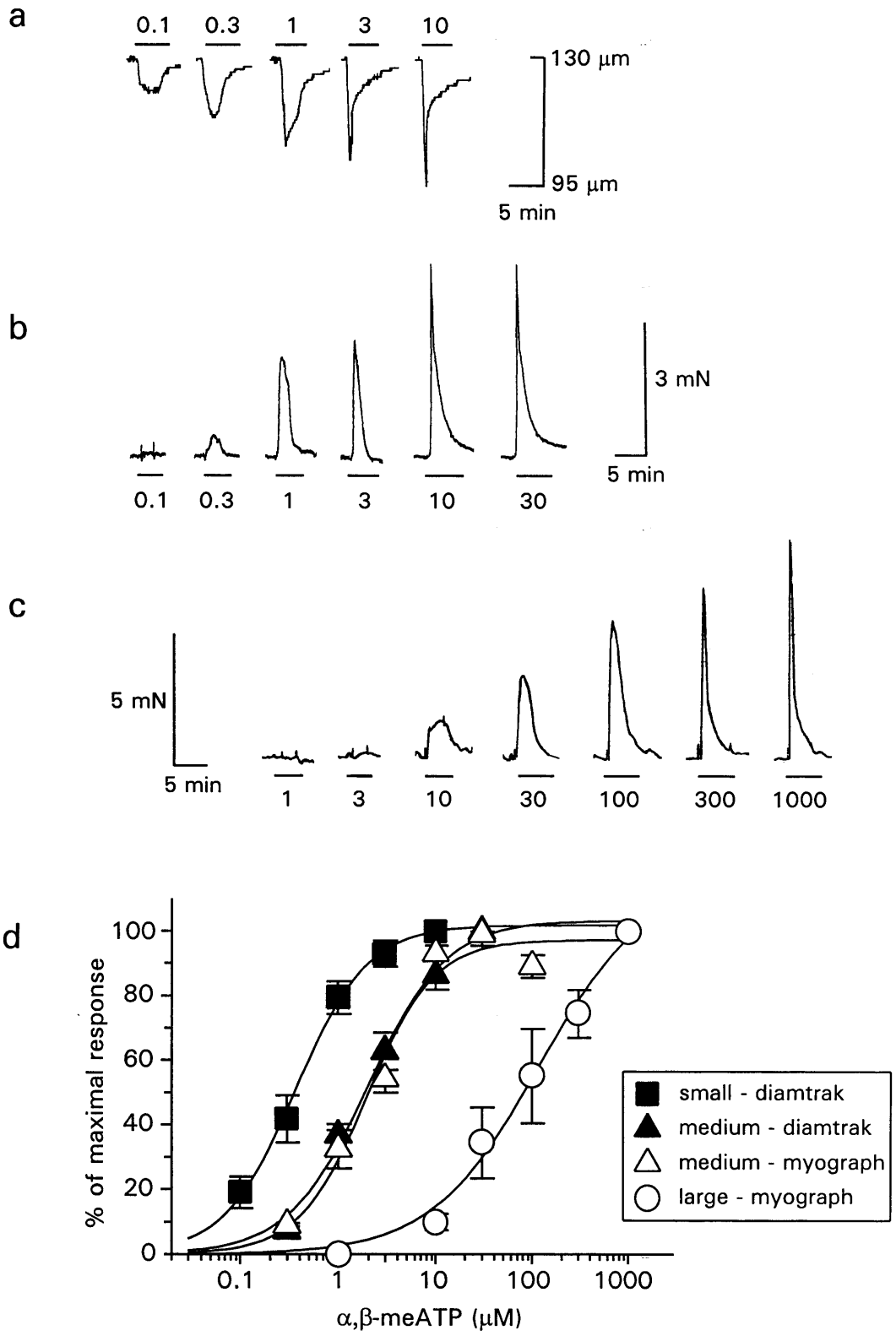


Figure 5 Characterisation of contractile responses to α,β-methylene ATP.(a), (b) and (c) show contractions in small, medium and large arteries respectively; values are concentration in µM and periods of agonist application are indicated by bars. Responses are clearly transient and concentration-dependent in all three vessel sizes. (d) Concentration-response curves showing different α,β-meATP sensitivity depending on vessel size: large arteries are significantly less sensitive than small or medium-sized arteries. The EC_{50} values for medium arteries determined using Diamtrak and myography are almost identical. Data are mean responses ± s.e.m. (n = 4 - 5) and are expressed as % of the maximal response to α,β-meATP in each tissue

It is possible that at the high range of concentrations used on large arteries, α,β-meATP may have contained significant amounts of contaminating ATP. Previous work has shown that commercially available ATP-based agonists can be substantially contaminated with ATP or related compounds (e.g. purity level of α,β-meATP is ~98%) producing potentially misleading results. If this were the case, contractions may have been caused by ATP acting through another, α,β-meATP-insensitive receptor. To test for this, the response evoked by ATP was determined at a putative contaminating level of 5 %. Such a protocol has been used previously to identify possible responses to contaminating agonists (Hartley *et al*, 1998). A 1 mM solution of α,β-meATP would therefore be contaminated with 50 μM ATP. This concentration of ATP however produced a response that was only 1.8 ± 1.9 % of that produced by 1 mM α,β-meATP (n = 4; Fig. 6). ATP was therefore not causing any contractions evoked in large arteries.

Calcium influx from the extracellular space has been shown to be essential for P2X receptor-mediated vasoconstriction (e.g. Galligan *et al*, 1995; McLaren *et al*, 1998a). It was therefore of interest to establish whether the requirement for extracellular calcium was the same in all three sizes of artery (Fig. 7). Removal of calcium from the extracellular solution abolished responses to 10 μM α,β-meATP in small and medium arteries (n = 4) and to 300 μM α,β-meATP in large arteries (n = 4).

3.3 PHENYLEPHRINE AND KCl

The reasons for this diameter-dependent difference in sensitivity of P2X receptor-mediated responses were unclear. On the one hand, the cause could have been differences specific to P2X receptors i.e. the number and subtypes of receptors expressed on arterial smooth muscle. There may however also be general differences in the properties of small and large arteries, such as calcium sensitivity or diffusional properties. In order to investigate this, concentration response relationships for other compounds mediating vasoconstriction were compared in small, medium and large mesenteric arteries. As noradrenaline is the other main transmitter co-released with ATP from sympathetic nerve terminals, it was of particular interest to compare the sensitivity of α-adrenoceptors in different arteries.

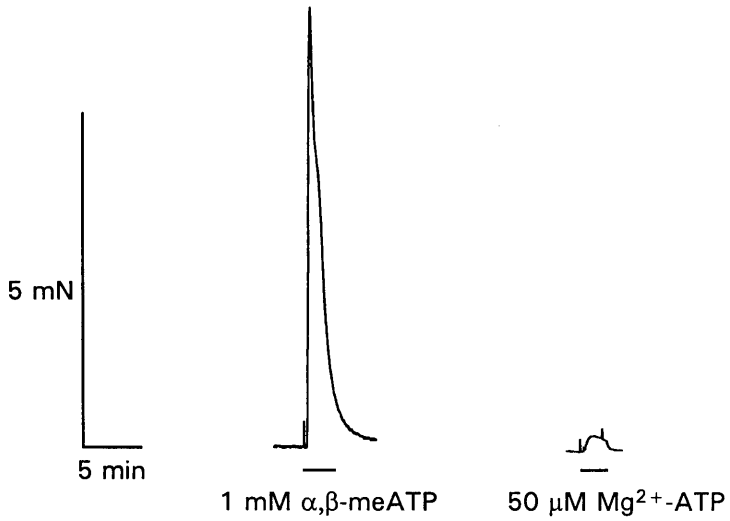


Figure 6 ATP is a weak agonist in large arteries. Comparing contractions shows that the response evoked by 50 μM ATP is only a fraction of that evoked by 1 mM α,β-meATP. Responses to high concentrations of α,β-meATP in large arteries are therefore not caused by contaminating ATP.

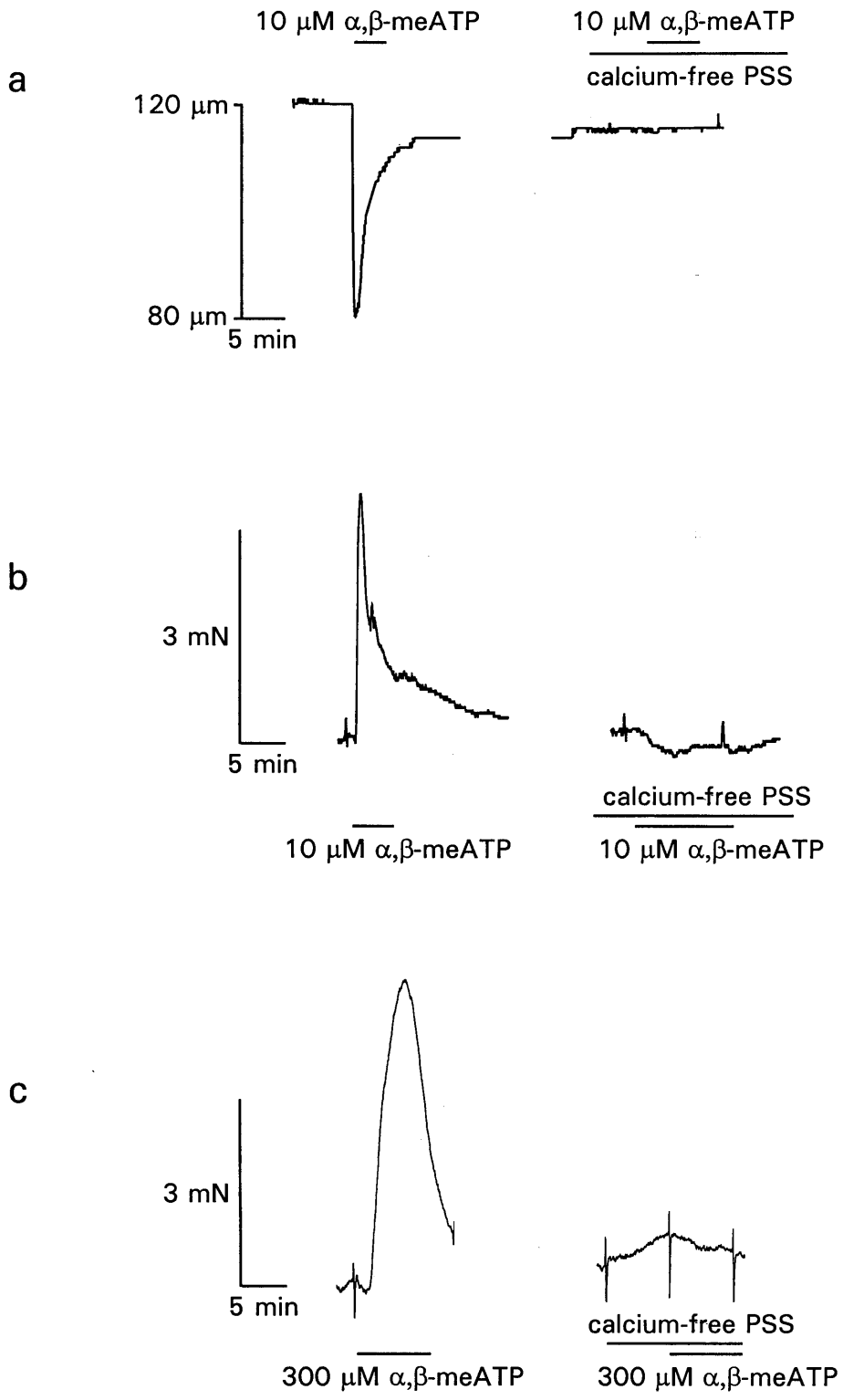


Figure 7 Contractions to α,β-meATP are abolished by removal of extracellular calcium. Typical responses to a near maximal concentration of α,β-meATP in small (a), medium (b) and large arteries (c); agonist applications is indicated by bars. Contractions in all vessels are abolished in calcium-free solution. Small deflections of the trace in (b) and (c) at solution changes are flow artifacts recorded by the force transducer.

The metabolically stable and α_1-adrenoceptor selective noradrenaline analogue phenylehprine evoked concentration-dependent contractions that were sustained throughout the five-minute application period. Responses were found to be very similar using either individual or cumulative additions, but in 90 % of experiments individual additions were made. The very similar potency of phenylephrine in all arteries suggests uniform expression of α_1-adrenoceptors throughout the mesenteric bed (Table 7; Fig. 8).

	EC_{50}	pA_{50}	Hill slope	n
Small	1.6 μM	5.9 ± 0.1	1.6 ± 0.4	4
Medium	3.0 μM	5.6 ± 0.2	4.2 ± 1.5	4
Large	2.9 μM	5.6 ± 0.2	1.8 ± 0.6	5

Table 7. Summary of data on potency of phenylephrine in mesenteric arteries. The differences in pA_{50} values were not significant suggesting uniform expression of α_1-adrenoceptors throughout arteries of the rat mesentery.

Substantially increasing the concentration of extracellular potassium ions depolarises the smooth muscle cell membrane. This opens voltage-gated calcium channels resulting in calcium influx and activation of the contractile machinery. Responses to a high concentration of KCl are therefore receptor-independent and provide a good indication of the general contractile behaviour of an artery. Substituted PSS containing 10 – 100 mM KCl evoked sustained concentration-dependent contractions. Concentration response curves were very similar for medium (n = 5) and large arteries (n = 4). For small arteries (n = 5) however, the concentration required to evoke 50 % of the maximal response was slightly but significantly lower in than in medium or large vessels ($p<0.05$). As part of the validation of techniques outlined in section 1.2.4, I also tested the sensitivity of medium-sized arteries using the Diamtrak system; concentration response curves obtained from both systems were virtually identical (n = 4; Fig. 9). Both the results for phenylephrine and KCl suggest that there is no anatomical difference between small and large arteries that might affect the diffusion of agonists through the tissue.

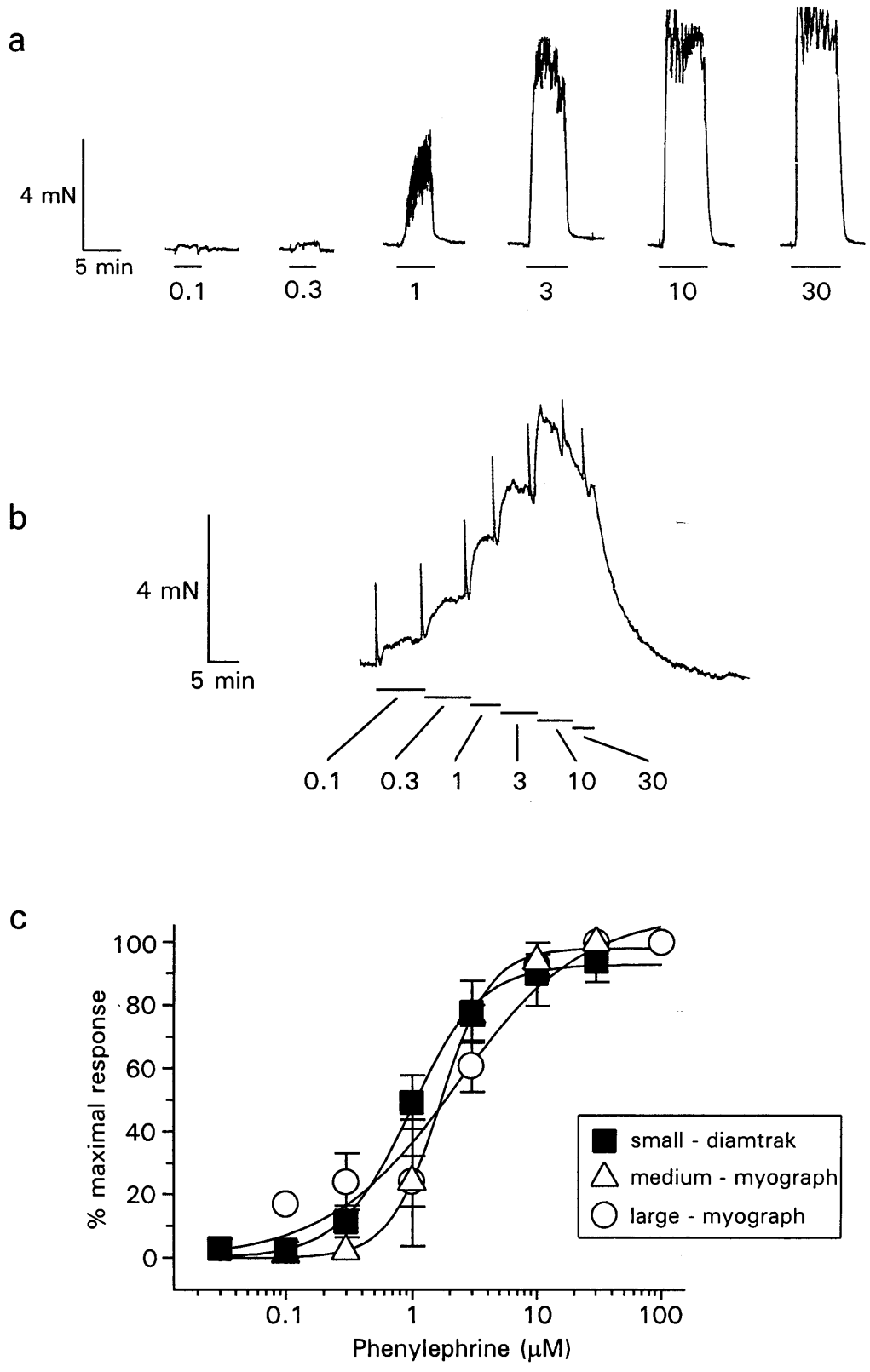


Figure 8 Phenylephrine is essentially equipotent in small, medium and large arteries. Maintained responses to individual and cumulative applications of phenylephrine in medium (a) and large arteries (b); values are concentration in μM and periods of agonist application are indicated by bars. (c) The three concentration response curves to phenylephrine show that the EC_{50} values are all approximately 1 - 2 μM. Data are mean responses ± s.e.m. (n = 4 - 5) and are expressed as % of the maximal response in each tissue.

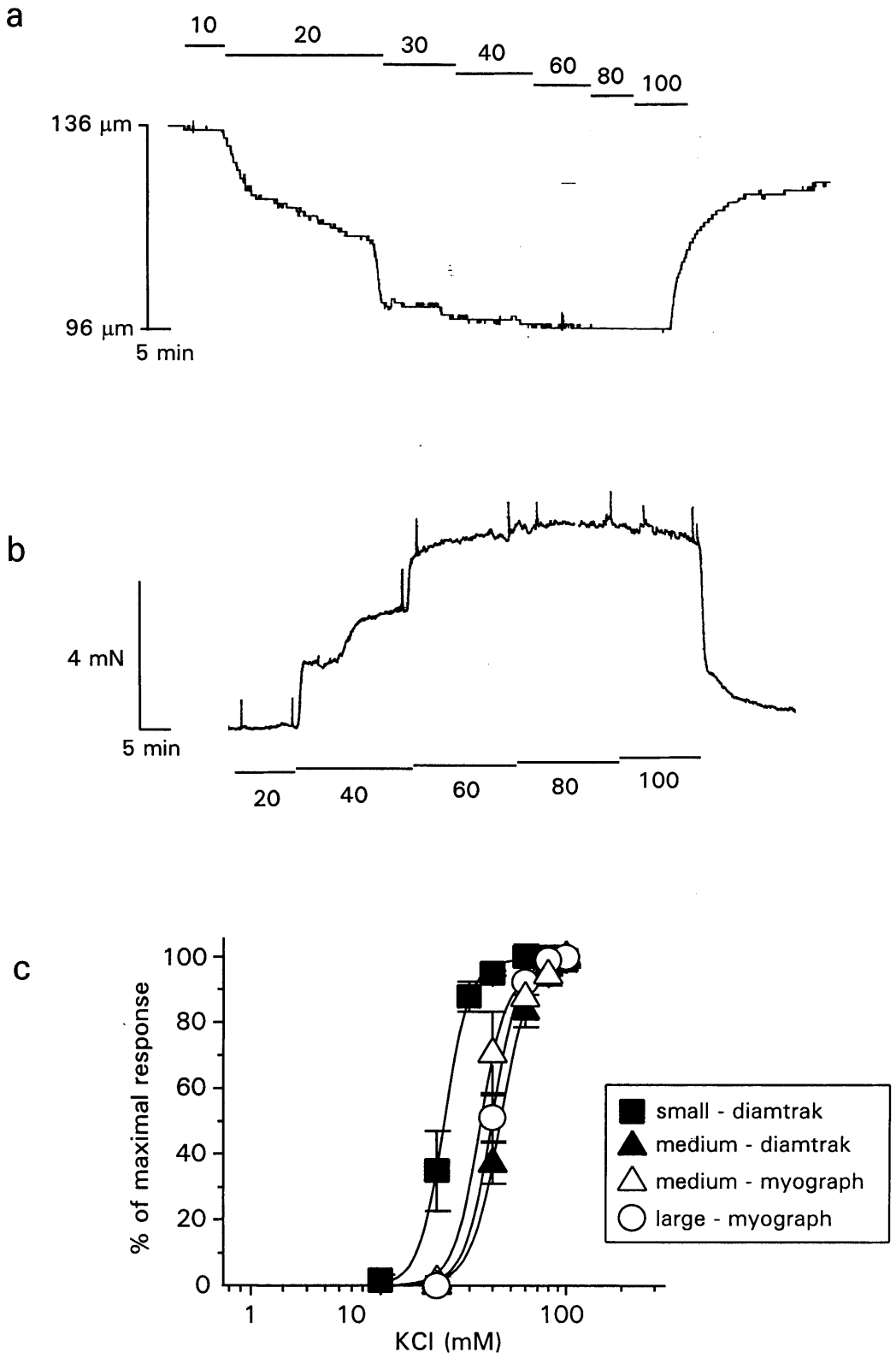


Figure 9 All arteries show similar sensitivity to KCl. Contractile responses to a solution containing a high concentration of KCl in small (a) and medium-sized arteries (b); values are concentration in mM and periods of agonist application are indicated by the bar. (c) Concentration effect curves showing the slightly higher sensitivity of small arteries. The experimental methodology again has no bearing on results in medium-sized arteries. Data are mean responses ± s.e.m. (n = 4 - 5) and are expressed as % of the maximal response in each tissue.

3.4 P2Y RECEPTOR AGONISTS

3.4.1 UTP

In the previous section I demonstrated significant differences in the properties of P2X receptors depending on the diameter of the vessel. If such regional variation exists for ionotropic purinoceptors the same may be true of metabotropic purinoceptors. Concentration effect relationships for a P2Y receptor agonist were therefore compared in the three sizes of mesenteric artery. UTP has been shown to evoke contractile responses in several vascular beds including the rat mesentery (Ralevic & Burnstock, 1996; Ohara *et al*, 1998). It is a potent agonist at $P2Y_2$ and $P2Y_4$ receptors, which may both be expressed on vascular smooth muscle cells (Kunapuli & Daniel, 1998).

UTP evoked concentration-dependent contractions in all arteries tested (Fig. 10). In small arteries responses were sustained over the five-minute period of application. Medium-sized and large arteries were found to be relatively insensitive to UTP and very high concentrations of UTP had to be used during these experiments. Despite increasing the concentration to 3 mM UTP, a clear maximum was not seen in the concentration response curves for these vessels and EC_{50} values had to be estimated (Table 8).

	EC_{50}	pA_{50}	Hill slope	n
Small	15 μM	5.0 ± 0.2	1.1 ± 0.3	5
Medium	1.6 mM	2.9 ± 0.1	0.9 ± 0.1	6
Large	1.4 mM	2.9 ± 0.2	1.3 ± 0.2	5

Table 8. Summary of data on the potency of UTP in mesenteric arteries. UTP was significantly more potent in small than in medium or large arteries ($p<0.005$).

The agonist was also tested on medium-sized arteries using Diamtrak, producing a mean EC_{50} value of 88 μM (pA_{50} = 4.1 ± 0.1 and Hill slope = 2.2 ± 0.3, n = 4). Unlike with α,β-meATP, there was thus a significant difference in the potency of UTP in medium arteries determined with Diamtrak compared to that determined with myography ($p<0.005$). Small arteries were however

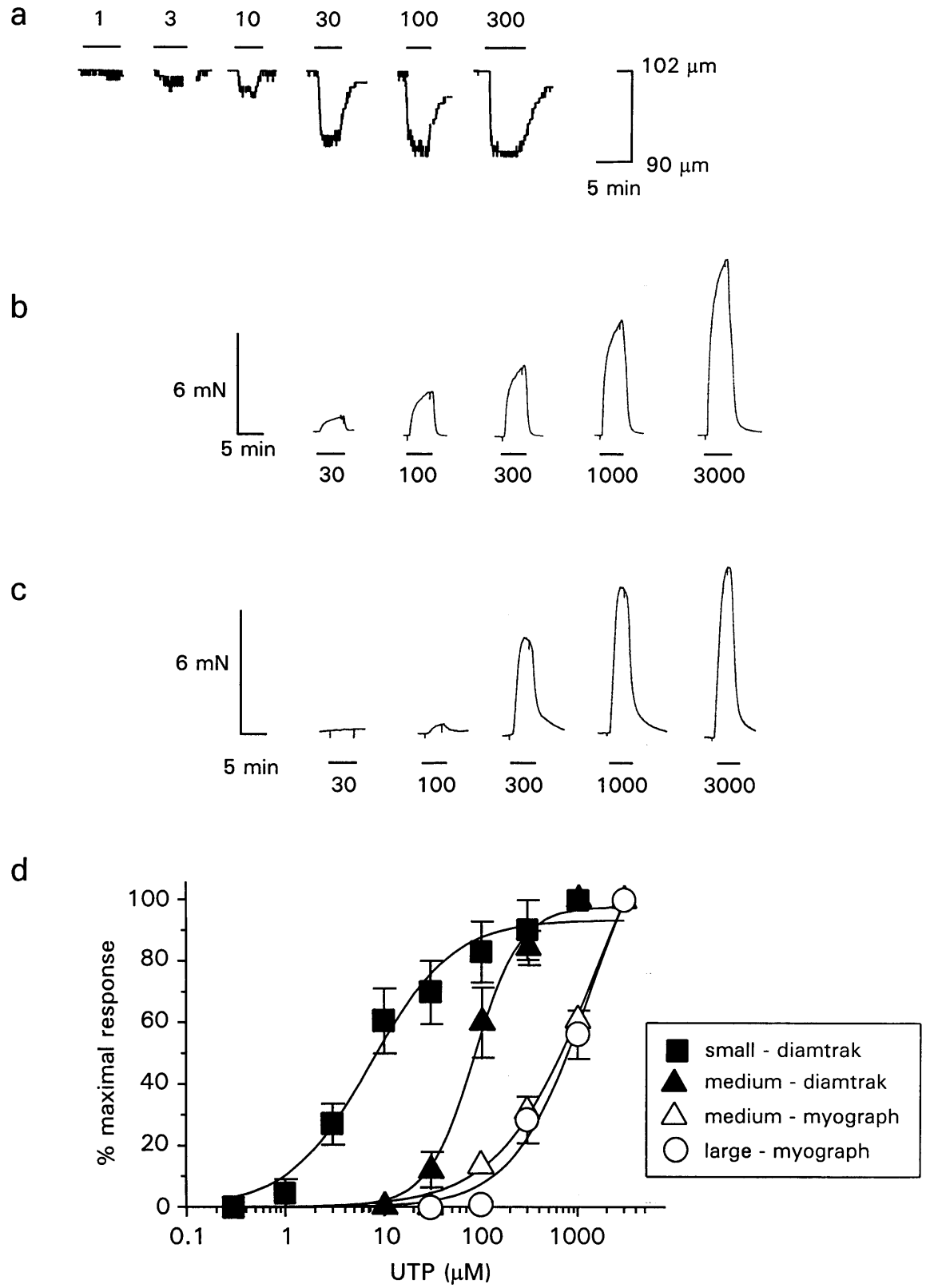


Figure 10 Characterisation of contractile responses to UTP. Typical responses in small (a), medium (b) and large (c) arteries, showing sustained constrictions to UTP; values are concentration in µM and periods of application are indicated by bars. Concentration response curves for UTP in the three sizes of mesenteric artery show substantial differences in sensitivity of the arteries depending on their size (d). In this case however large and medium vessels have a similar EC_{50} value while that of small vessels is approximately 100 times lower. There is also a significant difference in EC_{50} values determined for medium vessels depending on the experimental system used. Data points are mean responses ± s.e.m. (n = 5 - 7) and are expressed as a % of the maximal response in small arteries and as % of the response to 3 mM UTP in medium-sized and large arteries.

still significantly more sensitive to UTP than medium vessels when using the Diamtrak system ($p<0.02$).

3.4.2 UTP-γ-S

A possible reason for the increased potency of UTP in small arteries may have been a lower level of metabolic degradation. Such metabolic breakdown can be avoided by the use of agonist analogues that are resistant to enzymatic degradation. UTP-γ-S is one such analogue of UTP. Comparing responses to UTP and UTP-γ-S can give an indication of the level of breakdown occurring within arterial tissue. As the potency of UTP was almost equal in medium and large arteries, contractions to UTP and UTP-γ-S were only compared in small and medium arteries (Fig. 11). Expressed as a percentage of the response to 10 μM UTP-γ-S, contractions to 10 μM UTP were 99.1 ± 17.2 % in small (n = 4) and 17.2 ± 10.9 % in medium-sized arteries (n = 6). Expressed as a percentage of the maximal response to UTP, contractions to 10 μM UTP-γ-S were 21.2 ± 6.3 in small (n = 4) and 18.1 ± 3.1 % in medium-sized vessels (n = 6).

3.4.3 CROSS-DESENSITISATION

UTP has been shown to activate P2X receptors in dissociated smooth muscle cells of the rat tail artery, but was roughly 100 times less potent than ATP (McLaren *et al*, 1998b). It was therefore possible that UTP also activated P2X receptors in mesenteric arteries. In order to verify that in my experiments UTP was indeed acting through P2Y and not P2X receptors, I assessed whether α,β-meATP could cross-desensitise responses to UTP. Initially, applications of UTP were made until reproducible responses were obtained. A near maximal concentration of α,β-meATP (10 μM in small and medium arteries and 300 μM in large) was applied and vessel diameter/tone allowed to return to baseline during the continued presence of the agonist. UTP and α,β-meATP were then applied concomitantly (Fig. 12). Expressed as a percentage of the response prior to α,β-meATP application, the amplitude of UTP-evoked responses after α,β-meATP was 99.7 ±

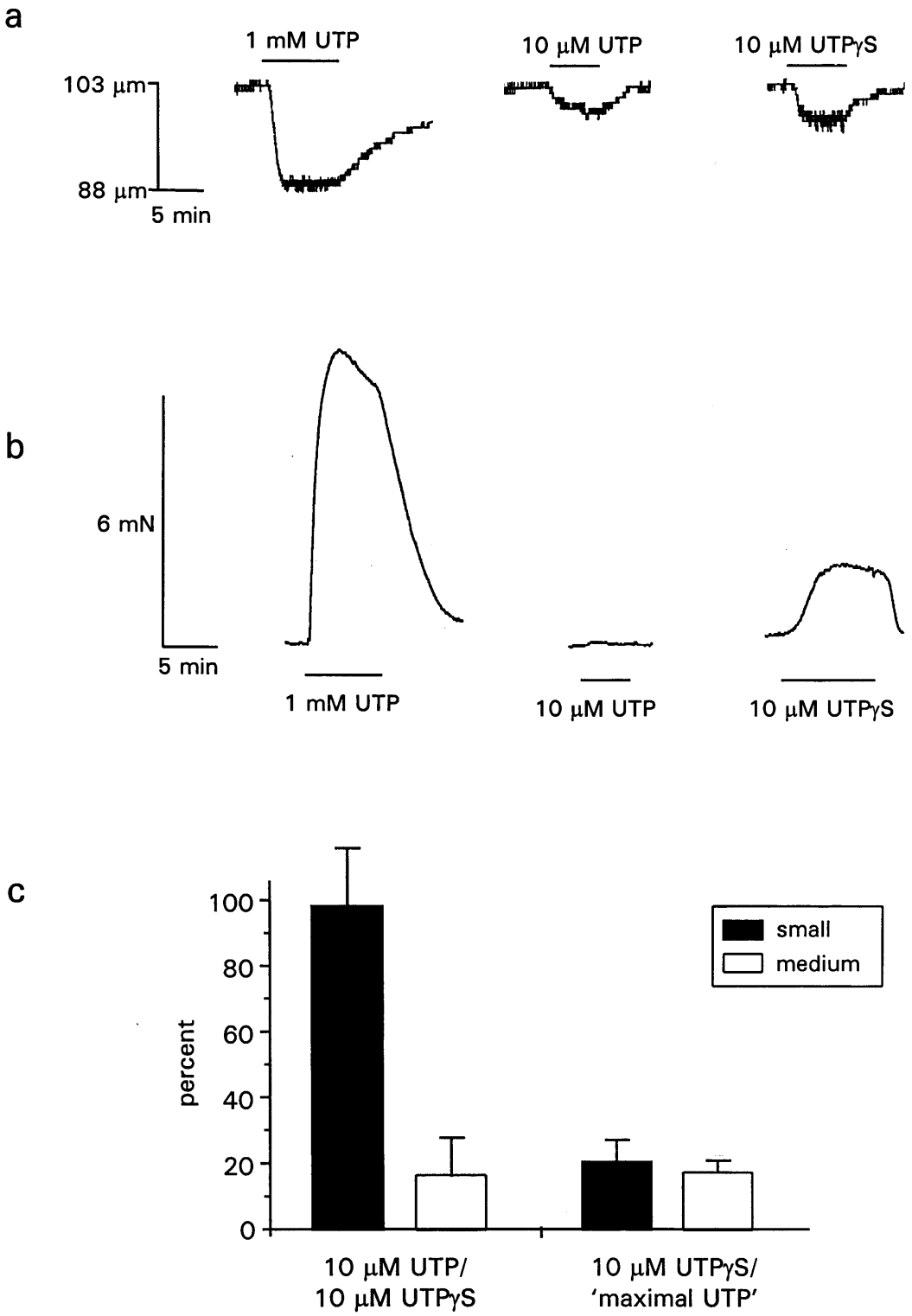


Figure 11 The stable UTP analogue UTPγS reveals substantial agonist breakdown in medium-sized arteries but very little in small arteries. Traces comparing the effects of 10 μM UTP and UTPγS as well as 1 mM UTP in small (a) and medium arteries (b). (c) UTPγS is much more potent than UTP in medium arteries while the two agonists are similarly potent in small arteries; responses to 10 μM UTP expressed as a percentage of the maximal response to UTP are similar in both vessels. Data are mean ± s.e.m (n = 4).

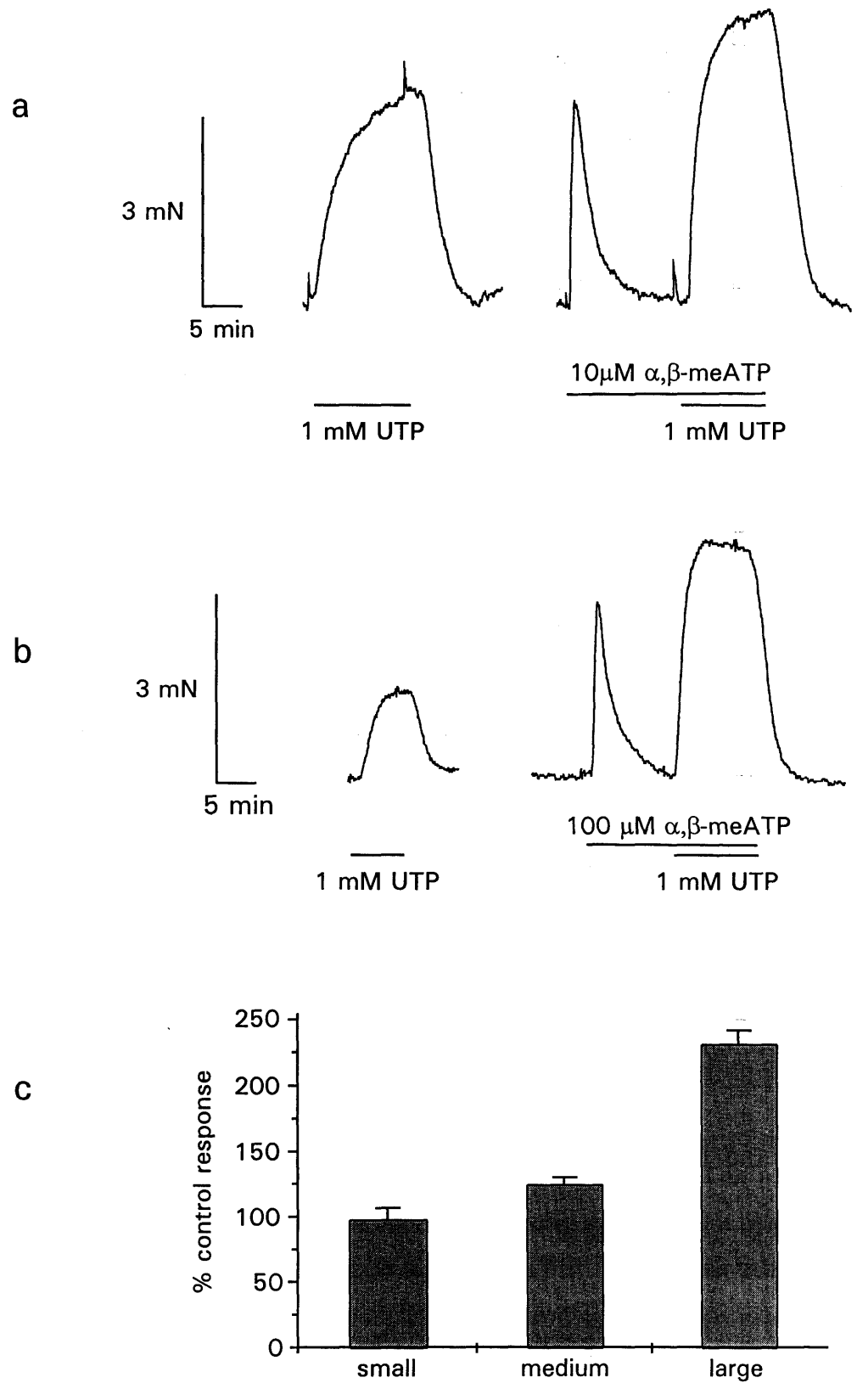


Figure 12 Contractions to α,β-meATP and UTP are mediated by different receptors. Typical cross-desensitisation experiments in medium (a) and large (b) arteries using near maximal concentrations of both agonists; agonist applications are indicated by bars. (c) Summary histogram showing no reduction of responses to UTP after desensitisation of $P2X_1$ receptors with α,β-meATP; responses in large arteries are even substantially enhanced. Data are mean ± s.e.m (n = 4 – 6) .

8.0 % (n = 6), 126.3 ± 5.2 % (n = 6) and 229.0 ± 10.7 % (n = 4) for small, medium and large arteries respectively. UTP therefore appears to be acting independently of P2X receptors.

3.5 OTHER P2X RECEPTOR AGONISTS

To further characterise responses at vascular P2X receptors I compared contractions evoked by α,β-meATP to those evoked by other putative P2X receptor agonists. These experiments were only conducted in medium-sized arteries and generally involved single applications rather than full concentration response curves

3.5.1 L-β,γ-meATP

L-β,γ-meATP is a metabolically stable, $P2X_1$ selective agonist (Trezise *et al*, 1995). A similar potency of α,β-meATP and L-β,γ-meATP in arterial smooth muscle would strongly support the view that contractions evoked through P2X receptors are mediated by the $P2X_1$ isoform. Assuming both compounds are full agonists, equal concentrations should produce comparable responses if they are both acting through the $P2X_1$ receptor. The fact that contractions were very similar in appearance and amplitude supports this conclusion (Fig. 13). Expressed in terms of mean responses to 10 μM α,β-meATP, contractions to 10 μM L-β,γ-meATP were 116.3 ± 18.6 % (n = 4).

3.5.2 DIADENOSINE POLYPHOSPHATES

Diadenosine polyphosphates are ATP-related compounds that are released into the circulation upon platelet activation (Floodgard & Klenow, 1982). They are naturally occurring ligands at P2 receptors that cause vasoconstriction by activating P2X receptors and vasodilatation by activating P2Y receptors (Ralevic *et al*, 1995; Steinmetz *et al*, 2000a). One of these compounds is Ap_5A, a metabolically stable and potent agonist at recombinant $P2X_1$ receptors (Bianchi *et al*, 1999). A concentration response curve for Ap_5A was constructed in order to compare its agonist

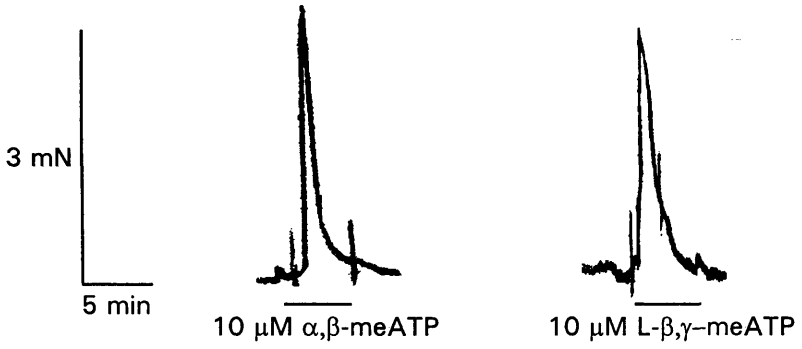


Figure 13 α,β-meATP and L-β,γ-meATP are equipotent in medium sized arteries. Sample traces showing the similar response to each agonist confirming the presence of $P2X_1$ receptors.

properties with those of α,β-meATP and to determine whether it is active at physiologically relevant concentrations. Contractions to Ap_5A were very similar in appearance and time course to those evoked by α,β-meATP (Fig. 14a) and Ap_5A was only slightly less potent: the mean EC_{50} value was 6.9 μM, the corresponding mean pA_{50} value was 5.2 ± 0.1 and the mean Hill slope was 1.2 ± 0.8 (n = 6; Fig. 14b).

To confirm that Ap_5A was evoking responses through P2X and not P2Y receptors, a cross-desensitisation experiment was conducted. Initially, Ap_5A (10 μM) was applied until responses were reproducible. An application of 10 μM α,β-meATP was made to desensitise arterial P2X receptors and continued until arterial tone had returned to baseline, which indicated that arterial P2X receptors were fully desensitised. 10 μM Ap_5A was then applied in the continued presence of α,β-meATP. Responses to Ap_5A were abolished (n = 4) using this protocol confirming that the agonist was also acting through P2X receptors (Fig. 14c).

The potential of other endogenously occurring Ap_nAs to cause vasoconstriction at physiological concentrations was also investigated. The potency of each compound was compared to that of α,β-meATP at a concentration of 10 μM. Ap_nAs with phosphate chain lengths from 2 to 7 were tested (Fig. 15). The results are summarised in Table 9.

Diadenosine Polyphsphate (10 μM)	Response as % of 10 μM α,β-meATP (n = 4)
Ap_2A	-5.7 ± 3.6
Ap_3A	-1.4 ± 1.4
Ap_4A	6.8 ± 0.5
Ap_5A	69.4 ± 10.6
Ap_6A	63.6 ± 5.6
Ap_7A	50.1 ± 11.2

Table 9. Comparison of contractile effect of diadenosine polyphosphates and α,β-meATP. Contractile potency rises with a chain length up to five phosphate groups and then drops as the number of groups increases beyond five.

As Ap_2A and Ap_3A had no intrinsic agonist activity of their own, it was of interest to determine whether they could modulate the activity of other P2X receptor agonists. Arteries were superfused with 10 μM Ap_2A or Ap_3A for 10 minutes followed by combined application of the

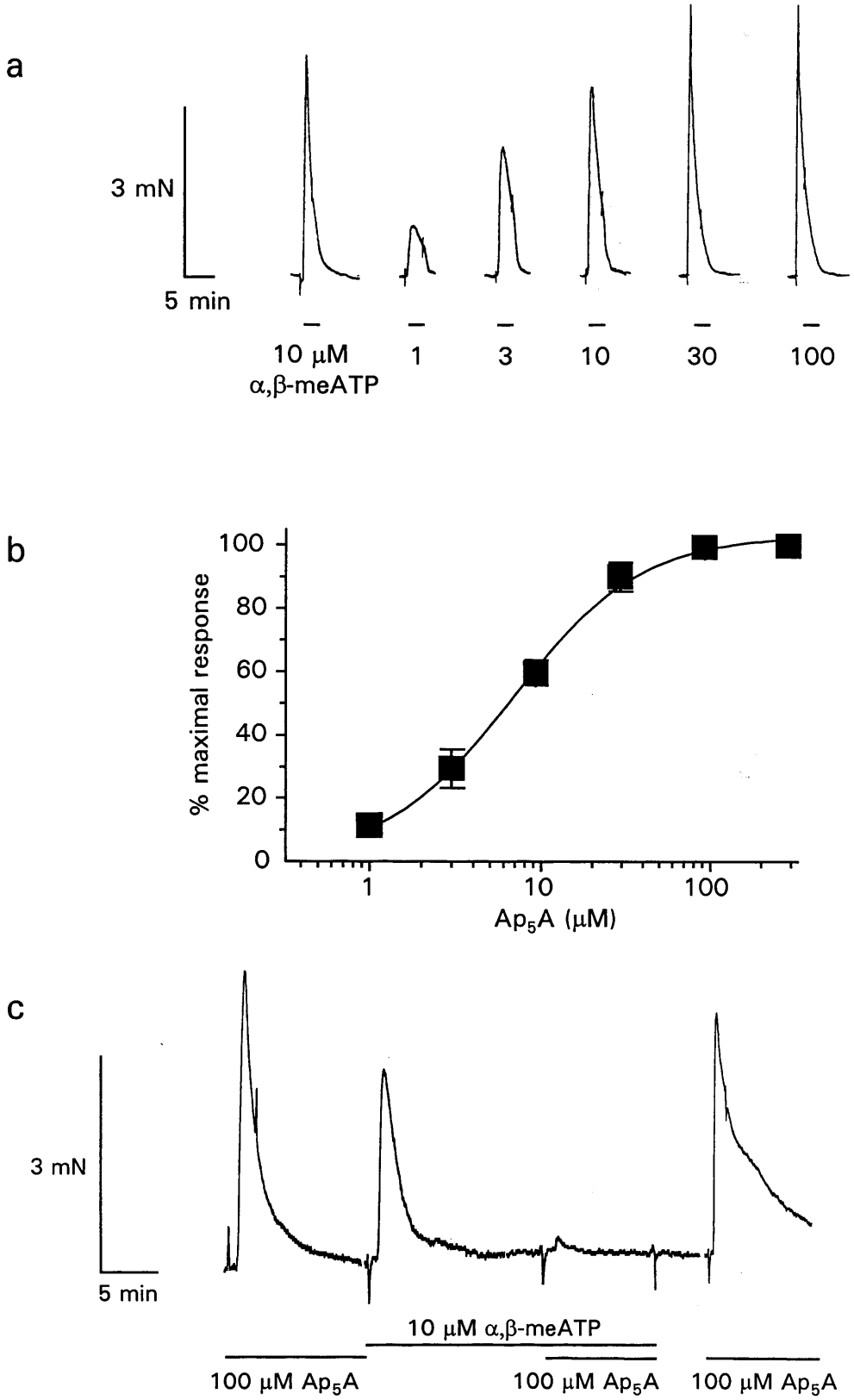


Figure 14 Ap_5A is a slightly less potent agonist than α,β-meATP in evoking contractions of medium-sized arteries. (a) Comparison of typical contractions reveals the similar time course and concentration dependence of responses to Ap_5A (concentrations are μM; the left-most trace is a response to 10 μM α,β-meATP in the same vessel). Agonist applications are indicated by bars. (b) Concentration response curve to Ap_5A producing a similar EC_{50} value to α,β-meATP. Data are mean ± s.e.m. (n = 6). (c) Cross-desensitisation experiment confirming that Ap_5A and α,β-meATP are acting at the same receptor: responses to Ap_5A are virtually abolished after $P2X_1$ receptor desensitisation with α,β-meATP.

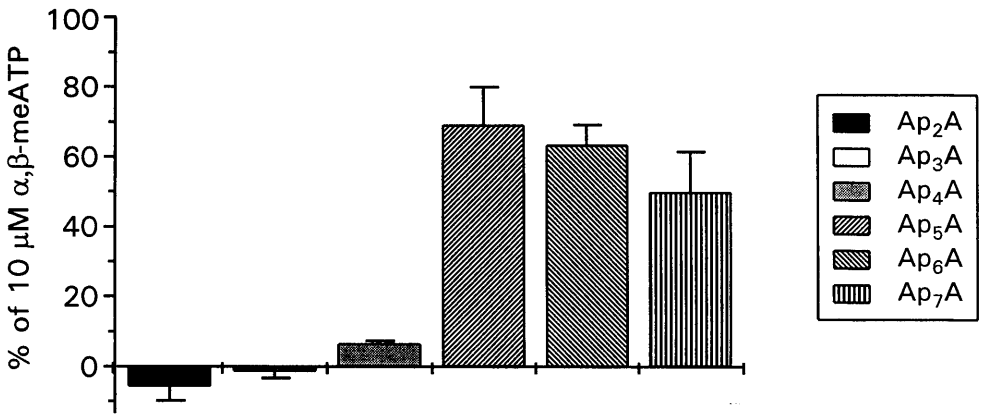


Figure 15 The contractile potency of diadenosine polyphosphates depends on the length of the phosphate chain. Histogram comparing responses to a range of Ap_nAs (10 μM) with those to α,β-meATP (10 μM). One can clearly see the increasing and then slightly decreasing contractile potency as the number of phosphate groups increases. Data are mean ± s.e.m. (n = 4).

Ap_nA and 10 μM α,β-meATP. Contractions to α,β-meATP remained essentially unaltered by Ap_2A but were significantly potentiated by Ap_3A. Expressed as a percentage of the control, responses in the presence of Ap_2A and Ap_3A were 102.9 ± 2.6 (n = 6) and 158.7 ± 15.0 (n = 6), respectively.

3.6 EFFECT OF P2 RECEPTOR ANTAGONISTS

Selective antagonists provide a useful means of distinguishing between different receptor subtypes. Suramin is a metabolically stable pseudo-competitive antagonist at both P2X and P2Y receptors with varying potencies depending on the receptor isoform. Another commonly used P2 receptor antagonist is iso-PPADS. This compound shows greater selectivity for P2X receptors than suramin (see Boarder & Hourani, 1998) and is effective at the same P2X isoforms. The inhibitory properties of a novel series of potentially subtype-selective P2X receptor antagonists were also tested.

3.6.1 P2X RECEPTOR-MEDIATED RESPONSES

Suramin and PPADS

Suramin is a potent antagonist at most P2X receptors but only a weak antagonist at $P2X_4$ and $P2X_6$ receptors. An effective way of quantifying the potency of a competitive antagonist is to determine its pA_2 value by Schild analysis, which provides a measure of the antagonist's affinity for the receptor in question (the theory behind Schild analysis is explained in section 2.7.2). Concentration response curves for all three sizes of artery were constructed in the absence of antagonist and in the presence of 10, 30 and 100 μM suramin. In medium-sized arteries, three concentrations of agonist were tested (1, 10 and 30 μM; n = 5 – 6) while in small and large vessels two concentrations were used (1 and 10 μM, n = 5 – 7 in small; 10 and 100 μM, n = 6 in large). A full Schild regression was therefore only performed for medium arteries and estimates of pA_2 values were made for small and large.

In small and medium-sized arteries suramin inhibited contractile responses to α,β-meATP in a concentration-dependent manner. Contractions were inhibited at all concentrations of suramin (10 – 100 μM) in small vessels, while 10 μM suramin caused a slight potentiation in medium arteries (Fig. 16). The antagonist produced parallel rightward shifts of the concentration response curve, which were used to calculate dose ratios and pA_2 values (Fig. 17). The pA_2 values obtained for small and medium-sized arteries were 5.14 and 5.08, respectively (Fig. 18). By contrast contractions in large arteries were essentially unaffected by suramin. Responses were slightly potentiated at 10 and 30 μM suramin and slightly inhibited at 100 μM. This suggests that in these vessels α,β-meATP is not acting through a suramin-sensitive P2X receptor.

To further characterise the antagonist profile P2X-mediated contractions in mesenteric arteries, the effect of iso-PPADS was investigated. As small and medium arteries were almost equally sensitive to suramin, iso-PPADS was only tested on medium and large vessels. Rather than performing complete Schild analysis, single concentrations of agonist and antagonist were chosen to determine the inhibitory properties of iso-PPADS. In each case the effect of a concentration of iso-PPADS shown to be effective in arteries (Ziganshin *et al*, 1994) was tested on an EC_{50} concentration of agonist. iso-PPADS (30 μM) abolished responses to 1 μM α,β–meATP in medium-sized arteries (n = 4) and reduced contractions to 300 μM α,β-meATP by 41.8 ± 14.2 % (n = 6) in large arteries (Fig. 19).

MRS compounds

The lack of subtype-selective antagonists has been a problem in the study of P2X receptor pharmacology. Compounds have however been synthesised which are suggested to be selective for certain P2X receptor subtypes. The MRS series is an example of such compounds. MRS-2179 and MRS-2220 have been reported to be antagonists selective for $P2X_1$ receptors over other P2X isoforms and MRS-2219 has been shown to selectively potentiate responses at $P2X_1$ receptors (Jacobson *et al*, 1998; Brown *et al*, 2000). The effect of each compound at a concentration of 10 μM was tested on responses to 10 μM α,β-meATP in medium-sized arteries (Fig. 20). Expressed as a percentage of control, contractions to α,β-meATP in the presence of

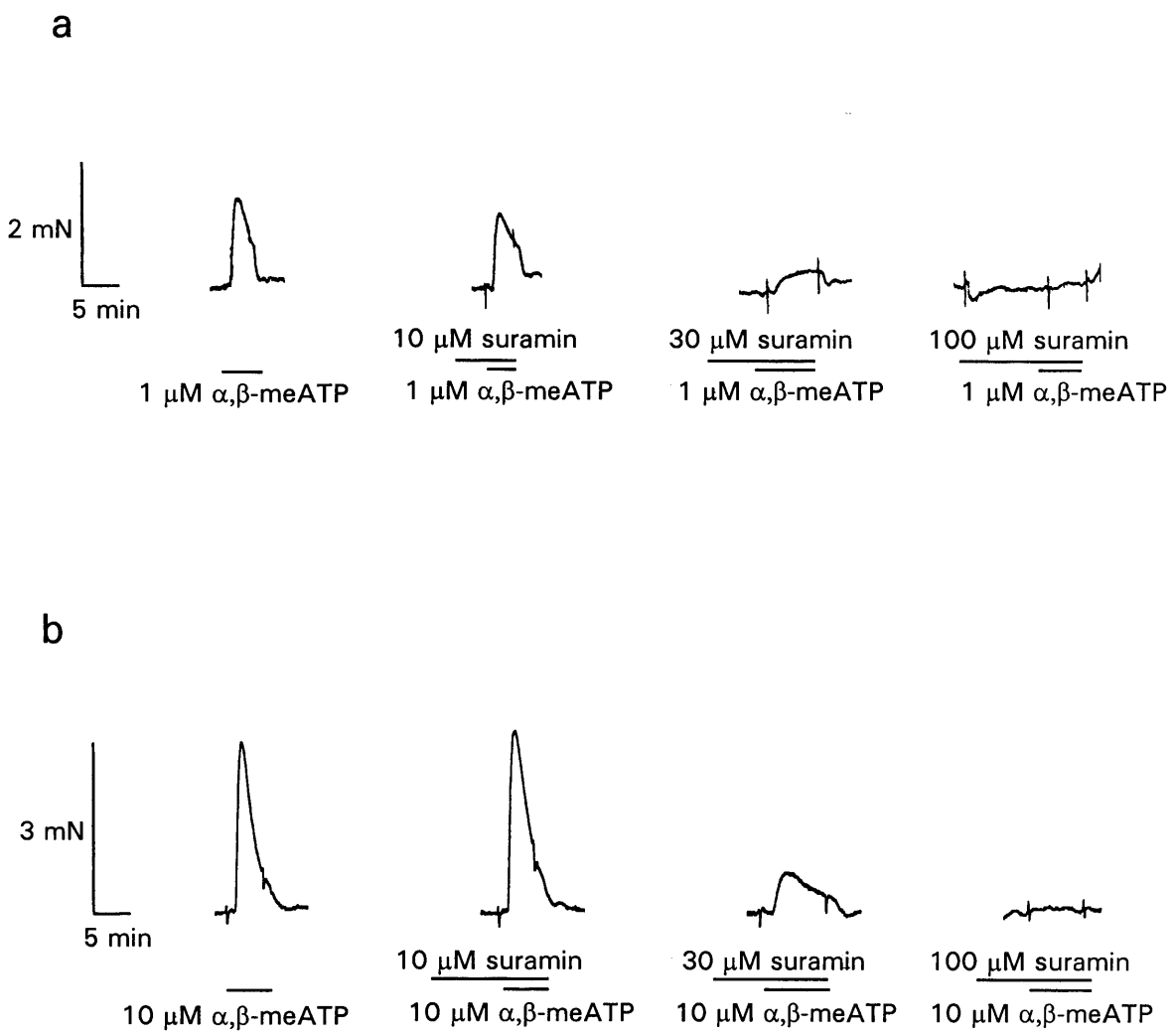


Figure 16 Suramin inhibits contractions to α,β-meATP in medium-sized arteries. Increasing concentrations of suramin (10, 30 and 100 μM) cause progressive inhibition of contractile responses to an EC_{50} concentration (1 μM; a) and to a near-maximal concentration of α,β-meATP (10 μM; b). Agonist and antagonist applications are indicated by bars. Small deflections are again changes in transducer output caused by solution exchange.

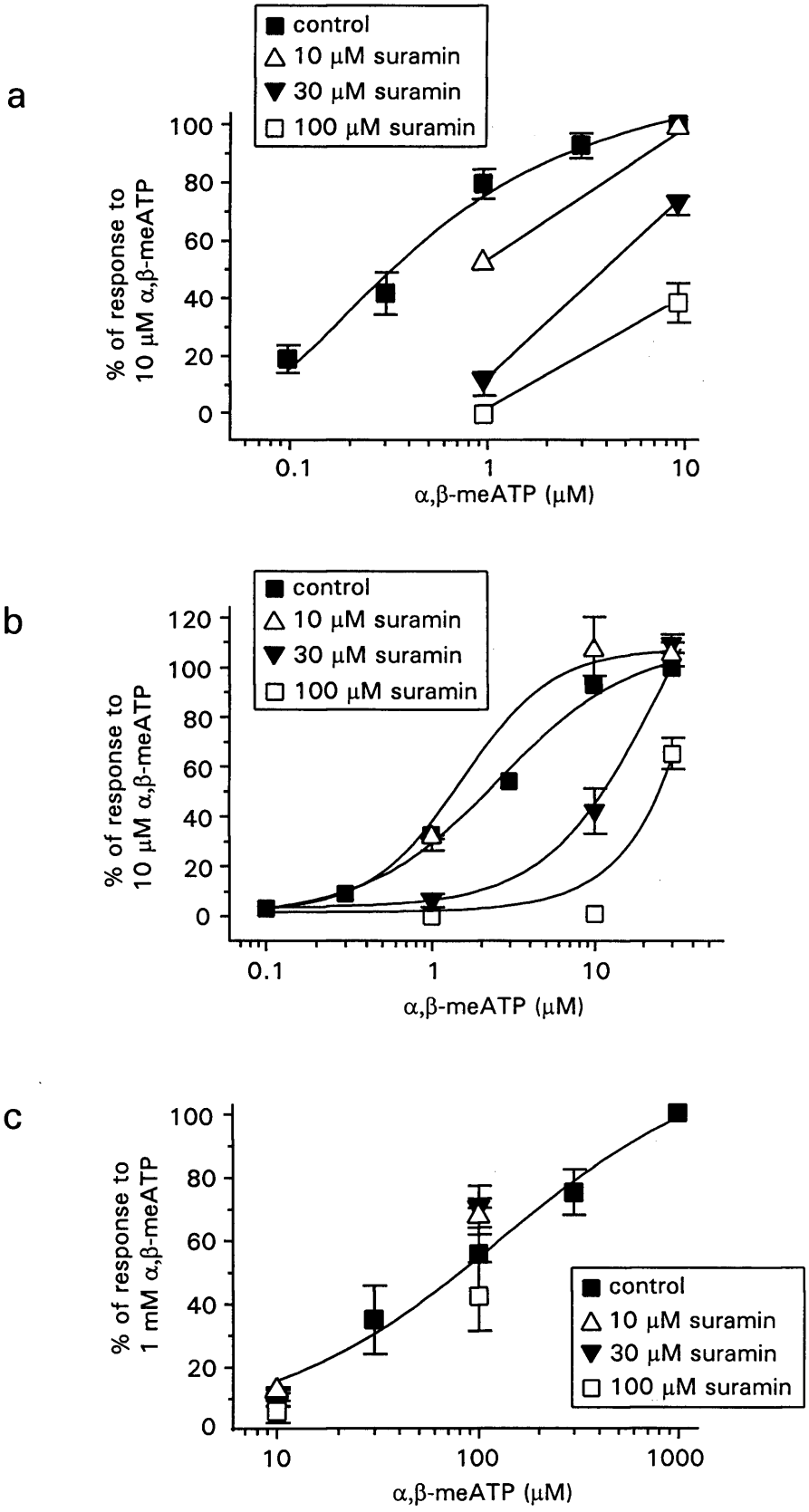
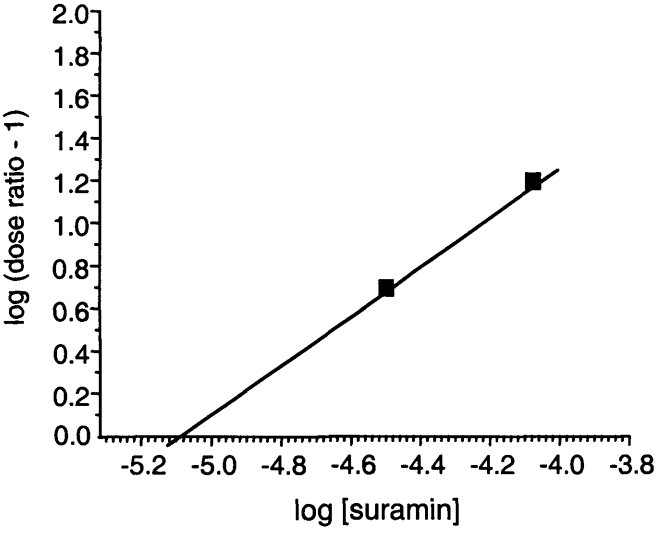


Figure 17 Suramin antagonises responses to α,β-meATP in small and medium-sized mesenteric arteries but not in large. Concentration response curves for responses to α,β-meATP alone and in the presence of suramin. A clear right-ward shift of the curve can be seen in small (a) and medium-sized (b) arteries but not in large (c). Data are mean responses ± s.e.m. (n = 5 - 7) and are expressed as % of the response to 10 μM α,β-meATP in small and medium arteries and % of the response to 1 mM α,β-meATP in large arteries.

a



b

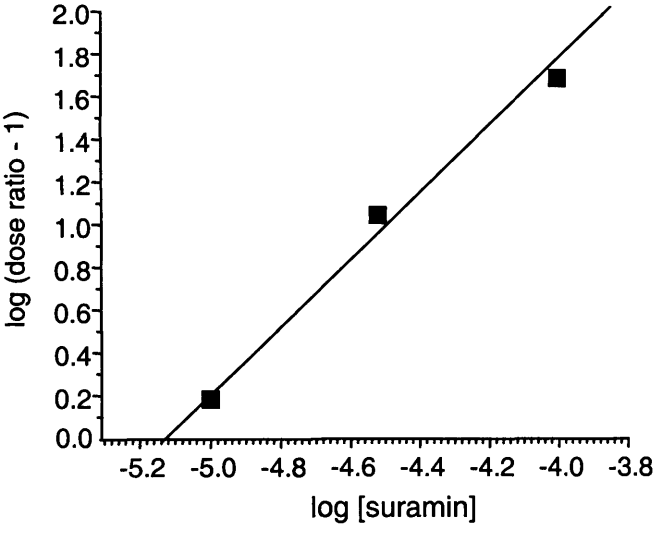


Figure 18 Suramin has a very similar pA_2 value in small and medium-sized arteries. Schild analysis of the antagonist potency of suramin at α,β-meATP-evoked contractions in small (a) and medium-sized arteries (b). The value in small arteries is not as accurate as the line was fitted to two rather than three data points. Nevertheless, suramin clearly has a very similar potency in both sizes of artery.

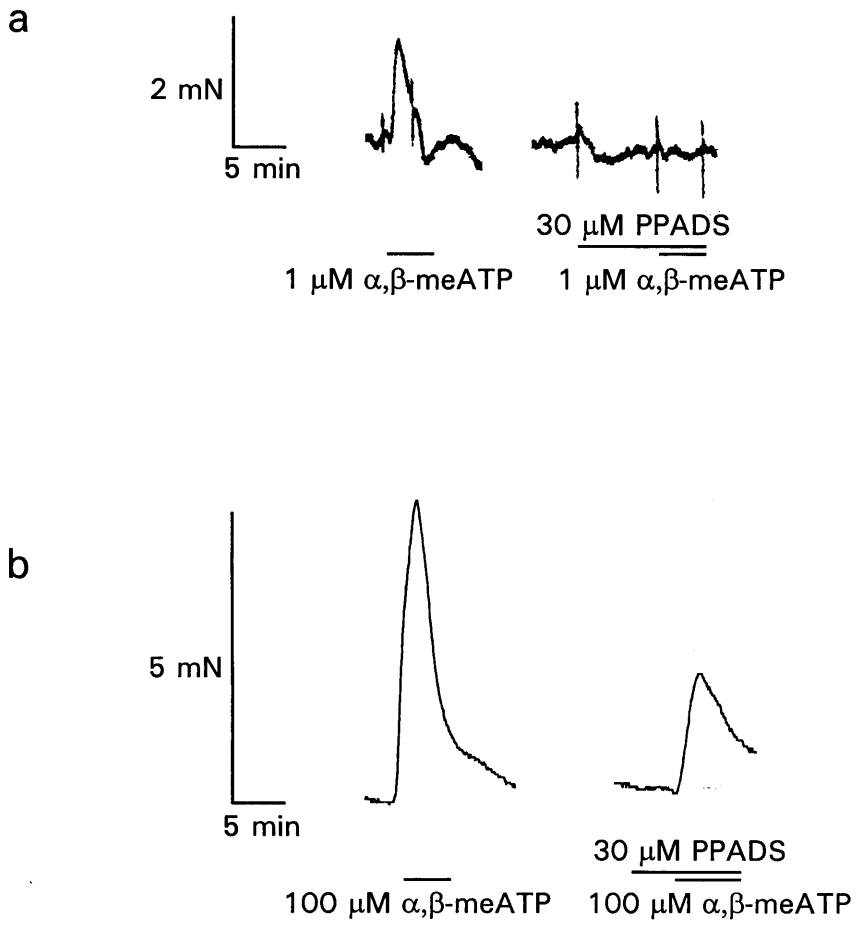


Figure 19 PPADS is significantly more potent in medium-sized arteries than in large arteries. Sample traces show that PPADS (30 μM) abolished responses to an EC_{50} concentration of α,β-meATP in medium-sized arteries (a) but only reduced them by roughly 40% in large vessels (b) agonist and antagonist applications are indicated by bars.

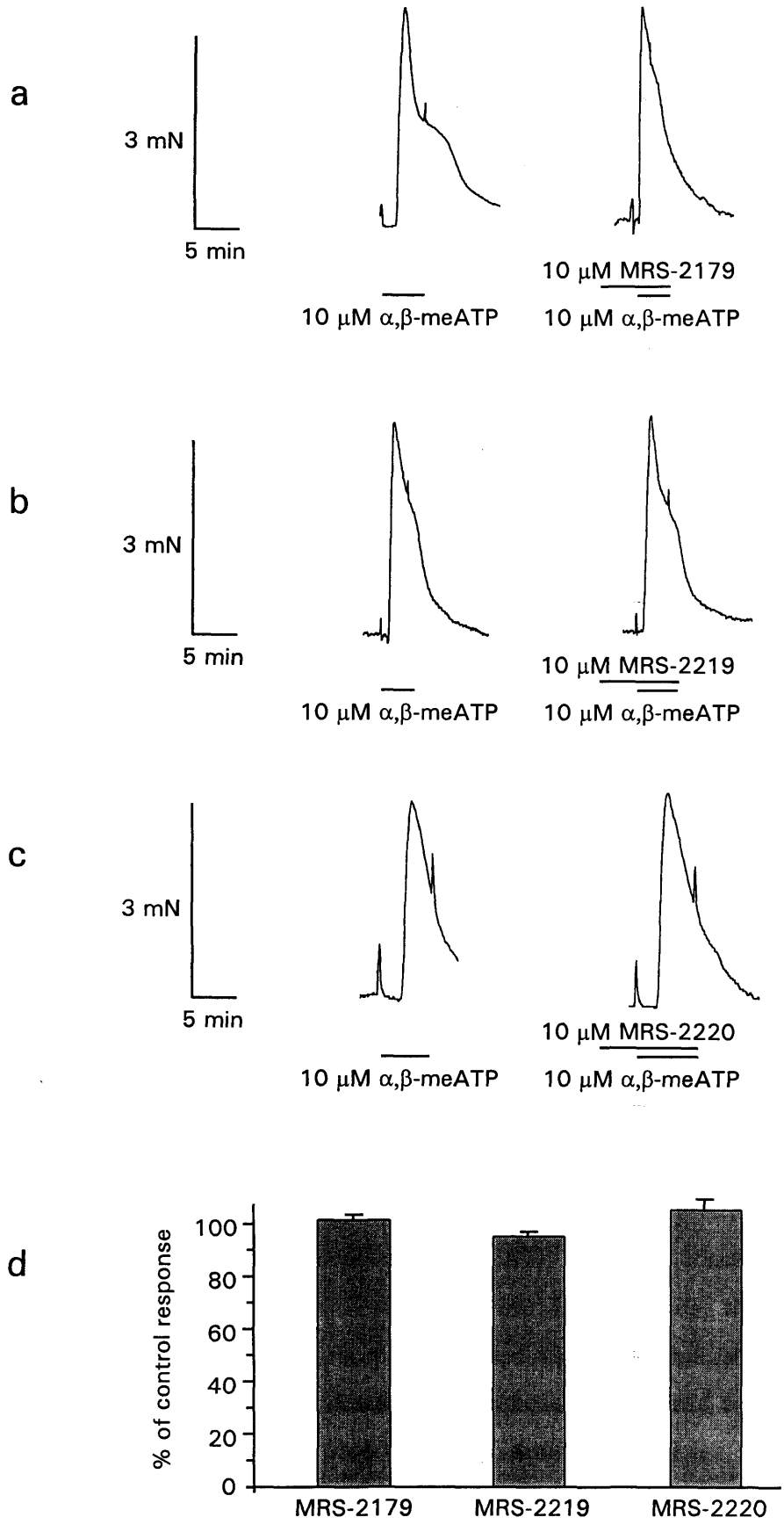


Figure 20 MRS-2179, MRS-2219 and MRS-2220 have no effect on contractile responses to 10 μM α,β-meATP. (a – c) Contractions in presence of MRS compounds (10 μM) are almost equal to control responses; agonist and antagonist applications are indicated by bars. (d) Summary histogram shows mean responses ± s.e.m. (n = 6).

MRS-2179, MRS-2219 and MRS-2220 were 101.9 ± 1.4 %, 95.8 ± 1.4 % and 105.8 ± 3.6 % respectively (n = 6).

3.6.2 P2Y RECEPTOR-MEDIATED RESPONSES

The P2 receptor antagonist suramin is also effective in distinguishing between P2Y receptor isoforms. Suramin is an antagonist at rat $P2Y_2$ receptors but not at rat $P2Y_4$ receptors both of which may mediate the vasoconstrictor effects of UTP. As the potency of UTP was almost identical in medium and large arteries when studied in the myograph, suramin was only tested on an EC_{50} concentration of UTP in small and medium-sized vessels. In medium-sized arteries, contractions to 1mM UTP were reduced by 48.8 ± 2.5 % and 65.5 ± 6.2 % in the presence of 30 μM and 100 μM suramin, respectively. In small arteries, the same concentrations of suramin reduced responses to 30 μM UTP by 64.3 ± 9.1 % and 96.3 ± 3.7 %, respectively (Fig. 21). UTP therefore appears to be acting through $P2Y_2$ receptors in both tissues.

3.7 DISCUSSION

The aim of the work described in this chapter was the characterisation of purinergic contractile responses in mesenteric arteries of the rat. My results show that there are substantial differences in the agonist and antagonist sensitivities of P2 receptors depending on the diameter of the vessel. The majority of this work has been published (Gitterman & Evans, 2000; Lewis *et al*, 2000a, see Appendix 1).

The metabolically stable ATP analogue α,β-meATP evoked concentration-dependent contractions in small and medium mesenteric arteries. In each case, the time course of contractions was characteristic of $P2X_1$ receptor-mediated responses: moderately desensitising at low agonist concentrations and rapidly desensitising approaching maximal concentrations. Such transient contractions are commonly observed when stimulating arteries with P2X receptor agonists. The EC_{50} values of 0.4 μM (pA_{50}: 5.6 ± 0.2) in small and 2.5 μM (pA_{50}: 6.4 ± 0.1) in medium arteries are also in agreement with data previously obtained in the same tissue. Two

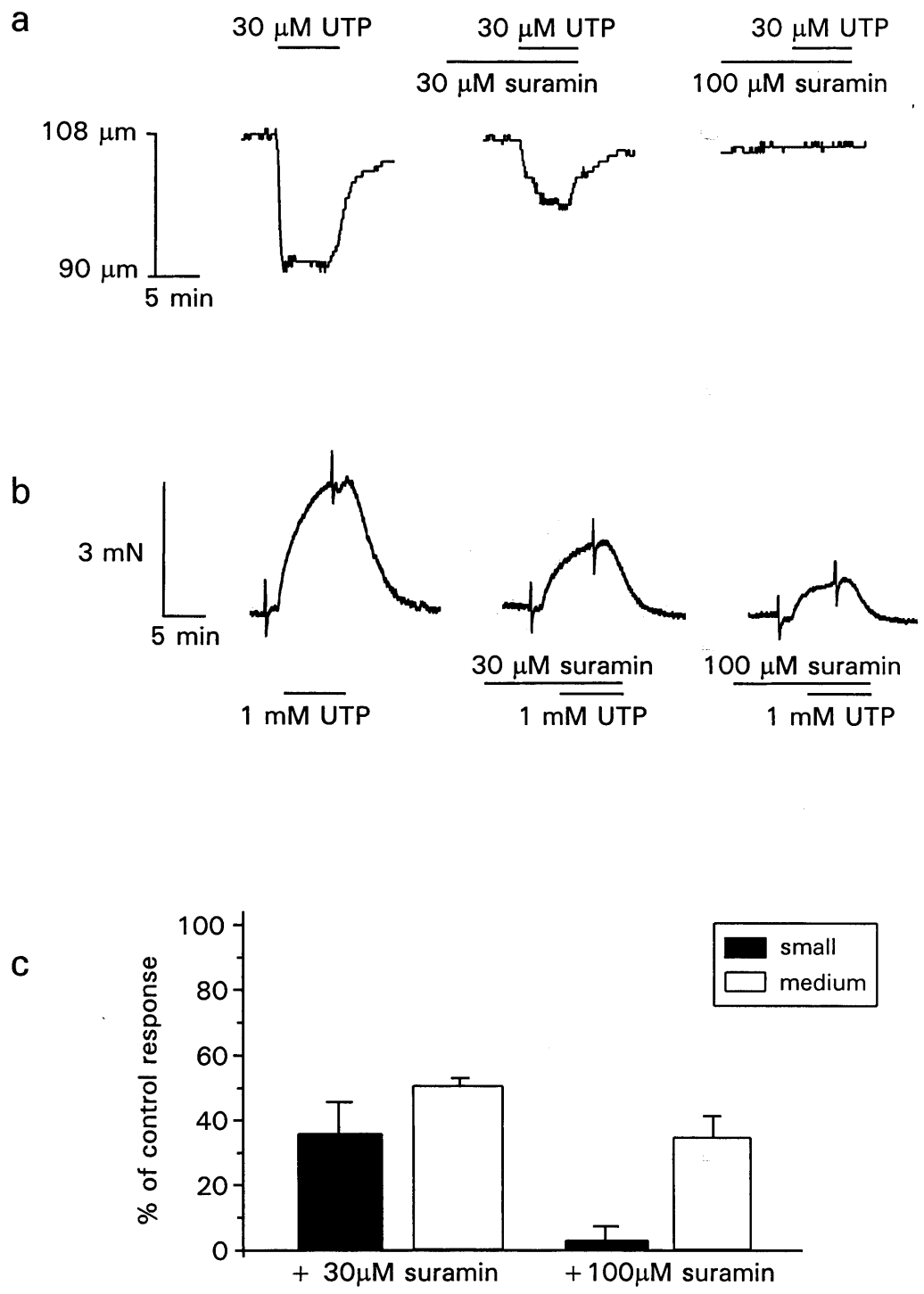


Figure 21 Suramin antagonises contractions to UTP in small and medium-sized arteries. Responses to an EC_{50} concentration of UTP are significantly reduced by 30 μM and 100 μM suramin in small (a) and medium (b) arteries; periods of agonist and antagonist application are indicated by bars. (c) Histogram of mean data ± s.e.m. (n = 5 – 6).

studies on medium-sized rat mesenteric arteries produced EC_{50} values of ~ 1 μM (Lagaud *et al*, 1996; Malmsjö *et al*, 1999b). A similar value was also obtained in the rat tail artery (Evans & Kennedy, 1994). Galligan *et al* (1995) characterised purinergic responses in guinea-pig submucosal arterioles and found a slightly higher potency of α,β-meATP (0.3 μM). Interestingly, this value is almost identical to the one I determined in the smallest arteries of the rat mesentery. Human bladder P2X receptors produced the same transient responses and were almost equally sensitive to α,β-meATP as medium-sized vessels in my study (EC_{50}: 2.3 μM). Although using pharmacological profiles to characterise receptors can be problematic, the similar properties of arterial tissue and recombinant $P2X_1$ receptors (Evans *et al*, 1995) provides further evidence that this is the key isoform in determining the vascular P2X phenotype.

Contractions of large arteries in response to α,β-meATP also showed the same transient time course. There was however a dramatic difference in the potency of the agonist compared to smaller vessels. With an EC_{50} value of 107 μM (pA_{50}: 4.0 ± 0.1), α,β-meATP is 40 – 200 times less potent in large than in smaller arteries. No other example of an artery that is so insensitive to α,β-meATP was found in the literature. A possible reason for this substantial difference in sensitivity may have been anatomical factors. Large arteries have a much thicker and more robust wall than smaller vessels with numerous layers of elastic laminae (see Chapter 5). These structural properties might limit access of the agonist to the receptor. If such a non-specific diffusional barrier existed, it should also affect agonists at other receptors. It was therefore of interest to compare the potency of other transmitters mediating vasoconstriction in the three sizes of mesenteric artery.

Noradrenaline is the other sympathetic transmitter co-released with ATP, and its postjunctional effects are mediated by α_1-adrenoceptors. As both transmitters are released from the same nerve terminals, they will diffuse through the same environment in order to reach their receptors. Structural characteristics of large arteries affecting the diffusion of α,β-meATP should therefore also affect the diffusion of noradrenaline. Phenylephrine, a stable and α_1-adrenoceptor selective analogue of noradrenaline, was equipotent in all three sizes of artery. The EC_{50} values for medium and large arteries were in fact virtually identical. These results argue against the possibility of a diffusion-based explanation for the above results. This conclusion is further

underlined by the concentration response data for potassium chloride; curves for medium and large arteries are almost identical. Interestingly, small arteries were slightly more sensitive to KCl than larger vessels: 20 mM KCl produced a response that is roughly 35% of maximal in small arteries but produced no response in medium arteries. Similarly, 40 mM KCl is near maximal in small arteries but evoked contractions that are only ~ 40 % of maximal in medium-sized vessels. The difference may reflect a greater sensitivity of the contractile machinery to changes in cytoplasmic calcium levels in small arteries. A recent study investigating the role of protein kinase C in determining the contractility of smooth muscle, found that the resting calcium sensitivity of contractile proteins increased as arterial diameter decreased (Akopov *et al*, 1998). PKC-mediated calcium sensitisation was also greatest in small arteries. Although these data were gathered in ovine cerebral vessels, the principles may well be generally applicable.

A higher sensitivity of the contractile machinery would also explain the slightly higher potency of α,β-meATP in small as compared to medium arteries in my studies. In addition, it is of interest to note that guinea-pig submucosal arterioles had an almost identical EC_{50} value for α,β-meATP to the one I determined in small mesenteric arteries of the rat (Galligan *et al*, 1995). This further supports the view that the smallest arteries within a vascular bed have the highest calcium sensitivity. In the case of noradrenaline however, all arteries have very similar EC_{50} values. A possible reason for this may be that slight differences in the receptor-effector coupling of adrenoceptors in small arteries counteract their inherently greater calcium sensitivity to produce a similar potency of phenylephrine in small and medium sized vessels. Another explanation would be heterogeneity of α1-adrenoceptor subtype expression. Heterogeneity of α1-adrenoceptors has indeed been found in rat mesenteric arteries (Hussain & Marshall, 2000) which could account for the slightly lower potency of phenylephrine in small arteries. A lower receptor reserve in these vessels would have the same effect.

Large arteries required very high concentrations of α,β-meATP to produce contractile responses. It was possible that under these conditions contractions may have been evoked at P2Y receptors where this agonist has only a low potency. In this case the calcium required for contraction would be provided by release from intracellular stores. However, the fact that all responses were abolished by the removal of extracellular calcium is not in line with such an explanation. An

equal requirement for calcium influx in all sizes of artery suggests that P2X receptors are mediating contractions in each case.

Another possible reason for the difference in potency of α,β-meATP between large and small arteries, may be that large arteries do not actually express α,β-meATP-sensitive receptors. The observed effect may have been caused by activation of a receptor different to the one mediating contractions in smaller vessels. At the high concentrations of agonist used in large arteries, sufficient amounts of contaminating ATP may have been present to produce an effect of its own by activating a different receptor. This is however unlikely as 50 μM ATP, simulating a 5 % contamination of 1 mM α,β-meATP, hardly evoked any response.

Data from experiments using other purinergic agonists also support the view that contractions evoked by P2X receptor activation are mediated by the $P2X_1$ isoform. Like α,β-meATP, the ATP analogue β,γ-meATP is an agonist at $P2X_1$ and $P2X_3$ receptors. Unlike α,β-meATP however, its optical isomers can distinguish between the two isoforms: D-β,γ-meATP is an agonist at both receptors whereas L-β,γ-meATP is only an agonist at the $P2X_1$ isoform (Trezise *et al*, 1995). In my experiments, α,β-meATP and L-β,γ-meATP evoked contractions of mesenteric arteries with almost equal potency. This is further evidence in favour of a crucial role for $P2X_1$ receptors.

To further characterise contractile responses in mesenteric arteries and to compare my findings with those of other groups, I studied responses evoked by diadenosine polyphosphates. The results of cross-desensitisation experiments between α,β-meATP and Ap_5A are in line with previously quoted data (Westfall *et al*, 1997; van der Giet *et al*, 1999). The clear implication is that both compounds are acting at the same receptor i.e. most probably $P2X_1$. The agonist order potency for Ap_nAs in my experiments agrees well with other reported data for α,β-meATP, Ap_3A, Ap_4A, Ap_5A and Ap_6A (Ralevic *et al*, 1995; Steinmetz *et al*, 2000a), underlining that the length of the phosphate chain is crucial in dictating agonist potency. The vasodilatory action seen for Ap_2A and Ap_3A confirms previous findings (Ralevic & Burnstock, 1996) although responses in my experiments were less pronounced. The relative potencies were however not the same.

While the potency of Ap_2A was greater than that of Ap_3A in my study, the opposite is true in the study by Ralevic *et al* (1995). The fact that the latter group used pre-contracted arteries and I did not, may have contributed to this difference in results.

Unfortunately, no another example of testing the antagonist effects of Ap_2A and Ap_3A was found in the literature. The difference in effect between the two compounds is curious; perhaps one would expect no significant effect as is the case with Ap_2A. The cause of the substantial potentiation of responses to α,β-meATP by Ap_3A is not clear. A possible reason may be a stabilisation of agonist-receptor binding. As α,β-meATP is a potent and efficacious agonist, it might not be expected to benefit from such an interaction. However, the presence of α,β-meATP may stabilise the binding of Ap_3A enhancing its efficacy as an agonist resulting in its own contractile effect. Another possible reason may be that Ap_3A is sufficiently similar in structure to the $P2X_1$ receptor's natural ligand that it does bind well at the agonist-binding site. Although it may not elicit a response of its own, it may prevent the receptor from interacting with endogenous ligand present in the tissue prior to addition of the exogenous agonist. This 'protects' the receptor from any desensitisation that may occur before being exposed to the agonist. The result is an enhanced response to the agonist. Ap_2A may be too dissimilar to ATP to produce such an effect.

Diadenosine polyphosphates are known to also activate P2Y receptors, for example Ap_4A. This can be seen be vasodilatory responses in raised tone preparations (Busse *et al*, 1988, Steinmetz *et al*, 2000b). When arteries are kept at resting tone however, this dinucleotide evokes vasoconstriction in most studies (see above). Interestingly, one report that disagrees with these findings demonstrates a dilatation of resting rabbit coronary arteries by Ap_4A (Pohl *et al*, 1991). The reason for this effect is unclear. The fact that inhibition of ecto-nucleotidase activity potentiated responses to Ap_4A is in agreement with data showing that this compound evokes proportionally greater currents in isolated cells than contractions in whole tissue experiments (compare Westfall *et al*, 1997 and Lewis *et al*, 2000a). This compound is therefore similar enough to ATP to also be a substrate for nucleotide degradation enzymes.

Although my data strongly suggest substantial differences in the properties of large and smaller arteries, there is one complication when using agonist sensitivities to characterise receptors in whole tissue experiments. Arterial contraction is only an indirect measure of receptor activation, as several steps in the process of excitation-contraction coupling are required to trigger the activation of contractile proteins. As a result, the contractile response is not a 1:1 reflection of agonist binding. More conclusive data can be generated using antagonists. If the antagonist is selective, one can be sure that any reduction in the observed response is solely due to a proportional reduction in receptor activation. The potency of an antagonist in contraction studies is therefore a direct measure of its affinity for a receptor. In my experiments, I used the P2 receptor antagonist suramin to complement the results from agonist studies.

Suramin is a non-selective, pseudo-competitive antagonist inhibiting both P2X and P2Y receptors. It reduced contractile responses to α,β-meATP in a concentration-dependent manner in both small and medium-sized arteries without affecting resting tone/diameter. The pA_2 value of 5.1 in both vessels is similar to that determined in other vascular smooth muscle preparations. A pA_2 value of 5.5 was determined for the same agonist in guinea-pig submucosal arterioles (Galligan *et al*, 1995) and 5.3 in rat vas deferens (Khakh *et al*, 1994). Suramin-mediated inhibition of α,β-meATP-induced responses has also been seen in numerous other preparations (e.g. rabbit ear artery: von Kügelgen & Starke, 1991; rat pulmonary artery: Hartley & Koslowski, 1997; rat renal artery: van der Giet *et al*, 1999). In addition, a similar potency was seen at recombinant $P2X_1$ receptors (Bianchi *et al*, 1999), which is consistent with this isoform playing an essential role in P2X-mediated contraction of small and medium-sized arteries. From my data, it is unclear whether suramin acts competitively or non-competitively, as maximal responses were not attained in the presence of the antagonist; previous studies have produced mixed results (Galligan *et al*, 1995; Evans *et al*, 1995). In stark contrast to smaller vessels, large arteries were largely unaffected by suramin up to a concentration of 100 μM. I have not found any other example in the literature of contractions evoked by α,β-meATP that were suramin-insensitive.

In an effort to address the lack of subtype-selective P2X receptor agonists and antagonists, a group at the NIH (Bethesda, USA) have synthesised the MRS series of compounds. Two of these, MRS-2219 and MRS-2220, are derivatives of pyridoxal phosphate and PPADS

respectively. MRS-2219 has been reported by the group to be a selective $P2X_1$ potentiator and MRS-2220 a selective $P2X_1$ antagonist (Jacobson *et al*, 1998). Another member of the series is MRS-2179, an adenosine derivative and putative antagonist selective for $P2X_1$ receptors over other P2X isoforms (Brown *et al*, 2000). Interestingly, none of these compounds had any significant effect when I tested them in mesenteric arteries. The basis for this discrepancy is unclear, although a possible reason may be the fact that the original experiments were conducted using recombinant receptors expressed in Xenopus oocytes. Discrepancies between data gathered in mammalian and amphibian cells have been reported previously (see Wildman *et al*, 1999 and Lewis *et al*, 2000a). In addition, MRS-compounds may undergo metabolic breakdown or modification in whole tissue experiments, although this may be unlikely for the two compounds based on metabolically stable PPADS. An example of the profound effect that metabolic breakdown can have on antagonist activity is TNP-ATP. This ATP derivative is a highly potent antagonist at $P2X_1$ and $P2X_3$ receptors in dissociated cells but is 10.000 times less potent in whole tissue experiments (Lewis *et al*, 1998).

From the data described so far, it is clear that there are substantial differences in the properties of large arteries as compared to small and medium: they are significantly less sensitive to both α,β-meATP and suramin. My results in smaller arteries confirm the accepted view that P2X-evoked contractions are mediated by the $P2X_1$ isoform. On the other hand, the phenotypic properties of large arteries indicate that this subtype is not involved in functional responses these vessels. Comparing the pharmacological properties observed with those of recombinant P2X receptors however reveals that no single isoform can account for the phenotype of large arteries: the rapidly desensitising responses are characteristic of $P2X_1$ and $P2X_3$ receptors, the relative insensitivity to α,β-meATP is seen in $P2X_{2,4,5,6}$ and $P2X_7$ receptors and the suramin insensitivity is a property of $P2X_4$ and $P2X_6$ receptors. The fact that no single P2X receptor can for the phenotype of large arteries raises the possibility of novel heteromultimer of P2X receptor subunits.

The possibility of heteromeric assembly of P2X receptor was first discovered by Lewis *et al* (1995). This study revealed that the mixed phenotype of sensory neuron P2X receptors was caused by $P2X_2$ and $P2X_3$ subunits forming functional receptors; the resulting channels were

sensitive to α,β-meATP ($P2X_3$) but were slowly desensitising ($P2X_2$). Several other possible examples have been found in the meantime. Le *et al* (1998) showed that $P2X_4$ and $P2X_6$ subunits form heteromeric channels with novel properties when co-expressed in Xenopus oocytes. The same has also been seen for $P2X_2$ and $P2X_6$ subunits (King *et al*, 2000). Both combinations of P2X isoforms were frequently found to co-localise in neuronal tissue suggesting a legitimate *in vivo* role for these heteromultimers. In addition, $P2X_1$ and $P2X_5$ subunits can co-assemble to form novel functional channels when expressed in human embryonic kidney cells (Haines *et al*, 1999; Surprenant *et al*, 2000). It therefore appears that heteromeric assembly of P2X receptors may be common. The only such heteromers that can accommodate all the properties of large mesenteric arteries are $P2X_{1/4}$ and $P2X_{1/6}$ receptors. $P2X_6$ protein is however almost exclusively associated with neuronal tissue (Collo *et al*, 1996) and has not been detected in studies identifying the isoforms expressed in vascular smooth muscle (Nori *et al*, 1998; Lewis & Evans, 2000a). The most probable candidate is therefore a $P2X_{1/4}$ receptor.

In a recent study investigating the diversity of potential heteromeric assembly of P2X subunits, it was however found that $P2X_1$ and $P2X_4$ subunits do not readily co-assemble (Torres *et al*, 1999b). These results were obtained using co-immunoprecipitation of recombinant P2X subunits expressed in HEK 293 cells and did not examine the functional responses of heteromeric channels. Although this would seem to discount the possibility of $P2X_{1/4}$ channel, the situation may be different in a native setting. The interaction may be too weak to survive the immunoprecipitation protocol. Another explanation may be that certain cellular constituents, which are absent in a recombinant system, are required to stabilise the interaction. These may be cytoplasmic components or additional accessory P2X receptor subunits that are not essential for homomeric channel assembly. Furthermore, this conclusion was drawn from investigating pairwise interactions. If three or more different subunits could co-assemble into functional receptors, additional combinations may be possible, for example $P2X_{1/4/4}$ or $P2X_{1/1/4}$.

Another reason for the functional differences between large and small arteries may be expression of splice variants. Splice variants have been documented for $P2X_2$ (Simon *et al*, 1997; Brändle *et al*, 1997) and $P2X_4$ (Dhulipala *et al*, 1998; Carpenter *et al*, 1999) receptors. In the case of $P2X_2$

receptors, splice variant displayed currents that were smaller and desensitised more slowly than full-length channels. A recent study has also identified splice variants of the $P2X_1$ receptor in rat mesenteric artery smooth muscle (Ohkubo *et al*, 2000). In this case, the spliced form of the channel was not trafficked to the cell membrane and therefore did not form functional channels. Other $P2X_1$ splice variants may also exist that have different functional properties to the unspliced channel as in $P2X_2$ receptors.

In addition to P2X receptors, arterial smooth muscle cells also express P2Y receptors capable of inducing vasoconstriction. UTP has been shown to evoke contractile responses acting either through $P2Y_2$ or $P2Y_4$ receptors (Juul *et al*, 1993; Malmsjö *et al*, 2000a). The properties of smooth muscle P2Y receptors in different-sized arteries could therefore be characterised by comparing responses evoked by UTP. As with the P2X receptor agonist α,β-meATP, there were diameter-dependent differences in the potency of UTP. In this case however, small arteries were much more sensitive than medium or large vessels. The fact that UTP was equally potent in medium and large arteries when studied in the myograph, supports the conclusion reached above that there is no barrier to agonist diffusion in large arteries. The sustained responses and very low potency of UTP that I observed are consistent with other studies in the rat mesentery (Juul *et al*, 1993; Lagaud *et al*, 1996; Malmsjö *et al*, 2000a) and other vascular beds (Inscho *et al*, 1998; Hartley *et al*, 1998). The literature also reveals that in certain studies similar difficulties in obtaining a maximal response were experienced (Hartly *et al*, 1998; Rubino *et al*, 1999). By contrast, concentration response curves did saturate between 1 μM and 3 μM UTP in the rat vas deferens (Bültmann *et al*, 1999a). Lopez *et al* (1998) found a very similar potency of UTP in the rat aorta to that which I determined in medium and large mesenteric arteries. In this study the concentration was increased until a maximal contraction was achieved at 30 mM. The resulting EC_{50} value of ~ 1 mM suggests that my estimates of EC_{50} values in medium and large arteries may be accurate.

Small arteries on the other hand were significantly more sensitive to UTP. Only one other example of arteries with a similarly high sensitivity to UTP was found in the literature, the rat renal vasculature (Inscho *et al*, 1998). Interestingly, these vessels were also very small, probably corresponding to pre-capillary resistance arterioles (inside diameter 20 – 30 μm). Further

investigation might reveal that arterioles are generally more sensitive to UTP than larger arteries. A possible reason for this may be the increased calcium sensitivity of small arteries discussed earlier. Differences in the rate of metabolic breakdown of UTP could also be a contributing factor. My experiments using the non-hydrolysable UTP analogue UTP-γ-S (Lazarowski *et al*, 1996) support this view. In small arteries the potencies of UTP and UTP-γ-S were very similar. In medium arteries however, 10 μM UTP produced no response while 10 μM UTP-γ-S evoked a substantial contraction (~ EC_{10}). Malmsjö *et al* observed a similar difference in potency of the two compounds, both in the same vascular bed (2000b) and in human coronary arteries (2000a). Larger arteries may therefore be less sensitive to UTP because of lower calcium sensitivity of the contractile apparatus and greater agonist breakdown. Further causes for the increased sensitivity of small arteries to UTP might be a greater receptor reserve or expression a novel P2Y receptor isoform.

A potential explanation for the differences in potency might have been endothelium-dependent effects. Numerous studies have demonstrated UTP mediated dilatation in raised tone arterial preparations (e.g. Garcia-Velasco *et al*, 1995; Matsumoto *et al*, 1997), including rat mesenteric arteries (Malmsjö *et al*, 2000b). A more pronounced dilatation in medium-sized and large arteries could account for the overall lower contractile potency of UTP in these vessels. However, the process of mounting arteries in the myograph almost certainly does some damage to the endothelium. The only vessels were an intact endothelium is guaranteed are therefore small arteries. But it is these vessels which are most sensitive to UTP. Endothelial factors can therefore be discounted as a cause of the differing potencies. One intriguing result is that medium-sized arteries are significantly more sensitive to UTP when studied with Diamtrak than when studied using myography. The cause of this difference is not entirely clear; differing levels of tension which the arteries are placed under are thought to be a contributing factor.

Although UTP is known to exert the vast majority of its effect through P2Y receptors, it has been shown to evoke inward currents in dissociated arterial smooth muscle cells (McLaren *et al*, 1998b). From the kinetics of current responses UTP was assumed to be activating P2X receptors. As in contraction studies it was at least two orders of magnitude less potent than 'conventional' smooth muscle P2X receptor ligands (ATP and α,β-meATP). The possibility therefore existed

that some of the contractile responses in my experiments were due to the activation of P2X and not P2Y receptors, particularly at high concentrations of UTP. The fact that in my cross-desensitisation experiment responses to UTP were not reduced, however confirms that UTP is acting solely through P2Y receptors. Studies in other tissues have found prior application of α,β-meATP had no effect on responses to UTP (Hartley *et al*, 1998; Ohara *et al*, 1998). What was somewhat unexpected, was the pronounced potentiation of contractions to UTP after treatment with α,β-meATP. This effect has in fact also been observed in porcine intrapulmonary arteries (McMillan *et al,* 1999). In this study it was argued that the potentiation was caused by inhibition of ectonucleotidase activity by α,β-meATP as described by Chen & Lin (1997). Less breakdown of UTP would therefore lead to a greater response. This argument also indirectly supports the theory that the increased sensitivity of small arteries to UTP is due to less ectonucleotidase activity in these vessels. If the potentiation of the UTP response in medium and large arteries is due to inhibition of these enzymes, one would expect responses to UTP in small arteries to be unaffected by desensitisation with α,β-meATP, as there is very little agonist breakdown to inhibit. The fact that contractions are indeed very similar before and after α,β-meATP application is consistent with this explanation.

Another cause of α,β-meATP-induced potentiation of UTP responses may be a sensitisation of intracellular calcium release channels. The P2X receptor is desensitised by prolonged application of α,β-meATP, but a contractile response with the associated influx of calcium is evoked initially. Although arterial tone returns to baseline during α,β-meATP application, it may be that the cytoplasmic calcium level is still sufficiently elevated to increase the sensitivity of IP_3 and ryanodine receptors. Renewed stimulation with UTP would thus lead to a larger response than prior to α,β-meATP treatment.

There is evidence that smooth muscle cells express $P2Y_2$ and $P2Y_6$ (Malmsjö *et al*, 2000a) and possibly also $P2Y_4$ receptors (Rubino *et al*, 1999). UTP could be an agonist at any of these isoforms (at $P2Y_6$ after breakdown to UDP). Of the three, only the $P2Y_2$ isoform shows significant sensitivity to the P2 receptor antagonist suramin. As responses in mesenteric arteries were suramin sensitive, UTP must be evoking contractions through $P2Y_2$ receptors in these vessels. Other research has also produced similar results; two recent papers have found suramin-

sensitive UTP mediated contractions, one in rat aorta (Lopez *et al*, 1998) and one in small rat pulmonary arteries (Hartley *et al*, 1998). There are however reports that disagree with my findings. Rubino *et al* (1999) found contractions evoked by UTP in rat pulmonary arteries were not reduced by the antagonist. Curiously, in direct contradiction of my data, Lagaud *et al* (1996) found that rat mesenteric arteries displayed suramin – resistant contractions to UTP. Both experiments were conducted using identical protocols and the reason for this discrepancy is not clear. Nevertheless, the fact that responses in my experiments were suramin-sensitive suggests that the same P2Y isoform is involved in all three arteries. The difference in UTP potency is therefore unlikely to result from P2Y receptor heterogeneity.

In summary, the experiments described in this chapter have demonstrated that there are significant differences in the P2 receptor phenotype of rat mesenteric arteries depending on vessel diameter. Large arteries are far less sensitive to the ATP analogue, α,β-meATP than small and medium-sized arteries. Moreover, P2X receptor-mediated contractions in small and medium arteries are suramin-sensitive while those in large are not. These results are not due to anatomical differences between the vessels and may be due heterogeneous distribution of P2X receptors throughout the vascular tree. Large arteries may express novel P2X subunits or a novel heteromeric P2X receptor. In contrast, UTP is more potent in small vessels than in medium or large. The fact that responses in both small and medium-sized vessels are suramin-sensitive suggests that similar receptors are expressed in each artery. Differential agonist breakdown and differences in receptor reserve are thought to be the key factors determining the observed pharmacological properties.

4 NEUROGENIC CONTRACTIONS AND SOURCES OF CALCIUM

4.1 INTRODUCTION AND AIMS

Short-term regulation of blood pressure is achieved is through the action of the sympathetic nervous system. ATP and noradrenaline are released from sympathetic nerve terminals and act on postjunctional P2 receptors and α-adrenoceptors expressed on the surface of smooth muscle cells (for review see Kennedy, 1996). Although both transmitters are important in the control of vascular tone, their relative contributions have been found to be variable, depending on the preparation and experimental conditions (e.g. Sjöblom-Widfeldt & Nilsson, 1990). If the currently available data is examined, there is some indication that ATP may be more important than noradrenaline as a transmitter in small resistance arteries (Evans & Surprenant, 1992). The studies in this field have however been conducted in several different preparations and under different conditions making it difficult to draw firm conclusions.

From the pharmacological characterisation described in Chapter 2, it was clear that there are differences in the α,β-meATP and suramin sensitivity of P2 receptors mediating contraction in mesenteric arteries. Using this information I went on to characterise and compare physiological responses in the different sizes of artery. In this case contractions were evoked by electrical stimulation of perivascular nerves which simulates the central regulation of arterial tone. In contrast to when agonists are applied exogenously, noradrenaline and ATP are released locally during neurogenic contraction. Receptors are exposed to transmitters in a quantity, ratio and duration that are equivalent to conditions *in vivo*. With this experimental strategy, the relative importance of purinergic and adrenergic transmission could be effectively studied. One of the aims of this chapter was to systematically characterise and compare the relative roles of ATP and noradrenaline in mediating sympathetic vasoconstriction in three different sizes of artery from a single vascular bed (the rat mesentery). Any differences in the contribution of each transmitter depending on the diameter of the artery were of particular interest. The purinergic and adrenergic components were quantified using antagonists to selectively block either α-adrenoceptors or P2X receptors.

A fundamental requirement for the contraction of a smooth muscle cell is an increase in the cytoplasmic level of calcium. Such an increase can be achieved either by calcium entry from outside the cell or by calcium release from intracellular storage organelles. It is accepted that calcium influx from the extracellular space is essential for P2X receptor-mediated smooth muscle contraction. This influx can occur either directly through the P2X channel or through voltage-dependent calcium channels; it is however not clear which route dominates. To address this question, the effect of blocking voltage-dependent calcium channels on contractions evoked by P2X receptor agonists and nerve stimulation was tested. To determine whether there is a role for additional calcium release secondary to influx, the potential contribution of intracellular calcium stores was also investigated.

4.2 CHARACTERISTICS OF NEUROGENIC RESPONSES

A prime concern in these experiments was to use parameters of electrical stimulation that approximated closely to conditions *in vivo*. It has been shown that sympathetic neurones fire in short bursts of activity rather than long sustained trains (Macefield *et al*, 1994). As a result it was decided to stimulate mesenteric arteries with a brief, ten pulse train of electrical field stimulation (frequency of 1 – 50 Hz). In all arteries tested, this protocol evoked reproducible contractile responses, which were rapid, monophasic and transient. In small and medium-sized vessels, changes in arterial tone or diameter rapidly reached a peak upon stimulation and returned to baseline equally rapidly once stimulation had ceased; the whole response lasted 5 - 7 seconds (Fig. 23a). Contractions in large arteries had a slightly longer time-course, as the decay phase of the response was slower than in smaller vessels (Fig. 23b).

Neurogenic responses rely on electrical stimulation of periarterial nerves. Nerves are however not the only excitable tissue in arteries; smooth muscle cells can also be electrically stimulated. For experiments characterising neurogenic responses, it is therefore of great importance to avoid direct electrical stimulation of smooth muscle cells. This is mainly achieved by using a short pulse width of stimulation. Very brief electrical pulses of less than 0.4 ms duration for example, will tend to stimulate nervous tissue but not smooth muscle cells; low voltages will also favour purely neurogenic responses. Tetrodotoxin (TTX) inhibits neuronal action potential propagation

and can be used to confirm that contractions are neurogenic in origin. If responses are abolished in the presence of TTX, they are entirely neurogenic and not due to direct stimulation of the smooth muscle. The voltage and pulse width of stimulation used at the start of an experiment were therefore adjusted during TTX application until contractions were abolished (Fig. 22). Parameters were chosen to produce large responses, clearly visible above background noise that were abolished by TTX (10 – 40 V and a pulse width of 0.2 – 0.3 ms). The experiment was not continued until contractions were shown to be neurogenic. After TTX application, responses returned to the previous amplitude within 10 - 30 minutes. In approximately 1/3 of tissues responses after TTX treatment were larger than prior to treatment. This probably reflects a non-specific increase in the responsiveness of the vessel over time; it is unlikely to be a consequence of TTX application.

4.3 FREQUENCY-RESPONSE RELATIONSHIPS

In order to compare the properties of sympathetic transmission in the three sizes of artery, frequency response relationships were studied. Similar frequency-response relationships in all arteries would suggest that the properties of transmitter release are similar in each case. Short bursts of 10 pulses were delivered at frequencies ranging from 1 Hz to 50 Hz. 10 pulses at 1 Hz evoked small but measurable responses in all arteries, and the size of contractions increased in a frequency-dependent manner up to the maximal response at 20 Hz. At 30 and 50 Hz, responses decreased in amplitude. When normalised to the maximal response, the relative amplitude of contractions was very similar for all three vessels up to a frequency of 20 Hz (n = 5 – 6). Only at higher frequencies was a slight divergence seen; there was a greater decrease in the size of responses in small and medium-sized arteries than in large vessels (Fig. 23c). In subsequent experiments I chose to stimulate with 10 pulses at 10 Hz as this always produced a robust but sub-maximal response. These parameters also reflect the short, high frequency bursts of sympathetic nerve activity seen *in vivo*.

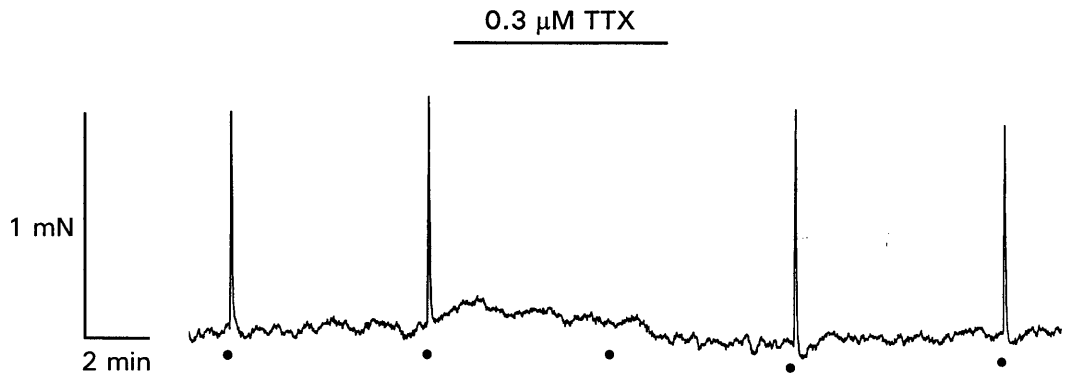


Figure 22 Tetrodotoxin abolishes contractions to electrical stimulation. Sample trace from a medium-sized artery showing the complete block of contractile responses by TTX (0.3 μM), confirming their neurogenic origin. This vessel recovers quickly and responses return within five minutes. Drug application is indicated by the bar and nerve stimulation by the circles.

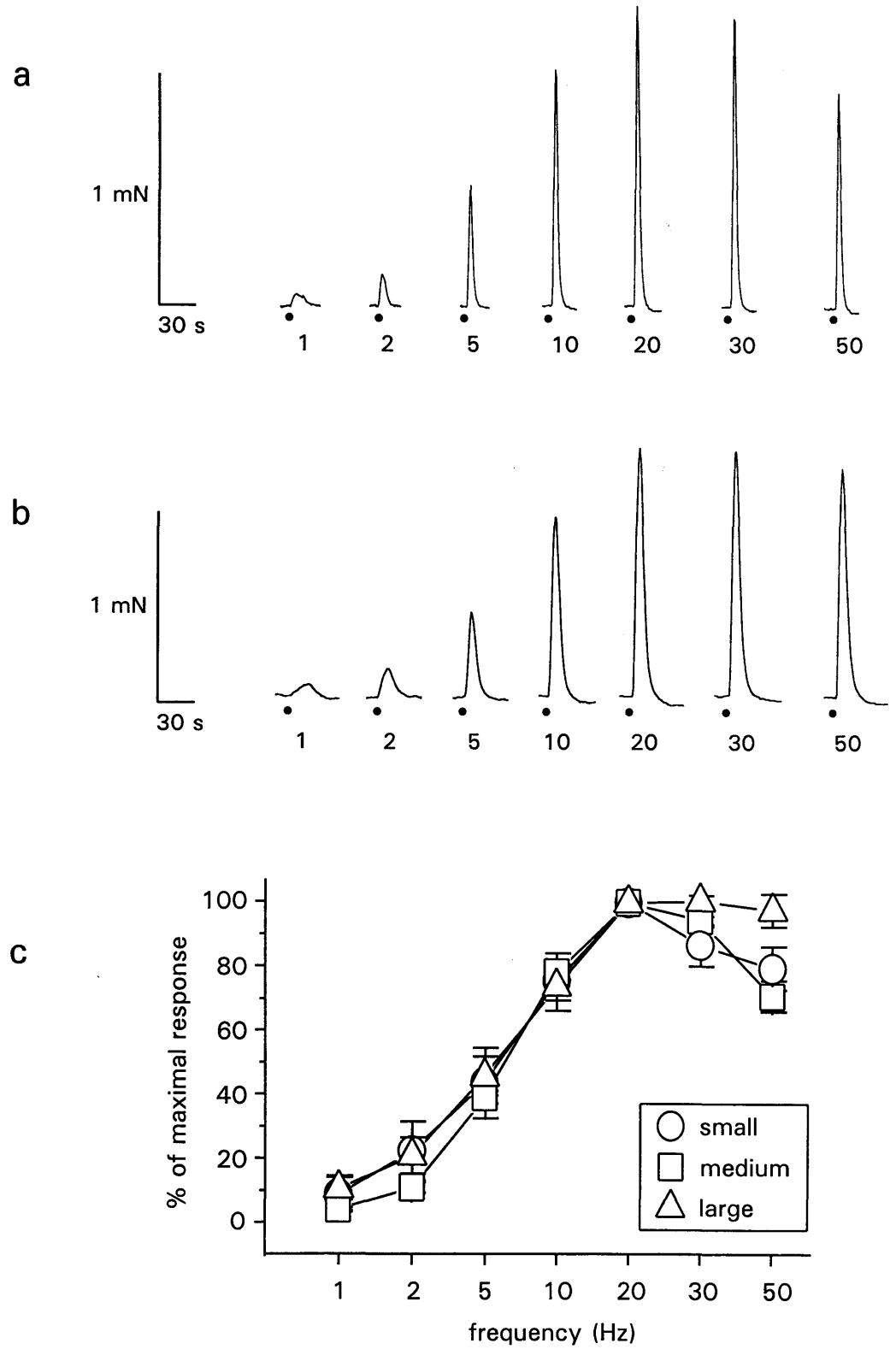


Figure 23 Characterisation of nerve-evoked contractions in small, medium and large arteries. Responses are evoked by 10 pulses of stimulation at frequencies of 1 – 50 Hz indicated by the circle. Panels (a) and (b) show contractions in medium-sized and large arteries, respectively. (c) Frequency response relationships are similar for all three sizes of vessel. Data are mean ± s.e.m. (n = 5 – 6) and normalised to the maximal response in each vessel.

4.4 PURINERGIC AND NORADRENERGIC COMPONENTS OF CONTRACTION

Surveying the literature reveals that previous experiments determining the relative contribution of ATP and noradrenaline to sympathetic control of arterial tone have produced very varied results. As well as being affected by the choice of animal and tissue, the proportions of contraction mediated by the two transmitters was found to depend strongly on the parameters of electrical stimulation. Short, low frequency stimulation appears to favour purinergic transmission, while longer, high frequency trains of stimulation favour noradrenergic transmission (e.g. Evans & Cunnane, 1992). There is also some indication that the size of the vessel also plays a role, with the purinergic component dominating in small arteries. This has however not yet been systematically investigated. In this study selective antagonists were used to compare the contribution of the purinergic and adrenergic components in small, medium and large arteries.

The adrenergic component of contraction was blocked with the α_1-selective adrenoceptor antagonist prazosin. Prazosin (0.1 μM) reached its maximal inhibitory effect 5 – 7 minutes after application in all arteries tested. Responses were reduced by 32.6 ± 2. 6 % (n = 4) and 27.0 ± 1.5 % (n = 6) in small and medium-sized arteries, respectively (Fig. 24). In large arteries, contractions were virtually abolished, being reduced by 97.0 ± 1.9 % (n = 6). This suggests a substantial non-adrenergic component in small and medium-sized arteries but not in large vessels.

In section 2.6.1 suramin was shown to be a potent inhibitor of P2X receptors. It would therefore be expected to effectively antagonise the purinergic portion of neurogenic responses at a concentration of 100 μM, revealing the remaining adrenergic component. Suramin was slower to act than prazosin, reaching its maximal effect after 15 – 20 minutes. It substantially inhibited responses in small and medium arteries, reducing them by 65.3 ± 7.4 % (n = 6) and 82.7 ± 3.3 % respectively (n = 6); the difference in effect was not significant. By contrast, in large vessels contractions were only reduced by 3.1 ± 6.1 % (n = 6). For each vessel, the suramin-resistant component (i.e. the implied adrenergic portion of the response) is roughly equivalent to that determined by blockade of α_1-adrenoceptors with prazosin. This means that the two elements of

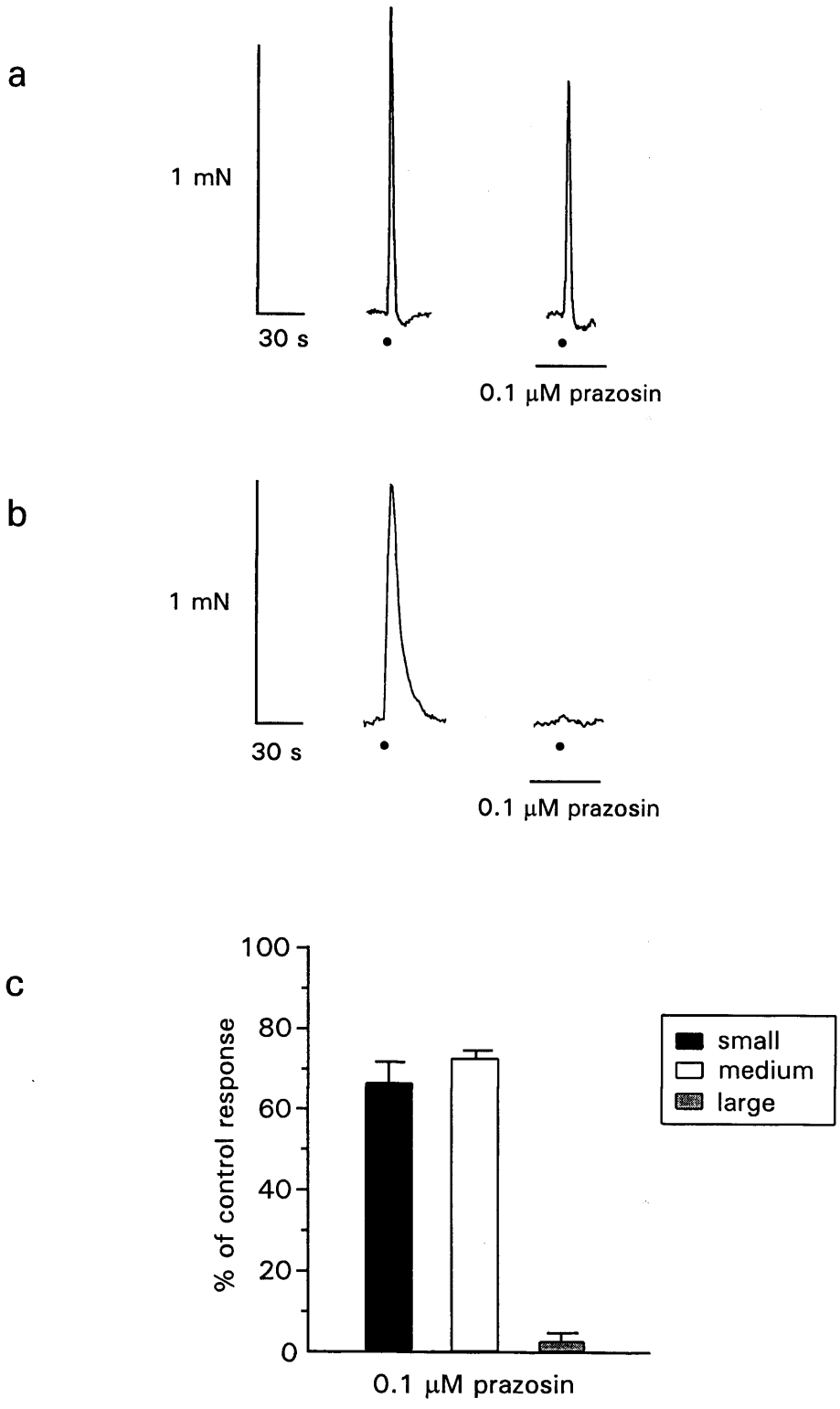


Figure 24 Contractions are reduced by prazosin in small and medium arteries but almost abolished in large arteries. Typical traces showing the dramatic difference in the effect of prazosin (0.1 μM) on responses evoked by 10 pulses of stimulation at 10 Hz between medium (a) and large (b) arteries. Prazosin application is indicated by the bar and nerve stimulation by circles. (c) Data are mean response remaining after drug application ± s.e.m. (n = 4 – 6)

the response combine to approximately 100% (97.9 %, 109.7 % and 100.1 % for small, medium and large vessels respectively) in all three sizes of artery. Concomitant application of suramin and prazosin abolished all responses (Fig. 25), confirming that suramin was effectively blocking the action of ATP. This result remains the same regardless of which order the antagonists are applied in.

A further way of inhibiting the purinergic component of contraction is to desensitise the P2X receptor with a near maximal concentration of α,β-meATP, blocking its subsequent activation. This approach has been used in numerous other studies (e.g. Allcorn *et al*, 1986; Bao & Stjärne 1993). α,β-meATP (10μM for small and medium and 100μM for large) was applied during nerve stimulation and arterial tone/diameter was allowed to return to baseline in the continued presence of the agonist. The neurogenic response that remained during continued α,β-meATP application was resistant to P2X receptor desensitisation and was thus taken to be the adrenergic component of constriction. This proportion of constriction was 52.9 ± 9.1 % (n = 4), 92.5 ± 2.6 % (n = 6) and 98.4 ± 14.0 % (n = 6) in small, medium and large arteries respectively, suggesting a much larger adrenergic component in medium and large vessels than that determined with suramin (Fig. 26). To confirm that no other transmitters were involved in mediating sympathetic vasoconstriction, both the purinergic and noradrenergic components were blocked simultaneously. Concomitant administration of prazosin and α,β-meATP abolished all neurogenic responses (Fig. 26), and as with suramin, the order of application did not alter the result.

There are however difficulties with using α,β-meATP to block P2X receptor activation. Firstly, the diameter of vessels studied on Diamtrak rarely returned all the way to their initial baseline value during α,β-meATP application, which may have led to an over-estimation of the purinergic component of the response. In addition, responses in medium and large arteries were reduced to a much smaller degree than would be expected if there was a substantial purinergic component of constriction. This finding is further highlighted by the fact that the effects of α,β-meATP and prazosin are not additive. When the purinergic and adrenergic components determined in small and medium arteries in this experiment are combined, the result is 85.4 % and 119.5 %, respectively (not 100%). If ATP and noradrenaline were the only transmitters

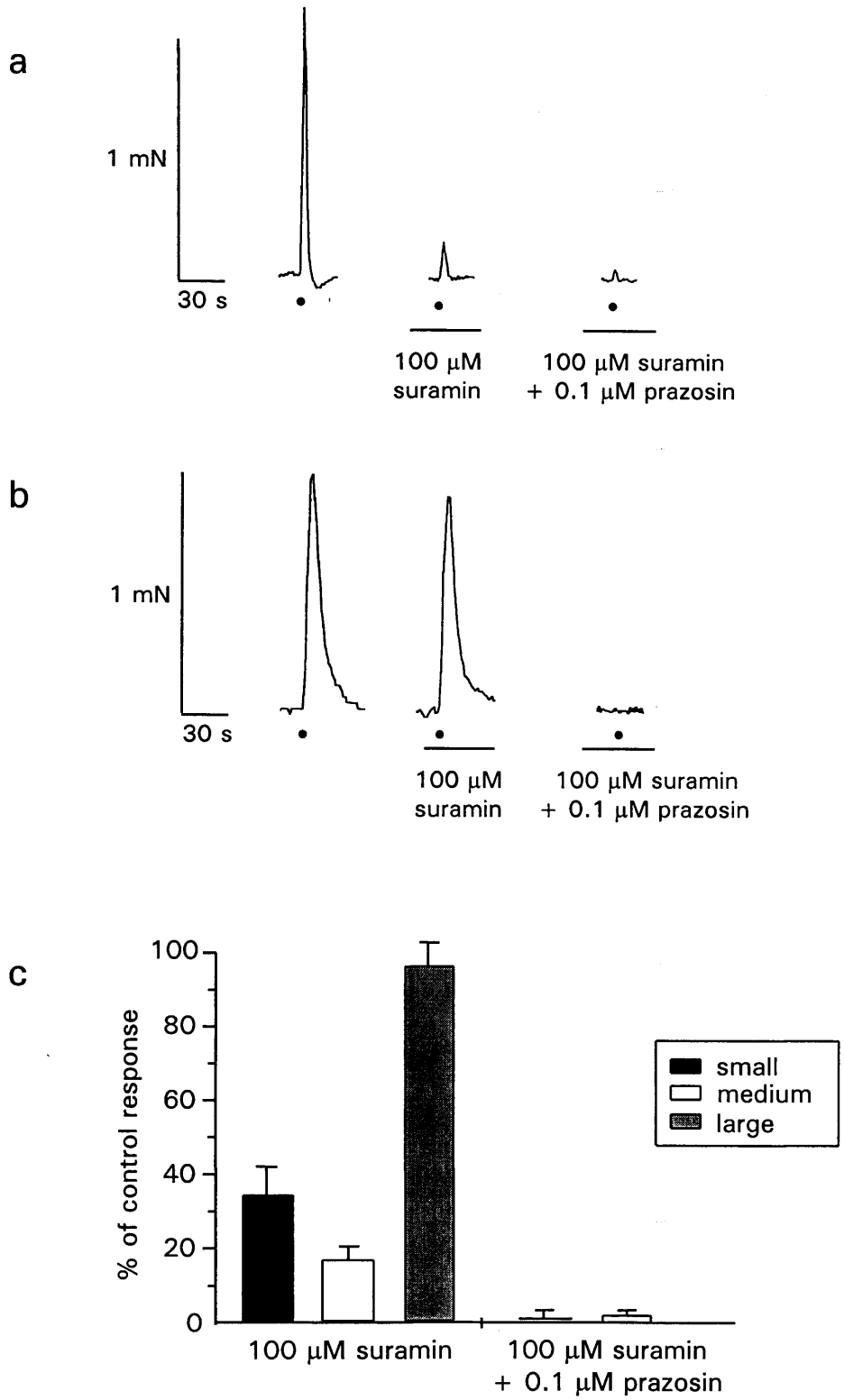


Figure 25 Suramin substantially reduces contractions in small and medium-sized arteries but has virtually no effect in large arteries. Representative traces for medium (a) and large (b) arteries; in each case the left most trace is a control response. The difference in effect of suramin (100 μM) on neurogenic contractions in the two vessels is clearly visible. Drug applications are indicated by bars and nerve stimulation by circles. (c) Summary data are mean ± s.e.m. (n = 6). Small arteries are slightly less sensitive to suramin than medium. Combined application of suramin (100 μM) and prazosin (0.1 μM) abolishes responses in all vessels.

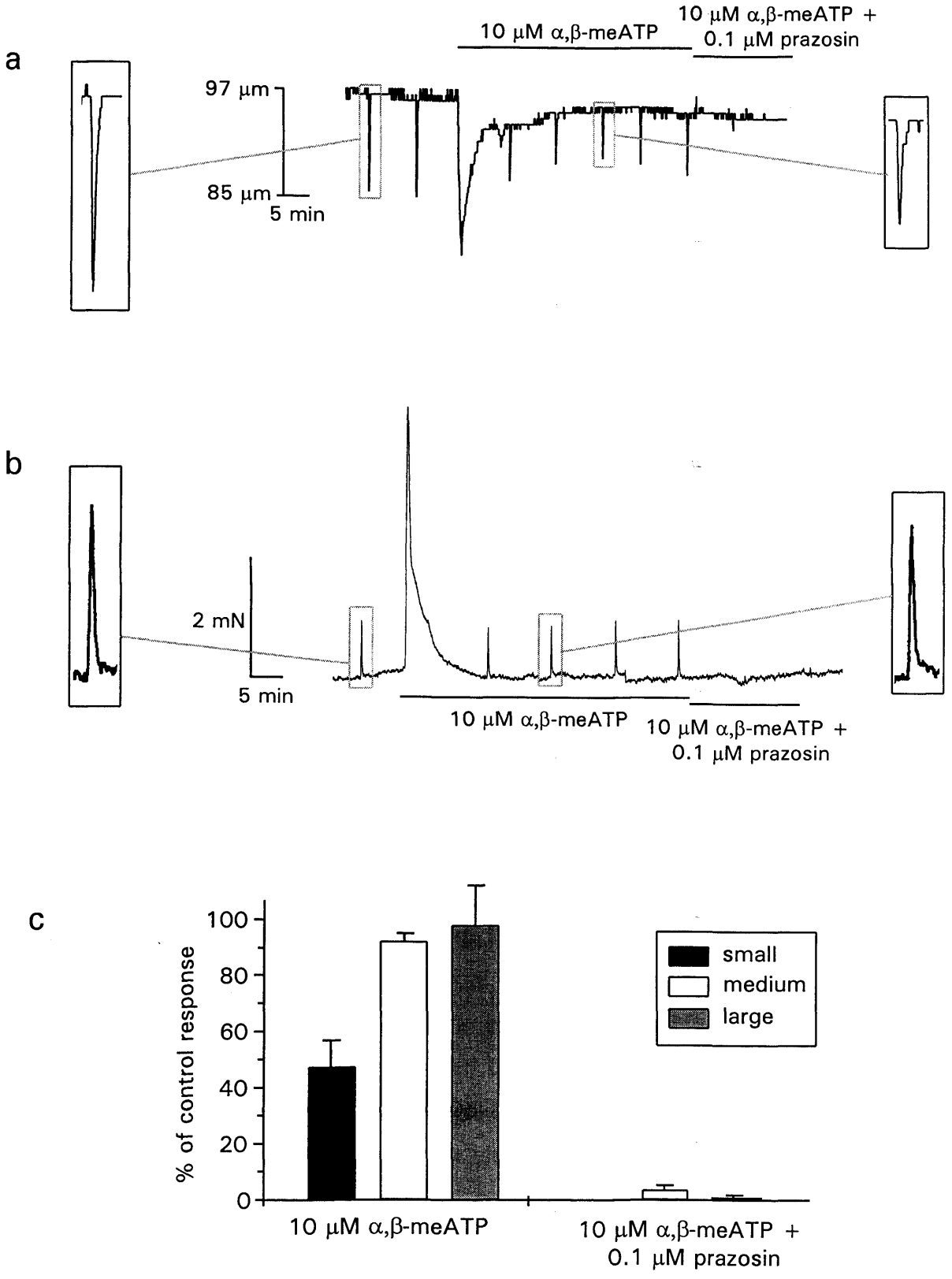


Figure 26 α,β-meATP only reduces neurogenic responses in small arteries. Repetitive contractions are evoked by 10 pulses of stimulation at 10 Hz. Application of α,β-meATP (10 μM) significantly reduced responses in small arteries (a) but not in medium (b); combined α,β-meATP and prazosin (0.1 μM) application abolished nerve-evoked contractions. Boxes either side of traces show responses before and after α,β-meATP application at 3 x magnification; drug applications are indicated by bars. (c) Histogram of mean data ± s.e.m. (n = 4 – 6).

mediating vasoconstriction one would expect the two components to combine to 100 % of the response. Possible explanations for the observed effects are discussed in Section 4.7.

4.5 COMPARISON OF RESPONSES TO DIFFERENT TRAIN LENGTHS OF STIMULATION

Previous studies have found that increasing the duration of nerve stimulation increases the noradrenergic component of the response in medium to large arteries of the rat mesentery and other vascular beds (e.g Sjöblom-Widfeldt *et al*, 1990; Evans & Cunnane, 1992). Long trains of stimulation could therefore lead to an overestimation of the adrenergic component of nerve evoked contractions. To test whether this was also true for smaller vessels, I conducted a series of experiments comparing responses to 10 pulses at 10 Hz (1-second train) with responses to 100 pulses at 10 Hz (10 second train).

The mean amplitude of responses to 100 pulses at 10 Hz was 147 ± 8.2 % of response to 10 pulses at 10 Hz (n = 6). In addition to being larger, responses to 100 pulses at 10 Hz had a different time course. When stimulated with 10 pulses at 10 Hz arteries relaxed almost as quickly as they constricted (3 – 4 seconds). Responses to 100 pulses at 10 Hz however had a much longer decay times; the vessel gradually relaxed over a period of 20 – 40 seconds (Figure 27a). As for short trains of stimulation the adrenergic component of contraction was determined with the α_1-adrenoceptor antagonist prazosin (Fig. 27 b,c). Responses to 100 pulses at 10 Hz were reduced by 68.8 ± 1.9 % in the presence of 0.1 μM prazosin (n = 6), confirming that the adrenergic component is indeed significantly greater with these stimulation parameters (32.6 ± 2.6 % with 10 pulses at 10 Hz; $p < 0.001$). To verify that only ATP and noradrenaline were mediating vasoconstriction, suramin (100 μM) was applied in combination with prazosin. In the presence of both antagonists responses were virtually abolished (reduced by 96.4 ± 2.5 %; Fig. 27 c).

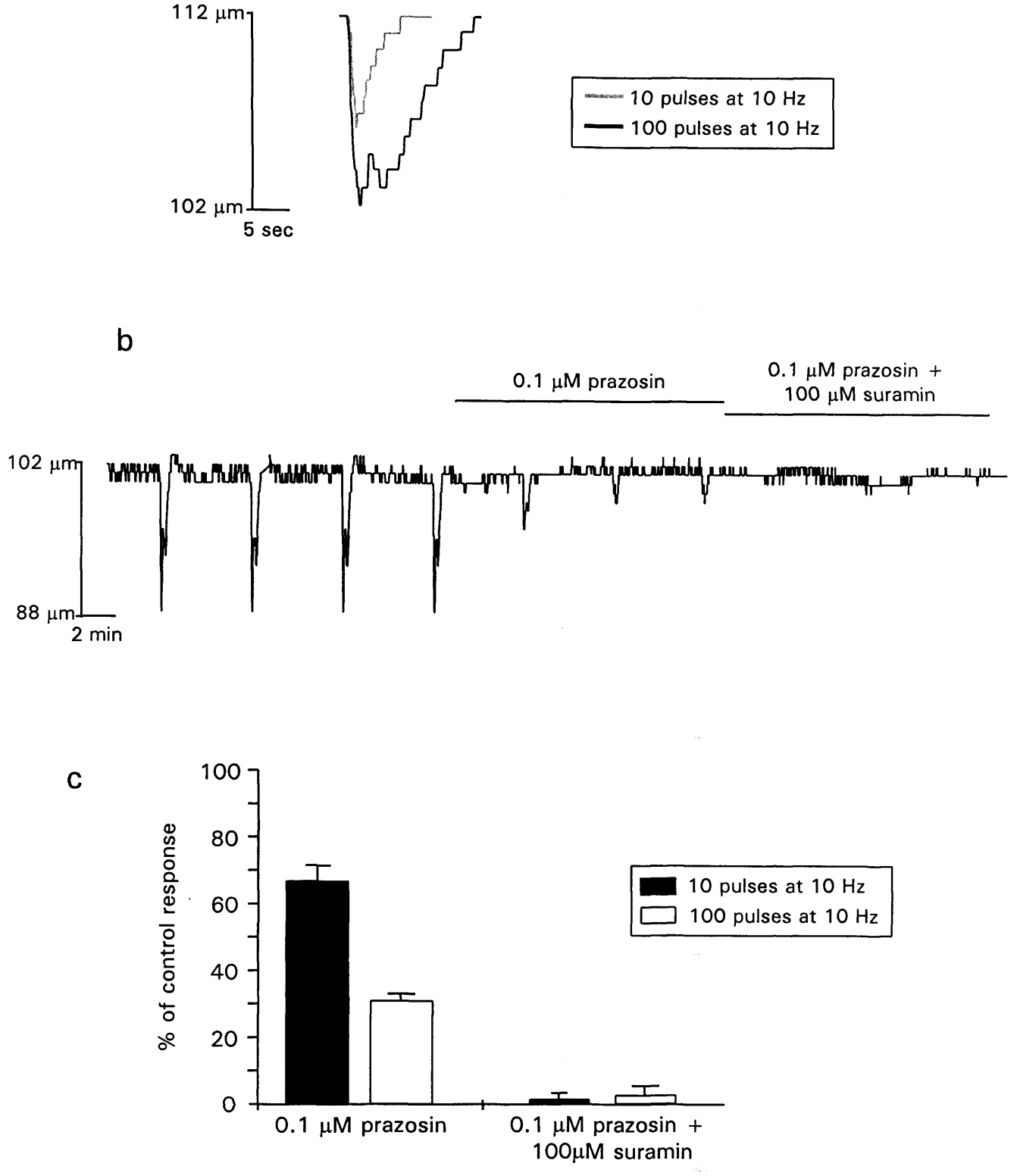


Figure 27 The adrenergic component of neurogenic contraction in small arteries increases with longer durations of stimulation. (a) Typical responses of small vessels to 100 pulses of stimulation at 10 Hz. Prazosin (0.1 μM) substantially reduces contractions and concomitant application of suramin (100 μM) abolishes them. (b) Comparison of the effect of prazosin and suramin on responses evoked by short and long trains of stimulation in small arteries; data are mean ± s.e.m. (n = 4 – 6).

4.6 SOURCES OF CALCIUM FOR P2X MEDIATED CONTRACTION

An increase in the cytosolic level of calcium is essential for smooth muscle contraction. In Chapter 3, I have shown that calcium influx from the extracellular space is essential for P2X receptor-mediated vasoconstriction, confirming results obtained in earlier studies e.g. Galligan *et al* (1995). This influx can occur either through the P2X receptor channel or through voltage-gated calcium channels, and it is unclear which route dominates. Although calcium channels are believed to mediate substantial calcium influx, there is evidence that suggests these channels may play a more limited role (Galligan *et al*, 1995). I therefore extended my initial findings by comparing the effect of blocking calcium influx through voltage-gated calcium channels in small, medium and large mesenteric arteries and assessing the contribution of other potential sources of calcium for contraction.

4.6.1 EFFECT OF NIFEDIPINE ON AGONIST-EVOKED RESPONSES

Extracellular calcium was shown to be essential for α,β-meATP – mediated vasoconstriction in Section 3.2. Although calcium must enter the cell in order for P2X receptor activation to trigger smooth muscle contraction, it is not clear by which route this influx occurs. Calcium can enter the cell through the P2X receptor channel, which is itself permeable to the ion (Benham & Tsien, 1987). Activation of the receptor however also leads to the depolarisation of the cell, which can subsequently cause opening of voltage-gated calcium channels. The following experiments were designed to determine the relative importance of these two routes is in rat mesenteric arteries.

L-type voltage-gated calcium channels are thought to be the predominant calcium channel expressed in vascular smooth muscle (Gollasch & Nelson, 1997). The importance of L-type calcium channels was studied by testing the effect of the selective antagonist nifedipine (1 μM) on contractions evoked by an EC_{50} concentration of α,β-meATP (1 μM in small and medium; 100 μM in large). Responses to α,β-meATP were not reduced in small arteries (n = 4) and were reduced by 2.0 ± 8.7 % (n = 4) and 15 ± 7.3 % (n = 6) in medium and large arteries, respectively (Fig. 28). These results suggest that L-type channels play only a minor role in mediating calcium influx.

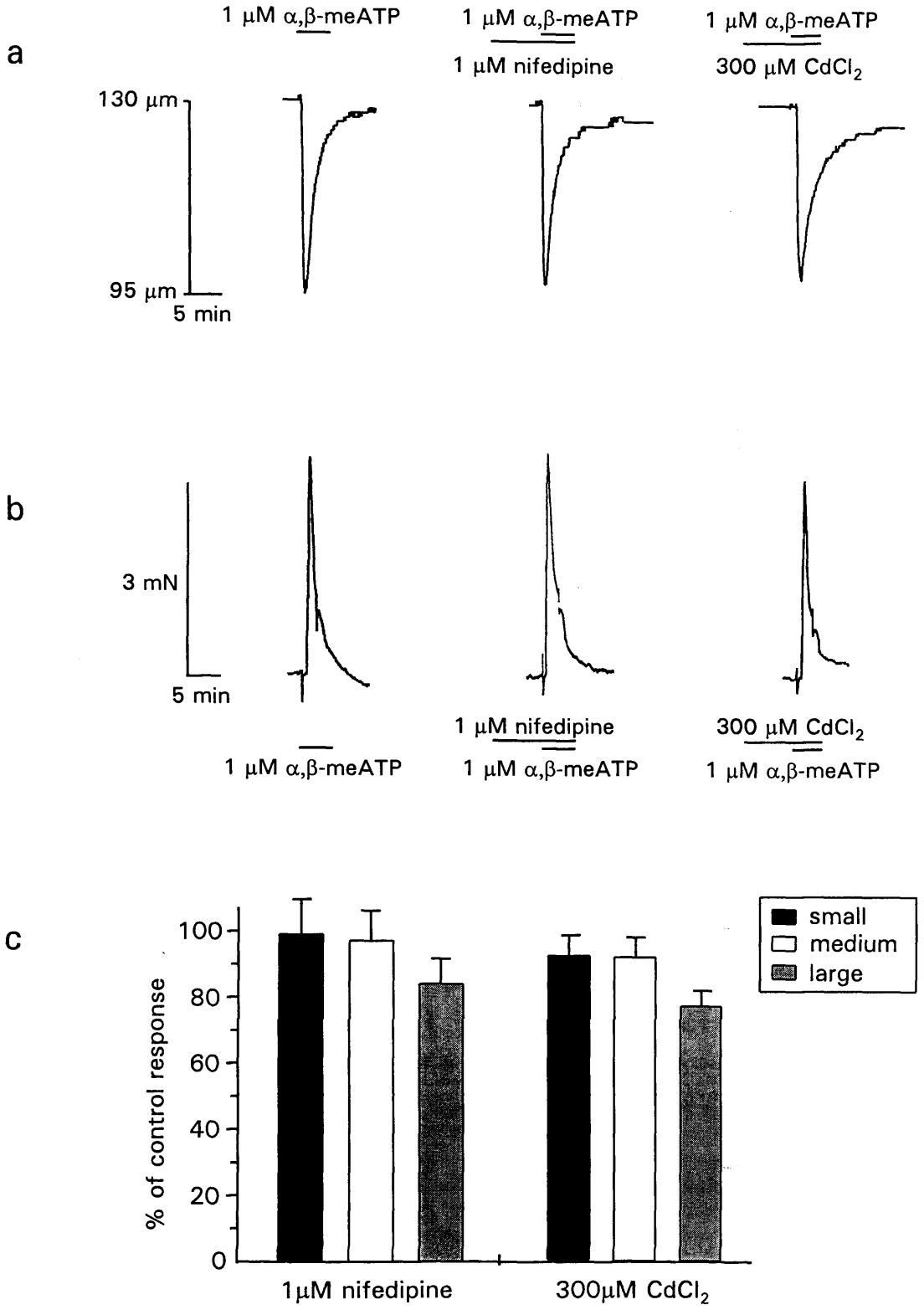


Figure 28 Nifedipine and cadmium have little effect on contractions evoked by α,β-meATP. Blockade of L-type calcium channels with nifedipine (0.1 μM) caused almost no reduction of contractions to an EC_{50} concentration of α,β-meATP (1 μM) in small (a) and medium (b) arteries; cadmium (300 μM) produced a very similar effect. Responses were reduced slightly more in large arteries. Drug applications are indicated by bars. (c) Histogram summarising mean data ± s.e.m. from all arteries. Contractions to α,β-meATP (300 μM) are reduced slightly more in large arteries.

The lack of effect seen with nifedipine made it important to confirm that the antagonist was effective in the tissue studied; a suitable control experiment therefore had to be performed. Exposing smooth muscle to a high concentration of potassium ions depolarizes the cells causing the opening of voltage-gated calcium channels. Contractions evoked by a 'high potassium' solution were therefore used as control responses because they rely on calcium influx through voltage- dependent channels. Responses to 60 mM KCl, a near maximal concentration in all arteries, were abolished by 1 μM nifedipine in medium-sized and large arteries (n = 4, Fig. 29 b). Interestingly, in small vessels, contractions were only reduced by 52.8 ± 6.9 % (n = 4; Fig. 29 a).

The nifedipine-resistant component of constriction to 60 mM KCl suggested the presence of a further 'non-L-type' calcium channel in small arteries. Other types of voltage-dependent calcium channel may therefore also have been present on medium and large arteries, which might have explained the limited effect of nifedipine on contractions evoked by α,β-meATP. To determine whether any such channel was involved in constrictions evoked by α,β-meATP, the effect of cadmium was tested. This metal is a non-specific blocker of all types of calcium channel. A similar pattern of effects on α,β-meATP evoked contractions was seen as with nifedipine although responses were reduced slightly more by cadmium (Fig. 28). $CdCl_2$ (300 μM) caused a mean reduction of responses of 8.4 ± 5.4 % (n = 4), 9.1 ± 5.0 % (n = 4) and 22.8 ± 4.0 % (n = 5) in small, medium and large arteries, respectively. Cadmium abolished control responses to KCl in all arteries (n = 4). These results appear to confirm data obtained with nifedipine suggesting that voltage-gated calcium channels do not play a significant role in mediating calcium influx in response to P2X receptor activation. It also appears that, there is an additional non-L-type calcium channel in small arteries, which is however not involved in P2X receptor-mediated vasoconstriction.

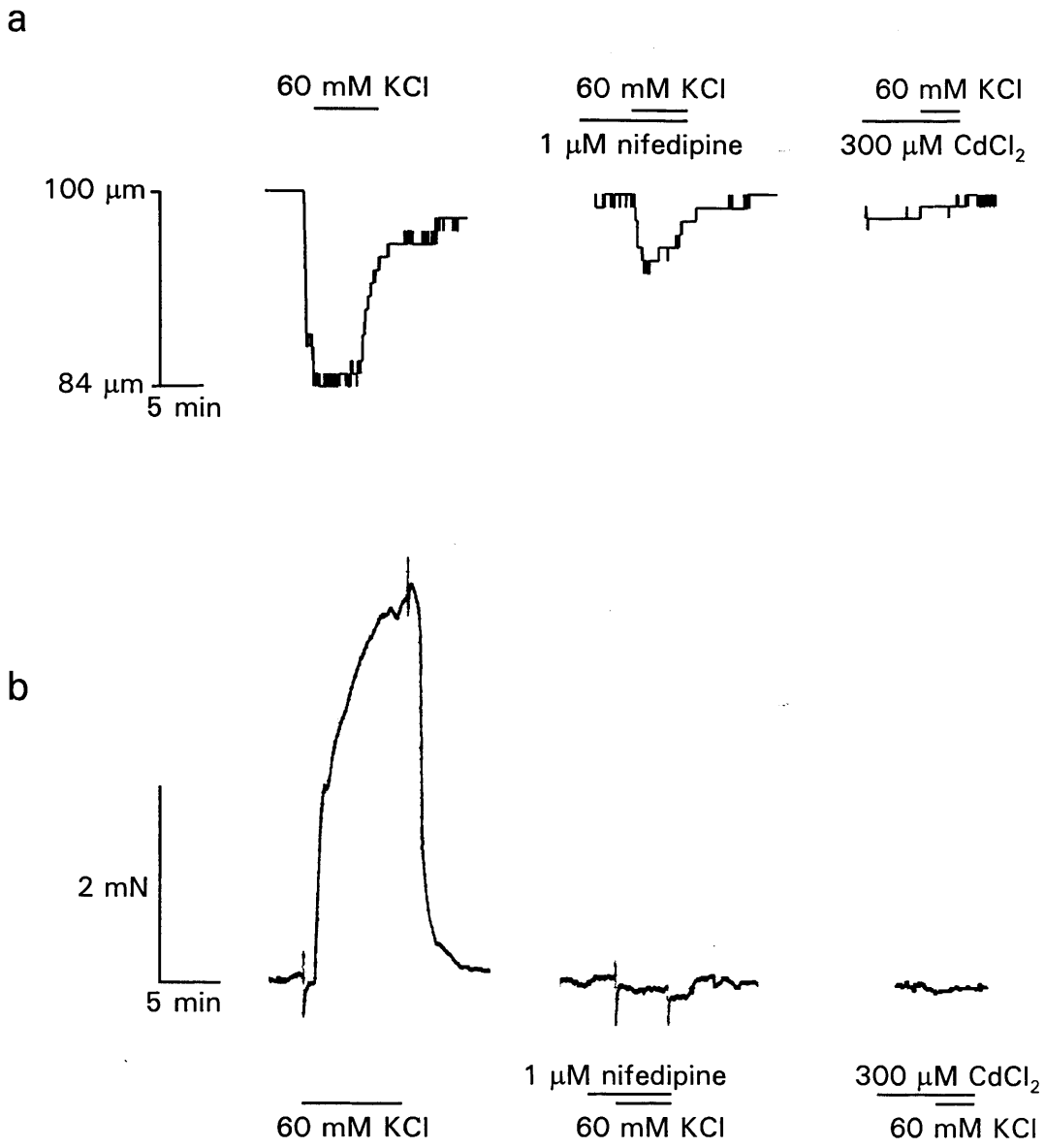


Figure 29 Control responses to KCl are reduced or abolished by nifedipine and cadmium. Typical contractions evoked by 60 mM KCl in small (a) and medium-sized (b) arteries. Nifedipine (0.1 μM) abolishes responses in medium arteries but a component of contraction remains in small vessels. Contractions in all arteries are abolished by cadmium (300 μM). Small deflections of traces in (b) at solution exchange are flow artifacts.

4.6.2 ROLE OF VOLTAGE-GATED CALCIUM CHANNELS IN NEUROGENIC RESPONSES

The above findings suggested that L-type calcium channels play a minor role in mediating calcium influx in response to P2X receptor activation. It was then of interest to determine whether similar results were obtained when contractions were evoked under conditions approximating closer to those found *in vivo*. The effect of nifedipine was therefore tested on neurogenic contractile responses.

Nifedipine (1 μM) reduced neurogenic responses by only 2.8 ± 3.3 % (n = 5), 10.0 ± 3.7 % (n = 6) and 13.5 ± 2.7 % (n = 6) in small, medium and large arteries, respectively (Fig. 30). This implies that calcium entry through voltage-gated channels also plays only a minor role in neurogenic vasoconstriction. In addition, I was particularly interested in examining the effect of nifedipine on just the purinergic component of neurogenic vasoconstriction as this could be compared directly with the results from exogenous application of α,β-meATP. When the adrenergic component was first blocked with 0.1 μM prazosin, nifedipine reduced contractions by 4.6 ± 7.9 % (n = 5), 14.3 ± 2.0 % (n = 6) and 3.0 ± 1.9 % (n = 6) in small, medium and large vessels respectively (Fig. 30). Even for P2X receptor-mediated contraction in response to nerve stimulation, voltage-gated calcium channels therefore appear not to be essential for calcium influx.

4.6.3 ROLE OF CALCIUM-INDUCED CALCIUM RELEASE

It thus appears that the majority of calcium influx required for P2X receptor-mediated smooth muscle contraction occurs directly through the P2X receptor itself. It is however unclear whether this influx is sufficient to sustain the whole contractile response or whether additional amplification of the calcium signal is required. A rise in the cytosolic level of calcium can trigger the liberation of further calcium from the sarcoplasmic reticulum by the process of calcium-induced calcium release (CICR). This would substantially enhance the rise in calcium levels produced by influx from the extracellular space. To test for a putative role for CICR, I examined the effect of depleting intracellular stores of all their calcium with cyclopiazonic acid (CPA).

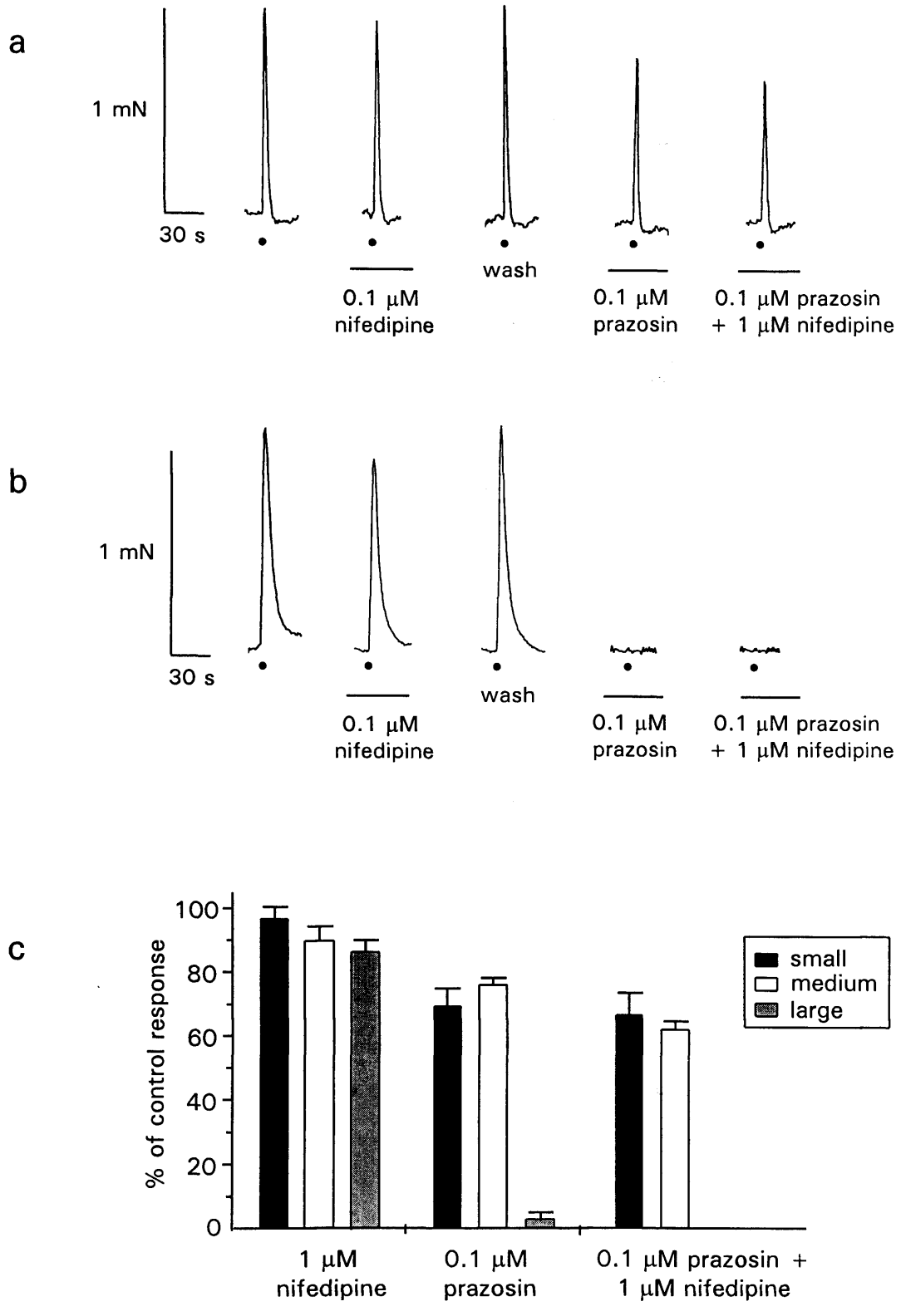


Figure 30 Nifedipine has little effect on both the overall neurogenic response and on the purinergic component of contraction. Responses evoked by 10 pulses of stimulation at 10 Hz are reduced to a small degree in medium-sized (a) and large (b) arteries. When the adrenergic component of the response is first blocked with prazosin (0.1 μM), nifedipine still has only a limited blocking effect in medium-sized arteries. Drug applications are indicated by bars and nerve stimulation by circles. (c) Histogram of mean data ± s.e.m. (n = 5 – 6) showing similar results for small and medium vessels.

This compound is an antagonist of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and prevents refilling of the calcium stores. As calcium is lost passively across the SR membrane, CPA will gradually cause the complete emptying of the stores.

Initially, the ability of CPA to deplete intracellular calcium stores had to be verified. Caffeine releases calcium from the internal stores of smooth muscle cells, which results in a transient contraction in arteries. CPA abolished contractions to caffeine (n = 4 – 5), and calcium stores were therefore assumed to be depleted after CPA treatment. Using the same protocol as for caffeine, CPA was found to have no inhibitory effect on contractions evoked by an EC_{50} concentration of α,β-meATP (1 μM in small and medium, 100 μM in large). Expressed as a percentage of control, responses in the presence of CPA were 101.3 ± 6.4 % (n = 4), 101.3 ± 3.5 % (n = 6) and 117.3 ± 9.5 % (n = 6) in small, medium and large arteries, respectively (Fig. 31).

If one assumes that 30 mM caffeine liberates all calcium from the sarcoplasmic reticulum, the amplitude of contractile responses evoked can be used to estimate the size of the intracellular calcium stores. Comparing contractions produced by 30 mM caffeine to responses evoked by other agonists can provide an indication of how much calcium is released upon store emptying. For example, a maximal contraction in response to KCl can serve as a good indication of the overall contractility of the vessel. Expressing responses to 30 mM caffeine in terms of responses to KCl can therefore give an estimate of what contribution calcium release from internal stores is able to make toward vasoconstriction. As a percentage of responses to a near maximal concentration of KCl (60 mM), contractions to 30 mM caffeine were 88.7 ± 7.5 % (n = 4), 51.7 ± 10.9 % (n = 4) and 30.9 ± 9.4 % (n = 5) for small, medium and large arteries respectively. Expressed in terms of contractions to α,β-meATP (1 μM in small and medium, 100 μM in large), responses were 90.8 ± 5.1 % (n = 4), 41.8 ± 5.8 % (n = 4) and 50.4 ± 19.3 % (n = 5), respectively.

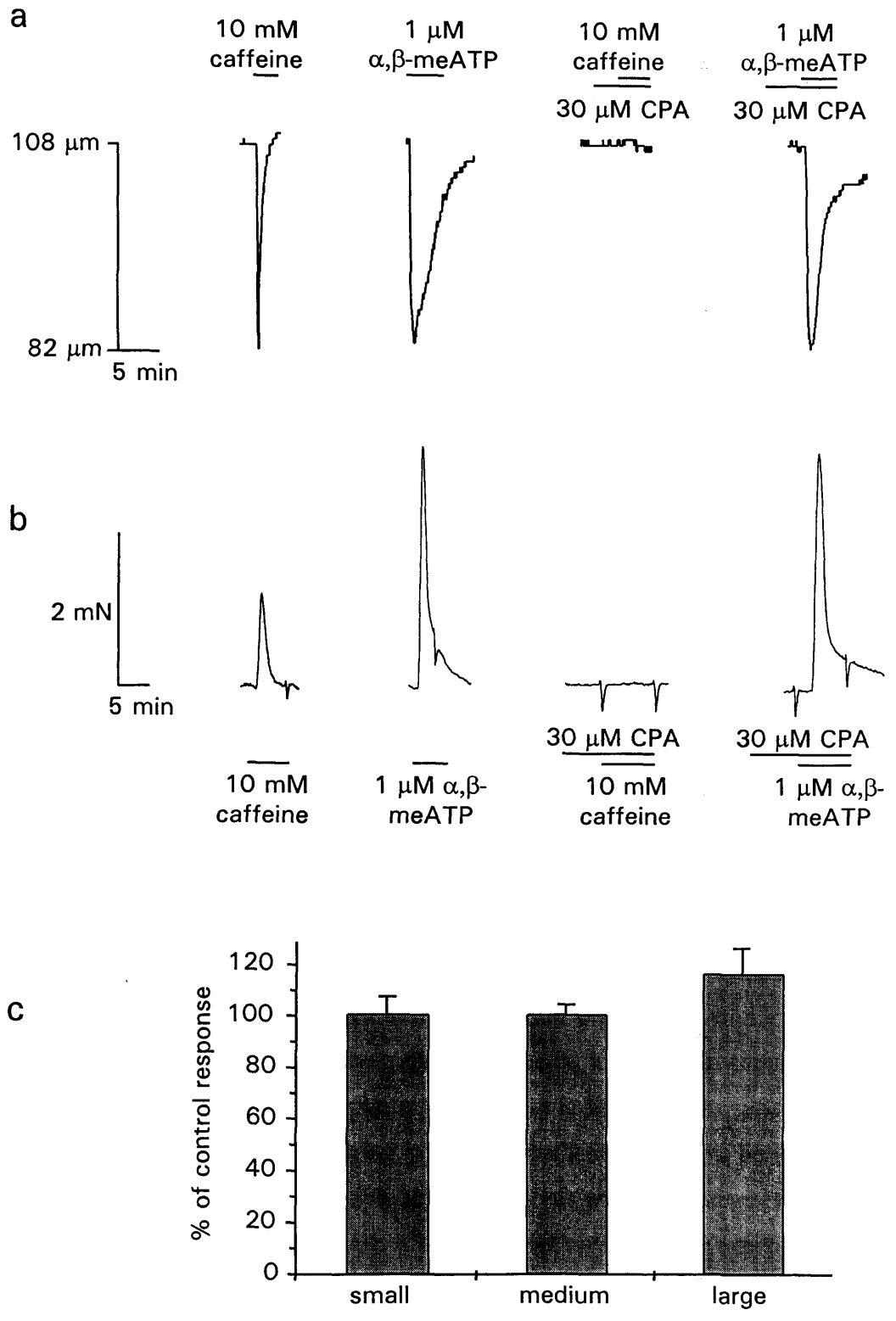


Figure 31 Contractile responses evoked by α,β-meATP are not reduced by CPA. Depletion of intracellular calcium stores with CPA (30 μM) abolishes control responses to caffeine in both small (a) and medium-sized (b) arteries but has no inhibitory effect on constrictions to α,β-meATP (1 μM in small and medium arteries and 100 μM in large). Note also the substantially larger response to caffeine as a proportion of α,β-meATP in small vessels. Drug applications are indicated by bars. (c) Data are mean ± s.e.m. (n = 4 – 6). Small deflections of traces in (b) are flow artifacts.

4.7 DISCUSSION

There is evidence to suggest that the relative contributions of ATP and noradrenaline toward sympathetic control of arterial tone vary depending on the diameter of the vessel. The results described here clearly demonstrate such variation within different-sized arteries of the same vascular bed. They show that while purinergic transmission dominates in small and medium-sized arteries, adrenergic transmission dominates in large arteries. In addition, my data indicate that L-type calcium channels are not essential in mediating calcium influx associated with contraction. It appears that all the calcium required for the contractile response enters the cell directly through the P2X receptor channel. This work has been published (Gitterman & Evans, 2001; Appendix 1).

One of the main concerns in these experiments was to choose parameters of electrical stimulation that approximate closely to neurotransmission *in vivo*. A large number of studies have been conducted on sympathetic neuroeffector transmission and many different patterns of stimulation have been used. The type of stimulation however significantly affects which transmitters mediate postjunctional responses. A question of great importance is therefore: what pattern of stimulation reflects the *in vivo* activity of perivascular sympathetic nerves? Although much research has investigated the properties of sympathetic transmission, very few studies have focused on the patterns of sympathetic nerve activity *in vivo*.

In one of the first studies examining the behaviour of individual sympathetic fibres *in situ*, Johnson & Gilbey (1994) found that firing frequently occurs in rhythmic bursts separated by relative silence rather than stochastic trains of continual impulses. In certain fibres this rhythm was found to depend on central respiratory activity as indicated by its synchrony with the firing pattern of the phrenic nerve. The firing rate of sympathetic fibres was between 0.8 and 0.9 Hz, each discharge being a burst of multiple action potentials with an intraburst frequency of up to 20 Hz or more (Johnson & Gilbey, 1996). A potential weakness of these experiments is the fact that they were carried out in the rat tail artery, a special thermoregulatory blood vessel. It could therefore be argued that they are not representative of the general vasculature. Similar results have however also been obtained in human muscle tissue. Again sympathetic vasoconstrictor nerve fibres were seen to fire in bursts, in this case with a mean frequency of 0.47 Hz. The mean

intraburst firing rate was also ~ 20 Hz but occasionally frequencies of up to 50 Hz were observed (Macefield *et al*, 1994). These data suggest that a normal pattern of sympathetic activity might be a brief 20 Hz burst of impulses every 2 seconds. Based on these studies, I have chosen stimulation parameters that are practical to use and that simulate one such burst of sympathetic activity: 10 pulses at a frequency of 10 Hz.

Frequency-response relationships were comparable for each artery. That is, for a given pattern of stimulation, the contractile response expressed as a proportion of the maximal response was very similar in all vessels. This shows that the properties of transmitter release are very similar in each case. In addition, it demonstrates that the difference in experimental methodologies (Diamtrak versus myography) would not be expected to influence the results observed. The fact that contractions were abolished in the presence of tetrodotoxin confirms that they were entirely neurogenic in origin. Interestingly, responses in large arteries had a noticeably longer time course than in small and medium-sized vessels. A possible reason for this may be the substantially thicker wall of large arteries containing large amounts of collagen and several layers of elastic laminae i.e. they are more rigid than smaller vessels and may therefore return to their baseline tone more slowly.

One of the main aims of this study was to determine the importance of purinergic transmission in neurogenic control of vascular tone. As mentioned in Section 1.3.3.2 a large body of research on the relative roles of ATP and noradrenaline has accumulated over recent years. In spite of this, there is little consistency in the data generated. Results have been found to depend on the species and the vascular bed studied. For example, two studies using a similar pattern of stimulation produced different results in different animals: ~ 70 % of the response to 40 pulses at 8 Hz was adrenergic in the rabbit ear artery, (Garcia-Villalon *et al*, 1997) and only 40 % was adrenergic in the dog ear artery (Haniuda *et al*, 1997). In two other studies, identical stimulation parameters (64 pulses at 64 Hz) were used in different rabbit arteries. An adrenergic component of 20 – 30 % was determined in the hepatic artery (Brizzolara & Burnstock, 1990), while one of 50 % was determined in the saphenous artery (MacDonald *et al*, 1992). A good example of the inconsistency of results is provided by two studies in identical tissues that used identical parameters to evoke contractions, but which still did not produce the same results (Burnstock &

Warland, 1987 and MacDonald et al, 1992); this discrepancy may be due to slight differences in the protocols used. Comparing results from different studies is therefore problematic, making it difficult to establish clear patterns in the properties of sympathetic transmission.

The most important factor affecting results however is the choice of stimulation parameters. This is in fact a rare point of agreement throughout studies on neurogenic vasoconstriction: in a given tissue, increasing the train length of stimulation will increase the adrenergic proportion of contraction. Increasing the frequency of stimulation also produces a similar change but the effect is not as consistent as when increasing the train duration. Many studies have used a range of different patterns of stimulation, and the resulting change in purinergic and noradrenergic components can readily be seen (Kennedy *et al*, 1986; Sjoblom-Widfeldt *et al*, 1990; Todorov *et al*, 1999; Yang & Chiba, 2000). This is also evident from my data in small arteries where increasing stimulation from 1 to 10 seconds doubled the adrenergic portion of the response. Using long trains of stimulation can therefore lead to a large adrenergic component of contraction. Considering the evidence described above that sympathetic nerves are most likely to fire in short bursts, this has significant implications for experimental results. The long duration of stimulation often used may therefore result in a substantial overestimation of the adrenergic (and hence underestimation of the purinergic) component of contraction.

The difficulty in drawing firm conclusions about the relative roles of ATP and noradrenaline in mediating sympathetic vasoconstriction is therefore clear. Although certain studies suggest that the purinergic component dominates in resistance arteries, no systematic comparisons of different-sized vessels from the same vascular bed using the same experimental conditions have been conducted. The present study was undertaken to fill this important gap in experimental data. Selective antagonism of P2X receptors and α-adrenoceptors revealed that purinergic transmission accounted for approximately 70 % of the response in small and medium-sized arteries. This is in keeping with the notion that ATP is more important in mediating contraction in smaller arteries. Other investigations using similar stimulation parameters have produced similar results. One second of stimulation at either 8 Hz or 10 Hz evoked responses that were between 80 % and 100 % purinergic in rabbit saphenous arteries (Burnstock & Warland, 1987),

rabbit jejunal arteries (Evans & Cunnane, 1992) and rabbit splenic arteries (Ren & Burnstock, 1997).

In stark contrast to this, responses in large mesenteric arteries are almost exclusively adrenergic. Equivalent results using a similar duration and frequency of stimulation have been observed in the rat tail artery (Bao & Stjärne, 1993) and may be a general characteristic of large conduit arteries. Unfortunately, no experiments were carried out in smaller branches of the tail artery so comparisons could not be made (this illustrates the value of comparing different vessels from the same vascular bed). One study contradicting the theory that small arteries display mainly purinergic contractions was conducted by Morris (1999). Here, a 30-second train of stimulation at 10 Hz evoked responses in small resistance arteries of the guinea-pig ear, that were entirely adrenergic. It must however be added that such a continual high frequency train of impulses probably does not correspond to the intermittent pattern of sympathetic firing observed *in vivo*. Nevertheless, an interesting comparison would be to assess the effect of using these parameters on small arteries in my studies.

A possible explanation for the difference in the relative contribution of noradrenaline and ATP toward sympathetic vasoconstriction could be different thresholds for noradrenaline action. Todorov *et al* (1999) found that in guinea-pig vas deferens, inhibition of noradrenaline release through prejunctional α_2-adrenoceptors is overcome the higher the frequency of stimulation is. As a higher frequency of stimulation releases more transmitter, this implies that a certain quantity of noradrenaline is required to produce a post-junctional effect. By the same token, noradrenaline in the nerve terminal might need to reach a certain level in order to overcome removal by the transporter uptake 1 and activate post-junctional α_1-adrenoceptors. Both these effects suggest a threshold for noradrenaline: no post-junctional effect will be produced until the required concentration of noradrenaline is reached at the post-junctional receptor. A possible cause of this may be the location of receptors at the neuroeffector junction: large arteries may have a much larger adrenergic component because post-junctional α_1-adrenoceptors are located closer to the nerve terminal. A similar explanation was given for variation of adrenergic and purinergic components between the rabbit ear and jejunal arteries (Goncalves & Guimaraes, 1991). This would minimise the effect of re-uptake and inhibition of noradrenaline release by

pre-junctional α_2-adrenoceptors (Figure 32). In small arteries however, α_1-adrenoceptors may be further from the junctional cleft (Figure 32). As a result, a higher level of stimulation may be required to release the transmitter in sufficient quantities to overcome the two limiting effects. Only then will a contractile response be evoked. The fact that increasing the train length from one to ten seconds doubled the adrenergic component in small arteries in my study would support this explanation. A recent study in canine splenic arteries suggests differential localisation of α_1-adrenoceptor subtypes in the sympathetic neuroeffector junction: α_{1B} and α_{1D} adrenoceptors were said to be located within the junction, while α_{1A} adrenoceptors were found to be extrajunctional (Yang & Chiba, 2001). A similar distribution pattern may be present in the rat mesentery; my results could be explained by a low density of junctional adrenoceptor subtypes in small and medium-sized arteries and a high density in large arteries. The above study was based on a pharmacological approach to locate receptors. A more direct approach might be to use sub-type specific antibodies and confocal microscopy to determine the precise location of adrenoceptors.

Another possibility is a difference in receptor density of either adrenoceptors or P2X receptors. Diameter-dependent differences in the density of P2X receptors have previously been elucidated using autoradiography (e.g. Neely et al, 1996). Using the same technique, Bo & Burnstock (1993) found that P2X receptor density decreased as arterial diameter increased. This might account for the small purinergic component of contraction I found in large arteries. On the other hand adrenoceptor density may be higher in large arteries, although the similar potency of noradrenaline in contraction studies might argue against this (Section 3.3). Different ratios of noradrenaline and ATP release in different vessels could also produce the observed results, i.e. less ATP release in large arteries results in a small purinergic component of neurogenic contraction. There are several examples of dissociated release of noradrenaline and ATP from sympathetic nerves (e.g. Goncalves *et al*, 1996; Todorov *et al*, 1996; Msghina *et al*, 1999).

In my experiments, the components of contraction inhibited by suramin and prazosin combine to ~ 100 % of the response. This implies that there is no synergistic interaction between the two transmitters as has been previously suggested for this and other tissues (Ralevic & Burnstock, 1990; Todorov *et al*, 1999). In addition, all responses are abolished by combined application of

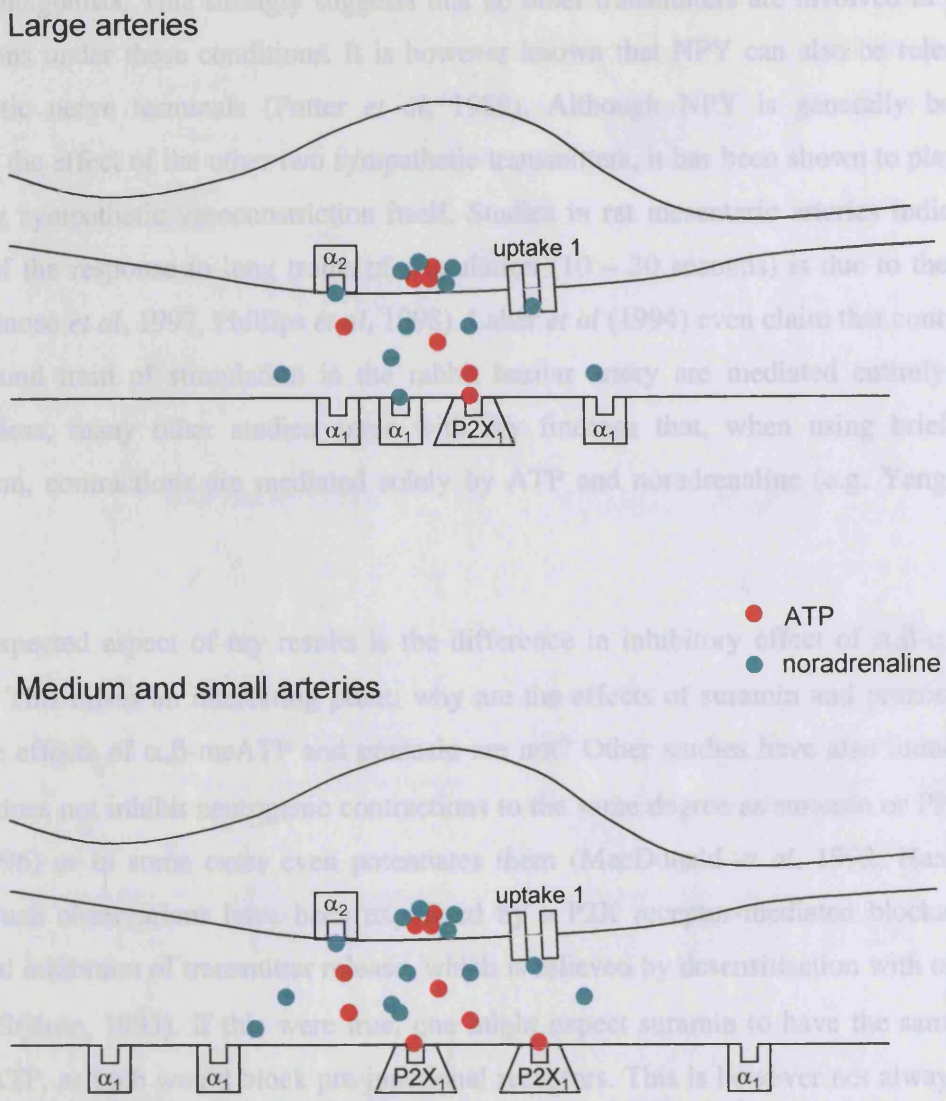


Figure 32 The location of adrenoceptors may explain the difference in adrenergic and purinergic components of nerve-evoked responses in large and small arteries. In large arteries, post-junctional α_1-adrenoceptors may be located within the neuroeffector junction thus limiting the effect of α_2-adrenoceptor mediated auto-inhibition and noradrenaline re-uptake. Noradrenaline has a substantial post-junctional effect and mediates the majority of sympathetic vasoconstriction. In small and medium arteries however, α_1-adrenoceptors are extra-junctional. As a result the concentration of noradrenaline in the junction has to be high to overcome the aforementioned effects limiting the post-junctional response. In this case the purinergic component of sympathetic vasoconstriction dominates.

the two antagonists. This strongly suggests that no other transmitters are involved in producing contractions under these conditions. It is however known that NPY can also be released from sympathetic nerve terminals (Potter *et al*, 1989). Although NPY is generally believed to modulate the effect of the other two sympathetic transmitters, it has been shown to play a role in mediating sympathetic vasoconstriction itself. Studies in rat mesenteric arteries indicate that a portion of the response to long trains of stimulation (10 – 30 seconds) is due to the action of NPY (Donoso *et al*, 1997, Phillips *et al*, 1998). Laher *et al* (1994) even claim that contractions to a 20 second train of stimulation in the rabbit basilar artery are mediated entirely by NPY. Nevertheless, many other studies agree with my findings that, when using brief trains of stimulation, contractions are mediated solely by ATP and noradrenaline (e.g. Yang & Chiba, 1998).

One unexpected aspect of my results is the difference in inhibitory effect of α,β-meATP and suramin. This raises an interesting point: why are the effects of suramin and prazosin additive while the effects of α,β-meATP and prazosin are not? Other studies have also found that α,β-meATP does not inhibit neurogenic contractions to the same degree as suramin or PPADS (Ren *et al*, 1996) or in some cases even potentiates them (MacDonald *et al*, 1992; Haniuda *et al*, 1997). Such observations have been explained by a P2X receptor-mediated blockade of pre-junctional inhibition of transmitter release, which is relieved by desensitisation with α,β-meATP (Bao & Stjärne, 1993). If this were true, one might expect suramin to have the same effect as α,β-meATP, as both would block pre-junctional receptors. This is however not always the case. Another explanation, relies on α,β-meATP mediated inhibition of ecto-nucleotidase activity as demonstrated by Chen & Lin (1997). A consequence of this would by elevated levels of ATP in the junctional area upon nerve stimulation. The concentration of ATP may therefore rise sufficiently to activate stimulatory pre-junctional P2 receptors, which are insensitive to α,β-meATP and would not be in the desensitised state. This would result in enhanced transmitter release leading to a greater post-junctional response mediated by noradrenaline (see Figure 33).

One interpretation, which would also account for my results with α,β-meATP, is the sensitisation of calcium release channels by raised intracellular calcium. As explained in section 3.7, the initial activation of the P2X receptor by α,β-meATP allows influx of extracellular

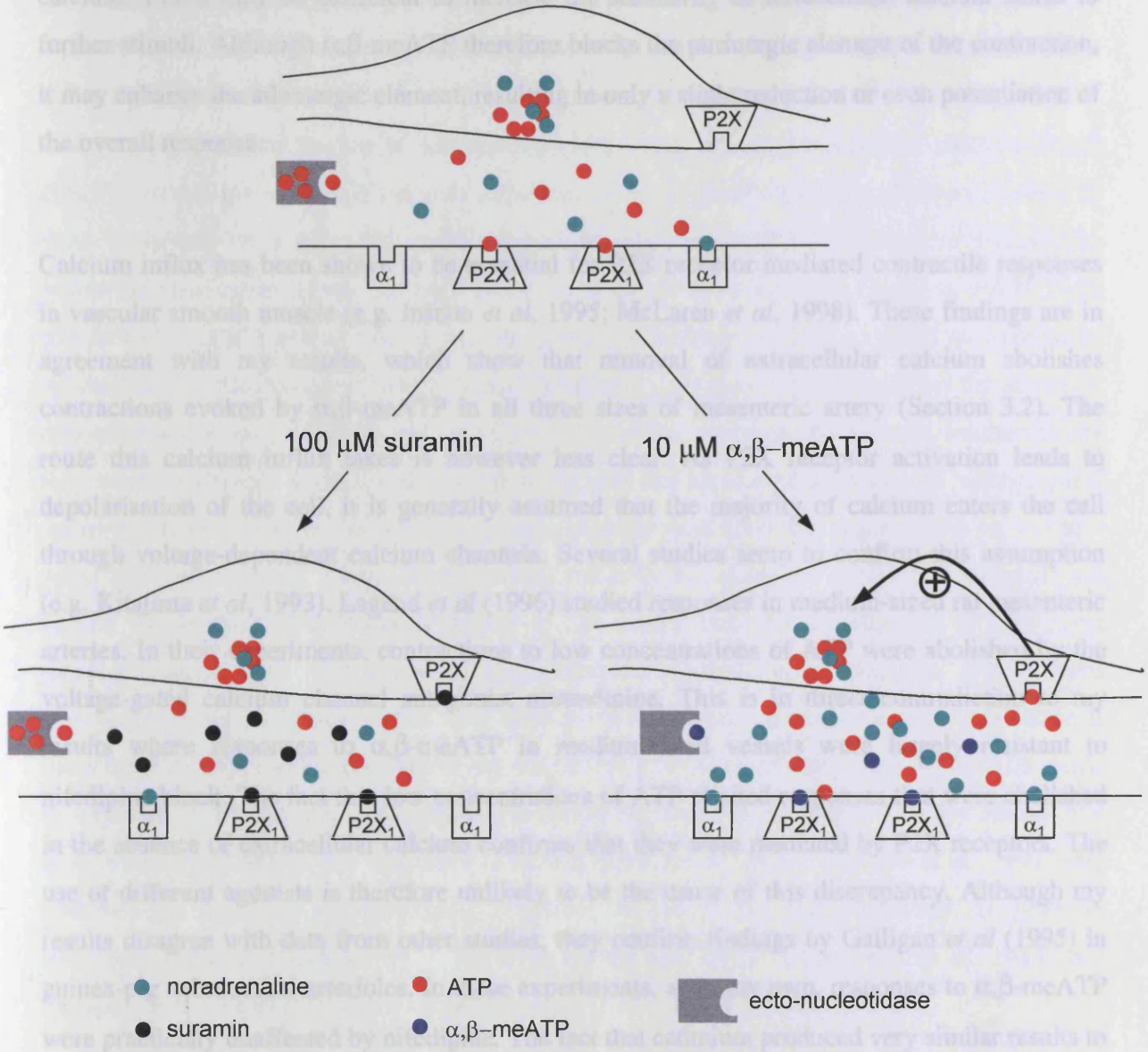


Figure 33 Putative scheme explaining the difference in effect of suramin and α,β-meATP on neurogenic vasoconstriction. In the presence of suramin, pre-and postjunctional P2X receptors are antagonised leaving only the adrenergic component of the response. In the presence of α,β-meATP however, only post-junctional P2X receptors are blocked. In addition the activity of ecto-nucleotidases is inhibited, raising the junctional concentration of ATP. Although ATP has no effect on desensitised smooth muscle P2X receptors, it can activate stimulatory pre-junctional P2X receptors. The result is greater transmitter release which increases the still functional adrenergic component.

calcium, which may be sufficient to increase the sensitivity of intracellular calcium stores to further stimuli. Although α,β-meATP therefore blocks the purinergic element of the contraction, it may enhance the adrenergic element, resulting in only a slight reduction or even potentiation of the overall response.

Calcium influx has been shown to be essential for P2X receptor mediated contractile responses in vascular smooth muscle (e.g. Inscho *et al*, 1995; McLaren *et al*, 1998). These findings are in agreement with my results, which show that removal of extracellular calcium abolishes contractions evoked by α,β-meATP in all three sizes of mesenteric artery (Section 3.2). The route this calcium influx takes is however less clear. As P2X receptor activation leads to depolarisation of the cell, it is generally assumed that the majority of calcium enters the cell through voltage-dependent calcium channels. Several studies seem to confirm this assumption (e.g. Kitajima *et al*, 1993). Lagaud *et al* (1996) studied responses in medium-sized rat mesenteric arteries. In their experiments, contractions to low concentrations of ATP were abolished by the voltage-gated calcium channel antagonist nitrendipine. This is in direct contradiction to my results where responses to α,β-meATP in medium-sized vessels were largely resistant to nifedipine block. The fact that low concentrations of ATP elicited responses that were abolished in the absence of extracellular calcium confirms that they were mediated by P2X receptors. The use of different agonists is therefore unlikely to be the cause of this discrepancy. Although my results disagree with data from other studies, they confirm findings by Galligan *et al* (1995) in guinea-pig submucosal arterioles. In these experiments, as in my own, responses to α,β-meATP were practically unaffected by nifedipine. The fact that cadmium produced very similar results to nifedipine in my study shows that no other voltage-dependent calcium channel is involved in mediating calcium entry. One unexpected result was that control responses to KCl were abolished by nifedipine in medium and large arteries but not in small. This nifedipine resistant component was however abolished by cadmium, suggesting the presence of a non-L-type calcium channel in these vessels. A comparison of these data with calcium currents recorded in single cells follows in Chapter 6.

It could be argued that assessing the effect of nifedipine on agonist-evoked contractions is not an accurate reflection of events *in vivo*. Under these circumstances, the system is 'flooded' with α,β-meATP, exposing every cell to the agonist. A very large proportion of P2X receptors will therefore be activated by the constantly present agonist, allowing substantial calcium influx directly through the receptor's intrinsic ion channel. *In vivo*, only a small proportion of cells is in close proximity to sympathetic varicosities and will therefore be exposed to transmitter. Moreover, transmitter release has been shown to be intermittent (Brock & Cunnane, 1987). Even if a smooth muscle cell is near a varicosity it may therefore still be exposed to transmitter infrequently. In this case, excitation of smooth muscle cells may rely far more on the spread of depolarisation from one cell to the next through electrical coupling. As a result, calcium influx may be more dependent on depolarisation of the cell and associated activation of voltage-gated calcium channels. The use of exogenous agonists may thus mask the requirement for voltage-gated calcium channels. These considerations can only be taken into account if responses are evoked by electrical stimulation of the nerve. The results from my investigation of neurogenic responses agree well with those of agonist-evoked contractions. Nifedipine also reduced contractions only marginally when they were evoked by nerve stimulation. The implication is therefore that L-type calcium channels are not essential for calcium entry, even when cells would be expected to rely on electrical coupling to spread the wave of excitation. The few studies that have been conducted in this area have produced quite mixed results. Bulloch *et al* (1991) also observed no reduction of neurogenic responses in the rabbit ileocolic artery. In contrast, nifedipine reduced responses by ~ 70 % in the rat tail artery (Surprenant *et al*, 1983) and systemic blood pressure by 50 % in the pithed rat (Bulloch & McGrath, 1988).

One means of directly comparing purinergic agonist and nerve-evoked contractions is by first blocking the adrenergic component of the neurogenic response with prazosin. Contractions are then mediated solely by P2X receptors, a situation analogous to exogenous application of α,β-meATP. Any noradrenaline-induced rise in calcium levels will therefore be abolished. The fact that nifedipine again had only a limited effect confirms that L-type calcium channels play a minor role in mediating calcium entry in response to P2X receptor activation. Using an identical protocol, Bulloch *et al* (1991) obtained similar results in the rabbit ileocolic artery, although

responses to longer trains of stimulation were much more sensitive to nifedipine. In dog mesenteric arteries, the purinergic component of neurogenic contractions was abolished by nifedipine (Omote *et al*, 1989). However, the duration of stimulation here was five times longer than in my experiments. It therefore appears that, in rat mesenteric arteries, L-type calcium channels are not required for calcium influx; almost all calcium entry associated with P2X receptor-mediated smooth muscle contraction enters the cell directly through the receptor's intrinsic ion channel.

Calcium entering smooth muscle cells can trigger a regenerative process of calcium release from intracellular stores called calcium-induced calcium release (CICR; Gregoire *et al*, 1993). In certain vascular tissues, this process has been shown to play a significant role in P2X receptor-mediated elevation of cytoplasmic calcium. The majority of this work appears to have been conducted in veins rather than arteries. Loirand & Pacaud (1995) demonstrated that blocking CICR reduced the ATP induced calcium rise by over 70 % in smooth muscle cells isolated from saphenous vein. Similar results were obtained in the rat portal vein (Pacaud *et al*, 1994). These results contrast with my findings where depletion of intracellular calcium stores with cyclopiazonic acid had no effect on contractions evoked by α,β-meATP in any size of artery. The difference in results may simply be due to the different tissues used. Another factor may be that I examined functional responses whereas the other two studies measured currents in isolated cells. An interesting aspect of the studies by Pacaud and colleagues is that responses were said to be mediated by P2X receptors. Expression of P2X receptors is generally associated with arteries and not with veins. One study that does however agree with my findings was conducted in vas deferens (Bourreau *et al*, 1991). Based on the lack of effect of CPA and the minimal effect of nifedipine on neurogenic contractions one can draw the following conclusion: under physiological conditions, the rise in cytosolic calcium associated with P2X receptor-evoked contractions is achieved principally by calcium influx directly through the P2X receptor.

This chapter has therefore established the following points: over 70 % of the nerve-evoked contractile response of small and medium-sized rat mesenteric arteries is mediated by ATP. In contrast, contractions recorded under the same conditions in large arteries are almost entirely

adrenergic. Nifedipine has only a limited effect on purinergic agonist-evoked contractions. Cadmium produces a similar pattern of inhibition, which suggests that no other voltage-dependent calcium channels are involved in calcium influx. Nifedipine also has a limited effect on nerve-evoked responses; L-type calcium channels therefore do not play a significant role when conditions approximate to those found *in vivo*. The same is true when the adrenergic component is first blocked by prazosin. This suggests that all calcium influx for the purinergic component of nerve stimulation occurs through the P2X receptor. In addition, there seems to be no requirement for additional calcium release. The great majority of calcium required for smooth muscle contraction therefore appears to come from P2X receptor-mediated calcium entry.

5 IMMUNOHISTOCHEMISTRY AND MOLECULAR BIOLOGY

5.1 INTRODUCTION AND AIMS

One of the aims of this thesis was to characterise purinergic contractile responses in different-sized mesenteric arteries. The data from contraction experiments described in Chapters 3 and 4 show clear differences in the properties of these responses depending on the diameter of the vessel. A possible explanation for the observed results is the heterogeneous expression of P2 receptor isoforms in different arteries. In order to correctly interpret the data, it is therefore essential to identify the P2 receptor isoforms expressed in each type of vessel. A major difficulty faced when studying purinergic transmission is however the lack of potent, subtype-selective agonists and antagonists. The identification of specific receptor isoforms using a classical pharmacological approach is therefore very difficult. A more effective and conclusive strategy is to use molecular techniques that rely on differences in the nucleotide sequence of P2 receptor genes or amino acid sequence of channel proteins. The aim of the work in this chapter was to use an approach based on molecular characteristics of P2 receptors to identify which P2X and P2Y isoforms are expressed in small, medium and large mesenteric arteries. P2X receptor protein was visualised by incubating sections of mesenteric artery with specific antibodies raised to each receptor isoform. In addition, total RNA was isolated from arterial tissue and reverse transcriptase-PCR (RT-PCR) used to characterise the distribution of P2X and P2Y receptor mRNA transcripts. The lack of anti-P2Y receptor antibodies meant that expression of these receptors could only be detected by RT-PCR.

5.2 IMMUNOHISTOCHEMISTRY

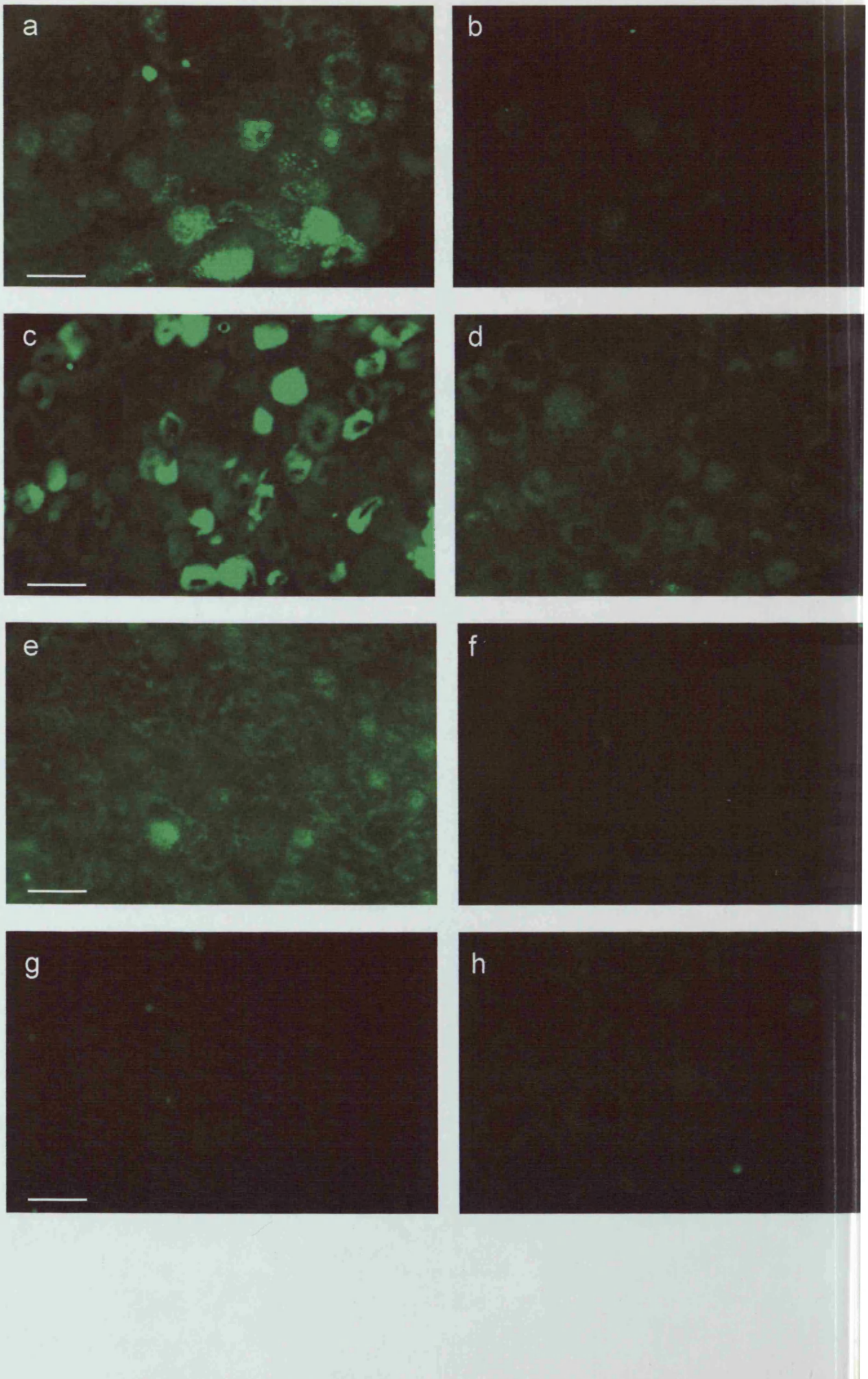
5.2.1 POSITIVE CONTROLS FOR ANTIBODY FUNCTION

All immunohistochemical experiments used the same protocol: tissue sections were first labelled with P2 receptor subtype-specific primary antibodies raised in rabbit, followed by incubation with fluorescine labelled secondary antibody to allow detection of the primary antibody under epifluorescence. Before the seven antibodies could be used to identify the distribution of P2X

subunits in arteries, control experiments had to be conducted to ensure they worked and to determine a suitable dilution factor. For each antibody, a control tissue was chosen that is known to express the desired isoform at high levels. $P2X_1$ and $P2X_4$ receptors are expressed in arterial smooth muscle; the arteries themselves were therefore positive controls for the anti-$P2X_1$ and anti-$P2X_4$ antibodies. The antibodies and respective tissues are listed below:

$P2X_1$	Arterial smooth muscle
$P2X_2$	Dorsal root ganglion
$P2X_3$	Dorsal root ganglion
$P2X_4$	Arterial smooth muscle
$P2X_5$	Spinal cord
$P2X_6$	Dorsal root ganglion
$P2X_7$	Dorsal root ganglion

A clear fluorescence signal can be seen in each of the control tissues (Fig. 34). Strong $P2X_2$ and $P2X_3$ immunoreactivity is mainly seen in small diameter neurones of the dorsal root ganglion. $P2X_6$ receptors are found at much lower levels in both small and large diameter neurones. Reactivity to $P2X_7$ receptors is evenly distributed throughout the ganglion tissue making it difficult to discern individual large and small diameter neurones. Where a blocking peptide was available, fluorescence was abolished. The lack of a blocking peptide for anti-$P2X_3$ and anti-$P2X_6$ antibodies could make interpretation of the data slightly problematic. However, as there was clear staining above background fluorescence in each case, one can be confident that the antibodies detect their target protein. All images of positive control experiments are shown in Figure 34. Once positive controls had established that each antibody functioned, experiments using arterial tissue were conducted (the dilution factors are given in Table 4). Table 10 summarises the results from these immunohistochemical studies conducted in three animals.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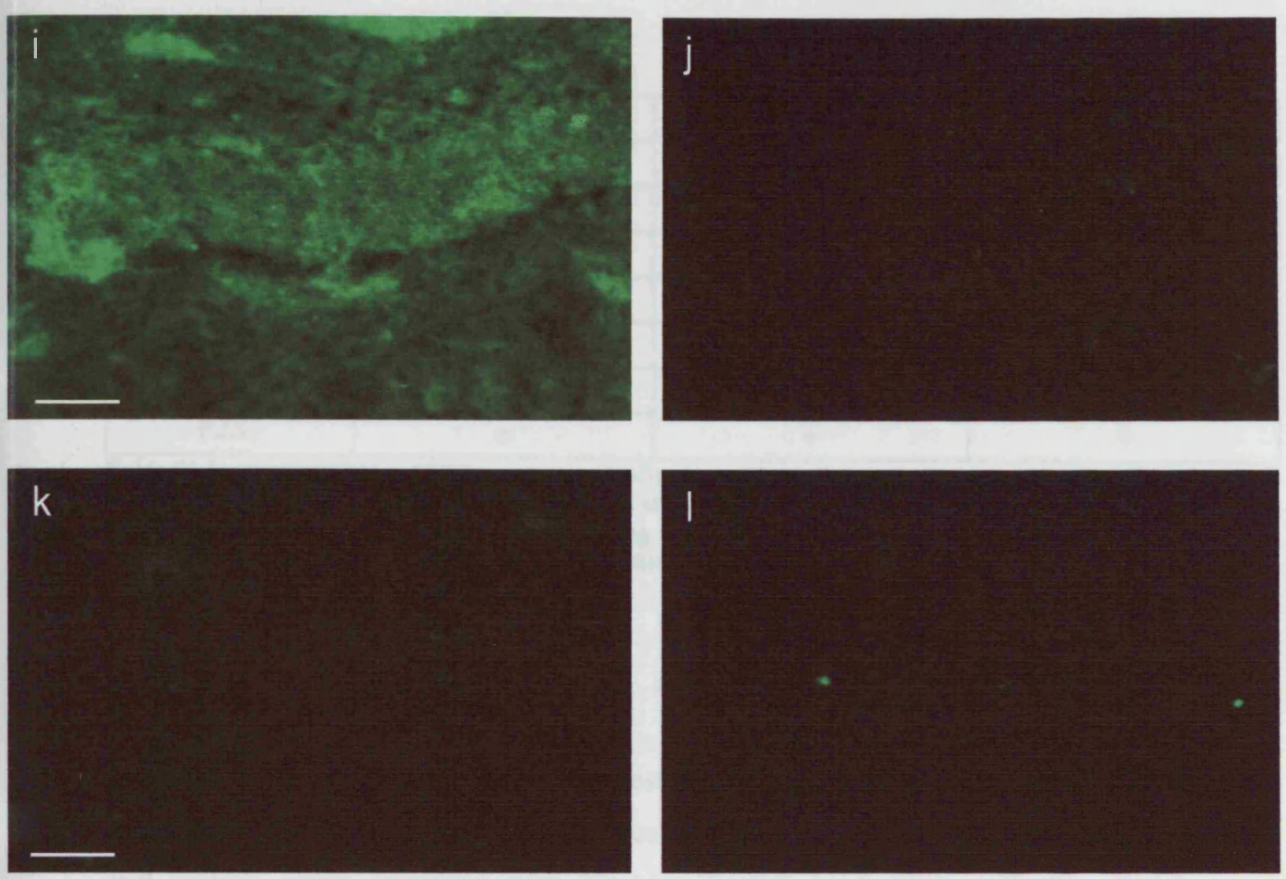


Figure 34 Control experiments for immunohistochemistry testing P2X receptor antibody function. (a) Dorsal root ganglia showing strong anti-$P2X_2$ immunoreactivity, which is abolished when the antibody is pre-incubated with its specific blocking peptide (b). Anti-$P2X_3$ (c) and anti-$P2X_6$ (d) staining of the same tissue. More uniform fluorescence is seen with the anti-$P2X_7$ antibody (e), which is also lost in the presence of the blocking peptide (f). (g) and (h) are control slides incubated with secondary antibody only and no antibody, respectively. (i) Prominent staining of spinal cord with anti-$P2X_5$ antibody. (j) Immunoreactivity is lost in the presence of the blocking peptide. (k) and (l) are control slides incubated with secondary antibody only and no antibody, respectively. Scale bar is 50 μm for (a) to (h) and 100 μm for (i) to (l).

Antibody	small	medium	large
$P2X_1$	+++	+++	++
$P2X_2$	-	-	-
$P2X_3$	-	-	-
$P2X_4$	±	+/++	+
$P2X_5$	±	±	±
$P2X_6$	-	-	-
$P2X_7$	±	+	+

Table 10 Immunoreactivity of P2X receptor antibodies in small, medium and large arteries. Expression of $P2X_1$ is the strongest in each size of vessel. $P2X_4$ and $P2X_7$ show clear staining, while reactivity to $P2X_5$ barely detectable. No fluorescence was seen with any of the remaining antibodies. Expression levels are denoted by symbols: +++ = strong; ++ = moderate; + = weak; ± = barely detectable; - = no expression

5.2.2 $P2X_1$

Anti-$P2X_1$ antibodies produced the strongest immunoreactivity in every artery examined (Fig. 35a). There was always a dramatic difference in the fluorescence intensity between sample slides and controls either incubated with blocking peptide or lacking antibodies. Staining was distributed throughout the smooth muscle layer and was strongest toward the luminal side of the artery wall. In vessels from one animal there was a punctate increase in fluorescence in small and medium-sized arteries, but it is unclear whether this represents a clustering of receptors or an unwanted aggregation of antibody molecules. Such points were not seen in other tissues. The level of fluorescence was roughly equal in small and medium arteries and slightly lower in large vessels.

5.2.3 $P2X_2$

No immunoreactivity was seen in any arterial sections incubated with anti-$P2X_2$ antibody (Fig. 35b).

5.2.4 $P2X_3$

Incubation with anti-$P2X_3$ produced no fluorescence in any tissue sections tested (Fig. 35c).

5.2.5 $P2X_4$

Immunoreactivity to $P2X_4$ antiserum was the most variable of the seven antibodies tested. In each case, fluorescence was evenly dispersed throughout the smooth muscle layer of the artery. Staining in small arteries ranged from no clear difference between sample and control, to a clear but faint increase in fluorescence of the sample slide as compared to the blocking peptide control slide. In medium-sized vessels there was always clearly more fluorescence in the sample slide, although the intensity varied from weak to moderate depending on the tissue. The strength of staining was the same in each section from large arteries examined, clearly visible but weak fluorescence (Fig. 35d).

5.2.6 $P2X_5$

The evaluation of these slides was made more difficult by a poor quality blocking peptide that generally produced as much fluorescence as the antibody itself. Although faint fluorescence was observed in arterial smooth muscle in nearly all samples, this was also seen in the corresponding blocking peptide control slide. In only one section of medium-sized artery was there slightly more staining in the sample slide than the control. For this reason, no clear expression of $P2X_5$ could be discerned in any size of artery (Fig. 35e).

5.2.7 $P2X_6$

As with the $P2X_3$ antibody, no blocking peptide was available for the $P2X_6$ antibody. No fluorescence was seen in any slide when compared to controls lacking primary and secondary

antibody. I therefore concluded that there is no expression of $P2X_6$ in any of the arteries examined (Fig. 35f).

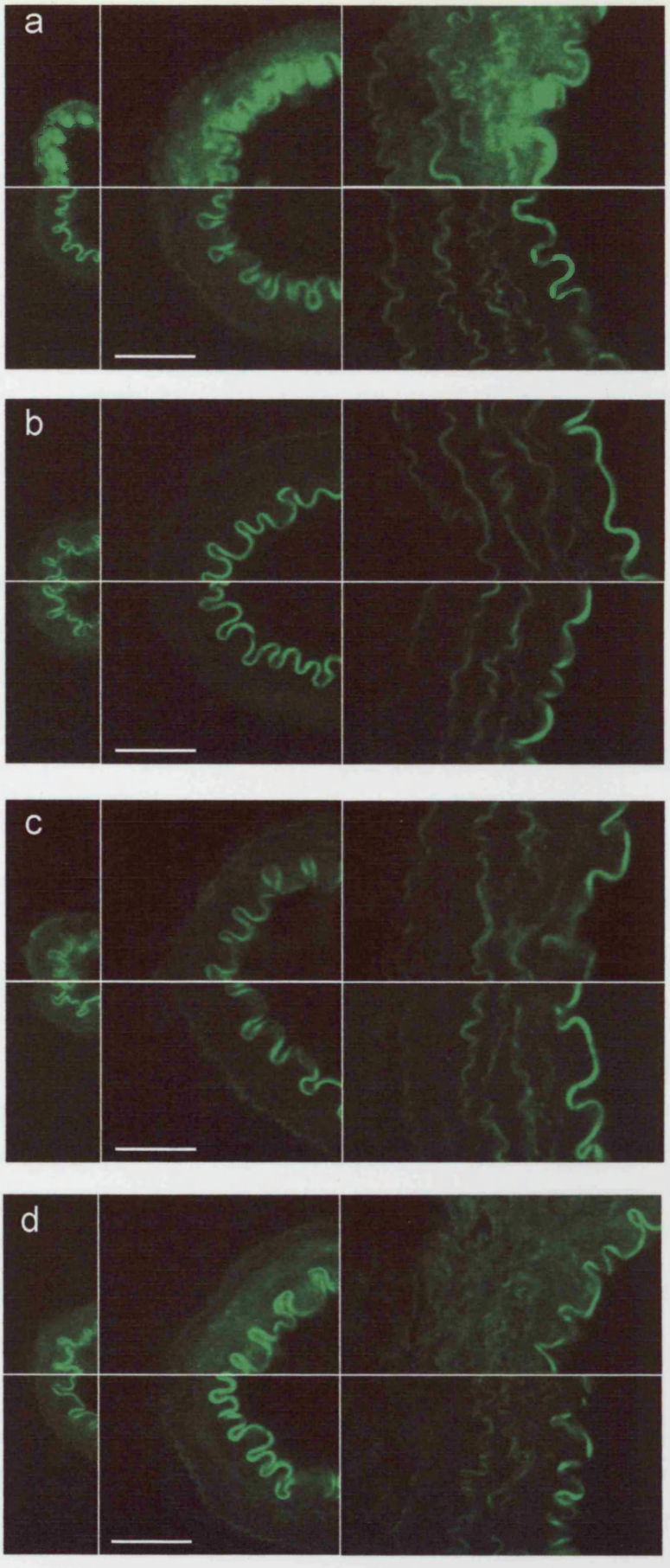
5.2.8 $P2X_7$

There was clearly detectable immunoreactivity in all medium and large arteries and faint reactivity in small arteries. In large vessels staining was diffuse throughout the smooth muscle layer but was clearly visible when compared to control slides. There was however a marked difference in the distribution of fluorescence in small and medium-sized arteries. In addition to faint fluorescence distributed throughout the smooth muscle layer, strong reactivity was found in discrete points along the outer periphery of medium-sized arteries. In one animal only this peripheral staining was seen. A similar pattern of fluorescence was observed in small vessels although it was weaker than in medium-sized arteries. In sections of small artery from one animal no clear reactivity was seen (Fig. 35g).

5.3 REVERSE TRANSCRIPTASE – PCR

To complement the identification of P2X receptor protein, P2X messenger RNA transcripts were detected by reverse transcriptase-PCR. This was also the only means of determining P2Y receptor distribution as no anti-P2Y antibodies were available. Total RNA was isolated from homogenised samples of arterial tissue and reverse transcribed into cDNA. PCR amplification with primers specific for each isoform was then used to detect the presence of $P2X_1$ – $P2X_7$ as well as $P2Y_{1,2,4}$ and $P2Y_6$ cDNA.

Unfortunately, this process had only very limited success. The distribution of P2X and P2Y receptor transcripts was only demonstrated in one sample of large artery. One problem was the homogenisation step of tissue preparation. Tissue samples from small and medium-sized arteries were so small that they did not get caught in the blade of the homogeniser. Increasing the quantity of tissue used did not solve the problem, as each vessel was still small enough to survive homogenisation without significant disruption. Additional centrifugation of the sample through


a
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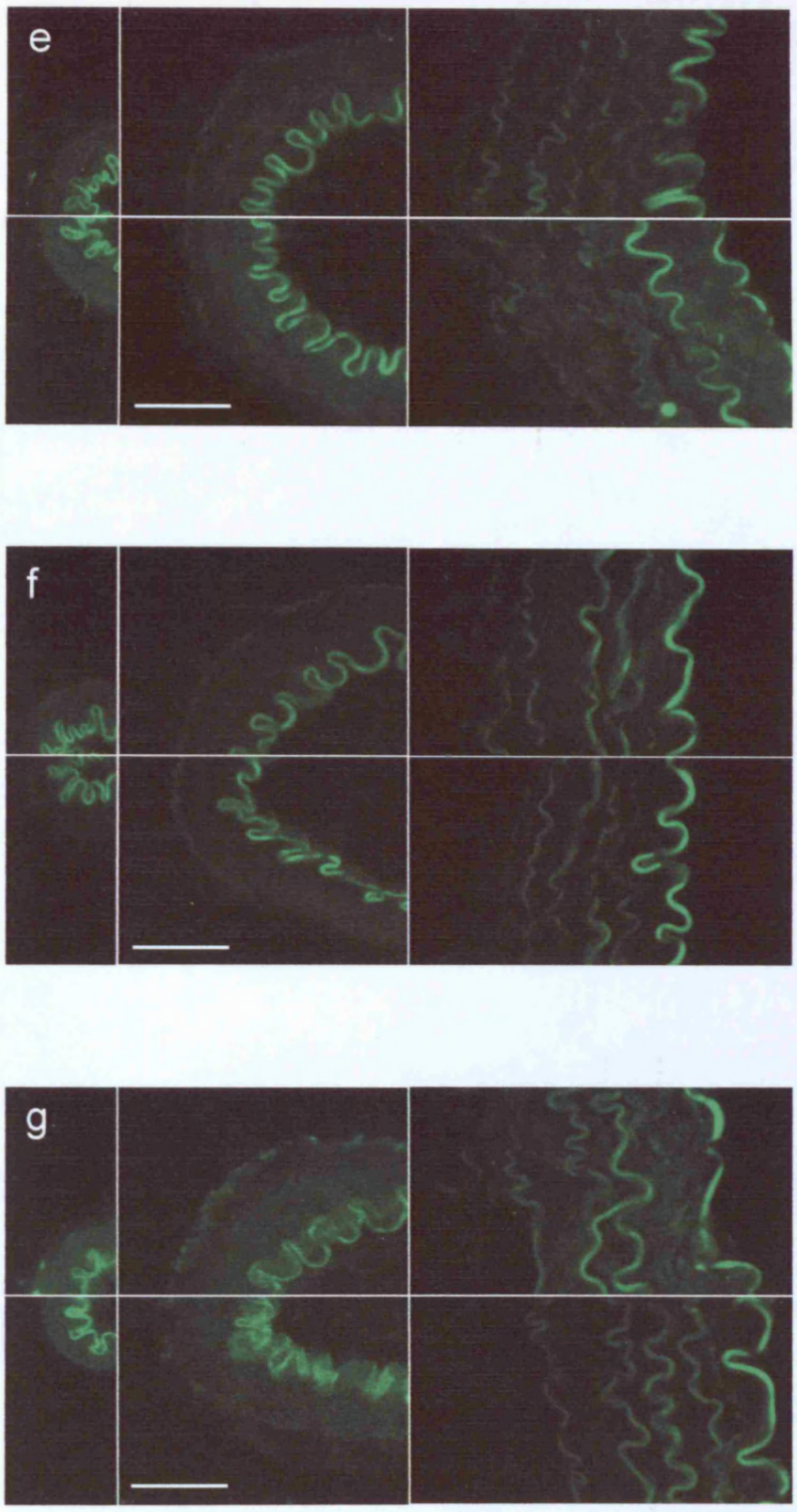

Figure 35 Immunohistochemical visualisation of P2X receptor distribution in mesenteric arteries. Each panel consists of images of small, medium and large arteries. Top images are vessels stained with antibody and bottom images are control experiments where the specific immunoreactivity of the antibody is blocked by pre-incubation with the blocking peptide antigen: (a) $P2X_1$, (b) $P2X_2$, (d) $P2X_4$, (e) $P2X_5$ and (g) $P2X_7$. Where no blocking peptide was available, bottom control images are tissue incubated with secondary antibody only: (c) $P2X_3$ and (f) $P2X_6$. The autofluorescence of the elastic laminae is clearly visible in all vessels. Scale bars are 50 μm.

'shredder columns' did not improve the situation. As a result, no RNA was extracted from the several samples; an example is shown in Figure 36a. Large arteries on the other hand, were big enough to be properly broken down.

A further problem was contamination of the extracted RNA with genomic DNA. This is indicated by bands in lanes where reverse transcriptase was not included in the reaction mixture. In these experiments where a band was produced even without enzyme, it was not clear whether the product of reactions containing reverse transcriptase originated from cDNA or from contaminating genomic DNA. Every gene is present in genomic DNA regardless of whether it is transcribed or not. Any information about tissue-specific expression of mRNA is therefore lost (Fig. 36b).

The successful determination of P2X and P2Y receptor transcript distribution in large arteries is shown in Figure 37. A strong band was seen for $P2X_1$ and a moderate band for $P2X_4$; no other P2X transcripts were detected (Fig. 37a). Regarding P2Y receptors, a very faint band was seen for $P2Y_1$ and moderate bands for $P2Y_2$ and $P2Y_6$ receptors; $P2Y_4$ mRNA was not present (Fig. 37b). All positive controls using brain tissue worked well.

5.4 DISCUSSION

The results of my immunohistochemical studies show similar expression of $P2X_{1,4,5}$ and $P2X_7$ isoforms in all rat mesenteric arteries. These findings confirm those made by Lewis & Evans (2000a) vessels identical to medium-sized arteries in the present study. In every size of artery, the most abundantly expressed isoform was the $P2X_1$ receptor. This is consistent with the observation that the phenotype of most arteries corresponds very closely to the properties of recombinant $P2X_1$ receptors. Several other studies have also found strong $P2X_1$ immunoreactivity in smooth muscle of vascular tissue (Chan *et al*, 1998; Bo *et al*, 1998; Hansen *et al*, 1999, Galligan *et al*, 2001). Prominent $P2X_1$ receptor expression has also been demonstrated by autoradiographic studies using radiolabelled α,β-meATP which binds to this isoform with high affinity (Bo & Burnstock, 1993; Zhao *et al*, 1996; Neely *et al*, 1996).

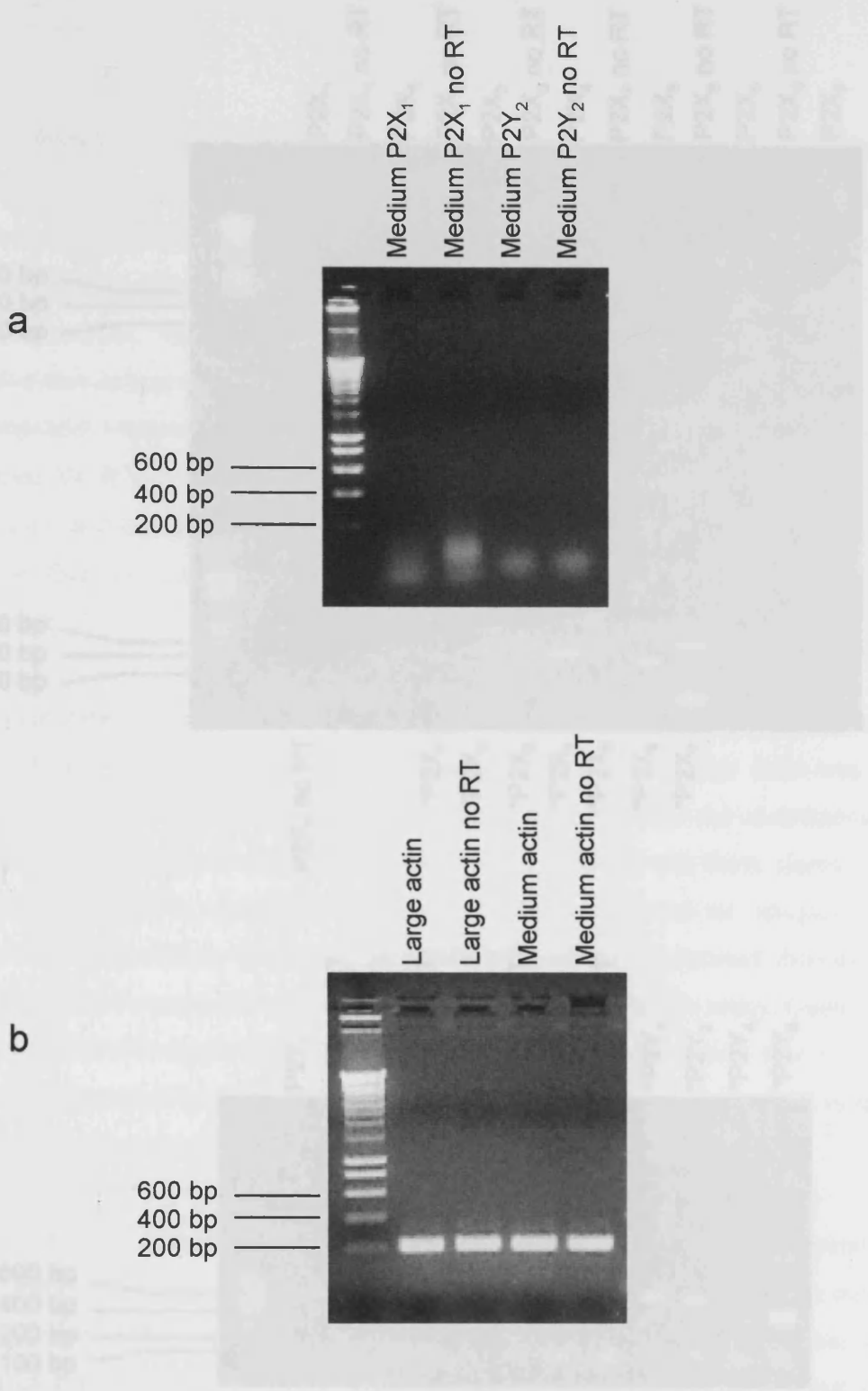

Figure 36 Unsuccessful attempts at RNA isolation for use in the detection of P2 receptor isoforms. (a) The RNA isolation step did not yield sufficient template to perform PCR: no product is seen for either $P2X_1$ or $P2Y_2$ in sample lanes; faint low molecular weight bands correspond to primers. (b) Non-optimal RNA isolation lead to contamination of the sample with genomic DNA. As a result, bands for the ubiquitous cellular protein actin are equally strong for experiments containing and lacking reverse transcritpase. It is therefore not clear whether the product in lanes containing the enzyme corresponds to cDNA transcribed from RNA or contaminating genomic DNA.

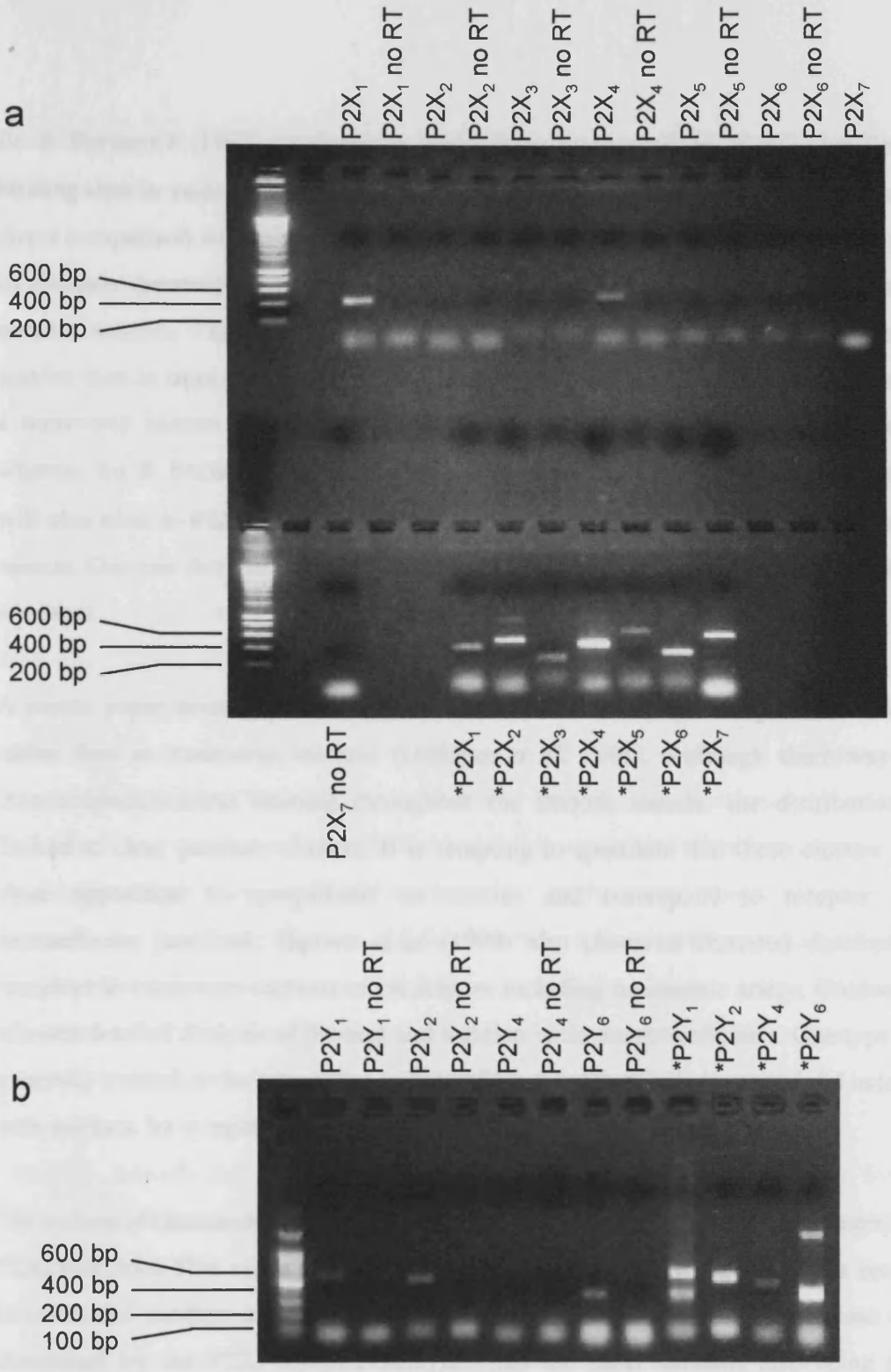

Figure 37 Successful RT-PCR experiment in large arteries. For each receptor isoform one reaction containing reverse transcriptase and one control reaction lacking reverse transcriptase (no RT) was performed. Asterisks denote positive control reactions using RNA isolated form brain. (a) A strong band is seen for $P2X_1$ and a weaker band for $P2X_4$; no product is seen for any other P2X receptor isoform. Control experiments using RNA isolated from brain yielded product for all isoforms. (b) A very faint band is produced for $P2Y_1$ and moderate bands for $P2Y_2$ and $P2Y_6$. Control experiments in brain again produced bands for all P2Y isoforms.

Bo & Burnstock (1993) conducted a comprehensive comparison of radio-labelled α,β-meATP binding sites in various rodent blood vessel. Data described for the rabbit femoral artery allows a direct comparison with my results as receptor density was characterised along the same artery as its diameter decreased. They found a substantial increase in the density of receptors as the artery became smaller. This is in line with my results, which show stronger immunoreactivity in smaller than in large arteries. One point the two studies disagree on is receptor distribution across a transverse section of artery. I found greater staining toward the luminal side of the wall, whereas Bo & Burnstock (1993) found the opposite. It should be mentioned that α,β-meATP will also bind to $P2X_3$ receptors; however, this isoform has not been found in arterial smooth muscle. One can therefore assume, as the authors have, that binding of the ligand indicates $P2X_1$ receptors.

A recent paper investigated the distribution of $P2X_1$ receptors along the surface of an artery rather than in transverse sections (Galligan *et al*, 2001). Although there was some diffuse immunohistochemical staining throughout the smooth muscle, the distribution was mainly limited to clear punctate clusters. It is tempting to speculate that these clusters are located in close apposition to sympathetic varicosities and correspond to receptor expression at neuroeffector junctions. Hansen *et al* (1999) also observed clustered distributions of $P2X_1$ receptors in transverse sections of rat arteries including mesenteric artery. Confocal microscopy allowed detailed analysis of the size and location of the receptor clusters. One type of cluster was generally located at the adventitial surface of the smooth muscle layer and did indeed co-localise with markers for sympathetic varicosities.

The pattern of immunoreactivity indicates expression of $P2X_{4,5}$ and $P2X_7$ receptors in addition to $P2X_1$ receptors. This raises questions as to the functional significance of these receptor subunits in small and medium-sized arteries. If the pharmacological properties of these vessels are so dominated by the $P2X_1$ receptor subtype, why are other subunits also being expressed? At present it is not clear whether these subunits actually form functional receptors, either as homomeric or heteromeric channels. Moreover, they may be expressed at such low levels compared to $P2X_1$ subunits, that any effect they have is masked by the more abundant isoform. The additional presence of $P2X_4$ subunits would have provided a very good explanation for the

contractile properties of large arteries. A $P2X_{1/4}$ heteromer could mix the transient responses of $P2X_1$ receptors with the α,β-meATP and suramin – insensitivity of $P2X_4$ receptors. However, as stated in section 3.7, a $P2X_{1/4}$ receptor is unlikely to form. Moreover a $P2X_{1/4}$ would not account for currents recorded in single cells (see Chapter 6). It would also be difficult to explain why the same subunits are expressed in small and medium-sized arteries but do not assemble to form heteromeric channels in these vessels.

Although positive staining for $P2X_7$ receptors was somewhat surprising, this isoform has previously been found in smooth muscle of toad stomach (Ugar *et al*, 1997) and human saphenous vein (Cario-Toumaniantz *et al*, 1998). In the latter study, $P2X_7$ receptor transcripts were detected and functional responses characteristic (cell lysis) of this channel were observed upon exposure of cells to BzATP. The authors concluded that the receptor's prime function may be in disease states. Increased and sustained levels of extracellular ATP can be produced by hypoxia, inflammation and stress, which would lead to $P2X_7$-mediated cell lysis and subsequent tissue damage (Cario-Toumaniantz *et al*, 1998). Although discussed in terms of venous disease, such conditions could equally apply to arterial disease states. It should be added that the method used in the above study could not specify where in the artery receptors were being expressed. Large arteries in my experiments did show staining throughout the smooth muscle layer but in smaller vessels reactivity was confined to punctate clusters within the adventitia. The significance of this peripheral distribution is unclear but could suggest involvement in sympathetic transmission.

The $P2X_1$ isoform also produced the brightest band in RT-PCR performed on tissue from large arteries, although a moderate band was also seen for $P2X_4$. No other isoforms were detected with this method. Two studies that conducted a complete analysis of P2X receptor transcripts found all seven isoforms to be present (Phillips *et al*, 1998; Lewis *et al*, 2000b). In the latter instance, a pial sheet was used. Cerebral arteries could therefore not be separated from other brain tissue during isolation, which explains the presence of several additional isoforms not associated with smooth muscle. The presence of non-vascular tissue would presumably also explain the data from Phillips *et al* (1998). In this sense, it is perhaps surprising that I only detected $P2X_1$ and $P2X_4$ transcripts. RNA was isolated from whole arteries, which would also be expected to

contain neuronal tissue and therefore neuronal P2X isoforms (e.g. $P2X_2$ and $P2X_3$). If any other transcripts were present in the samples, they were obviously not abundant enough to form a visible band.

A further study of mRNA distribution used *in situ* hybridisation to test for the presence of $P2X_{1,2}$ and $P2X_4$ mRNA in several rat arteries (Nori *et al*, 1998). In most arteries positive results were seen for $P2X_1$ and $P2X_4$ isoforms, which agrees with my data. Curiously, the only vessel to show no reactivity at all was the superior mesenteric artery, which is identical to large arteries in my experiments. A further difference is that Nori *et al* (1998) detected $P2X_2$ mRNA in the smooth muscle layer of the remaining arteries tested. In contrast, I found no evidence for expression of this isoform either in immunohistochemical or PCR experiments. Moreover, this subunit is more generally associated with neuronal rather than smooth muscle tissue (Vulchanova *et al*, 1996). Although different techniques were used, it is difficult to explain these direct contradictions between the two studies.

One interesting aspect of my results is the discrepancy between the data obtained with immunohistochemistry and RT-PCR. Unfortunately, no comparisons could be made for small and medium arteries. In the case of large arteries the two methodologies are in agreement for $P2X_1$ and $P2X_4$. However, despite clear immunoreactivity for $P2X_7$ subunits, no transcripts for this isoform were detected. A possible explanation might be a very low cytoplasmic level of $P2X_7$ mRNA. This may be because protein translation follows on very rapidly from mRNA transcription. Another reason could be a low level of receptor turnover. Messenger RNA is only present in a cell if the protein it codes for is being actively synthesised. If it is not, one would not expect the protein's mRNA to be detectable. It is also possible that translation of neuronal proteins occurs in the cell body and not in axons. As a result, the tissue samples I used, which are only innervated by distal processes would not contain any mRNA, even if the corresponding protein was present. This would also explain the lack of other neuronal P2X mRNA one might expect to find in the whole artery samples.

The lack of P2Y receptor antibodies means the distribution of these receptors must be determined by detecting mRNA rather than protein. As described earlier, RT-PCR data is only

available for large arteries. Comparisons with other data in the literature will therefore be based only on this vessel. Few studies have investigated the distribution of P2Y receptors in vascular smooth muscle. The results from one study in the same tissue are in exact agreement with my data: transcripts for only $P2Y_{1,2}$ and $P2Y_6$ subunits with a weaker band for $P2Y_1$ than the other two isoforms (Lewis *et al*, 2000b). A very similar pattern of bands found using human coronary artery (Malmsjö *et al*, 2000a). However, in this study a faint band was also seen for $P2Y_4$ mRNA. This difference may reflect a slight species-dependent variation in the complement of arterial P2Y receptors. Experiments by Hartley and colleagues only tested for the $P2Y_6$ isoform but also obtained a strong band in rat pulmonary arteries (Hartley *et al*, 1998). All these results agree with many studies, which have shown that $P2Y_2$ and $P2Y_6$ receptors are functionally expressed in arterial smooth muscle. Although there is limited data which suggests expression of $P2Y_4$ receptors, my own and the above-mentioned data, do not agree with these findings. The band for $P2Y_1$ receptors can easily be explained by the presence of endothelial cells in arterial tissue, which express this isoform at high levels.

As data was only available for large arteries, it was not possible to assess whether differences in the expression pattern of P2Y isoforms caused the difference in UTP potency. The fact that responses in both small and medium-sized arteries were suramin-sensitive however, suggests that the same isoform was mediating contractile responses in each case; suramin sensitive contractions evoked by UTP are characteristic of $P2Y_2$ receptors. Of the P2Y isoforms detected by PCR, only $P2Y_2$ and $P2Y_6$ are activated by uridine nucleotides and only $P2Y_2$ receptors are significantly antagonised by suramin. It is therefore probable that $P2Y_2$ receptors are the dominant smooth muscle P2Y receptors in all sizes of artery, a conclusion in line with the results from my PCR studies. Potential reasons for the difference in potency of UTP in my contraction studies are discussed in Section 3.7.

In summary, all three sizes of artery show a similar pattern of P2X receptor immunoreactivity, $P2X_1$ being the most abundantly expressed isoform followed by $P2X_4$. Weak expression of $P2X_7$ and possibly $P2X_5$ receptors was also detected. In large arteries, $P2X_1$ and $P2X_4$ receptors were also identified using RT-PCR; the same technique revealed expression of $P2Y_{1,2}$ and $P2Y_6$ receptors. These results are in agreement with most data available on the smooth muscle

expression pattern of P2 receptor isoforms. They are also in agreement with the functional data from whole artery experiments described in Chapter 3.

6 ELECTROPHYSIOLOGY

6.1 INTRODUCTION AND AIMS

The studies described in Chapters 3 and 4 focus on contractile responses of whole artery segments. Such experiments provide valuable information on the physiological role receptors play in regulating vascular tone *in vivo*. There are however some limitations to this approach when attempting to characterise the sensitivity of a receptor to a given agonist. When a ligand binds to a receptor that causes vasoconstriction, several steps of cellular metabolism are required to trigger contraction of the smooth muscle cell. Calcium entering the cytoplasm stimulates myosin light chain kinase, one of the key enzymes regulating the activity of the contractile machinery. Whether the cell is under tension or relaxed depends on the activity of myosin light chain kinase and the phosphorylation state of the contractile proteins. These two factors are regulated independently of agonist binding and receptor activation. As a result, the functional response of an artery is not an exact reflection of the properties of agonist binding: activation of the receptor may not necessarily lead to vasoconstriction. In order to study the effects of agonist binding one must look directly at the activity of the ion channel using electrophysiological techniques on dissociated smooth muscle cells.

The data from my contraction studies show clear differences in the contractile properties of whole artery segments, depending on vessel diameter. The much lower sensitivity of large arteries to α,β-meATP and suramin suggests that the number and subtypes of P2X receptors expressed on these vessels may not be the same as in small and medium-sized arteries. One of the aims of this work was to study membrane currents evoked by P2X receptor activation in single smooth muscle cells obtained from different-sized arteries. These results will then be compared to contractile responses seen in whole tissue experiments. With this approach it is possible to look directly at agonist-induced activation of the receptor without the complication of functional responses. My contraction experiments also suggested possible differences in the expression of voltage-gated calcium channels between the different sizes of artery. A further aim of this chapter was therefore to characterise and compare calcium currents in single smooth muscle cells from small, medium and large mesenteric arteries.

6.2 P2X RECEPTOR CURRENTS

The differences in contractile properties of whole arteries could be explained by differences in the underlying membrane currents evoked by P2X receptor activation. Currents produced by application of α,β-meATP were therefore compared in cells dissociated from the three sizes of artery. One difficulty when studying currents in response to α,β-meATP using the whole-cell patch clamp configuration is that they decrease in amplitude with successive agonist applications. This phenomenon is termed tachyphelaxis or 'run-down', which has been reported to be caused by cytoplasmic constituents dialysing out of the cell and into the patch electrode (Evans & Kennedy, 1994; Khakh *et al*, 1995). Multiple applications of α,β-meATP are therefore not possible when making 'whole-cell' recordings. As a result, full concentration response curves were not constructed as this would have required use of the perforated patch technique (Lewis & Evans, 2000a). Instead, responses to concentrations of α,β-meATP that were maximal (10 μM) and half-maximal (1 μM) in contraction studies in small and medium arteries were recorded. This was sufficient to reveal any substantial differences in agonist sensitivity. Because of run-down, the first response of a cell to α,β-meATP was always the largest and was taken as the cell's 'true response'; the results in this chapter all represent the first current recorded in a cell. In addition, the effect of suramin at a concentration known to antagonise responses in whole arteries (100 μM) was tested on currents evoked by both concentrations of α,β-meATP.

6.2.1 PEAK CURRENT AMPLITUDES

α,β-meATP evoked rapid inward currents in all cells studied. Responses to 10 μM α,β-meATP were strongly inactivating in all cells and fully returned to baseline during the presence of the agonist. Currents evoked by 1 μM α,β-meATP also desensitised but to a much smaller extent; they did not return to baseline during the time of application. When current amplitudes to 1 μM α,β-meATP were very small, only a slight decline from the peak amplitude was seen. Mean inward currents were largest for both 1 μM and 10 μM α,β-meATP in cells from medium-sized arteries. Currents from large arteries were significantly smaller, while responses in small arteries

were the smallest at both concentrations of agonist. The results are shown in Table 11 and Figure 38.

	1 μM α,β-meATP (pA)	10 μM α,β-meATP (pA)
Small	223.6 ± 51.3 (n = 7) **	1288.9 ± 187.5 (n = 23) **
Medium	1556.5 ± 170.0 (n = 25) **	3533.7 ± 433.9 (n = 24) **
Large	742.9 ± 112.7 (n = 20) **	2010.7 ± 199.7 (n = 31) *

Table 11. Comparison of maximal currents evoked by 1 and 10 μM α,β-meATP. Significant differences are represented by * where $p < 0.05$ and by ** where $p < 0.005$. Comparisons between small and medium are indicated by red, between medium and large by green and between small and large by black.

A key factor affecting the size of whole cell currents is the number channels activated. Assuming the density of channels is constant, this will depend on the size of the cell. Differences in cell size can be corrected for by dividing the peak current by the cell capacitance. The cell capacitance (units: Farads, F) is directly proportional to the total surface area of the cell and is therefore a measure of cell size. The mean capacitance of smooth muscle cells was 8.7 ± 0.4 pF, 12.9 ± 0.6 pF and 10.7 ± 0.4 pF for small, medium and large arteries, respectively. When expressed as pA/pF, recordings describe the 'current density' of the cell. The same trend as for peak amplitudes applies when responses are expressed in terms of current density (Table 12):

	1 μM α,β-meATP (pA/pF)	10 μM α,β-meATP (pA/pF)
Small	28.2 ± 4.2 (n = 7) **	174.1 ± 40.0 (n = 23) **
Medium	140.1 ± 18.4 (n = 25)	298.3 ± 22.9 (n = 24) *
Large	109.7 ± 13.7 (n = 20) **	220.4 ± 22.3 (n = 31)

Table 12. Peak currents to α,β-meATP expressed as current density. Current densities in medium-sized arteries are largest, despite the greater size of these cells. Significant differences are represented by * where $p < 0.05$ and by ** where $p < 0.005$. Comparisons between small and medium are indicated by red, between medium and large by green and between small and large by black.

Another way of comparing current densities recorded in different cells is to express the current densities for responses to 1 μM α,β-meATP as a percentage of responses to 10 μM α,β-meATP. The values for small, medium and large arteries are 17.3 %, 44.0 % and 36.9 %, respectively.

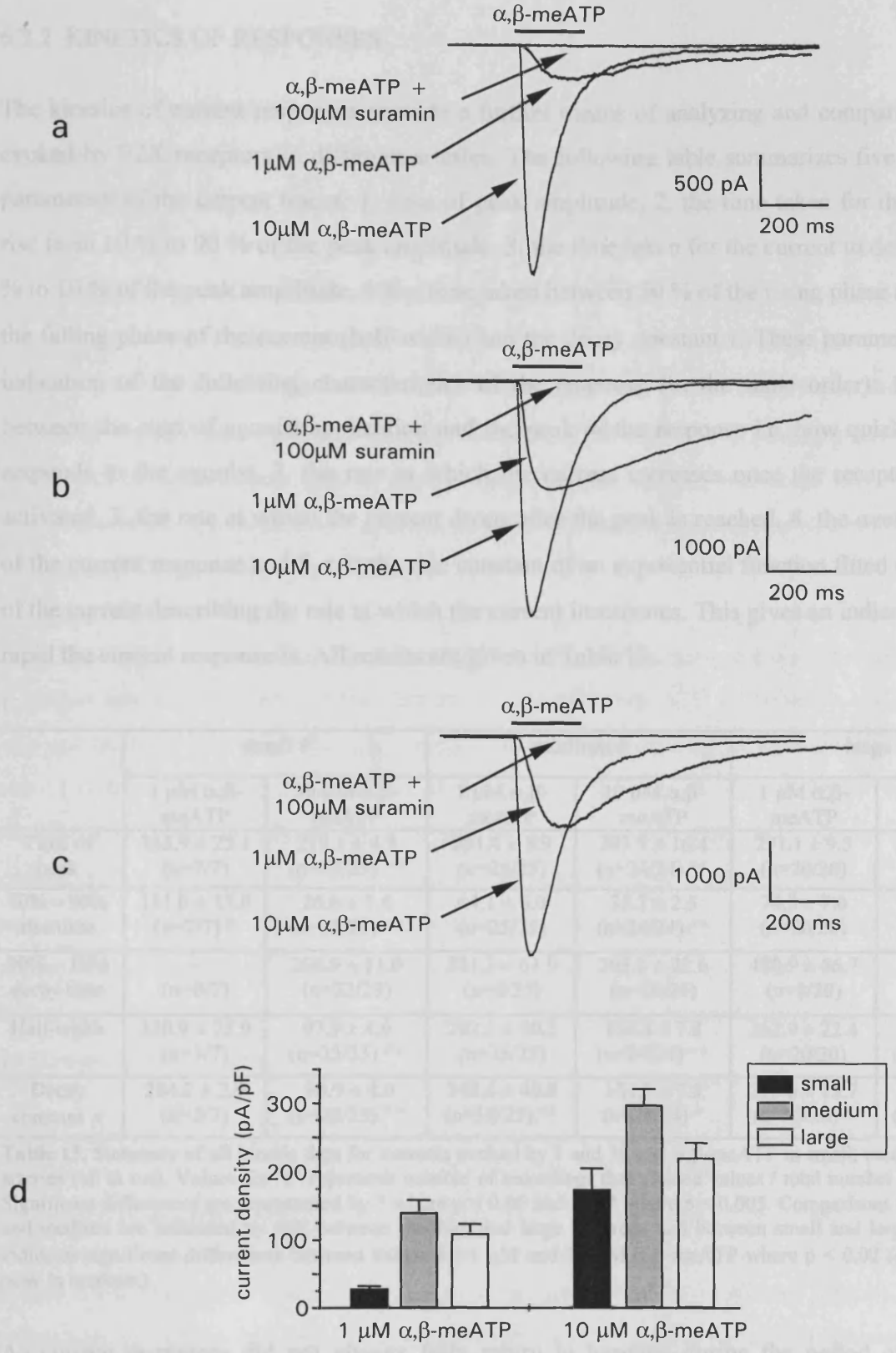

Figure 38 Characterisation of current responses to α,β-meATP and effect of suramin. α,β-meATP evokes transient responses in small (a), medium (b) and large (c) arteries that desensitise during agonist application; desensitisation is rapid at 10 μM α,β-meATP and substantially slower at 1 μM. Responses to both concentrations of agonist are abolished by suramin (100 μM). Figures show superimposed traces from different smooth muscle cells. (d) Summary of current density data (mean ± s.e.m. , n = 7 – 31). Note the especially small responses to 1 μM α,β-meATP in small arteries.

6.2.2 KINETICS OF RESPONSES

The kinetics of current responses provide a further means of analyzing and comparing currents evoked by P2X receptors in different arteries. The following table summarizes five key kinetic parameters of the current traces: 1. time of peak amplitude, 2. the time taken for the current to rise from 10 % to 90 % of the peak amplitude, 3. the time taken for the current to decay from 90 % to 10 % of the peak amplitude, 4. the time taken between 50 % of the rising phase and 50 % of the falling phase of the current (half-width) and the decay constant τ. These parameters give an indication of the following characteristics of the response (in the same order): 1. the delay between the start of agonist application and the peak of the response i.e. how quickly the cells responds to the agonist, 2. the rate at which the current increases once the receptor has been activated, 3. the rate at which the current drops after the peak is reached, 4. the overall duration of the current response and 5. τ is the rate constant of an exponential function fitted to the decay of the current describing the rate at which the current inactivates. This gives an indication of how rapid the current response is. All results are given in Table 13.

	small #		medium #		large #	
	1 μM α,β-meATP	10 μM α,β-meATP	1 μM α,β-meATP	10 μM α,β-meATP	1 μM α,β-meATP	10 μM α,β-meATP
Time of peak	345.9 ± 25.1 (n=7/7)	219.1 ± 4.3 (n=23/23) **	291.4 ± 8.9 (n=25/25)	293.9 ± 16.4 (n=24/24) **	291.1 ± 9.5 (n=20/20)	227.5 ± 2.4 (n=31/31)
10% – 90% rise time	111.9 ± 15.0 (n=7/7) *	26.6 ± 1.4 (n=23/23) **	64.1 ± 6.0 (n=25/25)	35.7 ± 2.6 (n=24/24) **	74.3 ± 7.0 (n=20/20)	26.8 ± 1.2 (n=31/31)
90% – 10% decay time	-- (n=0/7)	206.9 ± 11.0 (n=22/23)	531.3 ± 61.9 (n=4/25)	303.5 ± 22.6 (n=20/24)	480.9 ± 66.7 (n=8/20)	274.8 ± 7.3 (n=27/31)
Half-width	330.9 ± 25.9 (n=3/7)	97.9 ± 4.9 (n=23/23) **	292.1 ± 40.2 (n=25/25)	156.4 ± 7.8 (n=24/24) **	262.9 ± 22.4 (n=20/20)	126.6 ± 3.8 (n=31/31) **
Decay constant τ	284.2 ± 2.9 (n=2/7)	90.9 ± 4.0 (n=23/23) **	342.4 ± 40.8 (n=18/25) **	134.7 ± 7.8 (n=24/24) *	215.6 ± 15.7 (n=15/20) *	115.7 ± 3.1 (n=31/31) **

Table 13. Summary of all kinetic data for currents evoked by 1 and 10 μM α,β-meATP in small, medium and large arteries (all in ms). Values for 'n' represent: number of recordings that yielded values / total number of recordings. Significant differences are represented by * where $p < 0.05$ and by ** where $p < 0.005$. Comparisons between small and medium are indicated by red, between medium and large by green and between small and large by black. # indicates significant differences between values for 1 μM and 10 μM α,β-meATP where $p < 0.02$ (except time of peak in medium).

As current responses did not always fully return to baseline during the period of recording, particularly for applications of 1 μM α,β-meATP, values for decay time, half-time and decay

constant τ could not always be calculated. Where this is the case, mean values must be interpreted with some caution, as they are not representative of the whole sample. The true mean would invariably be higher.

From the appearance of current recordings alone, it is clear that responses to 10 μM α,β-meATP always have a more rapid time course than those to 1 μM α,β-meATP (Fig. 38 a-c). For the parameters rise time, decay time, half-width and τ, values were always lower for applications of 10 μM α,β-meATP than 1 μM α,β-meATP. The same applies for time of peak in small and large arteries. An exception is the time of peak in medium-sized arteries, where the values for both concentrations of agonist are very similar. All other differences between the values for the two concentrations are significant ($p < 0.02$).

There are also substantial differences in the time course of responses to the same concentration of agonist between different arteries. Currents to 10 μM α,β-meATP are fastest in small arteries followed by large and then medium vessels. This order is reflected in all kinetic parameters, which confirms the rapid time course of these currents in small vessels. Most differences between arteries are significant for comparisons of every parameter except decay time. For applications of 1 μM α,β-meATP, the order of τ values is large < medium < small. All other parameters also confirm that currents in large arteries are more rapid than in medium. In small arteries, values from all cells were only available for 'time of peak' and 'rise time'. Despite this lack of a complete data set for other parameters, it is clear that responses to 1 μM α,β-meATP are slowest in small arteries.

6.2.3 EFFECTS OF SURAMIN

Results from my whole tissue experiments show that unlike in small and medium arteries, contractions evoked by α,β-meATP in large vessels were resistant to suramin antagonism. It was of interest to determine whether this was also reflected in membrane currents recorded in single smooth muscle cells. Current 'run-down' mentioned in section 6.2 made the protocol used during

contraction studies unsuitable when recording current responses. A 'receptor-protection' protocol (Evans & Kennedy, 1994) was therefore used instead. Initially, cells were superfused with 100 μM suramin solution for 5 minutes. Once the whole-cell configuration was attained, 10 μM α,β-meATP was applied in the presence of 100 μM suramin to determine the effect of the antagonist. Responses to α,β-meATP alone and those to α,β-meATP in the presence of 100 μM suramin were recorded in different cells, using only the first recording as a data point in each case. This protocol was used on applications of both 10 μM α,β-meATP and 1 μM α,β-meATP. Suramin abolished responses to 10 μM (n = 3, 7, 7 for small, medium and large, respectively) and 1 μM α,β-meATP (n = 3, 4, 4 for small, medium and large, respectively) in all cells tested (Fig 38).

6.3 CALCIUM CURRENTS

Voltage-gated calcium channels are one of the main routes by which calcium enters a cell from the extracellular space. They play an important role in determining the cytosolic level of calcium in smooth muscle cells and hence contribute to the regulation of vascular tone. Differences in calcium channel expression could therefore lead to different properties of contraction. Data I obtained from whole artery experiments showed a component of depolarisation-induced contraction that was resistant to L-type channel blockade in small vessels. Functional responses are however again only an indirect measure of calcium channel activity. A more direct approach is to measure calcium currents in individual smooth muscle cells. Any differences in the types of channels expressed throughout the vascular tree should be reflected in currents measured at the single cell level, i.e. cells from small vessels should also display nifedipine-resistant calcium currents. Currents were characterised and compared in the three sizes of artery with the aim of investigating such potential differences. Throughout these experiments, barium was used as the charge carrier as current amplitudes are greater than when the extracellular solution is calcium-based. In addition, responses do not inactivate as rapidly or run down as much when calcium is replaced by barium.

6.3.1 CURRENT-VOLTAGE RELATIONSHIPS

In order to determine the activation profile of calcium channels in small, medium and large arteries, current voltage relationships were plotted for each size of vessel. Cells were held at a potential of –60 mV and subjected to 10 increasingly positive voltage steps of 10 mV up to a command potential of +30 mV (Fig. 39). Currents were not always measurable and only a proportion of cells tested could be used (8/12, 6/7 and 6/19 for small, medium and large respectively). A possible source of error when recording very small currents is 'leak-current'. Poor seal formation can lead to a loss of injected current between the patch electrode and the cell membrane. As a result more current than required may be injected into the cell in response to ion channel activation, as part of it is lost across the seal. This would lead to an overestimation of the current flowing through the channel. Since calcium currents are very small, the problem of 'leak-currents' had to be addressed. This was done by always making additional recordings in the presence of cadmium, which blocks all voltage-gated calcium channels. The two currents were then subtracted to reveal the cadmium-sensitive component (Fig. 39 a-c).

Cells from medium-sized arteries produced the largest peak currents both in absolute terms and when corrected for the size for the cell: 3.78 ± 0.68 pA/pF (n = 8), 4.28 ± 0.74 pA/pF (n = 6) and 2.39 ± 0.36 pA/pF (n = 6) in small, medium and large respectively. It is however of interest to note that clear inward currents were never recorded in cells from large arteries; the current trace never went more negative than the baseline value prior to depolarisation. The only way to reveal a response was to superimpose a trace from a recording made in the presence of cadmium (Fig. 39c).

Plots of the current voltage relationship for voltage steps from –60 mV to +30 mV in small and medium arteries were bell-shaped and very similar in appearance. The threshold for channel activation was –30 mV in small and –40 mV in medium arteries; the peak current was recorded at a command potential of +10 mV in small and 0 mV in medium arteries (Fig. 40 a,b). At more positive potentials, current amplitudes declined. The I-V plot for large arteries however, had a very different, more linear appearance. Rather than currents reaching a peak around 0 mV or +10 mV and then declining, they continued to increase up to the last voltage step of +30 mV (Fig. 40). A possible explanation for this may have been a different calcium channel that is activated

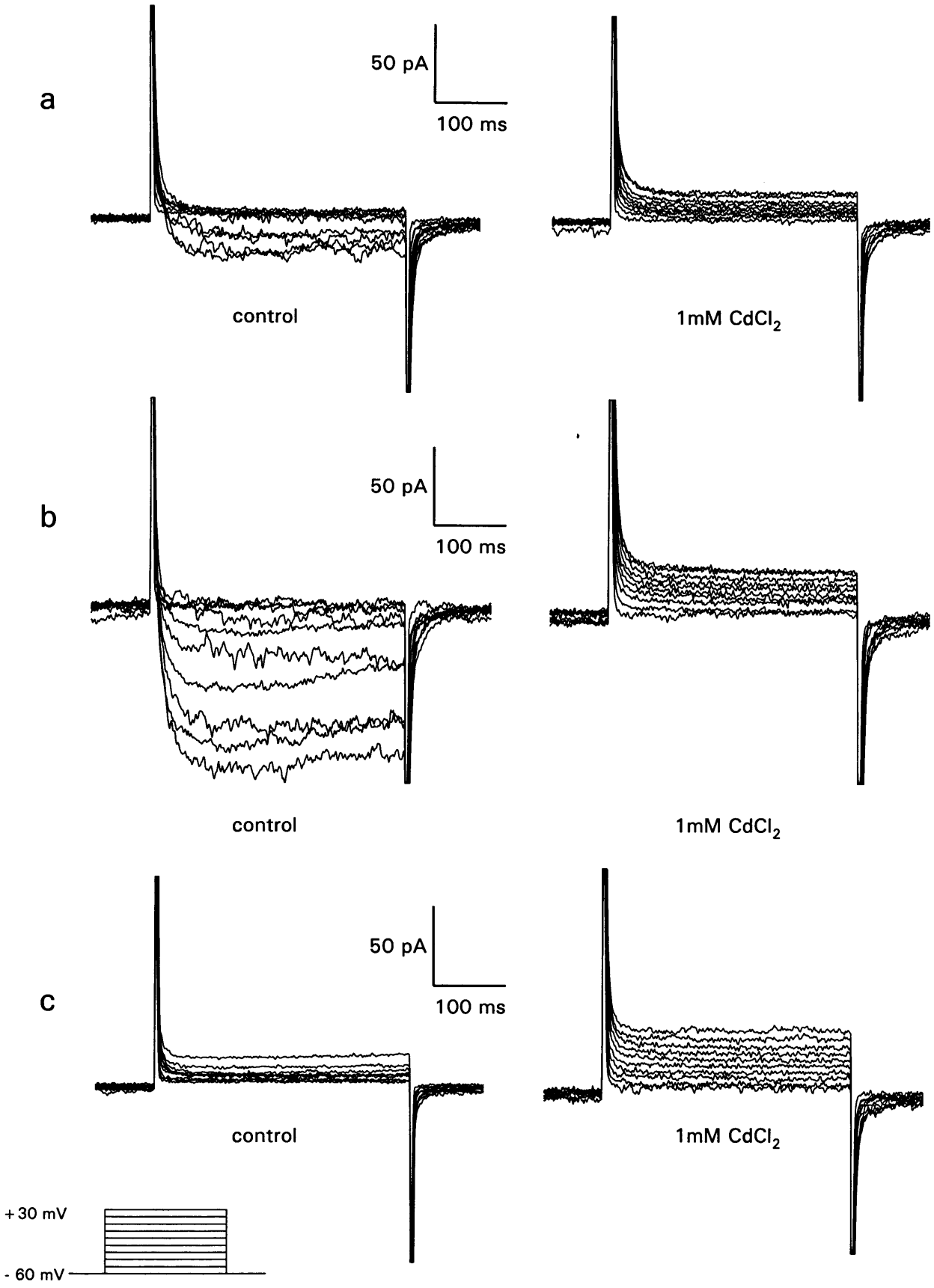

Figure 39 Step depolarisations for construction of calcium current-voltage relationships. Cells are depolarised in 10 mV steps from a holding potential of – 60 mV to a final command potential of +30 mV (see waveform). The lack of clear inward currents in large arteries (c) as compared to small (a) and medium (b) is particularly well illustrated by the range of depolarisations. Responses in the presence of cadmium, i.e. when currents are blocked, are again used as reference points for the calculation of calcium current amplitudes.

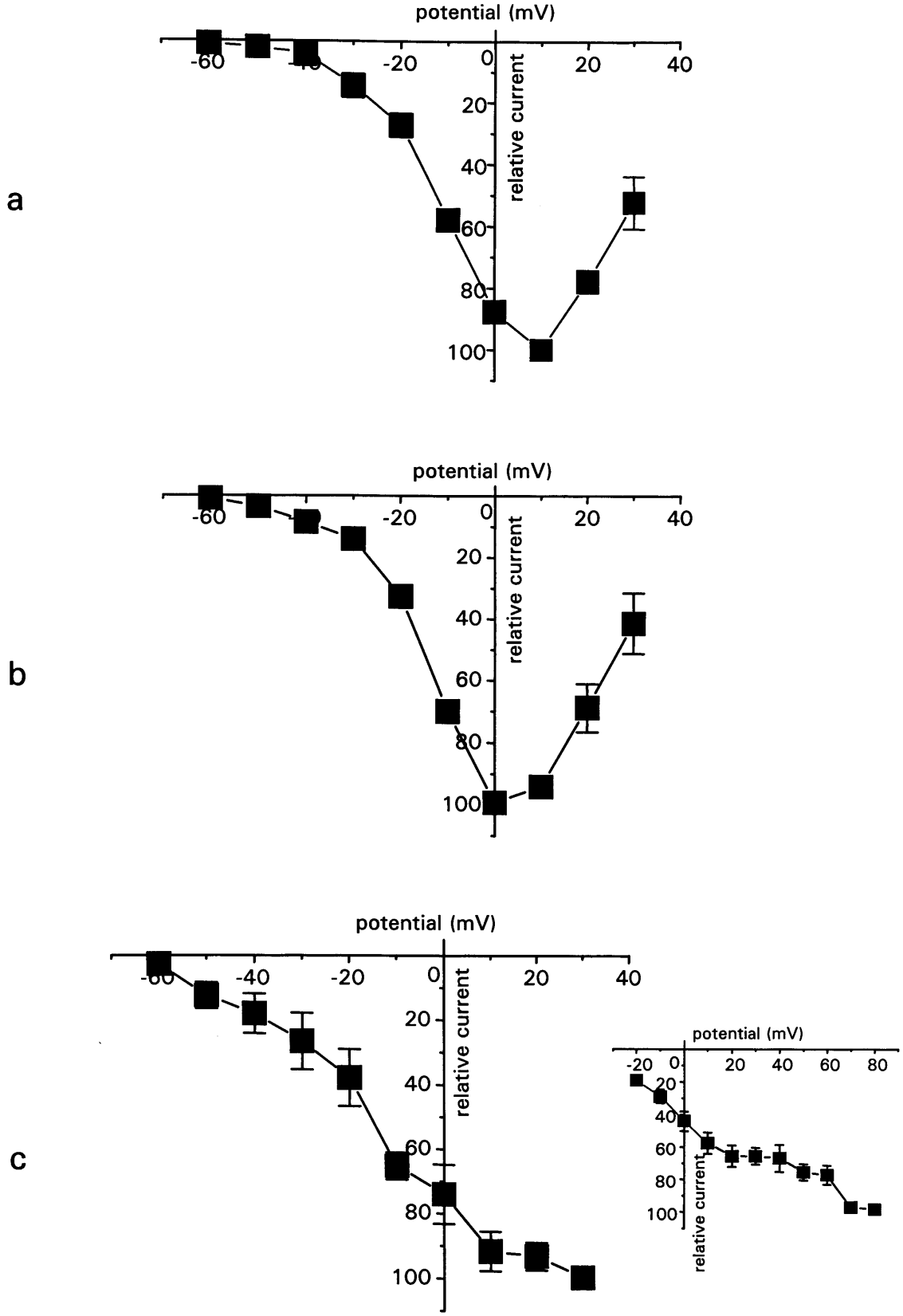

Figure 40 Calcium current-voltage relationships reveal anomalous result in large arteries. Data from Fig. 39 were used to construct I-V plots for the three sizes of artery. Plots for small (a) and medium-sized arteries (b) are characteristic of L-type calcium currents, activating maximally between 0 mV and +10 mV. (c) Currents in large arteries however continue to rise at potentials more positive than +10 mV. Inset shows that this carries on up to +80 mV. These responses appear not to be mediated by an L-type calcium channel. Data are currents normalised to the maximal response ± s.e.m. in each vessel (n = 6 – 9).

at more positive potentials than L-type channels. I therefore constructed another I-V plot for potentials from –20 mV to + 80 mV, which also was linear (Fig. 40 c).

6.3.2 EFFECTS OF NIFEDIPINE

Contractions evoked by 60 mM KCl in small arteries were partially resistant to nifedipine and abolished by cadmium. This suggests that a 'non-L- type' calcium channel is also present in these arteries. This putative calcium channel should also evoke nifedipine-resistant currents in single smooth muscle cells. The effect of nifedipine on calcium currents was therefore compared in cells from small, medium and large arteries.

The current-voltage relationships show that the largest calcium current amplitudes are evoked at command potentials of 0 mV or +10 mV. In experiments testing the effect of nifedipine, cells were therefore depolarised to 0 mV and to +10mV from a holding potential of –60 mV. The quality of recordings was found to depend strongly on the quality of cells. A number of cells were tested from each size of artery, of which approximately half yielded measurable currents: 9/21 cells from small, 9/18 cells from medium and 6/11 cells from large arteries.

All calcium currents recorded had a similar appearance: they activated rapidly, reached a plateau level and then inactivated slightly during the recording period (Fig. 41 a-c). For all three sizes of artery, calcium currents were largest for the depolarisation to +10mV (Table 14).

	0 mV	+10 mV
Small	21.3 ± 3.7 pA (n = 9)	30.4 ± 4.8 pA (n = 9)
Medium	30.0 ± 9.5 pA (n = 9)	37.6 ± 11.2 pA (n = 9)
Large	14.9 ± 2.7 pA (n = 6)	17.8 ± 2.4 pA (n = 6)

Table 14. Peak calcium current in small, medium and large arteries. In absolute terms, currents appear to be largest in medium-sized arteries. Currents in small arteries are significantly larger than in large arteries at 10 mV ($p < 0.05$)

Correcting for the size of the cell reveals that calcium currents seem to be largest in cells from small arteries (Table 15).

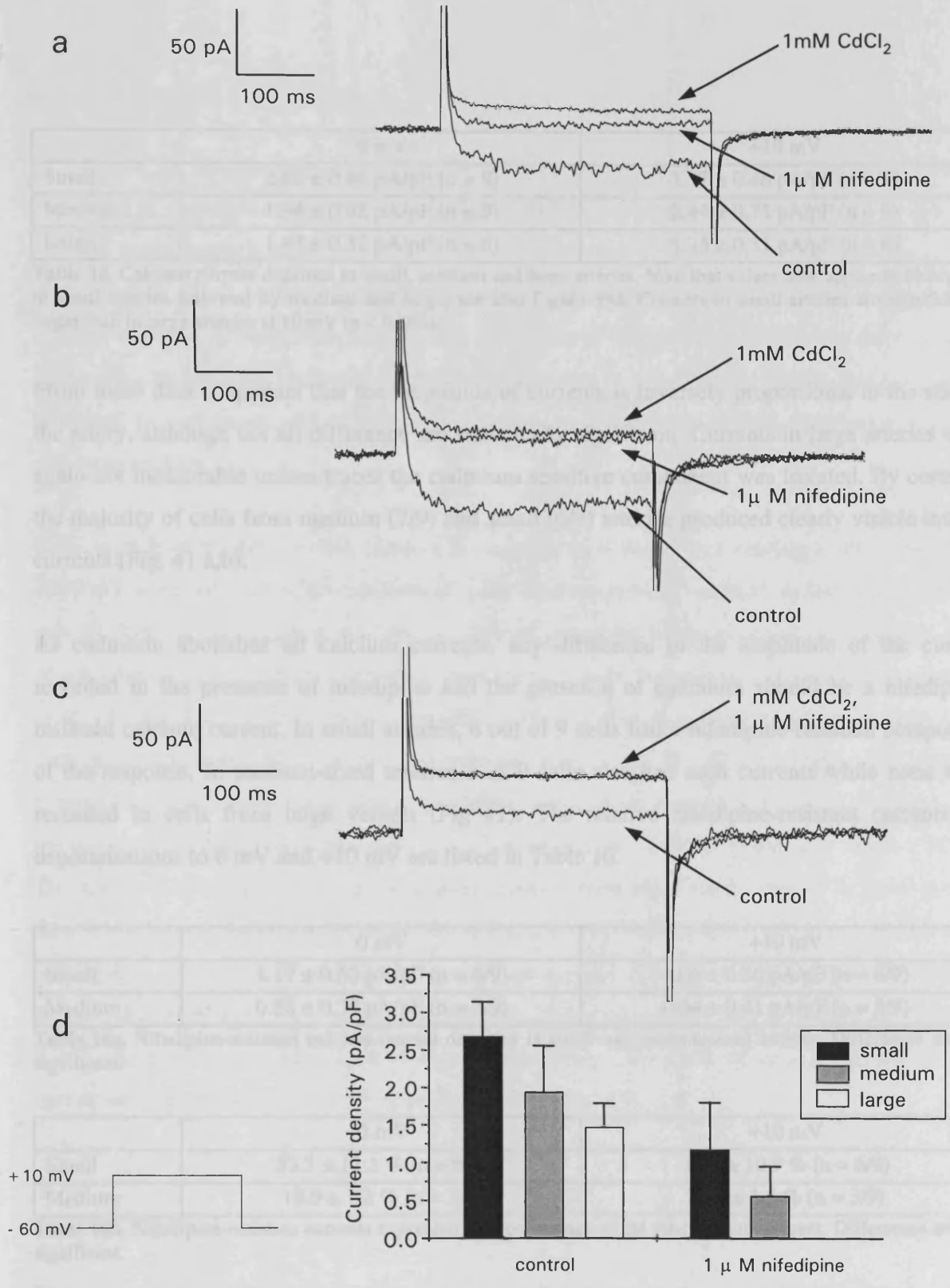

Figure 41 Small and medium-sized arteries display nifedipine-resistant calcium currents. Calcium currents recorded in response to a step depolarisation from –60 mV to +10 mV (see waveform). Sustained inward currents are seen in small (a) and medium (b) vessels. Recordings after blockade of voltage-dependent calcium currents with cadmium are reference points for amplitude measurement. The difference between responses recorded in the presence of cadmium and nifedipine are non-L-type calcium currents. (c) In large arteries however, no clear inward current is recorded and responses are abolished by the L-type channel blocker nifedipine. (d) Data are mean current density ± s.e.m. (n = 6 – 9).

	0 mV	+10 mV
Small	2.68 ± 0.46 pA/pF (n = 9)	3.77 ± 0.46 pA/pF (n = 9)
Medium	1.94 ± 0.62 pA/pF (n = 9)	2.44 ± 0.73 pA/pF (n = 9)
Large	1.47 ± 0.32 pA/pF (n = 6)	1.75 ± 0.31 pA/pF (n = 6)

Table 15. Calcium current densities in small, medium and large arteries. Note that values now appear to be highest in small arteries followed by medium and large; see also Figure 39d. Currents in small arteries are significantly larger than in large arteries at 10 mV ($p < 0.005$).

From these data is appears that the amplitude of currents is inversely proportional to the size of the artery, although not all difference are statistically significant. Currents in large arteries were again not measurable unless traces the cadmium sensitive component was isolated. By contrast, the majority of cells from medium (7/9) and small (6/9) arteries produced clearly visible inward currents (Fig. 41 a,b).

As cadmium abolishes all calcium currents, any difference in the amplitude of the current recorded in the presence of nifedipine and the presence of cadmium should be a nifedipine-resistant calcium current. In small arteries, 6 out of 9 cells had a nifedipine-resistant component of the response. In medium-sized arteries 3 of 9 cells also had such currents while none were recorded in cells from large vessels (Fig 41). The relative nifedipine-resistant currents for depolarisations to 0 mV and +10 mV are listed in Table 16.

	0 mV	+10 mV
Small	1.17 ± 0.63 pA/pF (n = 6/9)	1.05 ± 0.50 pA/pF (n = 6/9)
Medium	0.56 ± 0.39 pA/pF (n = 3/9)	0.64 ± 0.41 pA/pF (n = 3/9)

Table 16a. Nifedipine-resistant calcium current densities in small and medium-sized arteries. Differences are not significant.

	0 mV	+10 mV
Small	33.3 ± 11.1 % (n = 6/9)	26.7 ± 10.9 % (n = 6/9)
Medium	19.0 ± 1.2 % (n = 3/9)	20.4 ± 1.5 % (n = 3/9)

Table 16b. Nifedipine-resistant currents expressed as a percentage of the total calcium current. Differences are not significant.

6.4 POTASSIUM CURRENTS

In addition to studying specific channels, it was also of interest to examine more general properties of smooth muscle cells. Voltage-gated potassium channels are not directly involved in calcium homeostasis but are also of importance in regulating the excitability of smooth muscle cells (Nelson *et al*, 1990). Potassium currents would therefore give a broader indication of whether there are general differences in the expression of ion channels in different sized arteries. To investigate possible diameter-dependent differences in potassium channels, current-voltage relationships and amplitudes of potassium currents were compared in cells from small, medium and large arteries.

Cells were held at –60 mV and currents in response to voltage steps ranging from –120 mV to +100 mV were recorded. Currents from all cells displayed strong outward rectification with little current flowing at negative potentials. At positive potentials robust outward currents were seen which grew in amplitude at increasingly positive potentials. Responses had a similar appearance in all vessels. The current increased rapidly, reached a plateau and remained at the same level until the voltage returned to the holding potential after 50 ms (Fig. 42 a-c). There were however slight differences in the rate at which the plateau phase of the current was reached. In most cells the current increased over a period of 20 – 30 ms, while in 1 cell this phase only lasted ~ 10 ms. The mean peak amplitudes of leak-subtracted currents were significantly smaller in small arteries than in medium or large vessels (300.6 ± 38.0 pA, n = 5; 768.0 ± 228.6 pA, n = 4 and 614.5 ± 168.7 pA, n = 4 in small, medium and large respectively). However, when expressed as current density, the differences are no longer significant: 37.4 ± 6.9 pA/pF, 48.5 ± 12.5 pA/pF and 51.5 ± 6.2 pA/pF for small medium and large, respectively. The plots of voltage against relative current are almost identical for all three vessel sizes (Fig. 43).

6.5 DISCUSSION

The experiments in this chapter were designed to compare responses to P2X receptor activation in different arteries at the single cell level. My data show rapidly desensitising and suramin-sensitive currents evoked by the ATP analogue α,β-meATP in cells from all three arteries,

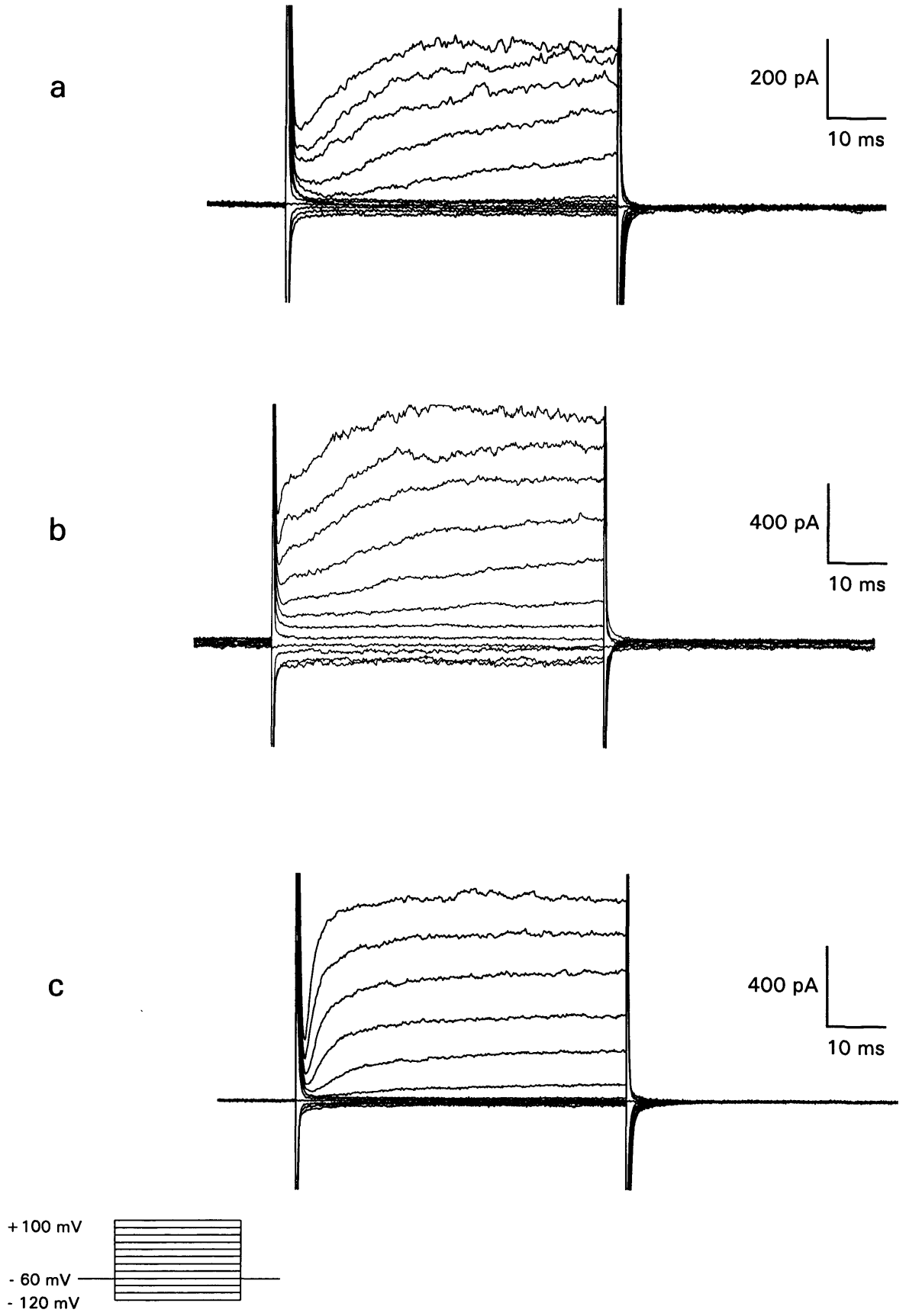

Figure 42 Voltage-dependent potassium currents are roughly equal in medium (b) and large (c) arteries but smaller in small vessels (a). Cells were held at –60 mV, hyperpolarised to –120 mV and then depolarised in 20 mV steps to a final command potential of +100 mV (see waveform). Currents have a similar appearance in all arteries but absolute current amplitudes in small vessels are roughly half of those in medium and large.

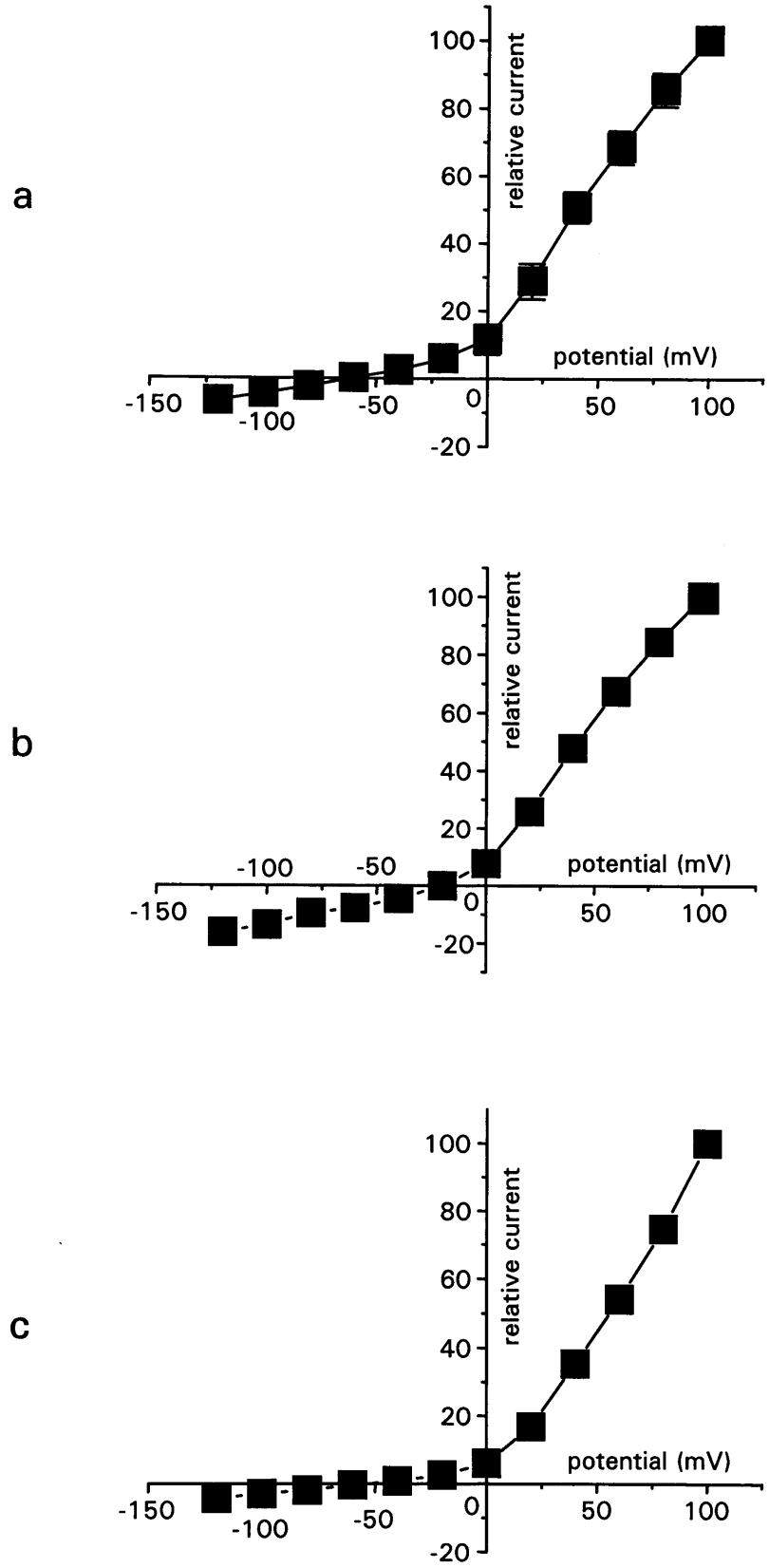

Figure 43 Current-voltage relationships for voltage-gated potassium currents. When currents in each vessel size are normalised to the maximal response, I-V plots are virtually identical. Responses are mean in small (a), medium (b) and large arteries (c) ± s.e.m. (n = 4 – 5).

properties characteristic of $P2X_1$ receptors. There were however significant differences in the densities of α,β-meATP-evoked currents depending on the size of the vessel. In addition, there was a trend indicating that calcium current density was inversely proportional to arterial diameter. The majority of cells from small arteries displayed partially nifedipine-resistant responses. Such currents were also seen in a proportion of cells from medium-sized vessels, while none were observed in large arteries.

The $P2X_1$ receptor is believed to be the isoform responsible for all P2X receptor-mediated effects of ATP in arterial smooth muscle. This view is strengthened by recent work in transgenic mice lacking functional $P2X_1$ receptors: experiments showed that ATP-evoked currents were lost in smooth muscle cells from vas deferens (Mulryan *et al*, 2000), bladder (Vial & Evans, 2000) and arteries (personal communication from C. Vial). The similarity between vas deferens and arterial smooth muscle suggests the same will be true in the latter. Currents evoked by α,β-meATP that desensitise in the continued presence of the agonist are characteristic of responses at $P2X_1$ receptors. Such currents are seen in all other studies on vascular smooth muscle cells (e.g. Evans & Kennedy, 1994). Similar transient responses to ATP have been recorded at recombinant $P2X_1$ receptors expressed either in HEK-293 cells or Xenopus oocytes (Evans *et al*, 1995; Stoop *et al*, 1997; Parker, 1998; Bianchi *et al*, 1999). Regarding currents recorded in native smooth muscle cells, the literature reveals a range of peak amplitudes from various cell types. The fact that responses are rarely expressed as current density does however make direct comparisons somewhat difficult. Nevertheless, responses in some arteries are clearly substantially smaller than the values I obtained in the rat mesentery, e.g. rat pulmonary arteries (Hartley & Kozlowski, 1997). In contrast, $P2X_1$ receptor agonists evoked currents in cells from the rat vas deferens that are comparable to responses I obtained in small mesenteric arteries (Khakh *et al*, 1995 and Trezise *et al*, 1995). The size of currents evoked in smooth muscle cells from mouse bladder were also roughly similar to my data (Vial & Evans, 2000). In one instance where current densities are quoted (human, porcine and guinea-pig bladder) values are roughly one order of magnitude smaller than those recorded in the present study (Inoue & Brading, 1991).

A small number of studies have also been conducted in smooth muscle cells obtained from the rat mesenteric bed; in each case vessels equivalent to medium-sized arteries in my experiments

were used. Interestingly, the peak current amplitudes were generally smaller than in my experiments, typically about 1 nA for 10 μM α,β-meATP (Lewis *et al*, 1998; Lewis & Evans, 2000a). Only one report arrived at a comparable current density in the same artery (~ 340 pA/pF), although in this case α,β-meATP was applied at a concentration of 300 μM (Lewis and Evans, 2000b). Unfortunately, the potency of α,β-meATP cannot be directly compared with values obtained in other studies, as I have not constructed full concentration response curves. A possible reason why P2X receptor-mediated currents were frequently smaller than my recordings may be due to different recording configurations. Smaller responses might for example be expected when using the perforated patch technique, because of slight run-down and more limited electrical access to the cell cytosol.

The lack of data obtained in native smooth muscle cells also means there is limited scope for comparing the kinetic parameters I have measured with results obtained in other studies. Values quoted for rise time or decay constant τ, range from substantially slower than the values I obtained (Khakh *et al*, 1995; Lewis *et al*, 2000b) to roughly similar (Evans & Kennedy, 1994; Lewis & Evans, 2000a) and significantly faster (McLaren *et al*, 1998). The most likely explanation for the differences observed between different studies is a combination of the following factors: differences in methodology, slight differences in the properties of smooth muscle cells from different vessels and the quality of cells obtained after enzymatic dissociation. The latter can have a profound effect on the quality of data generated. In contrast, the effect of suramin on currents evoked by ATP or α,β-meATP are the same in all studies. Both in smooth muscle cells and at recombinant receptors, suramin always abolished responses up to the concentrations of agonist I used in my experiments (Evans *et al*, 1995; Khakh *et al*, 1995; McLaren *et al*, 1998; Lewis *et al*, 2000b).

A further interesting point arising from my electrophysiological data is the difference in contractile responses and current responses recorded in large arteries. In contraction studies large arteries are relatively insensitive to α,β-meATP, and suramin did not substantially reduce responses evoked by this agonist. However, when measuring currents in single cells, α,β-meATP evoked responses with a high potency which were abolished by suramin. A possible reason for the differing potencies of α,β-meATP may be agonist breakdown. Although α,β-meATP is said

to be metabolically stable, there is evidence which shows that can indeed be broken down in whole tissue (Cascalheira & Sebastiao, 1992). It is possible that ectonucleotidases are more potent in large arteries than in smaller vessels. Such metabolic differences could also explain the small purinergic component of neurogenic responses: hydrolytic enzymes may be potent enough to destroy most of the ATP before it has any post-junctional effect. Another explanation may lie in a concomitant vasodilatory response in large arteries. α,β-meATP has indeed been reported to cause vasorelaxation in pre-contracted rat mesenteric arteries (Juul *et al,* 1993; Steinmetz *et al,* 2000a). P2X receptor expression has been reported in the endothelium of several arteries (Loesch & Burnstock, 2000; Yamamoto *et al,* 2000) including $P2X_1$ receptors on rat mesenteric artery endothelial cells (Hansen *et al,* 1999). It is therefore possible that in large arteries, α,β-meATP mediates endothelium-dependent vasodilatation which counteracts the vasoconstrictor effect, resulting in a lower overall contractile potency. The difference in suramin sensitivity between whole tissue and single cell studies in large arteries may be a reflection of the higher concentrations of agonist that had to be used in large arteries to evoke contractile responses.

To investigate the possible presence of nifedipine-resistant calcium channels, as indicated by my contractions studies, and to compare calcium channels in all sizes of artery, underlying calcium currents were characterised. The current voltage relationships produced show maximal activation of calcium currents at between 0 mV and +10 mV. This agrees well with several previous studies (Bowles *et al,* 1998; Simard *et al,* 1998), although there are reports where peak currents are recorded at slightly more positive potentials (Ohya *et al,* 1998). There is also some variation in the literature regarding the densities of whole cell calcium currents. In rat basilar artery, a peak current density of ~15 pA/pF was recorded (Simard *et al,* 1998). Another study used rat mesenteric arteries equivalent to medium-sized vessels in my experiments and determined a value of ~11 pA/pF (Ohya *et al,* 1998). The latter results are directly comparable to mine and it is unclear why there is a four-fold difference in the current densities; this may be a reflection of differences in the experimental protocol or in recording conditions. In contrast, similar current densities were observed in pig (Bowles *et al,* 1998) and human coronary arteries (Quignard *et al,* 1997). A further difference is that currents in my experiments appear to be somewhat more sustained than in other studies.

Comparing the calcium current densities in the three sizes of artery reveals a possible diameter dependent trend. Although the difference in current densities was only significant between small and large arteries, the implication is: the smaller the artery, the larger the current. This demonstrates that the density of L-type calcium channels may be inversely proportional to the size of the artery. The same trend was seen by Bowles *et al* (1997) studying pig coronary arteries. Indeed, the effect is shown more conclusively in this study as robust calcium currents were evoked in all sizes of artery and differences were significant. For reasons which are not apparent, no clearly visible currents were seen in large arteries in my experiments. This is underlined by a very unexpected, linear current-voltage relationship, which is not characteristic of voltage-dependent calcium currents. A potential reason for this may be a very low density of calcium channel expression. It is possible that no real calcium currents were actually being measured in large arteries, a theory supported by the anomalous I-V plot. The fact that currents in large arteries appeared to be cadmium sensitive however contradicts this; these data suggest calcium channels are involved in mediating the observed currents. In addition, my contraction studies show that KCl has a similar 'potency' in large arteries as in smaller vessels (Section 3.3). As these responses rely on calcium influx through voltage-dependent calcium channels, this suggests a similar density of these channels in all three arteries.

The implication of such calcium channel heterogeneity would therefore be that small arteries are more sensitive to changes in physiological stimuli than larger vessels. This argument seems plausible: as the smallest arteries in a vascular bed are most important in regulating blood flow and blood pressure, they would need to be more responsive to metabolic changes. The fact that voltage-gated potassium currents are similar in all sizes arteries (as previously found in coronary arteries by Quayle *et al*, 1996) suggests that the observed differences in calcium currents are a specific property of these channels and not a general feature of smooth muscle cells.

Many studies support the view that L-type calcium channels are the most important voltage-gated calcium channel in vascular smooth muscle (as reviewed by Gollasch & Nelson, 1997). This is confirmed by reports where nifedipine abolishes either arterial contractions induced by depolarisation with KCl (e.g. Criddle *et al*, 1997) or calcium currents in isolated smooth muscle cells (e.g. Bowles *et al*, 1997). Most of these studies have however been conducted in arteries I

would describe as medium or large. My results however reveal that there are substantial nifedipine resistant currents in small mesenteric arteries. This is consistent with my results from contraction studies where small arteries exhibited a nifedipine-resistant component of contraction to 60 mM KCl. Interestingly, a proportion of cells from medium-sized arteries also had small nifedipine-resistant currents despite contractions in these vessels being abolished. It may therefore be that a minimum proportion of cells must have nifedipine-resistant responses before the tissue as a whole shows this phenotype. This threshold may be attained in small arteries but not in medium.

A recent study in guinea-pig terminal mesenteric arteries, equivalent to my 'small arteries', has reported similar findings (Morita *et al*, 1999). Here, medium-sized mesenteric arteries had only a small nifedipine-resistant component while almost the entire current in small arteries and submucosal arterioles was nifedipine-resistant. One substantial difference between the two studies is the degree of resistance. Currents in small arteries in my experiments were ~ 30 % resistant, while in the above study the proportion was closer 90 %. The implication in this study is that every cell showed some degree of resistant current but it is not unequivocally stated. It would be of interest to clarify this, as I only found such currents in a proportion of cells tested. Another substantial difference was the time course of currents. In the present study they were very sustained, inactivating only slightly during the recording period. In contrast, currents recorded by Morita *et al* (1999) inactivated substantially, almost returning to baseline level before the end of the voltage step. The experimental conditions appear to have been very similar (Ba^{2+} as the charge carrier in each case), so this cannot be the cause of this discrepancy.

The above investigation rigorously attempted to identify the channel responsible for the observed results using detailed pharmacological, kinetic and molecular analysis of nifedipine resistant currents, experiments beyond the scope of my work. They reach the conclusion that their observations cannot be accounted for by any one of the existing voltage-gated calcium channels. A previously unknown splice-variant of existing calcium channel α subunits or a novel α subunit was thought to be the only plausible explanation. A similar approach would be required to accurately identify the nifedipine-insensitive channel in my experiments. It has been suggested that T-type calcium channels may play an important role in regulating smooth muscle calcium

levels (Sarsero *et al*, 1998). These channels activate at more negative potentials than L-type channels and are characterised by transient current responses. This transient nature of currents recorded by Morita *et al*, (1999) would therefore point toward substantial expression of T-type channels. However, the sustained nature of nifedipine-resistant currents in my study seems to argue against involvement of T-type channels in these responses.

The electrophysiological studies described in this chapter have charcterised currents mediated by P2X recpetors and calcium channels in three sizes of mesenteric artery. Currents evoked by both 1 μM and 10 μM α,β-meATP are largest in medium-sized arteries followed by large and small. Responses to 1 μM α,β-meATP in small arteries were significantly smaller than in the other two sizes of vessel. Currents in response to both concentrations are abolished by suramin in all cells. With respect to calcium currents there seems to be a diameter-dependent trend: as arteries become smaller, calcium current density increases. Current-voltage relationships were very similar in small and medium arteries, producing the characteristic bell-shaped curve. However, in large vessels the I-V plot was linear, suggesting a lack of true calcium currents. Small arteries produced the highest proportion of cells with nifedipine-resistant currents, although such responses were also seen in medium-sized arteries.

7 TRANSGENIC MOUSE STUDIES

7.1 INTRODUCTION AND AIMS

The most effective way to determine the physiological role of a receptor or ion channel is with the use of highly selective agonists and antagonists. If the application of such a compound has a physiological effect, this is a strong indication that the receptor it is selective for participates in the observed response. Similarly, if the function of the receptor-subtype is known but its identity is not, these compounds can aid in identification. One of the major problems in characterising the physiological role of different P2X receptors has been the lack of sub-type selective agonists and antagonists. As a result, P2X receptor isoforms mediating responses to ATP have been identified indirectly. This has relied on comparing the pharmacological properties of a tissue with those of recombinant P2X receptors. Although this strategy can be effective, particularly when using antagonists, it has limitations.

It is generally accepted that the P2X receptor isoform mediating the effects of ATP in vascular smooth muscle is $P2X_1$ (MacKenzie *et al*, 1999). This is based on the fact that arteries are α,β-meATP and suramin sensitive and display rapidly desensitising responses to P2X receptor agonists (all properties of recombinant $P2X_1$ channels). This evidence is however only sufficient to conclude that the receptor is $P2X_1$-like, not to categorically state it is a $P2X_1$ receptor. Data from immunohistochemical studies also suggests that the $P2X_1$ receptor is the predominant isoform. However, the only way to provide conclusive proof that the $P2X_1$ isoform is responsible for all P2X receptor-mediated responses, is to delete the receptor from the cell using a molecular approach and observe the resulting effects. A transgenic mouse was therefore generated where the $P2X_1$ receptor gene had been modified, preventing the expression of functional receptors.

The aims of this study were to determine the role that $P2X_1$ receptors play in the function of smooth muscle. It was noticed during breeding of transgenic mice, that $P2X_1$-deficient males were infertile. It therefore seemed appropriate to test the function of the vas deferens as this tissue is involved in reproduction and contains smooth muscle expressing high levels of the $P2X_1$ receptor. The contractile and pharmacological properties of vas deferens from $P2X_1$-deficient mice were characterised and compared with those of heterozygous and wild-type animals. An additional advantage of this tissue is that vas deferens are much larger and more

robust than arteries and reliably produce large contractile responses. Most of this work has been published (Mulryan *et al*, 2000, Appendix 1).

7.2 CONTRACTION STUDIES ON VAS DEFERENS FROM $P2X_1$-DEFICIENT MICE

7.2.1 AGONIST-EVOKED CONTRACTIONS

The $P2X_1$ receptor is believed to be the P2X isoform mediating the contractile effects of ATP in vas deferens. Removal of functional $P2X_1$ channels should therefore have a profound effect on responses to purinergic agonists known to evoke contractions in the wild-type. To test this hypothesis, concentration response curves for α,β-meATP were constructed in wild-type, heterozygote and knock-out vas deferens.

In all wild-type vessels, α,β-meATP evoked concentration-dependent contractions typical of $P2X_1$-mediated responses: transient with tension returning toward baseline in the continued presence of the agonist. Similar contractile responses were seen in tissue from heterozygote animals (Fig. 44 b,c). The potency of α,β-meATP was very similar for both types of mouse and concentration-response curves were of a similar shape (Fig. 44 d). EC_{50} values were 1.6 μM and 2.0 μM for wild-type and heterozygote, respectively (corresponding pA_{50} values 5.8 ± 0.1, n = 7 and 5.7 ± 0.1, n = 6). By contrast, α,β-meATP failed to produce any contractile response in tissue from $P2X_1$-deficient mice, up to a concentration of 100 μM (Fig. 44 a). Single concentrations of ATP and L-β,γ-meATP were also compared in knock-out and wild-type animals; the latter is selective for smooth muscle $P2X_1$ receptors (Trezise *et al*, 1995). At a concentration of 10 μM, both agonists produced large contractions in wild-type tissue, while no responses were seen in knock-out animals (Fig 45).

It is possible that the lack of response to α,β-meATP in $P2X_1$ -/- mice was caused not only by the lack of $P2X_1$ receptors but also by a general loss of contractility in vas deferens from knock-out animals. To test for this, the potency of noradrenaline was compared in the three types of mouse. This transmitter is co-released with ATP from sympathetic nerves which control vas deferens function. Noradrenaline evoked transient, concentration-dependent

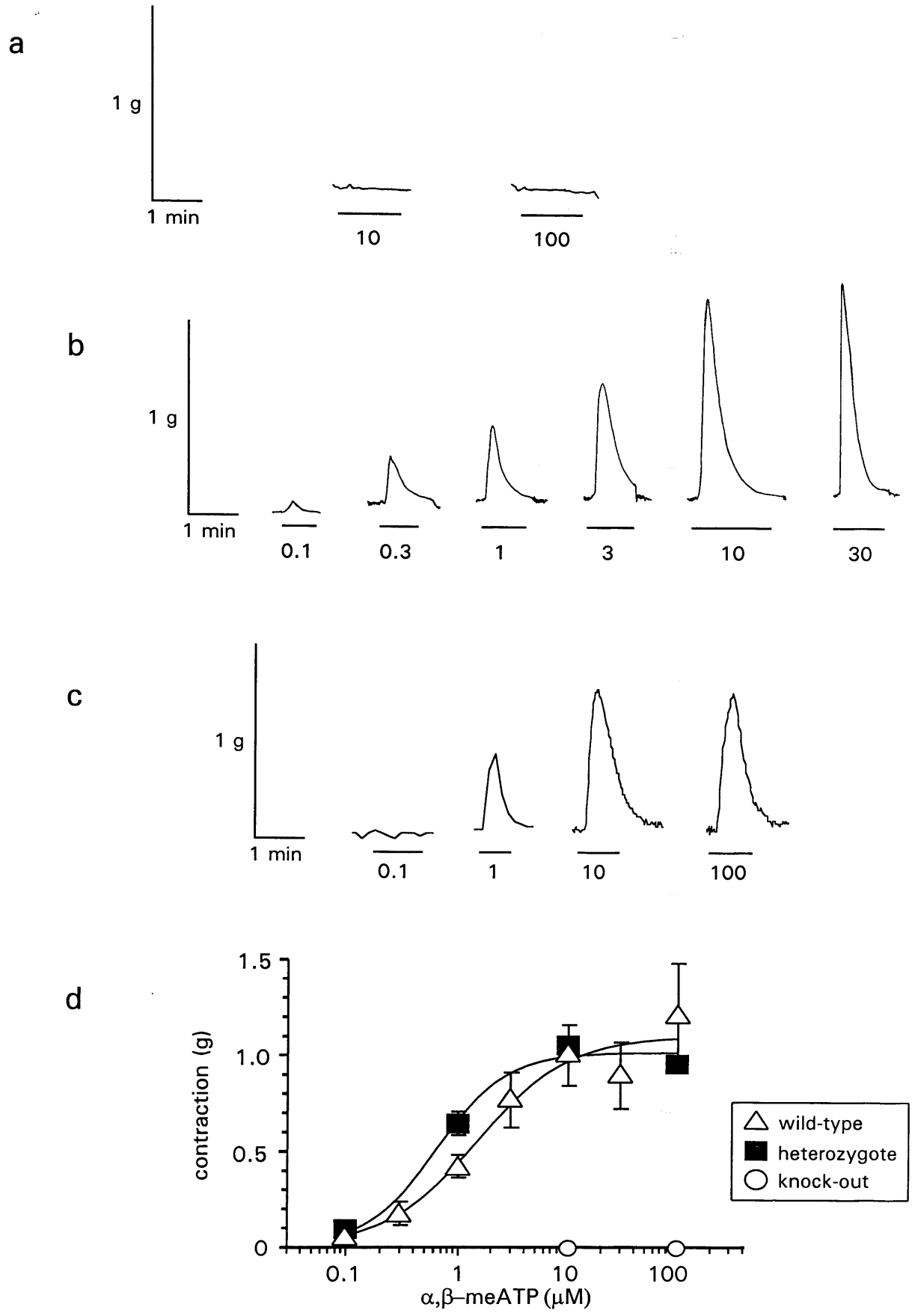

Figure 44 Characterisation of contractile responses to α,β-meATP. No responses to α,β-meATP are seen in knock-out animals up to a concentartion of 100 μM (a). In both wild-type (b) and heterozygote (c) animals, contractions are transient and fade in the continued presence of the agonist; characteristics of responses mediated by $P2X_1$ receptors. Values are concentration in μM and bars indicate agonist applications. No responses were seen in knock-out animals. (c) Concentration response curves are similar for wild-type and heterozygote tissue. Data are mean ± s.e.m. (n = 6 – 7).

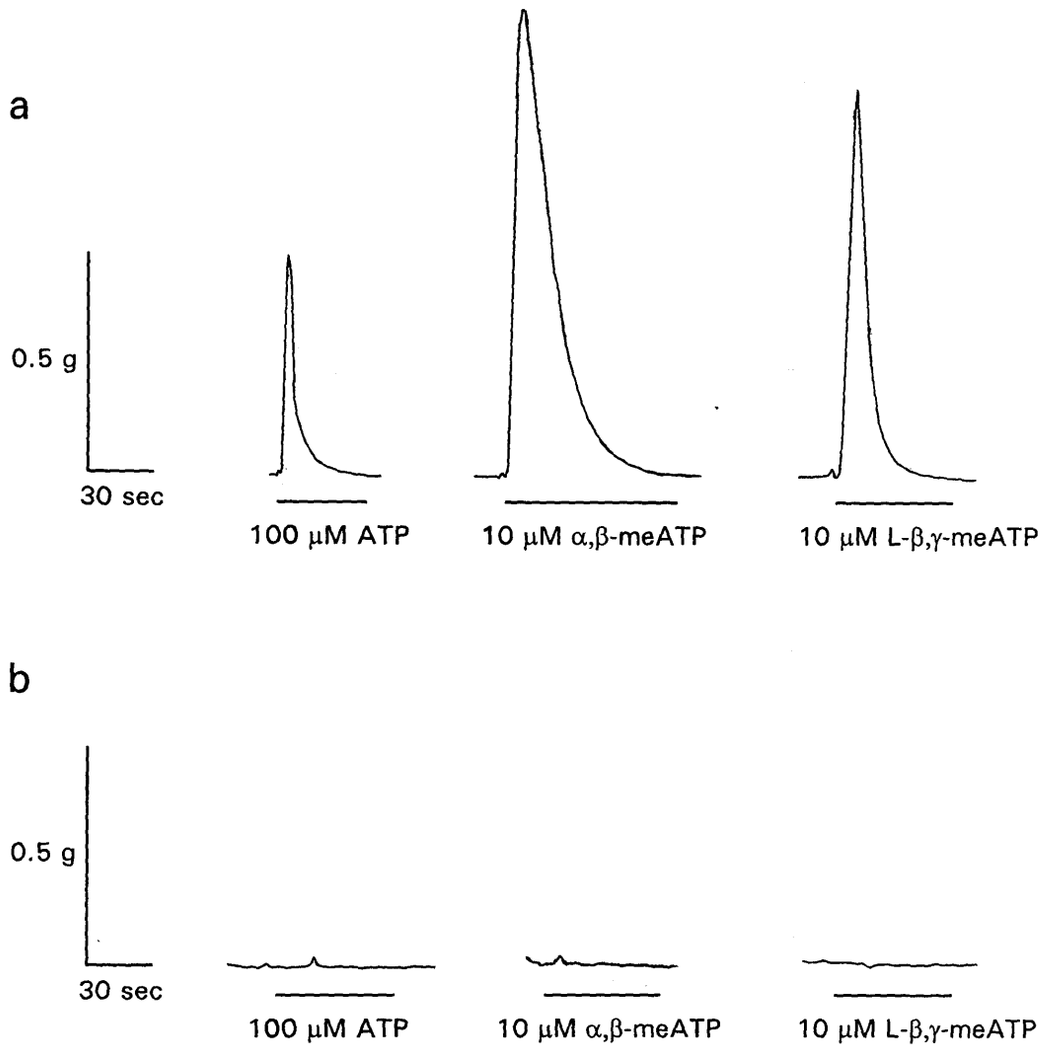

Figure 45 $P2X_1$ receptor agonists have no effect in knock-out animals. Typical transient contractile reponses to ATP, α,β-meATP and L-β,γ-meATP in vas deferens from wild-type animals (a). In tissue from knock-out mice, no contractions are seen to any of these agonists (b). The lack of response to ATP confirms that no other P2X receptor isoform mediates contractions in this tissue.

contractions in all tissues tested (Fig. 46 a,b). Concentration response curves for wild-type and heterozygote vas deferens were almost superimposable (Fig. 46 c). The EC_{50} values were 15.8 μM and 7.9 μM and corresponding pA_{50} values were 4.8 ± 0.1 (n =6) and 5.1 ± 0.3 (n = 6), respectively. Tissue from $P2X_1$ receptor-deficient mice were however more sensitive to noradrenaline ($p = 0.005$), $EC_{50} = 2.0$ and $pA_{50} = 5.7 \pm 0.2$ ($n = 6$).

Another means of assessing the contractility of smooth muscle is to evoke responses with KCl. A high concentration of KCl depolarises the tissue leading to calcium influx through voltage-dependent channels and contraction. These responses are independent of any receptor and give a more general indication of the responsiveness of a tissue. 100 mM KCl evoked contractions of a similar amplitude in vas deferens of wild-type and knock-out mice: 1.36 ± 0.10 g (n = 14) in wild-type and 1.17 ± 0.10 g (n = 17) in $P2X_1$ knock-out mice.

7.2.2 NERVE-EVOKED CONTRACTIONS

Vas deferens function is regulated by the sympathetic nervous system. ATP and noradrenaline are co-released from sympathetic nerve terminals and both contribute to the control of vas deferens smooth muscle tone. If the effects of ATP are mediated solely through $P2X_1$ receptors, then ATP-based transmission should be lost if this receptor is deleted. It therefore seems probable that the properties of neurogenic contractions will be different in knock-out mice as compared to wild-type animals. Contractile responses evoked by nerve stimulation were compared in wild-type, heterozygote and knock-out mice.

7.2.2.1 Frequency response relationships

As an initial step in the characterisation of neurogenic contractions in the three types of animal, I investigated the frequency-response relationship of nerve-evoked responses. The total duration of mouse copulation has been reported to be between 1 and 3 seconds (Yang & Clemens, 1998); vas deferens activity would therefore be expected to last no longer than 3 seconds. Three second trains of stimulation were given at frequencies ranging from 3 to 20 Hz. Contractions increased in amplitude in a frequency dependent manner and had a similar time course for each genotype of animal, rapidly reaching a peak and inactivating during

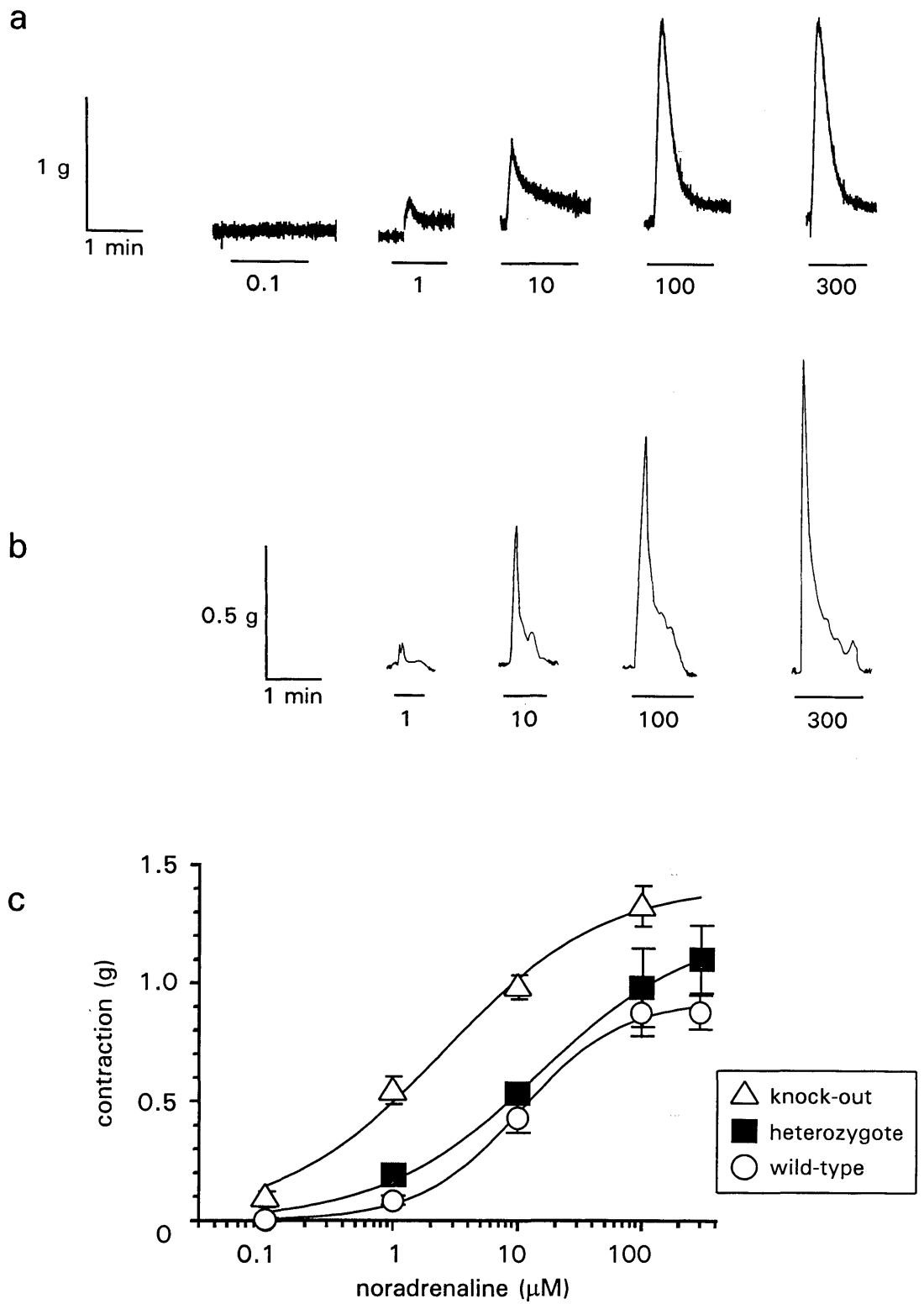

Figure 46 Noradrenaline is more potent in knock-out animals than in wild-type or heterozygote. Transient contractions to noradrenaline in wild-type (a) and knock-out tissue (b); values are concentration in μM and agonist applications are indicated by bars. (c) Concentration response relationships show leftward shift of curve in knock-out animals illustrating greater sensitivity of this tissue to noradrenaline. Data are mean ± s.e.m. normalised to the maximal response in each vessel (n = 6).

electrical stimulation (Fig. 47 a,b). After three seconds of stimulation, tension had almost returned to the baseline value. When normalised to the maximal response for each genotype (60 pulses at 20 Hz), frequency response relationships were roughly similar for all three types of mouse (Fig. 47 c). A slight divergence was seen at 15 pulses at 5Hz, where relative responses in knock-out mice were slightly larger (n = 4 – 6). The fact that contractions were abolished in the presence of 0.3 μM tetrodotoxin or 3 μM guanethidine (n = 4), confirmed that they were evoked by sympathetic nerves.

7.2.2.2 Responses to 3 second trains of stimulation

For experiments to determine the purinergic and adrenergic components of neurogenic contraction, responses were evoked with 60 pulses at 20 Hz. These stimulation parameters yielded reproducible contractions in vessels from all three types of animal. The overall mean amplitude of contractions for wild-type, heterozygote and knock-out mice was 1.16 ± 0.08 g (n = 21), 1.22 ± 0.09 g (n = 16) and 0.70 ± 0.04 g (n = 36), respectively. To ascertain the purinergic component of the contractile response, 10 μM α,β-meATP was applied to desensitise $P2X_1$ receptors. The response that remained when vas deferens tone had returned to baseline during continued α,β-meATP application was taken to be the adrenergic component. Responses were reduced by 79.0 ± 2.6 % (n = 8), 59.9 ± 8.0 % (n = 6) and 9.8 ± 2.6 % (n = 10) in wild-type, heterozygote and knock-out animals respectively (Fig. 48). The adrenergic component of the response expressed in absolute terms was 0.213 ± 0.062 g, 0.314 ± 0.045 g and 0.488 ± 0.097 g for wild-type, heterozygote and knock-out, respectively. Combined application of 0.1 μM prazosin and 10 μM α,β-meATP to block both the adrenergic and purinergic components of contraction abolished responses in tissues from all animals (Fig. 48d).

An alternative way of determining the purinergic component of contraction is to antagonise the $P2X_1$ receptor rather than desensitise it. The pyridoxal phosphate derivative iso-PPADS is a P2X receptor antagonist. iso-PPADS (10 μM) reduced responses in wild-type, heterozygote and knock-out mice by 66.0 ± 7.9 % (n = 4), 66.6 ± 4.2 % (n = 4) and 35.3 ± 7.9 % (n = 6) respectively (Fig. 49). The remaining response, expressed in absolute terms was 0.434 ± 0.083 g, 0.453 ± 0.019 g and 0.438 ± 0.104 g, respectively. Concomitant application of 10μM

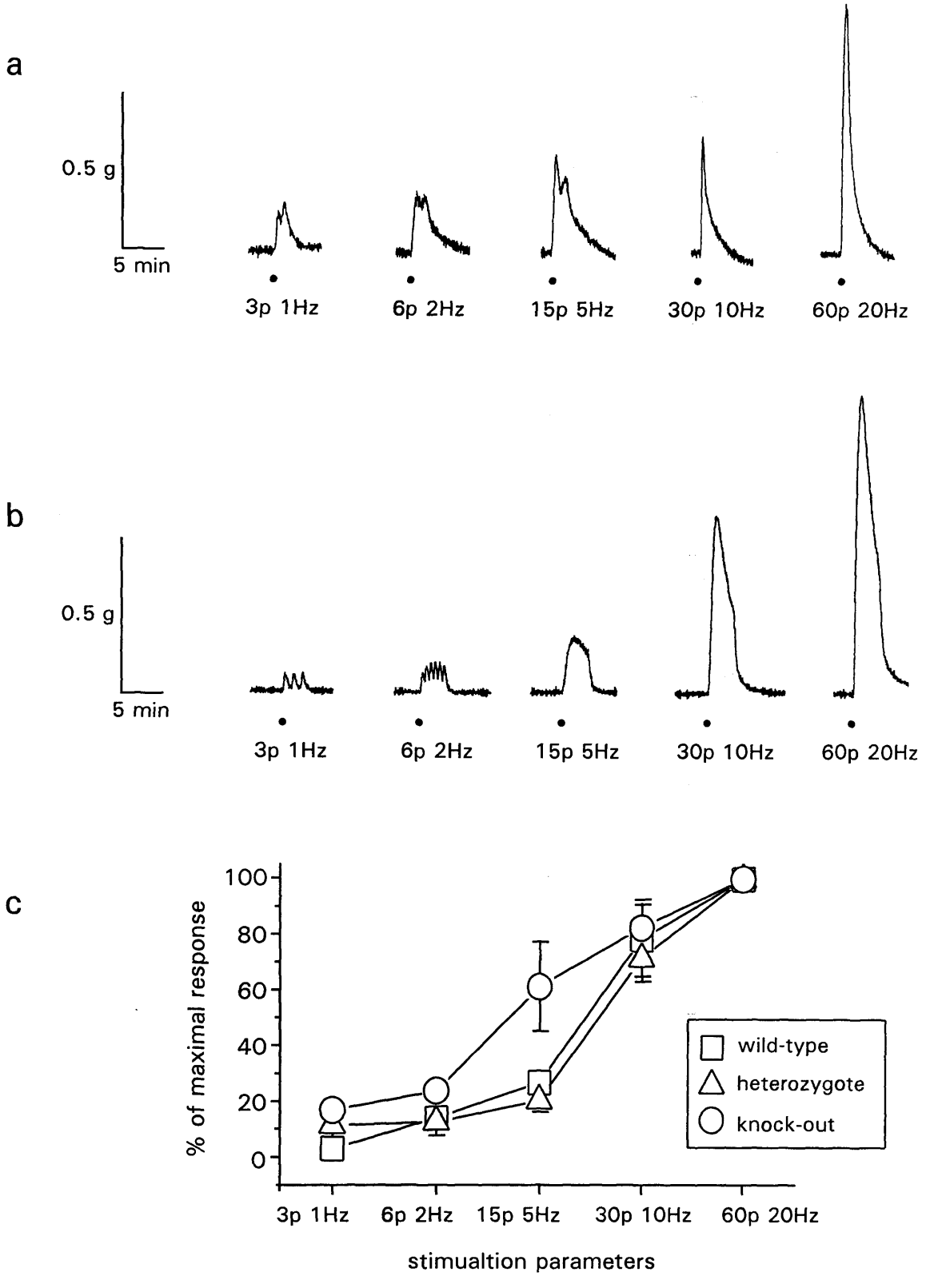

Figure 47 Responses to nerve stimulation in all three types of animal show a similar frequency dependence. Contractile responses in knock-out (a) and wild-type (b) animals to a 3 second train of stimulation at frequencies ranging from 1 Hz to 20 Hz. In wild-type tissue, contractions to each stimulus can be see at low frequencies; at higher frequencies these summate to yield one peak response. Circles indicate beginning of nerve stimulation (c) Frequency-response relationships are very similar for wild-type and heterozygote. Knock-out tissues show a slightly greater relative response to 15 pulses at 5 Hz. Data are mean contractions normalised to the maximal response in each animal ± s.e.m. (n = 4 – 6).

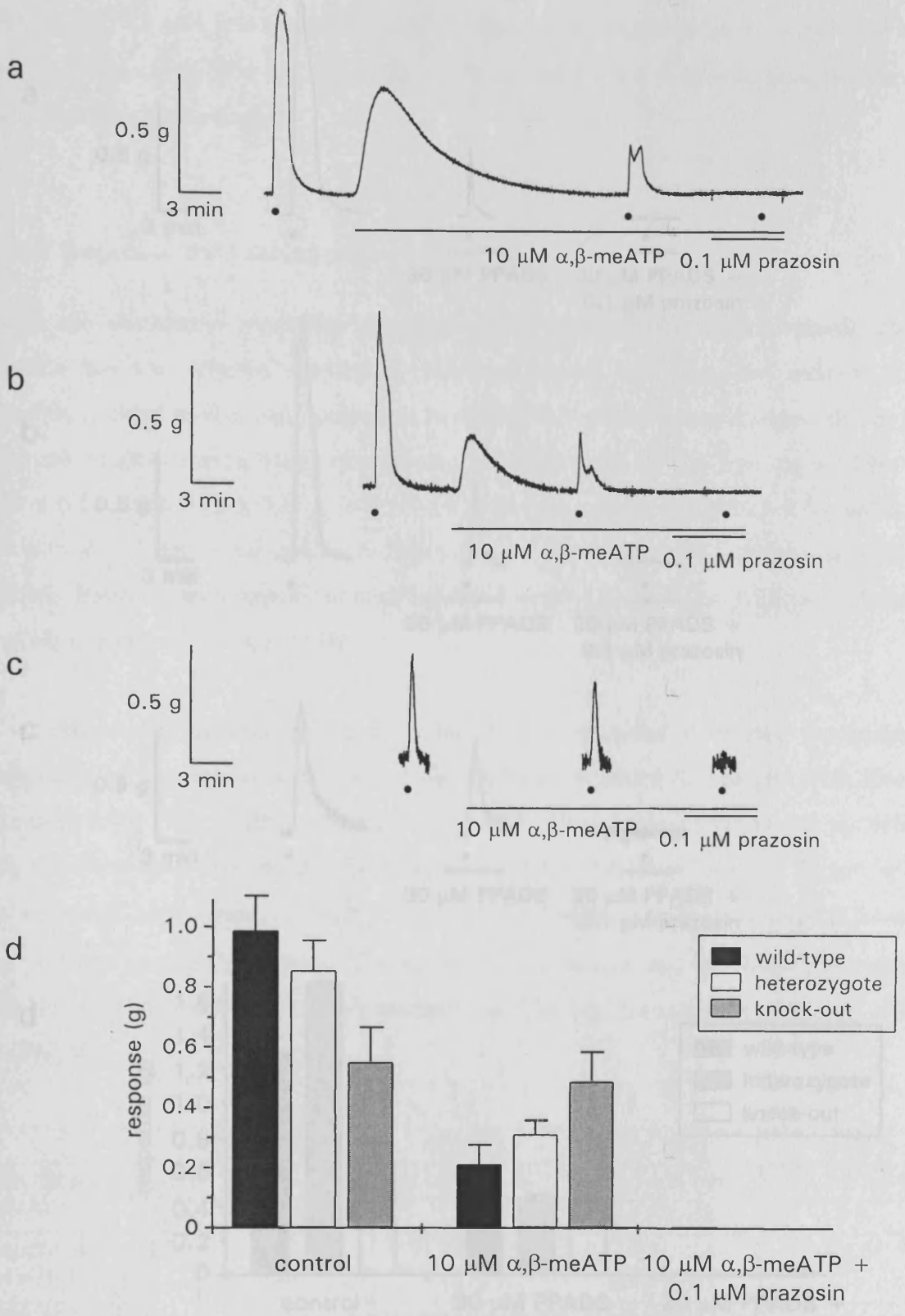

Figure 48 α,β-meATP substantially reduces responses to nerve stimulation in wild-type and heterzygote animals but has little effect in the knock-out. Sample traces from experiments on wild-type (a), heterozygote (b) and knock-out animals (c). Leftmost traces are control responses. Contractions in the continued presence of α,β-meATP (10 μM) represent the adrenergic component, a small proportion of the overall response in wild-type and heterozygote animals. Contractions in knock-out animals are however only slightly reduced under these conditions. Concomitant application of α,β-meATP (10 μM) and prazosin (0.1 μM) abolishes responses in all animals. Bars indicate agonist application and circles indicate beginning of nerve stimulation. (d) Data are mean ± s.e.m. (n = 6 – 10).

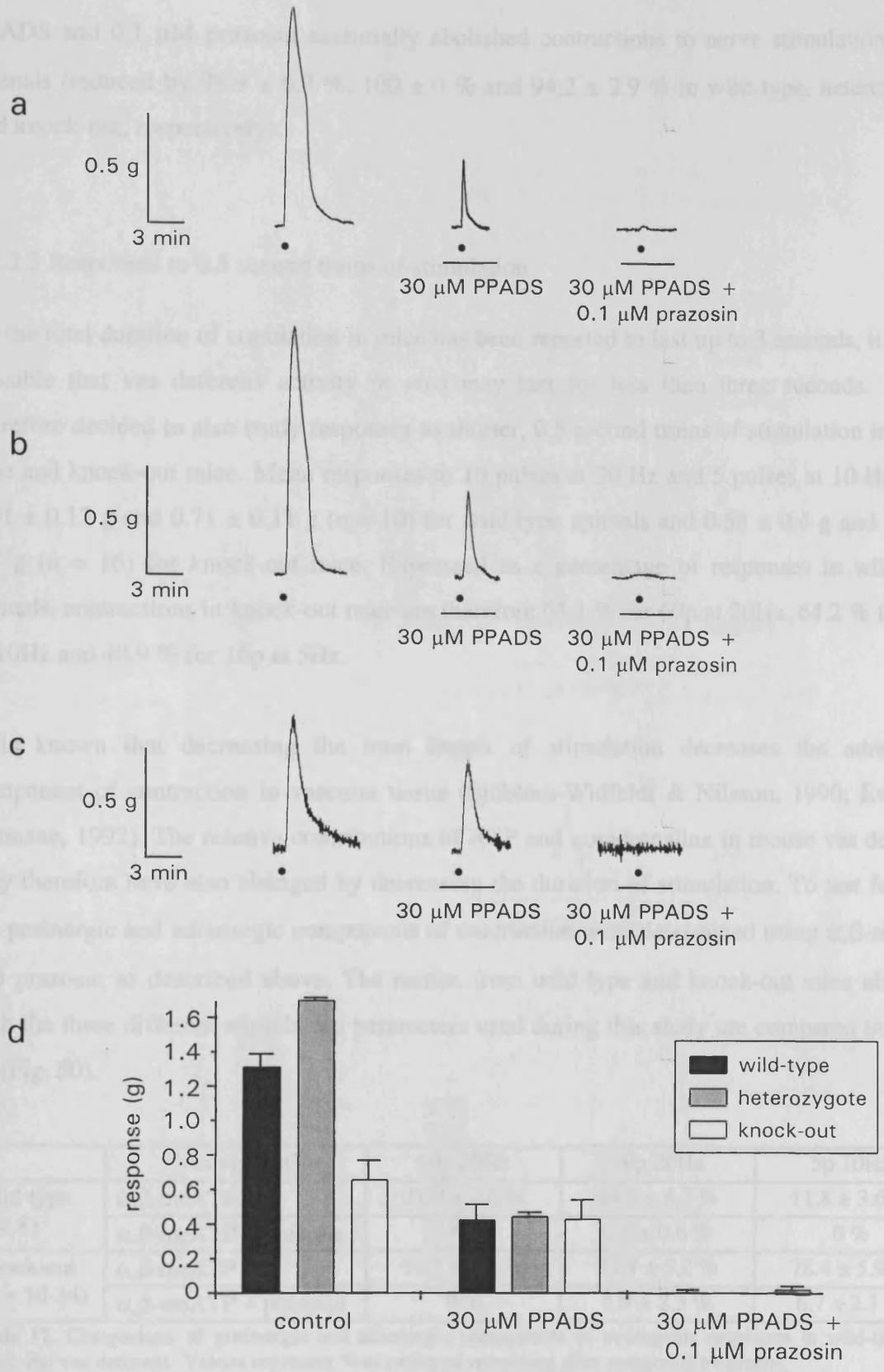

Figure 49 PPADS significantly inhibits neurogenic responses in wild-type and heterozygote animals but only slightly in knock-out animals. Results with PPADS (10 μM) for wild-type (a) heterozygote (b) and knock-out (c) are comparable to those obtained with α,β-meATP (Fig. 48). Combined application of PPADS and prazosin (0.1 μM) also abolishes contractions in each animal. Bars indicate agonist application and circles indicate beginning of nerve stimulation. (d) Data are mean ± s.e.m. (n = 4 – 6). A clear difference to the results obtained with α,β-meATP is that responses remaining in the presence of PPADS in are very similar when expressed in absolute terms.

PPADS and 0.1 μM prazosin essentially abolished contractions to nerve stimulation in all animals (reduced by 98.9 ± 0.7 %, 100 ± 0 % and 94.2 ± 2.9 % in wild-type, heterozygote and knock-out, respectively).

7.2.2.3 Responses to 0.5 second trains of stimulation

As the total duration of copulation in mice has been reported to last up to 3 seconds, it seems possible that vas deferens activity *in vivo* may last for less than three seconds. It was therefore decided to also study responses to shorter, 0.5-second trains of stimulation in wild-type and knock-out mice. Mean responses to 10 pulses at 20 Hz and 5 pulses at 10 Hz were 0.91 ± 0.12 g and 0.71 ± 0.11 g (n = 10) for wild-type animals and 0.58 ± 0.4 g and 0.33 ± 0.4 g (n = 16) for knock-out mice. Expressed as a percentage of responses in wild-type animals, contractions in knock-out mice are therefore 64.1 % for 60p at 20Hz, 64.2 % for 20p at 10Hz and 40.9 % for 10p at 5Hz.

It is known that decreasing the train length of stimulation decreases the adrenergic component of contraction in vascular tissue (Sjöblom-Widfeldt & Nilsson, 1990; Evans & Cunnane, 1992). The relative contributions of ATP and noradrenaline in mouse vas deferens may therefore have also changed by decreasing the duration of stimulation. To test for this, the purinergic and adrenergic components of contraction were determined using α,β-meATP and prazosin as described above. The results from wild-type and knock-out mice obtained with the three different stimulation parameters used during this study are compared in Table 17 (Fig. 50).

	'Antagonist'	60p 20Hz	10p 20Hz	5p 10Hz
wild-type (n= 8)	α,β-meATP	21.0 ± 2.6 %	24.3 ± 6.3 %	11.8 ± 3.6 %
	α,β-meATP + prazosin	0 %	0.9 ± 0.6 %	0 %
knock-out (n = 10-14)	α,β-meATP	91.2 ± 2.6 %	73.7 ± 5.2 %	78.4 ± 5.9 %
	α,β-meATP + prazosin	0 %	8.9 ± 2.5 %	6.7 ± 2.7 %

Table 17. Comparison of purinergic and adrenergic components of neurogenic responses in wild-type and knock-out vas deferens. Values represent % of response remaining after antagonist treatment.

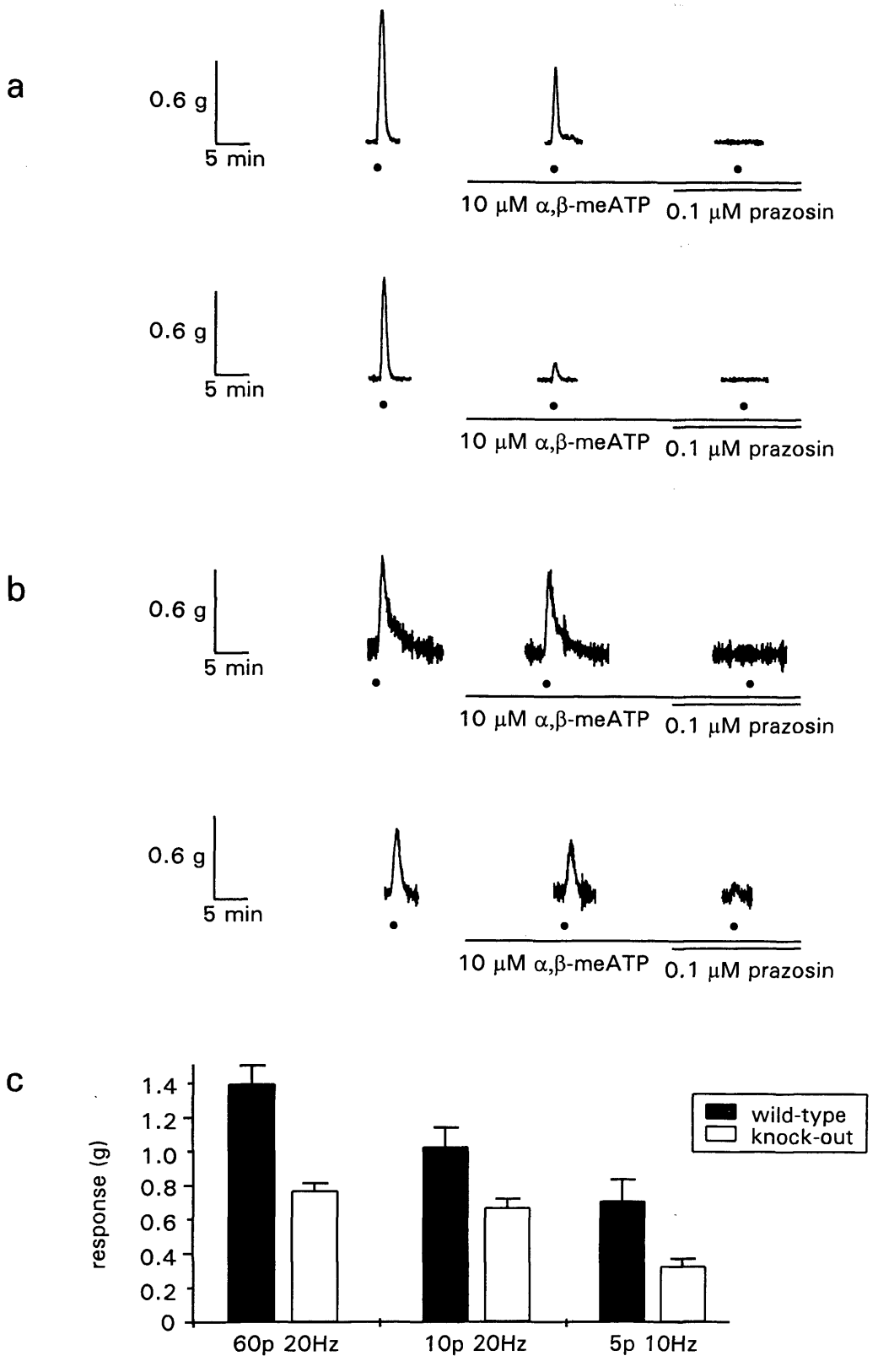

Figure 50 Decreasing the duration of simulation from 3 s to 0.5 s causes a greater reduction in neurogenic responses in knock-out than in wild-type tissue; the purinergic component of contraction in the wild-type is also increased under these conditions. Sample traces from experiments with wild-type (a) and knock-out tissue (b); in each case, top traces are responses to 10 pulses at 20 Hz and bottom traces are responses to 5 pulses at 10 Hz. Note the very large reduction of contractions to 5 pulses at 10 Hz in the presence of α,β-meATP (10 μM) in wild-type animals. α,β-meATP has virtually no effect in the knock-out. Combined application of α,β-meATP (10 μM) and prazosin (0.1 μM) abolishes responses in all tissues. Bars indicate agonist application and circles indicate beginning of nerve stimulation. (c) Data are mean ± s.e.m. comparing contractions in wild-type and heterzygote animals at the three stimulation parameters used (n = 8 – 14).

7.3 MOUSE TAIL GENOTYPING

The recombinant construct that was synthesised and inserted into the $P2X_1$ gene has a nucleotide sequence that is very different to the wild-type. In addition to a portion of the wild-type $P2X_1$ sequence, it contains a *lacZ* gene and a neomycin resistance gene (neo^R). These markers can be used to select for successful genetic modification of cells and to unambiguously determine whether an animal is wild-type, heterozygote or knock-out. Although the genotype of a $P2X_1$ -/- was clear from the pharmacological characterisation of vas deferens tissue, it was nevertheless necessary to determine it directly by testing a sample of DNA. Using primers specific for each sequence, PCR reactions were performed on mouse DNA to detect the wild-type $P2X_1$ or the neomycin resistance gene. Wild-type animals contained only the intact $P2X_1$ gene, knock-out animals contained only the neo^R gene and heterozygotes contained both. This molecular approach was the only way to distinguish between wild-type and heterozygous animals as their phenotypes were virtually identical. Figure 51 shows four examples of such genotyping experiments.

7.4 DISCUSSION

The experiments described in this chapter clearly support the theory that the $P2X_1$ receptor is the P2X isoform that mediates the contractile effects of ATP in vas deferens smooth muscle. This has been demonstrated in experiments comparing contractions evoked by exogenous agonists and nerve stimulation in vas deferens from wild-type, heterozygote and knock-out mice. Contractions to α,β-meATP, L-β,γ-meATP and ATP were abolished in $P2X_1$-deficient mice and the purinergic component of neurogenic contractions was dramatically reduced. The fact that this receptor is present on wild-type vas deferens smooth muscle but absent in knock-out tissue has also been demonstrated using $P2X_1$-specific antibodies (Mulryan *et al*, 2000).

The dramatic effect of deleting functional $P2X_1$ receptors is demonstrated in Figure 45. α,β-meATP and L-β,γ-meATP evoke large and roughly equal contractions in wild-type tissue. ATP, which is much less potent in whole tissue experiments, evokes a smaller response. It contrast, none of these agonists evoke any response in vas deferens from $P2X_1$-deficient

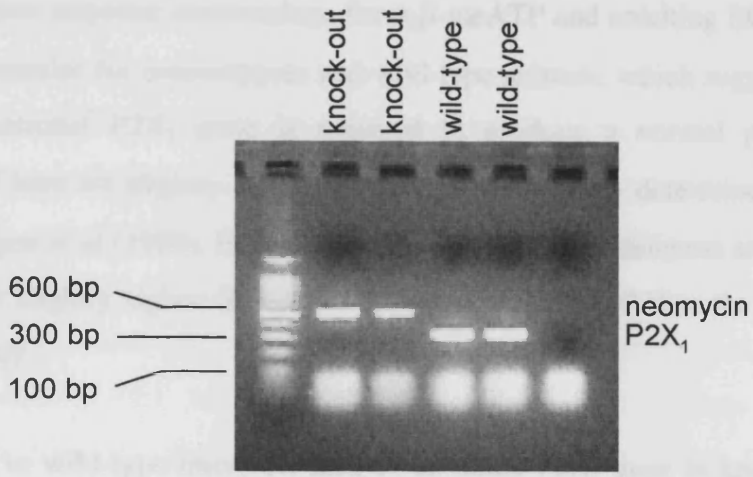

Figure 51 Example of mouse genotype determination by PCR. This procedure allowed the genotype of an animal to be unambiguously identified. PCR was performed using primers specific for either the wild-type $P2X_1$ gene or the neomycin resistance gene which was engineered into the modified $P2X_1$ construct. Of the four animals tested two are wild-type and two are knock-out. A heterozygote animal would have contained one wild-type gene and one modified gene and would therefore have produced two bands.

mice. The fact that ATP is also inactive is very significant and suggests that no other P2X isoforms are involved in mediating its contractile effects. The $P2X_1$ receptor therefore appears to be the only functional P2X isoform expressed in vas deferens smooth muscle. This is further underlined by other data obtained in P2X -/- vas deferens. Electrophysiological studies on single smooth muscle cells showed that all ATP-evoked currents were abolished in this tissue (Mulryan *et al*, 2000).

Concentration response relationships for α,β-meATP and resulting EC_{50} values (pA_{50} values) were very similar for heterozygote and wild-type animals, which suggests that only one copy of the functional $P2X_1$ gene is required to produce a normal phenotype. The figures determined here are slightly higher than 0.7 μM previously determined in the same tissue by von Kügelgen *et al* (1990). EC_{50} values reported in rat vas deferens are identical to (Khakh *et al*, 1995) or slightly higher (Bültmann & Starke, 1993, 1994) than the those determined in the present study.

In contrast to wild-type mice, the lack of an intact $P2X_1$ gene in knock-out animals clearly leads to complete insensitivity to α,β-meATP. The fact that responses to 100 mM KCl were not significantly different between knock-out and wild-type mice shows that deletion of the $P2X_1$ gene did not affect the general contractility of the tissue. One interesting finding is the increased sensitivity of knock-out vas deferens to noradrenaline. The reason for this is unclear but could be due to developmental differences between the animals. It is possible that the lack of functional post-junctional $P2X_1$ receptors leads to an compensatory up-regulation in the expression of post-junctional α_1-adrenoceptors during embryonic development. Knock-out tissue may therefore have a higher density of α_1-adrenoceptors making it more sensitive to noradrenaline.

The fact that vas deferens of knock-out mice was insensitive to ATP suggested that responses of this tissue to nerve stimulation would also be affected by deletion of the $P2X_1$ gene. This was tested by comparing neurogenic contractions in wild-type and knock-out animals. The relative contractile responses show a similar frequency dependence in each tissue, suggesting similar characteristics of transmitter release. Interestingly, the slightly different shape of the curve between knock-out and wild-type animals is reproduced in a recent study by Guitart *et*

al (1999), where the epididymal and prostatic portion of the rat vas deferens were investigated separately. The epididymal section is known to exhibit mainly adrenergic transmission while the prostatic half is mainly purinergic; the former is therefore equivalent to tissue from a knock-out mouse while the latter is more similar to wild-type vas deferens. Indeed, the slightly greater responses seen at low frequency stimulation are both seen in epididymal and knock-out vas deferens but not in prostatic or wild-type. The reason for this slight difference is therefore most likely due to the differential dominance of the two transmitters.

When expressed in absolute terms it is however clear that the same stimulation parameters evoke much smaller contractions in knock-out vas deferens than in wild-type tissue. The reduction is presumably due to the loss of ATP-mediated transmission in $P2X_1$-deficient mice. The fact that PPADS significantly reduced contractions in wild-type but was less effective in knock-out animals supports this conclusion. PPADS has previously been shown to be an effective P2X receptor antagonist in rodent vas deferens (McLaren *et al*, 1994; Khakh et al, 1994; Westfall *et al*, 1997; Knight & Burnstock, 1998). Combined application of PPADS and prazosin abolishes all responses, which shows that all post-junctional effects of ATP were blocked by PPADS.

Another means of blocking $P2X_1$ receptors is by desensitisation with α,β-meATP. The difference between wild-type and knock-out animals was in fact more pronounced when using α,β-meATP than with PPADS: a substantial reduction in wild-type and little effect in knock-out. The reason for greater inhibition of wild-type responses may lie in the differing potencies of the two compounds: α,β-meATP is a potent and selective $P2X_1$ receptor agonist and will very efficiently desensitise this receptor. PPADS is a general P2X antagonist and has a lower affinity for the $P2X_1$ receptor (compare Michel & Humphrey, 1993 and Khakh *et al*, 1994). However, in the case of knock-out animals, α,β-meATP was less potent than PPADS. This suggests that PPADS-sensitive isoforms other than $P2X_1$ may be present on vas deferens smooth muscle; although the fact that α,β-meATP abolished neurogenic responses when combined with prazosin would argue against additional receptors. Another possible reason may lie in the fact that P2 receptor antagonists have been shown to not only inhibit P2 receptors but also ectonucleotidase enzymes in rat vas deferens (Bültmann *et al*, 1999b). This

might explain the larger responses of wild-type animals in the presence of PPADS compared to α,β-meATP. PPADS may also be having prejunctional effects, which may account for the observed difference in effects. Experiments using either PPADS or α,β-meATP demonstrate that the purinergic component of neurogenic contraction is virtually absent in mice lacking $P2X_1$ receptors. This agrees with other data, which shows that EJPs evoked by electrical stimulation of vas deferens tissue are abolished in animals lacking the receptor (Mulryan *et al*, 2000). Both results imply that ATP cannot exert any postjunctional effect in $P2X_1$ -/- tissue. This again lends support to the theory that this is the P2X isoform mediating all effects of ATP in vas deferens smooth muscle.

One interesting aspect of my results is the component of contraction remaining after antagonist application. When expressed in absolute terms, responses after PPADS treatment are similar in all three animals, while after α,β-meATP treatment, responses are smaller in wild-type than in knock-out animals. The results of inhibition using α,β-meATP are perhaps more expected: the adrenergic component remaining after treatment is largest in knock-out, followed by heterozygote and wild-type. Concentration response curves in $P2X_1$-deficient mice showed a clear increase in the sensitivity to noradrenaline. If the same amount of transmitter is released, responses in knock-out vas deferens will therefore be larger than in wild-type. When PPADS is the antagonist, the remaining contractions in wild-type and heterozygous animals are similar to those in knock-out animals. PPADS and α,β-meATP therefore do not have entirely the same effect; this may again be a reflection of pre-junctional factors. Selective antagonists would be very useful in this case as the contribution of different isoforms to the observed effect could be studied individually.

The three-second train of stimulation chosen may be too long in view of the total duration of copulation (see section 7.2.2.2). Responses to a shorter 0.5-second train were therefore characterised. It is known that decreasing the duration and frequency of sympathetic nerve stimulation decreases the adrenergic component of contraction allowing the purinergic component to become more dominant (Evans & Cunnane, 1992). As contractions in knock-out mice are entirely adrenergic, the amplitude of the contractile response might be expected to decrease more in knock-out mice than in wild-type animals. My results confirm that this is

the case. This is underlined by experiments in wild-type animals using α,β-meATP, which show that the purinergic component is greatest at stimulation with 5 pulses at 10 Hz.

The experiments discussed above provide clear evidence for the crucial role that $P2X_1$ receptors play in mediating P2X receptor-evoked smooth muscle contraction. The total lack of responsiveness to α,β-meATP in knock-out tissue confirms the lack of functional $P2X_1$ receptors in these animals. Similar sensitivity to KCl and noradrenaline in knock-out and wild-type tissue indicates that this effect is not caused by a general loss of contractility. The fact that ATP is inactive in $P2X_1$ -/- tissue indicates that this isoform mediates all post-junctional effects of ATP. Other data also supports this conclusion: ATP evokes no currents in vas deferens smooth muscle cells and no EJPs are evoked in vas deferens upon electrical stimulation. $P2X_1$-deficient mice also show a similar loss of ATP-mediated responses in arterial smooth muscle (personal communication from C. Vial). A slightly increased potency of noradrenaline in knock-out tissue may indicate developmental compensation for the lack of $P2X_1$ receptors. Loss of ATP-based sympathetic transmission is reflected by significantly smaller contractile responses to nerve stimulation in knock-out animals. This is also demonstrated by the modest reduction of responses caused by $P2X_1$ receptor desensitisation or antagonism. Further strong evidence for the importance of $P2X_1$ receptors in normal vas deferens function comes from the substantial difference in reproductive physiology between wild-type and knock-out animals: fertility in male $P2X_1$ -/- mice is reduced by over 90 % (Mulryan *et al*, 2000). This may have important implications for pharmacological interventions in human fertility (i.e. $P2X_1$ receptor antagonism for contraceptive purposes or enhancement of $P2X_1$ receptor activity for the treatment of infertility).

8 GENERAL DISCUSSION

One of the key factors controlling blood pressure is vascular tone which is in turn predominantly regulated by the sympathetic nervous system. This neuronal control of arterial smooth muscle tone is known to be mediated by both noradrenaline and ATP, which are co-released from sympathetic nerves (e.g. Sneddon & Burnstock, 1984b; Ren *et al*, 1996). The relative contribution of the two transmitters has been the subject of extensive investigation and has been found to vary depending on the vessel and experimental conditions (e.g. MacDonald *et al*, 1992; Todorov *et al*, 1999). There is evidence to suggest that purinergic component of the response increases as arterial diameter decreases (e.g. Evans & Surprenant, 1992) but the effect of vessel size *per se* has not yet been studied. Another important aspect concerning the regulation of arterial tone is the source of elevated intracellular calcium that is required for smooth muscle contraction. There are several processes which can produce this rise in cytosolic calcium concentration: calcium entry through the P2X channel, calcium entry through voltage-dependent calcium channels or calcium release from intracellular stores. Different studies into the relative roles of these pathways have produced a range of results, depending on the animal and the tissue studied. Certain data suggest that influx through the P2X receptor channel may be the principal means of raising calcium levels in resistance arteries (Galligan *et al*, 1995); however the effect of arterial diameter was again not studied. The literature therefore contains examples which suggest heterogeneity in the characteristics of neurogenic and purinergic vasoconstriction depending on the size of the artery, but there is a lack of data that specifically addresses this question. The experiments in this thesis were undertaken to substantiate this potential trend in the regulation of arterial function. The effect of arterial diameter on the above-mentioned properties of neuronal control of smooth muscle tone has been systematically studied, using the rat mesentery as a model vascular bed.

Numerous studies have demonstrated that the $P2X_1$ receptor is the P2X isoform that mediates the effects of ATP in smooth muscle. Early studies by Benham & Tsien (1987) show the characteristic transient current responses to ATP in single cells and experiments in whole arteries confirmed that contractions were evoked through an α,β-meATP and suramin sensitive P2X receptor (Evans & Surprenant, 1992; Khakh *et al*, 1995). All these properties are characteristic of

$P2X_1$ receptors. These findings are supported by work on cloned (Valera *et al*, 1994; Evans *et al*, 1995; Bianchi *et al*, 1999) as well as native channels (Lewis & Evans, 2000a). My experiments have provided further strong evidence for the central role of the $P2X_1$ receptor in mediating the effects of ATP in arterial smooth muscle:

i) The pharmacological analysis of functional responses at the single cell level showed characteristic transient currents in response to α,β-meATP that were suramin sensitive in all arteries.

ii) Immunohistochemical determination of the expression patterns of P2X isoforms revealed high levels of $P2X_1$ protein in all vessels; in large arteries these results were also confirmed by RT-PCR.

iii) P2X receptor-mediated responses were lost in vas deferens from $P2X_1$ -/- transgenic mice. (this data is supported by similar results in mesenteric arteries; personal communication from C. Vial)

From these data it is therefore clear that the $P2X_1$ receptor is the crucial P2X isoform in arterial smooth muscle function.

These data contrast with whole artery studies where the characterisation of functional responses revealed a different pattern. While small arteries retained the characteristic properties of $P2X_1$ receptors, large arteries were relatively insensitive to both α,β-meATP and suramin. This diameter-dependent difference in functional profile is underlined by the fact that neurogenic contractions in large arteries are almost entirely adrenergic while those in smaller vessels are mainly purinergic. Clear differences therefore exist between the contractile phenotype of large and small arteries.

Such functional heterogeneity is perhaps not surprising. Large conduit arteries and resistance arteries perform different functions in the circulation and might therefore be expected to have different phenotypic properties. The literature contains several other examples of such regional variation. Experiments in rat pulmonary arteries showed that calcium activated chloride currents are more prominent in large conduit arteries than small resistance vessels (Clapp *et al*, 1996). Diameter-dependent differences have also been described for L-type calcium currents (Bowles *et al*, 1997) and inward rectifier potassium currents (Quayle *et al*, 1996) in porcine coronary

arteries. There are also examples of such effects in purinergic control of arterial smooth muscle tone. An early example is the finding by Krishnamurty & Kadowitz (1983) that the degree of ATP-induced relaxation of rabbit mesenteric arteries pre-contracted with 60 mM KCl varied with the diameter of the vessel. Shinozuka *et al* (1991) found that sympathetic transmission in small rabbit pulmonary arteries was much less susceptible to purinergic modulation than in the main pulmonary artery. Other observations include differing contributions of NO and EDHF to P2Y receptor-mediated vasodilatation in rat cerebral arteries (You *et al*, 1999) and differential effects of diadenosine polyphosphates in pre-contracted mesenteric arteries (Steinmetz *et al*, 2000b); both dependent on arterial diameter. Diameter-dependent differences in function responses have therefore been demonstrated in several vascular beds.

A possible reason for the observed differences may be as follows: the small purinergic component of sympathetic vasoconstriction in large arteries as well as their insensitivity to $P2X_1$ receptor agonists may be important in injury. Under normal conditions, the tone of resistance arteries is under the continual control of sympathetic nerves. Large arteries however, do not contract in response to the low concentrations of ATP liberated from sympathetic nerve terminals. In addition, arterial smooth muscle cells are shielded from circulating nucleotides by the intact endothelium. However, during injury the blood vessel and surrounding tissue is damaged and disruption of the endothelium allows nucleotides in the circulation to interact directly with the smooth muscle layer. As large quantities of ATP are released from damaged cells and activated platelets, the concentration of ATP at the site of injury will rise dramatically. Large arteries will now contract in response to high local concentrations of ATP with the aim of limiting blood loss from the injury. The elevated levels of ATP associated with tissue damage may have the additional effect of activating P2X receptors on sensory neurons to convey nociceptive signals to the CNS (Burnstock, 1996; Bland-Ward & Humphrey, 2000). This may be one of the mechanisms that makes injury painful.

Small arteries are known to be most important in controlling blood flow and blood pressure (Mulvany *et al*, 1996). The fact that ATP-mediated transmission dominates in small rather than large vessels therefore lends great support to the notion that ATP plays a key role in regulating blood pressure. As a result, disruption of purinergic transmission in blood vessels could have

substantial anti-hypertensive effects, a theory confirmed by *in vivo* studies. Dalziel *et al* (1990) found that intravenous administration of α,β-meATP in pithed rats, substantially inhibited the increase in blood pressure caused by stimulation of pre-ganglionic sympathetic nerves. More physiologically relevant evidence comes from experiments on conscious, freely moving rats. Here, α,β-meATP (after an initial increase) caused a substantial drop in mean arterial blood pressure (Tarasova *et al*, 1998). This study is of some interest as the effect of P2X receptor desensitisation was assessed on normally fluctuating blood pressure rather than on changes in blood pressure induced by nerve stimulation. It suggests that blocking transmission through $P2X_1$ receptors will reduce steady state blood pressure *in vivo* (Curiously, PPADS had no such lowering effect; possible reasons for this may be prejunctional effects of PPADS or insufficient dosage).

In our studies in mice, deletion of functional $P2X_1$ receptors had no substantial effect on mean arterial blood pressure (Mulryan *et al*, 2000), despite the loss of purinergic transmission in arteries from P2X -/- animals (personal communication from C. Vial). This finding appears to contradict the view that $P2X_1$ receptors are important in regulating blood pressure; it can however be explained by developmental compensation. In the transgenic mouse, $P2X_1$ receptors are absent from the outset, and as a result, processes can be initiated from the earliest stages of development to counteract this deficiency. The fact that noradrenaline is more potent in vas deferens from $P2X_1$ -/- mice (Chapter 7) for example, suggests a higher density of α_1-adrenoceptors in this tissue (although such changes were not seen in arteries; personal communication from C. Vial). The results from Tarasova *et al* (1998) however imply that antagonising the $P2X_1$ receptor can be effective when no such compensation has occurred. Judging by the loss of all post-junctional responses to ATP in vas deferens from $P2X_1$ -/- mice, blockade of this receptor should be sufficient to abolish the purinergic component of sympathetic vasoconstriction.

In Chapter 4, I have shown that calcium entry through voltage-dependent calcium channels is negligible compared to P2X receptor-mediated influx. Other studies in different vessels have however shown significant inhibition of purinergic contractions by nifedipine. It is therefore of interest to determine whether sufficient calcium can enter the cell through P2X receptors to

evoke contraction. It has been estimated that roughly 6 % of the current through P2X receptors is carried by calcium (Benham & Tsien, 1987), while essentially all of the current through voltage-gated calcium channels is carried by calcium. Comparing the current amplitudes can therefore give some indication as to how much calcium flows through P2X receptors. Using medium-sized arteries as an example, the maximal calcium current density is 3.8 pA/pF. The mean current density for 10 μM α,β-meATP in the same tissue is 298 pA/pF and 6 % of the current carried by calcium would be equivalent to 17.9 pA/pF. The calcium component of the P2X current is therefore substantially larger than the voltage-gated calcium current. This is of course a very crude approximation which does not take several considerations into account: the ratio of calcium to P2X channels may not be the same in the rabbit ear artery (Benham & Tsien, 1987) as in the rat mesenteric artery; the *in vivo* concentration of ATP is probably higher than 10 μM; not all cells will be exposed to transmitter *in vivo* as they are during agonist application; *in vivo* the mainly sodium-mediated depolarisation will reduce the driving force for calcium entry; *in vivo* depolarisation will be counter-acted by voltage-gated potassium channels resulting in less calcium entry. Despite these limitations, this rough estimate does however show that calcium influx through L-type calcium channels may not be required because substantial calcium entry can occur through P2X receptors. Previous estimates also suggest that P2X receptors can mediate significant calcium influx (Benham, 1989).

In summary, one can therefore highlight potential clinical implications of the data presented in this thesis. My results indicate firstly, that purinergic transmission is substantially more important than adrenergic transmission in mediating sympathetic vasoconstriction of small resistance arteries and secondly, that the great majority of calcium required for arterial smooth muscle contraction is provided by calcium entry through the P2X channel. The L-type voltage-dependent calcium channel blocker nifedipine is a commonly used anti-hypertensive drug and α-adrenoceptor antagonists have also had some therapeutic applications. My data suggest that these two approaches may not be as useful as previously thought. A selective $P2X_1$ receptor antagonist on the other hand may potently reduce blood pressure. It will therefore be of interest to monitor the development of more potent and selective pharmacological tools for P2X receptors, especially if the are shown to be active after oral administration. Clearly, my findings cannot be considered universally applicable. They were obtained in one vascular bed that was used as a

model system. Different conditions may apply in different parts of the vasculature and results in humans may be different to those obtained in rats. However, they do demonstrate the greater importance of purinergic transmission in resistance arteries and suggest that this area merits further investigation. I have therefore answered the key question posed at the outset of my PhD studies. I have confirmed the hypothesis that there is a heterogeneity in the role of ATP in mediating arterial smooth muscle contraction depending on the diameter of the vessel. My results begin to give a pattern to the rather confused and inconclusive data in the literature and underline the substantial influence of vessel size in studies investigating the regulation of arterial smooth muscle tone.

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APPENDIX 1

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Effects of diadenosine polyphosphates (Ap_nAs) and adenosine polyphospho guanosines (Ap_nGs) on rat mesenteric artery P2X receptor ion channels

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1 Diadenosine polyphosphates (Ap_nAs, $n=3-7$) and adenosine polyphospho guanosines (Ap_nGs, $n=3-6$) are naturally occurring vasoconstrictor substances found in platelets. These vasoconstrictor actions are thought to be mediated through the activation of P2X receptors for ATP. The effects of Ap_nAs and Ap_nGs at P2X receptors on rat mesenteric arteries were determined in contraction studies and using the patch clamp technique on acutely dissociated artery smooth muscle cells.

2 $P2X_1$ receptor immunoreactivity was detected in the smooth muscle layer of artery rings. The sensitivity to α,β-methylene ATP and desensitizing nature of rat mesenteric artery P2X receptors correspond closely to those of recombinant $P2X_1$ receptors.

3 Ap_4A, Ap_5A and Ap_6A evoked concentration dependent P2X receptor inward currents which desensitized during the application of higher concentrations of agonist. The agonist order of potency was $Ap_5A \geqslant Ap_6A \geqslant Ap_4A >> Ap_3A$. Ap_2A and Ap_7A were ineffective. Similar results were obtained in contraction studies except for Ap_7A which evoked a substantial contraction.

4 Ap_nGs ($n=2-6$)(30 μM) evoked P2X receptor inward currents in mesenteric artery smooth muscle cells. Ap_nGs ($n=4-6$) were less effective than the corresponding Ap_nA.

5 This study shows that at physiologically relevant concentrations Ap_nAs and Ap_nGs can mediate contraction of rat mesenteric arteries through the activation of $P2X_1$-like receptors. However the activity of the longer chain polyphosphates ($n=6-7$) may be overestimated in whole tissue studies due to metabolic breakdown to yield the P2X receptor agonists ATP and adenosine tetraphosphate.

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Keywords: P2X receptors; artery; diadenosine polyphosphates; $P2X_1$ receptors; vasoconstriction

Abbreviations: α,β-meATP, α,β-methylene ATP; Ap_nAs, diadenosine polyphosphates; Ap_nGs, adenosine polyphosphoguanosines; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline

Introduction

Platelets store and release a range of vasoactive nucleosides including diadenosine polyphosphates (Ap_nAs, $n=3-7$) and adenosine polyphospho guanosines (Ap_nGs, $n=2-6$) which can act through P2X receptors for ATP to mediate vasoconstriction (Busse *et al.*, 1988; Schlüter *et al.*, 1994; 1998; Jankowski *et al.*, 1999). P2X receptors are ligand gated cation channels and their activation leads to membrane depolarization, calcium influx and smooth muscle contraction (Evans & Surprenant, 1996). Seven genes encoding P2X receptors have been isolated ($P2X_{1-7}$) (Collo *et al.*, 1996; Surprenant *et al.*, 1996; Ralevic & Burnstock, 1998). Artery smooth muscle P2X receptors are sensitive to the metabolically stable ATP analogue α,β-methylene ATP (α,β-meATP) and responses rapidly desensitize during the continued presence of agonist. The $P2X_1$ receptor is the predominant isoform expressed by smooth muscle and its properties are similar to those of the native arterial smooth muscle P2X receptor (Valera *et al.*, 1994; Collo *et al.*, 1996; Evans & Surprenant, 1996; Lewis *et al.*, 1998; McLaren *et al.*, 1998).

The vasoconstrictor effects of a range of naturally occurring Ap_nAs and Ap_nGs have been determined in a limited number of studies on perfused vascular beds. This work has shown that the activity of these compounds is dependent on the length of the polyphosphate chain (Ralevic *et al.*, 1995; van der Giet *et al.*, 1997; Schlüter *et al.*, 1998). Similar results have been reported for the actions of Ap_nAs at recombinant $P2X_1$ receptors (Evans *et al.*, 1995; Wildman *et al.*, 1999; Bianchi *et al.*, 1999). However a direct quantitative comparison of the effects of Ap_nAs at arterial smooth muscle and recombinant $P2X_1$ receptors is difficult to make as drugs were applied in a bolus in the perfused vascular bed experiments and thus the effective agonist concentration at the receptor was not determined. In addition the metabolism of nucleotides by ecto-enzymes can lead to underestimating agonist potency in organ bath studies (Benham & Tsien, 1987; Inoue & Brading, 1990; Evans & Kennedy, 1994; Trezise *et al.*, 1994). For example recent studies have suggested that Ap_4A may be subject to ecto-enzyme breakdown (Westfall *et al.*, 1997). Complications associated with agonist breakdown can be overcome in patch clamp studies on acutely dissociated smooth muscle cells where drugs can be applied rapidly under concentration clamp conditions (Evans & Kennedy, 1994). This system has the added advantage that currents recorded in response to drugs give a direct measure of activation of the P2X receptor channels. The primary aim of this study was to characterize the effects of a range of Ap_nAs, Ap_nGs and related compounds on rat mesenteric artery smooth muscle P2X receptors to allow comparisons to be made between native smooth muscle and recombinant $P2X_1$ receptors and to investigate further the structure activity relationships of these compounds.

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Methods

Male wistar rats (250–300 g) were killed by cervical dislocation and femoral exsanguination. The mesentery was removed and second and third order mesenteric arteries were dissected. For contraction experiments second order mesenteric artery rings were mounted in a Mulvany myograph (vessel diameter 226.5±11.4 μm, $n=8$) (Lagaud *et al.*, 1996). The arteries were superfused at 2 ml min^{-1} with a warmed physiological saline with the following composition (mM): NaCl 150, KCl 5, HEPES 10, $CaCl_2$ 2.5, $MgCl_2$ 1, (pH to 7.3 with NaOH). The organ bath temperature was 37°C. Drugs were added to the superfusate. The vasoconstrictor agonists evoked transient contractions of the artery that returned to baseline diameter in the continued presence of purinergic drugs (Figure 2a). Reproducible contractions were obtained when agonists were applied at 30 min intervals.

Acutely dissociated smooth muscle cells were prepared by a two step papain and collagenase/hyaluronidase enzymatic digestion (see Quayle *et al.*, 1996). Cells were plated onto glass coverslips, stored at 4°C and used within 2–36 h. For electrophysiological recording smooth muscle cells were superfused at 2 ml min^{-1} with physiological solution and drugs were applied rapidly using a U-tube perfusion system (Evans & Kennedy, 1994). Amphotericin permeabilized patch recordings were made with an Axopatch 200B amplifier and data was collected using pClamp6 software (Axon Instruments U.S.A.). Holding potential was −60 mV. Patch electrodes (2–5 MΩ) were filled with a solution of the following composition (mM): potassium gluconate 140, NaCl 5, HEPES 10, EGTA 9, (pH adjusted to 7.3 with KOH). Reproducible responses to agonists (200–500 ms pulse duration) were obtained when a 5 min interval was given between agonist applications.

Data are reported throughout as mean±s.e.mean, n=number of observations. Peak currents in response to drugs are expressed as percentage of the response to 10 μM α,β-meATP, this is a maximal concentration at rat mesenteric artery P2X receptors (Lewis *et al.*, 1998). It was not always possible to construct a concentration response curve in a single smooth muscle cell therefore pooled concentration response data for agonists were fitted by the least squares method using Origin software (Microsoft U.S.A.) with the equation; $response = \alpha[A]^H/([A]^H + [A_{50}]^H)$ where α and H are the asymptote and Hill coefficient, [A] is the agonist concentration, A_{50} is the agonist concentration producing 50% of the maximum agonist response (EC_{50}). Differences between means were determined by Student's unpaired t-test (two tailed) and were considered significant when $P<0.05$.

For immunohistochemical studies second and third order mesenteric arteries were dissected as for electrophysiology experiments and embedded in Tissue-Tek (Miles Inc, Elkhart, In.) and frozen over dry ice and hexane. Frozen transverse arterial 12 μm sections were cut and mounted on pre-subbed slides. The sections were fixed in a 2% paraformaldehyde solution in Sorensons buffer for 10 min. After washing in a phosphate buffered saline (PBS) solution the slides were incubated in a blocking solution (10% donkey serum (Jackson Immunoresearch), 0.5% Triton-X (Sigma) in PBS for 30 min at room temperature to permeabilize the tissue and reduce non-specific binding. Sections were then washed and incubated overnight in primary antisera (Anti-$P2X_1$±control antigen blocking peptide, Alomone Lab. Israel) at 4°C. Anti-$P2X_1$ is a polyclonal antibody raised in rabbit against the C-terminal residues 382–399 of the $P2X_1$ receptor. The anti-$P2X_1$ receptor antibody was used at a dilution of 1:200 in 10% donkey serum in PBS. When blocking peptides were used, the primary antibody and its corresponding antigen peptide were incubated together (1 mg of each) for 1 h at room temperature. After washing in PBS, sections were incubated in secondary antisera for visualization, which in each case was fluorescein isothiocyanate (FITC) labelled anti-rabbit IgG raised in donkey (Jackson Immunoresearch) (1:100 in 10% donkey serum in PBS) for 2 h at room temperature. As a control to determine the amount of non-specific binding, slides were incubated in 10% donkey serum in PBS only or secondary antisera only. Slides were washed in PBS and mounted in Citifluor (UKC Chem Lab, U.K.). Arterial sections were examined under epifluorescence with neutral density filters. Images were captured using Scionimage software.

Drugs: papain, dithioerythritol, collagenase, hyaluronidase, α,β methylene ATP (α,β-meATP, Ap_2A, Ap_3A, Ap_4A, Ap_5A, Ap_6A, GTP, adenosine tetraphosphate, tetrapolyphosphate (Hexa-ammonium salt), and trisodium trimetaphosphate (Sigma). HPLC analysis of diadenosine polyphosphates from Sigma has shown that they are of high purity and are not contaminated with ATP (Pintor *et al.*, 1996). Ap_7A, Ap_2G, Ap_3G, Ap_4G, Ap_5G, and Ap_6G (synthesized and HPLC purified by H. Schlüter).

Results

Effects of Ap_nAs on acutely dissociated rat mesenteric smooth muscle cells

The diadenosine polyphosphates Ap_4A, Ap_5A and Ap_6A evoked concentration dependent inward currents which declined during the continued presence of high concentrations of agonist (Figure 1a). Ap_5A evoked responses had an EC_{50} of 6.7 μM (pA_{50} 5.23±0.12) and a slope of 1.1±0.23, the maximal response to Ap_5A was 80.8±7.2% of the response to 10 μM α,β-meATP ($n=5$). The mean concentration response relationship for Ap_6A gave an EC_{50} of ~13 μM and a maximum of 43±7.5% of the response to 10 μM α,β-meATP ($n=5-7$) (Figure 1a,b). No clear maximum response to Ap_4A was recorded at concentrations up to 300 μM ($n=4$) (~50 μM Ap_4A evoked a response equivalent to 50% of the maximal α,β-meATP response). Ap_3A evoked a small current at 100 μM which was 21.3±5.7% of the maximal α,β-meATP evoked response. In cross desensitization studies following α,β-meATP (10 μM) treatment no response to Ap_nAs (all 30 μM, $n=3-6$) was recorded indicating that all these agonists acted at the same receptor. No response was recorded to applications of Ap_2A or Ap_7A (30 and 100 μM) ($n=4-6$).

Comparison of Ap_nA evoked contractions in intact arteries and currents recorded from isolated smooth muscle cells

The majority of studies on arterial smooth muscle P2X receptors have recorded contractile responses. These whole tissue pharmacological studies may be complicated by problems associated with agonist metabolism by ecto-enzymes and the contractile response only gives an indirect measure of P2X receptor activation. It was therefore of interest to compare Ap_nA mediated P2X receptor inward currents and contractions in the rat mesenteric artery. Ap_5A evoked rapid, concentration dependent, transient constrictions of rat mesenteric artery wings which returned to baseline during the continued presence of the agonist (Figure 2a). The EC_{50} values for evoking contraction and inward currents were essentially the same (6.5 and 8.5 μM respectively, Figure 2b). The

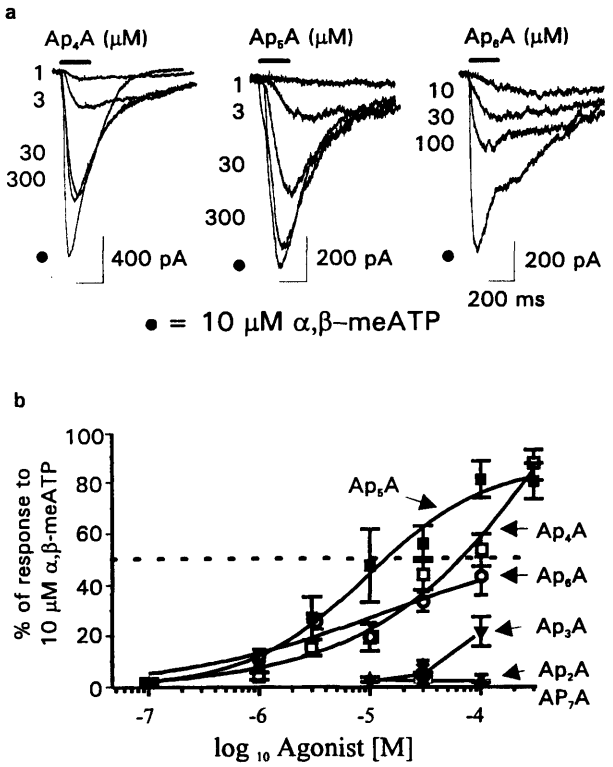

Figure 1 P2X receptor mediated inward currents evoked by Ap_nAs in acutely dissociated rat mesenteric smooth muscle cells. (a) Representative traces of inward currents evoked by Ap_4A, Ap_5A and Ap_6A compared to the current evoked by 10 μM α,β-meATP. Drugs were applied for 200 ms indicated by the bar. (b) Concentration response relationships for inward currents evoked by Ap_nA ($n=2-8$). Each point is the mean percentage of the response to 10 μM α,β-meATP ($n=4-7$ for each point).

maximal constrictor response was $113\pm11.6\%$ of the response to 10 μM α,β-meATP compared to $80.8\pm7.2\%$ for inward currents. Following desensitization of smooth muscle P2X receptors in the continued presence of 10 μM α,β-meATP the response to 100 μM Ap_5A was abolished ($n=4$) (Figure 2c). These cross desensitization experiments suggest that Ap_5A and α,β-meATP are acting through the same P2X receptor to mediate contraction.

The effects of 10 μM Ap_nAs ($n=2-6$) in contractile and patch clamp studies are shown in Figure 2d. Ap_2A (upto 100 μM) and Ap_3A (upto 30 μM) did not evoke contractions or inward currents in the mesenteric artery. Ap_2A (10 μM) had no effect on contractions evoked by an EC_{50} concentration of α,β-meATP (1 μM) (responses $103\pm2.6\%$ of control, $n=4$). In contrast Ap_3A (10 μM) potentiated by $59\pm15\%$ ($n=6$) contractions evoked by α,β-meATP (1 μM). Ap_4A appeared less effective in mediating vasoconstriction than inward currents ($8.8\pm1.3\%$ and $19.1\pm5.2\%$ respectively of the response to 10 μM α,β-meATP, $n=6$). Although this effect is not significant, given that Ap_5A and Ap_6A were more effective in evoking contractions than inward currents, it may indicate that Ap_4A is subject to ecto-enzyme breakdown in whole tissue studies as has been reported previously (Westfall *et al.*, 1997). Ap_6A was significantly more effective in mediating vasoconstriction than inward currents ($50.3\pm4.3\%$ and $21.2\pm4\%$ of the response to 10 μM α,β-meATP $n=6$ and 5 respectively $P<0.005$). Ap_7A was an effective agonist in mediating transient contractions (response to 10 μM Ap_7A was $50.1\pm11.2\%$ of response to 10 μM α,β-meATP, $n=4$) (Figure 2d). In cross desensitization studies following pre-treatment with α,β-meATP (10 μM) contractile responses to Ap_7A were abolished ($n=3$). These results indicate there is a major difference in the activity of Ap_7A in contractile and patch clamp studies and suggest that Ap_nAs may be subject to metabolism in whole tissue studies.

Effects of Ap_nGs on acutely dissociated rat mesenteric smooth muscle cells

The Ap_nGs mediate vasoconstriction in the rat isolated perfused kidney preparation and have been suggested to mediate this response through the activation of P2X receptors (Schlüter *et al.*, 1998). In the present study Ap_nGs ($n=2-6$, 30 μM) evoked P2X receptor mediated inward currents in isolated smooth muscle cells (Figure 3). Ap_3G was significantly more effective than Ap_3A in evoking inward currents ($P<0.001$). In contrast Ap_4G, Ap_5G and Ap_6G were significantly less effective at evoking inward currents than the corresponding diadenosine polyphosphate compound ($P<0.05$). In cross desensitization studies following α,β-meATP treatment (10 μM) Ap_nAs ($n=4-6$, 30 μM) had no effect on holding current indicating that they were acting through the same P2X receptor ($n=3-6$). In whole tissue experiments Ap_nGs ($n=4-6$) were effective in evoking contractions, the response to 30 μM Ap_nG ($n=4-6$) were 7.3 ± 1.6, 44.1 ± 10.3 and $55.4\pm7.9\%$ respectively of the contractile response to 10 μM α,β-meATP ($n=4$). Thirty μM Ap_3G failed to evoke a contractile response ($n=4$).

Effects of other 'polyphosphate' compounds on P2X receptors

The action of Ap_nAs and Ap_nGs at P2X receptors raises questions about ligand recognition by P2X receptors and possible metabolism of these compounds in whole tissue studies. In order to investigate this further we have determined the effects of a number of related 'polyphosphate' compounds. GTP, trimetaphosphate and tetrapolyphosphate (100 μM) were ineffective as agonists at mesenteric artery P2X receptors ($n=4-6$) and ineffective as antagonists (Cook & Evans, 1999 unpublished observations) (Figure 4a). These results show that an adenosine moiety and a phosphate chain are required for ligand recognition by mesenteric artery P2X receptors. Adenosine tetraphosphate evoked similar currents to those seen with α,β-meATP (Figure 4b,c) with an EC_{50} of 0.51 μM. Responses to adenosine tetraphosphate (10 μM) were abolished following desensitization with 10 μM α,β-meATP ($n=3$). At adenosine tetraphosphate concentrations ≥1 μM recovery from receptor inactivation was prolonged, for example following an application of 1 μM adenosine tetraphosphate a subsequent response to 10 μM α,β-meATP required 15 min to return to pre adenosine tetraphosphate control levels, and at concentrations of 3 and 10 μM adenosine tetraphosphate responses to α,β-meATP (10 μM) were only $\sim70\%$ of control values after 30 min ($n=4$). The maximal adenosine tetraphosphate response was 159.5% of the response to 10 μM α,β-meATP.

Immunohistochemical localization of $P2X_1$ receptors in rat mesenteric artery smooth muscle cells

A polyclonal antibody raised against the carboxy terminus of the $P2X_1$ receptor produced selective staining of the smooth muscle cell layer of mesenteric artery rings that was abolished by pre-incubation of the antibody with a sequence specific blocking peptide, similar detection of $P2X_1$ receptor immuno-

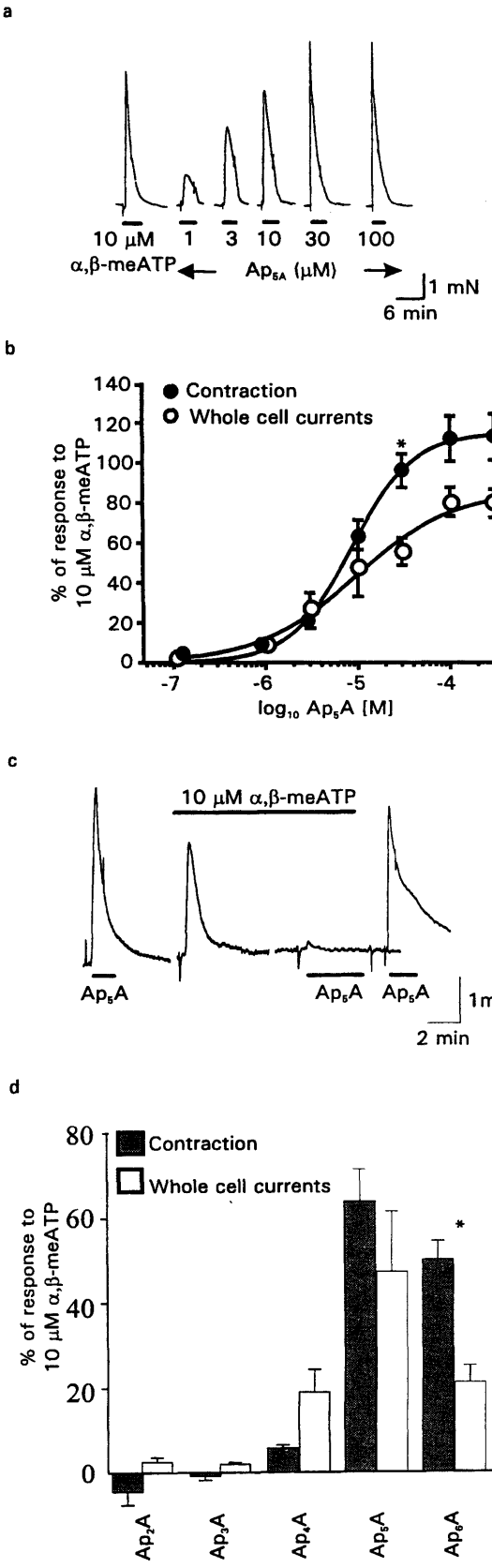

Figure 2 Comparison between responses to Ap_nAs in whole mesenteric artery contraction studies and patch clamp recordings from acutely dissociated mesenteric artery smooth muscle cells. (a) Ap_5A evoked concentration dependent contractions which fade during the continued presence of the agonist. A response to 10 μM α,β-meATP in the same vessel is shown. Drugs were applied for the time indicated by bars. (b) Comparison of concentration response relationships for contractions and whole cell currents evoked by Ap_5A. Each point is the mean percentage of the response to 10 μM α,β-meATP ($n=4-6$ for each point). (c) Ap_5A (100 μM) evoked transient contraction of the rat mesenteric artery, similar responses were evoked by α,β-meATP (10 μM). In the continued presence of α,β-meATP the response to Ap_5A was reduced by >95%. Drug application periods indicated by bars. (d) Comparison between contractions and whole cell currents evoked by 10 μM Ap_nAs ($n=2-7$). Each column is the mean response as a percentage of the maximal response to 10 μM α,β-meATP ($n=4-7$). ($^*P<0.05$, $^{**}P<0.01$).

reactivity in smooth muscle has been detected previously (Vulchanova *et al.*, 1996) (Figure 5). A high level of background autofluorescence was associated with the endothelial cell layer (Figure 5b).

Discussion

This study has shown that a range of Ap_nAs evoked inward currents in rat mesenteric artery smooth muscle cells through the activation of P2X receptor cation channels and that activity of these compounds is dependent on the length of the phosphate chain. The study extends on previous work and documents the effective concentration range of agonists and presents new data on the structure activity relationships of P2X receptors. These data show that at concentrations likely to be present physiologically Ap_nAs and Ap_nGs may contribute to the control of blood pressure through the direct activation of $P2X_1$-like receptor cation channels.

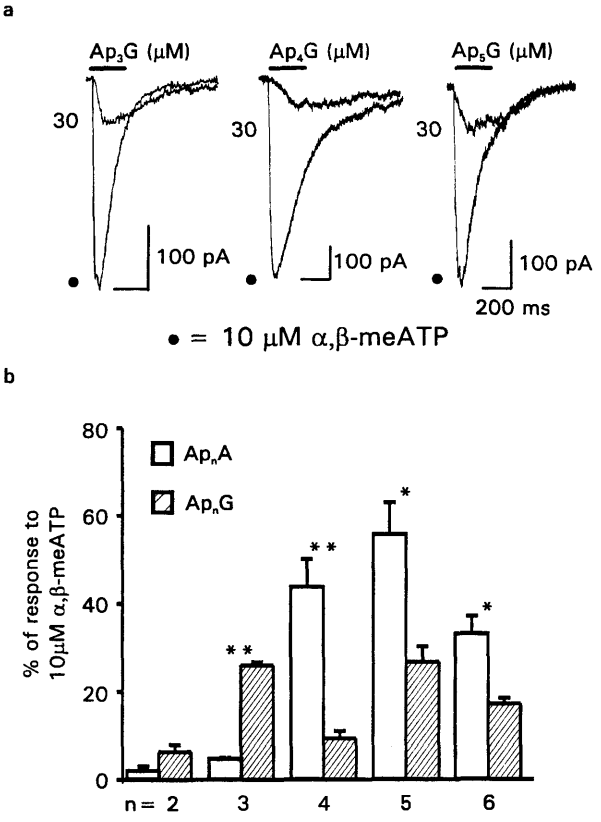

Figure 3 P2X receptor mediated currents evoked by Ap_nGs in isolated rat mesenteric arterial smooth muscle cells. (a) Representative traces of inward currents evoked by Ap_3G, Ap_4G and Ap_5G (all 30 μM) compared to currents evoked by 10 μM α,β-meATP (•) in individual cells. Drugs were applied for 200 ms as indicated by the bar. (b) Comparison between current amplitude of responses evoked by the diadenosine phosphates (30 μM) and the adenosine polyphospho guanosines (30 μM). Each column is the mean response as a percentage of the maximal response to 10 μM α,β-meATP ($n=4-7$). ($^*P<0.05$, $^{**}P<0.01$).

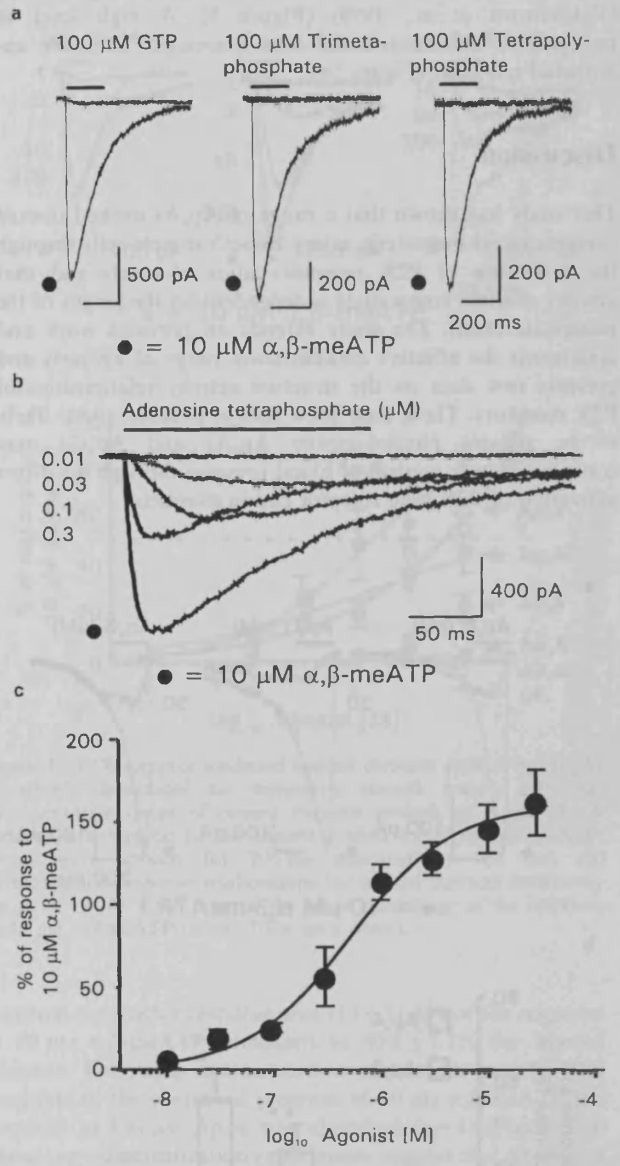

Figure 4 Effects of nucleotides and polyphosphate compounds on dissociated rat mesenteric artery smooth muscle cells. (a) GTP, trimetaphosphate and tetrapolyphosphate (all 100 μM) had no effect on the holding current in cells where α,β-meATP (10 μM, indicated by •) evoked an inward current. (b) Adenosine tetraphosphate evoked concentration dependent inward currents, the response to 10 μM α,β-meATP (indicated by •) is shown for comparison. (c) Concentration dependence of inward currents evoked by adenosine tetraphosphate. Each point is the mean percentage of the response to 10 μM α,β-meATP ($n=4-7$ for each point). Drugs were applied for 200 ms as indicated by the bar.

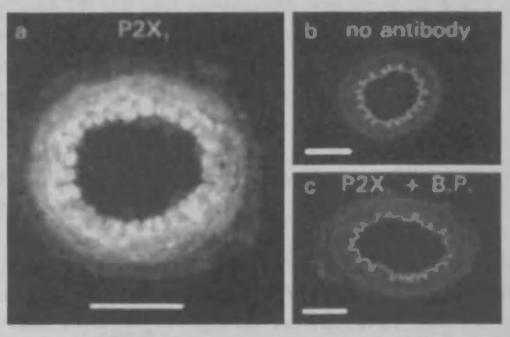

Figure 5 Immunohistochemical localization of $P2X_1$ receptors on rat mesenteric artery rings. (a) $P2X_1$ receptor specific immunoreactivity is localized to the smooth muscle layer of mesenteric artery rings. (b) Autofluorescence of the endothelial cell layer in fixed sections with no antibody. (c) $P2X_1$ receptor specific immunoreactivity is blocked by pre-incubation of the antibody with antigen blocking peptide (B.P.). Scale bar = 50 μm.

The use of patch clamp methods in this study has allowed a direct comparison of the effects of Ap_nAs between P2X receptor mediated currents in rat mesenteric smooth artery cells and at recombinant $P2X_1$ receptors (Evans *et al.*, 1995; Wildman *et al.*, 1999; Bianchi *et al.*, 1999) to be made. Previous comparisons of native smooth muscle and recombinant $P2X_1$ receptors have been based on the timecourse of response and sensitivity to the agonists α,β-meATP, 1-β,γ-methylene ATP and the antagonist trinitrophenyl ATP. We extended this to compare further native smooth muscle P2X receptors and recombinant $P2X_1$ receptors. Our results are similar to those reported for human $P2X_1$ receptors expressed in mammalian HEK293 or 1321N1 astrocytoma cells (Evans *et al.*, 1995; Bianchi *et al.*, 1999). There are however marked differences in the actions of Ap_4A and Ap_6A in rat mesenteric artery smooth muscle cells and at recombinant rat $P2X_1$ receptors expressed in *Xenopus* oocytes (Wildman *et al.*, 1999). A degree of caution however must be used in drawing conclusions based on these findings as discrepancies between the properties of recombinant P2X receptors expressed in mammalian and amphibian systems have been reported (e.g. comparison of the studies of Brake *et al.*, 1994; Evans *et al.*, 1995; Pintor *et al.*, 1996). Thus it remains to be confirmed whether the native rat mesenteric artery smooth muscle P2X receptor is different from recombinant rat $P2X_1$ receptors or if the differences can be accounted for by the difference in the expression system. However the general properties of rat mesenteric artery P2X receptors and the presence of $P2X_1$ receptor immunoreactivity on these vessels suggest strongly that the $P2X_1$ receptor characteristics dominate this native phenotype (Evans & Surprenant, 1996; Lewis *et al.*, 1998).

Ap_7A has been shown recently to be released by platelets and has a vasoconstrictor action in the isolated perfused rat kidney (Jankowski *et al.*, 1999). In the present study we show that Ap_7A evokes α,β-meATP sensitive contractions of the rat mesenteric artery. However Ap_7A failed to evoke inward currents in patch clamp studies. Similarly Ap_6A was more effective in evoking contractions than P2X receptor currents. These results suggest that in whole tissue studies Ap_6A and Ap_7A may be subject to metabolism, as has been shown for Ap_4A (Westfall *et al.*, 1997), and that when added under concentration clamp conditions in patch clamp studies the true potency of the agonist is revealed. Cleavage of the phosphate chain could give rise to ATP and adenosine tetraphosphate (Mateo *et al.*, 1997) which then act locally to activate P2X receptor channels (Evans *et al.*, 1995 and this study). As Ap_6A is an agonist of P2X receptor currents it is likely that in contraction studies the response results from activation of the P2X receptor by both Ap_6A and metabolic products. Thus the activity of Ap_6A and particularly Ap_7A directly at P2X receptors may be overestimated in whole tissue studies due to metabolism.

The Ap_nGs evoked inward currents and contractions through mesenteric artery P2X receptors. Previously Ap_nGs have been shown to mediate vasoconstriction in the perfused kidney preparation (Schlüter *et al.*, 1998) however this is the first time a direct role for P2X receptors in this process has been demonstrated. Ap_2G and Ap_3G were more effective than the corresponding Ap_nA at evoking inward currents. The

increased efficacy of Ap_3G relative to Ap_3A may reflect that binding of the γ-phosphate to the P2X receptor is influenced by the chemical group substituted at the ester oxygen. When this group is a phosphate i.e. adenosine tetraphosphate the potency is equivalent to α,β-meATP. The potency order of the chemical groups at this position is phosphate > > guanosine > adenosine. In contrast Ap_4G, Ap_5G and Ap_6G were less effective than the corresponding Ap_nA. The reduced potency of Ap_nG ($n=4-6$) compared to the corresponding Ap_nA may simply reflect the reduced probability of the adenosine moiety binding with the P2X receptor.

A number of 'polyphosphate' compounds including Ap_2A, Ap_3A, GTP, trimetaphosphate and tetrapolyphosphate were ineffective as agonists at the P2X receptor. When these compounds were co-applied with α,β-meATP (1 μM, an EC_{50} concentration) they either had no effect on the amplitude of the P2X receptor mediated responses or slightly potentiated them. These results demonstrate that binding of an adenine group in the case of Ap_2A or a triphosphate group in the case of GTP is ineffective in activating the receptor. The fact that these compounds are ineffective as antagonists suggests that for high affinity binding at the P2X receptor both an adenine group and a triphosphate moiety are required.

The activation of platelets leads to the release of a variety of vasoactive nucleotides. For example Ap_nAs and Ap_nGs have been estimated to be present at 0.5–3 μM in the supernatant following platelet aggregation (Schlüter *et al.*, 1998). Recent studies on platelets have shown that it is likely that the nucleotide concentration at the local microenvironment of the cell surface may be at least an order of magnitude higher than that measured in the supernatant (Beigi *et al.*, 1999). Ap_nAs and Ap_nGs may therefore be present locally in the 5–30 μM range, concentrations that were effective in activating P2X receptors in this study. When the endothelium is damaged there may be a local rise in Ap_nAs and Ap_nGs following platelet activation resulting in the activation of P2X receptors and subsequent vasoconstriction.

In summary these results have shown that diadenosine polyphosphates and adenosine polyphospho guanosines at physiologically relevant concentrations can act directly at $P2X_1$-like receptor ion channels to mediate vasoconstriction in rat mesenteric artery. The activity of these compounds is dependent on the length of the polyphosphate chain. In addition it is possible that Ap_nAs and Ap_nGs ($n=6-7$) may be metabolised in whole tissue studies to yield the P2X receptor agonists ATP and adenosine tetraphosphate that may mediate some of their vasoconstrictor actions.

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Properties of P2X and P2Y receptors are dependent on artery diameter in the rat mesenteric bed

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1 P2 receptor mediated contractile responses have been characterized in different diameter arteries from the rat mesenteric arterial vasculature (first, second to third and fifth to sixth order for large, medium and small arteries) using wire myograph and diamtrak video imaging.

2 α,β-methylene ATP (α,β-meATP) evoked transient concentration-dependent contractions in mesenteric arteries with EC_{50} values of 0.4, 2.5 and 107 μM for small, medium and large arteries respectively.

3 Suramin (10–100 μM) produced substantial parallel rightward shifts of the concentration-response curve to α,β-meATP in small and medium-sized arteries with pA_2 of 5.1. Responses in large vessels were unaffected by suramin.

4 Immunohistochemical analysis of arterial sections revealed no substantial differences in expression patterns of P2X receptors between different sizes of artery. $P2X_1$ receptors were expressed at high levels, $P2X_4$ and $P2X_5$ receptors were also detected on smooth muscle. The P2X receptor response is dominated by $P2X_1$ receptor in small and medium arteries but the nature of the receptor mediating the suramin insensitive α,β-meATP mediated response in large arteries is unclear.

5 The P2Y receptor agonist UTP was significantly more potent in small than in medium or large arteries (EC_{50} values: 15.0 μM small, 88.5 μM diamtrak medium 1.6 mM myography medium and 1.4 mM large). Responses in both small and medium-sized vessels were reduced by suramin (30–100 μM). The sensitivity to UTP and suramin indicates the presence of $P2Y_2$ receptors.

6 This study shows that P2 receptors do not have a homogenous phenotype throughout the mesenteric vascular bed and that the properties depend on artery size.

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Keywords: P2X receptors; P2Y receptors; artery; suramin; immunohistochemistry

Abbreviations: iso-PPADS, iso-pyridoxalphosphate-6-azophenyl-2′-5′-disulphonate; α,β-meATP, α,β-methylene ATP

Introduction

P2 receptors are activated by extracellular nucleotides and play an important role in the control of vascular tone and blood pressure (Boarder & Hourani, 1998). They can be divided into two classes, ionotropic P2X receptors and metabotropic P2Y receptors (Abbracchio & Burnstock, 1994; Ralevic & Burnstock, 1998). P2X receptors for ATP are ligand-gated cation channels and are found on many different cell types, including vascular smooth muscle, neurones and blood cells (Buell *et al.*, 1996; Collo *et al.*, 1996; Surprenant *et al.*, 1996; Vulchanova *et al.*, 1996). ATP is co-stored and co-released with noradrenaline from sympathetic nerves and in arteries P2X receptors mediate membrane depolarization and a component of constriction associated with sympathetic nerve stimulation (Burnstock, 1997). Following P2X receptor activation calcium can enter the cell either directly through the calcium permeant P2X receptor channel (Benham & Tsien, 1987; Valera *et al.*, 1994) or through voltage-gated calcium channels opened in response to P2X receptor mediated membrane depolarization (Bulloch & McGrath, 1988).

Genes encoding seven different P2X receptors ($P2X_{1-7}$) have so far been identified (Burnstock, 1997). They can form either homomeric or heteromeric channels with at least three subunits giving rise to a wide range of receptor phenotypes (Nicke *et al.*, 1998; North & Surprenant, 2000). In vascular smooth muscle the α,β-methylene ATP (α,β-meATP) and L-β,γ-methylene ATP sensitive rapidly desensitizing P2X receptor phenotype corresponds most closely to that of recombinant $P2X_1$ receptors (Valera *et al.*, 1995; Evans & Surprenant, 1996). This is consistent with studies showing that the $P2X_1$ receptor is the predominant P2X receptor isoform expressed in smooth muscle (Collo *et al.*, 1996) and that P2X receptor mediated responses are absent in vas deferens smooth muscle from $P2X_1$-receptor-deficient knock-out mice (Mulryan *et al.*, 2000). However there is some evidence to suggest that there may be a heterogenous distribution of P2X receptor isoforms and P2X receptor properties throughout the arterial vasculature (Nori *et al.*, 1998; Lewis & Evans, 2000a).

Metabotropic G-protein coupled P2Y receptors can also contribute to the control of vascular tone. At least four different isoforms of the P2Y receptor have been cloned in the rat corresponding to $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Burnstock, 1997). $P2Y_1$ receptors are activated by ADP and ATP, and in the vasculature they are found on endothelial cells where they mediate vasodilation (Webb *et al.*, 1993; Burnstock, 1997). In contrast $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are expressed on smooth muscle, are activated by uridine nucleotides and mediate vasoconstriction (Urquilla, 1978; von Kugelgen & Starke, 1990; Hartley & Kozlowski, 1997; Erlinge *et al.*, 1998; Hartley *et al.*, 1998).

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Small arteries and arterioles are known to account for over 45% of peripheral resistance to blood flow through the vasculature and therefore substantially contribute to systemic blood pressure. It seems possible that as large conduit arteries and small resistance arteries perform different functions they may also have different P2 receptor mediated properties. Indeed, variations in distribution or properties depending on arterial size have already been demonstrated for inward rectifier potassium channels (Quayle *et al.*, 1996), L-type calcium channels (Bowles *et al.*, 1997) and calcium-activated chloride channels (Clapp *et al.*, 1996). No systematic comparison of P2 receptor-mediated contractile responses in different sizes of artery has yet been carried out. In the meseneteric circulation P2X and P2Y receptors can mediate artery constriction (Sjoblom-Widfeldt, 1990; Ralevic *et al.*, 1995; Lagaud *et al.*, 1996; Lewis *et al.*, 1998) and vessels can be studied from the main conduit superior mesenteric artery down to small resistance arteries allowing direct comparisons of responses between different sized vessels. The aim of this study was thus to characterize the properties of P2 receptors in different calibre arteries using the rat mesenteric arcade as a model vascular bed.

Methods

Adult male Wistar rats (200–300 g) were killed by cervical dislocation or CO_2 asphyxiation followed by femoral artery exsanguination. A portion of the gut with attached mesenteric arcade was removed and mesenteric arteries of different diameters were dissected; large vessels from the superior mesenteric artery, medium-sized vessels were from second or third order branches and small vessels correspond to fifth or sixth order arteries.

Contraction studies

Large and medium artery rings (537±23 μm, range 386–806 μm and 215±7 μm, range 131–342 μm internal diameter, $n=30$ and 40 arteries respectively) were mounted in a Mulvany myograph using standard procedures (Lagaud *et al.*, 1996); changes in arterial tone were recorded and analysed using a MacLab data acquisition system. The lower limit of vessel that can be mounted in the myograph is dependent on the ability to insert two 40 μm tungsten wires through the lumen therefore the theoretical limit is a vessel with an internal diameter of at least 80 μm. In addition it is technically very difficult to work on small arteries in the myograph without damaging them. As a result we have studied small resistance arteries using the video imaging diamtrak method. In order to control for differences in methodology between myography and diamtrak we have collected data for medium arteries with both systems. We are therefore able to make direct comparisons between small/medium and medium/large vessels. Small arteries were dissected carefully cleaning away all connective tissue and pinned out (stretched to approximately 150% of their resting length) in a Sylgard coated organ bath (volume 2 ml). The organ bath was placed on the stage of an inverted microscope and changes in external arterial diameter were analysed using Diamtrak software as previously described (Neild, 1989). This method uses a video camera to produce a digitized image of the blood vessel, this is then analysed on line by a computer programme (Diamtrak) that measures the outside diameter (dark edge) of the artery. The outside diameter of the medium arteries was 293±5 μm (range 278–321, wall thickness accounts for ~40% giving an internal diameter of ~180 μm), and for small arteries was 102±3 μm range (69–135 μm, $n=40$ arteries) (wall thickness accounts for ~40% giving a mean internal diameter of ~60 μm).

Tissues were superfused with a physiological saline solution (composition in mM): NaCl 150, KCl 2.5, HEPES 10, $CaCl_2$ 2.5, $MgCl_2$ 1, pH to 7.3 with NaOH. Experiments were conducted at 32–34°C for diamtrak and 34–36°C for myography. Drugs were made as concentrated stock solutions in MilliQ distilled water and added to the superfusate at the required final concentration, in general only one agonist was tested per artery. Reproducible responses to agonists were obtained when applications were separated by 30 min intervals. In experiments testing the effect of antagonists, suramin was pre-superfused for 30 min before being added in combination with the agonist. The procedure for the UTP cross-desensitization experiment was as follows: an application of UTP was made; this was followed by an application of α,β-meATP allowing the response to fully return to baseline tone; in the continued presence of α,β-meATP a second application of UTP was then made. The two contractile responses for UTP were then compared.

Data analysis

Data are expressed as mean±s.e.mean throughout and $n=$number of animals, unless otherwise stated. Concentration-response data were fitted by the least squares method using Microcal Origin software with the following equation:

$$\text{response} = \alpha[A]^{n_H}/([A]^{n_H} + [A_{50}]^{n_H}) \quad (1)$$

where α is the asymptote, n_H is the Hill coefficient, [A] the agonist concentration and A_{50} the agonist concentration producing a half maximal response (EC_{50}), $pA_{50} = -\log EC_{50}$. Differences between means were tested using either a 2 sample or paired, two tailed *t*-test, as appropriate. A P value of <0.05 was considered statistically significant. pA_2 values for suramin were estimated using Schild analysis for competitive receptor antagonists. A full Schild regression was made for medium-sized arteries, while in small vessels two data points were used to estimate a pA_2.

Immunohistochemical studies

Mesenteric arteries were dissected as above and immunohistochemical analysis of P2X receptor expression was performed as described previously (Lewis & Evans, 2000). Briefly, embedded tissues were cut into 12 μm transverse sections and mounted on pre-subbed slides. Sections were fixed in paraformaldehyde, permeabilized with a 0.5% Triton-X (Sigma) solution and incubated with primary and secondary antisera. Anti-$P2X_1$, $P2X_2$, $P2X_4$ and $P2X_7$ antibodies (Alomone, Israel) were all used at a dilution of 1:200. Anti-$P2X_5$ and $P2X_6$ antibodies (gift from Roche Bioscience) were used at 1:1000 and anti-$P2X_3$ was used at 1:5000 (gift from Dr L. Vulchanova, University of Minnesota, U.S.A.). The secondary antibody was in each case fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG raised in donkey (Jackson Immunoresearch) used at a 1:100 dilution. All dilutions were made using 10% donkey serum (Jackson Immunoresearch) in phosphate buffered saline (PBS). When blocking peptides were used, the antibody was pre-incubated with its corresponding antigen peptide for 1 h at room temperature. To test for non-specific antibody

binding, control slides were incubated with secondary antisera only and non-immune donkey serum only. Tissue sections mounted in Citifluor (UKC Chem Lab, U.K.) were examined under epifluorescence and images were captured using Scionimage software. Immunohistochemical studies were conducted on at least three arteries from different animals. The level of immunoreactivity for a given P2X receptor subunit seen between animals was reproducible. The level of immunoreactivity was estimated by eye and assigned to the following categories; + + + = strong expression, + + = moderate expression, + = weak expression, ± = barely detectable expression, − = no expression.

Drugs

α,β methylene ATP, suramin, phenylephrine, UTP (Sigma, U.K.). iso-pyridoxalphosphate-6-azophenyl-2′-5′-disulphonate (iso-PPADS) (Tocris Cookson, U.K.).

Results

Sensitivity to $P2X_1$ receptor agonist, α,β-meATP

The metabolically stable ATP analogue α,β-meATP evoked concentration-dependent constrictions of rat mesenteric arteries. At higher concentrations, responses rapidly reached a peak and declined toward baseline in the continued presence of the agonist (Figure 1a–c). There was a marked difference in sensitivity to α,β-meATP based on the diameter of the vessel (Figure 1). The mean EC_{50} values for small, medium and large arteries were ~0.4, 2.5 and 107 μM (coresponding pA_{50} values were 6.4±0.1 small, 5.7±0.1 medium diamtrak 5.6±0.1 medium myography, and large 4.0±0.1; n=4-5, and Hill slopes were 1.5±0.2, 1.2±0.1 diamtrak and 1.2±0.2 myography, and 0.9±0.1, respectively). These correspond to significant differences in sensitivity to α,β-meATP between small and medium ($P<0.005$) and medium and large ($P<0.005$) vessels. The sensitivity to α,β-meATP was the same for medium arteries whether determined using diamtrak or myography techniques.

The differences in potency of α,β-meATP may have been caused by anatomical differences between large and smaller arteries, affecting access of the agonist to the receptor. To test for such a putative diffusional barrier, contractile responses to the α_1-adrenoceptor agonist phenylephrine were studied. There was no significant difference between mean EC_{50} values for phenylephrine in all three vessel sizes (Figure 2a); 1.6, 3.0 and 2.9 μM (pA_{50} = 5.9±0.1, 5.6±0.2 and 5.6±0.2, n=3–4) for small, medium and large respectively. The Hill slopes for small, medium and large were 1.6±0.4, 4.2±1.5 and 1.8±0.6, respectively. In addition 'agonist-independent' constrictions were evoked by superfusion with a solution containing a high concentration of potassium ions (KCl). There was no difference in response to KCl between medium (diamtrak or myography) and large arteries. The concentration of KCl required to evoke ~30% maximum response was however significantly lower for small arteries ($P<0.05$) (Figure 2b).

It is possible that at the very high concentrations of α,β-meATP used in large arteries, the constrictions may have been caused by small amounts of contaminating ATP activating a P2Y receptor. The effect of ATP was therefore tested at a concentration equivalent to a putative 5% contamination of the α,β-meATP, i.e. a 1 mM solution of

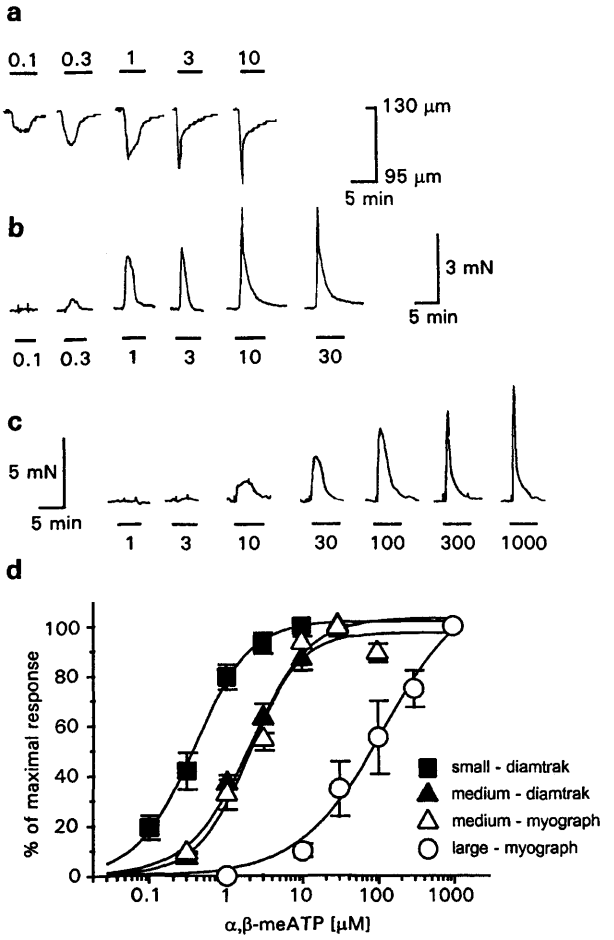

Figure 1 Characterization of contractile responses to α,β-methylene ATP. (a), (b) and (c) show contractions in small, medium and large arteries respectively; periods of application are indicated by the bar. The transient nature of contractile responses can be clearly seen in all three vessel sizes. (d) Concentration-response curves showing different α,β-meATP sensitivity depending on vessel size. Data are mean responses ± s.e.mean (n=4–5) and are expressed as per cent of the maximal response to α,β-meATP in each tissue.

α,β-meATP would thus contain 50 μM contaminating ATP. This concentration of ATP however produced a contraction that was only 1.8±1.9% of the response to 1 mM α,β-meATP (n=3), demonstrating that the observed responses were evoked by α,β-meATP and not due to ATP contamination.

Effect of removal of extracellular calcium on constrictions evoked by α,βmeATP

To test whether the requirement for calcium influx from the extracellular space for smooth muscle contraction was the same in all sizes of artery, calcium was removed from the extracellular solution. Under these conditions contractile responses to 10 μM α,β-meATP were abolished in small and medium-sized arteries and constrictions to 300 μM α,β-meATP were abolished in large arteries (n=3–4).

Sensitivity of α,β-meATP mediated responses to the P2 receptor antagonists

Due to the lack of subtype-selective agonists for different P2X receptors, one way of distinguishing between isoforms is to determine antagonist sensitivity. In the rat, suramin is a potent antagonist at the $P2X_{1,2,3}$ and $P2X_5$ receptor isoforms

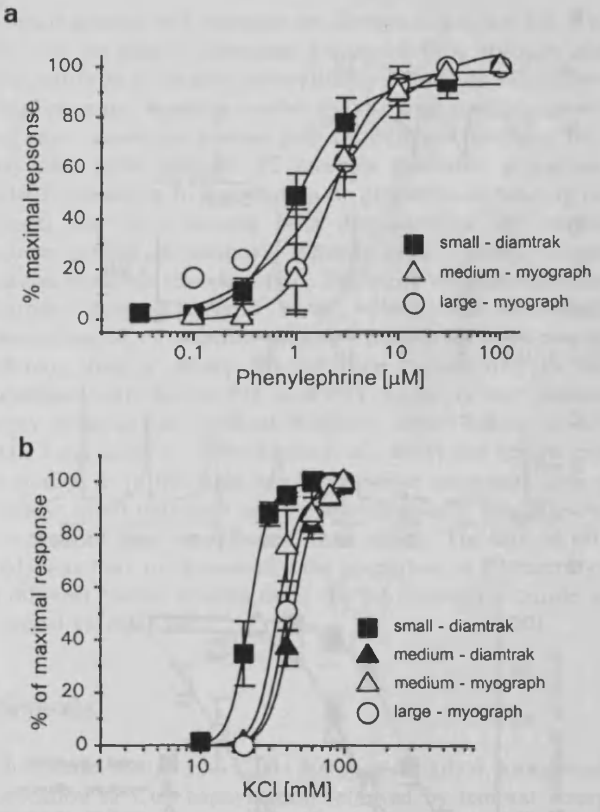

Figure 2 Small, medium and large arteries all display similar sensitivity to phenylephrine, and KCl is more potent on small arteries. The three concentration response curves to phenylephrine show that the EC_{50} values are all approximately 2 μM (a). Contractile responses to a solution containing a high concentration of KCl, small arteries were more sensitive (b). Data are mean responses ± s.e.mean ($n = 3-4$) and are expressed as per cent of the maximal response in each tissue.

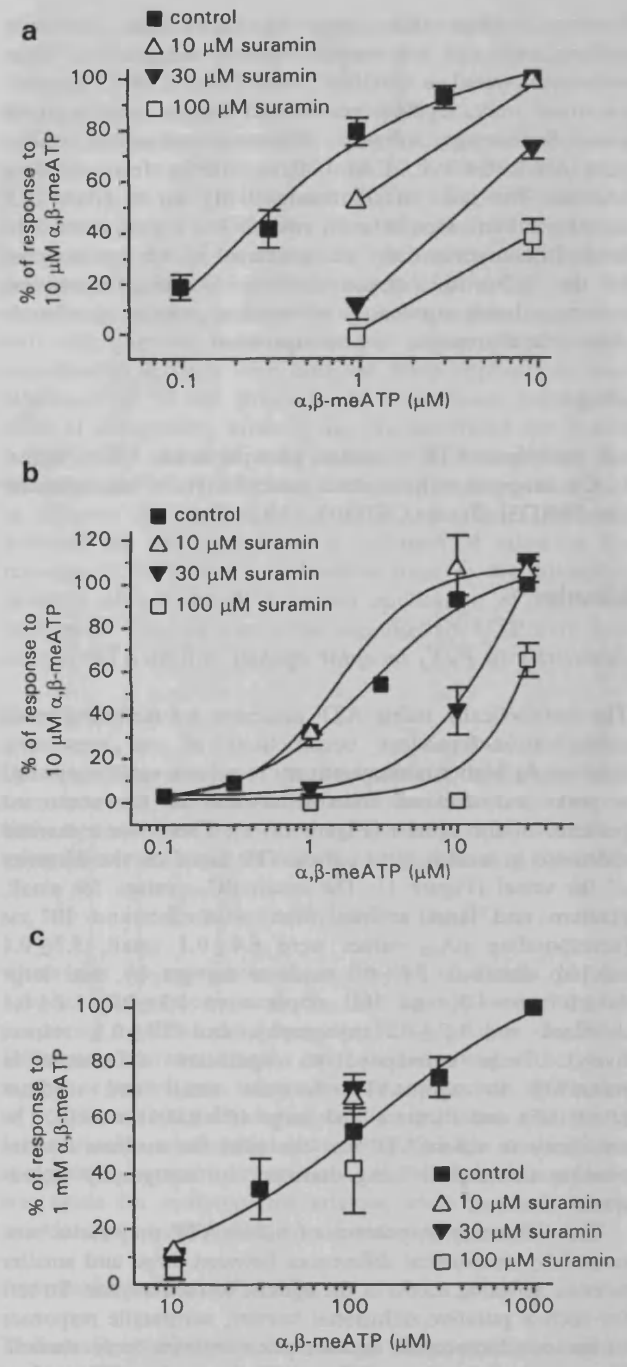

Figure 3 Suramin sensitivity of contractile responses to α,β-meATP in different-sized mesenteric arteries. Concentration response curves for responses to α,β-meATP alone and in the presence of suramin. A clear right-ward shift of the curve can be seen in small (a) and medium-sized (b) arteries but not in large (c). Data are mean responses ± s.e.mean ($n = 3-6$) and are expressed as per cent of the response to 10 μM α,β-meATP in small and medium arteries and per cent of the response to 1 mM α,β-meATP in large arteries.

and a weak antagonist at $P2X_4$ and $P2X_6$ receptors. Suramin had no effect on the resting tone of the arteries but produced substantial rightward shifts of the concentration response curve for α,β-meATP in the small and medium arteries (Figure 3a,b). These data were used to estimate a pA_2 value using Schild analysis. The values obtained for small and medium-sized arteries were 5.14 and 5.08, respectively. In contrast, suramin had no effect on responses to α,β-meATP in large arteries (Figure 3c), suggesting that the agonist is not acting at suramin sensitive P2X receptors in this tissue. Iso-PPADS is an effective antagonist at P2X receptors in medium mesenteric arteries (Lagaud *et al.*, 1996; Lewis & Evans, 2000b). In large arteries the P2 receptor antagonist iso-PPADS (30 μM) inhibited responses to α,β-meATP (300 μM) by 41.8 ± 14.2%, $n = 3$).

Immunohistochemical detection of P2X receptor subunits

The expression of defined P2X receptor isoforms was determined using receptor specific antibodies (Table 1) and extends a previous study characterizing P2X receptor expression in medium mesenteric arteries (Lewis & Evans, 2000b). There is substantial expression of $P2X_1$ receptor subunits in all vessel sizes (Figure 4). The $P2X_4$ receptor is expressed in the smooth muscle of medium and large arteries and weakly in small vessels, $P2X_5$ receptor immunoreactivity was weak/barely detectable in all arteries. $P2X_7$ is weakly expressed in the outer non-smooth muscle layers of medium-sized arteries and in the smooth muscle layer of large arteries. $P2X_{2,3}$ and $P2X_6$ receptor immunoreactivity was below the limit of detection in arterial tissue.

Characterization of contractile responses to UTP

Our results from experiments with α,β-meATP suggested there is heterogeneity in P2X receptor expression dependent on the size of the artery. In order to test whether such variation was also found for P2Y receptors, concentration-

Table 1 Immunohistochemistry on sections of small, medium and large mesenteric arteries.

	Small	*Medium*	*Large*
$P2X_1$	+ + +	+ + +	+ +
$P2X_2$	−	−	−
$P2X_3$	−	−	−
$P2X_4$	±	+/+ +	+
$P2X_5$	±	±	±
$P2X_6$	−	−	−
$P2X_7$	±	+	+

+ + + = strong expression, + + = moderate expression, + = weak expression, ± = barely detectable expression, − = no expression.

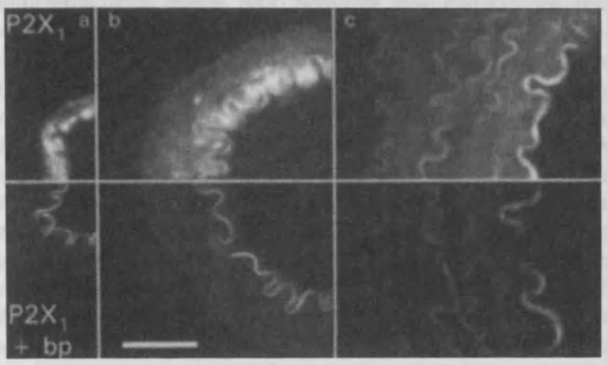

Figure 4 Immunohistochemical visualization of $P2X_1$ receptors in rings of small (a), medium (b) and large (c) mesenteric arterial rings. $P2X_1$ receptor-specific immunoreactivity is associated with the smooth muscle layer of all three sizes of artery (top panels). Specific immunoreactivity is blocked by pre-incubation of the antibody with its specific antigen blocking peptide, leaving only the autofluorescence of the elastic laminae (bottom panels). Calibration bar 50 μm.

response relationships for UTP were constructed. UTP evoked sustained contractions of mesenteric arteries (Figure 5). The EC_{50} values for UTP were 15 μM, 88 μM, 1.6 mM and 1.4 mM ($pA_{50} = 5.0 \pm 0.2$, 4.1 ± 0.1, 2.9 ± 0.1 and 2.9 ± 0.2, $n = 4-6$) for small, medium (diamtrak), medium (myography) and large arteries, respectively (Figure 4). The slopes of concentration responses curves were 1.1 ± 0.3, 2.2 ± 0.3, 0.9 ± 0.1 and 1.3 ± 0.2, respectively. In diamtrak studies on medium arteries UTP was significantly more potent at small compared to medium arteries ($P < 0.02$). In dimatrak studies the potency of UTP was significantly higher than that estimated in myograph studies ($P < 0.005$).

To ensure that observed responses were actually being mediated by P2Y and not P2X receptors, a cross-desensitization experiment was conducted. Responses to UTP prior to and following desensitization of the P2X receptor with α,β-meATP were compared. The second response to UTP expressed as a percentage of the first was 99.7 ± 8.0, 126.3 ± 5.2 and $229.0 \pm 10.7\%$ for small, medium and large arteries respectively ($n = 3-6$) (Figure 5a,b). This confirms that UTP is acting at a different receptor to α,β-meATP. The potentiation of UTP responses in medium and large arteries following α,β-meATP treatment may reflect a sensitization of the responses to subsequent agonist stimulation.

In order to determine which P2Y receptor subtype UTP was acting at, the effect of suramin was tested. In the rat, $P2Y_2$ receptors are sensitive to suramin while $P2Y_4$ receptors are not. In medium sized arteries 30 μM (Figure 5c) and 100 μM suramin reduced responses to 1 mM UTP by 48.8 ± 2.5 and $65.5 \pm 6.2\%$ respectively ($n = 4$). In small

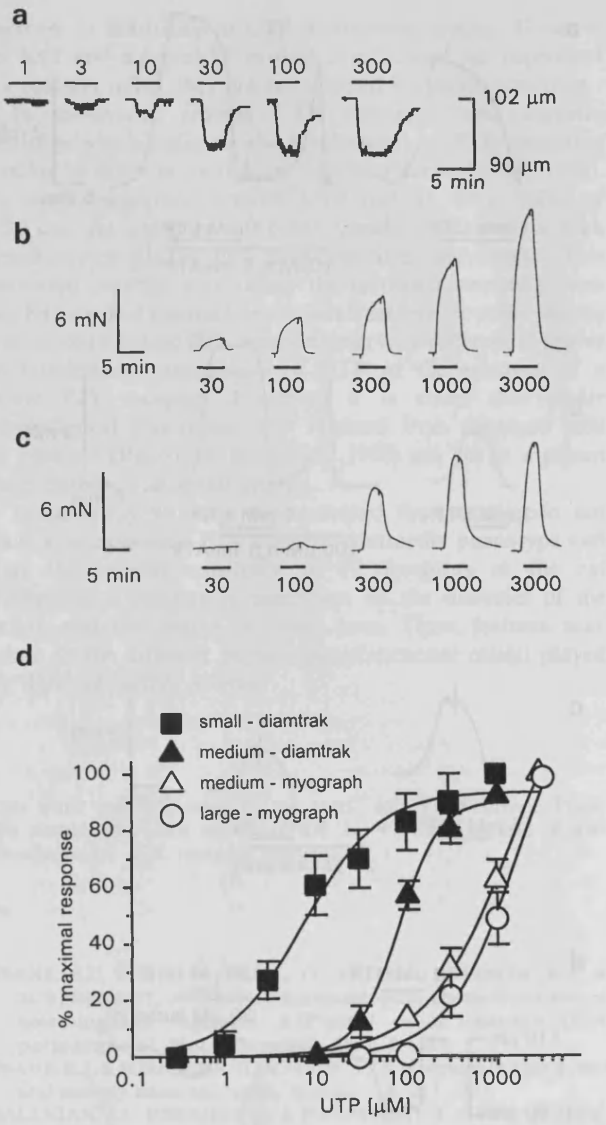

Figure 5 Characterization of contractile responses to UTP. Typical responses in small (a), medium (b) and large (c) arteries, showing sustained constrictions to UTP; periods of application are indicated by the bar. Concentration-response curves for UTP in the three sizes of mesenteric artery show substantial differences in sensitivity of the arteries depending on their size or method of recording (d). Data points are mean responses ± s.e.mean ($n = 4-6$) and are expressed as a per cent of the maximal response in small arteries and as per cent of the response to 3 mM UTP in medium-sized and large arteries.

vessels, contractions to 30 μM UTP were reduced by $64.3 \pm 9.1\%$ (Figure 5d) and $96.3 \pm 3.7\%$ respectively ($n = 5$).

Discussion

In this study we have shown that the properties of P2 receptors in rat mesenteric arteries depend on the diameter of the vessel. For P2X receptors the differences in receptor function seem to result from the differences in contractile sensitivity and expression of different receptor subtypes. Small and medium vessels share a similar α,β-meATP and suramin sensitive phenotype while the large vessels show a relatively α,β-meATP insensitive and suramin insensitive phenotype. In contrast P2Y receptors in the medium and large vessels share similar properties while UTP is more potent in smaller diameter arteries.

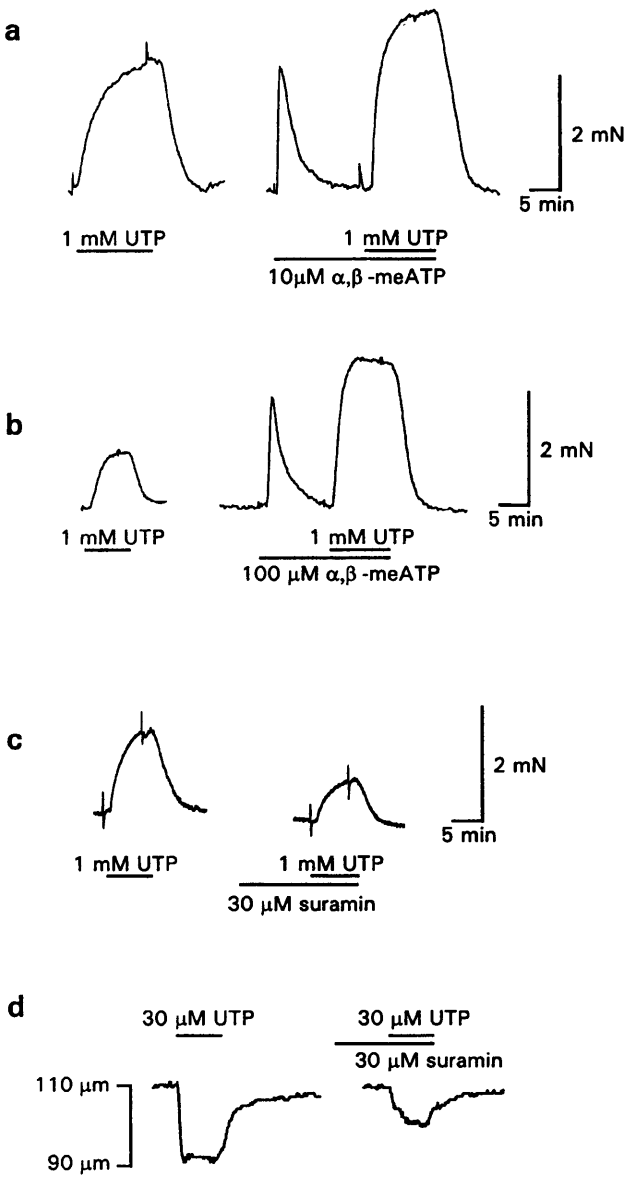

Figure 6 Contractions to UTP are mediated through receptors other than $P2X_1$ and are suramin sensitive. Cross-desensitization experiments in medium-sized (a) and large (b) arteries. Responses to UTP are no smaller after the $P2X_1$ receptor has been desensitized with α,β-meATP. Suramin (30 μM) reduced responses to UTP by approximately 50% in both medium-sized (c) and small (d) arteries.

The metabolically stable ATP analogue α,β-meATP produced concentration-dependent contractile responses in all arteries. Ca^{2+} influx was essential for vasoconstriction and indicates that the responses are mediated by P2X receptor ion channels and not by metabotropic P2Y receptors. There was however a significant difference in the potency of the agonist depending on the diameter of the vessel. Small and medium-sized arteries had EC_{50} values of 0.4 and 2.5 μM respectively. The small difference in sensitivity to α,β-meATP can be accounted for by an increased calcium sensitivity of the muscle in small arteries as indicated by the increased sensitivity of small arteries to KCl. For example a concentration of KCl (20 mM) failed to evoke a contraction in medium but evoked ~35% of maximum response in the small arteries, 40 mM KCl evoked ~40% contraction in medium vessels and ~95% in small arteries. If we compare the shifts in the amplitude of the response for α,β-meATP evoked responses we see a similar pattern e.g. 0.3 μM α,β-meATP evoked ~5% max response in medium compared to ~40% in small and 1 μM α,β-meATP evoked ~37% in medium compared to ~80% in large. So the effective change in per cent response between small and medium for a given concentration of KCl or α,β-meATP are the same and are likely to result from a change in calcium sensitivity of the contractile machinery. The lack of increased potency of phenylephrine in small compared to medium arteries may indicate differences in receptor expression and/or coupling. The EC_{50} values for α,β-meATP are similar to values obtained in other arteries and smooth muscle preparations (von Kugelgen *et al.*, 1990; Galligan *et al.*, 1995; Lagaud *et al.*, 1996).

Contractions of small and medium arteries were suramin sensitive and the estimates of pA_2 values for small and medium arteries are identical and agree well with those previously quoted for vas deferens and submucosal arterioles (von Kugelgen *et al.*, 1990; Khakh *et al.*, 1994; Galligan *et al.*, 1995). These properties bear the hallmark of recombinant $P2X_1$ receptors (Valera *et al.*, 1994) and are consistent with the immunohistochemical studies showing that the $P2X_1$ receptor is expressed at high levels in the smooth muscle layer. We have recently shown that the $P2X_1$ receptor is essential for P2X receptor mediated smooth muscle contraction in the vas deferens (Mulryan *et al.*, 2000), and it therefore appears that responses in small and medium-sized mesenteric arteries are dominated by the $P2X_1$ receptor phenotype. The role of $P2X_4$ and $P2X_5$ receptor subunits expressed on mesenteric arteries however remains unclear.

Two key features distinguished P2X receptor mediated responses in large arteries from smaller vessels: (1) the sensitivity of large arteries to α,β-meATP was approximately 25–100 fold lower and (2) the sensitivity to the P2 receptor antagonist suramin. A diffusional barrier limiting agonist access to large vessels is unlikely to account for these differences as the sensitivity to KCl, phenylephrine and UTP was the same for medium and large vessels in the myograph. Ionic currents recorded from recombinant $P2X_1$ receptors have shown that maximal stimulation of the $P2X_1$ receptor is achieved at a concentration of 10–30 μM α,β-meATP (Evans *et al.*, 1995). This concentration of agonist however produces contractions in large vessels that are equivalent to only 10% of the maximal response. There are a number of caveats to classifying receptors based on agonist sensitivities and the use of antagonists is often more conclusive. Suramin was an effective antagonist at small/medium arteries (pA_2 5.1) but had no effect on α,β-meATP evoked responses in large arteries. Taken together these results provide compelling evidence that the contractile P2X phenotype in large mesenteric arteries is not $P2X_1$-like and that a different P2X receptor mediates the α,β-meATP evoked contraction.

Comparing the pharmacological properties of large arteries with those of the recombinant receptors reveals a mixture of phenotypes: the rapidly desensitizing response to agonists is characteristic of $P2X_1$ and $P2X_3$ receptor isoforms, the relative insensitivity to α,β-meATP is seen in the $P2X_{2,4,5,6}$ and 7 receptors and the lack of effect of suramin is shared by the $P2X_4$ and $P2X_6$ receptor isoforms. The fact that no single receptor can account for the phenotype of the large mesenteric artery P2X receptor raises the possibility of a novel heteromultimer of P2X receptor subunits. More than one type of isoform forming functional heteromeric receptors has been reported on several occasions (Lewis *et al.*, 1995; Le *et al.*, 1998; Haines *et al.*, 1999). However immunohistochemical analysis revealed no major differences in patterns of P2X receptor isoform expression between the three sizes of

vessel and may suggest that a novel additional subunit is expressed in large arteries.

In addition to testing agonists at P2X receptors, we also characterized constrictions to UTP in the mesenteric circulation. This pyrimindine has been shown to have a contractile effect in numerous arterial preparations (von Kugelgen & Starke, 1990; Ralevic & Burnstock, 1991; Hartley *et al.*, 1998) that is mediated by either $P2Y_2$ or $P2Y_4$ receptors present on the surface of smooth muscle cells. In the rat, $P2Y_2$ receptors are suramin sensitive, while $P2Y_4$ receptors are suramin insensitive (Boarder & Hourani, 1998). In myograph studies the potency of UTP was the same for medium and large arteries and was essentially the same as that reported recently (Malmsjo *et al.*, 2000). The sensitivity to UTP was increased ~20 fold for medium arteries when contractions were studied using the diamtrak technique. In contrast there was no change in the sensitivity between diamtrak or myograph studies for responses to KCl or α,β-meATP. It has previously been shown in rabbit mesenteric arteries that the sensitivity of contractions to other G-protein coupled receptors is dependent on the degree of vessel tone/pressurization or depolarization with potassium (Dunn *et al.*, 1994). It is known that stretching vessels also results in membrane depolarization. Recent work has shown that oscillations in calcium release from intracellular stores following P2Y receptor activation can be potentiated by membrane depolarization (Mason *et al.*, 2000). Thus one possible explanation for these findings is that in mesenteric arteries stretch induced depolarization associated with pinning out vessels for diamtrak analysis accounts for the increase in sensitivity to UTP in diamtrak studies. However as KCl and α,β-meATP evoked contractions are dependent on calcium influx they are not affected by vessel stretching.

In mesenteric arteries UTP responses were suramin sensitive which indicates the involvement of $P2Y_2$ receptors similar to those in pulmonary arteries (Hartley *et al.*, 1998). In small mesenteric arteries UTP had an EC_{50} value of 15.0 μM, no evidence of other vessels with such a high sensitivity to UTP has been reported previously. This increased potency may reflect the increased sensitivity seen for KCl evoked contractions in small arteries. It could also be due to an increased P2Y receptor reserve, a differential degree of nucelotidase metabolism of UTP or the existence of a novel P2Y receptor. However, it is clear, that under physiological conditions UTP released from damaged cells or platelets (Ralevic & Burnstock, 1998) can act as a potent vasoconstrictor at small arteries.

In summary we have demonstrated that arteries do not have a homogenous P2X receptor contractile phenotype and that the agonist sensitivity of P2 receptors in the rat mesenteric circulation is dependent on the diameter of the artery and the degree of vessel tone. These features may relate to the different physiological/functional role(s) played by different calibre arteries.

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Reduced vas deferens contraction and male infertility in mice lacking $P2X_1$ receptors

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$P2X_1$ receptors for ATP are ligand-gated cation channels, present on many excitable cells including vas deferens smooth muscle cells[1–5]. A substantial component of the contractile response of the vas deferens to sympathetic nerve stimulation, which propels sperm into the ejaculate, is mediated through P2X receptors[1]. Here we show that male fertility is reduced by ~90% in mice with a targeted deletion of the $P2X_1$ receptor gene. Male mice copulate normally—reduced fertility results from a reduction of sperm in the ejaculate and not from sperm dysfunction. Female mice and heterozygote mice are unaffected. In $P2X_1$-receptor-deficient mice, contraction of the vas deferens to sympathetic nerve stimulation is reduced by up to 60% and responses to P2X

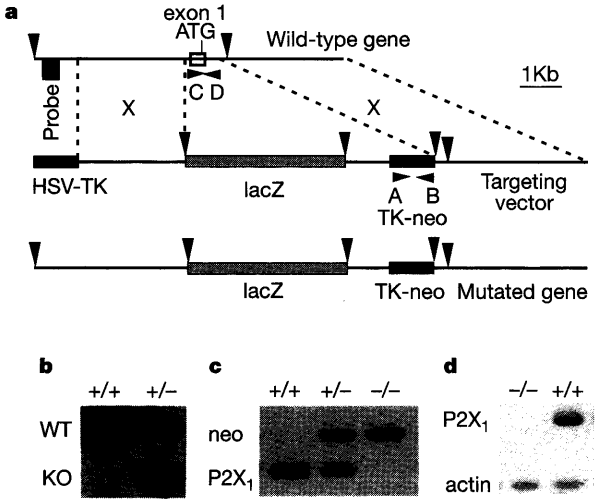

Figure 1 Generation of $P2X_1$-receptor-deficient mice. **a**, Genomic maps of the wild-type gene, targeting vector and mutated gene. *Bam*HI sites (indicated by arrows) and the probe used for detection of the homologous recombination events by Southern analysis are shown. Polymerase chain reaction (PCR) primers used for genotyping of mouse-tail DNA are indicated (A–D). **b**, Southern blot analysis of tail genomic DNA from +/+ and –/– animals. Genomic DNA was digested with *Bam*HI and hybridized with the probe indicated in **a** which detects a 4.8-kb band in +/+ DNA and a 3.7-kb band in –/– DNA. WT, wild-type; KO, knock-out. **c**, PCR genotyping of mouse-tail DNA. Primers A, B, C and D were used in one PCR reaction to genotype mouse-tail genomic DNA. Primers A and B amplify a 519-bp product from the neo^R gene, whereas primers C and D amplify a 317-bp product from the deleted region of the $P2X_1$ receptor gene. **c**, RT-PCR analysis. A PCR product of 442 bp from the $P2X_1$-receptor gene was amplified from bladder complementary DNA from a +/+ animal but not from bladder cDNA of a –/– animal. As a control, amplification of 199-bp product from the actin gene was detected in both samples.

receptor agonists are abolished. These results show that $P2X_1$ receptors are essential for normal male reproductive function and suggest that the development of selective $P2X_1$ receptor antagonists may provide an effective non-hormonal male contraceptive pill. Also, agents that potentiate the actions of ATP at $P2X_1$ receptors may be useful in the treatment of male infertility.

ATP is co-released with noradrenaline from sympathetic nerves and acts through P2X receptors on smooth muscle to mediate membrane depolarization and contraction[1]. Seven P2X receptors have been identified at the molecular level[1]. P2X receptors were originally isolated from the rat vas deferens and can account for the rapidly inactivating α,β-methylene ATP (α,β-meATP)-sensitive native vas deferens P2X receptor phenotype[6]. In smooth muscle the $P2X_1$ receptor is the dominant form expressed[5]. Owing to the lack of potent and subtype-selective P2X-receptor antagonists the physiological role of $P2X_1$ receptors has been difficult to determine. To overcome these problems we have generated a $P2X_1$-receptor-deficient mouse.

The $P2X_1$-receptor-targeting vector was designed to remove the first 45 amino acids of the $P2X_1$ receptor (exon 1) and results in the functional inactivation of the $P2X_1$ receptor gene (Fig. 1). Male and female mice heterozygous for the $P2X_1$ receptor deficiency (+/−) are phenotypically normal and when inter-crossed produced progeny with a mendelian genotype distribution of 1+/+ : 2.1+/− : 1−/− (sex ratio 1.05 male : 1 female, $n = 320$) indicating that there is no selective fertilization or mortality *in utero*. Confirmation of the deficiency of the $P2X_1$ receptor in homozygous mutant mice was obtained at the messenger RNA and protein levels. Polymerase chain reaction after reverse transcription of RNA (RT-PCR), using primers specific for a region 3′ to that deleted by the targeting vector, amplified a 442-base pair (bp) product from +/+ mice but not from −/− mice, indicating that mRNA for a truncated form of the $P2X_1$ receptor is not produced by −/− mice (Fig. 1d). A $P2X_1$ receptor antibody raised against the carboxy terminus of the protein produced high levels of immunoreactivity in the smooth muscle layer of +/+ vas deferens as reported previously[7]. However, no $P2X_1$ receptor immunoreactivity was detected in the vas deferens of −/− mice (Fig. 2a and b).

Interbreeding of mutant −/− mice did not result in pregnancy ($n = 8$) even though coitus had taken place. Mutant −/− female mice produced litters when mated with +/+ ($n = 5$) or +/− males ($n = 3$). When −/− males were mated with +/+ females only 13.7% of matings resulted in pregnancy ($n = 51$ matings of 22 −/− males) compared to a 100% pregnancy rate for +/− males ($n = 12$). These results indicate that the reduced fertility was specific to the male $P2X_1$-receptor-deficient mice. The mean litter size was also reduced for −/− males by 30% (mean litter size −/− 6.6 ± 1.0, $n = 6$; +/− 9.5 ± 0.46, $n = 12$; $P < 0.05$). Taking together the reduction in pregnancy rate and litter size, the fecundity of −/− males is reduced by 90.4%.

A vaginal coagulum/plug is formed following copulation. This results from the action of enzymes in the seminal plasma secreted from the male sex accessory glands. Coagulum weight was essentially the same following mating with +/+ or +/− mice (47.2 ± 2.7 mg, $n = 5$) or −/− males (57.3 ± 3.7 mg, $n = 7$) indicating that similar ejaculate volumes were produced. Given that the fluids secreted from accessory sexual glands account for ~80% of total semen volume these findings suggest that the contraction and function of these glands is unaffected in the $P2X_1$-receptor-deficient mouse. The reduction in male fertility could result from problems with spermatogenesis or sperm quality and/or the reduction of sperm numbers in the ejaculate. $P2X_1$ receptor immunoreactivity was detected in blood vessels in the +/+ testis; however, no immunoreactivity was detected in the seminiferous tubules (Fig. 2c), indicating that $P2X_1$ receptors are not involved in spermatogenesis. In addition, haematoxylin- and eosin-stained sections of testis from −/− mice appeared normal. Vas deferens from +/+, +/− and −/− mice appeared equally full of sperm. Motile sperm were recovered from +/+, +/− and −/− epididymis and vas deferens in similar numbers (epididymal count $(3.6 \pm 0.8) \times 10^6\ ml^{-1}$ for +/+ and +/−, $(5.1 \pm 1.6) \times 10^6\ ml^{-1}$ for −/−, $n = 5$–6) and were equally effective at fertilizing ova *in vitro* (Fig. 2b and d, % success at 4 days 70.0% ± 2.6% for +/+ and 65.5% ± 5.9% for −/−, $n = 3$). Thus infertility does not result from

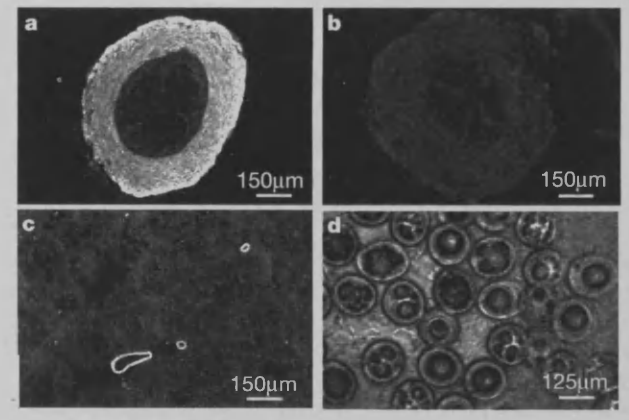

Figure 2 Immunohistochemical detection of $P2X_1$ receptors and *in vitro* fertilization studies. **a**, **b**, Confocal images of transverse sections of vas deferens show immunohistochemical detection of the $P2X_1$ receptor protein in the smooth-muscle layers of the wild-type vas deferens (**a**); no immunoreactivity was present in the −/− mutant animal (**b**). **c**, Confocal image of a transverse section of testis shows $P2X_1$-receptor immunoreactivity associated with blood vessels, but no reactivity with the seminiferous tubules. **d**, Fertilization of ova *in vitro* by sperm extracted from −/− epididymis, photomicrograph taken at 4 days.

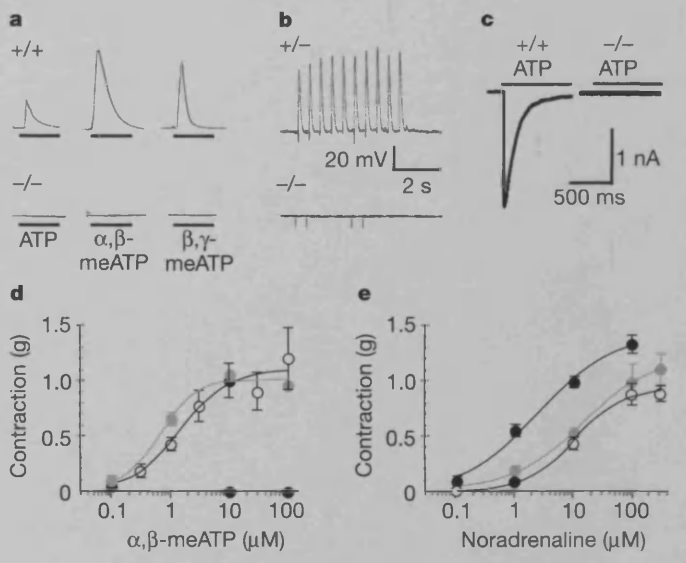

Figure 3 Response to purinergic agonists, nerve stimulation and noradrenaline of wild-type and $P2X_1$-receptor-deficient mouse vas deferens. **a**, ATP (100 μM), α,β-meATP and 1-β,γ-meATP (both 10 μM) evoked transient contractions of +/+ vas deferens but had no effect on the tone of −/− vas deferens. **b**, Intracellular membrane potential recordings from vas deferens smooth-muscle cells. Sympathetic nerve stimulation (10 pulses at 10 Hz, 0.5 ms pulse width) evoked excitatory junction potentials in +/− but not −/− vas deferens (resting membrane potential −87.7 ± 1 mV and 87.6 ± 1.7 mV for +/− and −/− mice respectively, $n = 16$ for each). **c**, Whole-cell patch-clamp recordings from acutely dissociated vas deferens smooth muscle cells. ATP evoked a rapidly inactivating inward current in +/+ but had no effect on −/− acutely dissociated vas deferens smooth muscle cells (holding potential −60 mV). (**a**, **c**, Agonist applications indicated by bar, 60s for **a**). **d**, **e**, Concentration–effect relationship for α,β-meATP (**d**) and noradrenaline (**e**) for +/+ (open circles), +/− (shaded circles) and −/− (solid circles) vas deferens segments.

a sperm dysfunction. Similarly reduced fertility is unlikely to have been caused by blockage of the vas deferens as this results in degeneration of sperm in the epididymis[8]. Analysis of lavage from the uterus, taken at the junction with the coagulum, showed that sperm were abundant in mice mated with +/+ ($n = 3$) or +/− mice ($n = 2$), but no sperm could be detected in the lavage from matings with −/− males ($n = 7$). These results show that the reduction in −/− male fertility results from a reduced sperm count.

Firing of sympathetic nerves associated with ejaculation results in contraction of the vas deferens and emission of sperm into the semen. A substantial component of this sympathetic-nerve-evoked contraction is mediated through the activation of P2X receptors[9]. $P2X_1$-receptor-deficient mice may therefore be compromised in vas deferens function and this could explain the absence of sperm in the ejaculate. In the +/+ vas deferens the purinergic agonists ATP, α,β-meATP and 1-β,γ-meATP evoked transient contractions through the activation of P2X receptors, which declined to baseline during the continued presence of the agonist as described previously[10,11] (Fig. 3a). The pEC_{50} values of α,β-meATP for evoking contraction of the vas deferens for +/+ and +/− mice are 5.83 ± 0.05 and 5.72 ± 0.04, respectively (Fig. 3d). P2X receptors are thought to underlie the excitatory junction potentials (EJPs) recorded from smooth muscle[12]. Spontaneous EJPs and EJPs evoked by sympathetic nerve stimulation were recorded from all impalements of the +/− vas deferens ($n = 16$ impalements from four vasa deferentia) (Fig. 3b). In patch clamp studies on acutely dissociated +/+ vas deferens smooth muscle cells α,β-meATP and ATP evoked transient inward currents (amplitude 2861 ± 410 pA, $n = 8$ and 2096 ± 175 pA, $n = 3$, respectively) with latency <10 ms and with 10%–90% rise times of 22.7 ± 7.7 ms ($n = 4$), demonstrating the direct activation of a ligand-gated P2X-receptor channel[3,11] (Fig. 3c). In contrast, the purinergic agonists α,β-meATP and ATP failed to evoke contractions (Fig. 3a, d) or inward currents (Fig. 3c) and spontaneous or evoked EJPs were not detected (Fig. 3b) ($n = 16$ impalements from four vasa deferentia) from the vas deferens of the −/− $P2X_1$-receptor-deficient mouse. It has been suggested, on the basis of contraction studies, that there may be multiple P2X receptor subtypes in the vas deferens (see references in ref. 13). In the present study vas deferens P2X receptors showed the hallmark properties of homomeric $P2X_1$ receptors (transient α,β-meATP and 1-β, γ-meATP sensitive responses). The lack of functional P2X receptors in the −/− vas deferens shows that if subtypes do exist the expression of $P2X_1$ receptors is essential for the production of functional receptors.

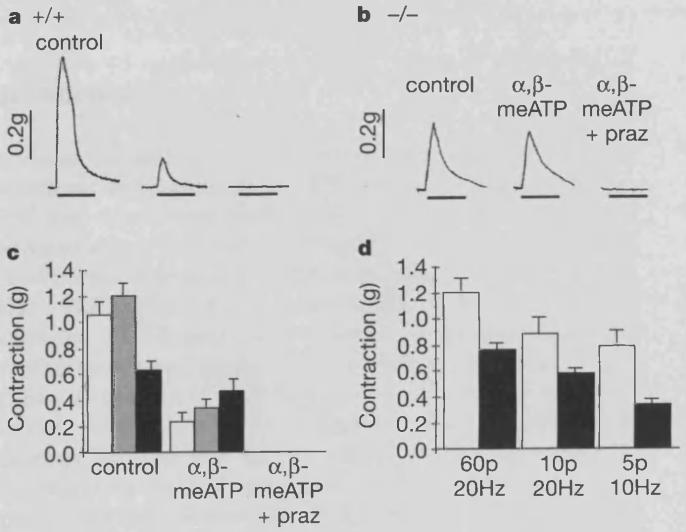

Figure 4 Sympathetic nerve-mediated contraction of +/+ and −/− $P2X_1$-receptor-deficient vas deferens. **a**, **b**, Nerve stimulation, 60 pulses at 20 Hz (period indicated by bar) evoked contractions of +/+ (**a**) and −/− (**b**) vas deferens. Following desensitization of P2X-receptor-mediated responses with α,β-meATP (10 μM), the residual contraction was abolished by the α_1-adrenoceptor antagonist prazosin (0.1 μM). **c**, Summary of the effects of P2X-receptor-desensitization with α,β-meATP and the α_1-adrenoceptor antagonist prazosin on neurogenic contractions of the mouse vas deferens; +/+ (white columns), +/− (grey columns) and −/− (black columns). **d**, Effects of stimulus frequency and duration on neurogenic contractions of the mouse vas deferens. Histogram shows peak amplitude of contraction to stimulation with 60 pulses at 20 Hz, 10 pulses at 20 Hz and 5 pulses at 10 Hz for +/+ (open columns) and −/− (black columns) mice.

The lack of contraction of the −/− vas deferens to purinergic agonists does not result from an impairment of the contractile function, as responses evoked by 100 mM potassium chloride were the same for +/+ and −/− $P2X_1$-receptor-deficient mice (1.36 ± 0.12 g and 1.17 ± 0.08 g, respectively; $n = 14$–17). In addition, +/+, +/− and −/− vas deferens contracted in response to noradrenaline (Fig. 3e). The vas deferens of the −/− mice were more sensitive to noradrenaline than the +/+ (pEC_{50} values of 5.65 ± 0.15 and 4.79 ± 0.09, respectively, $P = 0.005$, $n = 4$–6) and the +/− mice had an intermediate sensitivity with a pEC_{50} of 5.1 ± 0.27 ($n = 5$). The increase in sensitivity to noradrenaline in −/− animals suggests a compensatory change in −/− animals which could be accounted for by an increase in α_1-adrenoceptor numbers[14].

The lack of P2X receptor-mediated contractions in −/− vas deferens suggests that contractions in response to sympathetic nerve stimulation may also be affected. In rats the duration of copulation from the first pelvic thrust to dismount is 1–3 s (ref. 15). To determine the response of the vas deferens to nerve stimulation the vas deferens was stimulated with a train of 60 pulses at 20 Hz (train duration 3 s) (Fig. 4). Electrically evoked responses were mediated through the activation of sympathetic nerves, as they were abolished by tetrodotoxin (0.3 μM) or the adrenergic neuron blocker guanethidine (3 μM) ($n = 4$). The peak amplitude of contraction of +/+ mouse vasa deferentia was 1.16 ± 0.08 g ($n = 21$). Desensitization of P2X receptors with α,β-meATP revealed that the P2X-receptor-mediated contraction accounted for 79 ± 5.5% of the response ($n = 7$). The residual response was abolished by the α_1-adrenoceptor antagonist prazosin (Fig. 4a and c). Similar responses were recorded from +/− males (Fig. 4c). In contrast, the peak amplitude of contractions of −/− vas deferens was only ~60% (0.7 ± 0.04 g, $n = 36$) of the +/+ response ($n = 16$), was reduced by <10% by α,β-meATP treatment and was abolished by prazosin ($n = 4$) (Fig. 4b and d). This increase in magnitude of the α_1-adrenoceptor-mediated component in −/− mice, like the response to exogenously applied noradrenaline, can be accounted for by an increase in α_1-adrenoceptor number[14]. Long trains of high-frequency stimulation are known to favour the noradrenergic-mediated component of smooth-muscle contraction; however, they probably do not reflect the *in vivo* nerve traffic.

The physiological sympathetic nerve firing pattern resulting in ejaculation remains to be determined. It is likely that the burst of sympathetic nerve activity to the vas deferens associated with ejaculation is considerably shorter than the duration of copulation. We therefore determined the effects of 0.5-s trains of stimuli at different frequencies. The magnitude of the contractile response was frequency dependent (Fig. 4d) and in response to 10-Hz stimulation the resonse of −/− vas deferens was only 40% of the +/+ response. A similar decrease in ejaculate sperm count results in infertility[16]. Thus, this reduction in the neurogenic vas deferens contraction can account for the decrease in male fertility rate and suggests that in the majority of cases, *in vivo* contraction of the −/− vas deferens is below the threshold required to eject the sperm into the semen.

In addition to the vas deferens, $P2X_1$ receptors are also present on a variety of other smooth-muscle preparations, including the urinary bladder and arteries, as well as parts of the nervous system. There was no obvious effect on the behaviour of $P2X_1$-receptor −/− mice, and heart rate (572.2 ± 20.3 beats per minute for −/− and 556 ± 19 beats per minute for +/+, $n = 8$ for each), and bladder function all appeared normal. There was, however, a small

increase in systolic blood pressure at rest (115.8 ± 2 mm Hg for −/− and 108 ± 1.8 mm Hg for +/+, as measured by the tail cuff method; $P = 0.016$, $n = 8$ for each).

We have shown that $P2X_1$ receptors are involved in the contraction of the vas deferens and that $P2X_1$ receptor deficiency results in a 90% decrease in male fertility through a reduction in sperm in the ejaculate associated with a decrease in neurogenic vas deferens contraction. In mice it appears that the residual α_1-adrenoceptor-mediated neurogenic vas deferens contraction is insufficient for normal ejaculatory function. Selective α_1-adrenoceptor antagonists do not cause azoospermia and infertility in man[17] and contractile studies have indicated the presence of a substantial non-adrenergic component of contraction of prostatic portions of the human vas deferens[18]. This suggests that $P2X_1$-receptor antagonists may provide a target for the development of a non-hormonal male contraceptive pill. In addition, agents that potentiate the actions of ATP at $P2X_1$ receptors may be useful in the treatment of male infertility[19]. □

Methods

Generation of $P2X_1$ receptor-deficient mice

The targeting vector includes 5.8 kilobases (kb) of $P2X_1$-receptor genomic DNA, the *lacZ* gene, the *neo*^{R} gene driven by the TK promoter and the HSV-tk gene. Homologous recombination of this vector with the wild-type gene results in deletion of 350 bp of DNA which includes exon 1 and the initiating ATG. The deleted 350 bp are replaced by the *lacZ* gene and the *neo*^{R} gene of the targeting vector. The targeting vector was electroporated into E14.1a embryonic stem (ES) cells derived from the 129Ola mouse strain, and colonies were selected with G418 and gancyclovir. Positive colonies were identified by the presence of the 3.7 kb band in *Bam*HI-digested genomic DNA. Four ES colonies containing the targeting event were microinjected into F1 (CBA × C57BL/6) blastocysts and chimaeras were derived. Germline transmission of the targeted allele was obtained from all four ES colonies upon mating to MF-1 animals. The mice analysed here have 129Ola-MF-1 genetic backgrounds.

Immunohistochemistry

Detection of the distribution of the $P2X_1$ receptor using an antibody raised against the C terminus of the receptor was as described[7]. *In vitro* fertilization was as described[20].

Physiological studies

Mice were produced by crossing +/− mice. Littermates were genotyped by PCR, animals were sexually mature (4–6 months old), weight was 37.5 ± 0.8, 39.6 ± 1.3 and 37.3 ± 1.0 g for wild type, +/− and −/−, respectively (n = 15–19). Mouse vasa deferentia were mounted in Ringers solution at 36 °C in 15-ml organ baths under an initial load of 1 g, and tension was monitored isometrically. Agonists were applied to the bath at 30-min intervals and removed by washing; this solution was also used for intracellular recordings from the vas deferens using standard methods. Trains of electrical stimuli were delivered through silver chloride electrodes, 20–40 V 0.5 ms pulse width. Vas deferens smooth-muscle cells were enzymatically dissociated and responses to purinergic agonists applied rapidly by a U-tube perfusion system were determined in voltage-clamp recordings as described[21].

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Nerve evoked P2X receptor contractions of rat mesenteric arteries; dependence on vessel size and lack of role of L-type calcium channels and calcium induced calcium release

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1 Contractile responses to short trains of nerve stimulation have been characterized in small, medium and large arteries from the rat mesenteric circulation (5th–6th, 2nd–3rd and 1st order, respectively). In addition, sources of calcium for smooth muscle contraction have been investigated.

2 Nerve stimulation (10 pulses at 10 Hz) evoked reproducible contractions. The P2 receptor antagonist suramin (100 μM) reduced constrictions by 65.3±7.4, 82.7±3.3 and 3.1±6.1% in small, medium and large arteries respectively. The α-adrenoceptor antagonist prazosin (0.1 μM) reduced responses by 32.6±2.6, 27.0±1.5 and 97.0±1.9% respectively.

3 The L-type calcium channel antagonist nifedipine (1 μM) reduced nerve-evoked contractions by 2.8±3.3, 10.0±3.7 and 13.5±2.7% in small, medium and large arteries respectively. When the adrenergic component of contraction was blocked by prazosin (0.1 μM) nifedipine reduced responses by 4.6±7.9, 14.3±2.0 and 3.0±1.9% respectively.

4 Contractile responses to exogenous α,β-meATP were unaffected by the depletion of calcium stores with cyclopiazonic acid (30 μM). This indicates that mobilization of calcium from internal stores is not required for P2X receptor mediated smooth muscle contraction.

5 We conclude that for neurogenic responses, the P2X receptor mediated component of constriction dominates in small mesenteric arteries (3rd –6th order) while in large arteries (1st order) noradrenaline mediates contraction. For P2X receptor mediated responses all the calcium required for smooth muscle contraction enters the cell directly through P2X receptor channels.

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Keywords: P2X receptors; artery; smooth muscle; calcium

Abbreviations: α,β-meATP, α,β-methylene ATP; CICR, calcium induced calcium release; CPA, cyclopiazonic acid; IP_3, inositol triphosphate; NPY, uropeptide Y

Introduction

Peripheral arterial tone is under the control of the sympathetic nervous system. ATP and noradrenaline are co-stored and co-released from sympathetic nerves and mediate vasoconstriction by acting at arterial P2X receptors and α-adrenoceptors (Sneddon & Burnstock, 1984; von Kugelgen & Starke, 1985). The relative contribution of each transmitter toward the overall contractile response is variable depending on the preparation, species and stimulation parameters used e.g. Bao & Stjarne (1993); Kennedy *et al.*, (1986); Sjoblom-Widfeldt (1990). We have recently shown differences, depending on the size of the vessel, in the sensitivity of P2X receptors in rat mesenteric arteries to exogenously applied agonists (Gitterman & Evans, 2000). This raised questions about the role of P2X receptors in mediating nerve-evoked contractions in different diameter mesenteric arteries. There is some indication that the size of the purinergic component of contraction is dependent on the diameter of the vessel, with the smallest arteries having the largest purinergic component of constriction (Evans & Surprenant, 1992; Ramme *et al.*, 1987). This is of interest as small arteries and arterioles are particularly important in determining vascular resistance and hence systemic blood pressure. The majority of work has however focused on medium and large arteries (e.g. Angus *et al.* (1988); Sjoblom-Widfeldt (1990)) with comparatively little research investigating small resistance arteries (Evans & Surprenant, 1992; Morris, 1999; Phillips *et al.*, 1998). The use of different vascular beds and different species has also made direct comparisons between large and small vessels difficult. In addition, the parameters of stimulation (frequency and train length) used can have a profound effect on contractile responses (Sjoblom-Widfeldt, 1990). We were interested in determining the relative importance of purinergic and adrenergic constriction using stimulation parameters that are thought to approximate to the symapthetic firing rate *in vivo*. Studies investigating the discharge pattern of sympathetic neurones *in vivo* have found that neuronal activity occurs in regular short bursts of high frequency firing rather than long sustained trains (Johnson & Gilbey, 1996). In this study we have used parameters of stimulation that reflect such a pattern of activity.

The trigger for contraction of smooth muscle is a rise in the cytosolic level of calcium. For noradrenaline it is known that activation of postjunctional α-adrenoceptors leads to the

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production of IP_3 and release of calcium from intracellular stores. For P2X receptors calcium influx is essential but the sources of calcium and whether additional amplification of the signal occurs is unclear. ATP released from sympathetic nerve terminals leads to the activation of postjunctional P2X receptors and direct influx of calcium (Benham & Tsien, 1987). The depolarization of the smooth muscle cell in response to P2X receptor activation opens voltage-dependent calcium channels thus providing an additional source of calcium influx. In large arteries the purinergic component of contraction has been shown to be sensitive to the L-type calcium channel blocker nifedipine (Bulloch *et al.*, 1991; Omote *et al.*, 1989; Surprenant *et al.*, 1983). By contrast, studies in small resistance arteries of the guinea-pig submucosa (Galligan *et al.*, 1995), established that nifedipine had no effect on contractile responses evoked by α,β-methylene ATP (α,β-meATP). This suggests that in small vessels, sufficient calcium enters the smooth muscle cell directly through the P2X receptor channel to produce contraction. It is however unclear whether the nifedipine resistance seen in response to exogenous agonists is also seen in nerve-evoked contractions. Moreover, in certain smooth muscle calcium entry is only the first step in producing a rise in the level of intracellular calcium (Ganitkevich & Isenberg, 1992). The initial signal of calcium entry can often trigger a process of amplification whereby further calcium is released from ryanodine-sensitive intracellular stores (Gregoire *et al.*, 1993). This process is termed calcium induced calcium release (CICR). It is unclear whether CICR is involved in achieving the necessary rise in intracellular calcium for P2X receptor-mediated contraction of vascular smooth muscle.

The aims of this study are twofold: (1) to systematically compare neurogenic vasoconstriction in small, medium and large arteries from the rat mesenteric bed, using stimulation parameters thought to approximate to conditions found *in vivo* and (2) to determine the relative roles of P2X receptors, L-type voltage-gated calcium channels and CICR in mediating the increase in intracellular calcium required for smooth muscle contraction in response to nerve stimulation.

Methods

Adult male Wistar rats (250–300 g) were killed by cervical dislocation or CO_2 asphyxiation followed by femoral artery exsanguination. A portion of the gut with attached mesenteric arcade was removed and mesenteric arteries were dissected; large vessels correspond to the superior mesenteric artery, medium-sized vessels were from second or third order branches and small vessels correspond to fifth or sixth order arteries.

Medium and large artery rings were mounted in a Mulvany myograph using standard procedures (Lagaud *et al.*, 1996) (internal diameters 252 ± 5 μm, range 231–315 μm, $n=23$, and 549 ± 28.0 μm, range 408–705 μm, $n=19$, respectively); changes in arterial tone were recorded and analysed using a MacLab data acquisition system. Small arteries were dissected carefully cleaning away all connective tissue and pinned out (stretched to approximately 150% of their resting length) in a Sylgard coated organ bath (volume 2 ml). The organ bath was placed on the stage of an inverted microscope and changes in external arterial diameter were analysed using Diamtrak software as previously described (Neild, 1989). Small arteries were studied using diamtrak video imaging microscopy because they were too small to be mounted in a myograph. In order to demonstrate that the different experimental methodologies had no bearing on our results we have previously compared the behaviour of medium-sized arteries using the two systems. The contractile responses to α,β-meATP and KCl were very similar in each case, producing almost identical concentration-response relationships when using either myography or Diamtrak (Gitterman & Evans, 2000). We are confident that the results we obtain with the two systems are directly comparable. The outside diameter of the vessels was 109 ± 6 μm, range 80–193 μm, $n=33$ (wall thickness accounts for $\sim$40% giving a mean internal diameter of $\sim$66 μm).

Nerve stimulation

Tissues were superfused with Ringer's solution (composition in mM) NaCl 120, Glucose 11, $NaHCO_3$ 22, KCl 5, NaH_2PO_4 1, $CaCl_2$ 2.5, pH to 7.3 with NaOH, kept at 35°C–37°C for myography and 32°C–34°C for Diamtrak and continually gassed with 95% O_2 and 5% CO_2. Magnesium was omitted from the solution as this has previously been found to increase the amplitude of contractile responses to nerve stimulation (Ramme *et al.*, 1987).

For myograph experiments, perivascular nerves were electrically stimulated by two platinum electrodes mounted in the jaws either side of the vessel. In Diamtrak studies, arteries were stimulated through a blunt glass microelectrode filled with bath solution and placed in close proximity to the artery about 1 mm from the diameter recording site. Electrical stimulation was delivered through an Applegarth Electronics stimulator (Oxford, U.K.); parameters were 10 pulses at 1–50 Hz (usually 10 Hz), 0.2–0.3 ms pulse width at 10–40 V. Stimulation delivered every 6 min gave reproducible responses. In each experiment 0.3 μM tetrodotoxin was applied to the preparation to confirm that contractile responses were neurogenic in origin. Antagonists were only applied once contractile responses were stable and applications continued until stable responses were obtained in the presence of the antagonist.

Agonist experiments

Tissues were superfused with a physiological saline solution (PSS; composition in mM): NaCl 150, KCl 2.5, HEPES 10, $CaCl_2$ 2.5, $MgCl_2$ 1, pH to 7.3 with NaOH. Experiments were conducted at 35°C–37°C for myography and 32°C–34°C for Diamtrak. Drugs were added to the superfusate at the required final concentration. Reproducible responses to agonists were obtained when applications were separated by 30 min intervals. In experiments testing the effect of nifedipine and $CdCl_2$ both antagonists were pre-superfused for 10 min prior to being added together with the agonist. In each case, contractions evoked by PSS containing 60 mM KCl (with a proportionally reduced concentration of NaCl) were used as a positive control to test for antagonist function.

In experiments determining the relative role of intracellular calcium stores caffeine (10 mM) was applied to evoke responses by releasing calcium from the sarcoplasmic reticulum. When contractions were reproducible, 30 μM

cyclopiazonic acid (CPA) was applied in zero calcium PSS for 15 min to deplete calcium stores. In the continued presence of CPA, the artery was then returned to normal calcium PSS for 90 s and caffeine subsequently applied. Responses to caffeine were abolished after this treatment and intracellular calcium stores considered to be fully depleted. The same depletion protocol was used to assess the contribution of intracellular calcium stores to contractions evoked by α,β-meATP.

Data analysis

Data are expressed as mean±s.e.mean throughout and n=number of arteries, number of animals. When more than one vessel was used from one animal, the data were averaged. The average value per animal was then used in calculating the overall mean and standard error. Differences between means were tested using either a two sample or paired, two-tailed t-test, as appropriate. A P value of <0.05 was considered statistically significant.

Drugs

α,β-methylene ATP (α,β-meATP), caffeine, $CdCl_2$, cyclopiazonic acid (CPA), nifedipine, prazosin and suramin (Sigma, U.K.).

Results

Frequency response relationship

Electrical stimulation of rat mesenteric arteries evoked reproducible constrictions in all vessels tested. Responses were fast, monophasic and transient, and arterial tone/diameter rapidly returned to the initial baseline value after stimulation ceased. In order to verify that the properties of sympathetic vasoconstriction were comparable in all sizes of artery, frequency response relationships were constructed. A short burst of 10 pulses was applied at frequencies ranging from 1–50 Hz (Figure 1). Relative contractile responses were very similar for all vessel sizes up to a frequency of 20 Hz (n=5–6 arteries from 3–6 animals), and only a slight divergence was seen at higher frequencies of stimulation (30 and 50 Hz). For the remaining experiments we chose to stimulate with 10 pulses at 10 Hz as these parameters always gave a robust, near maximal response and reflect the short bursts of sympathetic activity recorded under physiological conditions (Johnson & Gilbey, 1996).

Relative amplitude of purinergic and adrenergic neurogenic responses

To determine whether the proportion of purinergic and adrenergic components of sympathetic vasoconstriction changed with vessel diameter, the effects of selective antagonists were tested. The P2 receptor antagonist suramin (100 μM) inhibited the purinergic component of constriction and substantially reduced responses in both small and medium-sized arteries (pA2 5.2 for small and medium arteries, see Gitterman & Evans (2000) but had virtually no effect in large vessels (Figure 2); responses were reduced by 65.3±7.4, 82.7±3.3 and 3.1±6.1% (n=6 arteries from 3–5 animals) in small, medium and large arteries respectively. Prazosin (0.1 μM), a potent and selective antagonist of α adrenergic receptors, had only a limited effect on constrictions in small and medium sized arteries, reducing contractile responses by 32.6±2.6 and 27±1.5% (n=4–6 arteries from 3–4 animals) respectively. Contractions in large arteries were abolished by prazosin (reduced by 97.0±1.9%, n=6 arteries from three animals). When the two antagonists were applied concomitantly, responses to nerve stimulation were abolished in all arteries tested (n=6 arteries from 3–5 animals).

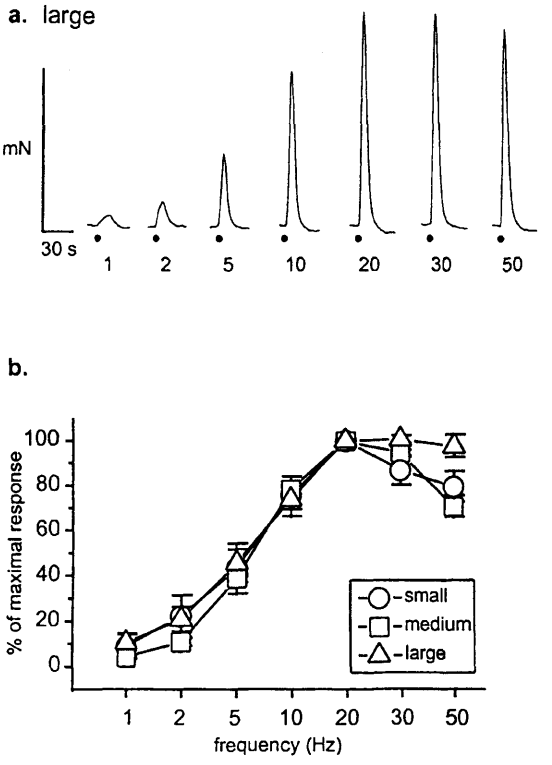

Figure 1 Nerve-evoked contractions of rat mesenteric arteries to 10 pulses of stimulation at a range of frequencies. (a) Responses of medium-sized mesenteric arteries; contractions are rapid and transient. Nerve stimulation is indicated by the circle. (b) Frequency-response relationships showing similar responses for all three sizes of artery.

Role of voltage-gated calcium channels

We have previously demonstrated that calcium influx is essential for P2X receptor-mediated constrictions in all sizes of mesenteric artery (Gitterman & Evans, 2000). Calcium influx in response to P2X receptor activation can occur either directly through the P2X receptor channel or through voltage-gated calcium channels. To investigate the role of the voltage-dependent calcium channels in neurogenic vasoconstriction, the effect of the selective L-type calcium channel antagonist nifedipine was tested on nerve-evoked contractions. Nifedipine (1 μM) had only a small effect on responses, with constrictions in small, medium and large vessels being reduced by only 2.8±3.3, 10.0±3.7 and 13.5±2.7% (n=5–6 arteries from 3–4 animals), respectively (Figure 3). To examine the effect of nifedipine on just the purinergic component of the response, prazosin was first

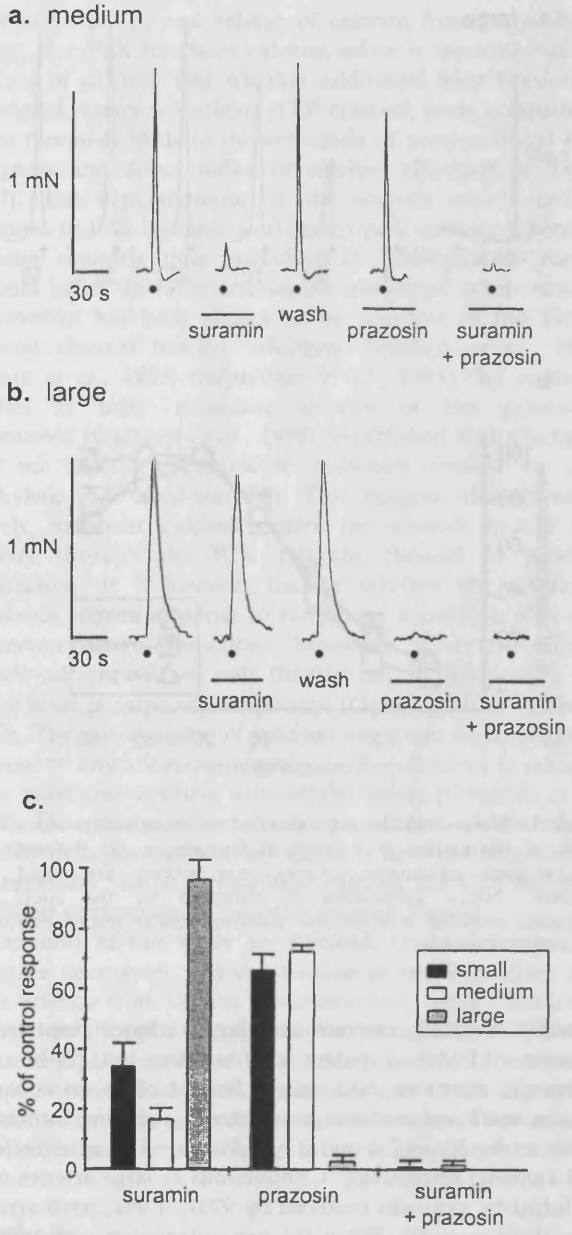

Figure 2 The purinergic component of constriction dominates in small and medium arteries while the noradrenergic component dominates in large. Effects of suramin (100 μM) and prazosin (0.1 μM) alone and in combination on responses to 10 pulses of stimulation at 10 Hz in medium (a) and large (b) arteries. Application of both antagonists abolishes responses in all arteries. Circle indicates electrical stimulation and bars indicate periods of antagonist application; traces without annotation are control responses. (c) Histogram shows percentage of control response in the presence of antagonist. Data are mean contarctions $\pm$ s.e.mean ($n=4-6$ arteries from 3–5 animals).

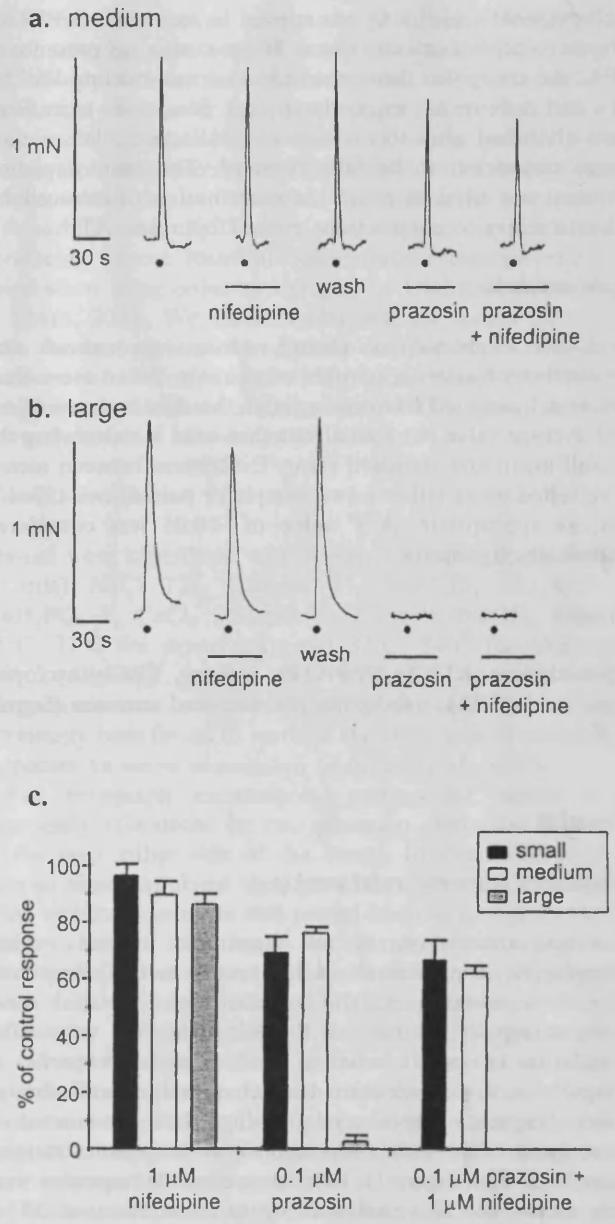

Figure 3 Nifedipine has only a small effect on nerve evoked constrictions. Typical responses in medium (a) and large (b) arteries. Nifedipine (1 μM) has little effect, both when applied alone, and when prazosin (0.1 μM) is first applied to block the adrenergic component of constriction. Circle indicates electrical stimulation and bars indicate period of antagonist application; traces without annotation are control responses. (c) Data are mean responses $\pm$ s.e.mean ($n=4-6$ arteries from 3–5 animals) expressed as per cent of control.

applied to block adrenergic transmission. Under these conditions, nifedipine also reduced responses only slightly: by 4.6 ± 7.9, 14.3 ± 2.0 and $3.0\pm1.9\%$ ($n=4-6$ arteries from 3–4 animals) respectively.

In control experiments, responses to 60 mM KCl were abolished by nifedipine (1 μM) in medium and large vessels but only reduced by $52.8\pm6.9\%$ ($n=4-5$ arteries from three animals) in small arteries (Figure 4). This nifedipine-resistant component of constriction to 60 mM KCl in small vessels suggested that other calcium channels may be present. We therefore also tested the effect of cadmium, a non-selective blocker of all voltage-dependent calcium channels. $CdCl_2$ (300 μM) abolished contractions to 60 mM KCl in all arteries ($n=4-5$ arteries from three animals).

The possibility therefore exists that, at least in small arteries, voltage-gated calcium channels distinct from L-type channels may be present. As cadmium blocks all voltage-dependent calcium channels, N-type calcium channels in the nerve terminal which are essential for the release of

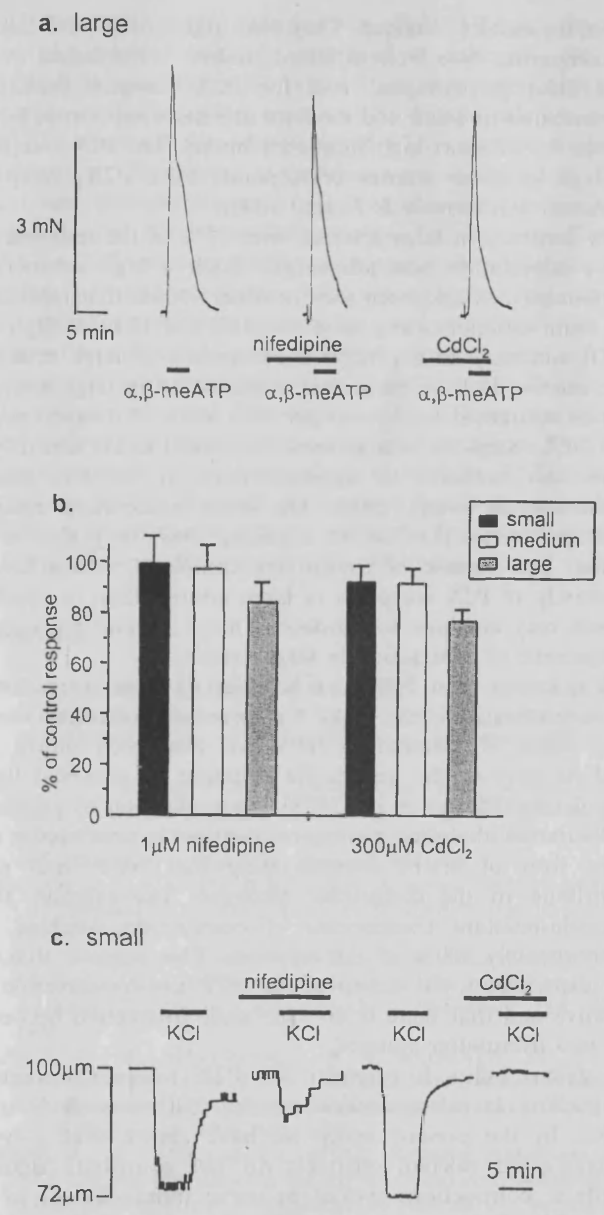

Figure 4 The calcium channel blockers nifedipine and cadmium have little effect on contractions to exogenous application of a P2X receptor agonist (a) Typical responses from a large artery showing the effect of nifedipine (1 μM) and $CdCl_2$ (300 μM) on responses to an EC_{50} concentration of α,β-meATP. (b) Histogram shows mean data$\pm$s.e.mean ($n=4-6$ arteries from 3–4 animals) for small, medium and large arteries. (c) Traces showing nifedipine resistance of control responses to 60 mM KCl in small arteries. The component of constriction resistant to nifedipine (1 μM) is abolished by $CdCl_2$ (300 μM). Periods of drug application are indicated by bar.

transmitters would also be blocked. We therefore stimulated arterial P2X receptors with applied agonist. Cadmium (300 μM) marginally reduced contractions to an $\sim EC_{50}$ concentration of α,β-meATP (1 μM for small and medium and 100 μM for large arteries see Gitterman & Evans (2000) in small and medium-sized arteries (reduced by 8.4 ± 5.4 and $9.1\pm5.0\%$, respectively; $n=4-5$ arteries from three animals), and reduced contractions by $22.8\pm4.0\%$ ($n=5$ arteries from three animals) in large vessels (Figure 4). To determine whether these reductions reflected the proportion of calcium influx through L-type calcium channels, nifedipine was also tested on responses to α,β-meATP. In this case, a similar pattern of results was seen as with cadmium although responses were slightly more resistant to blockade by nifedipine (Figure 4). Contractions were not reduced in small arteries and were reduced by 2.0 ± 8.7 and $15\pm7.3\%$ ($n=4-5$ arteries from three animals) in medium and large vessels respectively.

Effect of cyclopiazonic acid on purinergic constrictions

To investigate whether calcium-induced calcium release is involved in smooth muscle contraction, we tested the effect of depleting intracellular calcium stores with cyclopiazonic acid (30 μM). Ideally this experiment would have been conducted using nerve-evoked responses. It has however been shown that CICR is involved in transmitter release from sympathetic nerve terminals (Smith & Cunnane, 1996). We therefore tested the effects of CPA on contractions to applied agonists. For these experiments, responses to caffeine (10 mM), which causes contractions by releasing calcium from intracellular stores, were used to control for complete store depletion. The amplitude of responses to caffeine were also used to estimate the size of the intracellular pool of calcium. Responses to 10 mM caffeine were 88.7 ± 7.5, 51.7 ± 10.9 and $30.9\pm9.4\%$ ($n=4-7$ arteries from 3–4 animals) of contractions to 60 mM KCl and 90.8 ± 5.1, 41.8 ± 5.8 and $50.4\pm19.3\%$ of response to α,β-meATP (10 μM for small and medium 100 μM for large, $n=3-6$ arteries from three animals) in small, medium and large arteries, respectively. Treatment with 30 μM CPA abolished responses to caffeine but did not reduce responses to an EC_{50} concentration of α,β-meATP (1 μM for small and medium, 100 μM for large, Figure 5). Responses were, as a percentage of control, 101.3 ± 6.4, 101.3 ± 3.5 and $117.3\pm9.5\%$ ($n=4-7$ arteries from 3–4 animals) for small, medium and large arteries, respectively.

Discussion

In this study we have shown substantial differences in the relative roles of P2X receptors and α-adrenoceptors in mediating sympathetic control of arterial tone in the rat

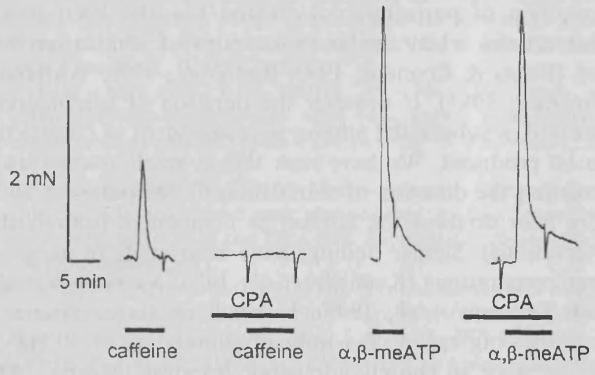

Figure 5 Contractions to α,β-meATP are unaffected by depletion of intracellular calcium stores with CPA. Typical traces of responses in medium arteries. Control contractions evoked by emptying intracellular calcium stores with caffeine (10 mM) are abolished by prior perfusion with CPA (30 μM) while contractions to α,β-meATP (1 μM) remain unaffected.

mesenteric bed depending on the diameter of the vessel. The purinergic component of transmission dominates in small-medium arteries (3rd–6th order), while contractions in large arteries (1st order) are almost entirely adrenergic. We have also shown that calcium entering the smooth muscle cell directly through the P2X receptor channel is sufficient to mediate contraction with little contribution from L-type voltage dependent calcium channels or CICR.

The variety of stimulation parameters used in studies investigating neurogenic vasoconstriction raises the question as to which pattern of stimulation corresponds to sympathetic transmission *in vivo*. Johnson & Gilbey (1996) showed that sympathetic neurones innervating rat arteries fire in rhythmic bursts separated by relative silence rather than sustained trains. The rhythm is often dictated by respiration as indicated by activity of the phrenic nerve. The firing rate of sympathetic neurones was found to be between 0.8 and 0.9 Hz, each discharge being a brief burst of multiple action potentials with an intraburst frequency of up to 20 Hz or more (Johnson & Gilbey, 1994). The parameters we chose were designed to represent one such burst of activity. Electrical stimulation of 10 pulses at 10 Hz evoked reproducible contractions in all arteries tested. Responses were neurogenic in origin as they were abolished by treatment with tetrodotoxin. Frequency response relationships were similar for all vessel sizes, indicating that the properties of neurotransmitter release are very similar. This further demonstrates that the different experimental methodologies which were used would not be expected to influence the results obtained.

Previous studies investigating the neurogenic control of arterial tone have revealed varying proportions of purinergic versus adrenergic components depending on the species, tissue and stimulation parameters used (Yang & Chiba, 1999, 2000). Although there is considerable evidence to suggest the purinergic component dominates in small resistance arteries (Evans & Surprenant, 1992; Ramme *et al.*, 1987), no systematic comparisons have been conducted on arteries from one vascular bed. To our knowledge, this is the first study directly comparing neurogenic contractions in three different sizes of artery from the same vascular bed. Selective blockade of purinergic and noradrenergic transmission revealed a P2X receptor mediated component of approximately 70% in small and medium arteries. A similar proportion of purinergic constriction has also been seen in other arteries when similar short trains of stimulation were used (Evans & Cunnane, 1992; Ren *et al.*, 1996; Warland & Burnstock, 1987). If however the duration of stimulation is increased, a substantial adrenergic component of constriction can be produced. We have seen this in small arteries where increasing the duration of stimulation to 100 pulses at 10 Hz more than doubled the adrenergic component (unpublished observations). Similar findings have been made in numerous other preparations (Kennedy *et al.*, 1986; Sjoblom-Widfeldt, 1990; Todorov *et al.*, 1999). In small resistance arteries of the guinea-pig ear, a 30 s train of stimulation at 10 Hz can even produce an entirely adrenergic response (Morris, 1999). However it is unclear what physiological firing patterns these long trains of high frequency correspond to. These examples demonstrate the great influence that stimulation parameters have on the contractile behaviour of blood vessels and underline how the choice of parameters can significantly affect the results obtained. They also highlight the difficulties in comparing data from different studies. Nevertheless there is a clear physiological role for P2X receptor mediated transmission in small and medium arteries when sympathetic nerves fire in short high frequency bursts. The P2X receptor subtype in these arteries corresponds to a $P2X_1$ receptor homomer (Gitterman & Evans, 2000).

By contrast, in large arteries, over 95% of the response to nerve stimulation was adrenergic. Such a high adrenergic component has also been seen in other vessels that represent the main conduit artery of a vascular bed (Bao & Stjarne, 1993) and may be a general characteristic of large arteries. The relative lack of purinergic transmission in large arteries can be accounted for by our previous work. We have shown that P2X receptors in large mesenteric arteries are about 100 times less sensitive to agonists than in smaller vessels (Gitterman & Evans, 2000). The lower innervation density in large arteries (Luff & McLachlan, 1989) may also be a factor. Less release of transmitter combined with a lower sensitivity of P2X receptors in large arteries than in smaller vessels may combine to produce a much smaller purinergic component of contraction in large vessels.

It is known that NPY can be released from sympathetic nerve terminals (Potter, 1988). Under certain conditions using long trains of stimulation NPY has also been shown to mediate part of the contractile response to electrical field stimulation (Phillips *et al.*, 1998). Co-application of prazosin and suramin abolished neurogenic contractile responses in all three sizes of artery demonstrating that NPY does not contribute to the constrictor response. The suramin and prazosin-resistant components of contraction combine to approximately 100% of the response. This suggests that in our experiments, the action of the ATP and noradrenline is additive and that there is no synergistic interaction between the two transmitter systems.

Calcium influx is essential for P2X receptor mediated contraction in rat mesenteric arteries (Gitterman & Evans, 2000). In the present study we have shown that L-type voltage-gated calcium channels do not contribute significantly to contractions evoked by nerve stimulation or to α,β-meATP. This extends on findings made in submucosal arterioles of the guinea-pig ileum (Galligan *et al.*, 1995). Our results suggest that all the calcium influx that occurs in response to P2X receptor activation following nerve stimulation is directly through the P2X receptor channel. Although evidence has been found for the involvement of calcium channels in purinergic vasoconstriction (Bulloch *et al.*, 1991; Omote *et al.*, 1989), these data are generally from large vessels, not small resistance arteries. The non-selective calcium channel blocker cadmium had a very similar effect to nifedipine on P2X receptor mediated contractions and confirms that there is no other voltage dependent calcium channel contributing to calcium influx. Interestingly, there was a discrepancy in the effects of calcium channel blockers on control responses to 60 mM KCl. Nifedipine and cadmium abolished responses in medium and large arteries, in contrast for small arteries there was a nifedipine-resistant component of contraction to 60 mM KCl that was abolished by cadmium. This suggests the presence of a non-L-type calcium channel in small arteries, however it does not appear to be involved in P2X receptor-mediated contraction.

CPA inhibits the sacrolemmal Ca^{2+}-ATPase depletes internal calcium stores and was used to determine the role of CICR in amplifying the calcium rise associated with P2X receptor activation. CPA treatment to deplete calcium stores had no effect on the amplitude of P2X receptor mediated contractions. Responses to caffeine were abolished by CPA treatment demonstrating that all releasable calcium had been depleted from the intracellular stores. The lack of effect of CPA on P2X receptor mediated responses demonstrates that calcium release from intracellular stores is not required for P2X receptor mediated contraction; and combined with the data with nifedipine and cadmium suggests that all the calcium required for contraction enters directly though the P2X receptor channel. The responses to caffeine can also be used to estimate the size of internal calcium stores. Our data show that relative to contractions evoked by KCl, calcium stores are largest in small arteries and become progressively smaller with increasing arterial diameter. This is intriguing as it seems to contradict other studies which have found that the relative importance and size of intracellular calcium stores is greatest in large conduit arteries (van Breemen & Saida, 1989; Ashida *et al.*, 1988).

In summary we have shown that the relative proportion of purinergic versus adrenergic component of contractile responses to nerve stimulation are dependent on artery size. In large arteries the response is essentially adrenergic, while in small and medium arteries, that play a key role in the control of blood pressure, the response is predominantly mediated through the activation of P2X receptors. We have shown that P2X receptor mediated neurogenic contractions are resistant to commonly used anti-hypertensive thereapies i.e. α-adrenoceptor and calcium channel antagonists. This suggests that P2X receptors may be novel targets for the treatment of hypertension.

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