STUDIES ON THE INTERACTION OF STEROIDAL NEUROMUSCULAR BLOCKING DRUGS WITH RECOMBINANT MUSCARINIC RECEPTORS

Thesis submitted for the degree of
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by

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Studies on the interaction of steroidal neuromuscular blocking drugs with recombinant human m1-m5 muscarinic receptors expressed in Chinese hamster ovary cells.

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Steroidal neuromuscular blocking drugs (NMBDs) produce a range of effects on the cardiovascular system encompassing both bradycardia and tachycardia. Opinion is divided as to how these side effects occur. Logical targets, however, include the muscarinic receptor in the heart (M2) and/or modulation of noradrenergic (NAdr) transmission in this tissue. In an attempt to address these two possible "side effect target sites" this thesis represents a detailed and systematic examination of the effects of a range of steroidal neuromuscular blocking drugs on recombinant human muscarinic receptors (assessed in [^{3}H]N-methyl scopolamine (NMS) binding studies using Chinese hamster ovary cells transfected with M1-M5 muscarinic receptors and cAMP/[Ca^{2+}]$_i$ measurements) and noradrenaline uptake-release mechanisms in simple cellular systems (using [^{3}H]NAdr in SH-SY5Y neuroblastoma cells and HEK293 cells expressing uptake$_1$). Four main NMBDs have been chosen; pancuronium (an agent that produces a tachycardia), vecuronium (an agent that produces a bradycardia), rocuronium (an agent with variable effects) and gallamine, a well documented muscarinic antagonist (an internal positive control). [^{3}H]-NMS binding was displaced in a concentration dependent manner from m1-m5 receptors by all NMBDs. Further kinetic studies with pancuronium, vecuronium and gallamine indicated an allosteric action at M2 receptors. Methacholine inhibition of cAMP formation at the M2 receptor was reversed by pancuronium. Rocuronium and vecuronium produced a small but significant shift in the methacholine response. At clinically achievable concentrations no NMBD affected [Ca^{2+}]$_i$. No NMBD affected either the uptake or release of [^{3}H]NAdr in SH-SY5Y or uptake of [^{3}H]NAdr 293hNET cells. Collectively these data conclusively identify pancuronium (at clinical concentrations) as a moderately selective M2 receptor antagonist with an additional allosteric mode of action. The actions of vecuronium and rocuronium at the cellular level could not be fully ascribed. Studies such as these are important to enable clinicians to make rationale evidence based choices in their use of neuromuscular relaxants.

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Abbreviations.

α	Alpha
AV	Atrio-ventricular
AC	Adenylate cyclase
ACh	Acetyl choline
AR	Adrenoceptor
ATP	Adenosine triphosphate
β	Beta
BP	Blood pressure
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular calcium
cAMP	Cyclic adenosine 3'5' monophosphate
CHO	Chinese hamster ovary
COMT	Catechol-O-methyl transferase
COOH	Carboxy group
4-DAMP	4-diphenylacetoxy-N-methyl piperidine
DAG	Di-acyl glycerol
DOPA	Di-hydroxyphenylalanine
d-TC	d-Tubocurarine
EDTA	Ethylenediaminetetra acetic acid
EGTA	Ethyleneglycol-di-(aminoethyl)-N,N,N',N'-tetra acetic acid
Fura2-AM	Fura2-acetoxymethyl ester
γ	Gamma
G protein	Guanine nucleotide binding protein
G_i	Inhibitory G protein (linked to AC)
G_q	Stimulatory G protein (linked to PLC)
G_s	Stimulatory G protein (linked to AC)
GTP	Guanine triphosphate
^3H	Tritium
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HR	Heart rate

$[Ins(1,4,5)P_3]$	Inositol 1,4,5-triphosphate
iu	International units
K^+	Potassium
λ	Lambda (wavelength)
MAO	Mono-amine oxidase
mAChR	Muscarinic ACh receptor
METH	Methoctramine
Na^+	Sodium
nAChR	Nicotinic ACh receptor
NAdr	Noradrenaline
NaOH	Sodium hydroxide
NH_2	Amino group
NMBD	Neuromuscular blocking drug
NMS	N-methyl scopolamine
PDE	Phosphodi-esterase
PI	Phosphoinositide
PIP_2	Phosphatidylinositol (1,4)bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PZP	Pirenzepine
SA	Sino-atrial
SNARE	Solublised NSF attachment receptor
SR	Sarcoplasmic reticulum
TROP	Tropicamide
VOCC	Voltage operated calcium channel

Units

ml	Millilitres
μl	Microlitres
l	Litres
g	Grammes

mg	Milligrammes
μg	Microgrammes
sec	seconds
min	Minutes
M	Molar
mM	Millimolar
μM	Micromolar
nM	Nanomolar
pmol	Picomolar
nm	nanometres
g	Gravity
pH	Log hydrogen ion concentration
SEM	Standard error of the mean
p	Probability

Binding

NSB	Non-specific binding
K_i	Dissociation constant
pK_i	log dissociation constant
K_d	Equilibrium dissociation constant
pK_i	Log equilibrium dissociation constant
B_{max}	Maximum binding sites
IC_{50}	50% inhibitory concentration
pIC_{50}	Log 50% inhibitory concentration
EC_{50}	50% effective concentration
pEC_{50}	Log 50% effective concentration

CHAPTER 1

Introduction

1.1. History.

The first "muscle relaxant" to be discovered was curare, the 'blow-dart' poison that tribes in South America used for hunting. It was not until 1942, however, that the use of curare as a muscle relaxant was first documented by Griffiths and Johnson in Canada. Curare has since been replaced by other muscle relaxants including the benzylisoquinoliniums (e.g. cis-atracurium) and the steroidal neuromuscular relaxants (e.g. rocuronium). Malouetine, extracted from *Malouetia bequaertiana*, served as the base product for steroidal NMBDs (Huu-Laine, et al., 1964), and benzylisoquinoliniums were extracted from the Chondodendron and Strychnos plants. Alkaloids extracted from these plants represent the first muscle relaxants used in the operating theatre (Wintersteiner, et al., 1943).

When these curare-like drugs were first used, their actions were unpredictable. Modern neuromuscular blocking drugs are designed with speed of onset, ease of reversal and the absence of side effects in mind. This thesis considers one side effect target: the cardiovascular system.

1.2. Synaptic transmission.

The nervous system is subdivided into central and peripheral branches with the former encompassing the brain and spinal cord and the latter encompassing the autonomic (sympathetic and parasympathetic) and somatic systems. In the peripheral nervous system, preganglionic fibres leaving the cord synapse at ganglia and utilise ACh as the transmitter. In postganglionic fibres sympathetic neurones utilise noradrenaline and parasympathetic fibres use ACh as the transmitter. These postganglionic fibres synapse at the neuroeffector junction; e.g., the heart. As will be described in detail later at the heart, release of noradrenaline from the sympathetic nerves increases heart rate and contractile force whilst ACh release from the parasympathetic nerve decreases heart rate and force of contraction. In the somatic nervous system a single neurone leaving the cord synapses with skeletal muscle and uses ACh as the transmitter

The process of neurotransmission at the ganglion (nerve-nerve), at the neuroeffector junction (e.g., the heart) and at the neuromuscular junction share a number of common features. Presynaptically there is propagation of an action potential involving Na^+ and

K^+ movements, presynaptic depolarisation enabling voltage sensitive Ca^{2+} channels to open and Ca^{2+} entry also occurs. This Ca^{2+} influx facilitates the movement of granules containing the appropriate transmitters. Depending on the site of release the transmitter will vary (see above). At autonomic ganglia ACh is released where it activates postganglionic nicotinic receptors to depolarise and propagate the signal (neurotransmission). At the neuromuscular junction ACh is released where it also activates postsynaptic nicotinic (muscle type) receptors resulting in contraction (neuromuscular transmission). In the heart release of noradrenaline (for example) from postganglionic fibres activates β receptors (neuroeffector transmission) on the heart to increase the rate and force of contraction.

1.3. The autonomic nervous system.

The autonomic nervous system and the endocrine system co-ordinate the function of different organs for homeostasis and specific functions such as a response to injury. Both systems work using chemical transmitters. Endocrine glands release hormones directly into the blood stream. Autonomic nerve endings release their transmitters at the target organs. Neurotransmitters released from autonomic nerve endings are rapidly inactivated or removed from the site of action whereas hormones are removed from circulation by metabolism in the liver, or excretion in the urine. The autonomic nervous system provides a rapid and sensitive control, e.g. BP or HR, and the endocrine system provides a slower control, e.g., salt and water balance. The autonomic system regulates the motor and secretory functions of the gut, contraction of the heart, blood vessel tone, blood flow, blood pressure, contraction of the bladder and bronchioles and thermoregulation (fig. 1.1). The hypothalamus, brain stem and the spinal cord are the higher centres responsible for autonomic nervous system control. The autonomic nervous system consists of two major divisions; the sympathetic and parasympathetic nervous systems. Both systems are made up of afferent and efferent nerves. The efferent neurones of both the sympathetic and parasympathetic systems consist of preganglionic neurones which connect the CNS to the postganglionic neurone. ACh is released from all preganglionic neurones to interact with specific receptors on the postganglionic membrane. Postganglionic parasympathetic neurones also secrete ACh, but some secrete other transmitters, e.g. vasoactive intestinal peptide. Most sympathetic postganglionic neurones release NAdr. Synthesis of ACh and NAdr

occurs in the neurones (figs. 1.3 and 1.4 respectively). The adrenal medulla, responsible for adrenaline and noradrenaline synthesis, is also part of the sympathetic nervous system. The secretory activity of the adrenal medulla is controlled by the preganglionic neurones of the splanchnic nerves (Emslie-Smith et al, 1988; Berne & Levy, 1990)

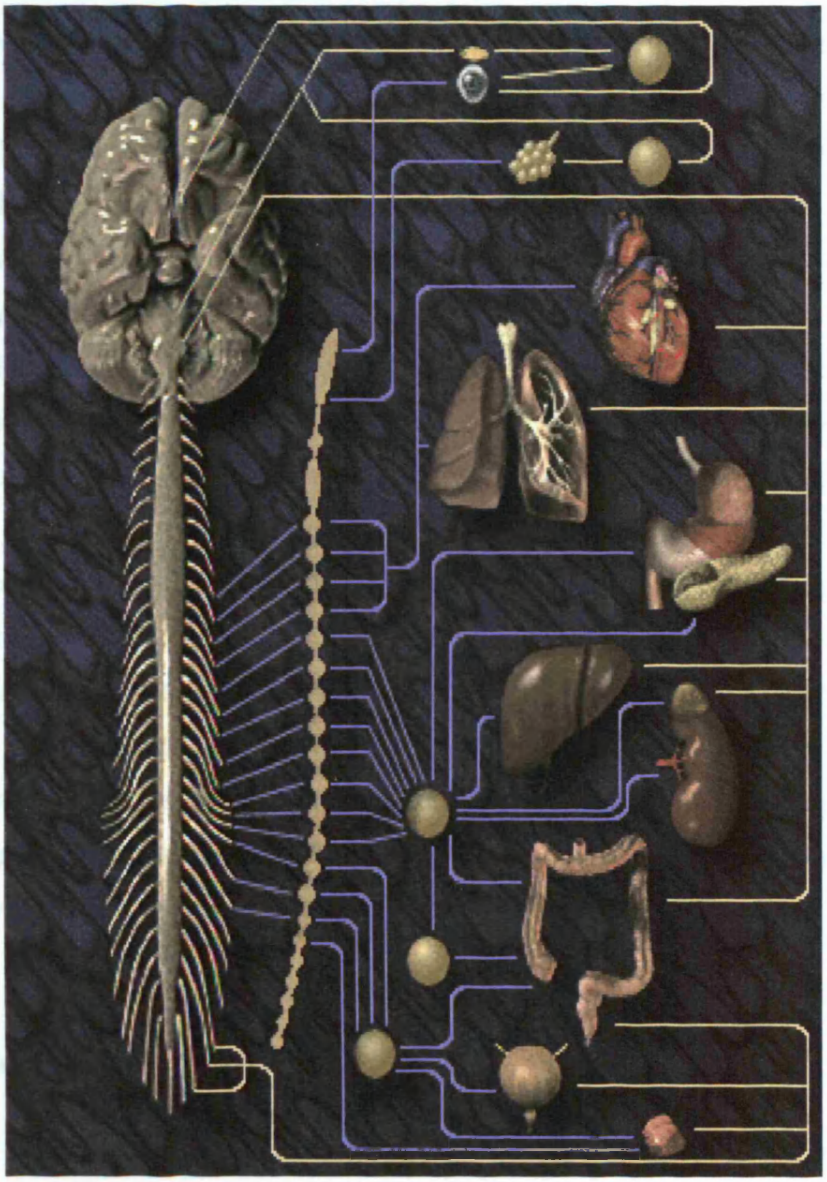


Figure 1.1. An overview of the autonomic nervous system showing the interaction between the sympathetic nervous system (blue lines) and the parasympathetic nervous system (gold lines).

1.4. Release of neurotransmitters.

ACh is considered further. Regulation of the secretion of ACh from the nerve terminal is governed by a specific cycle that includes assembly, filling, transport, tethering and fusion of vesicles containing ACh.

SNAREs (solublised NSF attachment receptor) are a class of proteins thought to be partly responsible for the movement and fusion of vesicles to the terminal axon membrane. Specific vesicle associated SNAREs (vSNAREs) and target membrane localised SNAREs (tSNAREs) are present in a single membrane compartment suggesting that they specify vesicle targeting. vSNAREs linked together with tSNAREs induce membrane fusion and subsequent release of vesicle content outside of the axon terminal membrane.

vSNAREs associate with myosin light chain kinase to enable vesicles to be transported around the axon terminal. Targeting of the vesicles to the membrane occurs via a protein complex termed the sec6/8. The sec6/8 complex targets vesicles via cytoskeletal interactions. Tethering of the vesicle requires low molecular weight GTPases (rabs) and soluble factor Uso1p/p115. These two components promote a reversible attachment that precedes the formation of the SNARE complex. Once this complex has been formed, vesicle fusion can occur and the contents of the vesicle can be expelled. GTP and calcium have also been implicated in the fusion of the vesicle to the membrane to allow its contents to be ejected into the synaptic cleft. The process of vesicle movement and fusion is outside the scope of this thesis and the reader is directed to the following excellent review (Bajjalieh, 1999).

1.5. The neuromuscular junction and ACh.

Motor nerves are large myelinated fibres that form terminal branches at the ends to couple to individual muscle cells. Nerves do not make contact with muscle cells. There is a small gap of approximately 60nm called the junctional cleft between nerve and muscle (Hirokawa and Heuser, 1982). The prejunctional nerve cell is filled with vesicles containing ACh. The postjunctional cell has many folds in the membrane packed with nAChR that respond to the released ACh. The nAChR is a pentameric receptor made up of $\alpha 2\beta\gamma\varepsilon$ subunits, the α subunits being identical, and form the basis

of binding to ligands (Lee 1972; Neubig and Cohen, 1979). Figure 1.2 shows the neuromuscular junction.

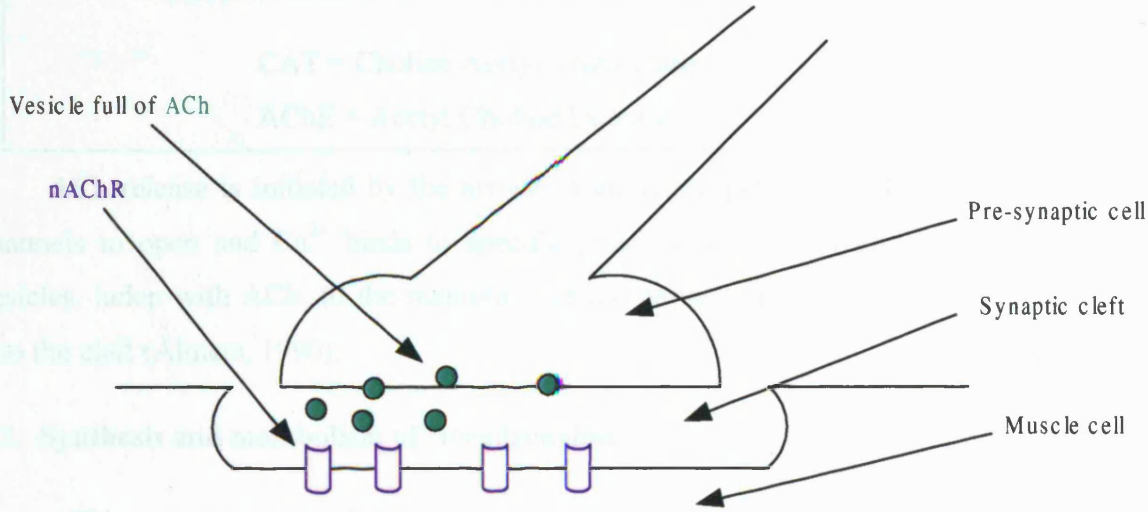
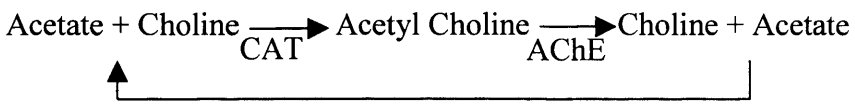


Figure 1.2. Diagram of neuromuscular junction. ACh is released from vesicles and crosses the synaptic cleft to bind with nAChR. Interference with this interaction is the basis of neuromuscular blockade.

1.6. Synthesis of ACh.

At the neuromuscular junction the pre-synaptic cell is responsible for synthesis, packaging and re-uptake of the breakdown products of ACh (Fig. 1.3). ACh is synthesised from choline and acetate. Acetate is provided in the axon terminal by Acetyl CoA, and is combined with choline using choline acetyl transferase to produce ACh. ACh is degraded by ACh esterase to form the choline and acetate. The choline and acetate are recycled and re-uptaken into the axon terminal to be used again in the transmission process. ACh-esterase is an area which can be targeted for the reversal of neuromuscular blockade (Standaert, 1986).

Figure 1.3. Synthetic pathway of acetyl choline.



CAT = Choline Acetyl Transferase

AChE = Acetyl Choline Esterase

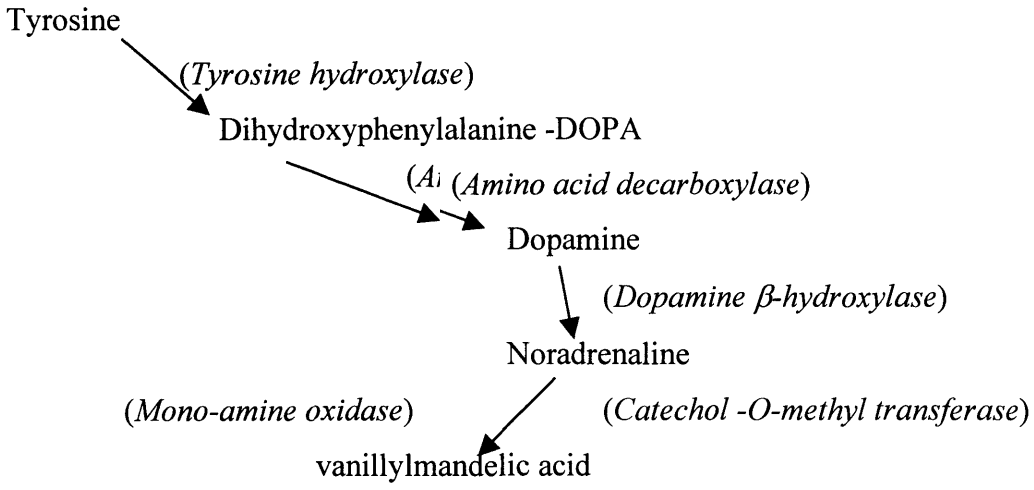
ACh release is initiated by the arrival of an action potential. This causes Ca^{2+} channels to open and Ca^{2+} binds to specific proteins which allows the transport of vesicles, laden with ACh, to the membrane of the nerve and their subsequent release into the cleft (Almers, 1990).

1.7. Synthesis and metabolism of Noradrenaline.

The neurotransmitter NAdr is known to control heart rate via its release from sympathetic neurons. Noradrenaline is synthesised from L-Tyrosine in the neuron. Tyrosine is converted to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. DOPA is converted to dopamine by DOPA decarboxylase (amino acid decarboxylase), and dopamine is converted to noradrenaline by dopamine β-hydroxylase (see figure 1.4). Tyrosine hydroxylase is the rate limiting enzyme in this synthetic pathway. Acute stimulation of sympathetic ganglia activates the initial rate limiting reaction of tyrosine conversion to DOPA. Chronic stimulation causes the concentration of tyrosine hydroxylase and amino acid decarboxylase to increase thereby maintaining noradrenaline production if in continuous demand. Noradrenaline is broken down by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) to form vanillylmandelic acid, which is excreted in the urine. Less than 5% of noradrenaline is excreted unchanged in the urine as the cycle of breakdown of noradrenaline is continuous. The regulation of the concentration of NAdr in the synaptic cleft is crucial for the regulation of heart rate. NAdr is an adrenoceptor agonist. Agonist effects at the β adrenoceptor on the heart leads to an increase in heart rate. NAdr also has a negative feedback effect on β adrenoceptors which leads to adrenoceptor phosphorylation, and thereby preventing further NAdr release. Control of the synaptic concentration of NAdr may be regulated by rate of release from or, rate of uptake back into the neuron (Emslie-

Smith et al., 1988; Rang, et al, 1995). In addition presynaptic α_2 receptors also control the secretory process (Docherty, 1998).

Figure 1.4. Synthetic pathway of noradrenaline.



1.8. Receptors as targets for neuromuscular blocking drugs.

1.8.1. Basic receptor characteristics.

Neuromuscular relaxation occurs due to the NMBD preventing neurotransmission. This is done by either prolonged activation of nAChRs, (depletion of neurotransmitter stores) and preventing the membrane repolarising (e.g. depolarising NMBD-succinylcholine); or by blockade of the nACh receptors and preventing ion channel activation by ACh (e.g. non-depolarising NMBD-pancuronium).

ACh interacts with both nicotinic and muscarinic acetylcholine receptors. Nicotinic AChRs are ligand gated ion channels that are activated by ACh to allow the passage of Na^+ and K^+ ions during neurotransmission.

NAdr interacts with α and β adrenoceptor subtypes. Activation of β adrenoceptors causes an increase in cAMP, activation of α_1 adrenoceptors leads to increased $[Ins(1,4,5)P_3]$ and $[Ca^{2+}]_i$ (Berne and Levy, 1990). Activation of α_2 leads to a decrease in cAMP (Docherty, 1998).

Nicotinic acetylcholine receptors are a family of ligand gated ion channels that are classified on the basis of their activation by nicotine despite that ACh is the endogenous activating ligand. These receptors are pentameric, ion conducting,

membrane spanning channel selective for Na^+, K^+, and Ca^{2+}. The binding site of the channel is formed between the α subunit and adjoining subunits. Table 1.1 describes the various forms of human nACh receptors (Aglan, et al, 1995).

Table 1.1. Types of human nicotinic ACh receptors.

Name	Neuronal	Ganglionic	Muscular
Subunits (pentameric)	α4β2 (major) α3β4	α7 (homomers) α3α5β4 α3α5β2β5	α1β1γδ(ε, neonate)
Receptor Selective Agonists	Epibatidine Anatoxin-a	Epibatidine DMPP	Epibatidine Anatoxin-a
Receptor Selective Antagonists	Mecamylamine Erysodine	Hexamethonium	α-bungarotoxin

(Taken from Watling, 1998)

Muscarinic receptors are members of the family of G-protein coupled receptors. These receptors mediate the actions of ACh in the CNS as well as other non-neuronal tissue innervated by the parasympathetic nervous system. Each of the 5 human muscarinic receptors is encoded by a separate gene, but show a high degree of sequence homology with each other. All the human mACh receptors display the seven trans-membrane spanning structure with most of the variation occurring in the extracellular NH_2 tail and the 3rd intracellular loop, thought to be the site of coupling with the G protein (Figure 1.5) (Bonner, 1989).

The muscarinic acetylcholine receptor was first discovered based on its ability to bind muscarine, derived from the *Amanita muscaria* mushroom (see Felder, 1995). Table 1.2 describes the distribution of muscarinic receptors about the human body. Table 1.3 describes the subtype agonists and antagonists available for experimentation. Muscarinic receptors are classed depending on their ability to activate certain second messenger systems and their differing affinities for various ligands.

Historically, the first two muscarinic receptors identified were the M1 and M2 subtypes. These were classified by their differing affinities for pirenzepine. It was later noted that M1 receptors were linked to PLC resulting in IP_3 and DAG formation and M2 receptors were linked to AC, whose stimulation led to a decrease in the production of cAMP (see Felder, 1995). The role of the M2 muscarinic receptor in regulating the heart rate is well documented in the literature (Weishaar, et al, 1988). Cyclic AMP has been shown to be intimately linked with changes in cardiac activity, namely affecting cardiac contractility by altering intracellular Ca^{2+} movements and other Ca^{2+} related events (Weishaar, et al 1988).

The muscarinic receptor family, now comprising 5 sub-types, are coupled to guanine nucleotide (G) binding proteins and display subtype selective second messenger coupling. Type M2 and M4 muscarinic receptors are linked via G_i to AC, activation of which causes a decrease in cAMP and activation of an inwardly rectifying K^+ channel to produce hyperpolarisation (Felder 1995, Caulfield 1993) and reduce Ca^{2+} influx through voltage sensitive Ca^{2+} channels. Type M1, M3 and M5 muscarinic receptors are linked to PLC via G_q to cause an increase in IP_3 which leads to an increase in $[Ca^{2+}]_i$ and DAG. Type M2 muscarinic receptors are found to be in a single population on the human heart (Caulfield 1993).

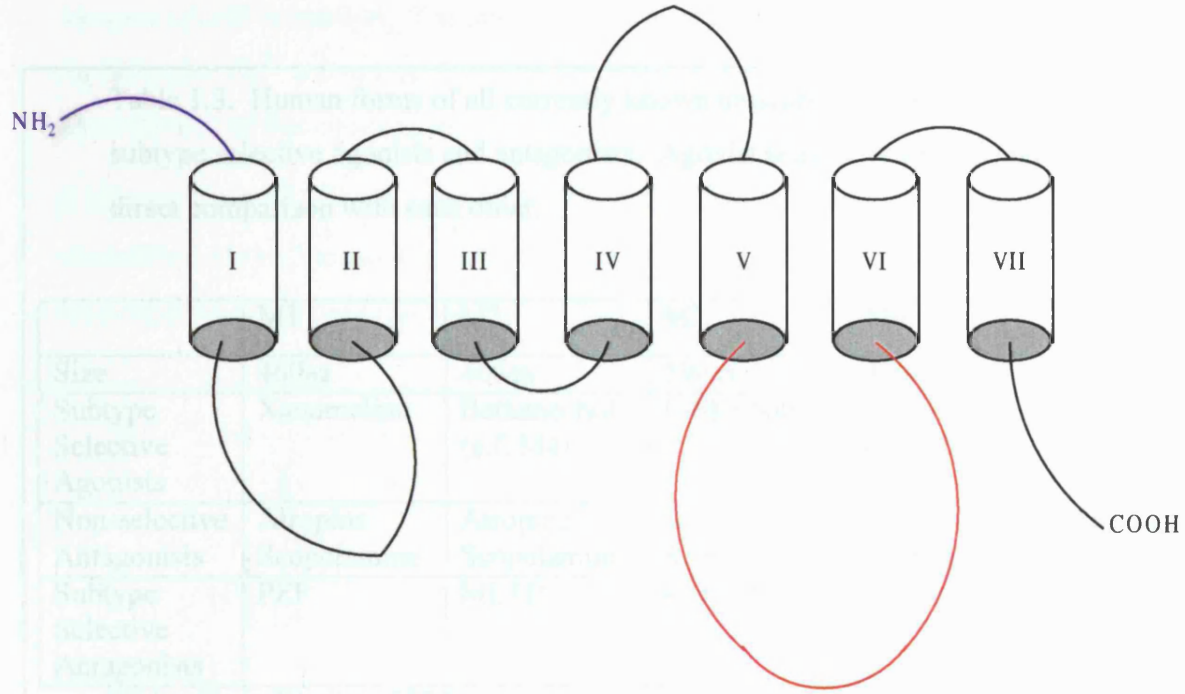


Figure 1.5. Typical muscarinic receptor showing large 3rd intracellular loop believed to be invloved with coupling to G proteins, and the NH_2 domain that incorporates much of the variety between muscarinic receptor subtypes. Cylinders denote trans-membrane sequences.

Table 1.2 shows the distribution of muscarinic receptors around the human body.

	M1	M2	M3	M4	M5
Hypothalamus		✓			✓
Cerebellum	✓	✓	✓	✓	
Hippocampus	✓	✓	✓	✓	✓
Olfactory bulb	✓	✓	✓	✓	
Thalamus		✓	✓	✓	✓
Substantia nigra					✓
Amygdala	✓				✓
Heart		✓			
Lung			✓	✓	
Vas deferens	✓				
Sympathetic ganglion	✓	✓	✓	✓	

(Adapted from Caulfield, 1993)

Table 1.3. Human forms of all currently known muscarinic receptors showing size, subtype selective agonists and antagonists. Agonist selectivity for M2 and M4 is in direct comparison with each other.

	M1	M2	M3	M4	M5
Size	460aa	466aa	590aa	479aa	532aa
Subtype Selective Agonists	Xanomeline	Bethanechol (c.f. M4)	L-689-660	McN-A-343 (c.f. M2)	None
Non-selective Antagonists	Atropine Scopolamine	Atropine Scopolamine	Atropine Scopolamine	Atropine Scopolamine	Atropine Scopolamine
Subtype Selective Antagonists	PZP	METH	4-DAMP	TROP	None

(Taken from Watling, 1998)

The physiological consequence of ACh binding to the muscarinic receptor is determined by the muscarinic receptor subtype. The receptor subtype determines the type of G protein/effector component.

1.9. Adenylate cyclase.

Muscarinic M2 and M4 receptors are linked via G_i (inhibitory G protein) to adenylate cyclase (Figure 1.6). Adenylate cyclase (AC) is the enzyme responsible for converting ATP to cAMP. Activation of G_i leads to a lowering of intracellular cAMP levels. Muscarinic M2 receptors also activate inwardly rectifying K^+ channels leading to hyperpolarisation of the cell membrane and a decrease in intracellular Ca^{2+} via closing of voltage sensitive calcium channels (Felder , 1995; Caulfield, 1993). Cyclic AMP has been shown to be linked with alterations of cardiac activity. Cyclic AMP affects cardiac activity by changing intracellular Ca^{2+} movement and other Ca^{2+}/cardiac muscle events (Weishaar, et al., 1988).

Whilst it is generally accepted that only m2 and m4 receptors couple to AC (inhibitory), M1, M3 and M5 (PI linked) receptors expressed in CHO cells stimulate AC to

increase cAMP formation. The mechanisms of this stimulatory coupling are probably secondary to Ca^{2+} activation of the Ca^{2+} sensitive isoform of AC. The functional consequence of this coupling are unclear.

β adrenoceptors (βAR) are coupled to AC via G_s (a stimulatory G protein). Agonist stimulation of the β adrenoceptor leads to an elevation in cAMP levels. For further discussion of cAMP and cardiac contractility see section 1.17, figure 1.12.

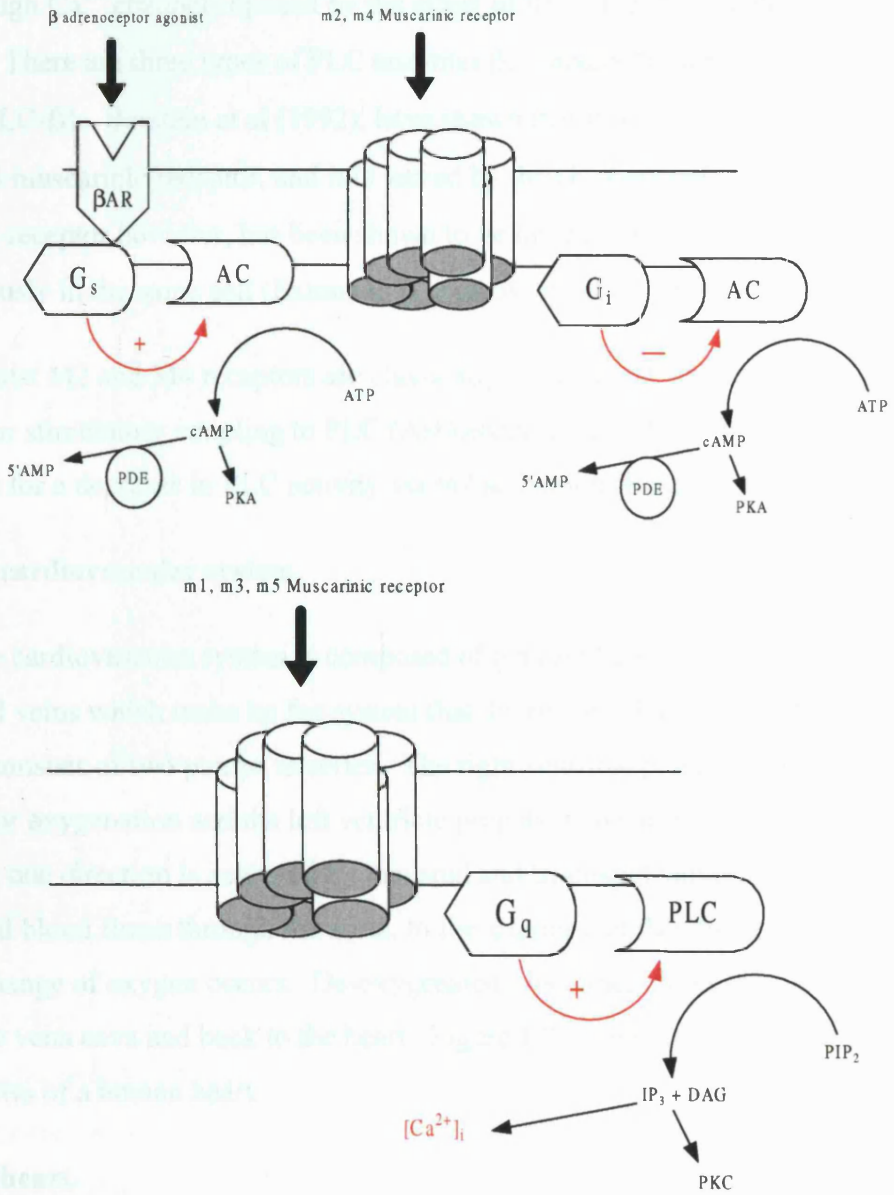


Figure. 1.6. Type 2 and 4 muscarinic receptor couple to AC via G_i and activation leads to a decrease in cAMP levels. Activation of the β AR leads to an increase in cAMP via stimulation of AC via G_s (Top). PKA = Protein kinase A.
Type m1, m3 and m5 couple to PLCβ1 via G_q. Activation of the odd numbered muscarinic receptors leads to an increase in $[Ca^{2+}]_i$ (Bottom). PKC = Protein kinase C.

1.10. Phospholipase C.

The M1, M3 and M5 muscarinic receptors are found in the brain, gut and salivary glands. These are linked via G_q (stimulatory G protein) to activate phospholipase C that cleaves PIP_2 (phosphotidyl inositol 1,4-biphosphate) to DAG (di-acyl glycerol) and IP_3 (Inositol triphosphate) (figure 1.6). This in turn leads to an increase in intracellular calcium concentration by release of Ca^{2+} from intracellular stores through Ca^{2+} channels opened by the effect of IP_3 at the IP_3 receptor (Rhee, et al., 1992). There are three types of PLC enzymes β, γ, and δ, (Exton, 1997) and G_q is linked to PLC-β1. Berstein et al (1992), have shown that PLC-β1 is linked to the M1 and the M3 muscarinic receptor, and it is served by the G_q α-subunit. The M5 muscarinic receptor however, has been shown to be linked to PLC-β and PLC-γ simultaneously in the same cell (Exton, 1997; Gusovsky, et al., 1993).

Whilst M2 and M4 receptors are classically linked to AC there is also evidence for stimulatory coupling to PLC (Ashkenazi, et al., 1987). In addition, there is evidence for a decrease in PLC activity via m2 activation (Bizzari, et al., 1990).

1.11. The cardiovascular system.

The cardiovascular system is composed of a muscular pump and a series of arteries and veins which make up the system that distributes blood around the body. The heart consists of two pumps in series. The right ventricle pumps blood around the lungs for oxygenation and the left ventricle propels blood around the body. Flow of blood in one direction is achieved by bicuspid and tri-cuspid valves in the heart. Oxygenated blood flows through the aorta, to the arteries and then to the capillaries where exchange of oxygen occurs. De-oxygenated blood then flows through various veins to the vena cava and back to the heart. Figure 1.7 shows simple exterior and interior views of a human heart.

1.12. The heart.

The heart is made up of bundles of cardiac myocytes, which are different from skeletal and smooth muscle cells. The contractile mechanisms within the cells are made up of actin and myosin. Shortening (contraction) of the cardiac myocyte occurs

by the sliding filament mechanism, actin filaments slide along adjacent myosin filaments by cycling of the intervening crossbridges.

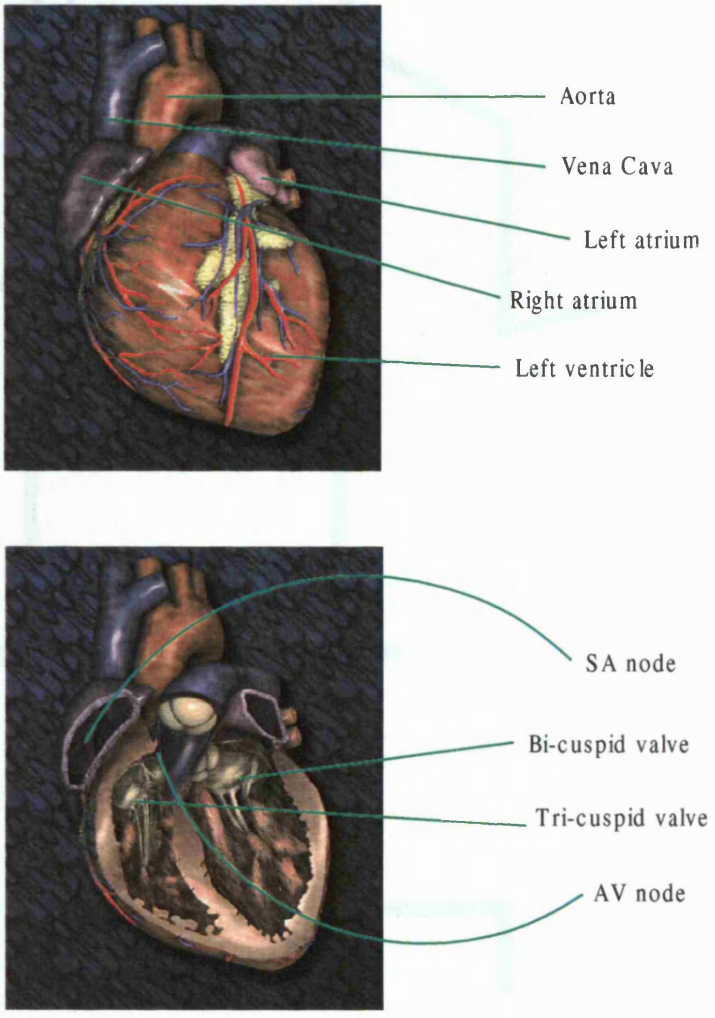


Figure 1.7. Simple exterior (top) and interior (bottom) views of the human heart describing approximate locations of blood vessels and nodes.

Cardiac muscle differs from other excitable cells in that it has a spontaneous rhythm generated by SA and AV nodes. Its action potential has a long duration and there is a long recovery period where the cell membrane is resistant to excitation. The action potential of a typical cardiac myocyte can be divided into 5 phases. Figure 1.8 describes the cardiac action potential and corresponding ion flow during the action potential.

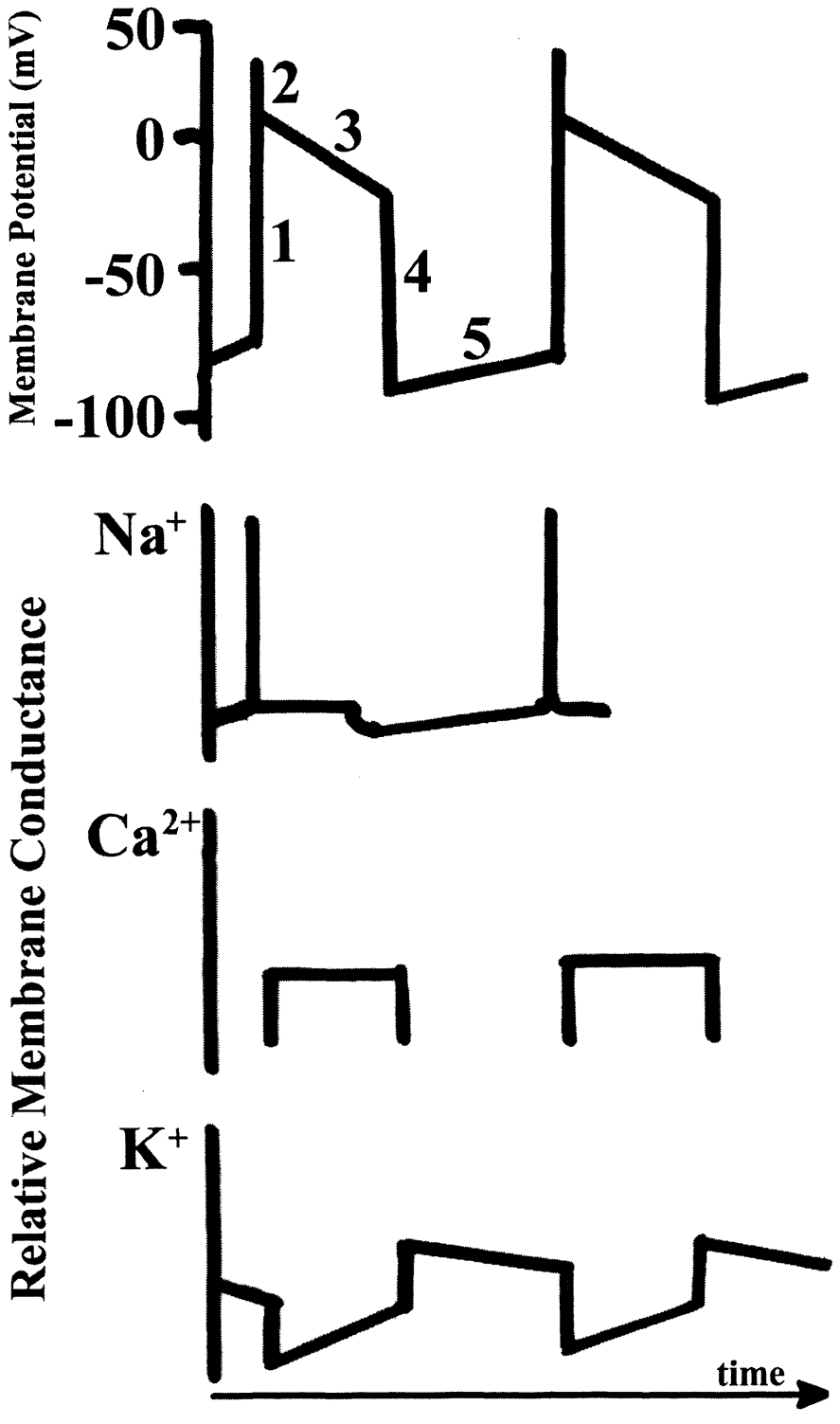


Figure 1.8. Cardiac action potential showing the five phases of membrane potential change (Top).
Corresponding ion fluxes for Na^+, Ca^{2+}, K^+ showing the relative changes in ion movement that affects the membrane potential during the cardiac action potential (Adapted from Rang, et al, 1995).

The first phase is the rapid depolarisation of the myocyte membrane. This depolarisation occurs when the inward flow of Na^+ ions becomes large enough to produce an all or nothing depolarisation. If the flow of Na^+ ions is transient then the ion channels close and becomes inactivated and are unavailable for the initiation of another action potential until the membrane repolarises.

Partial repolarisation (phase 2) occurs as the inward flow of Na^+ ceases and the Na^+ current is inactivated.

The plateau phase (phase 3) of the cardiac action potential results from the slow influx of Ca^{2+} into the cardiac myocyte. The Ca^{2+} current is similar in action to the Na^+ current but the activation and inactivation of Ca^{2+} channels occurs over a greater time period. Increasing Ca^{2+} flow into the cell is also responsible for the initiation of contraction. This plateau phase is prolonged by the lowering of K^+ conductance of the membrane. This prevents repolarisation of the membrane while the Ca^{2+} plateau occurs.

Phase 4 is the repolarisation of the cardiac myocyte membrane. As the Ca^{2+} current inactivates, K^+ conductance through the membrane increases and this restores the membrane potential to normal resting levels ready for another depolarisation.

The final phase (phase 5) is the gradual depolarisation of the membrane due to a slow influx of Na^+. This activity is normally restricted to nodal and conducting tissue in the heart as this tissue provides the pacemaker rhythm for the remainder of the heart (Rang, et al, 1995).

1.13. Contraction.

For adequate control of contractility the heart requires tight control of Na^+, K^+ and Ca^{2+} ions. Very high levels of K^+ cause the heart cells to depolarise and the heart will halt in systole. Very high concentrations of Ca^{2+} produce a similar action. If Ca^{2+} is removed from the heart, it will stop in diastole as the contractile force will decrease.

Removal of Na^+ from the heart will prevent excitation (i.e. no action potentials). Contraction starts with an initial wave of excitation spreading along the myocardial sarcolemma passing along the cardiac myocytes. During the cardiac action potential, Ca^{2+} enters the cardiac myocyte through the sarcolemma (see section 1.17, figure 1.12). Ca^{2+} entering the cell is not sufficient to cause contraction itself, but it enables the release of Ca^{2+} from the sarcoplasmic reticulum. Cytosolic Ca^{2+}

concentrations increase rapidly and Ca^{2+} binds to troponin C. The Ca^{2+}/troponin complex, interacting with tropomyosin, acts to unblock active sites between the actin and myosin filaments. Unblocking of these active sites allows the crosslink recycling between the actin and myosin to cause contraction.

Ca^{2+} entry into the cell ends when contraction ceases and release from the SR is ended. Phosphorylation of the ATP-Ca^{2+} pumps enables Ca^{2+} to be taken back into the SR. This decrease in Ca^{2+} concentration reverses the binding of Ca^{2+} to troponin C, blocks the active sites on the actin/myosin filaments and allows relaxation of the heart to occur.

1.14. Neuronal regulation of the heart.

The neuronal regulation of the heart involves many reflexes, receptors for which are located in blood vessels, the skin and the heart itself. Vagal (parasympathetic) and sympathetic nerve fibres directly innervate the heart and are influenced by the higher centres in the brain. Figure 1.9 describes the neuronal regulation of heart rhythm.

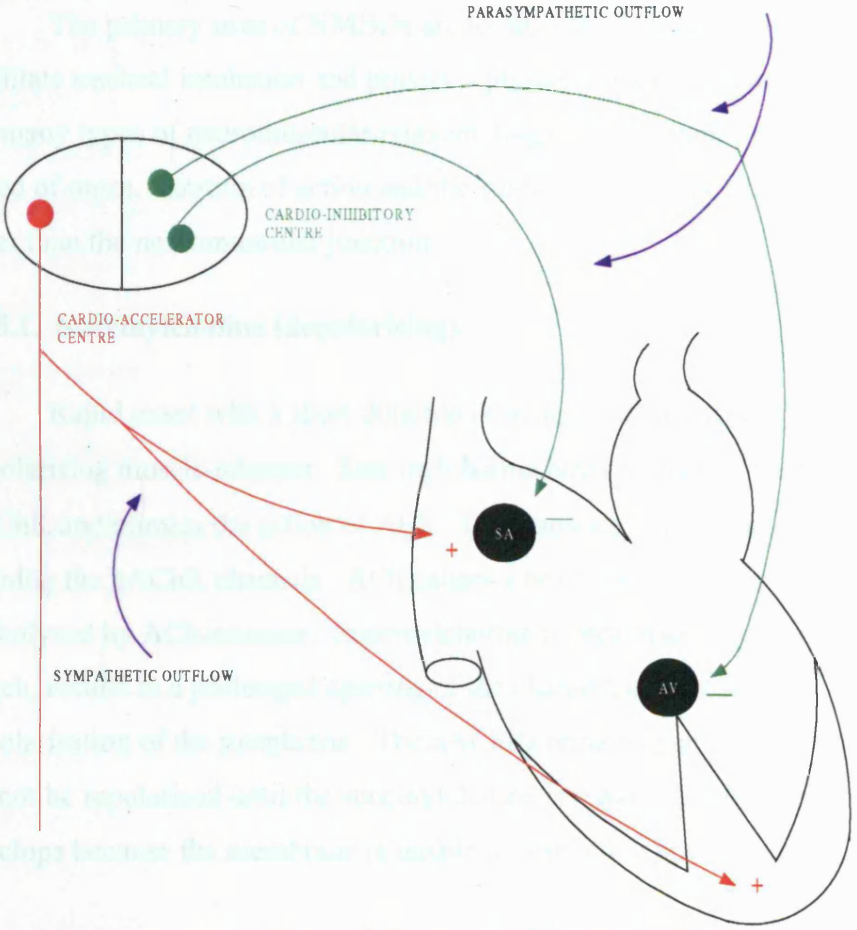


Figure 1.9. Nervous regulation of the action of the heart. The cardio-accelerator centre is responsible for sympathetic stimulation of the nodes leading to an increase in HR (red). The cardio-inhibitory centre is responsible for para-sympathetic stimulation of the nodes leading to a reduction in HR (green). SA=Sino-atrial node. AV=Atrio-ventricular node (Adapted from Mackenna, et al, 1990).

Vagal nerves supply the sino-atrial (SA) node, the atrioventricular (AV) node and the muscles of the heart. Stimulation of the vagal nerves leads to a release of ACh and causes a decrease in heart rate. Sympathetic nerves also supply the SA, AV nodes and the heart muscles. Stimulation of sympathetic nerves leads to NAdr release, which in turn leads to an increase in heart rate.

The contraction of the heart is self initiated but contraction is finely adjusted by a balance between the parasympathetic and sympathetic outflows from higher cardio-control centres in the brain and spinal cord. Sympathetic stimulation of the heart leads to acceleration by affecting the SA node and the heart muscle directly. Parasympathetic stimulation of the heart leads to a deceleration by affecting the SA and AV nodes. At rest, parasympathetic action is dominant (Berne and Levy, 1990)

1.15. Neuromuscular blocking drugs (see fig. 1.10).

The primary uses of NMBDs are to provide adequate skeletal muscle relaxation to facilitate tracheal intubation and provide optimum conditions available for surgery. There are many types of neuromuscular relaxant drugs and the choice of use is influenced by speed of onset, duration of action and the side effects that the drugs may have on sites other than the neuromuscular junction.

1.15.1. Succinylcholine (depolarising).

Rapid onset with a short duration of action is a hallmark of succinylcholine, a depolarising muscle relaxant. Succinylcholine binds to each of the two α subunits of the nAChR and mimics the action of ACh. This causes a depolarisation of the membrane by opening the nAChR channels. ACh causes a brief opening of the channels before it is hydrolysed by ACh-esterase. Succinylcholine is broken down more slowly than ACh which, results in a prolonged opening of the channel, and causes a prolonged depolarisation of the membrane. The nAChRs remain open for longer and the membrane cannot be repolarised until the succinylcholine is removed. The neuromuscular blockade develops because the membrane is unable to respond to any subsequent release of ACh.

Succinylcholine is broken down by plasma cholinesterase. Agents that decrease plasma cholinesterase activity are likely to increase the duration of action of succinylcholine (Viby-Morgensen, 1980).

1.15.2. Steroidal NMBDs.

A longer duration of action is provided by other muscle relaxants such as the steroidal muscle relaxants, pancuronium, pipecuronium, vecuronium and rocuronium. The steroidal muscule relaxants are non-depolarising. The basic structure of the steroidal NMBD contains an androstan skeleton with amino alcohol moieties introduced into the skeleton. These moieties are important for the interaction at the ACh receptor (Bucket, et al., 1973). It is essential to have at least two nitrogen atoms in the molecule with at least one of them having a positive charge. NMBDs with a low potency have a faster onset than relaxants that have a higher potency (Naguib, et al., 1995). NMBDs cause muscle relaxation by binding to the α sub-units, competing with ACh, without activating the receptor. Prevention of activation of the nAChR by ACh by the NMBDs is the basis of the competitive blockade of the membrane. All of the steroidal NMBDs are positively charged cations because of the positively charged quaternary nitrogen atoms. The drug molecules also contain many hydroxyl and methoxy groups. These groups render the steroidal NMBDs relatively water soluble and highly lipophilic (Savarese, et al, 1990). Hence, NMBDs are not easily reabsorbed through renal membranes, so that which is not broken down in the liver is excreted unchanged in the urine.

1.15.2.1. Pancuronium and pipecuronium.

Pancuronium, first used in 1967, was initially praised for its cardiovascular stability (Santoli, et al, 1975) when compared with its main predecessor and rival d-TC (d-tubocurarine). Pancuronium is a long acting drug, ideally suited to operations of 3-4 hours in length and is mainly deacetylated in the liver and excreted in the urine, with the 3-OH metabolite being approximately 50% less potent than pancuronium itself (Miller, et al., 1978). Pancuronium has a mild vagolytic effect and , coupled with other mechanisms, leads to an increase in HR, BP and cardiac output (Hunter J, 1995). Pipecuronium, which has a similar duration of action to pancuronium, was reported to be without undesirable side effects (Foldes, et al., 1990)(see section 1.17 for side effects). Pipecuronium is a

derivative of pancuronium and is 20-30% more potent than pancuronium. It is also suited to operations of 3-4 hours in duration (Hunter, 1995).

1.15.2.2. Vecuronium.

Vecuronium was released in 1983 and was initially reported as being free from associated cardiovascular phenomena (Husby, et al, 1996). Vecuronium is also deacetylated in the liver to produce a 3-OH metabolite (as with pancuronium), and this metabolite has approximately 80% the potency of vecuronium, and so may contribute to the muscle relaxant properties (Savarese, et al, 1990). Vecuronium is 20 times weaker as a vagolytic substance than pancuronium, and ganglion and histamine effects are both absent with vecuronium use (Savarese, et al, 1990).

1.15.2.3. Rocuronium.

Rocuronium was released into clinical practice in 1995 and a major selling point was the fast onset of action (Motsch, et al., 1995; Cooper, et al., 1992). It has a pharmacodynamic profile similar to vecuronium and a duration of action similar to vecuronium (Hunter, 1995). Rocuronium, however, is much less potent than vecuronium and pancuronium (Hunter, 1996).

1.15.2.4. ORG 9487 (Rapacuronium).

ORG 9487 (Rapacuronium) is characterised by a rapid onset similar to succinylcholine, has an intermediate duration of action and is easily reversed, even at high levels of neuromuscular block (Wierda, et al., 1995; Van Den Broek, et al., 1994). ORG 9487 is less potent than rocuronium and reverse of block may be able to be carried out after a few minutes after administration (Hunter, 1996). Due to a potency that is even lower than rocuronium, slight side effects have surfaced, such as a slight tachycardia, possibly caused by the same mechanism as rocuronium (Osmer, et al, 1998).

1.15.3. Other neuromuscular blocking drugs.

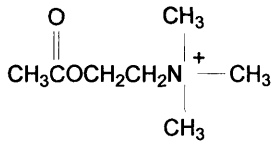
Tubocurarine is the oldest representative of all the muscle relaxants ever used, and was marketed as Intocostrin and used by Griffiths and Johnson in 1942. Derivatives from this are atracurium, cis-atracurium and mivacurium. Disadvantages of atracurium are

histamine release (Basta, et al, 1983), hypotension and tachycardia (Hosking, et al., 1988; Waldman, et al, 1986), but it does have a short elimination half life. Cis-atracurium is more potent than atracurium and has a similar duration of action (Meretoja, et al., 1995). As with atracurium, cis-atracurium is also degraded by Hoffmann elimination and by ester hydrolysis. Mivacurium is a short acting relaxant that has a relatively slow onset (Savarese, et al., 1988), but is relatively free of side effects (Stoops, et al., 1989).

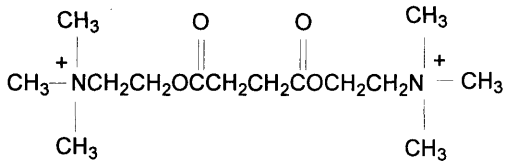
Gallamine is a non-natural trisquaternary muscle relaxant that has a strong vagolytic effect resulting in a marked tachycardia (Ramzan, et al., 1981).

With the exception of gallamine these compounds are not considered further in this thesis.

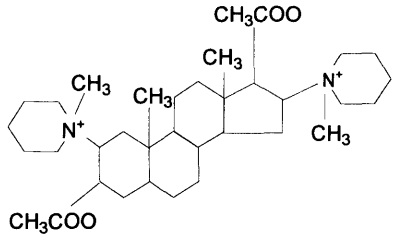
Neuromuscular blocking drugs.



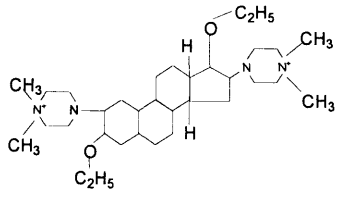
Acetyl choline



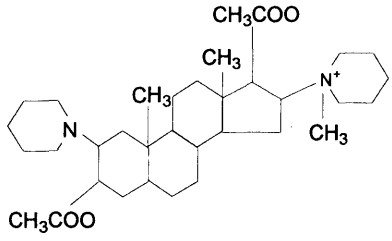
Succinylcholine



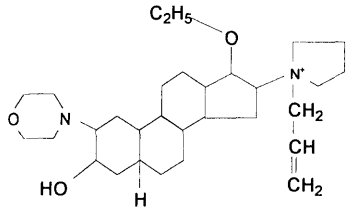
Pancuronium



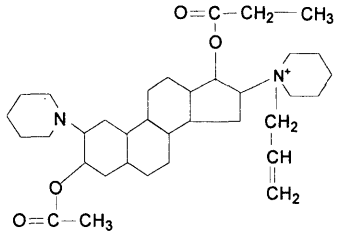
Pipecuronium



Vecuronium



Rocuronium



ORG 9487 (Rapacuronium)

Figure 1.10. Structural representation of ACh, succinylcholine and steroidal neuromuscular blocking drugs.

1.16. Cardiovascular side effects of steroidal NMBDs.

The steroidal NMBDs used in this thesis include pancuronium, pipecuronium, vecuronium and rocuronium. NMBDs are used clinically to produce muscle relaxation suitable to facilitate tracheal intubation, ventilation of the lungs and subsequent surgery (Miller, et al., 1990). These agents are either depolarising (e.g., succinylcholine, Puhringer, et al., 1992) or non-depolarising and whilst their precise mode of action differs, the net result is reduced neuromuscular transmission. As noted non-depolarising neuromuscular blockers are potent inhibitors of the muscle type nicotinic receptor (see Hunter, 1995). With the exception of pipecuronium (Larijani et al, 1989, Foldes et al, 1990), all are associated with effects on the cardiovascular system. Pancuronium and rocuronium are associated with tachycardia (Stevens et al, 1997, see figure 1.11), and vecuronium, bradycardia (Inoue et al, 1988, Stevens et al, 1997). In general pancuronium and rocuronium produce a cardiac 'stimulant' and vecuronium a cardiac 'depressant' side effect profile. The side effect profile of pancuronium includes increased heart rates and arrhythmias (Lee Son, et al, 1981; Parmentier, et al, 1979; Brichard, 1973). Rocuronium, along with pancuronium, is also associated with a statistically significant increase in HR (Stevens, et al., 1997). A report showed that asystole and life threatening bradycardias was associated with the use of vecuronium (Inoue, et al., 1988; Starr, et al, 1986; Milligan, et al, 1985). Possible mechanisms underlying these effects include direct and indirect vagolysis and sympathomimetic activity (Roizen, et al, 1979). More specifically, interaction with muscarinic (M2) and/or modulation of catecholaminergic transmission is suspected and forms the major investigation in this thesis. Animal studies have shown a vagolytic effect of pancuronium in cats, dogs, and guinea pigs (Vetterman, et al, 1988). Okanlami et al., (1996) showed that pancuronium and pipecuronium were antagonists of m2 receptors. Moreover, Kobayashi et al., (1987) reported that pancuronium exerted a pre-synaptic anti-muscarinic atropine like effect by preventing the inhibitory action of muscarinic receptor stimulation on noradrenaline release from sympathetic nerve endings. In addition, Narita et al., (1992) suggested that the parasympatholytic or vagolytic effects of these drugs are mediated by both muscarinic and neuronal nicotinic receptors in hearts (of anaesthetised dogs). The vagolytic activity of pancuronium relative to its neuromuscular blocking activity has been shown to correlate with its ability to produce hypertension and tachycardia in man (Du et al, 1996).

Marshall et al., (1980) showed in dogs that vecuronium (ORG NC45) produced a transient and small decrease in arterial pressure (<0.4 kPa) accompanied by a slight tachycardia in the dose range 0.1-1 mg kg^{-1}, whilst larger doses (3-30 mg kg^{-1}) produced dose dependent decreases in arterial pressure and bradycardia. In addition, their work in cats showed that at high doses (3-30 mg kg^{-1} ORG NC45), a dose dependent, though transient decrease in arterial pressure of between 2.4-12.4 kPa accompanied by transient bradycardia of between 20-58 beats min^{-1} was produced. Work on pithed rats (Marshall, et al., 1980) indicated that vecuronium was 15-29 times less active than pancuronium in stimulating the heart but had a similar neuromuscular blocking potency. They concluded that "vecuronium is 300 times less potent than pancuronium in blocking the bradycardia produced by vagal stimulation in pithed rats, and in guinea pig atria it has only 1/5 the affinity of pancuronium for cardiac muscarinic receptors subserving the 'chronotropic responses'. In anaesthetised cats it is, '35 times less active in blocking cardiac responses to vagal stimulation'".

Animal studies have shown rocuronium to have minimal cardiovascular activity. A study by Muir et al., (1989) reported that vagal blockade with rocuronium may be seen at doses of 4 (pigs) to 7 (cats) times the ED_{90} with ganglion blockade at several times the dose required to produce neuromuscular block. Studies in dogs (Cason, et al., 1990) reported no changes in haemodynamic parameters at up to 5 times the ED_{90}. In a study using "opioid free anaesthesia" patients, pancuronium caused a tachycardia and high doses of rocuronium (1200μg kg^{-1}) caused a significant increase of HR from baseline (see figure 1.11) (Stevens et al, 1997).

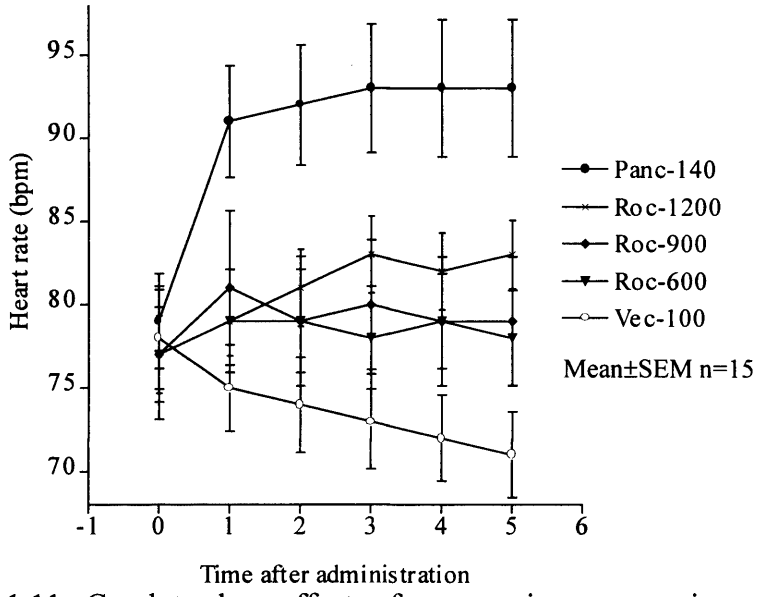


Figure 1.11. Graph to show effects of pancuronium, rocuronium and vecuronium on heart rate during the first 5 minutes of administration of NMBD. (Modified from table 2, Stevens et al, 1997) (doses = μg/kg).

The cardiovascular effects of the steroidal NMBDs may also result from have an effect on the sympathetic nervous system. Re-uptake of noradrenaline is one process, which may affect heart rate. Inhibition of this process ($uptake_1$) may be partly responsible for the tachycardia that is observed and may also lead to an increase in BP. Many animal studies in the 1970s and the 1980s have shown that pancuronium does affect $uptake_1$ (Docherty, et al, 1978, Salt et al, 1980). Several studies have noted that pancuronium and gallamine affect the parasympathetic system by affecting ACh release from the vagus nerve by an interaction with M2 muscarinic receptors (Manabe et al, 1991, 1997).

1.17. Functional consequences of NMBDs at muscarinic receptors. (NMBDs, muscarinic receptors and the heart).

The mechanism behind the effect of NMBDs on the heart rate is not well understood. One effect of the steroidal NMBDs may be as a direct effect on the muscarinic receptor on the heart. Previous work by Appadu and Lambert (1994) has already shown that steroidal NMBDs interact with rat M2 cardiac muscarinic receptors.

Muscarinic (M2) receptors are intimately linked with cardiac function. The M2 muscarinic receptors present in cardiac myocytes are linked via G_i to adenylyl cyclase to

control contractility. Cyclic AMP has a profound effect on myocardial contractility by affecting 3 important regions in the cardiomyocyte, the myocardial filaments, the myocyte membrane and the sarcoplasmic reticulum. Figure 1.12 shows where cAMP has an effect on the cardiac myocyte. Cyclic AMP has a separate effect on each of these 3 areas, but together, an increase in cAMP levels lead to an increase in cardiac contractility via cAMP dependent protein kinase A (Katz, 1979, Tsien et al, 1986). Cyclic AMP also has a profound effect on the rate of muscle filament relaxation. Calcium uptake into the sarcoplasmic reticulum is regulated by cAMP dependent protein kinases that are linked to calcium dependent ATPases. These ATPases are responsible for muscle relaxation. Any increase in cAMP which causes an increase in contraction must also cause an increase in the rate of relaxation to provide an overall increase in the heart rate (Katz 1979, Hicks et al, 1979).

Detailed examination of both cAMP levels and intracellular Ca^{2+} may allow a greater understanding of the mechanism underlying the changes in heart rate observed in theatre when NMBDs are used.

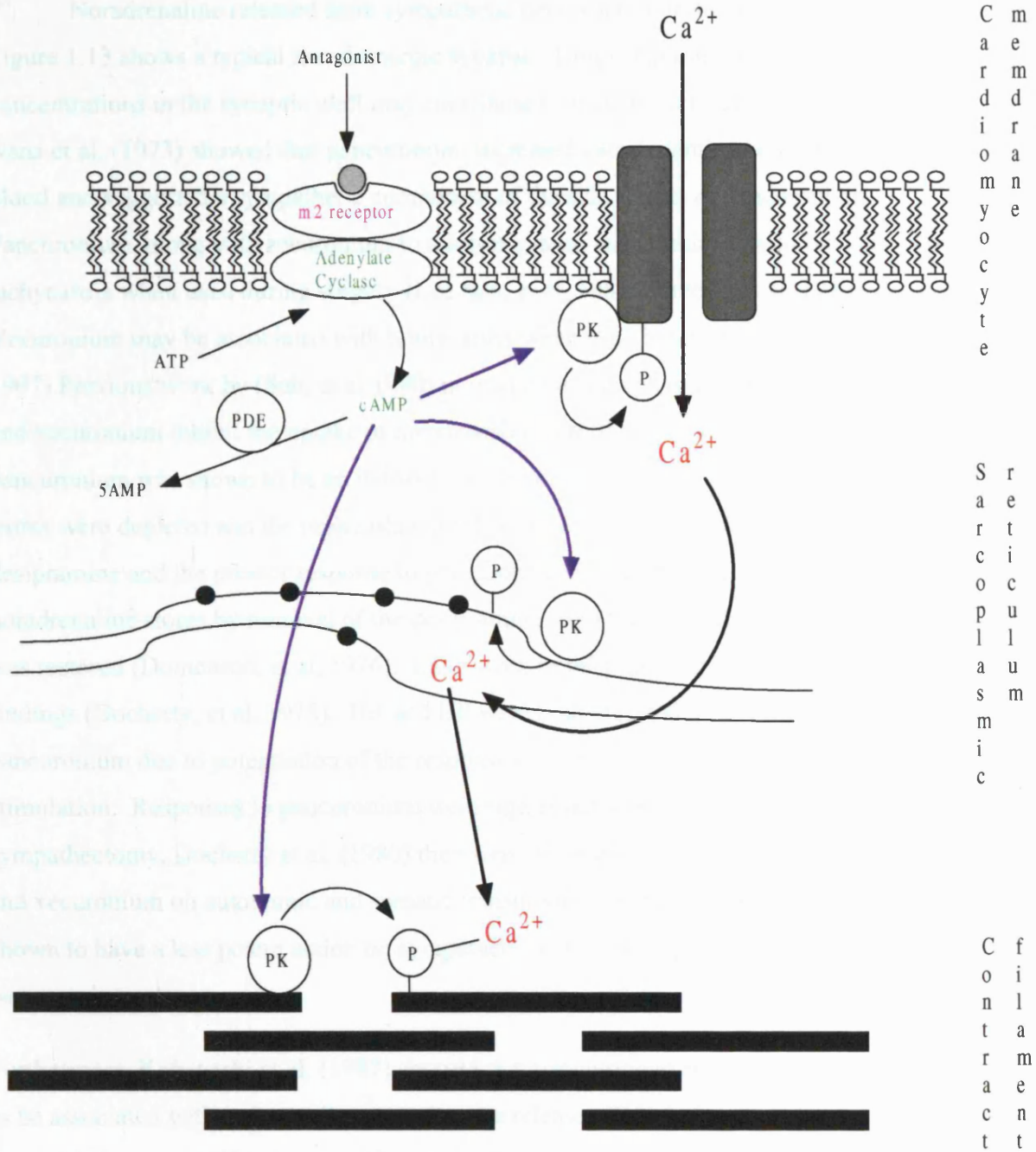


Figure 1.12. Diagram to show the effects of cAMP on the mechanism of contraction of a cardiac myocyte. Cyclic AMPactivates PK to phosphorylate the calcium channel and allow calcium to enter the myocyte. Once calcium has entered the cell, contraction can begin.

PDE, phosphodiesterase. PK, protein kinase. P, PO_4^-.

1.18. Effects of steroidal NMBD on noradrenergic synapses.

Noradrenaline released from sympathetic nerves has a profound effect on heart rate. Figure 1.13 shows a typical noradrenergic synapse. Drugs that interfere with these NAdr concentrations in the synaptic cleft may contribute to changes in heart rate seen in theatre. Nana et al, (1973) showed that pancuronium increased catecholamine concentrations in the blood and suggested a sympathetic component of the side effects of pancuronium. Pancuronium, along with rocuronium to a lesser extent, are associated with incidences of tachycardia when used during surgery (Lee Son, et al, 1981, Stevens, et al, 1997). Vecuronium may be associated with bradycardia when used in surgery (Stevens et al, 1997) Previous work by (Salt, et al 1980) in whole rat hearts has shown that pancuronium and vecuronium inhibit the uptake of noradrenaline into the sympathetic neurons. Indeed, pancuronium was shown to be an indirect sympathomimetic agent in dogs. Noradrenaline stores were depleted and the replenishment of noradrenaline stores was prevented by desipramine and the pressor response to pancuronium was prevented. On restoration of noradrenaline stores by removal of the desipramine from the heart, the pressor response was restored (Domenech, et al, 1976). Later work in the pithed rat confirmed these findings (Docherty, et al, 1978). HR and BP were both elevated on i.v. injection of pancuronium due to potentiation of the responses to cardiac and vasopressor sympathetic stimulation. Responses to pancuronium were significantly reduced following sympathectomy. Docherty et al, (1980) then went on to show the effect of pancuronium and vecuronium on autonomic and somatic transmission in the rat. Vecuronium was shown to have a less potent action on sympathetic and parasympathetic transmission than pancuronium.

Furthermore, Kobayashi et al, (1987) showed that pancuronium and gallamine, both known to be associated with tachycardia, potentiate the release of NAdr from guinea pig atrial sympathetic nerves. This is caused by the inhibition of prejunctional muscarinic receptors. They also showed, by their experimental design, that any interaction between the vagal and the sympathetic system could be investigated, as the sympathetic release of NAdr is under tonic muscarinic control of ACh released from the vagus nerve (Manabe et al, 1991). The release of NAdr from the heart has already been shown, by Muscholl, (1980) to be controlled by muscarinic receptors located on the sympathetic nerve terminals.

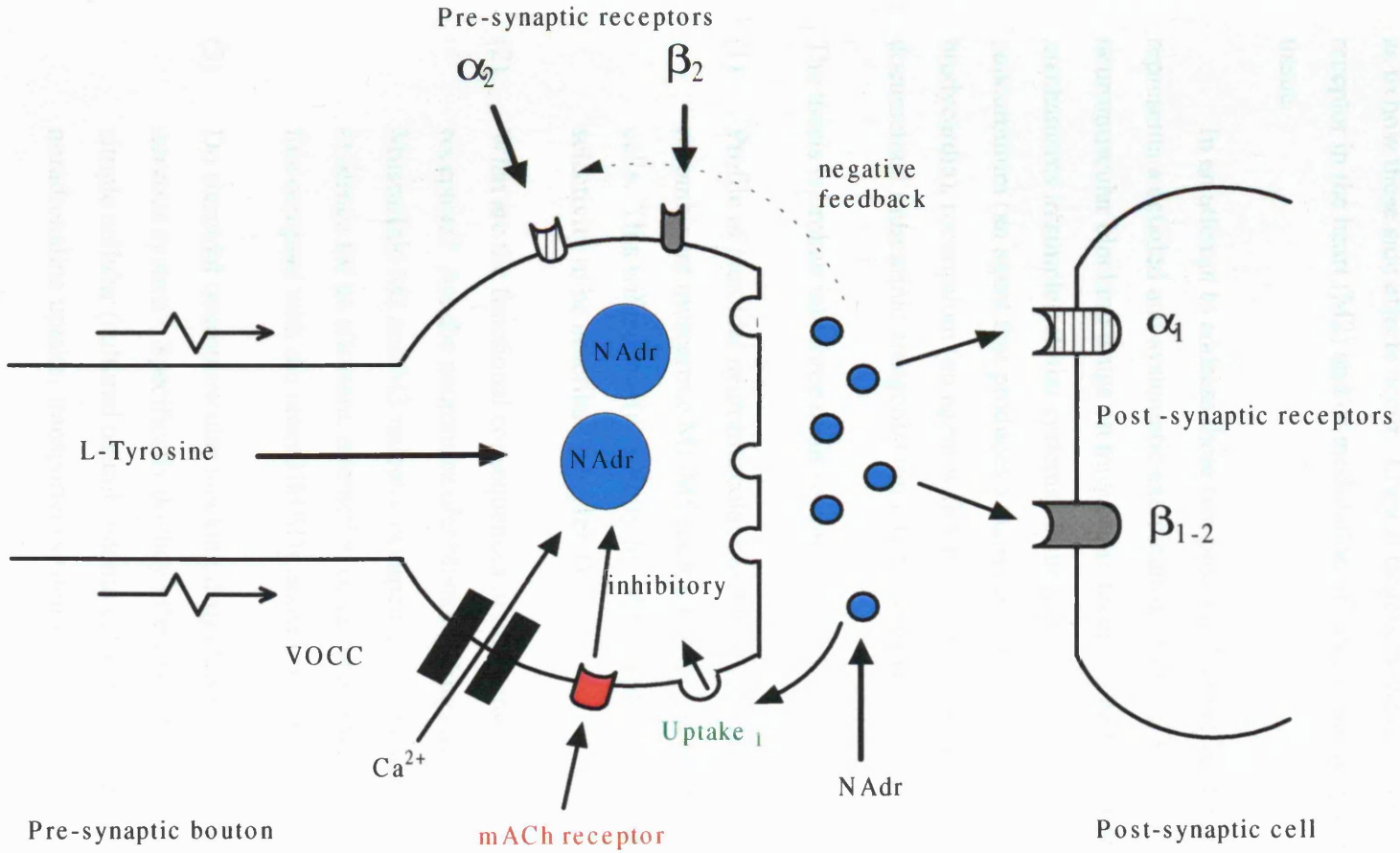


Figure 1.13. Diagrammatic representation of a typical noradrenergic synapse. NAdr is synthesised from L-Tyrosine as described in figure 1.3. NAdr is released from the presynaptic bouton by many processes, electrical and chemical, including the mACh receptor. Once in the cleft it activates the post-synaptic receptor to cause various effects, one being an increase in heart rate. Removal from the cleft is by re-uptake back into the bouton where it is broken down by MAO and COMT (see section 1.7).

1.19. Aims.

As described previously, steroidal NMBDs produce a range of effects on the cardiovascular system encompassing both bradycardia and tachycardia. Opinion is divided as to how these side effects occur. Logical targets, however, include the muscarinic receptor in the heart (M2) and/or modulation of catecholaminergic transmission in this tissue.

In an attempt to address these two possible "side effect target sites" this thesis represents a detailed and systematic examination of the effects of a range of steroidal neuromuscular blocking drugs on muscarinic receptors and noradrenaline uptake-release mechanisms in simple cellular systems. Four main NMBDs have been chosen; pancuronium (an agent that produces a tachycardia), vecuronium (an agent that produces a bradycardia), rocuronium (an agent with variable effects) and gallamine, a well documented muscarinic antagonist (an internal positive control).

The thesis is broken into three main sections:

(1) Profile of steroidal neuromuscular blocking drugs on a full spectrum of recombinant muscarinic M1-M5 receptors expressed in Chinese hamster ovary cells. This will enable (a) affinity for M2 receptors to be determined and (b) any selectivity to be described (chapter 4)

(2) What are the functional consequences of an interaction with recombinant M2 receptors? Are the neuromuscular blocking drugs agonists or antagonists? Muscarinic M2 and M3 receptor occupation is compared (chapter 5). There is evidence for an allosteric interaction of gallamine with the M2 receptor. How does this compare with the other NMBDs under investigation (chapter 6)?

(3) Do steroidal neuromuscular blocking drugs have an effect on the sympathetic nervous system? Specifically do they affect noradrenaline uptake or release in simple cellular (cultured neuroblastoma cells and cells expressing recombinant noradrenaline uptake$_1$ transporter) systems (chapter 7)?

CHAPTER 2

Methods and Materials.

(Experimental rationale can be found in specific chapter-aims sections)

2.1. Sources of Chemicals.

Sigma chemicals, Poole, Dorset, England.

Fura2-AM, Triton-X100, EDTA, EGTA, HEPES, Pancuronium bromide, pargyline, imipramine, atropine, forskolin, ascorbic acid, methacholine, 3-isobutyl-1-methyl xanthine, cAMP.

Fisher Chemicals/Scientific, Loughborough, Leics., England.

Sodium chloride, potassium chloride, magnesium chloride, calcium chloride, magnesium sulphate, sodium hydrogen carbonate, sodium hydroxide, hydrochloric acid, methanol, glucose, potassium dihydrogen phosphate, Folin and Ciocalteau's phenol reagent, Optiphase Hi-Safe 3 and Optiphase Safe.

Life Technologies, Paisley, Scotland.

α-Minimum Essential Medium (α-MEM), Dulbecco's modified eagle medium, fungizone, L-glutamine, penicillin/streptomycin, geneticin (G418), foetal calf serum, new born calf serum, trypsin.

Radiochemicals.

1-[N-Methyl -^{3}H] Scopolamine methyl chloride (^{3}H-NMS), L-[7,8-^{3}H]-Noradrenaline greater than 98% purity was obtained from Amersham International PLC, Bucks., England.

[2,8^{3}H]-cyclic Adenosine 3,5-Mono Phosphate greater than 98% purity was obtained from NEN du Pont.

Other chemicals.

Vecuronium bromide, rocuronium bromide, pipecuronium bromide – Organon Teknika, Newhouse, Scotland.

All other reagents were of the highest purity available.

2.2. Buffer compositions.

1. Harvest buffer for cell culture.
HEPES 10mM, EDTA 0.05%, NaCl 0.9% at pH 7.4.

2. Radioligand ([^{3}H]-NMS) binding buffer for cell membranes.
HEPES 20mM, $MgCl_2$ 1mM, pH 7.4.

3. Krebs/HEPES buffer for whole cell experiments.
NaCl 143mM, KCl 4.7mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, glucose 11.7mM, HEPES 10mM, $CaCl_2$ 2.6mM, pH 7.4.

4. Krebs/HEPES buffer for [^{3}H]-Noradrenaline uptake and release experiments.
HEPES 10mM, NaCl 143.3mM, KCl 4.7mM, $CaCl_2$ 2.6mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, glucose 11.7mM, ascorbic acid 0.2mM and pargyline 0.1mM, pH 7.4. The KCl concentration is increased to 100mM for the release buffer, and is osmotically balanced by a reduction in the NaCl concentration. Although 100mM K^+ is not a physiological depolarisation, it enables NAdr to be released form SH-SY5Y cells without the need for expensive electrical apparatus. The depolarisation is caused by the increased external concentration of K^+ thereby causing the membrane potential to become more positive. This change in potential causes Na+ channels to open and cause the depolarisation which causes the release of NAdr.

5. Cyclic AMP assay buffer.
50mM Tris, 4mM EDTA, pH 7.4.

2.3. Tissue Culture.

CHO cells expressing M1-M5 muscarinic receptors were selected because the receptors have been studied extensively in other binding studies enabling easier comparisons of data to be made. The cells were also obtained from one place (N.Buckley, UCL, London) enabling a standardisation of cloning and expression of each receptor subtype. Many other cell lines expressing muscarinic receptors have

been established, such as A9-L cells, NG108-15 cells, murine B82 fibroblast cells and SH-SY5Y cells (see Hulme et al, 1990; Caulfield, 1993).

2.3.1. CHOm1-m5 cells.

CHOm1-5 cells were routinely maintained in α-MEM supplemented with, 10% (v/v) new born calf serum, penicillin 100iu ml^{-1}, streptomycin 100μg ml^{-1} and 2.5μg ml^{-1} fungizone, in a humidified 5% CO_2/air atmosphere at 37°C. Stock cultures were fed twice weekly and passaged weekly. Cell monolayers were harvested with trypsin 0.05%/ EDTA 0.02% for maintenance only, and cultured until confluent. A photomicrograph of sub-confluent CHO cells is illustrated in figure 2.1.

2.3.2. SH-SY5Y cells.

SH-SY5Y human neuroblastoma cells were routinely maintained in minimal essential medium supplemented with L-glutamine 2mmol $litre^{-1}$, penicillin 100iu ml^{-1} streptomycin 100μg ml^{-1}, fungizone 2.5μg ml^{-1} and 10% (v/v) foetal calf serum in a humidified 5% CO_2/air atmosphere at 37°C. Stock cultures were fed twice weekly and passaged weekly. Cell monolayers were harvested with trypsin 0.05%/ EDTA 0.02% for maintenance only, and cultured until confluent. A photomicrograph of sub-confluent SH-SY5Y cells is illustrated in figure 2.1, note the extension of small processes.

2.3.3. 293-hNET cells.

293-hNET cells were routinely maintained in high glucose (4500μg ml^{-1}) Dulbecco's modified Eagle medium supplemented with 10% (v/v) foetal calf serum, L-glutamine 2mmol $litre^{-1}$, penicillin 100 iu ml^{-1}, streptomycin 100μg ml^{-1}. 293-hNET cells are human embryonic kidney cells transfected with, and expressing, the recombinant human noradrenaline $uptake_1$ transporter. Non-experimental stock cultures were maintained in media supplemented with geneticin (G418) 250μg ml^{-1} and further passages were sub-cultured from this stock. G418 is a protein synthesis inhibitor that prevents the growth of cells that do not express the plasmid containing the human $uptake_1$ transporter. 293-hNET cells were treated as SH-SY5Y cells in every other way. Structurally, 293-hNET cells are similar to CHO cells.

2.4. Harvesting and washing of CHO, SH-SY5Y and 293-hNET cells for experimentation.

Confluent CHOm1-m5, SH-SY5Y and 293-hNET cells were harvested using 5mls harvest buffer (sec 2.2, no. 1). After gentle agitation, the cells were detached and resuspended in 20 mls Krebs/HEPES buffer in a centrifuge tube. The suspension was again gently agitated to disturb clumps of cells. The suspension was centrifuged at ~450g, 4°C for 2 minutes in a Heraeus Labofuge 400R. The washing and centrifugation was repeated twice more until finally re-suspended in a final volume of Krebs/HEPES buffer suitable for experimentation.

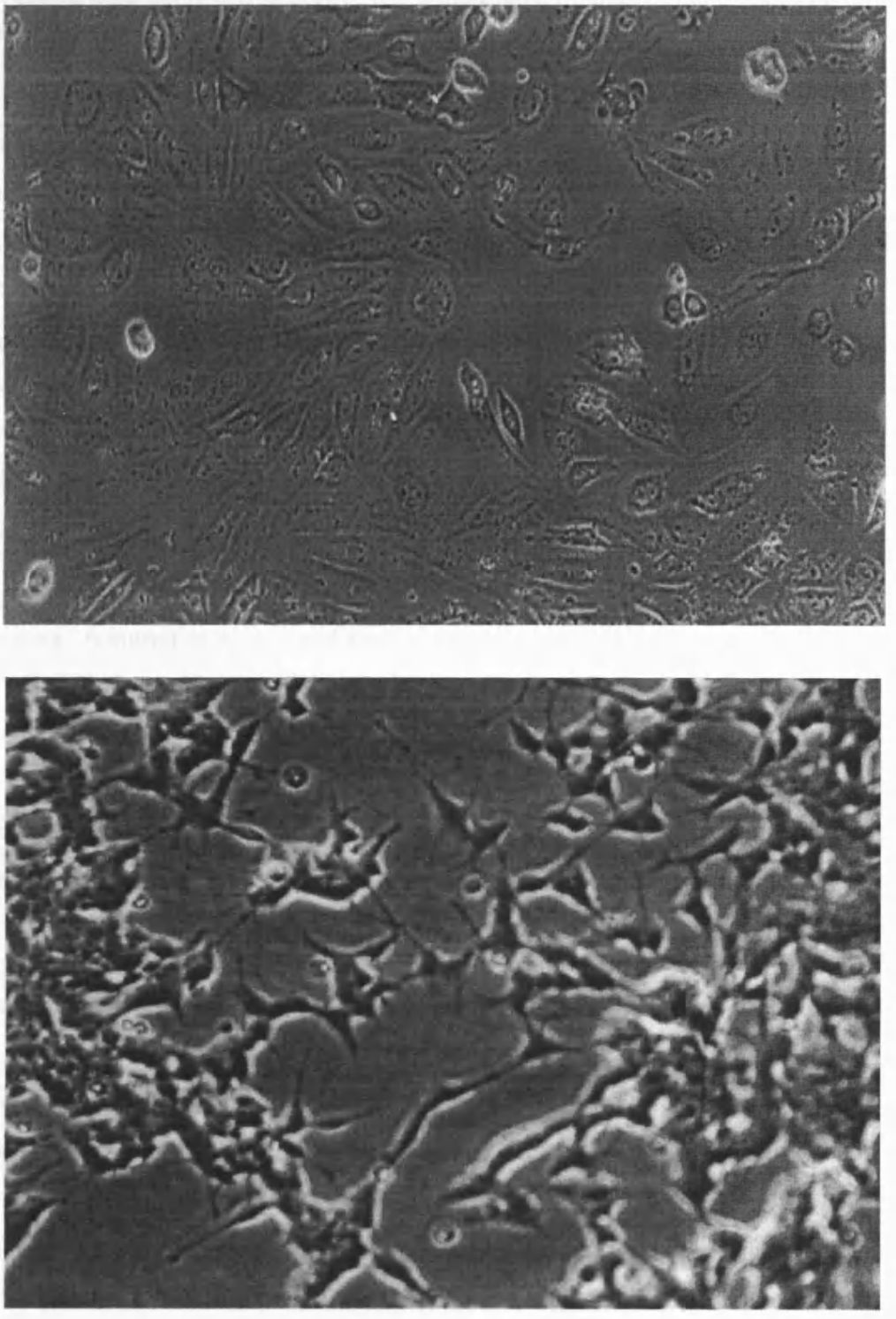


Figure 2.1. Photomicrographs of sub-confluent cultures of CHO (top) and SH-SY5Y (bottom) cells. Note the presence of short processes on the lower SH-SY5Y micrograph.

2.5. Preparation of CHOm1-m5 membranes for radioligand binding.

CHO cells were harvested as above, but resuspended in 5mls of radioligand binding buffer (2.2, no. 1). The cells were homogenised using an Ultra Turrax set at 13500 rpm for 3x10s bursts. The homogenate volume was made up to 45 mls with radioligand binding buffer before centrifugation at 20000g for 10 minutes at 4°C in a Biofuge 28RS Heraeus Sepatech centrifuge. The supernatant was removed, the resulting cell clump was re-homogenised in 5 mls of radioligand binding buffer and re-centrifuged and re-homogenised twice more. The final membrane pellet was re-suspended in radioligand buffer at a cell mass appropriate for the cell type and experimental protocol.

2.6. Radioligand binding assay.

2.6.1. Theory.

Radioligand binding assays provide useful indications of ligand-receptor affinities. A common radioligand for investigating muscarinic receptor binding is [^{3}H]-*N*-methyl scopolamine (^{3}H-NMS), which is a specific muscarinic receptor antagonist. Radioligand binding assays can assist in the identification of receptors and enable the properties of drug and receptor interactions to be studied. Binding studies are limited by their relative inability to determine whether any functional response has occurred.

Ligand binding to a receptor cannot be determined directly as the radioligand binds to the other cellular components (i.e. this is non-specific binding, NSB). NSB can be defined by adding excess of a competitive antagonist (e.g. atropine for muscarinic receptors) which blocks all receptor sites. Therefore any bound radioligand must be bound to non-specific sites. In practice, specific binding at a given radioligand concentration is determined by performing total binding (just radioligand), and NSB (radioligand + antagonist, e.g. atropine) in parallel, then subtracting NSB from total bound radioactivity.

Saturation experiments are used to determine the maximum number of binding sites (B_{max}) available and, the dissociation constant (K_d) of the ligand for the receptor by increasing the concentration of radioligand used until no more radioligand can be

bound. In this thesis B_{max} and K_d values were obtained using two analysis methods; sigmoid curve and Scatchard analysis of the resultant specific binding curves (see figure 2.2).

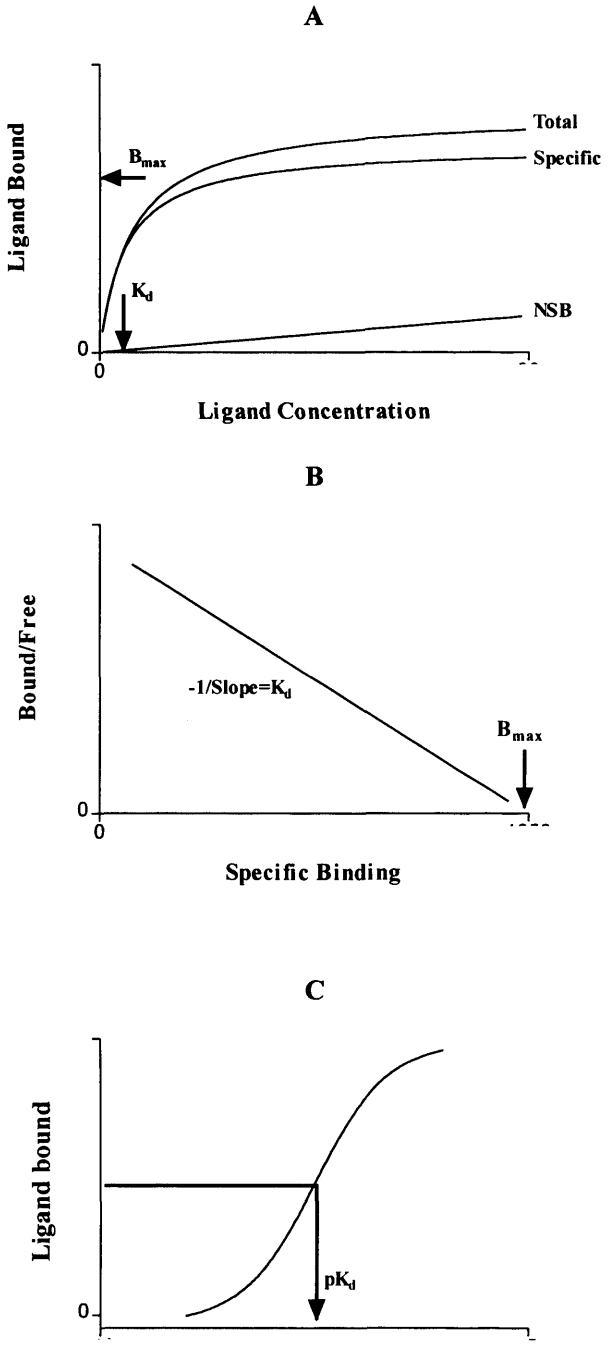


Figure 2.2. Theoretical radioligand saturation experiment (A) analysed according to Scatchard (B) and using a sigmoid curve (C). B_{max} (Maximum number of binding sites) and K_d/pK_d (radioligand equilibrium dissociation constant) are noted.

Displacement experiments are one method that can be used to determine the affinity of a non-radioactive ligand for a receptor using a radioligand. This is carried out by labelling a fixed amount of receptors with a fixed low concentration of radioligand and then increasing the concentration of drug that is being studied, this results in concentration dependent displacement. The concentration of displacer producing 50% displacement is related to the affinity (see below). The concentration of the radioligand being used affects the binding of the displacer (unlabelled ligand) to the receptor. Using the Cheng and Prussoff (1973) equation (Eqn. 2.1), the affinity (K_i) of the unlabelled ligand can be estimated. The K_i is the concentration of the displacing drug that causes 50% displacement of ligand binding when corrected for the competing mass of radioligand. As the concentration of the radioligand increases the concentration response curve of the displacer is shifted to the right (see fig 2.3). The Cheng and Prussoff equation (see below) takes the effect of the radioligand into account and shifts the concentration response curve of the displacer back to the left to a theoretical position in the absence of radioligand.

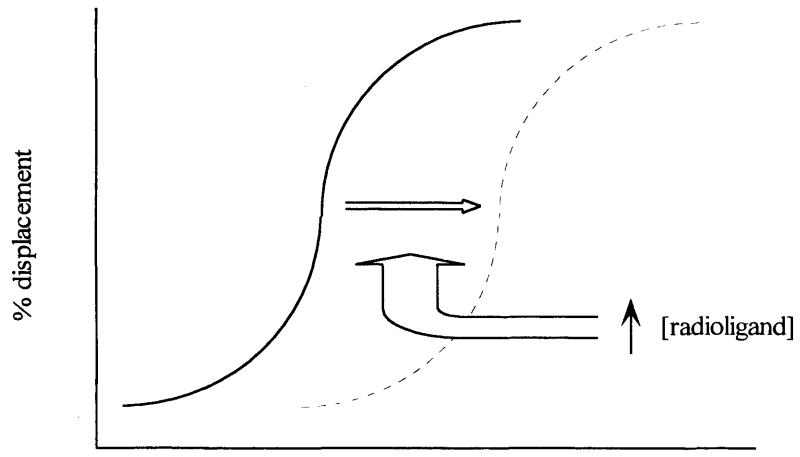


Figure 2.3. Diagrammatic representation of radioligand effects on displacing ligands. The original displacement of the radioligand by the displacer is shown by the solid curve. As the concentration of the radioligand increases the displacement curve is moved to the right as shown by the dotted curve. This shift occurs because the radioligand itself also competes with the displacer for the receptor.

Eqn. 2.1. $K_i = IC_{50} \div (1 + (L \div K_d))$

K_i = affinity of displacer

L = [radiolabeled compound] (concentration)

K_d = dissociation constant of radiolabel

IC_{50} = [displacer that causes 50% displacement of radioligand binding]

2.6.2. Method.

All radioligand binding assays were performed in 1 ml assay volumes. All experiments were performed using CHO cell membranes prepared in 20mM HEPES/1mM Mg^{2+} buffer (pH 7.4). Addition volumes for each experimental protocol are noted below. The reaction was always initiated by adding 100µl of an appropriate mass of cell membranes (table 2.1). Non-specific binding was determined using atropine (a specific muscarinic antagonist) at a final concentration of 10µM. Each tube was vortexed before incubation for 1 hour at 37°C. Bound and free radioactivity were separated by rapid vacuum filtration using a Brandel cell harvester onto Whatman GF/B glass fibre filters and washed twice more using 4ml ice cold HEPES/Mg^{2+} buffer. The filters were then placed in 4mls of Optiphase Safe scintillation fluid. Bound radioactivity was extracted for at least 8 hours in scintillation fluid, with the bound activity quantified by liquid scintillation spectroscopy on a Packard 1900 TR β counter.

Table 2.1. Approximate mass of cell membranes used in saturation experiments.

Cell type	CHOm1	CHOm2	CHOm3	CHOm4	CHOm5
[membranes]µg	~70	~200	~70	~120	~250

2.6.3. Saturation binding.

Assays were performed using a range of concentrations of [^{3}H]-NMS from 0.008-3.23nM in each of the total and non-specific binding tubes (see figure 2.2). Table 2.2 describes a typical saturation protocol.

Table 2.2. Typical protocol for saturation of CHOm1-m5 membranes using 0.008-3.23 nM [^{3}H]-NMS using a 1ml assay volume.

	Volumes (µl)			
	Membranes	Atropine	^{3}H-NMS	Buffer
Total NMS_{1-n}	100	0	200	700
NSB NMS_{1-n}	100	200	200	500

B_{max} (maximum number of binding sites, fmols/mg protein) and K_d (equilibrium dissociation constant, nM) were estimated from specific binding curves obtained from sigmoid curve and Scatchard (1949) analysis.

2.6.4. Displacement.

Assays were performed using a fixed concentration of [^{3}H]-NMS (~0.2nM) in all of the tubes. Total and non-specific binding tubes are made up as in the saturation binding assay. Table 2.3 describes a typical displacement protocol.

Table 2.3. Typical addition protocol for displacement of ~0.2nM ^{3}H-NMS from CHOm1-m5 membranes, where displacer = NMBDs and gallamine at various concentrations in a 1ml assay volume.

	Volumes (µl)				
	Membranes	Atropine	^{3}H-NMS	Buffer	Displacer
Total	100	0	200	700	0
NSB	100	200	200	500	0
$Ligand_{1-n}$	100	0	200	500	200_{1-n}

2.6.5. Allosteric interactions. (For theory see chapter 6, section 6.1).

Membranes were prepared from CHO cells expressing recombinant human m2 muscarinic receptors as above. Dissociation studies were performed using [^{3}H]-NMS as the radioligand of choice as above and adapted from a method by Mohr et al 1992. Table 2.4 describes the addition protocol. Receptors were labelled with

~0.2nM [^{3}H]-NMS and incubated to equilibrium for 60 mins as above. Ligand dissociation was initiated by the addition of atropine (10μM), or atropine + NMBD. The time course for dissociation was then determined. Reactions were terminated between 1 and 15 mins using rapid vacuum filtration and bound radioligand was then measured as above.

Table 2.4. Typical addition protocol for the displacement of ~0.2nM [^{3}H]-NMS from CHOm2 membranes, where displacer = atropine or atropine + NMBD combined in a 1 ml assay volume.

	Volumes (μl)			
	Membranes	[^{3}H]-NMS	Buffer	Displacer
Control	100	200	700	0
Atropine	100	200	500	200
Atropine + NMBD	100	200	500	200

2.7. Measurement of [^{3}H]-cAMP.

2.7.1. Theory.

In this assay (a competitive binding protein assay) a binding protein (essentially protein kinase A prepared, from bovine adrenal glands) is incubated with a fixed concentration of radiolabeled [^{3}H]-cAMP and unlabelled cAMP in a fashion essentially identical to the radioligand binding assay described earlier. The amount of binding protein and radioligand are constant and therefore, the unlabelled ligand (cAMP) will compete with the radioligand for the available binding sites on the binding protein. The amount of radioligand bound to the binding protein is inversely proportional to the amount of unlabelled ligand (cAMP) present. Increasing amounts of unlabelled ligand will displace increasing amounts of radioligand bound to the binding protein. Comparison with a set of known standards enables unknown cAMP mass to be estimated (see fig. 2.4).

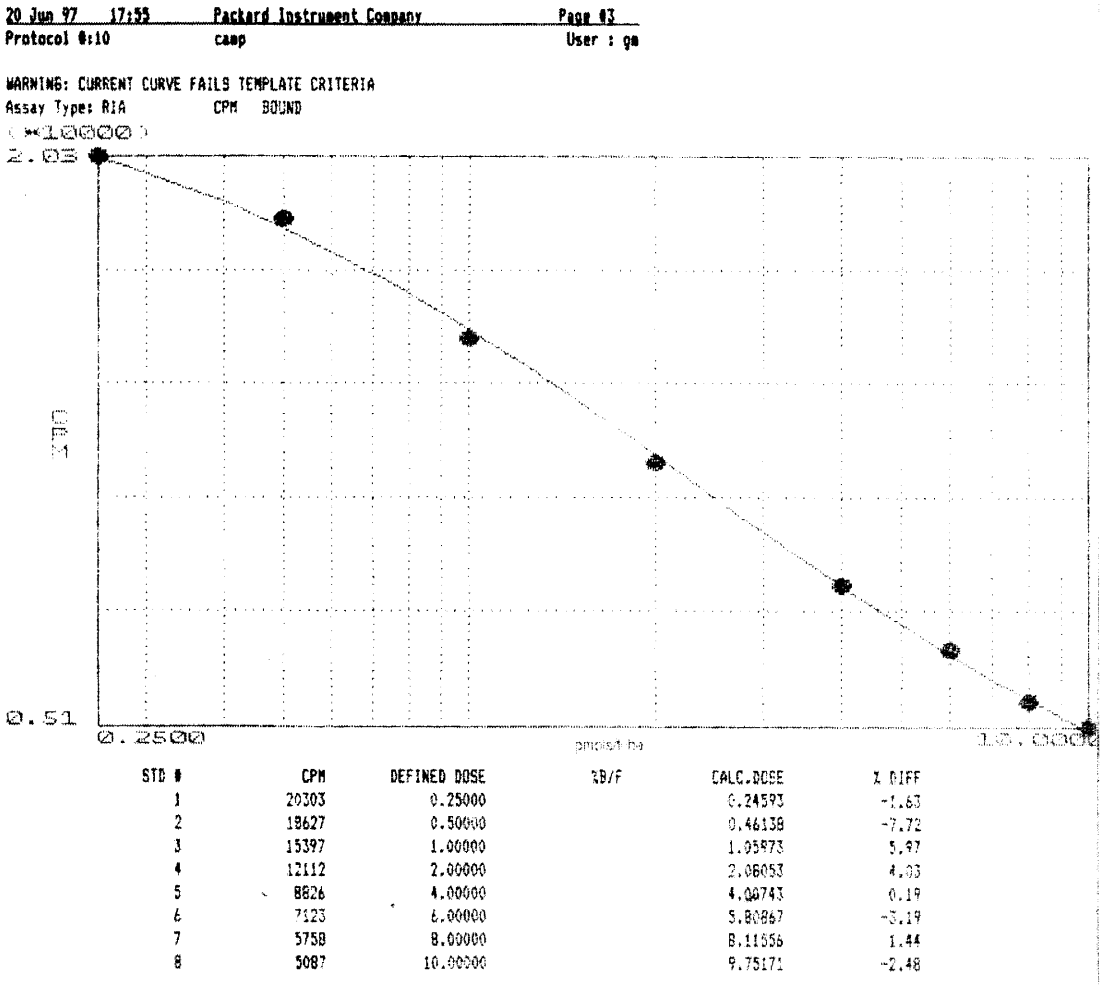
2.7.2. Experiment.

All cAMP studies were performed in 0.3ml volumes of Krebs/HEPES buffer. Experiments were performed at 37°C for 15 minutes using whole CHOm2 cells.

Whole cells were incubated with methacholine (10^{-8}-10^{-2}M), in the absence and presence of pancuronium (300nM), vecuronium (1μM) and rocuronium (1μM). Methacholine is a non-selective muscarinic agonist. Each drug was added in 20 μl volumes. 20 μl forskolin (1μM) provided direct stimulation of adenylate cyclase (see section 1.9) and 40μl 3-isobutyl-1-methylxanthine (1mM) was added as a phosphodiesterase inhibitor to prevent the breakdown of cAMP. 20 μl atropine (1nM) provided a positive control (Atropine antagonises the effect of methacholine at the m2 receptor to reverse the methacholine induced decrease in cAMP production). The remaining volume was made up with Krebs/HEPES buffer. Reactions were terminated with 10 μl of 10M HCl, neutralised with 10μl of 10M NaOH and buffered with 180μl of 1M Tris, pH 7.4.

2.7.3. [^{3}H]cAMP mass assay.

Samples were then centrifuged at 12000g for 2 mins to remove cell debris. To keep the experimental temperature at 4°C, the extraction of the supernatant was performed on a bed of ice. Supernatant was removed and 50μl was incubated with 100μl [^{3}H]-cAMP and 150μl binding protein made up in cAMP assay buffer for at least 2 hours at 4°C. Unbound [^{3}H]-cAMP was removed using 250μl of 1% activated charcoal, 0.4% BSA mix in cAMP assay buffer, incubated for 1min at 20°C and then centrifuged for 1 minute at 12000g. 400μl of the supernatant from each sample was removed and added to 1ml of Optiphase Hi-Safe 3 scintillation fluid. Samples were vigorously agitated and incubated at 20°C for at least 8 hours. Cyclic AMP levels were estimated on a Packard TR 1900 β-counter using a RIASMART program. Cyclic AMP was extrapolated from a standard curve using standards ranging from 0.5-10 pmol (see figure 2.4) (Brown, *et al.*, 1971, Hirst, *et al.*, 1995).



STD #	CPM	DEFINED DOSE	%B/F	CALC.DOSE	% DIFF
1	20303	0.25000		0.24593	-1.63
2	18627	0.50000		0.46138	-7.72
3	15397	1.00000		1.05973	5.97
4	12112	2.00000		2.08053	4.03
5	8826	4.00000		4.00743	0.19
6	7123	6.00000		5.80867	-3.19
7	5758	8.00000		8.11556	1.44
8	5087	10.00000		9.75171	-2.48

Figure 2.4 shows a concentration dependent displacement of [^{3}H]-cAMP bound with increasing concentration of unlabelled cAMP. Note the close agreement with defined and calculated "dose" parameters.

2.8. Measurement of $[Ca^{2+}]_i$ in CHOm3 cells.

2.8.1. Theory.

Intracellular calcium is measured using the dye Fura-2. Fura-2 is a calcium sensitive dye that fluoresces when bound to Ca^{2+} when it is stimulated by a particular wavelength of light. The fura-2 free acid is the form that is sensitive to calcium binding, but is however, unable to penetrate the cell membrane. Cells are thus loaded with fura-2 penta-acetoxymethyl (AM) ester. This form of the dye is membrane permeable but Ca^{2+} insensitive. Intracellular esterase enzymes cleave the AM group to leave the fura-2 free acid inside the cell, unable to leave, where it will bind Ca^{2+}. Fura-2 is a dual excitatory, single emission ratiometric dye. Fura-2 is stimulated both by 340 and 380 nm (figure 2.5).

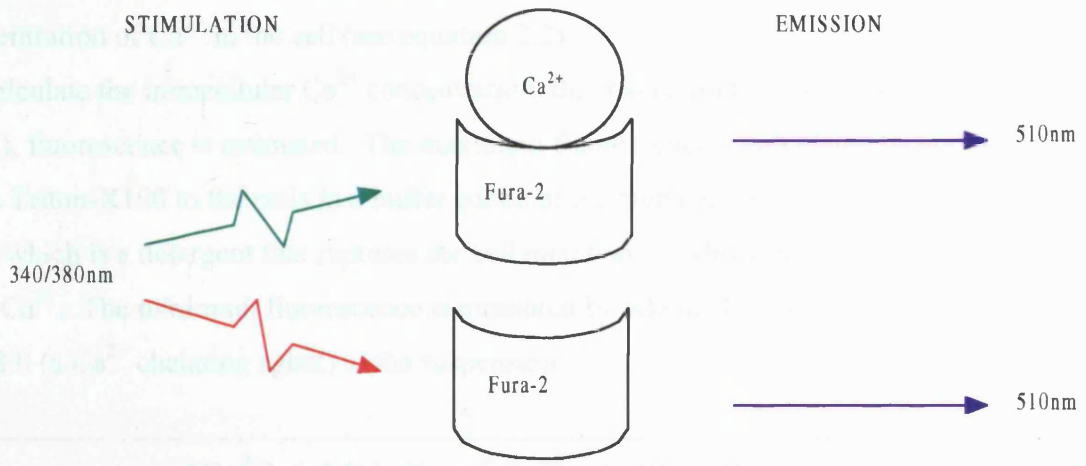


Figure 2.5. Stimulate bound and unbound fura-2 with dual λ of 340/380nm. Fura-2 is a Ca^{2+} sensitive, dual excitatory single emission dye. Bound (top) fura-2 and unbound (bottom) fura-2 have maximum emissions at λ of 510nm. Total Ca^{2+} measured is calculated from a ratio of the two emissions as described by equation 2.2.

Maximum fura-2 fluorescence occurs at an emission λ of 510nm, with fura-2 bound to Ca^{2+} observed at a λ of 340nm and fura-2 unbound at a λ of 380nm excitation (see figure 2.6). The Grynkiewicz (1985) equation describes the relationship

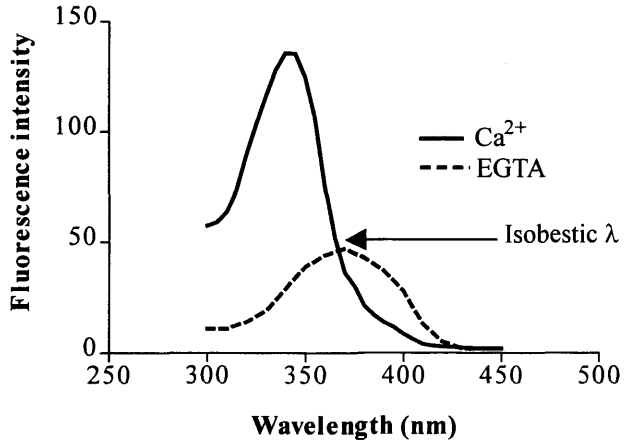


Figure 2.6. Fluorescence spectra of Fura-2 free acid when bound and unbound to Ca^{2+}. The isobestic λ is when there is no net change in fluorescence as $[Ca^{2+}]$ changes.

between the ratios of free and bound fura-2 and enable calculation of the concentration of Ca^{2+} in the cell (see equation 2.2).

To calculate the intracellular Ca^{2+} concentration, the maximum (R_{max}) and minimum (R_{min}), fluorescence is estimated. The maximum fluorescence is calculated by adding 0.1% Triton-X100 to the cells in a buffer containing a high concentration of Ca^{2+}(which is a detergent that ruptures the cell membrane), which saturates the dye with Ca^{2+}. The minimum fluorescence is measured by adding 4.5mM EGTA at pH>8.0 (a Ca^{2+} chelating agent) to the suspension.

$$[Ca^{2+}]_i\ (nM) = K_d \times [(R-R_{min})\div(R_{max}-R)] \times Sfb$$

K_d = 225nM (Ca^{2+} binding to fura-2 at 37°C).

R = 340nm/380nm ratio.

R_{max} = 340/380 ratio under Ca^{2+} saturating conditions.

R_{min} = 340/380 ratio under Ca^{2+} free conditions.

Sfb = ratio of baseline fluorescence (380nm) under Ca^{2+} free and Ca^{2+} bound conditions.

Equation 2.2. The Grynkiewicz equation.

2.8.2. Method.

Confluent CHOm3 cells were harvested and washed as in section 2.4. 3mls of cell suspension was incubated with 3μM fura2-AM for 30 minutes at 37°C in Krebs/HEPES buffer. The cells are then centrifuged at 1500rpm for 2 minutes in a Heraeus Labofuge 400R centrifuge. The pellet is then resuspended in Krebs/HEPES buffer and left in the dark for 20 minutes at room temperature to allow the de-esterification of the AM groups from fura2. The cell preparation is then re-pelleted twice as before and then finally resuspended in a volume of Krebs/HEPES buffer appropriate for the experiment. 2mls of cell suspension was then placed in a quartz cuvette, containing a magnetic stirrer and then placed in the fluorimeter (Perkin Elmer LS50B) at 37°C. Fluorescence at 510nm is measured with excitation at 340 and 380nm. The changeover time between 340 and 380 occurs 1 second per ratio pair. Ratio derivation and Ca^{2+} calculation was performed automatically using machine specific software (FLDM).

2.9. Measurement of [^{3}H]-NA uptake and release.

2.9.1 Measurement of ^{3}H-noradrenaline:uptake (SH-SY5Y and 293-hNET cells).

Experiments were performed in 5ml polystyrene test tubes containing whole SH-SY5Y and 293-hNET cells suspended at a concentration of ~180μg ml^{-1} and 120μg ml^{-1} respectively. Assays were performed in 0.5ml volumes of Krebs/HEPES at 37°C for 30 mins in the presence and absence of vecuronium, pancuronium, rocuronium and pipecuronium (10^{-8}-10^{-4} M). Non-specific uptake was defined in the presence of imipramine 50μM.

The reaction was started with the addition of a fixed concentration of [^{3}H]-noradrenaline (~41nM (≡ 1μCi/ml) allows maximum uptake of [^{3}H]-NAdr in minimum time) and incubated for a further 5 mins. Reactions were terminated by rapid vacuum filtration using a Brandel cell harvester onto Whatman GF/B filter papers with ice cold Krebs/HEPES buffer and rinsed twice more with 4mls ice cold buffer. Filter retained [^{3}H]-noradrenaline was extracted overnight in Optiphase 'Safe'

scintillation fluid and measured using liquid scintillation spectroscopy on a Packard 1900 TR β-counter.

2.9.2. Measurement of ^{3}H-noradrenaline release: basal (SH-SY5Y cells only).

Assays were performed using whole SH-SY5Y cells. Cells were seeded into 12 well multitrays at a concentration of ~1-1.5 x10^{-5} cells/well. When confluent, cells were loaded with ~40nM [^{3}H]-noradrenaline in 0.75ml volumes for 60 mins. The monolayers received 3, 20 minute washes with 1 ml of Krebs/HEPES buffer. The resulting monolayers were then challenged with 1ml Krebs/HEPES buffer for basal release and either rocuronium, vecuronium, pipecuronium or pancuronium (100 μM) for 3 mins. 200μl samples of supernatent were removed and placed in scintillation fluid to measure released radioactivity. [^{3}H]-NAdr was extracted overnight and radioactivity was quantified by liquid scintillation spectroscopy. Remaining [^{3}H]-noradrenaline was extracted from the monolayer using 1ml 0.4M perchloric acid and a 200μl sample was treated as above.

2.9.3. Measurement of ^{3}H-noradrenaline release: stimulated (SH-SY5Y cells only).

Cells were treated as above but the resulting monolayers were then challenged with 1ml Krebs/HEPES buffer for basal release or 100mM K^+ with or without either rocuronium, vecuronium, pipecuronium or pancuronium (100 μM) + 100mM K^+ for 3 mins. NMBD was pre-incubated in the last 20mins wash. 100mM K^+ was also used as a test for the cells responsiveness. Released radioactivity was measured as above and calculated using equation 2.2.

Equation 2.2. Calculation of [^{3}H]-NAdr release

$$[^3H]\ \text{released}\ \% = \frac{[^3H]_S}{[^3H]_T + [^3H]_S} \times 100$$

$[^3H]_S$ = dpm radioactivity in 200μl supernatent

$[^3H]_T$ = dpm radioactivity in 200μl monolayer extract.

2.10. Lowry protein assay.

Proteins were diluted 1:20 and 1:50 (25 and 10 µl respectively) in 500µl of 0.1 M NaOH. NaOH solublises the protein. Protein standards were made from bovine serum albumin (BSA) at 0, 50, 100, 150, 200 and 250µg/ml in 0.1M NaOH. Protein assay reagents: Protein A: 2% Na_2CO_3 in 0.1M NaOH; Protein B: 1% $CuSO_4$; Protein C: 2% Na^+K^+ tartrate. These three solutions were mixed in the ratio of 100:1:1 of A B: C respectively. 2.5mls of A: B: C is then added to each of the 500µl standards and unknown protein samples, and incubated at 20°C for 10 minutes. Folin and Ciocalteau's phenol reagent was diluted 1:4 with distilled H_20. 250µl of this solution is then added to each of the standard and unknown protein samples. The tubes are then vortexed and incubated at 20°C for 30 minutes to allow the colour to develop. 3mls of each of the standard and unknown samples are then measured separately in plastic cuvettes in a Corning colour spectrophotometer at 750nm. A standard curve is then generated from the protein standards and the unknown protein samples can be extrapolated from this graph (see fig 2.7) (Lowry et al 1951).

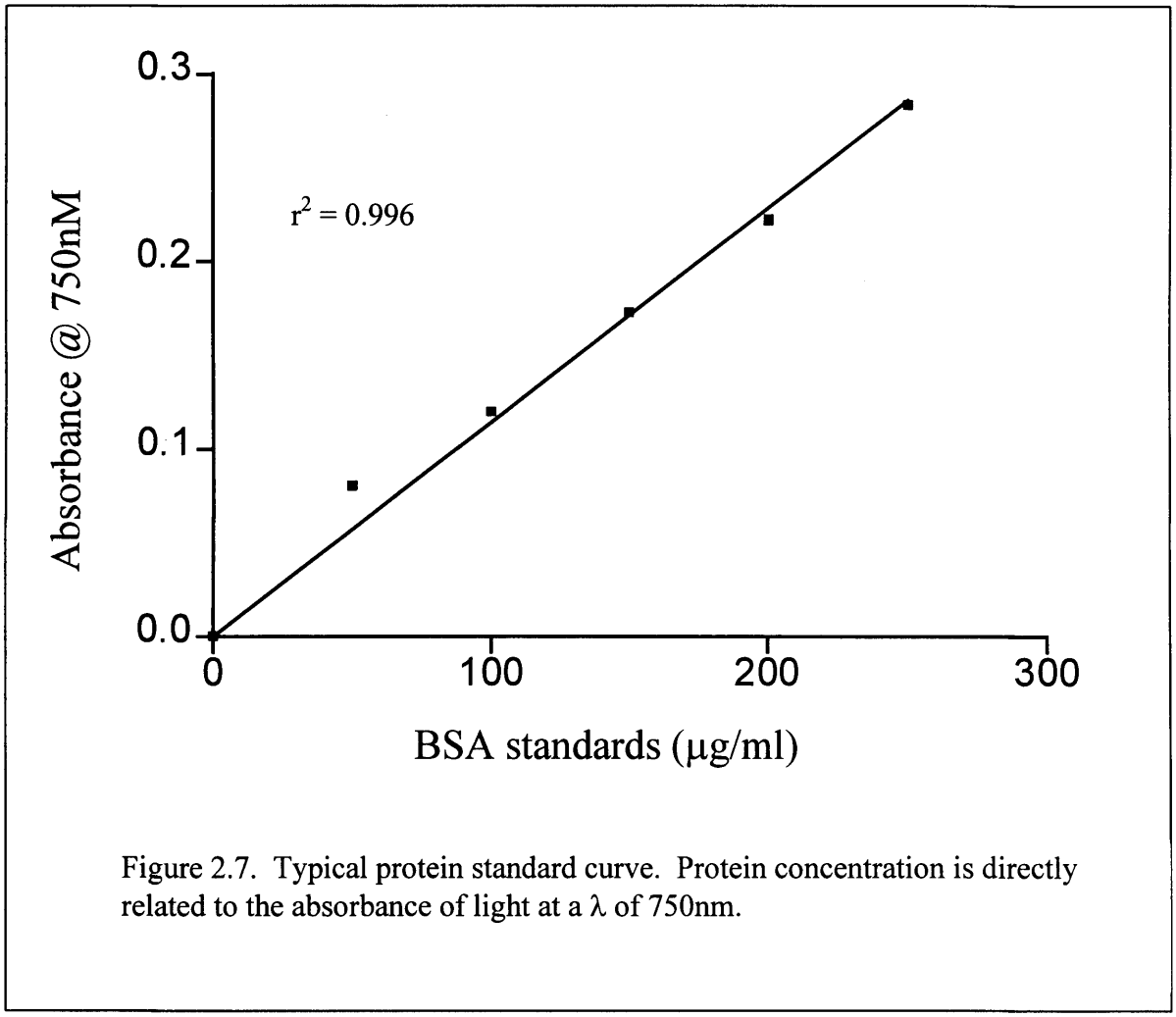


Figure 2.7. Typical protein standard curve. Protein concentration is directly related to the absorbance of light at a λ of 750nm.

2.11. Data analysis and statistics.

All data, unless otherwise stated, are expressed as mean and standard error of the mean (SEM) of at least 5 independent determinations. pIC_{50}, pEC_{50} and slope factors were all obtained by computer-assisted curve fitting using Graphpad Prism.

Both pEC_{50} and pIC_{50} values were obtained by fitting sigmoidal curve with a variable slope. pK_i values were obtained from displacement curves by modeling via correction of pIC_{50} values using Cheng and Prussof equation (see equation 2.1). Rate of radioligand dissociation (K_{off}) was obtained using one-phase exponential decay equation on Graphpad Prism.

Where appropriate, statistical comparisons were made using Student's paired or un-paired t-test (following analysis of variance (ANOVA) where appropriate) and considered significant when $p<0.05$.

CHAPTER 3

Basic pharmacological characterisation of CHOm1-m5 cells

3.1 Introduction.

Muscarinic receptors have been studied extensively over the years by using tissue prepared from animals (Caulfield & Birdsall, 1998; Felder, 1995) with molecular biology confirming the existence of 5 different types of human muscarinic receptor (Bonner, 1989). The studies have also shown that these receptors exist in mixed populations, for example, muscarinic receptors types m1 and m3 existing in the cerebral cortex, and m1, m3 and m4 existing together in the hippocampus (Caulfield, 1993). The m2 muscarinic receptor is predominantly found in the heart but is also present in the brain and elsewhere. The m1, m4 and m5 muscarinic receptors are present in high numbers in the brain but not in the heart whilst the m3 muscarinic receptors are present in great numbers in the CNS and the ileum but also not in the heart (Bonner, 1989, Caulfield 1993).

Mixed populations of receptors make interpretation of experimental results difficult, as any effect cannot be assigned to a single receptor type. Buckley et al (1989), have overcome this problem by stably transfecting each of the muscarinic receptors into the CHO-K1 (Chinese hamster ovary) cell line to produce the CHOm1-m5 cells. CHO cells were used because they lack endogenous muscarinic receptors, and therefore affinities of antagonists and other ligands derived using radioligand-binding studies can be clearly assigned to the cloned exogenous muscarinic receptor.

3.2 Aims.

The aims of this chapter are:

To determine the B_{max} and K_d for each of the receptor subtypes of muscarinic receptors using ^{3}H-NMS;

To use subtype selective compounds in order to verify the homogeneous expression of each of the subtypes of muscarinic receptor expressed in each CHO cell line.

3.3 Methods.

See section 2.6 for a detailed explanation of the theory and method used in this chapter.

3.3.1 Saturation.

See section 2.6.1 for a detailed protocol of the method used.

3.3.2 Displacement.

See section 2.6.2 for a detailed protocol of the method used.

3.4 Results.

3.4.1 Saturation.

In all cell lines [^{3}H]-NMS binding was concentration dependent and saturable. The B_{max} values varied from 127-2242 fmols/mg/protein in CHOm1-m5 cells and the K_d values varied from 0.11-0.22 nM in CHOm1-m5 cells (fig 3.1-3.5). [^{3}H]-NMS has between 1.09 fold greater affinity for m1 muscarinic receptors over m3 and m4 muscarinic receptors, to 2-fold greater affinity for m1 muscarinic receptors over m5 muscarinic receptors.

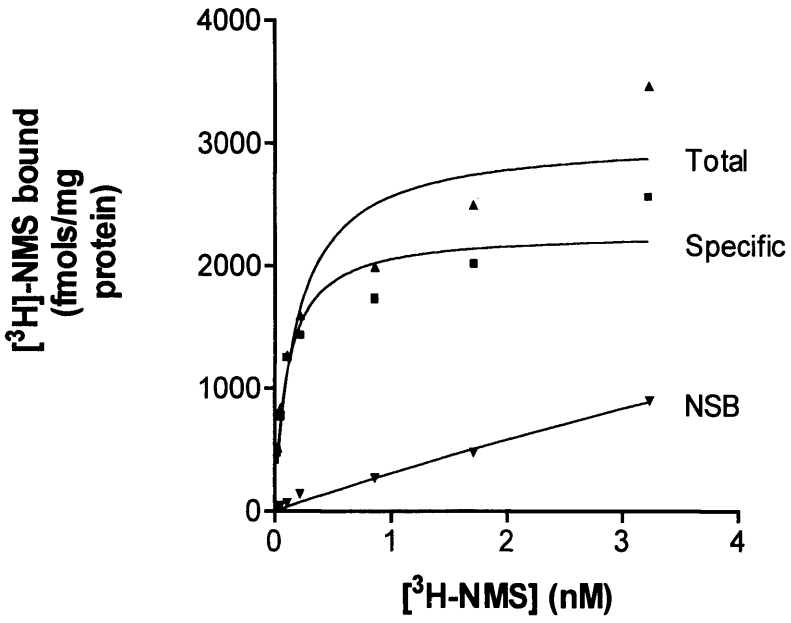
The rank order of B_{max} values for CHOm1-m5 muscarinic receptors was m1>m3>>m4>m2>m5. There was no significant difference between the B_{max} values derived by Scatchard and Sigmoid Curve analysis except for CHOm1 ($p<0.05$). There was no significant difference between the K_d values for CHOm1, m2, m3 and m4 however, there was a significant difference between the CHOm1-m4 and CHOm5 K_d value ($p<0.05$). The rank order of K_d values for CHOm1-m5 muscarinic receptors was m1>m3, m4>m2>m5. There was no significant difference between the K_d values derived by Scatchard and S.C. analysis. The data is summarised in table 3.1.

The slope factor values (derived from sigmoid curve analysis) varied from 0.88-1.13 in CHOm1-m5 cells (table 3.1) with the only differences occurring between the m2 and m4 receptors compared with the m3 receptor ($p<0.05$). The slope factor values are all tending towards 1 which indicates that in each of the cell lines there is a single class of [^{3}H]-NMS binding sites, i.e. homogeneous expression.

Table 3.1. Table showing saturation data of m1-m5 human muscarinic receptors according to Scatchard (1949) and analysis by sigmoid curve (S.C.). Sigmoid analysis gives slope factor and hence information on binding homogeneity.

	m1	m2	m3	m4	m5
Scat B_{max} (fmols/mg protein)	2242±75	165±13	1877±33	458±30	127±2
Scat K_d (nM)	0.11±0.02*	0.15±0.01*	0.12±0.01*	0.12±0.01*	0.22±0.01
S C B_{max}	1792±159**	150±18	1953±41	569±26	130±10
S C K_d	0.08±0.01*	0.13±0.02*	0.13±0.001*	0.14±0.02*	0.24±0.03
S C slope factor	0.98±0.11	1.09±0.07•	0.88±0.01	1.13±0.19•	1.03±0.08

(*$p<0.05$ c.f.m5. **$p<0.05$ c.f. Scatchard. •$p<0.05$ c.f. m3)



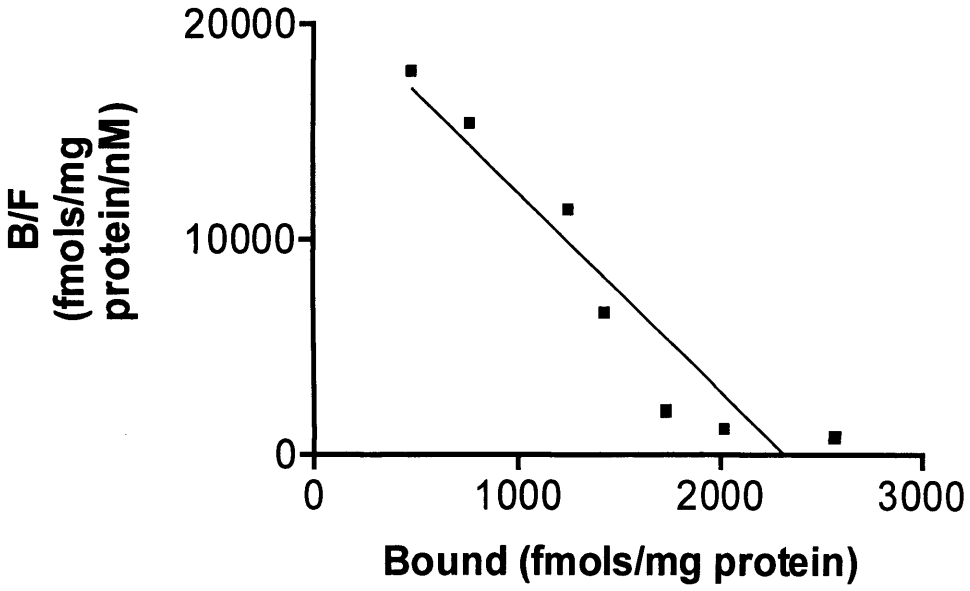
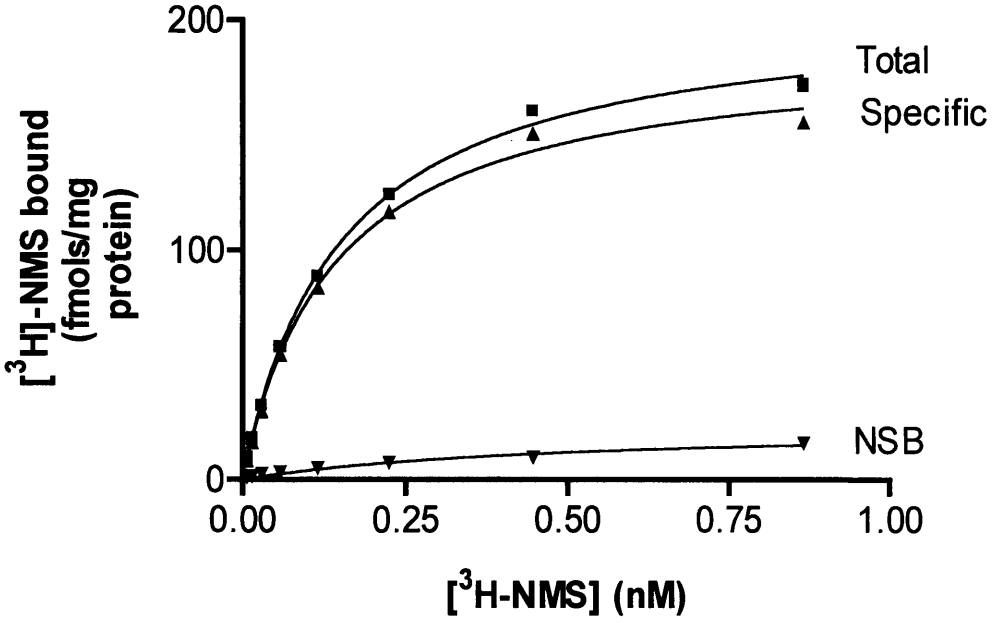


Figure 3.1. Rectangular hyperbole and Scatchard analysis of saturation of CHOm1 membranes as described in 3.4.1.



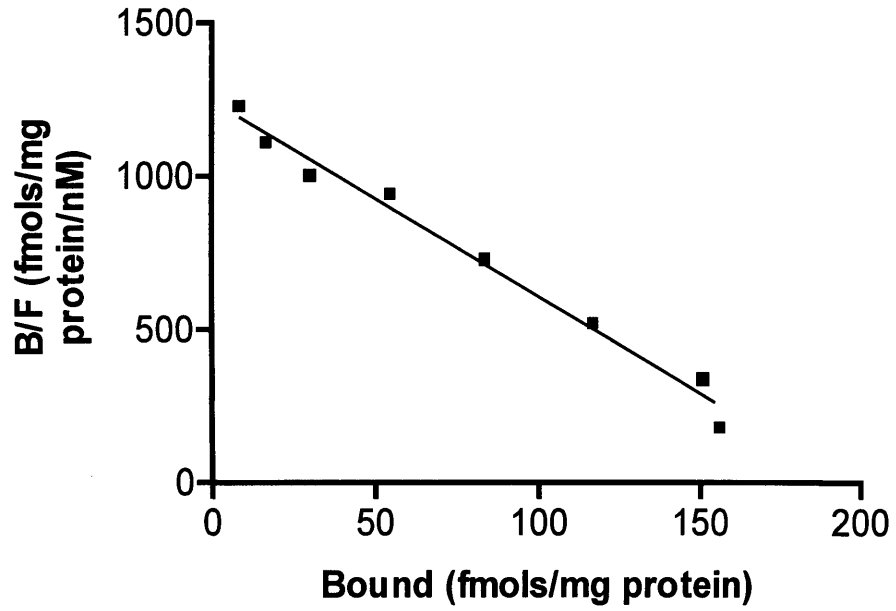
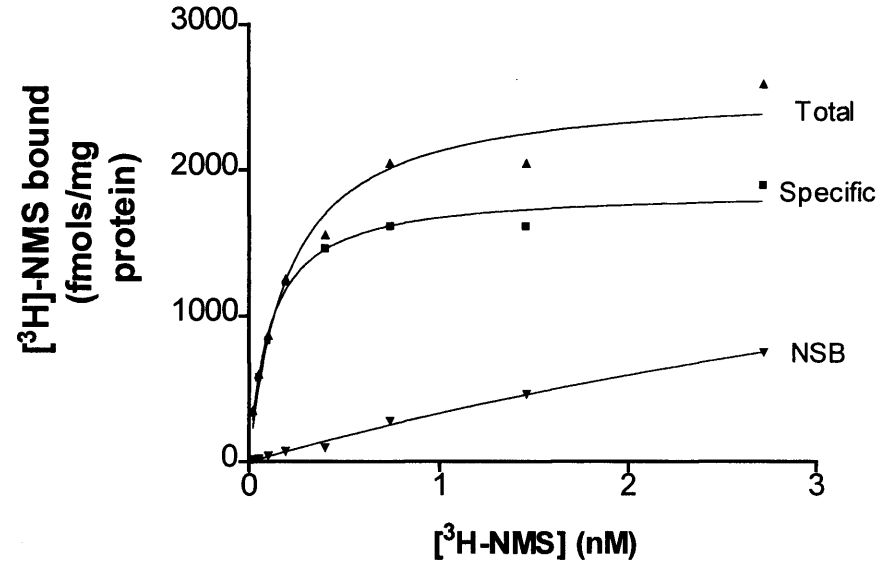


Figure 3.2. Rectangular hyperbole and Scatchard analysis of saturation of CHOm2 membranes as described in 3.4.1.



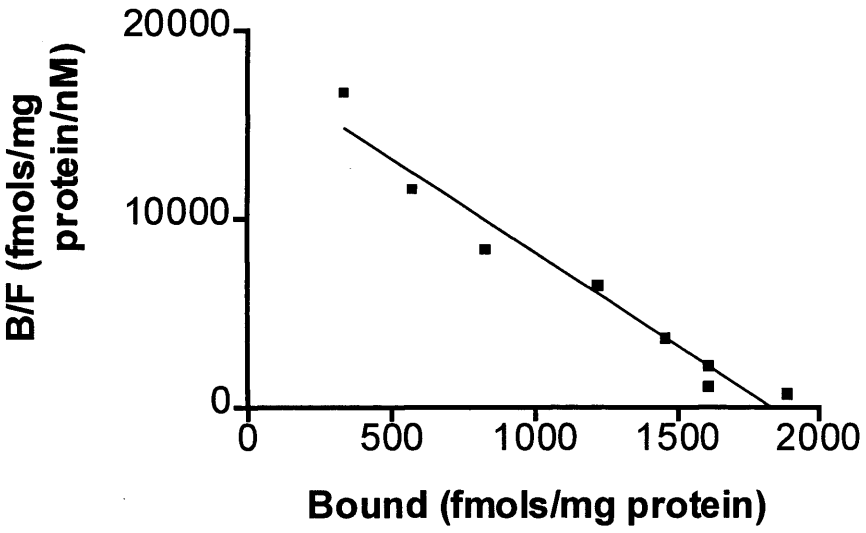


Figure 3.3. Rectangular hyperbole and Scatchard analysis of saturation of CHOm3 membranes as described in 3.4.1.

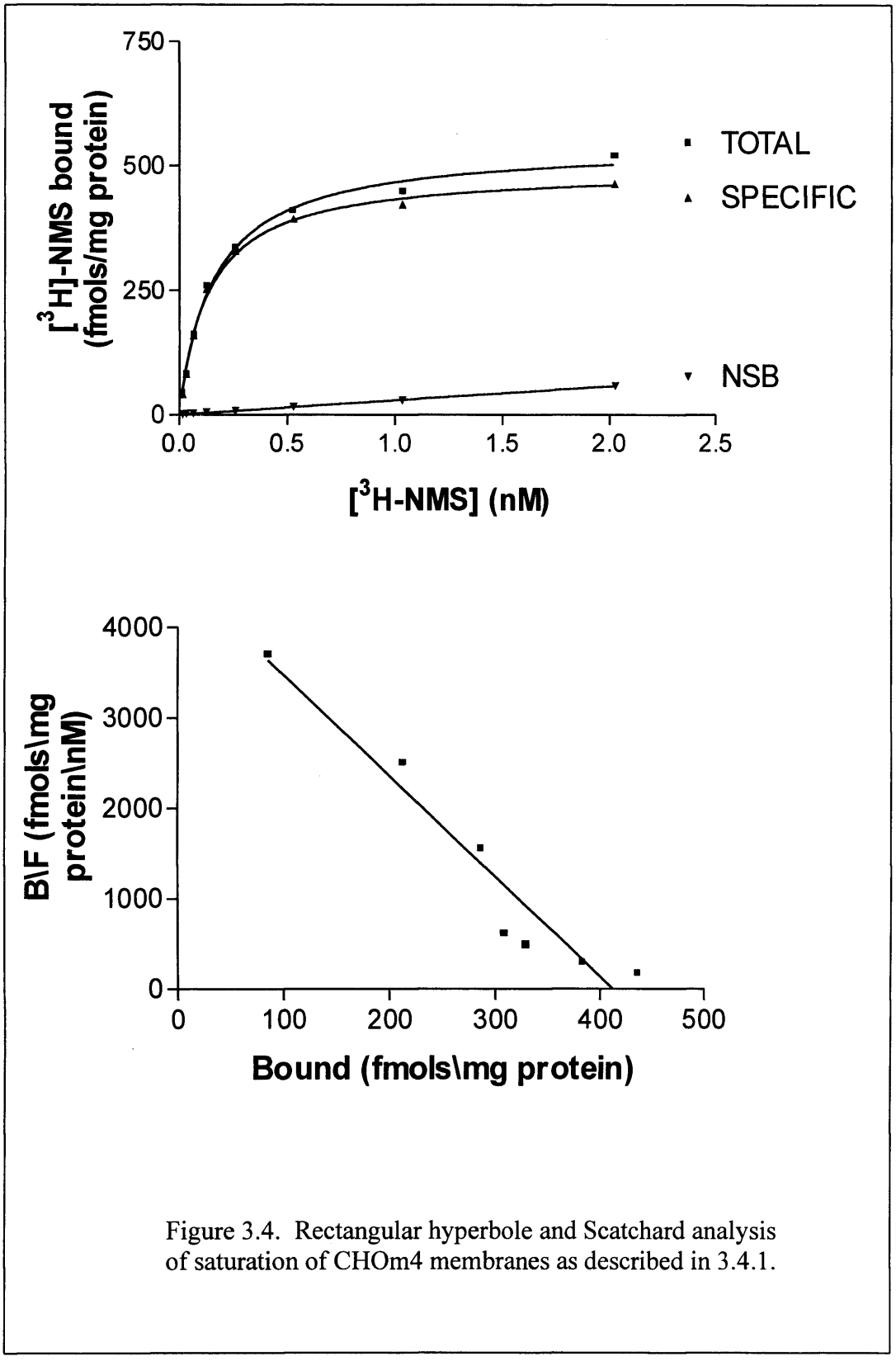


Figure 3.4. Rectangular hyperbole and Scatchard analysis of saturation of CHOm4 membranes as described in 3.4.1.

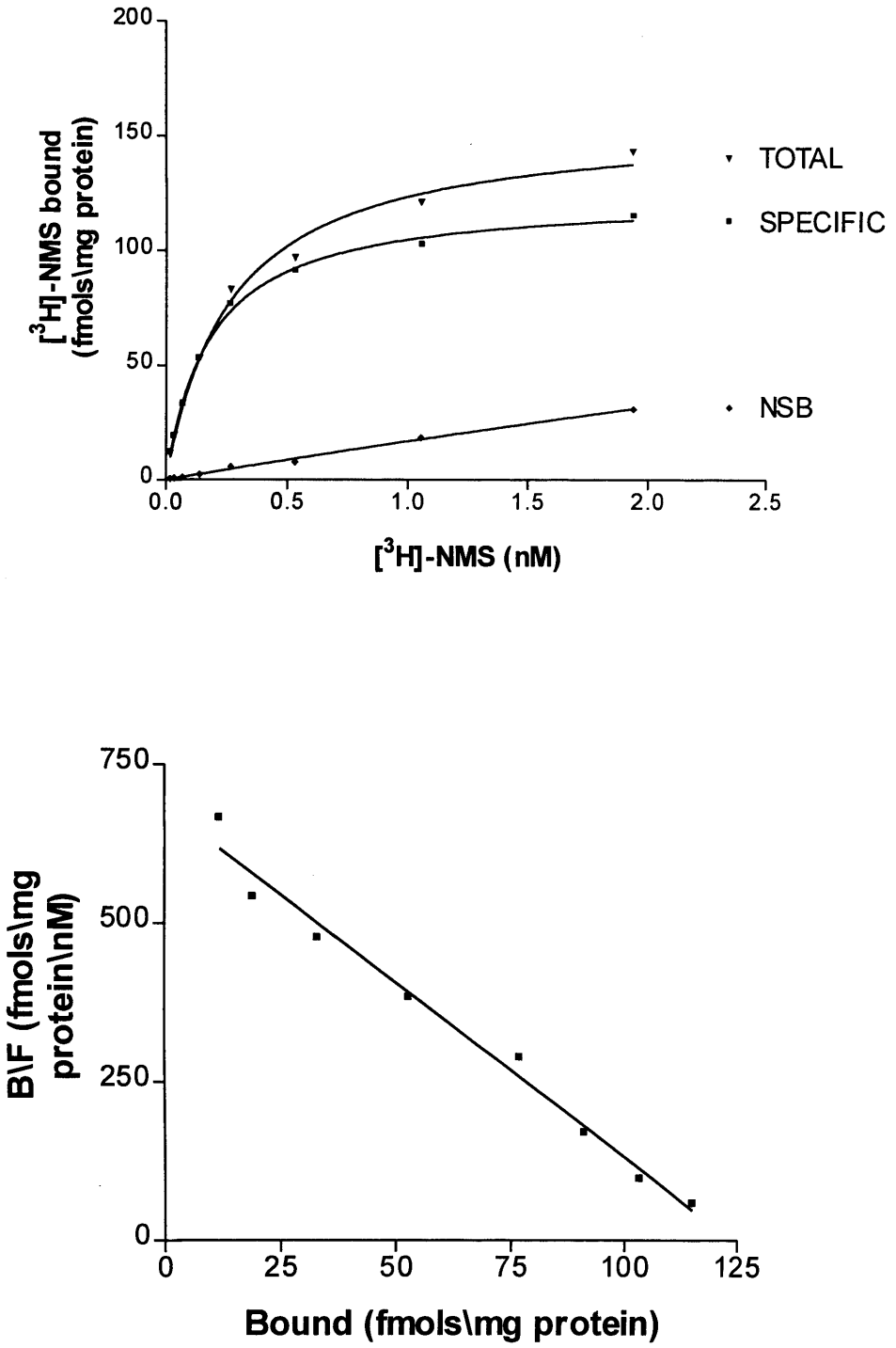


Figure 3.5. Rectangular hyperbole and Scatchard analysis of saturation of CHOm5 membranes as described in 3.4.1.

3.4.2 Displacement.

The binding of ~0.2nM ^{3}H-NMS was displaced concentration dependently by PZP in CHOm1 membranes to yield a slope factor of 0.90±0.01 and a pK_i of 7.97±0.04 which was significantly different form PZP binding to CHOm5 (*p<0.001*). PZP showed a ~24 fold greater selectivity for the m1 muscarinic receptor over the m5 muscarinic receptor. (fig. 3.6).

The binding of ~0.2nM ^{3}H-NMS was displaced concentration dependently by METH in CHOm2 membranes to yield a pK_i of 8.55±0.1 and a slope factor of 1.41±0.14 which was significantly different from METH binding to CHOm5 (*p<0.001*). METH showed a ~21 fold greater selectivity for the m2 muscarinic receptor over the m5 muscarinic receptor. (fig. 3.7).

The binding of ~0.2nM ^{3}H-NMS was displaced concentration dependently by 4-DAMP in CHOm3 membranes to yield a pK_i of 9.38±0.03 and a slope factor of 0.86±0.02. 4-DAMP showed a ~1.5 fold greater selectivity for the m3 muscarinic receptor over the m5 muscarinic receptor, but the difference was not significant (fig. 3.8).

The binding of ~0.2nM ^{3}H-NMS was displaced concentration dependently by TROP in CHOm4 membranes to yield a pK_i of 6.98±0.01 and a slope factor of 0.91±0.03. TROP showed a ~1.2 fold greater selectivity for the m4 muscarinic receptor over the m5 muscarinic receptor, but the difference was not significant (fig. 3.9).

The binding of ~0.2nM ^{3}H-NMS was displaced concentration dependently by PZP, 4-DAMP, METH and TROP in CHOm5 membranes to yield a pK_i values of 6.59±0.04, 9.20±0.14, 7.22±0.01, 6.89±0.05 and a slope factors of 0.95±0.01, 0.77±0.03, 1.16±0.03, 0.76±0.02 respectively (fig. 3.10).

The data is summarised in table 3.2.

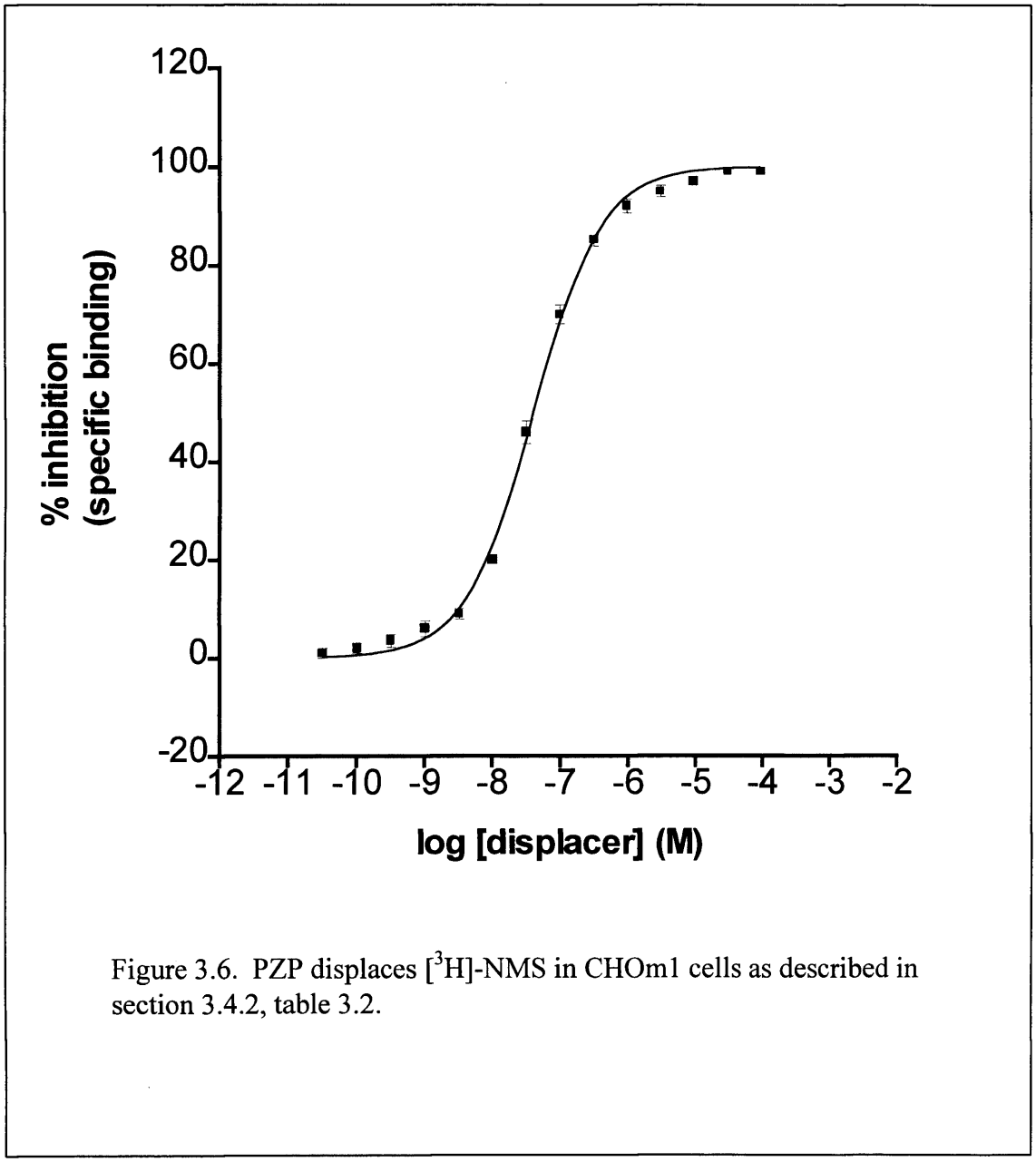


Figure 3.6. PZP displaces [^{3}H]-NMS in CHOm1 cells as described in section 3.4.2, table 3.2.

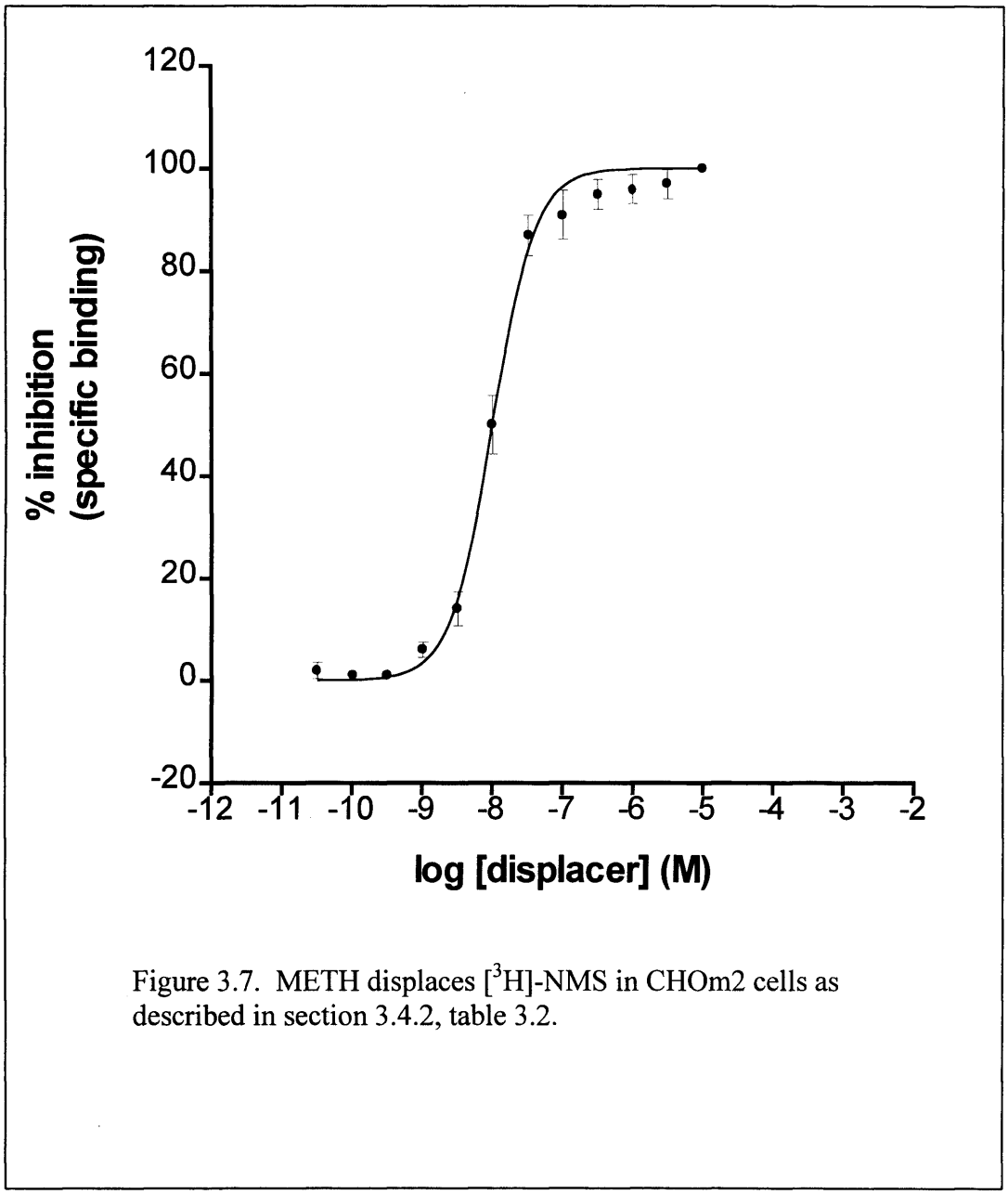


Figure 3.7. METH displaces [^{3}H]-NMS in CHOm2 cells as described in section 3.4.2, table 3.2.

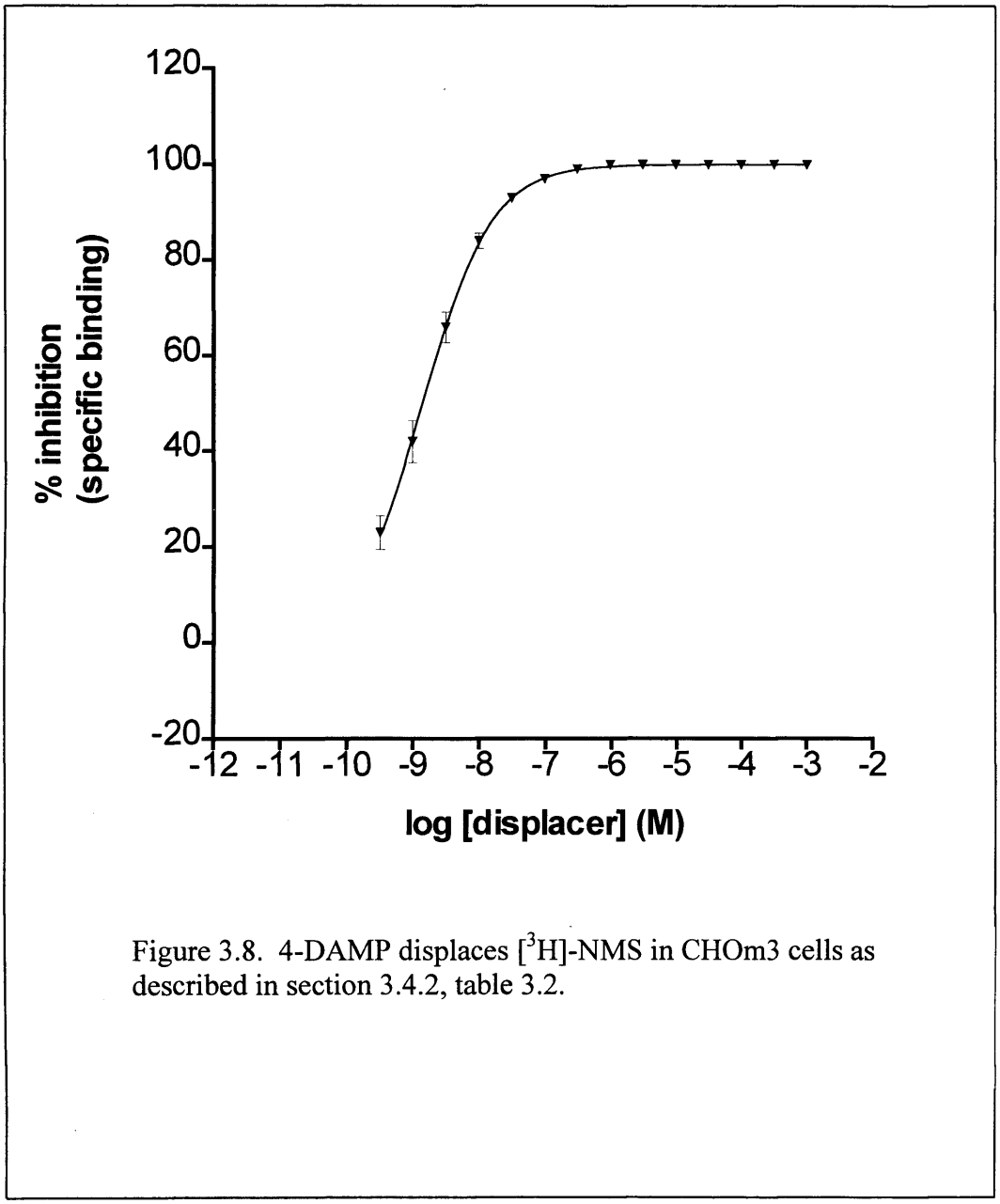


Figure 3.8. 4-DAMP displaces [^{3}H]-NMS in CHOm3 cells as described in section 3.4.2, table 3.2.

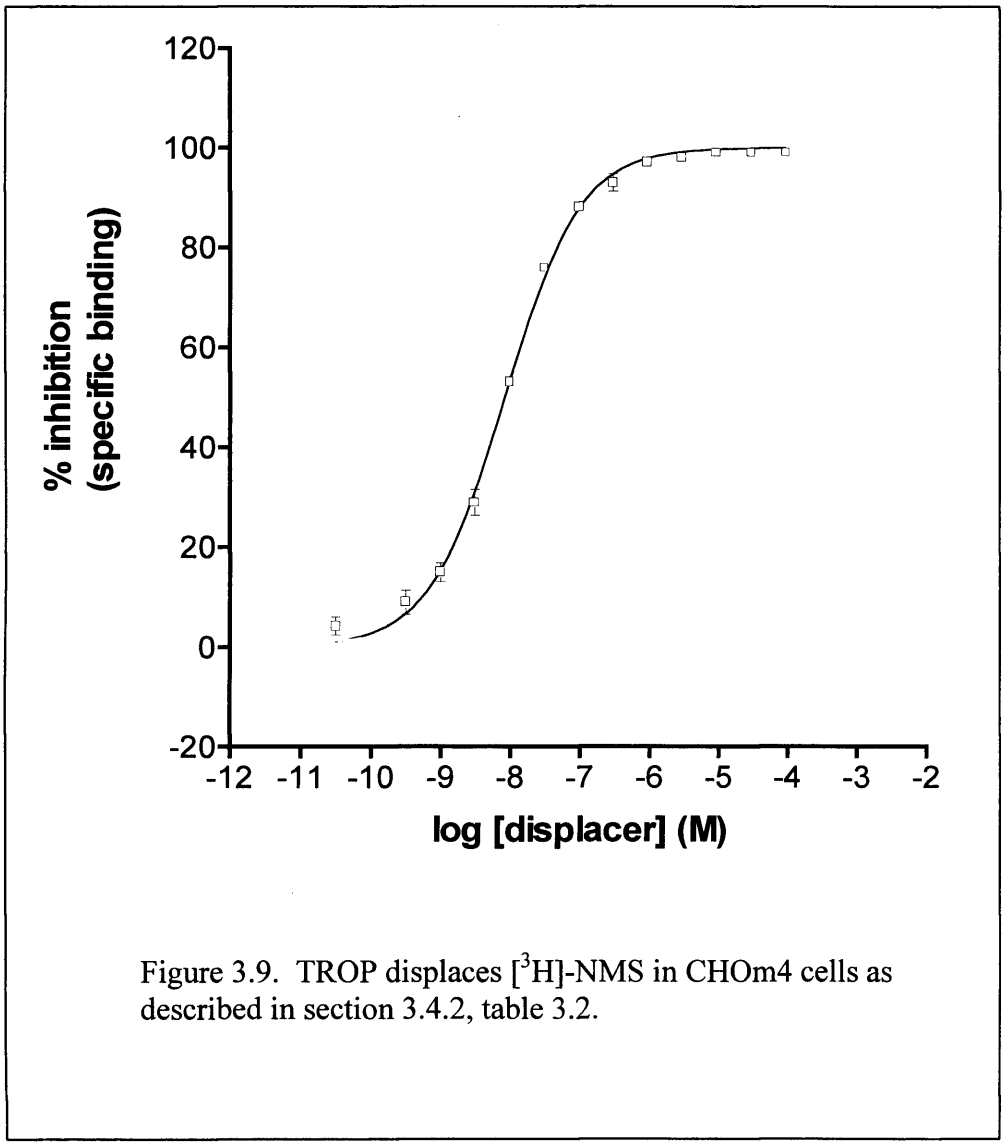


Figure 3.9. TROP displaces [^{3}H]-NMS in CHOm4 cells as described in section 3.4.2, table 3.2.

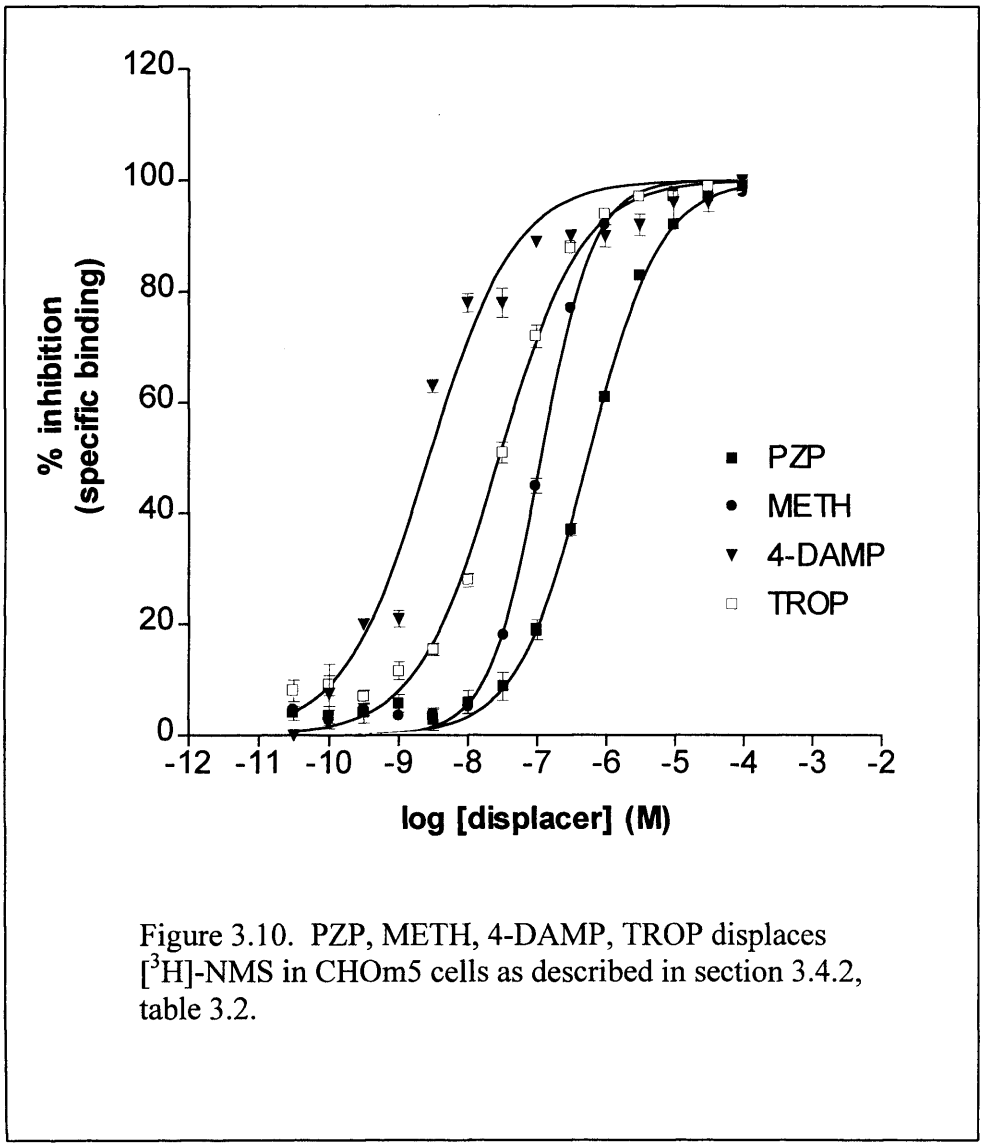


Figure 3.10. PZP, METH, 4-DAMP, TROP displaces [^{3}H]-NMS in CHOm5 cells as described in section 3.4.2, table 3.2.

3.5 Discussion.

I have shown in this chapter that there are single populations of each of the muscarinic receptors in each of the cell lines and, that the subtype selective antagonists have successfully verified the identity of these receptors. Table 3.2 compares experimental data, with data taken from the literature. The experimental data obtained shows good agreement.

Human mAChRs (muscarinic acetylcholine receptors) have been cloned and transfected into many different types of host cell to try and elucidate their function. CHO cells are one cell line in which all five types of human mAChR have been stably expressed (Buckley, et al., 1989). Expressing human receptors in immortal cell lines is a convenient method of examining the interaction of drugs with receptors. As the cell lines that were used express a single population of a particular receptor, this removes the ambiguity of responses that occur with heterogeneous populations of receptors and ligands that are not receptor type specific. Homogenous cell suspensions enable precise and simple monitoring of biochemical events.

The displacement of ^{3}H-NMS by subtype selective compounds in each of the M1-M5 cell lines provided values that are consistent with the literature, (Waelbrock et al., 1992, Buckley et al 1989, Caulfield 1993, Hulme et al 1990, Dorje et al 1991), but a value for TROP could not be found for the M5 receptor. Selective antagonists for the M5 receptor as yet do not exist with a high enough selectivity to discriminate from other subtypes. Using these other subtype selective antagonists on the CHOm5 receptor, a rank order of potency can be determined. The rank order of potency of PZP, TROP, 4-DAMP and METH for CHOm5 receptors is 4-DAMP>METH>TROP>PZP (Table 3.2). This rank order compares favourably to the data in the literature (see table 3.2), although again a value for TROP could not be found.

Binding constants only reveal an interaction between the ligand and the receptor, and not the nature of the interaction. The cardiovascular side effects of steroidal neuromuscular blocking drugs (NMBDs) may be linked to an effect on the M2 muscarinic receptor. The next chapter looks at the interaction of these drugs with recombinant receptors.

Table 3.2. Selective antagonist pK_i values for muscarinic subtypes taken from the literature.

Bold type denotes experimental values from this thesis.

	CHOm1	CHOm2	CHOm3	CHOm4	CHOm5
PZP	**7.97±0.04** 7.9-8.2 Dorje et al 1991 Hulme et al 1990 Buckley et al 1989				**6.59±0.04** 6.2-7.1 Dorje et al 1991, Hulme et al 1990 Buckley et al 1989
METH		**8.55±0.1** 7.8-8.3 Dorje et al 1991, Hulme et al 1990 Buckley et al 1989			**7.22±0.01** 6.9-7.2 Dorje et al 1991, Hulme et al 1990
4-DAMP			**9.38±0.03** 8.9-9.3 Dorje et al 1991, Hulme et al 1990		**9.2±0.14** 9.0. Dorje et al 1991, Hulme et al 1990 Buckley et al 1989
TROP				**6.98±0.01**	**6.89±0.05**

CHAPTER 4

The interaction of steroidal neuromuscular blocking drugs with recombinant human m1-m5 muscarinic receptors expressed in Chinese hamster ovary cells

4.1. Introduction.

As noted in Chapter 3, cells expressing a single receptor population offer tremendous advantages over conventional whole animal, ex vivo tissues and tissue homogenates for binding and functional studies. However, these simple cellular systems are not without their own drawbacks namely loss of tissue integrity. In order to gain a better understanding of whole systems the functions of the building blocks (e.g., receptors) must be understood.

The role of the M2 muscarinic receptor in regulating the heart rate is well documented (see section 1.16). Activation of M2 receptors in the heart leads to decreased heart rate and force of contraction and conversely inhibition of M2 muscarinic would lead to a reduction in parasympathetic "tone" and an increase in heart rate and force of contraction (Weishaar et al, 1988). As noted NMBDs produce both tachycardias (e.g., pancuronium) and bradycardias (e.g., vecuronium) and these actions could clearly result from antagonist (tachycardia) and agonist (bradycardia) action at the M2 receptor. To date there have been no systematic studies of the effects of steroidal neuromuscular blocking drugs on muscarinic receptors in simple cellular systems, specifically with recombinant M2 receptor proteins. In addition the effects of these agents at other muscarinic receptors M1,3-5 are unknown.

4.2. Aims.

The aim of this chapter is:

To investigate the interaction of steroidal NMBDs and gallamine with the M2 muscarinic receptor expressed in CHO cells and;

To compare the interaction of the M2 muscarinic receptor with M1, M3, M4 and M5 subtypes expressed in CHO cells

4.3. Methods.

See section 2.6 for a detailed explanation of the methods used for radioligand binding experiments.

4.4 Results.

Displacement of ^{3}H-NMS by steroidal NMBDs and gallamine was concentration dependent (figures 4.1-4.5) yielding pK_i values for CHOm1-m5 as shown in table 4.1.

Pancuronium displayed pK_i values ranging from 5.79-7.68 for CHOm1-m5 muscarinic receptors. Pancuronium showed ~77, ~18, ~14 and ~ 13 fold selectivity for M2 muscarinic receptors over M5, M1, M3 and M4 muscarinic receptors respectively.

Vecuronium displayed pK_i values ranging from 6.14-7.31 for CHOm1-m5 muscarinic receptors. Vecuronium showed ~6, ~5 and ~5 fold selectivity for M2 muscarinic receptors over M1, M3 and M5 muscarinic receptors respectively. Vecuronium showed a ~2.5 fold selectivity for the M4 muscarinic receptor over the M2 muscarinic receptor.

Pipecuronium displayed pK_i values ranging from 4.80-6.60 for CHOm1-m5 muscarinic receptors. Pipecuronium showed ~60, ~4 and ~2 fold selectivity for M2 muscarinic receptors over M5, M3 and M1 muscarinic receptors respectively. Pipecuronium showed relatively poor selectivity with similar pKi values for M1-M4. Binding to M5 was weak.

Rocuronium displayed pK_i values ranging from 4.34-5.40 for CHOm1-m5 muscarinic receptors. Rocuronium showed ~12, ~2 and ~2 fold selectivity for M2 muscarinic receptors over M3, M4 and M5 muscarinic receptors respectively.

Gallamine displayed pK_i values ranging from 5.34-7.67 for CHOm1-m5 muscarinic receptors. Gallamine showed ~218, ~42, ~30 and ~7 fold selectivity for M2 muscarinic receptors over M5, M3, M4 and M1 muscarinic receptors respectively.

Table 4.1. pK_i (K_i) values of NMBDs in CHO cells expressing recombinant m1-m5 muscarinic receptors.

	CHOm1	CHOm2	CHOm3	CHOm4	CHOm5
Pancuronium	6.43±0.12	7.68±0.02	6.53±0.06	6.56±0.03	5.79±0.10
	(371 nM)	(21 nM)	(295 nM)	(275 nM)	(1622 nM)
K_i cf m2*	17.6	1.0	14.0	13.1	77.2
Slope factor	0.89±0.03	0.61±0.02	0.97±0.08	0.68±0.03	0.72±0.06
Vecuronium	6.14±0.04	6.90±0.05	6.17±0.04	7.31±0.02	6.20±0.07
	(724 nM)	(126 nM)	(676 nM)	(49 nM)	(631 nM)
K_i cf m2*	5.7	1.0	5.4	**0.39**#	5.0
Slope factor	0.89±0.04	0.95±0.13	0.88±0.05	0.61±0.02	0.85±0.08
Pipecuronium	6.34±0.11	6.58±0.03	5.94±0.01	6.60±0.06	4.80±0.03
	(457 nM)	(263 nM)	(1148 nM)	(251 nM)	(15849 nM)
K_i cf m2*	1.7	1.0	4.4	0.95	60.3
Slope factor	0.93±0.04	0.98±0.02	0.85±0.02	0.59±0.01	0.76±0.03
Rocuronium	5.42±0.01	5.40±0.02	4.34±0.02	5.02±0.04	5.10±0.03
	(3801 nM)	(3981 nM)	(45709 nM)	(9550 nM)	(7943 nM)
K_i cf m2*	0.95	1.0	11.5	2.4	2.0
Slope factor	0.65±0.07	0.62±0.04	0.98±0.06	0.48±0.01	0.98±0.04
Gallamine	6.83±0.05	7.67±0.04	6.06±0.06	6.20±0.03	5.34±0.03
	(148 nM)	(21 nM)	(871 nM)	(631 nM)	(4571 nM)
K_i cf m2*	7.0	1.0	41.5	30.0	217.7
Slope factor	0.77±0.05	0.83±0.05	0.67±0.02	0.75±0.03	0.84±0.04

Data are mean±SEM ($n \geq 5$). *expressed as a ratio of K_i M2 (e.g., for pancuronium at m1 371nM/21nM=17.6). With the exception of vecuronium at M4 (#$p<0.05$) receptors all NMBDs displaced with equal or higher affinity at M2 subtype.

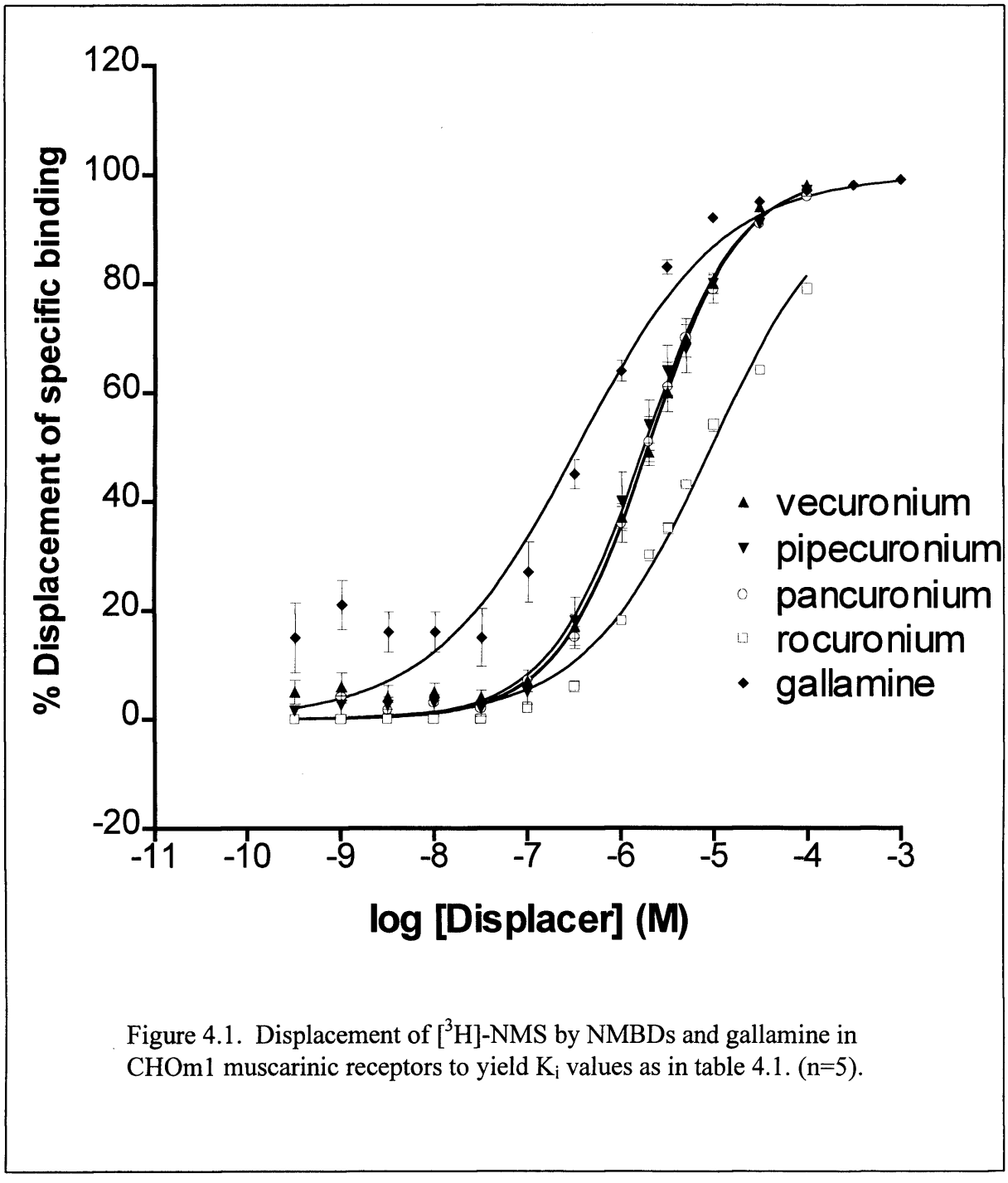


Figure 4.1. Displacement of [^{3}H]-NMS by NMBDs and gallamine in CHOm1 muscarinic receptors to yield K_i values as in table 4.1. (n=5).

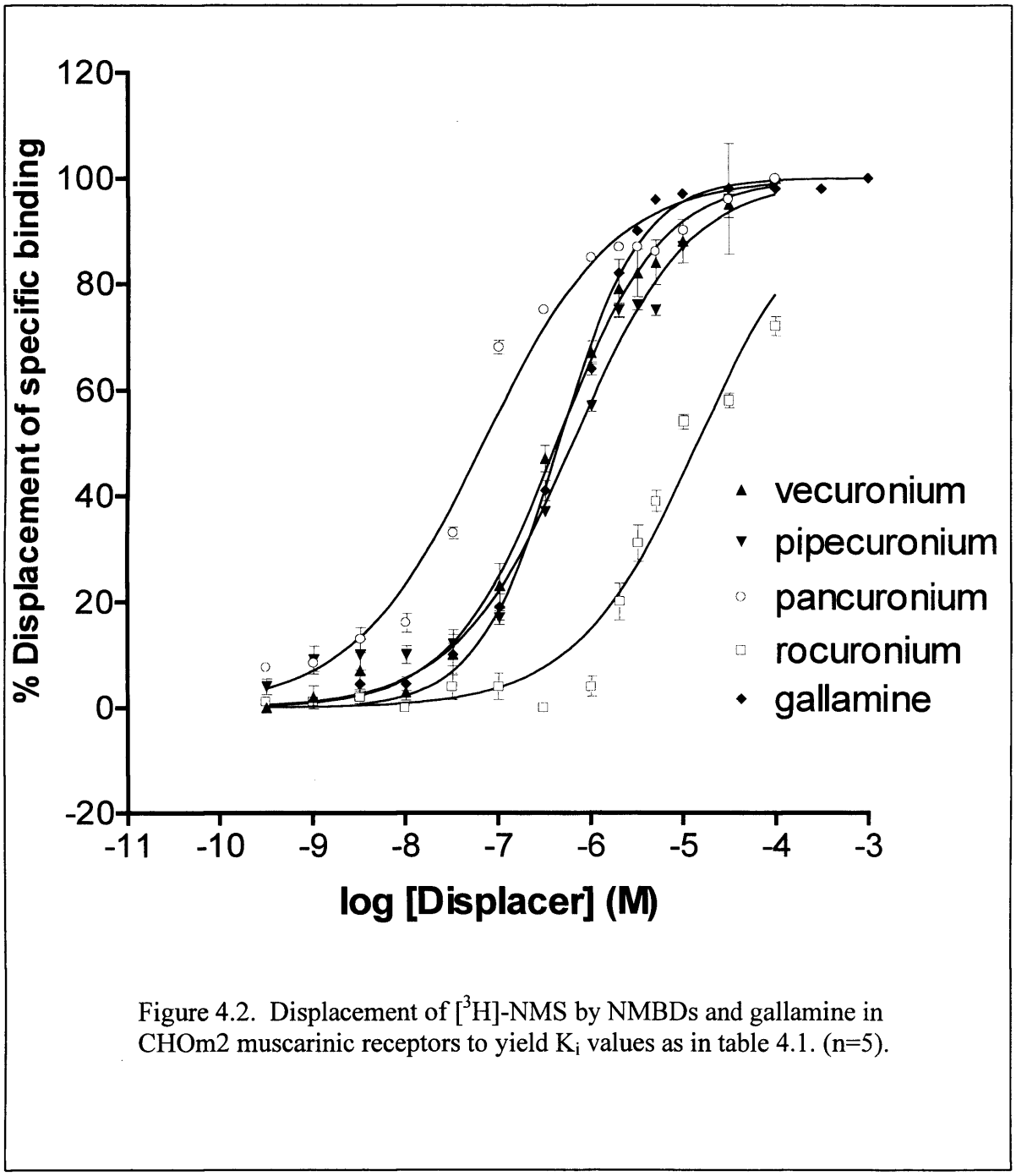


Figure 4.2. Displacement of [^{3}H]-NMS by NMBDs and gallamine in CHOm2 muscarinic receptors to yield K_i values as in table 4.1. (n=5).

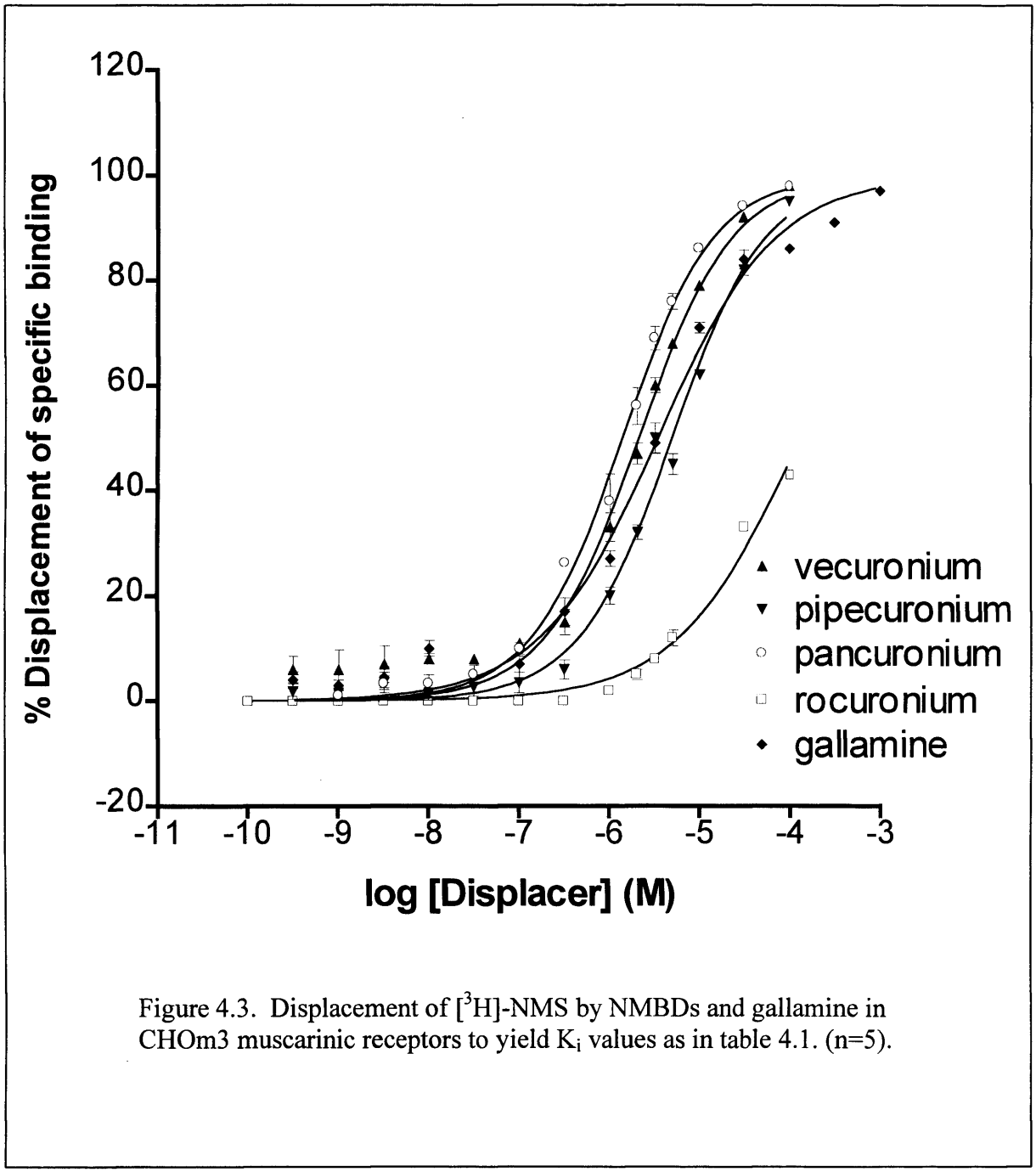


Figure 4.3. Displacement of [^{3}H]-NMS by NMBDs and gallamine in CHOm3 muscarinic receptors to yield K_i values as in table 4.1. (n=5).

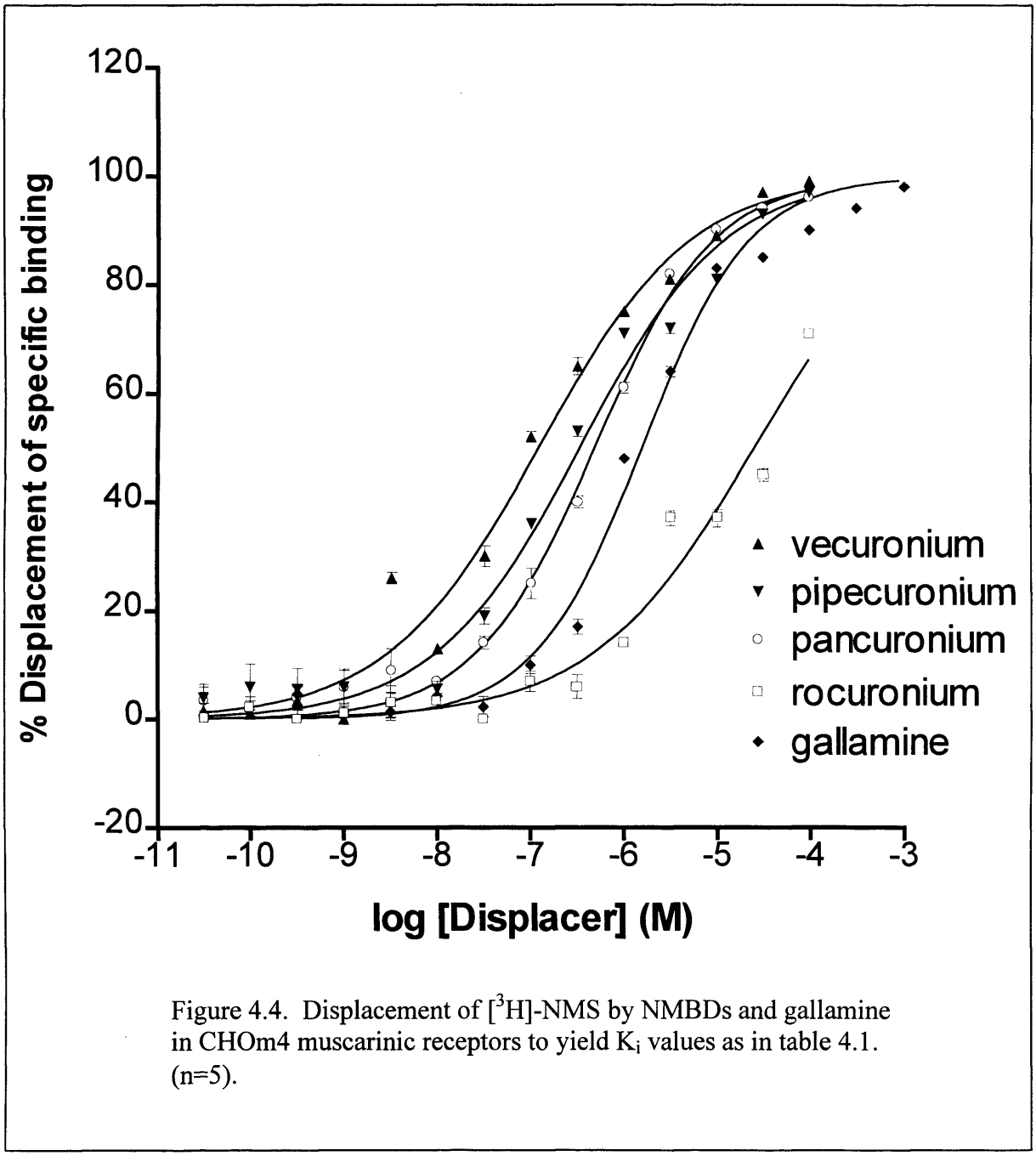


Figure 4.4. Displacement of [^{3}H]-NMS by NMBDs and gallamine in CHOm4 muscarinic receptors to yield K_i values as in table 4.1. (n=5).

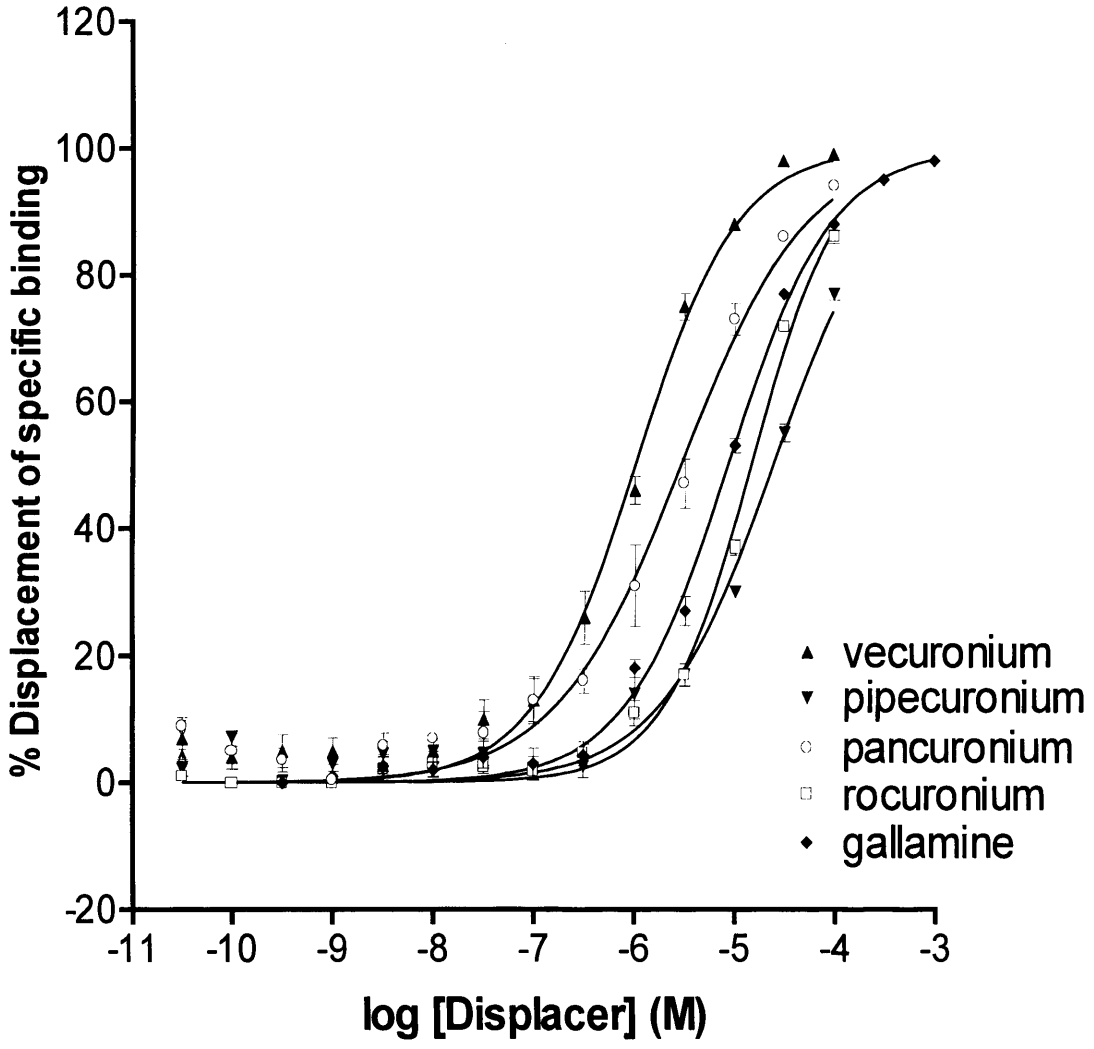


Figure 4.5. Displacement of [^{3}H]-NMS by NMBDs and gallamine in CHOm5 muscarinic receptors to yield K_i values as in table 4.1. (n=5).

4.5. Discussion.

In the present study using CHO cells expressing recombinant human m1-m5 muscarinic receptors we have shown that there is a clear interaction of a range of NMBDs with muscarinic receptors. As can be seen in table 4.1 there was a wide variation in K_i values. These studies represent the first complete comparison to be made. The protocol used was simple radioligand displacement, which enabled the binding affinity of the displacing drug (NMBD) to be determined. This interaction with the cardiac M2 receptor may explain the effects of these agents observed on the heart.

The use of recombinant muscarinic receptors offers several distinct advantages over conventional tissue homogenates in that a single subtype can be studied in isolation. Moreover, in contrast to our earlier preliminary investigation of the M2 receptor population endogenously expressed in rat heart (Appadu, *et al.*, 1994), in this study we have used the human isoform and can therefore eliminate any interpretation problems related to species differences.

As discussed at length in the Introduction (section 1.15) the cardiovascular side effects observed during surgery could potentially result from inhibition of noradrenaline reuptake, histamine release, interaction with cardiac muscarinic receptors or a combination of these effects (Miller, et al.,1990). As demonstrated in this chapter all the muscle relaxants examined interact with muscarinic receptors at differing concentrations. Of particular importance to the cardiovascular effects of these drugs is the interaction with the M2 receptor. The rank order K_i was; Panc=Gall>Vec>Pipe>>Roc (c.f. table 4.2). An important question is does the interaction occur at clinically achievable concentration. Typical plasma concentrations producing 50% depression of muscle twitch tension seen in humans are illustrated in Table 4.3. With the exception of rocuronium we would predict that a significant interaction with the M2 receptor would occur for all the NMBDs examined at clinically achievable concentrations. Interestingly (with the exception of vecuronium) all NMBDs tested showed some selectivity for the m2 receptor Table 4.3. Vecuronium displayed the highest affinity for the M4 receptor.

Table 4.2. Rank order of selectivity for NMBDs and gallamine for CHOm1-m5 muscarinic receptors.

Pancuronium	**M2**>M4>M3>M1>>M5
Vecuronium	**M2**=M4>M5>M3>M1
Pipecuronium	**M2**=M4>M1>M3>>M5
Rocuronium	**M2**=M1>M5>M4>M3
Gallamine	**M2**>M1>>M4>M3>>M5

Table 4.3. Plasma free fraction and plasma concentration producing 50% depression in muscle twitch tension (Cp_{50}) for gallamine, pancuronium, pipecuronium, vecuronium and rocuronium.

Blocker	Free fraction	$Cp_{50(total)}\mu g/ml$	$Cp_{50(free)}$
Gallamine	0.30	5.82	6.53μM
Pancuronium	0.93	0.193	245nM
Pipecuronium	0.75	0.067	68nM
Vecuronium	0.43	0.09	61nM
Rocuronium	0.75	1.22	1.5μM

Data are from Wierda, et al., (1995); Khahil, et al, (1994); Ornstein, et al (1992); Miller, (1990); Yajima, et al., (1990); Wood, et al., (1983); Tassonyi, et al., (1981); Skivington, (1972).

Pipecuronium is the only steroidal NMBD tested that is thought to be devoid of cardiovascular side effects (Larijani et al, 1989; Foldes et al, 1990). However, we have reported significant interaction with the M2 receptor.

Gallamine is a well characterised antagonist at M2 receptors (Ellis, et al., 1991; Dunlap, et al., 1983) with a reported K_i of 41.7nM in binding studies (Leppik, et al., 1994)

and was included in these studies as a reference compound. In this chapter a K_i value of 21nM was determined. This is in good agreement with the earlier published value. Clinically gallamine produces an anticipated tachycardia probably resulting from an atropine like block of M2 receptors (Eisle, et al., 1971; Brown, et al., 1970).

Table 4.1 reports binding slope factors. These give some simple indication of the number of "binding sites" involved in the interaction but cannot be used alone to report agonist or antagonist activity (see below). A low slope factor for a *known* antagonist is indicative of an interaction with more than one subtype of receptor and is the basis of drug selectivity (e.g., PZP is M1 selective. In a binding experiment such as reported here where there was a mixed population of M1 and M2 receptors the displacement curve would have a low slope factor with high affinity binding representing the M1 receptor). A low slope factor for a *known* G-protein coupled receptor agonist would indicate the presence of G-coupled and G-free receptor (G-coupled receptors have high affinity for agonist)

As we have reported that there is a significant interaction of NMBDs with other muscarinic receptors then we should suggest NMBD effects on other tissues including the brain (M1-M5), glandular tissue (M3), the stomach (M1) and the gut (mixed). At the time of preparation of this thesis I was unable to find any evidence for direct effects on muscarinic transduction in glandular or GI tissue. Experimentally this might be difficult to separate from any nicotinic effects of these drugs. An effect on the brain can be excluded as it is generally accepted that NMBDs do not pass the blood brain barrier (Fahey et al 1989).

This chapter has reported an interaction in a radioligand binding experiment with a range of muscarinic receptors. A functional effect has *not* been demonstrated. This is one of the major drawbacks of this type of study. In subsequent chapters attempts to determine the type of interaction (agonist, antagonist or allosteric regulator) have been made.

CHAPTER 5

The effect of steroidal neuromuscular blocking drugs on cAMP production in CHOm2 cells and on $[Ca^{2+}]_i$ in CHOm3 cells

5.1. Introduction.

Muscarinic receptors are coupled to guanine nucleotide binding (G) proteins and display subtype selective second messenger coupling. Muscarinic M2 and M4 receptors are linked via G_i to adenylate cyclase and activation lowers intracellular cAMP levels. This is in contrast to the βAR. This receptor is linked to AC via G_s, activation of which, by an agonist (typically NAdr) leads to an increase in cAMP levels. In addition M2/4 receptors also activate an inwardly rectifying K^+ channel to produce hyperpolarisation (Felder, 1995; Caulfield, 1993) and reduce Ca^{2+} influx through voltage sensitive Ca^{2+} channels. In contrast M1/3/5 receptors activate phospholipase C to increase IP_3 and diacylglycerol formation and subsequently increase intracellular Ca^{2+} (Caulfield, 1993; Bonner, 1989).

Chapter 4 has already shown that (with the exception of rocuronium) steroidal NMBDs show high affinity for M2 and moderate affinity for M3 muscarinic receptors. At a cellular level the functional consequences of these interactions are unknown.

Using CHOm2 cells, agonist/antagonist effects of pancuronium, vecuronium and rocuronium have been assessed. Functional responses in CHOm2 cells were measured using inhibition of cAMP formation. Methacholine is a muscarinic receptor agonist, which produces a decrease in cAMP formation in CHOm2 cells (Caulfield 1993). If the NMBD produces a drop in cAMP levels then agonist properties are suggested. If the NMBD reverses the inhibition of cAMP production by methacholine then antagonist properties are suggested.

There is evidence to suggest that steroidal NMBDs have a direct effect on M3 muscarinic receptors as antagonists (Okanlami et al 1996, Hou et al 1998). This was also investigated using human recombinant M3 muscarinic receptors stably expressed in CHO cells

5.2. Aims

Using CHOm2 cells to determine the effects of pancuronium (which produces a tachycardia), vecuronium (which produces a bradycardia) and rocuronium (an essentially neutral NMBD on the cardiovascular system), on cAMP formation (a) alone and (b) in combination with methacholine.

To determine any effects of the above NMBDs on $[Ca^{2+}]_i$ in CHOm3 cells. This protocol will enable selectivity between PI linked and AC linked receptors to be determined.

5.3. Methods.

5.3.1 [^{3}H]-cAMP mass assay.

The reader is directed to section 2.6 for a comprehensive description of the method used to measure cAMP.

5.3.2 $[Ca^{2+}]_i$ measurement.

The reader is directed to section 2.7 for a comprehensive description of the method used to measure $[Ca^{2+}]_i$.

5.4. Results.

5.4.1. cAMP measurements.

Formation of cAMP in CHOm2 cells was inhibited in a concentration dependent fashion by methacholine to produce pIC_{50} values shown in table 5.1. Methacholine pIC_{50} values in the presence of 300nM pancuronium, 1μM vecuronium and 1μM rocuronium are shown in table 5.1. See also figures 5.1-5.3. Pancuronium reversed the methacholine inhibition of cAMP production. Vecuronium and rocuronium produced a small rightward shift in the methacholine curve. As a control atropine (1nM) completely inhibited the methacholine inhibition of cAMP.

Antagonist affinities (K_i) derived using equation 5.1 were obtained from the IC_{50} values obtained from figs 5.2, 5.3 and 5.4.

When added acutely there was no direct action of any of the NMBDs tested on the formation of cAMP (i.e., the drugs did not display any agonist action)

Equation 5.1. K_i is derived from the concentration of drug required to cause 50% inhibition of cAMP production with and without the presence of the steroidal NMBD.

$$K_i = \frac{[NMBD]}{1+\left(\frac{IC_{50} + NMBD}{IC_{50} - NMBD}\right)}$$

Table 5.1. Log molar concentration of methacholine (Meth, agonist) that produces a half maximal inhibition (pIC_{50}) of cAMP formation with and without the steroidal neuromuscular blocking drugs pancuronium, vecuronium and rocuronium and derived K_i values. (n=5).

	Meth- NMBD	Meth + NMBD	K_i
Pancuronium	6.18±0.34	3.57±0.36*	0.73nM
Vecuronium	6.26±0.12	5.38±0.16*	114.9nM
Rocuronium	6.59±0.25	5.43±0.24*	64nM

* $p<0.05$ significant difference from Meth – NMBD.

5.4.2. $[Ca^{2+}]_i$ measurements.

Basal intracellular calcium concentrations in CHOm3 cells ranged from 79-197nM with a mean±SEM of 125±3nM. When methacholine (10μM) was added at 100s, a peak $[Ca^{2+}]_i$ was achieved approximately 10-15s later. Peak intracellular calcium concentrations ranged from257-1400nM with a mean±SEM of 739±40nM with 10μM methacholine. This marked variation in $[Ca^{2+}]_i$ is common for CHO cells but is relatively consistent within a single batch of cells. Hence paired data are analysed. When pretreated for 5 minutes with a particular NMBD or gallamine, $[Ca^{2+}]_i$ ranged from 85-406nM for basal values and 257-1546nM for peak values when challenged

with 10μM methacholine. The results are summarised in table 5.2 and shown in figs 5.4-5.8.

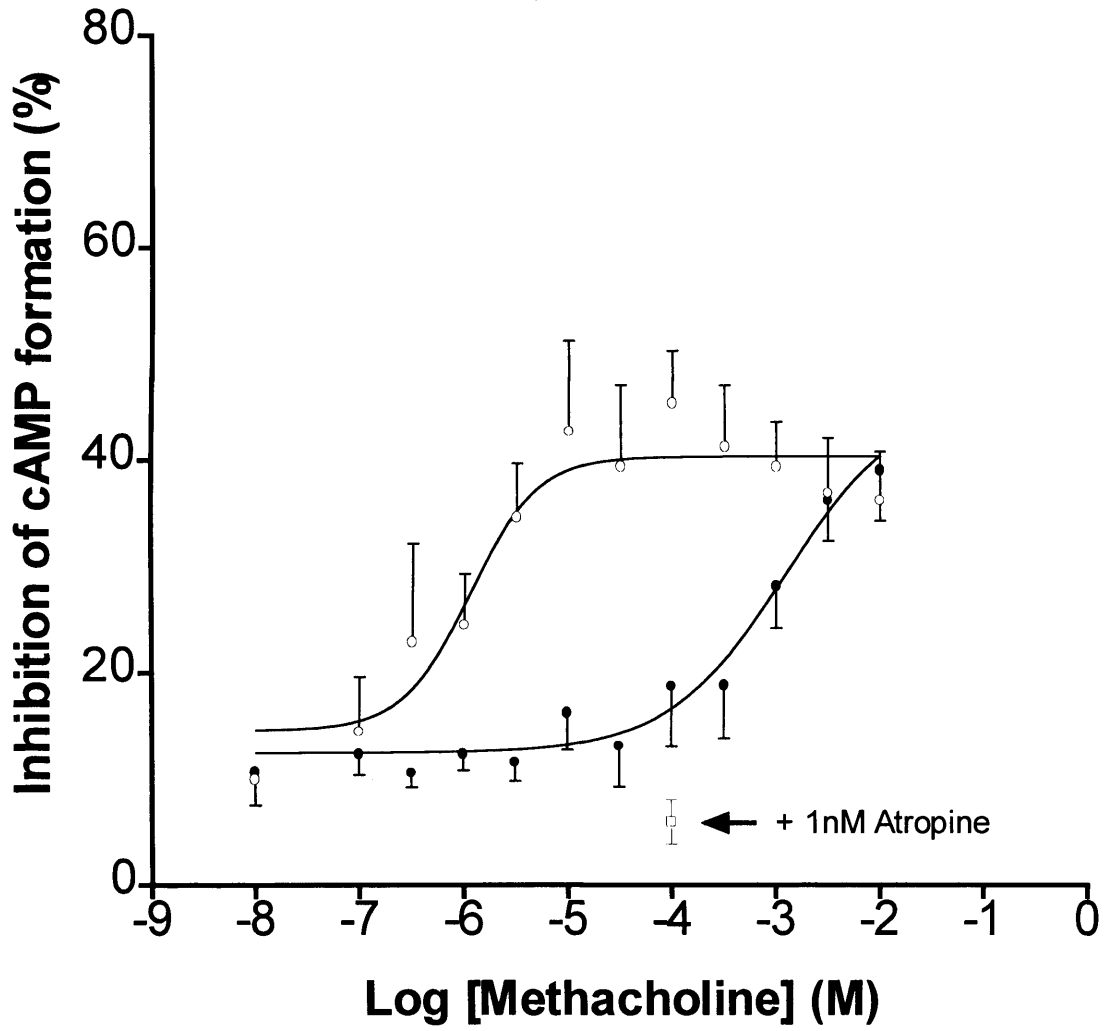


Figure 5.1. Reversal of methacholine inhibition in CHOm2 cells by pancuronium (300nM). Open circles depict methacholine only. Closed circles depict methacholine and pancuronium. Atropine is noted as an internal positive control.

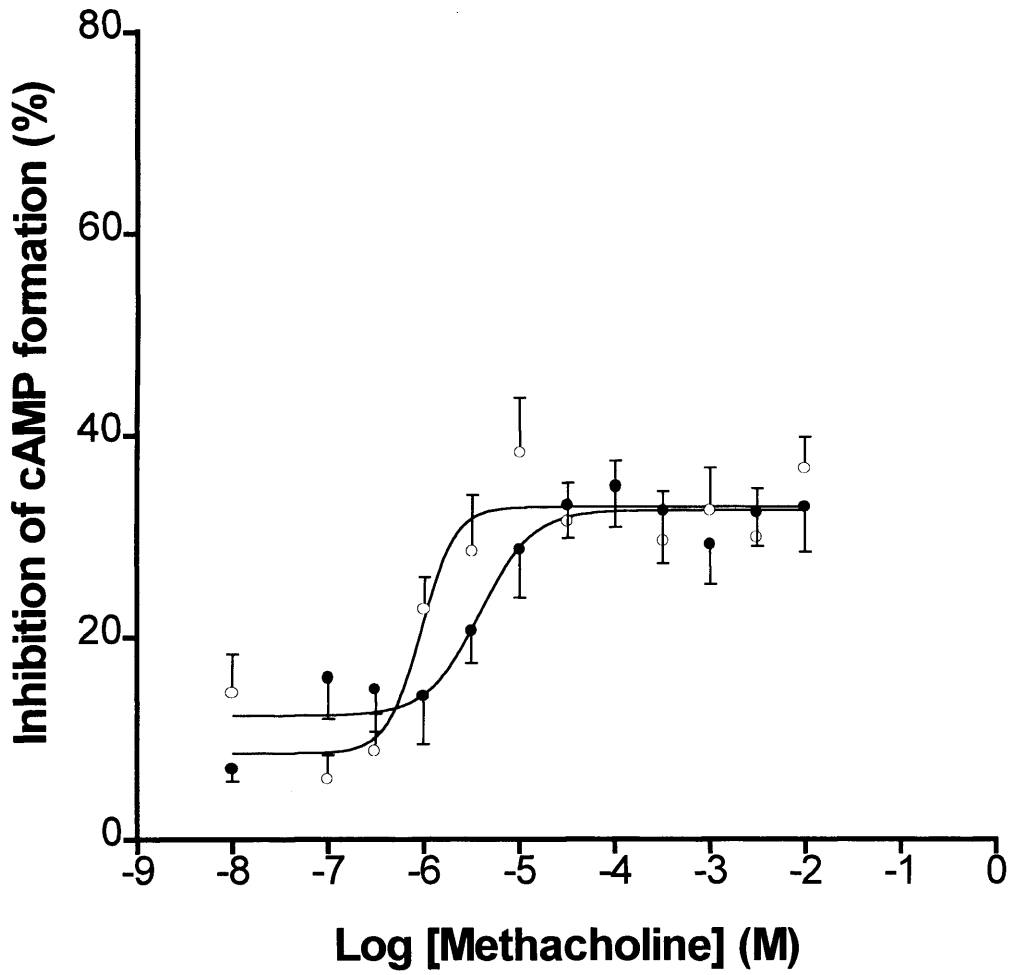


Figure 5.2. Reversal of methacholine inhibition in CHOm2 cells by vecuronium (1μM). Open circles depict methacholine only. Closed circles depicts methacholine and vecuronium.

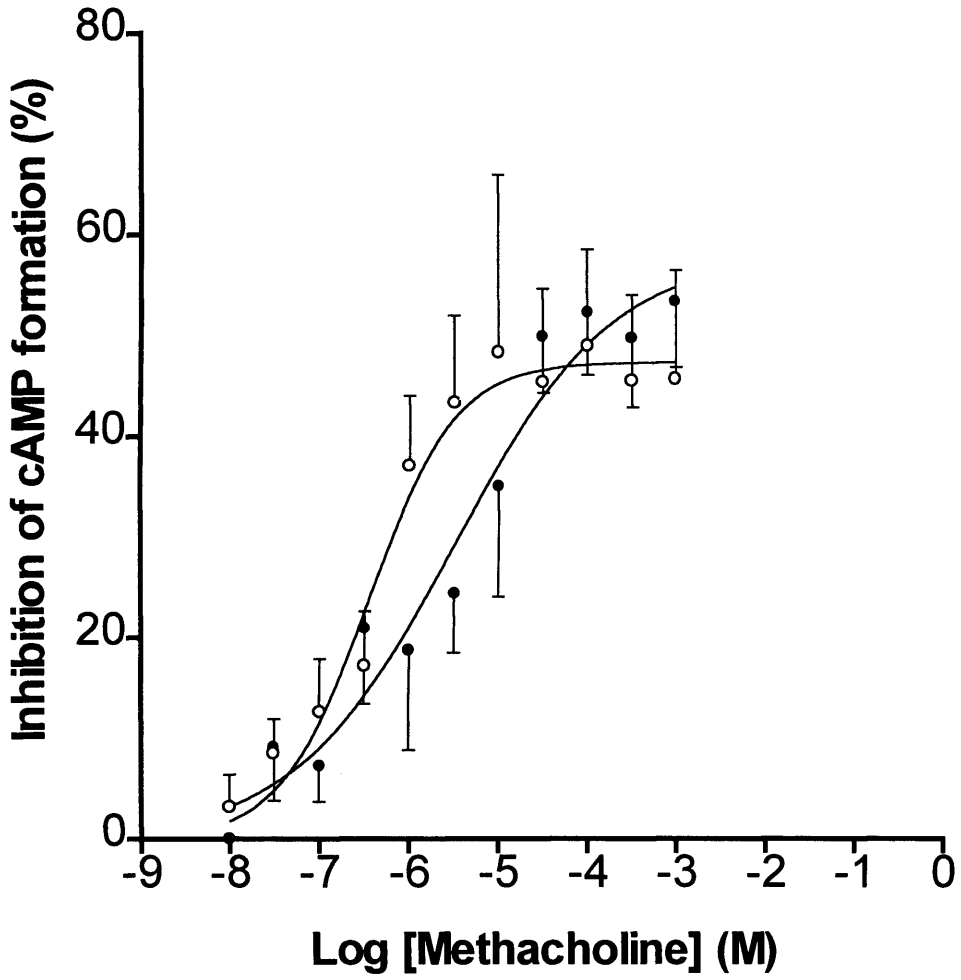


Figure 5.3. Reversal of methacholine inhibition in CHOm2 cells by rocuronium (1μM). Open circles depict methacholine only. Closed circles depict methacholine and rocuronium.

Table 5.2. Basal, Peak and changes in intracellular calcium concentrations in CHOm3 cells.

	Basal $[Ca^{2+}]_i$ (nM)	Peak $[Ca^{2+}]_i$ (nM)	Δ $[Ca^{2+}]_i$ (nM) (Peak-Basal)	$Peak_{NMBD}$ c.f. Peak $_{METH}$ (paired t-test)
Methacholine 10μM (n=55)	79-197 125±3	257-1400 739±40	614	
Gallamine 300nM (n=15)	102-406 151±22	623-1546 842±57	691	P=0.56 ns
Pancuronium 300nM (n=11)	87-201 120±10	278-386 329±12	209	P=0.24 ns
Rocuronium* 100μM (n=15)	95-131 112±2	463-1043 758±39	646	P=0.01 sig.
Vecuronium 100μM (n=10)	85-202 125±10	317-1153 652±78	527	P=0.56 ns
Vecuronium 10μM (n=10)	85-202 125±10	396-987 681±52	556	P=0.74 ns
Vecuronium 1μM (n=11)	85-202 125±10	358-918 636±51	511	P=0.39 ns

Methacholine peak and basal $[Ca^{2+}]_i$ are obtained from all 55 experiments. Each peak value obtained with the use of methacholine + NMBD was compared with the paired value obtained with methacholine alone for that experiment.

Only rocuronium significantly inhibited the methacholine response. All other NMBDs were ineffective at the M3 receptor. Figs 5.9 and 5.10 show that rocuronium, vecuronium and pancuronium alone were also ineffective on $[Ca^{2+}]_i$.

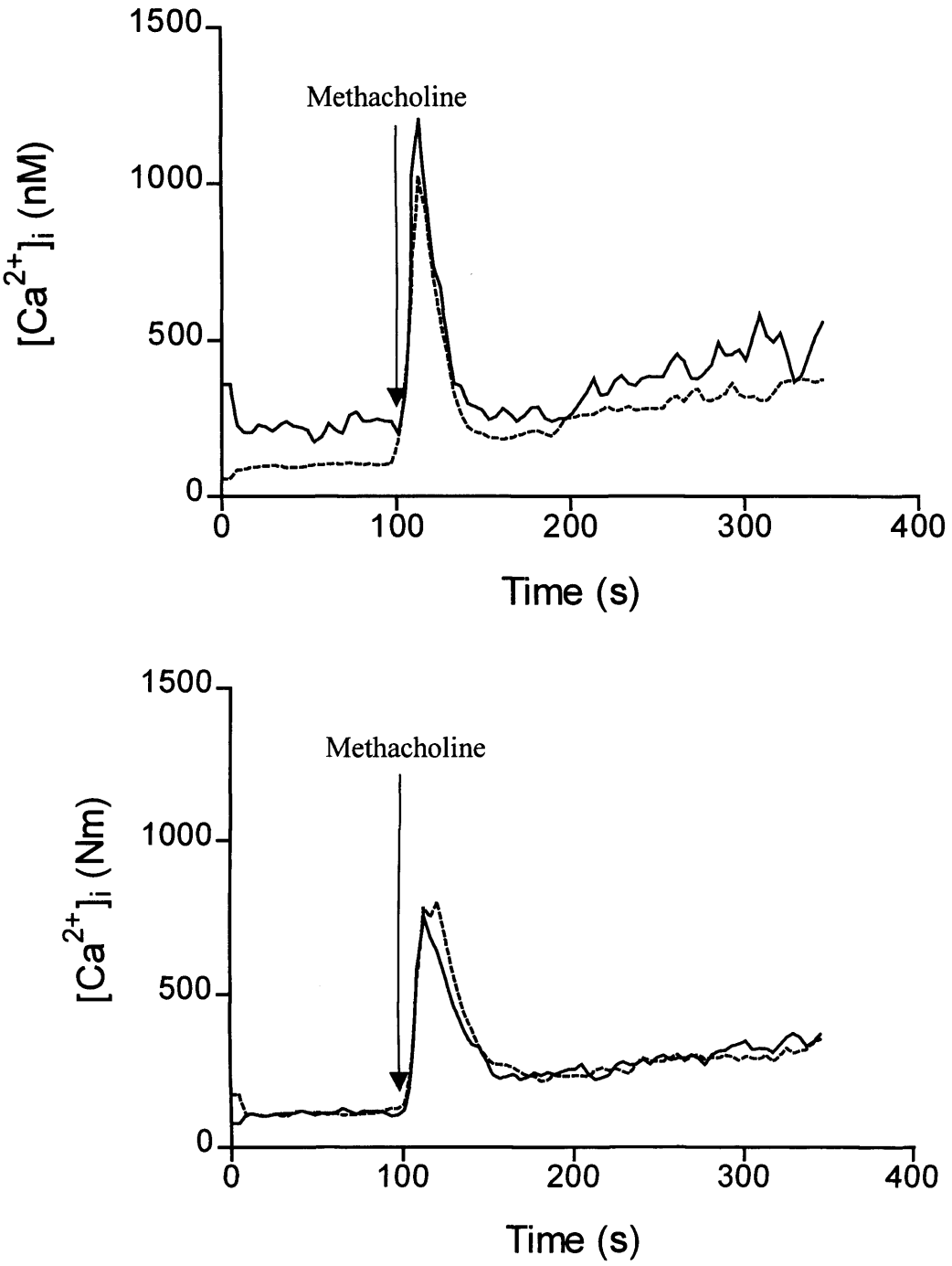


Figure 5.4. These graphs represent typical $[Ca^{2+}]_i$ in CHOm3 cells. Methacholine (10μM) was used to evoke calcium release form internal stores. Peak values ranged from 257-1546nM as summarised in table 5.2. In each panel duplicate traces are shown.

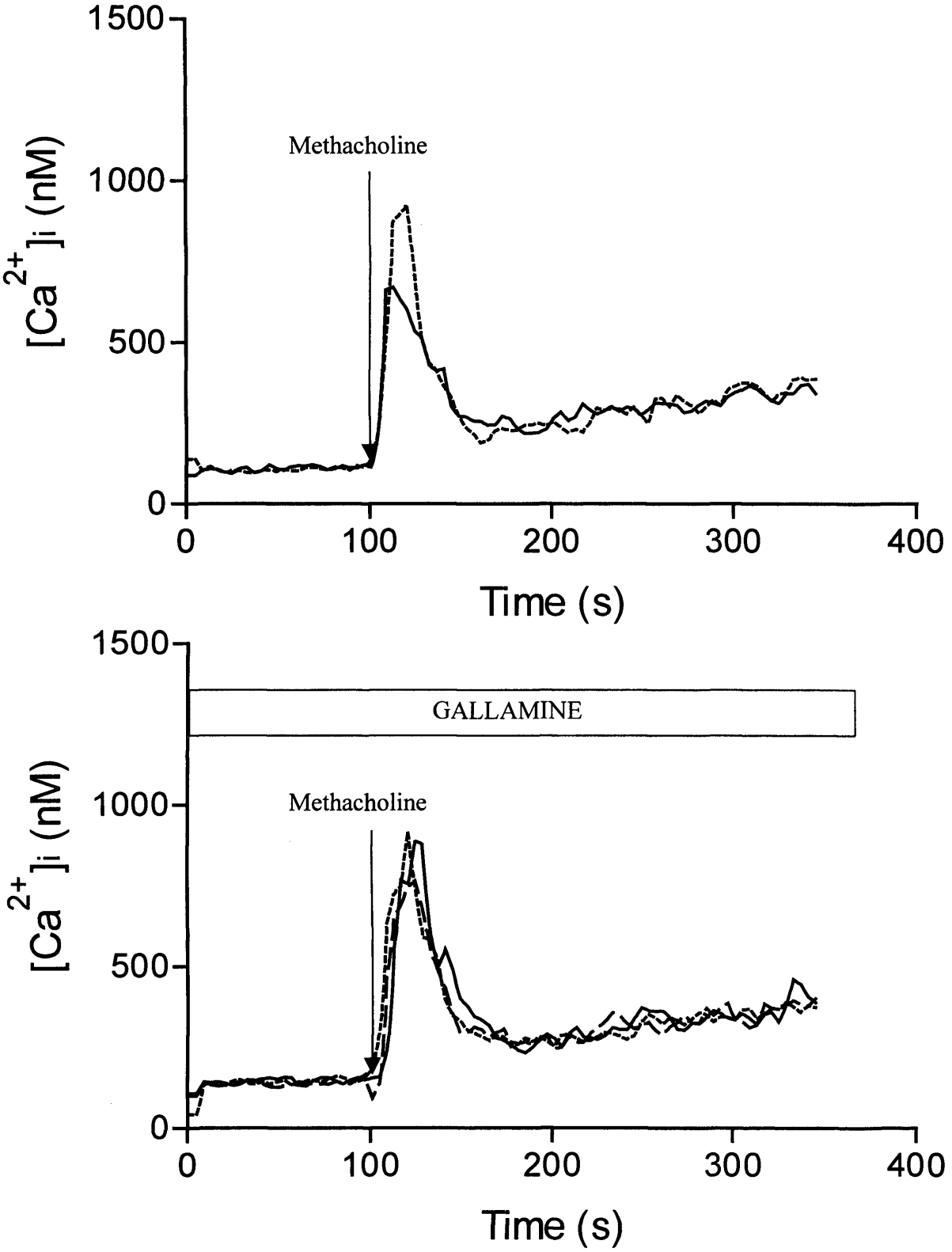


Figure 5.5. Typical $[Ca^{2+}]_i$ traces with methacholine (10μM) (top) and gallamine (300nM) (bottom) yielding peak values ranging between 653-1546nM and 623-1546nM for methacholine and gallamine respectively. The data is summarised in table 5.2. In the upper panel duplicate traces shown. In the lower panel triplicate traces are shown.

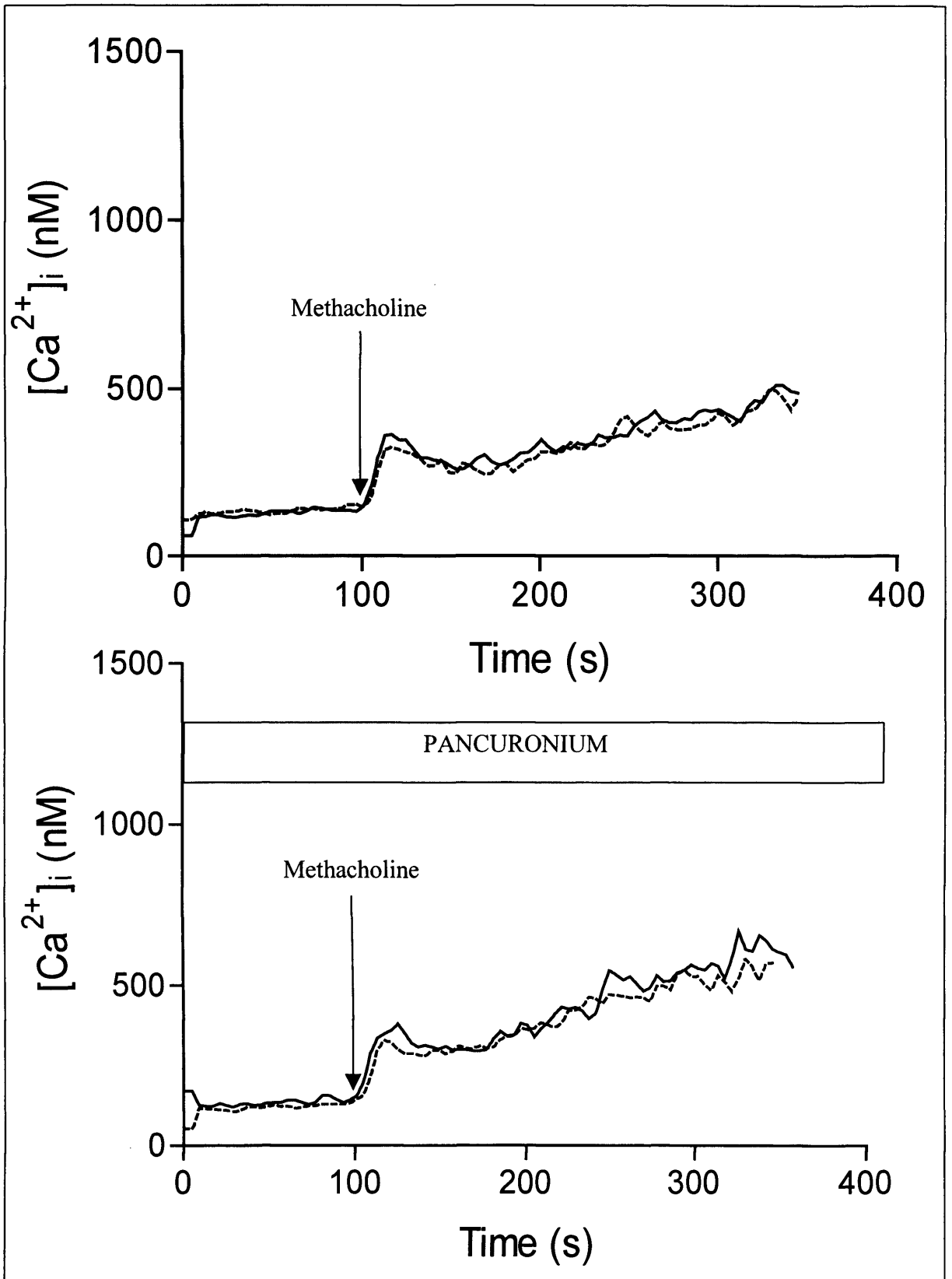


Figure 5.6. Typical $[Ca^{2+}]_i$ traces with methacholine (10μM) (top) and pancuronium (300nM) (bottom)yielding peak values ranging between 257-633nM and 278-386nM for methacholine and pancuronium respectively. The data is summarised in table 5.2. Duplicate traces are shown in both panels

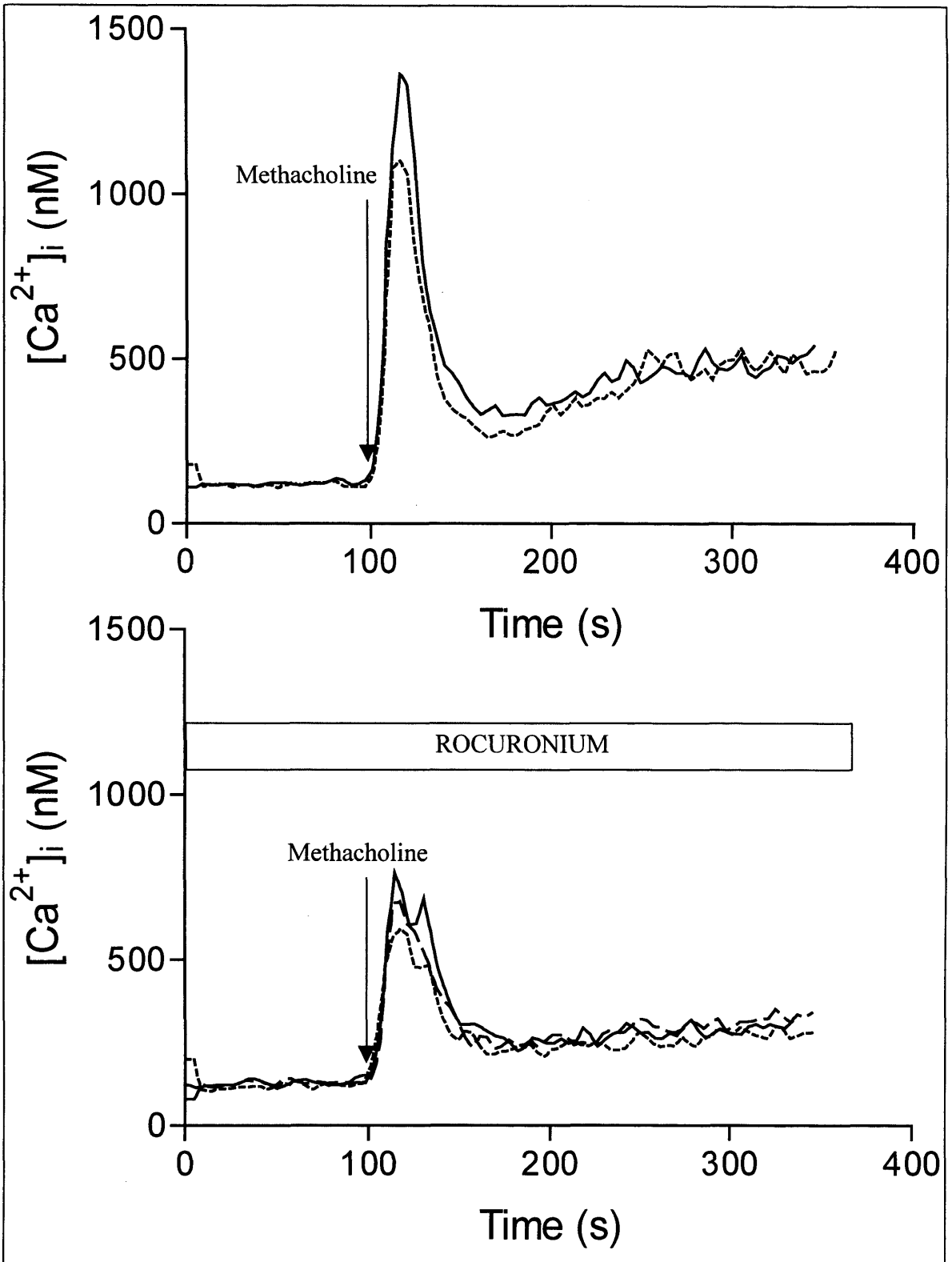


Figure 5.7. Typical $[Ca^{2+}]_i$ traces with methacholine (10μM) (top) and rocuronium (100μM) (bottom) yielding peak values ranging between 662-1400nM and 463-1043nM for methacholine and rocuronium respectively. The data is summarised in table 5.2.
In the upper graph duplicate traces shown and in the lower, triplicate traces shown.

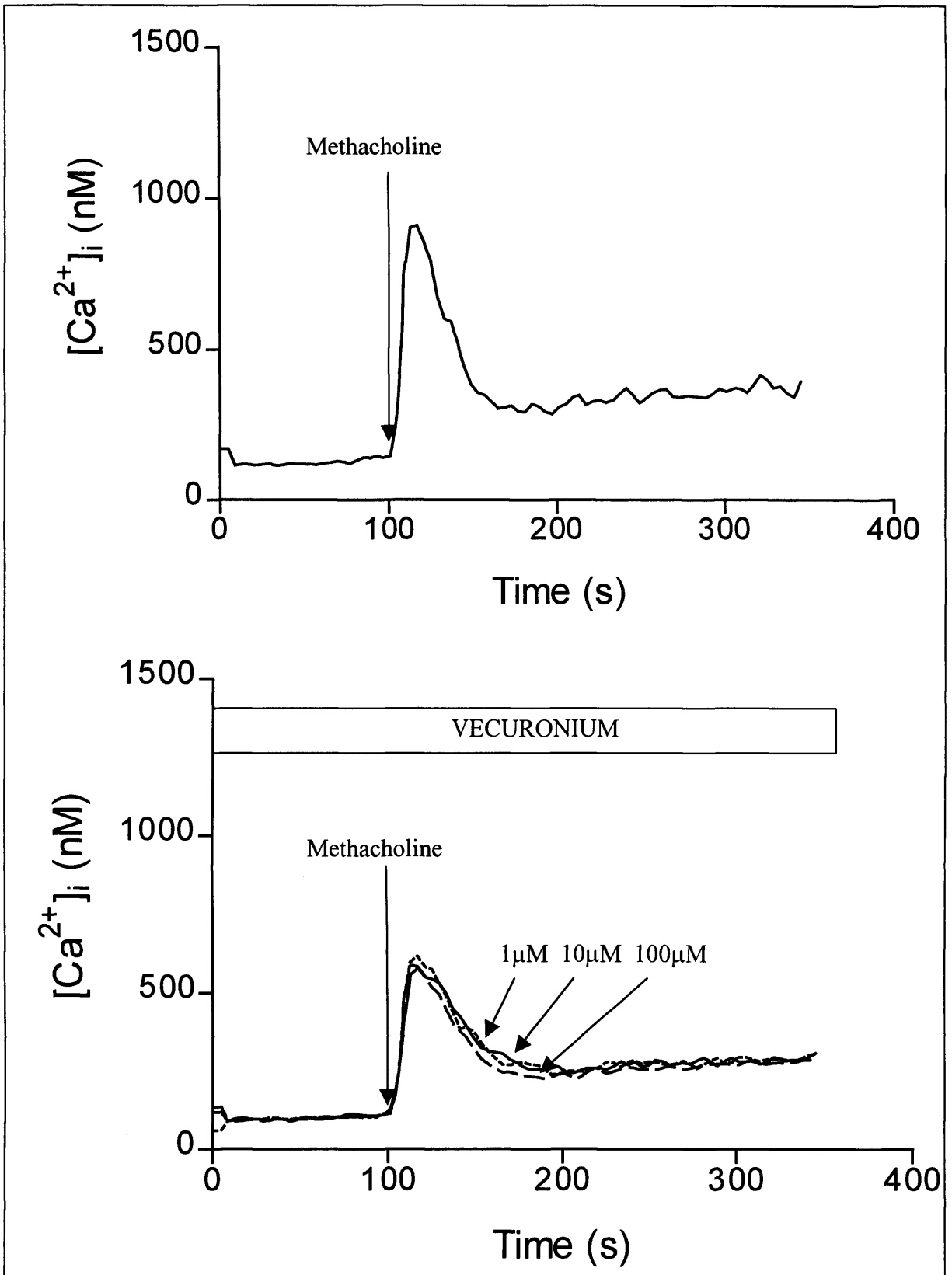


Figure 5.8. Typical $[Ca^{2+}]_i$ traces for methacholine (10μM) (top) and vecuronium (100, 10, 1μM) (bottom) yielding peak values ranging from 342-1337nM and 317-1153nM for methacholine and vecuronium respectively. The data is summarised in table 5.2.

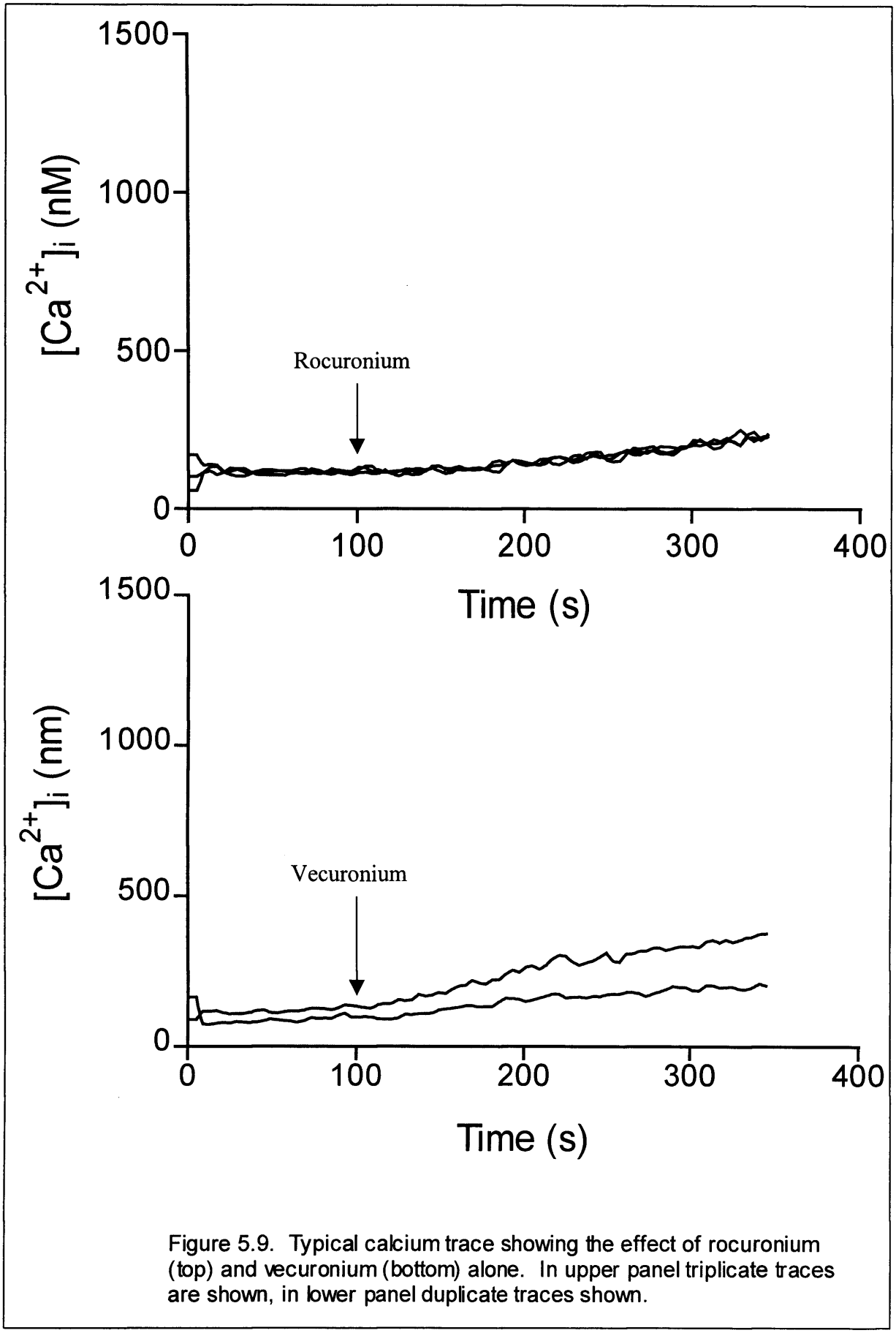


Figure 5.9. Typical calcium trace showing the effect of rocuronium (top) and vecuronium (bottom) alone. In upper panel triplicate traces are shown, in lower panel duplicate traces shown.

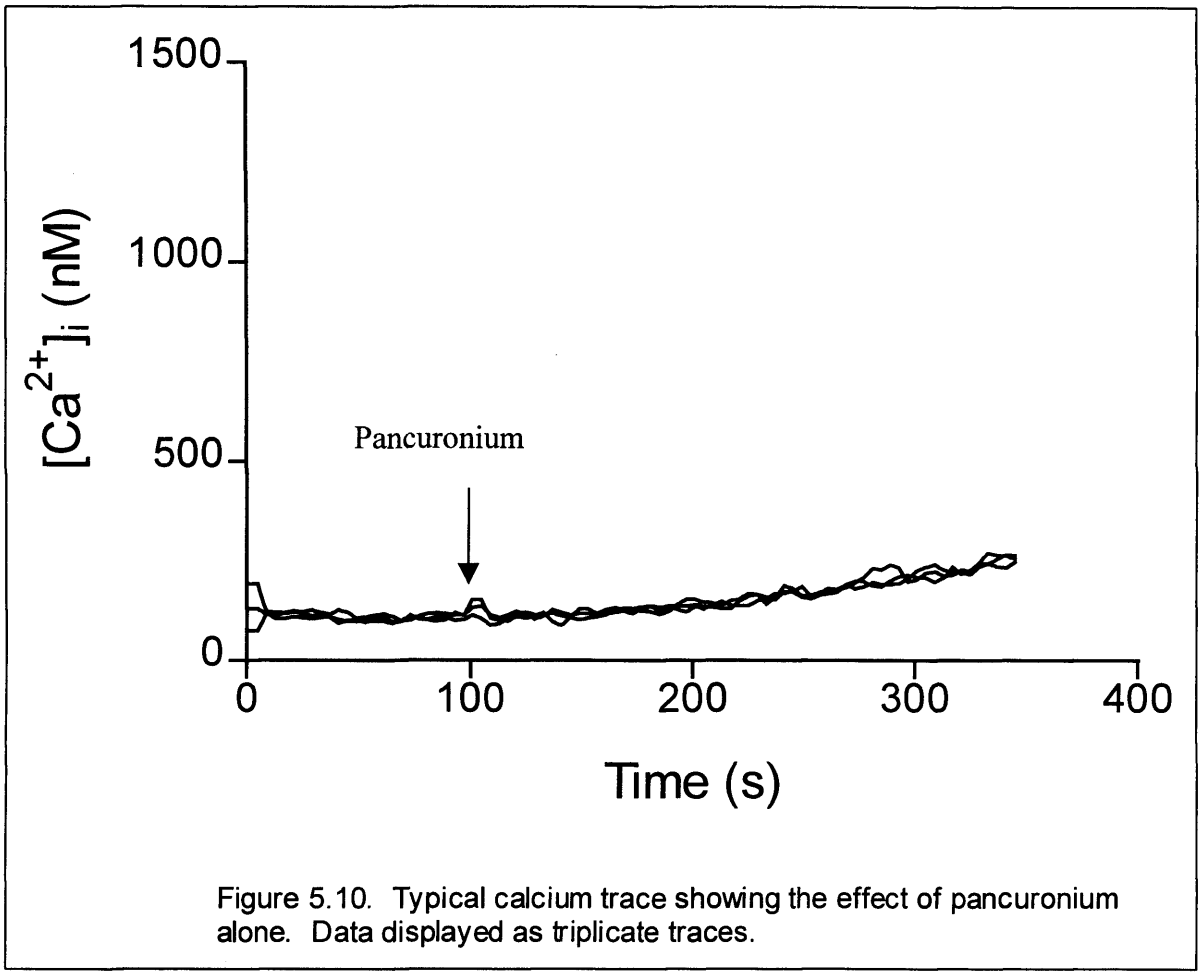


Figure 5.10. Typical calcium trace showing the effect of pancuronium alone. Data displayed as triplicate traces.

5.5. Discussion.

The data presented in the chapter has conclusively demonstrated an antagonistic profile for pancuronium. This is based on the parallel rightward shift in the concentration response curve for methacholine on cAMP inhibition. There was a small but significant shift in the concentration response curve to vecuronium and rocuronium. Pancuronium, vecuronium and gallamine do not affect basal of methacholine stimulated increase in $[Ca^{2+}]_i$ in CHOm3 cells. Rocuronium was shown to have a very weak antagonist action at M3 receptors.

5.5.1 cAMP formation

Pancuronium has been associated with tachycardia when used clinically (Lee Son 1981). Increases in heart rate are associated with an increase in cAMP levels (Weishaar 1988). Antagonist reversal of agonist interactions with the M2 muscarinic receptor may lead to an increase in cAMP. Therefore any tachycardia experienced in patients who have received pancuronium for muscle relaxation may be caused by the reversal of ACh dependent vagal tone on a whole system basis and via elevated cAMP levels at the cellular level.

Vecuronium produces a bradycardia when used clinically (Stevens et al, 1997) and could reasonably be expected to exert muscarinic agonist activity. This would be manifest in the test tube as a direct inhibition of cAMP formation (i.e., a methacholine like effect). Indeed, muscarinic agonists applied to M2 muscarinic receptors expressed in guinea pig atria produce a bradycardia (Caulfield 1993). In this study a direct (agonist) action of vecuronium was not observed.

Rocuronium produces a tachycardia when used clinically (Stevens et al 1997) and as noted above this would be consistent with an antimuscarinic action. In this study rocuronium produced a small but significant shift in the concentration response curve to methacholine, i.e., rocuronium was displaying weak antagonist effects at the M2 muscarinic receptor.

5.5.2 Intracellular Ca^{2+} measurements

There is a large variation of Ca^{2+} responses to methacholine in the CHOm3 cells between each group of drugs tested, however the results are consistent within each batch and paired statistics were used in all comparisons. This type of response variability with CHO cells in common in our laboratory.

The $[Ca^{2+}]_i$ responses reported in this chapter do not appear to be returning to basal levels due of fura2 'leaking' from the cell. This leakage possibly due to an efflux pump, P-glycoprotein (Brezden, et al., 1994). The pump may be responsible for expelling fura-2 from the cell and therefore increasing baseline measurements by virtue of exposure to saturating Ca^{2+} concentrations in the incubation medium. The fluorimetry protocol used in this study does not discriminate between intra- and extra-cellular Ca^{2+}. Probenecid, an organic anion transport inhibitor, has been used to reduce fura-2 leakage (Hirst, et al., 1999). However, probenecid was not used in these experiments due to possible interference with agonist responses (Harrison, personal communication, 1999, Hirst et al, 1999).

The lack of effect by the NMBDs at the M3 receptor suggests that their functional activity is restricted solely to the M2 muscarinic receptor. This is interesting in that these agents were shown in Chapter 4 to have affinity for the M3 receptor.

Chapter 6 examines any possible allosteric effects of NMBDs at the M2 muscarinic receptor.

CHAPTER 6

The effect of pancuronium, vecuronium and gallamine on atropine induced dissociation of [^{3}H]-NMS from recombinant human M2 muscarinic receptors expressed in CHO cells

6.1. Introduction.

The binding of steroidal NMBDs to M1-M5 receptors has been characterised in chapter 4 using displacement protocols. The nature of the interaction between the NMBD and the M2 receptor has not been fully explored. In this chapter allosterism is considered further. Allosterism in pharmacology was defined by Monod et al in the 1960's. According to Monod et al (1965), 'at least two conformational states are accessible to allosteric oligomers', and 'as a result, the affinity of one (or several) of the sites towards the corresponding ligand is altered when a transition occurs from one to the other state'.

Ligand binding to all muscarinic receptors can be modified in an allosteric manner but this phenomena has not been extensively examined using steroidal NMBDs. Allosteric interactions have been described using other neuromuscular blocking drugs such as alcuronium and gallamine (Tucek & Proska 1995, Ellis et al, 1991). Jakubik et al (1996), showed that alcuronium has a rank order of affinity for each of the muscarinic receptors expressed in CHO cells in the order M2>M4=M3>M1>M5. Positive allosteric effects of alcuronium were only shown at the M2 and M4 receptors (Jakubik et al 1996). The orthosteric binding site is located in a depression created by the 7 trans-membrane domains (see figure 6.1). Two binding sites for the M2 receptor have been suggested (Ellis, et al, 1992). The orthosteric binding site in the muscarinic receptor is the normal competitive binding site for ligands. A suggested position for the allosteric site locates it near to the orthosteric site, but at an extracellular location as compared to the orthosteric site (Proska, et al 1993, Tucek & Proska et al 1995). This positioning would make it an appropriate site for manipulation of the binding of ligands to the orthosteric site.

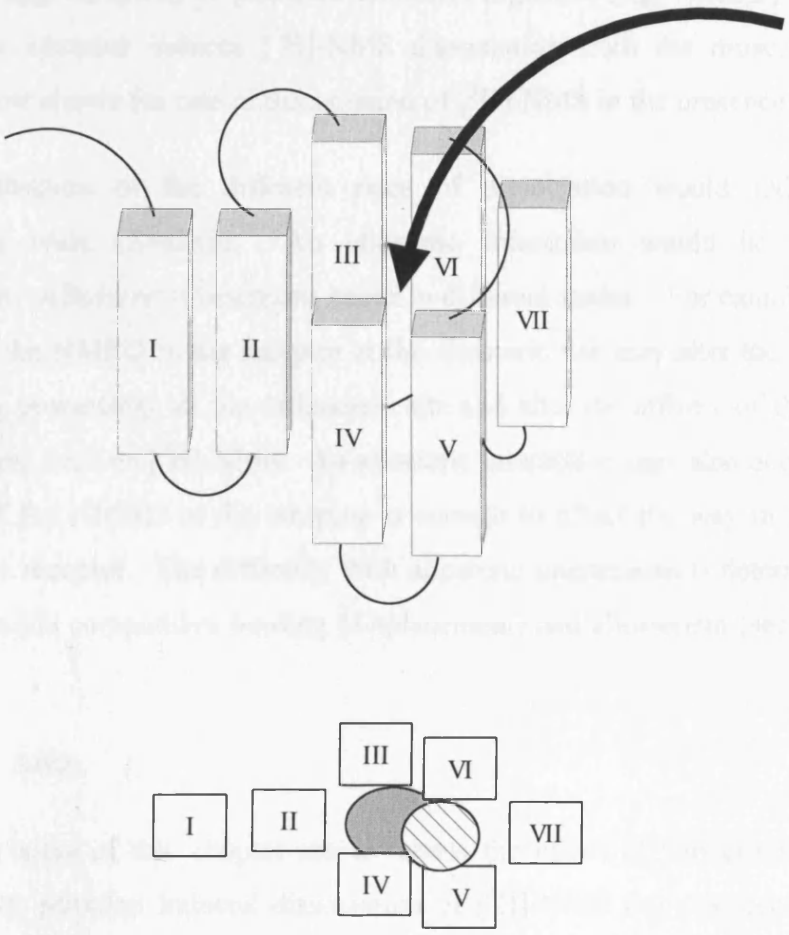


Figure 6.1. Schematic (top) and 'bird's eye view' (bottom) of a muscarinic receptor. The arrow in the top diagram shows where in the muscarinic receptor that the binding sites are likely to be found.
The bottom diagram shows the relative positions in the receptor of each of the binding sites. The allosteric binding site (lined) is positioned slightly nearer the extracellular surface of the receptor than the orthosteric site (grey), which is positioned further inside the receptor.

Direct measurement of allosteric interactions is difficult. Indirect assessment of allosteric interactions is more commonly performed using radioligand dissociation studies. Figure 6.2 displays a typical result expected from an allosteric interaction. The

control curve shows the radiolabel binding to a muscarinic receptor without the influence of an antagonist (e.g. atropine) or potential allosteric regulator (e.g. NMBD). The atropine curve shows how atropine induces [^{3}H]-NMS dissociation from the muscarinic receptor. The NMBD curve shows the rate of dissociation of [^{3}H]-NMS in the presence of NMBD.

Evaluation of the different rates of dissociation would indicate which of the interactions were allosteric. An allosteric interaction would be anticipated to slow dissociation. Allosteric interactions occur in different forms. For example it may be that the binding of the NMBD to the receptor at the allosteric site may alter the shape, (and therefore the binding properties), of the orthosteric site and alter the affinity of the orthosteric site for other ligands, such as [^{3}H]-NMS. An allosteric interaction may also occur when the physical presence of the NMBD or the atropine is enough to affect the way in which the [^{3}H]-NMS binds to the receptor. The difficulty with allosteric interactions is determining the difference between simple competitive binding (displacement) and allosterism (see general conclusions, chapter 8).

6.2 Aims.

The aims of this chapter are to assess the effect of pancuronium, vecuronium and gallamine on atropine induced dissociation of [^{3}H]-NMS from the recombinant human M2 muscarinic receptor. Gallamine has already been shown to be an allosteric modulator and is therefore used as a positive control in this chapter. Pancuronium has been shown to be an antagonist at the M2 receptor (chapter 5) and may also display allosteric properties. Vecuronium binds to the M2 receptor but as yet has an undetermined action.

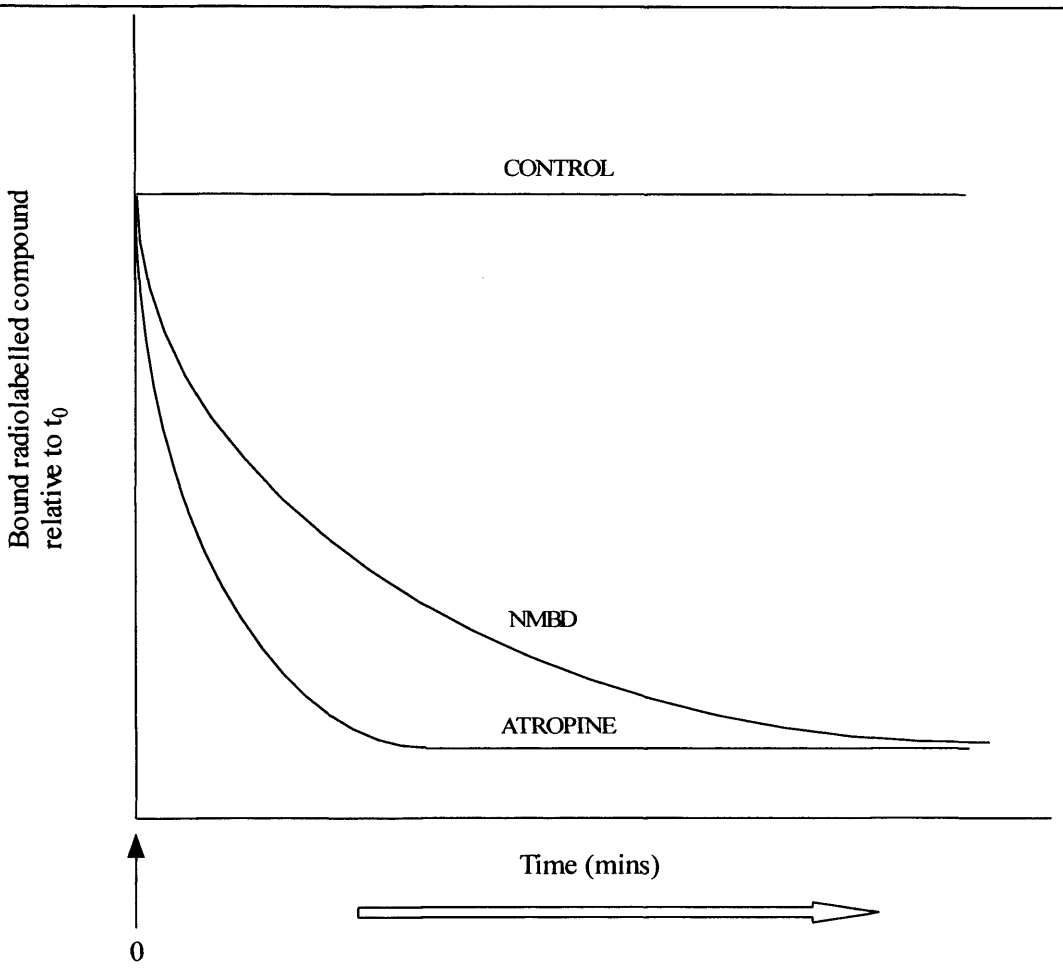


Figure 6.2. Illustration to show the allosteric effects of NMBDs on the dissociation of [^{3}H]-NMS from a theoretical muscarinic receptor. The CONTROL curve shows [^{3}H]-NMS binding to theoretical muscarinic receptors without the influence of allosteric agents. The NMBD curve shows atropine induced dissociation of [^{3}H]-NMS is affected by steroidal neuromuscular blocking drugs. The ATROPINE curve shows atropine induced dissociation of [^{3}H]-NMS without any NMBDs.

6.3. Method.

The reader is directed to section 2.6.3 for a comprehensive description of the method used to assess allosteric binding.

6.4. Results.

The binding of [^{3}H]-NMS to the M2 muscarinic receptor was time dependent reaching equilibrium at 45 minutes (see fig 6.3). Incubations were therefore performed for 60 minutes. Addition of atropine (10μM) produced a time dependent rapid dissociation of [^{3}H]-NMS. An apparent plateau (NSB) was reached at 10 minutes. Dissociation rate for atropine (K_{off}) was 0.43±0.04 min^{-1} (n=23), which is in good agreement with the literature, 0.5±0.08 min^{-1} (Proska et al, 1993).

Dissociation of [^{3}H]-NMS induced by atropine was significantly slowed by gallamine (fig.6.4, table 6.2). This is consistent with the literature (Ellis et al, 1989). In addition, there was a concentration dependent slowing of atropine induced dissociation by pancuronium (21 and 11nM). These data are illustrated in figure 6.5 and table 6.2. Moreover, vecuronium (125 and 62.5 nM) also slowed atropine induced dissociation of [^{3}H]-NMS from the m2 receptor (fig. 6.6) with K_{off} values shown in table 6.2.

Table 6.2. Gallamine, pancuronium and vecuronium all slowed atropine induced dissociation of [^{3}H]-NMS from the M2 muscarinic receptor with K_{off} values as shown.

Dissociation initiated with	K_{off} (min^{-1})
Atropine (10μM)	0.42±0.05
Atropine + Gallamine (21nM)	0.15±0.04*
Atropine (10μM)	0.53±0.17
Atropine + Pancuronium (21nM)	0.03±0.01*
Atropine (10μM)	0.39±0.12
Atropine + Pancuronium (11nM)	0.22±0.18*
Atropine (10μM)	0.45±0.06
Atropine + Vecuronium (126nM)	0.04±0.01*
Atropine (10μM)	0.35±0.02
Atropine + Vecuronium (62.5nM)	0.06±0.02*

Data are mean ± SEM ($n \geq 5$). ($p<0.001$, atropine + NMBD c.f. atropine).

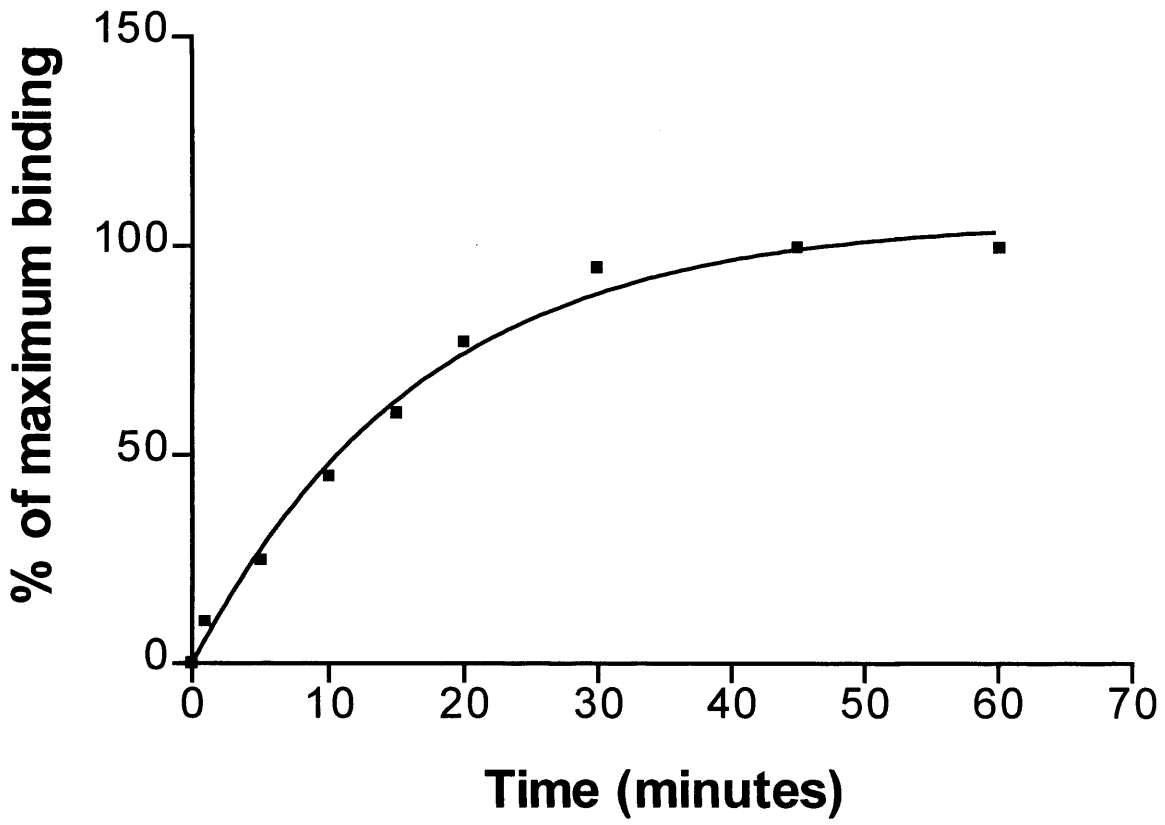


Figure 6.3. Time course (representative of 5 experiments) to show equilibration of binding of [^{3}H]-NMS to M2 muscarinic receptors. Equilibrium was reached at approximately 45 minutes.

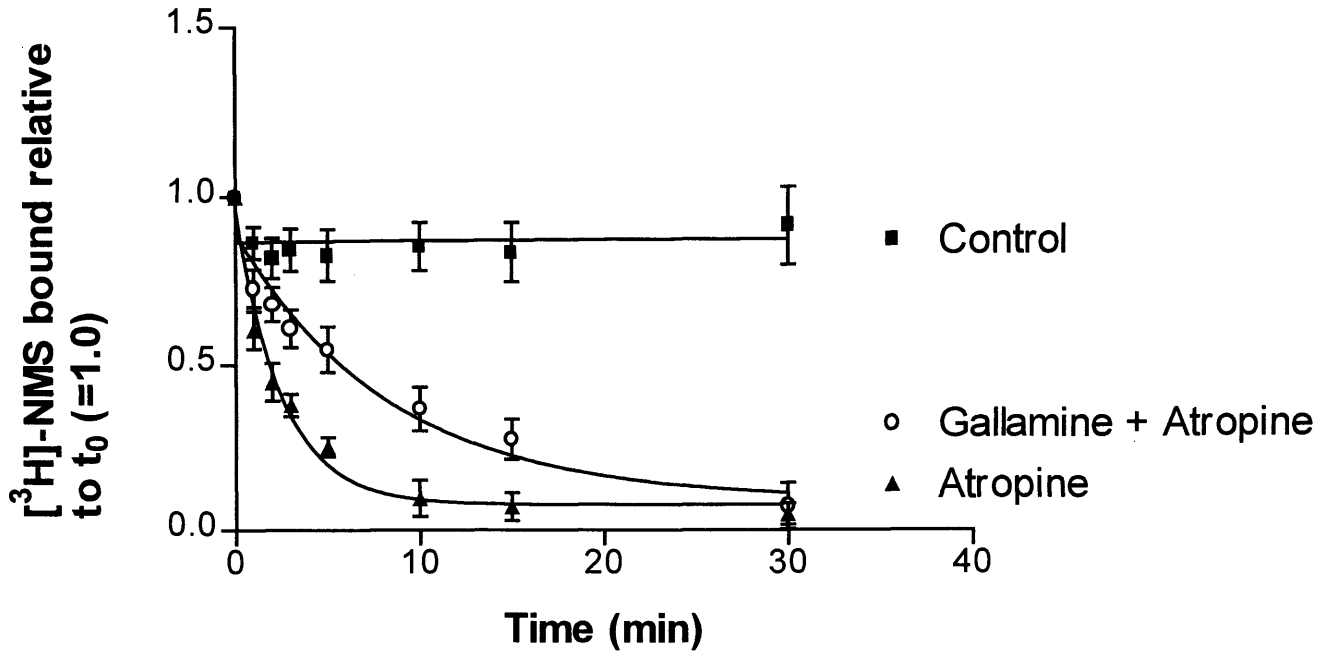


Figure 6.4. Effect of gallamine (21nM) on atropine (10μM) induced dissociation of [^{3}H]-NMS (n=5).

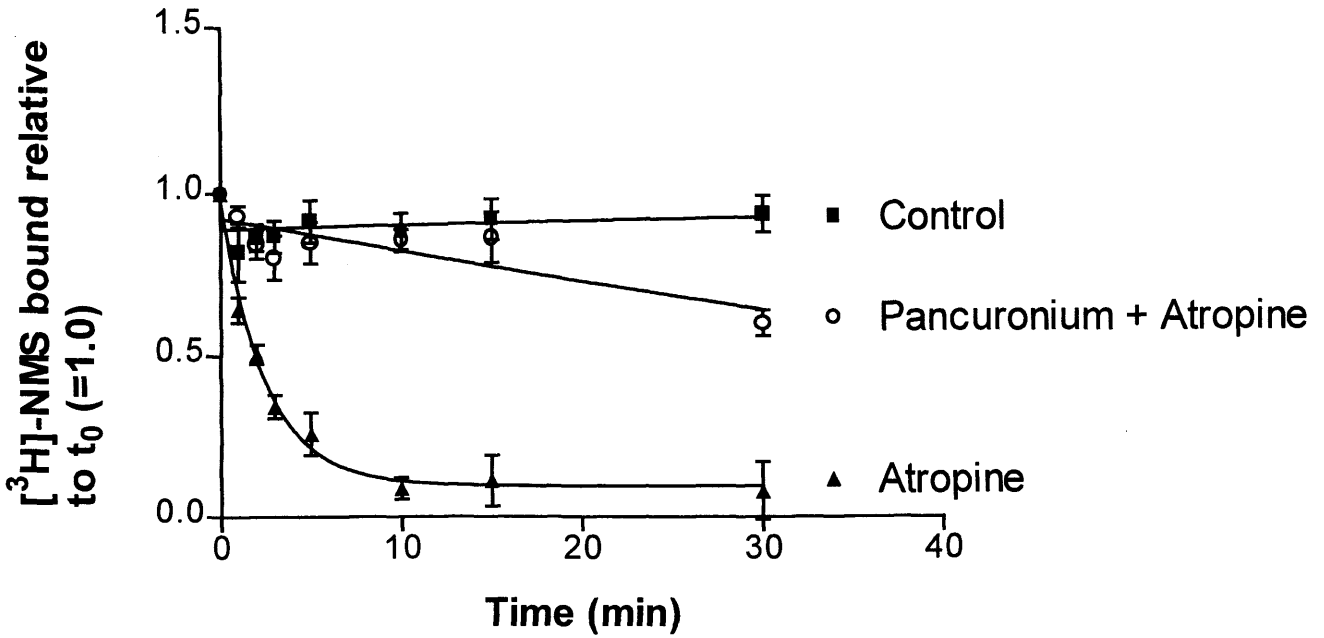


Figure 6.5. Effect of pancuronium (21nM) on atropine (10μM) induced dissociation of [^{3}H]-NMS (n=5).

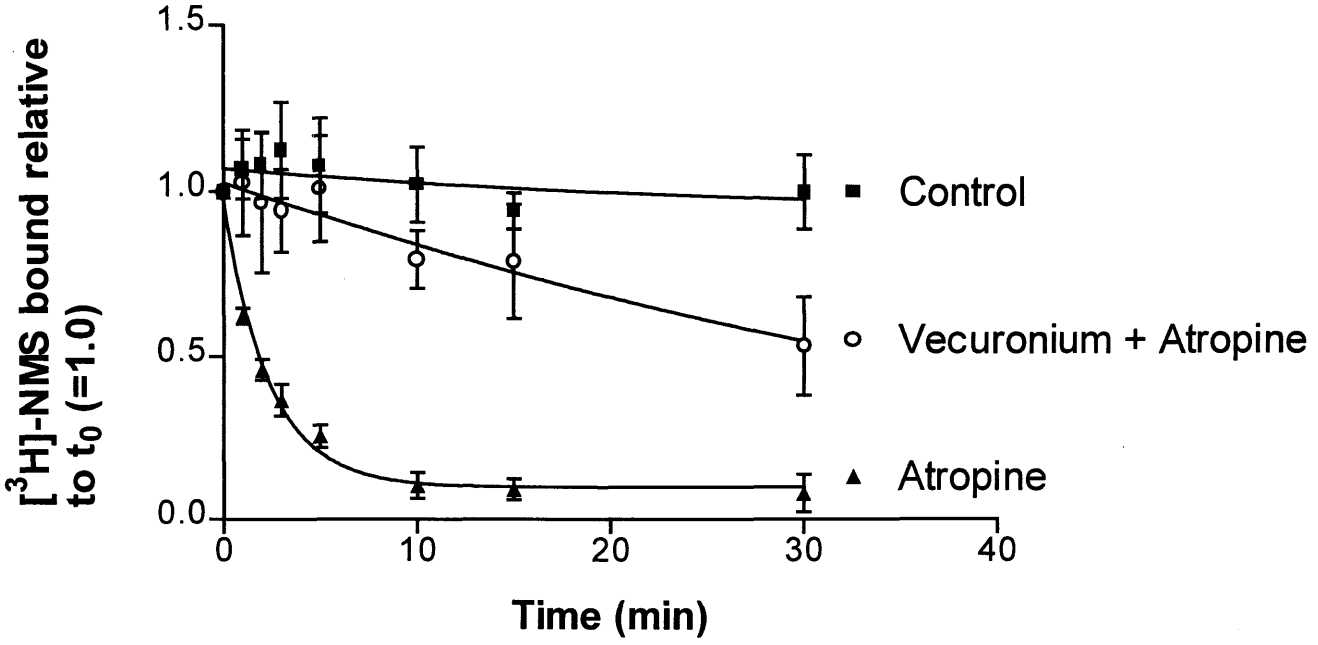


Figure 6.6. Effect of vecuronium (125nM) on atropine (10μM) induced dissociation of [^{3}H]-NMS (n=5).

6.5. Discussion.

The data described in this chapter indicates that gallamine, pancuronium and vecuronium all produce allosteric effects at the recombinant human M2 muscarinic receptor expressed in CHO cells. The good agreement between the experimental atropine induced dissociation rate of [^{3}H]-NMS and the literature value (Proska, et al, 1993) validates the technique as presented. For vecuronium and pancuronium, an increase in NMBD concentration leads to a further dramatic slowing in the dissociation rate of [^{3}H]-NMS from the M2 receptor. This may be due to the NMBD forming a stable ligand/antagonist complex by an allosteric mechanism which competes with the antagonist (atropine) and therefore prevents the [^{3}H]-NMS from dissociating from the receptor (see figure 6.2).

The interaction of NMBDs with the M2 receptor is complex. If the interaction between the antagonist and the NMBD was purely competitive as in chapter 4, then the dissociation rate would not be expected to change. NMBDs display competitive binding properties as well as allosteric binding properties. The NMBDs show competitive binding with the M2 receptor because they can displace [^{3}H]-NMS (chapter 4). NMBDs may also influence the binding of [^{3}H]-NMS to the receptor by binding at another site (the allosteric site) to alter the binding kinetics of [^{3}H]-NMS (slowing dissociation).

Gallamine has already been shown to be an allosteric modulator of binding to muscarinic receptors, and it shows a greater affinity for the M2 muscarinic receptor over all the other M1, M3-M5 muscarinic receptors (Ellis et al, 1991). Gallamine has also been shown to have an effect on cAMP levels in CHO cells expressing M2 muscarinic receptors. Gallamine causes an increase in cAMP levels without classical antagonists and this action is unaffected by agonists (Jakubik 1996). Pertussis toxin treatment of M2 receptors prevents G-protein modulation of agonist binding but does not have any effect on the ability of gallamine to modulate the G-protein in an allosteric manner (Stockton et al, 1983). This indicates that gallamine is able to interact with the allosteric site on the M2 receptor and cause activation of the G- protein without binding to the orthosteric site.

Allosterism is a relatively new phenomenon for the explanation of the mechanism of action of drugs. D-tubocurarine was first used in 1942, but its allosteric effects were only

brought to light in 1988 on the M2 cardiac receptor in rats (Waelbroeck, 1988). The list of drugs that have allosteric actions has grown. Methoctramine has been shown to produce an allosteric effect at the M2 muscarinic receptor (Giraldo, et al 1988), pirenzepine at the M1 muscarinic receptor (Roeske et al, 1984) and verapamil at the M2 and M3 muscarinic receptors (Ellis et al, 1991).

Most of the steroidal NMBDs have a higher or equal affinity for the M2 muscarinic receptor as compared to the M1, M3-M5 receptors (see table 4.1). Allosteric modulation of functional activity by gallamine has shown that M2 receptors can be activated at the allosteric site. These allosteric interactions may occur, in part, due to the cardiac M2 muscarinic receptor having two antagonist binding sites (Prakash, et al 1990). These two sites that are close to each other may also be bridged by one molecule of the NMBD to cause an allosteric effect (Hulme EC, et al 1990). See chapter 8 for further discussion.

CHAPTER 7

The effect of steroidal NMBDs on [^{3}H]-noradrenaline release/uptake from SH-SY5Y human neuroblastoma cells and uptake into 293-hNET cells

7.1. Introduction.

Noradrenaline is released from sympathetic nerve terminals to play an important role in the regulation of heart rate and force of contraction. NAdr is synthesised from L-Tyrosine in the axon terminal (see section 1.18, fig. 1.7; section 1.13, fig. 1.4). Interference with this mechanism by steroidal NMBDs may be a factor that causes a change in cardiovascular activity.

As mentioned previously in section 1.18, pancuronium and vecuronium inhibit the uptake of NAdr back into sympathetic neurons present in whole rat hearts (Salt, et al., 1980). Vecuronium was subsequently shown to have less effect on sympathetic transmission than pancuronium (Docherty, et al, 1980). Pancuronium also potentiates the release of NAdr from guinea-pig atrial sympathetic nerves. This release was caused by antagonism of presynaptic muscarinic receptors (Kobayashi et al., 1987). The effects of other steroidal NMBDs is unknown.

Two simple cellular systems will be examined in this chapter. SH-SY5Y cells (Gift from J Biedler USA) are human sympathetic neurones expressing the human NAdr uptake$_1$ transporter and also capable of releasing NAdr when stimulated. 293-hNET cells (Gift from R.D. Blakely, USA) are human embryonic kidney cells transfected with and expressing the human uptake$_1$ transporter.

7.2. Aims.

These studies were performed;

To investigate any possible direct effects of steroidal NMBDs on the uptake and release of [^{3}H]-NAdr from SH-SY5Y cells on the NAdr uptake$_1$ 293-hNET cells.

Inhibition of uptake or enhanced release would increase NAdr concentrations at the synapse and cause enhanced agonist effects of accumulated NAdr. Opposite effects would cause a reduction in NAdr signalling. Both scenarios could potentially have a significant effect on the heart.

7.3 Methods.

7.3.1. Measurement of [^{3}H]-noradrenaline:uptake.

The reader is directed to section 2.10.1 for a detailed description of the method used to measure [^{3}H]-NAdr uptake in SH-SY5Y and 293-hNET cells.

7.3.2. Measurement of [^{3}H]-noradrenaline:basal release.

The reader is directed to section 2.10.2 for a detailed description of the method used to measure [^{3}H]-NAdr release in SH-SY5Y cells.

7.3.3. Measurement of [^{3}H]-noradrenaline:stimulated release.

The reader is directed to section 2.10.3 for a detailed description of the method used to measure stimulated [^{3}H]-NAdr release in SH-SY5Y cells.

7.4. Results.

7.4.1. Results: uptake.

SH-SY5Y cells: [^{3}H]-noradrenaline uptake was inhibited by imipramine with a pIC_{50} of 7.3±0.05 (This served as internal positive control). Pancuronium, vecuronium and rocuronium produced a small inhibition of reuptake but pIC_{50} values could not be calculated. Pipecuronium had no effect on [^{3}H]-noradrenaline uptake. (Fig. 7.1)

293-hNET cells: [^{3}H]-noradrenaline uptake was inhibited by imipramine with a pIC50 of 6.7±0.2. Pancuronium and vecuronium produced a small inhibition of reuptake but pIC_{50} values could not be calculated. Pipecuronium and rocuronium had no effect on [^{3}H]-noradrenaline uptake. (Fig .7.2).

7.4.2 Results: release.

Table 7.1 shows basal and potassium stimulated [^{3}H]-NAdr release from SH-SY5Y cells. 100mM K^+ produced between 1.94-4.34 fold increases of [^{3}H]-NAdr release over basal levels.

Table 7.1. Mean and range values for basal and K^+ (100mM) evoked release of [^{3}H]-NAdr release from SH-SY5Y cells.

	n	mean±s.e.m	Range	Δ release
Basal	160	2.85±0.08	1.54-7.77	-
K^+	165	8.29±0.16*	4.44-14.42	5.53±0.18

($p<0.001$, K^+ release of [^{3}H]-NAdr c.f. basal release)

Release of [^{3}H]-NA from SH-SY5Y cells expressed as a percentage of total NAdr uptake is shown in figs. 7.3-7.6. NMBD did not affect basal or K^+ evoked [^{3}H]-NAdr release from SH-SY5Y cells. Although there is a wide variation in the release values of [^{3}H]-NAdr, values were consistent for each set of results provided as the experiments were performed in quadruplicate.

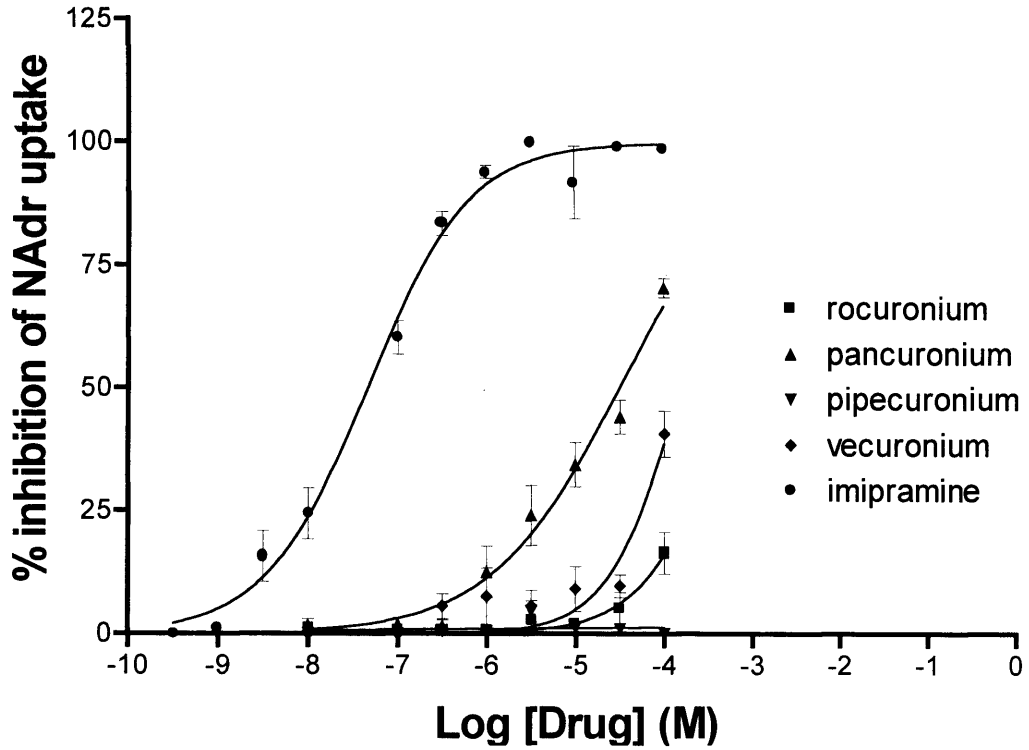


Figure 7.1. Inhibition of [^{3}H]-NAdr uptake into SH-SY5Y by steroidal NMBDs and imipramine (n=5).

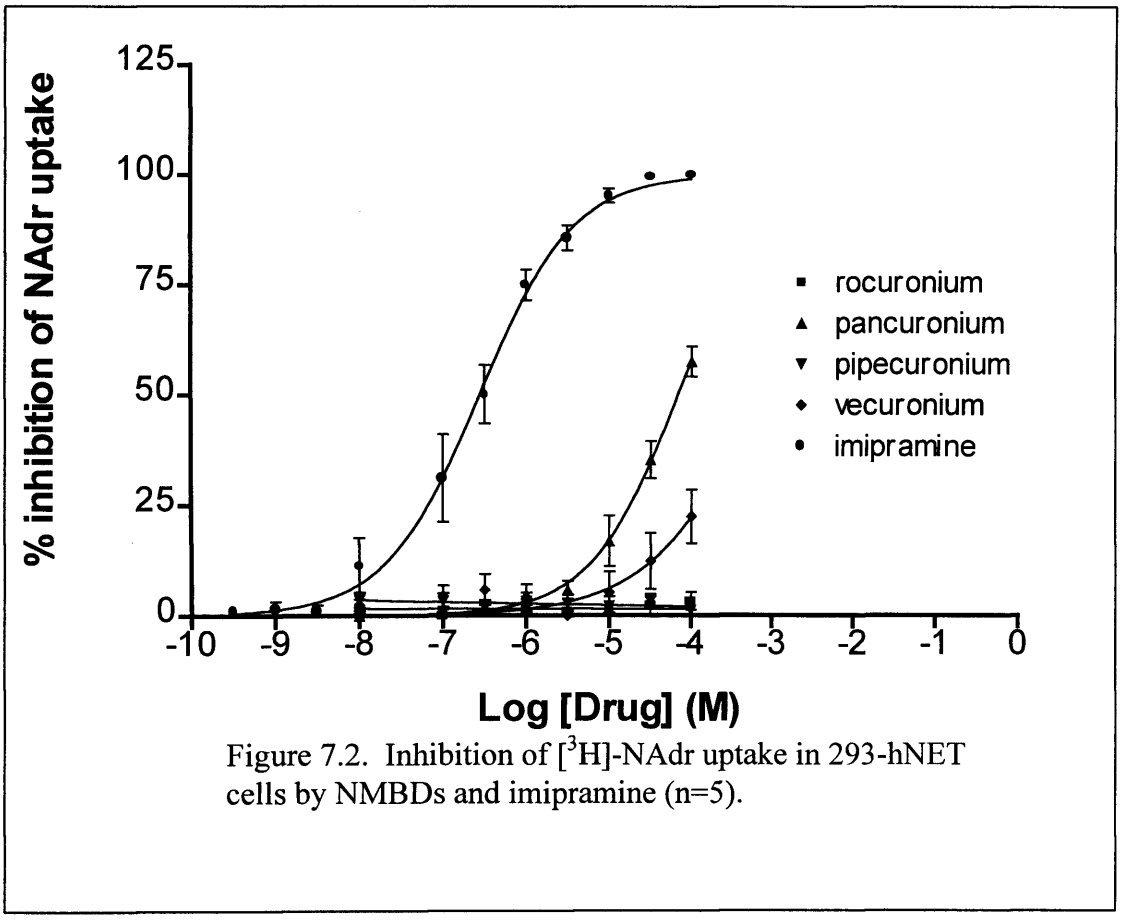


Figure 7.2. Inhibition of [^{3}H]-NAdr uptake in 293-hNET cells by NMBDs and imipramine (n=5).

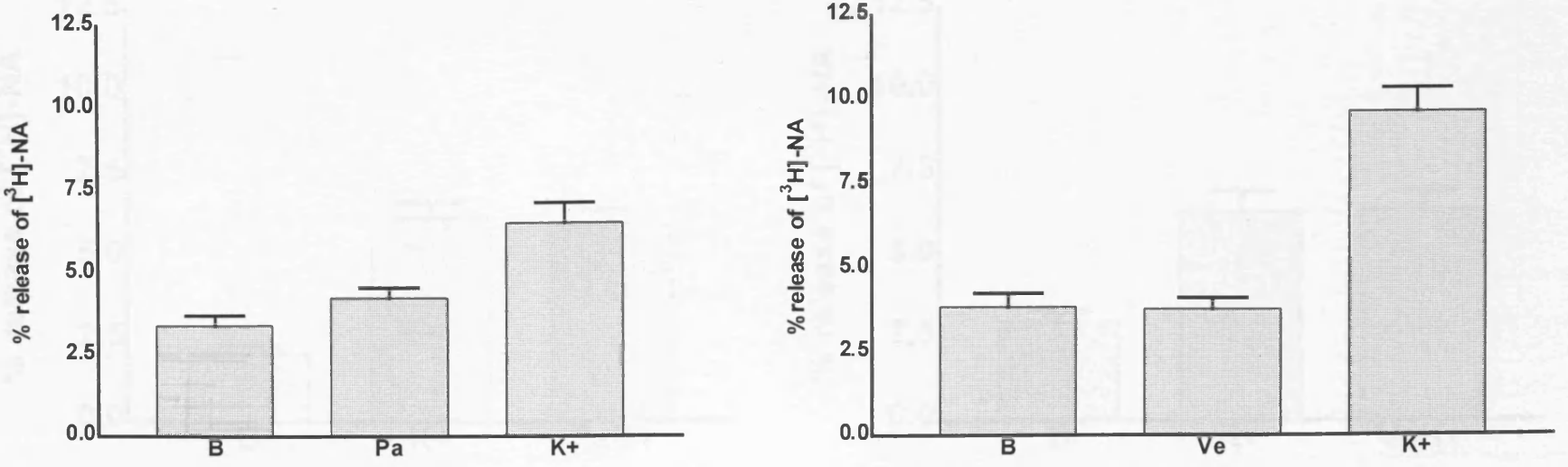


Figure 7.3. Effect of pancuronium (Pa) and vecuronium (Ve) on basal (B) release of [^{3}H]-noradrenaline from SH-SY5Y cells. 100mM K^+ stimulation ensured the cells were responding (n=6).

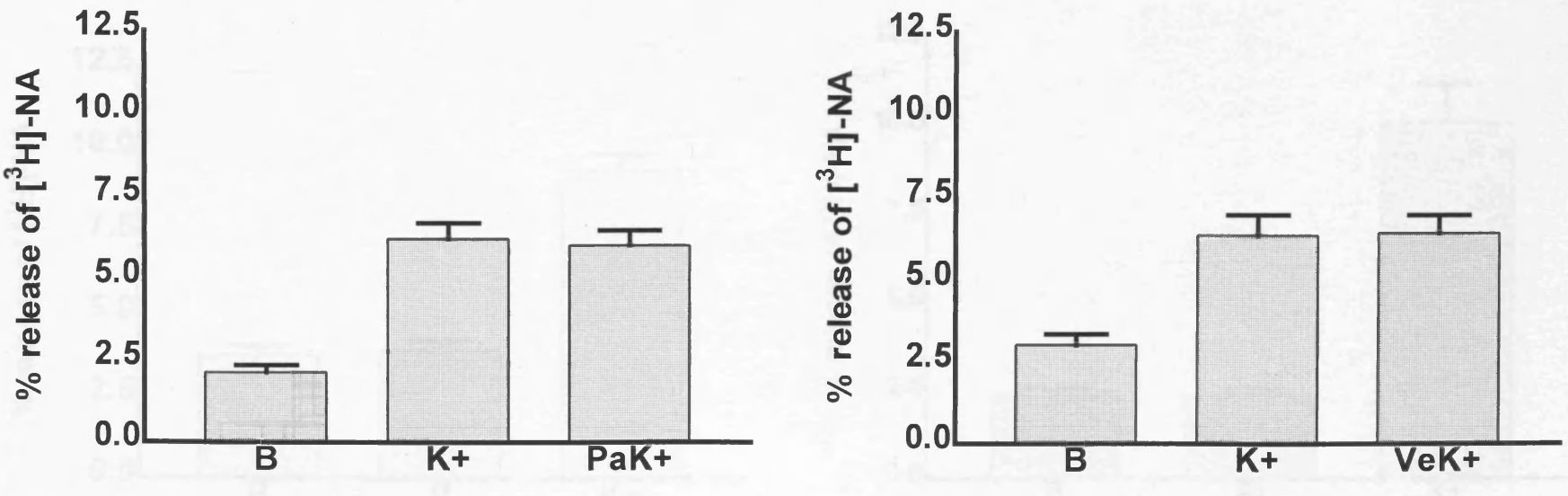
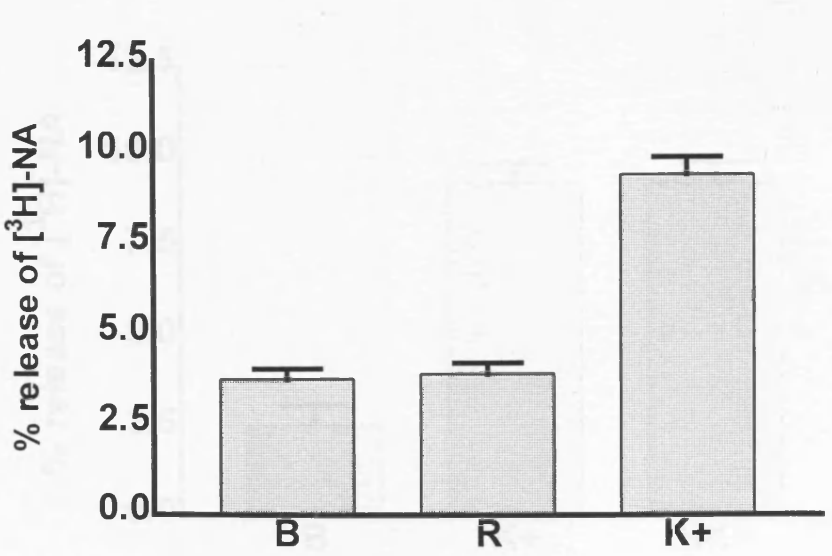


Figure 7.4. Effect of pancuronium (Pa) and vecuronium (Ve) on K^+ evoked release of [^{3}H]-noradrenaline from SH-SY5Y cells (n=6).



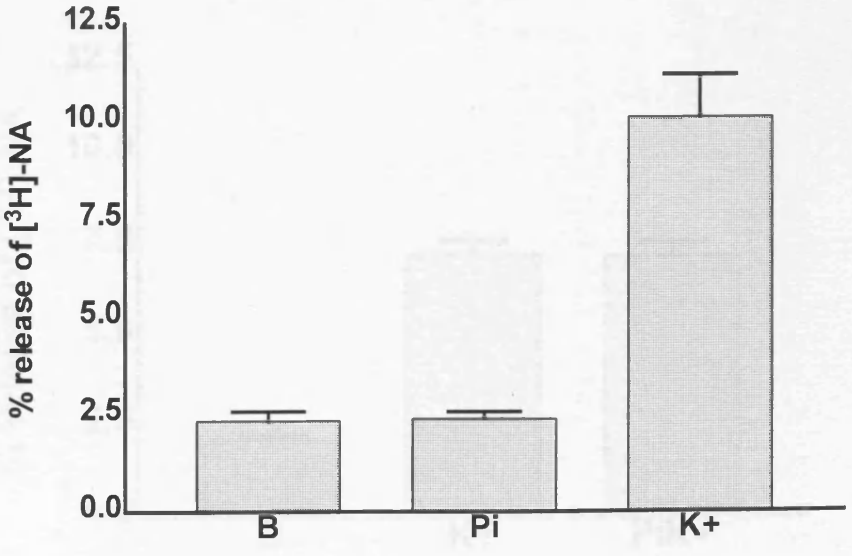


Figure 7.5. Effect of rocuronium (R) and pipecuronium (Pi) on basal (B) release of [^{3}H]-noradrenaline from SH-SY5Y cells. 100mM K^+ stimulation ensured the cells were responding properly (n=6).

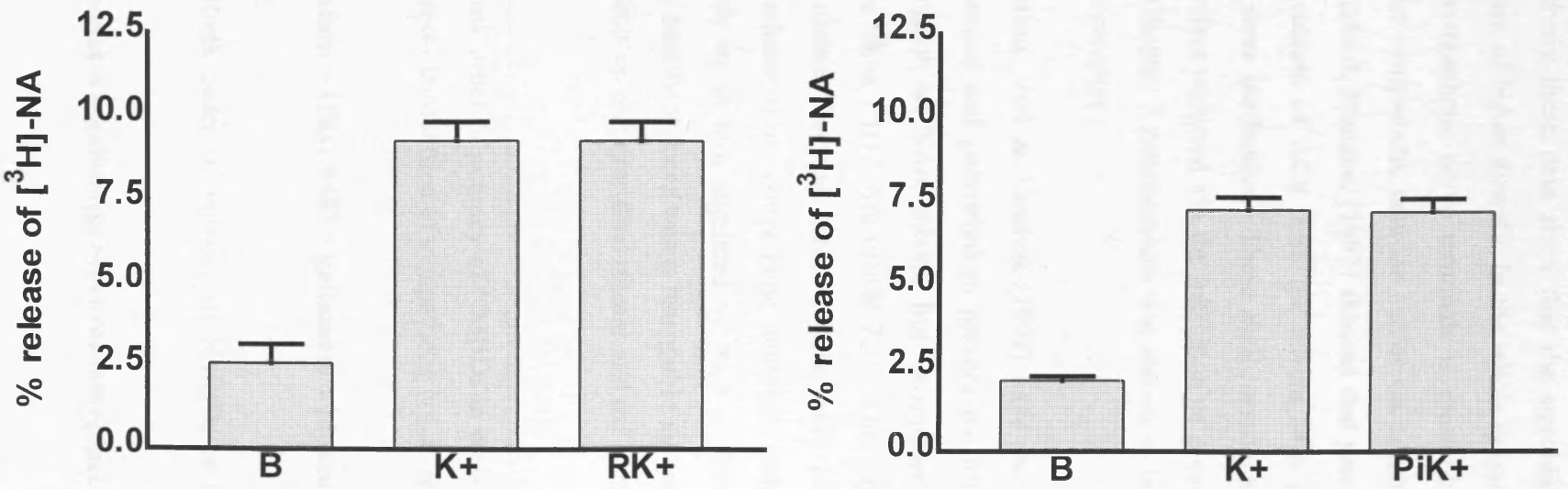


Figure 7.6. Effect of rocuronium (R) and pipecuronium (Pi) on K^+ evoked release of [^{3}H]-noradrenaline from SH-SY5Y cells (n=6).

7.5. Discussion.

Collectively these data show that the steroidal NMBDs do not affect the uptake or release of NAdr directly. In the whole animal inhibition of noradrenaline release from sympathetic nerve terminals is most likely to occur via muscarinic receptors on the sympathetic neurone and/or via control by ACh released from the vagus nerve. Indeed, Manabe, (1997) showed that pancuronium and gallamine both enhanced the release of ACh from the atrium of a guinea pig. Vecuronium and pipecuronium were ineffective. These data corroborate the above suggestion, that the observed effect occurred via the inhibition of presynaptic muscarinic receptors. Moreover, in Chapter 5 pancuronium was shown to be an antagonist at the human M2 muscarinic receptor .

In addition, Vizi & Lendvai (1997) have shown that a range of NMBDs including gallamine and pancuronium prevent the inhibitory effect of muscarinic receptor stimulation on NAdr release from sympathetic nerve endings in isolated guinea pig atria using [^{3}H]-NAdr (Table 7.2). This supposition could be consistent with the hypothesis that the cardiovascular side effects may be regulated by noradrenaline release from sympathetic neurones under the control of muscarinic receptors, which are in turn regulated by ACh released from the vagus. In partial support of this statement there was a reasonable agreement between the rank order potency for effects on noradrenaline release and m2 interaction (Table 7.2)

Table 7.2a. Rank order of potency of NMBDs in preventing the inhibitory effect of muscarinic receptor stimulation of sympathetic NAdr release (Vizi 1995).

Pancuronium > ORG 9487 > gallamine > pipecuronium > rocuronium.

Table 7.2b. Rank order of potency of NMBDs at the M2 muscarinic receptor (Chapter 4).

Pancuronium = gallamine>vecuronium>pipecuronium>rocuronium

CHAPTER 8

General conclusions.

In the clinical setting NMBDs are used to produce neuromuscular relaxation (Heining, et al, 1996). However, a range of cardiovascular side effects has been reported in the literature and these have been summarised in table 8.1. The program of research reported in this thesis represents an attempt to define the target site(s) and mode of action of these agents on the heart.

8.1. Summary of results.

In this thesis I have shown that NMBDs bind to all five subtypes of muscarinic receptors. With the exception of vecuronium all NMBDs shown some selectivity for the M2 subtype. This is important, as an interaction at this site is a logical target and could be used to explain the cardiovascular side effect profile for these agents.

In an attempt to determine any functional consequences of this M2 interaction receptor specific signal transduction studies were performed. Specifically inhibition of cAMP formation in M2 receptors and as a further control to determine selectivity increased $[Ca^{2+}]_i$ in M3 cells. In CHOm2 cells pancuronium produced a parallel rightward shift in the concentration response curve to methacholine indicating an antagonist action at this receptor. However, there was also some evidence for a weak antagonist action of vecuronium and rocuronium. It should be noted that the differences in the methacholine and methacholine +NMBD curve was only statistically significant when the IC_{50} values were compared. Comparison of the whole curves failed to show statistical significance. Therefore the antagonist action of vecuronium and rocuronium at the M2 receptor is questionable. At the M3 receptor only rocuronium at high concentrations affected the rise in $[Ca^{2+}]_i$ from the $Ins(1,4,5)P_3$ sensitive stores.

The interaction of these NMBDs may not be simple as has been suggested for gallamine where an allosteric mode of action has been reported (Ellis et al, 1991). Further kinetic studies with pancuronium (clinically producing a tachycardia), vecuronium (clinically producing a bradycardia) and gallamine (as a control) indicated that all three agents interacted with this receptor in an allosteric fashion.

Table 8.1. Cardiovascular effects of pancuronium (PANC), vecuronium (VEC) and rocuronium (ROC).

DRUG	HR	MAP	AP	PAP	PVR	CO	CI	DP	CVP	RPP	PCWP	SVI
PANC	⇑(2) (4) 20% (13)17%	⇑(1) 6%	⇑(2)	⇑(3)26%	⇑(3)31%	⇑(3)7%	⇑(2) (4)20%					
VEC	⇓(6)39% (7) (8)27% (10)7%	⇓(7) (11)20% (1)19%	⇓(1)15%	⇓(10)17 %				⇓(1)	⇓(10)15 %	⇓(10)9%		
ROC	⇑(11)7% (12)9% (5)5-10% (9)15% ⇓(9)10%	⇑(5)10-15% (10)5%					⇑(10)11 %				⇑(10)25 %	⇑(10)15 %

(1) Lavery et al, 1986; (2) Lienhart et al, 1983; (3) Du et al, 1996; (4) Niedhart et al, 1994; (5) Robertson et al, 1994; (6) Inoue et al, 1988; (7) Searle et al, 1994; (8) Couture et al, 1996; (9) Motsch et al, 1995; (10) McCoy et al, 1993; (11) Stevens et al, 1997; (12) Wierda et al, 1997; (13) Saxena et al, 1970.

Key: AP-arterial pressure, CI-cardiac index, CO-cardiac output, CVP-central venous pressure, DP-diastolic pressure, HR-heart rate, MAP-mean arterial pressure, PAP-pulmonary artery pressure, PCWP-pulmonary capillary wedge pressure, PVR-peripheral vascular resistance, RPP-rate pressure product, SVI-stroke volume index.

↑ denotes increase in effect.
↓ denotes decrease in effect.

123

An additional part of this study was to examine the effects of a range of NMBDs on catecholaminergic transmission in a simple cellular system. Clearly it is possible increased noradrenaline concentration at the heart would lead to a tachycardia and decreased concentrations would lead to a bradycardia. Catecholamine concentration can be modulated via interference with the reuptake or the release process (Emslie-Smith et al, 1988). Chapter 7 has clearly shown that there was no significant effects on either reuptake (in SH-SY5Y and 293-hNET cells) or release (SH-SY5Y cells) of [^{3}H]noradrenaline.

The main findings of this thesis are summarised in table 8.2

Table 8.2. Summary of functional and binding effects of steroidal NMBDs.

	M2 receptor*	CAMP	$[Ca^{2+}]_i$	NAdr release	NAdr uptake	Allosteric effect
Pancuronium	High	Antag.	No effect	No effect	No effect	Yes
Vecuronium	Moderate	Weak antag.	No effect	No effect	No effect	Yes
Rocuronium	Low	Weak antag.	Small ↓ @100μM	No effect	No effect	Untested
Pipecuronium	Moderate	Untested	Untested	No effect	No effect	Untested

(antag. = antagonist effect, * = binding affinity relative to each NMBD).

8.2. Do the observed effects occur at clinically achievable concentrations?

Comparing table 4.1 with the values noted in table 8.3 it can be clearly seen that with the exception of rocuronium a significant interaction with the M2 receptor for all the NMBDs tested would be anticipated. A general pattern can be seen for subtype specific effects of NMBDs. Firstly rocuronium does not appear to produce a significant interaction at any muscarinic receptor at clinically achievable concentrations. Secondly, the interaction appears to occur at clinically achievable concentrations for the M2 and M4 subtypes (these are the adenylate cyclase coupled subtypes). An interaction at clinically achievable concentration does not occur for the M1, M3 and M5 receptor (with some exceptions e.g., pancuronium and M1 and M3). These are the phosphoinositide pathway linked subtypes.

Table 8.3. Plasma free fraction and plasma concentration producing 50% depression in muscle twitch tension (Cp_{50}) for gallamine, pancuronium, pipecuronium, vecuronium and rocuronium.

Blocker	Free fraction	$Cp_{50(total)}\mu g/ml$	$Cp_{50(free)}$
Gallamine	0.30	5.82	6.53μM
Pancuronium	0.93	0.193	245nM
Pipecuronium	0.75	0.067	68nM
Vecuronium	0.43	0.09	61nM
Rocuronium	0.75	1.22	1.5μM

Data are from Wierda, *et al.*, (1995); Khahil, *et al*, (1994); Ornstein, *et al* (1992); Miller, (1990); Yajima, *et al.*, (1990); Wood, *et al.*, (1983); Tassonyi, *et al.*, (1981); Skivington, (1972).

8.3. Is there a correlation with the classical effects of NMBDs?

Figure 8.1 (top) compares the K_i values for the M2 muscarinic receptor with the Cp_{50} values in figure 8.3. The figure shows that there is no correlation between the affinity of the drug for the receptor and the amount of the drug that is available for binding to the receptor. Figure 8.1 (bottom) compares the K_i values for the M2 muscarinic receptor with the dose required for endotracheal intubation and indicates that there was no correlation for these effects.

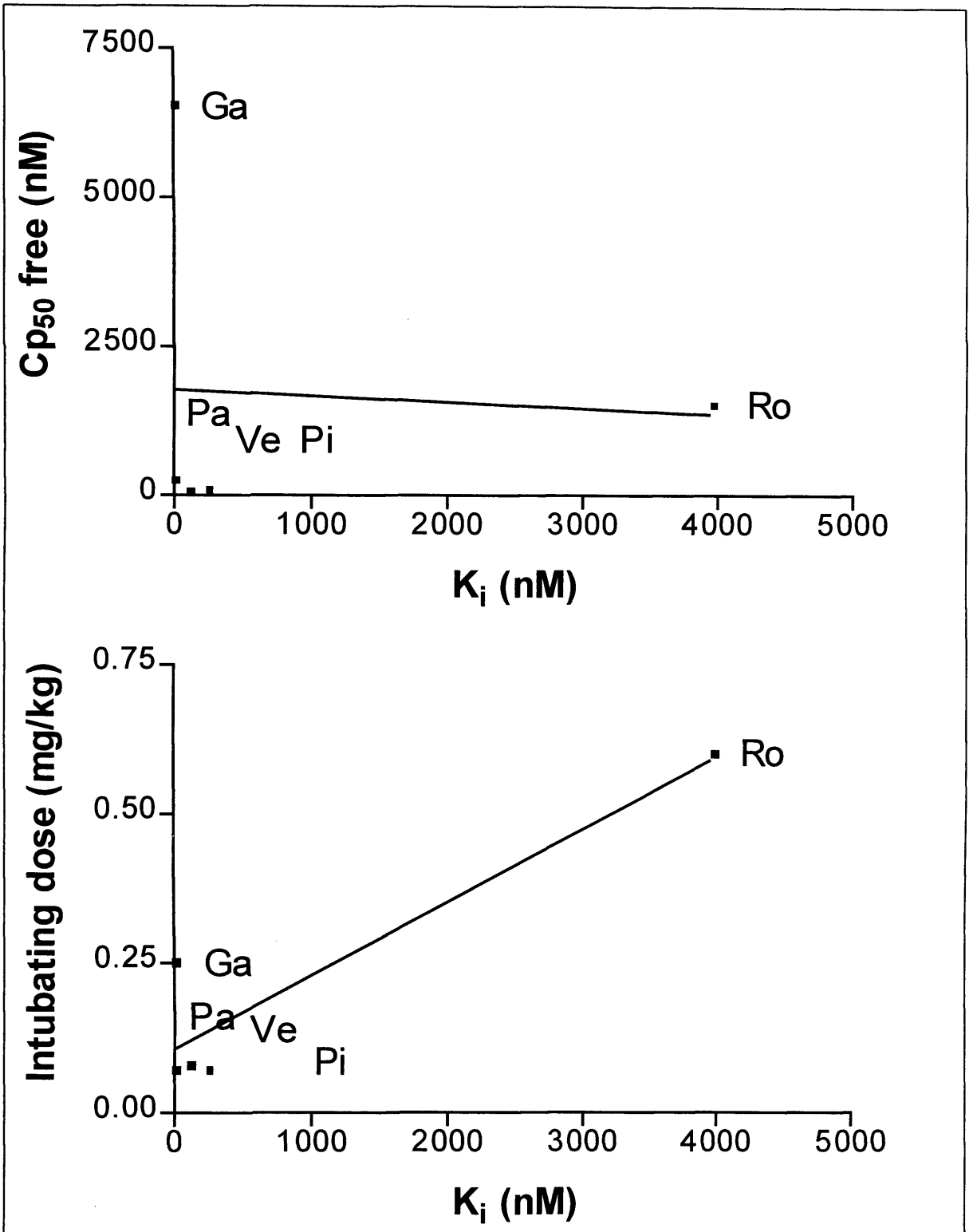


Figure 8.1. Comparison of K_i values (M2 muscarinic receptors) with Cp_{50} values for steroidal NMBDs and gallamine where r^2=0.004 and P=0.92 (top).
Comparison of K_i values (M2 muscarinic receptors) with intubation doses (data form ABPI) of steroidal NMBDs and gallamine where r^2=0.87 and P=0.02 (bottom).

8.4 Are the drugs agonists or antagonists?

From the data presented in this thesis it is clear that pancuronium is an antagonist and that this antagonism occurs at clinically achievable concentrations. An antagonist action in the 'test tube' agrees well with the actions seen in the clinical setting (Okanlami, et al, 1996). In addition gallamine also produces a tachycardia (Ramzan, et al, 1981) and this is a known muscarinic antagonist (Manabe et al, 1991). Unfortunately cAMP studies were not made using this drug but we would reasonably predict a parallel rightward shift in the concentration response curve. Moreover, pipecuronium was not examined in Chapter 5 ($cAMP/Ca^{2+}$) as this drug is thought to be relatively free from cardiac side effects. However, in Chapter 3 a significant interaction with the M2 receptor was reported. The functional consequences of this interaction are unclear but clearly insufficient to modulate cAMP or Ca^{2+} responses in CHOm2 or CHOm3 cells. As for vecuronium and rocuronium my data suggest weak antagonism but I feel that there would be little effect in the clinical setting. Again the functional consequences in whole animals of this interaction are unclear.

8.5 Competition compared with allosterism.

Allosterism in pharmacology is a relatively new concept. In these studies both competitive (Chapter 4) and allosteric interactions (Chapter 6) have been described. At first glance these may seem incompatible. How can a competitive displacer have an allosteric action? This would be possible is the NMBD bridged the orthosteric (competitive site) and allosteric site. In this model the radiolabel would have equal access to both sites. Therefore in an experiment designed to determine competitive binding (e.g., displacement) this could be observed and in an experiment designed to determine allosterism (e.g., kinetic) then this could also be observed. Figure 8.2 shows how the M2 receptor binding sites could be bridged by one molecule of NMBD. This type of interaction has been suggested for gallamine (Hulme, et al 1990). One unanswered question is the consequences of the allosteric interaction.

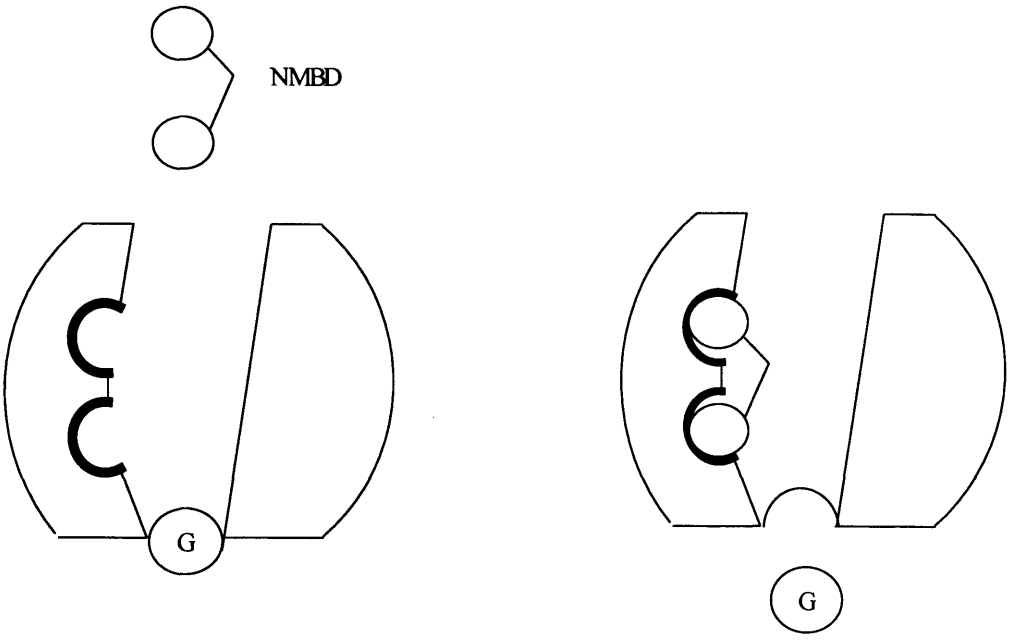


Figure 8.2..
Left: NMBD is not bound to either of the binding sites (bold lines) and the receptor remains unchanged
Right: NMBD binds to the receptor by bridging both sites, causing an allosteric change in the receptor and activating the G protein (G).
[^{3}H]-NMS has equal access to both of these sites and depending on the product used both competitive type binding and allosterism can be observed.

8.6. Catecholaminergic transmission, simple cellular systems compared with whole animals.

An interaction with catecholaminergic transmission has been suggested (Kobayashi et al, 1987) In this study such an interaction was not observed. NMBDs did not affect either the release or reuptake of catecholamines in two simple cellular systems (SH-SY5Y and 293hNET). We suspect that the lack of effect is a consequence (and indeed a drawback) of using such simple cellular systems. In the whole heart and more importantly the whole animal the complex interplay in the autonomic nervous system is retained. Whilst this thesis has not demonstrated an interaction I feel that in the whole animal there is likely to be an interaction. Whether this is direct or indirect via the muscarinic system remains to be determined. One further point of interest and like with section 8.4 is the observation that SH-SY5Y cells express M3 muscarinic receptors (Atcheson, et al, 1994; Lambert et al, 1990) and that activation of these receptors stimulated noradrenaline release. If the interaction of NMBDs at this receptor was agonist then a stimulation of release would be expected. This was not observed. Therefore functional effects associated with the interaction of NMBDs with M3 receptors (Chapter 4) is questionable.

8.7. Clinical relevance of NMBDs interactions.

Studies in isolated organs, tissues and single cells have shown the muscarinic receptor to be a potential target for NMBDs. These effects along with a modulation of sympathetic activity many be sufficient to explain the cardiovascular side effect profile described above for these agents. However, we should remember that in the majority of patients, these effects are of little clinical significance. Tachy- or brady-cardic episodes occur up to the first 5 minutes of application of the drug in theatre. High concentrations of the drugs occur in the heart before the NMBD is distributed around the body and this factor may validate effects seen in laboratories where the drugs are tested above the accepted blood concentrations that are recorded when the drugs are used in theatre. A clear mechanistic understanding of the action of these commonly used anaesthetic drugs may still give the clinician the necessary information to make a more rational, evidence based, choice of NMBDs. The influence of muscarinic receptors in anaesthesia is not widely known as the subject of an excellent review further discussing the role of muscarinic receptors (Lambert & Appadu, 1995).

8.8. Further work.

Using isolated spontaneously beating neo-natal rat cardiac myocytes the steroidal NMBDs, along with gallamine, could be tested to examine, if any, the effects on rate of contractility of the cardiac myocytes. These experiments would show that the steroidal NMBDs could have a **direct** effect on the rate of contraction of cardiac myocytes. If this could be shown then a direct link between HR and blockade of the M2 muscarinic receptor could be made.

CHAPTER 9

References.

AGLAN, M.Y., POLLARD, B.J. (1995). Molecular mechanisms of neuromuscular blocking agents: Is the increased understanding of importance to the practising anaesthestist? *Pharmac. Ther.*, **68 (3),** 365-383.

ALMERS W. (1990). Exocytosis. *Ann. Rev. Physiol.*, **52**, 607-624.

APPADU, B.L., LAMBERT, D.G. (1994). Studies on the interaction of steroidal neuromuscular blocking drugs with cardiac muscarinic receptors. *Brit. J. Anaes.*, **68,** 86-88.

ASHKENAZI A., WINSLOW J.W., PERALTA E.G., PETERSON G.L., SCHIMERLIK M.I., CAPON D.J., RAMACHANDRAN J. (1987). An m2 muscarinic receptor coupled to both adenylyl cyclase and phosphoinositide turnover. *Science*, **238**, 672-675.

ATCHESON R, LAMBERT D.G., HIRST R.A., and ROWBOTHAM D.J. (1994) Studies on the mechanism of [^{3}H]-noradrenaline release from SH-SY5Y cells: The role of Ca^{2+} and cyclic AMP. *Br. J. Pharmacol.* **111**, 787-792.

BAJJALIEH S.M. (1999). Synaptic vesicle and fusion. Current opinion in *Neurobiology*, **9** (**3**), 321-328.

BASTA, S.J., SAVARESE, J.J., ALI, H.H., MOSS, J., GIONFRIDDO, M. (1983). Histamine releasing potencies of atracurium, dimethyl tubocurarine and tubocurarine. *Brit. J. Anaes.*, **55**, 105S.

BERNE R.M., LEVY M.N. (1990). *Principles of Physiology*. International student edition, Wolfe Publishing Ltd.

BERSTEIN G., BLANK J.L., SMRCKA A., HIGASHIJIMA T., STERNWEIS P.C., EXTON J.H., ROSS E.M. (1992). Reconstitution of agonist stimulated phosphotidylinositol 4,5-bisphosphate hydrolysis using purified m1 muscarinic receptor, $G_{q/11}$, and phospholipase C-β1. *J. Biol. Chem.* **267**, 8081-8088.

BIZZARI C., GIROLAMO M.D., D'ORAZIO M.C., CORDA D. (1990). Evidence that a guanine nucleotide-binding protein linked to a muscarinic receptor inhibits directly phospholipase C. *Proc. Natl. Acad. Sci. USA.* **87**, 4889-4893.

BONNER, T.I. (1989). The molecular basis of muscarinic receptor diversity. *TINS*, **12 (4)**, 148-151.

BREZDEN C.B., HEDLEY D.W., RAUTH A.M. (1994). Constitutive expression of P-glycoprotein as a determinant of loading with fluorescent calcium probes. *Cytometry*, **17**, 343-348.

BRICHARD G. 1973. Arrhythmia inducing action of pancuronium in patients under halothane. *Anaes. Analg. Reanim.*, 30(5), 947-950.

BROWN, B.B., CROUT, J.R. (1970). The sympathomimetic effect of gallamine on the heart. *J. Pharm. Exp. Ther.*, **172**, 266-273.

BROWN, B.L., ALBANO, J.D.M., Ekins, R.D., SGHERZI, A.M. (1971). A simple and sensitive assay method for the measurement of adenosine 3':5'-cyclic monophosphate. *Biochem. J.*, **121**, 516-522.

BUCKET W.R., HEWET C.L., SAVAGE D.S. (1973). Pancuronium bromide and other steroidal neuromuscular blocking agents containing acetylcholine fragments. *J. Med. Chem.*, **16**, 1116-1124.

BUCKLEY, N.J., BONNER, T.I., BUCKLEY, C.M., BRANN, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.*, **35**, 469-476.

CASON B., BAKER D.G., HICKEY R.F., MILLER R.D., AGOSTON S. (1990). Cardiovascular and neuromuscular effects of three steroidal neuromuscular blocking drugs in dogs (ORG 9616, ORG 9426, ORG 9991). *Anesth. Analg.*, 70, 382-388.

CAULFIELD, M.P. (1993). Muscarinic receptors – Characterization, coupling and function. *Pharmac. Ther.*, **58**, 319-379.

CAULFIELD, M.P., BIRDSALL, N.J.M. (1998). International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *The American society for Pharmacology and Experimental Therapeutics*. **50(2)**, 279-290.

CHENG, Y.C., PRUSOFF, W.M. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099-3108.

COBBOLD P.H., RINK R.J. (1987). Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem. J.*, **248**, 313-328.

COOPER R., MIRAKHUR R.K., CLARKE R.S.J., BOULES Z.S. (1992). Comparison of intubation conditions after administration of ORG 9426 (rocuronium) and suxamethonium. *Brit. J. Anaesth.*, **69**, 269-273.

COUTURE, P., BOUDREAULT, D., GIRARD, D., PLANTE, F., MCKENTY, S., ST.PIERRE, J., POIRIER, N.L. (1996). Haemodynamic interaction of high dose fentanyl and increasing doses of vecuronium in patients undergoing myocardial revascularisation. *Acta Anaesthesiol. Scand.*, **40**, 32-38.

DOCHERTY J.R. (1998) Subtypes of functional alpha1- and alpha2-adrenoceptors. *Eur J Pharmacol.* **361,** 1-15.

DOCHERTY, J.R., MCGRATH, J.C. (1980). A comparison of the effects of pancuronium bromide and its monoquaternary analogue, ORG NC 45, on autonomic and somatic neurotransmission in the rat. *Br. J. Pharmac.* **71**, 225-233.

DOCHERTY, J.R., MCGRATH, J.C. (1978). Sympathomimetic effects of pancuronium bromide on the cardiovascular system of the pithed rat: a comparison with the effects of drugs blocking the neuronal uptake of noradrenaline *Brit. J. Anaes.*, **64**, 589-599.

DOMENECH, J.S., GARCIA, R.C., SASIAIN, J.M.R., LOYOLA, A.Q., OROZ, J.S. (1976). Pancuronium bromide: An indirect sympathomimetic agent. *Brit. J. Anaes.*, **48**, 1143-1148.

DORJE F., WESS J., LAMBRECHT G., TACKE R., MUTSCHLER E., BRANN M.R. (1991). Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J. Pharmac. Exp. Ther.* **256**, 727-733.

DU H., ORII R., YAMADA Y., HAYASHIDA M., KIN N., SUWA K., HANAOKA M., HAYASHIDA M. (1996). Pancuronium increases pulmonary arterial pressure in lung injury. *Brit. J. Anaesth.*, 77(4), 526-529.

DUNLAP, J., BROWN, J.H., (1983). Heterogeneity of binding sites on cardiac muscarinic receptors induced by the neuromuscular blocking agents gallamine and pancuronium. *Mol. Pharmacol.*, **24**, 15-22.

EISLE, J.H., MARTE, J.A., DAVIS, H.S. (1971). Quantitative aspects of the chronotropic and neuromuscular effects of gallamine in anesthetized man. *Anesthesiology,* **35**, 630-633.

ELLIS, J., HUYLER, J., BRANN, M.R. (1991). Allosteric regulation of cloned m1-m5 muscarinic receptor subtypes. *Biochem. Pharmacol.*, **42** (**10**), 1927-1932.

ELLIS J., SEIDENBERG M. (1992). Two allosteric modulators interact at a common site on cardiac muscarinic receptors. *Mol. Pharmacol.* **42**, 638-641.

ELLIS J., SEIDENBERG M. (1989). Gallamine exerts biphasic allosteric effects at muscarinic receptors. *Mol. Pharmacol.*, **35**, 173-176.

EMSLIE-SMITH D., PATERSON C.R., SCRATCHERD T., READ N.W. (1988). *Textbook of Physiology.* **11**th edition, Churchill Livingstone.

EXTON J.H. (1997). Cell signalling through guanine-nucleotide-binding regulatory proteins (G proteins) and phospholipases. *Eur. J. Biochem.*, **243**, 10-20.

FAHEY, M.R., SESSLER, D.I., CANNON, J.E., BRADY, K., STOEN, R., MILLER, R.D. (1989). Atracurium, Vecuronium and Pancuronium do not alter the minimum alveolar concentration of Halothane in humans. *Anesthesiology*, **71**, 53-56.

FELDER, C.C. (1995). Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB J.*, **9**, 619-625.

FOLDES, F.F., NAGASHIMA, H., NGUYEN, H.D., DUNCALF, D., GOLDINER, P.L. (1990). Neuromuscular and cardiovascular effects of pipecuronium. *Can. J. Anaesth.*, **5**, 549-555.

GIRALDO E., MICHELETTI R., MONTAGNA E., GIACHETTI A., VIGANO M.A., LADINSKY H., MELCHIORRE C. (1988). Binding and functional characterisation of the cardioselective muscarinic antagonist methoctramine. *J. Pharmacol. Exp. Ther.*, **244**, 1016-1020.

GRIFFITHS H.R., JOHNSON G.E. (1942). The use of curare in general anaesthesia. *Anesthesiology*, 3, 418-420.

GRYNKIEWICZ G., POENIE M., TSIEN R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3449.

GUSKOVSKY F., LEUDEN J.E., KOHN E.C., FELDER C.C. (1993). Muscarinic receptor mediated tyrosine phosphorylation of phospholipase Cγ. *J. Biol. Chem.* **264**, 18288-18295.

HEINING M.P.D., BOGOD D.G., AITKENHEAD A.R. (1996). Essential anaesthesia for medical students. **1st** edition Arnold publishing.

HICKS, M.J., SHIGEKAWA, M., KATZ, A.M. (1979). Mechanism by which cyclic adenosine 3',5'-monophosphate-dependent protein kinase stimulates calcium transport in cardiac sarcoplasmic reticulum. *Circ. Res.*, **44**, 384-391.

HIROKAWA N., HEUSER J.E. (1982). Internal and external differentiation of the post-synaptic membrane at the neuromuscular junction. *Jnl. Neurocytol.*, **48**, 487-510.

HIRST R.A., HARRISON C., HIROTA K., LAMBERT D.G. (1999). Measurement of $[Ca^{2+}]_i$ in Whole Cell Suspensions Using Fura-2. *Methods in Molecular Biology*, **114**, 31-39.

HIRST, R.A., LAMBERT, D.G. (1995). Adenylyl cyclase in SH-SY5Y human neuroblastoma cells is regulated by intra- and extra-cellular calcium. *Biochem. Pharmacol.*, **49**, 1633-1640.

HOSKING M.P., LENNONR.L., GRONERT G.A. (1988). Combined H1 and H2 receptor blockade attenuates the cardiovascular effects of high dose atracurium for rapid sequence endotracheal intubation. *Anesth. Analg.*, **67**, 1089-1092.

HOU V.Y., HIRSHMAN C.A., EMALA C.W. (1998). Neuromuscular relaxants as antagonists for m2 and m3 muscarinic receptors. *Anesthesiology*. **88**, 744-750.

HULME, E.C., BIRDSALL, N.J.M., BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Ann. Rev. Pharmacol. Toxicol.*, **30**, 633-673.

HUNTER, J.M. (1996). Rocuronium: the newest aminosteroid neuromuscular blocking drug. *Brit. J. Anaesth.*, **76**, 481-483.

HUNTER, J.M. (1995). New neuromuscular blocking drugs. *The New England Journal of Medicine,* **332 (25)**, 1691-1699.

HUSBY P., GRAINSTAD L., ROSLAND J.H., VAMNES J.S., SEGADAL L. (1996). Haemodynamic effects of high dose vecuronium compared with pancuronium in beta blocked patients with coronary heart disease during fentanyl-diazepam-N_2O anaesthesia. *Acta Anaesthesiol. Scand.*, 40(1), 26-31.

HUU-LAINE F.K., PINGO-SCOGNAMIGLIO W. (1964). Activite curarisante du dichlorure de 3β-20α bistrimethylammonium 5α pregane (malouetine) et de ses stereoisomeres. *Arch. Int. Pharmacodyn. Ther.*, **147**, 209-219.

INOUE K., EL-BANAYOSY A., STOLARSKI L., REICHELT W. (1988). Vecuronium induced bradycardia following induction of anaesthesia with etomidate or thiopentone with or without fentanyl. *Brit. J. Anaesth.* 60, 10-16.

JAKUBIK J., BACAKOVA L., LISA V., EL-FAKAHANY E.,. TUCEK S. (1996). Activation of muscarinic acetylcholine receptors via their allosteric binding sites. *Proc. Natl. Acad. Sci. USA,* **93**, 8705-8709.

KATZ, A.M. (1979). Role of contractile proteins and sarcoplasmic reticulum in the response of the heart to catecholamines: An historical review. *Adv. Cyclic Nucleotide Res.*, **11**, 303-340.

KHALIL, M., D'HONNEUR, G., DUVLDESTIN, P., SLAVOV, V., HYS, C. DE, GOMENI, R. (1994). Pharmacokinetics and Pharmacodynamics of Rocuronium in patients with cirrhosis. *Anesthesiology*, **80 (6)**, 85-86.

KOBAYASHI O., NAGASHIMA H., DUNCALF D., CHAUDRY I.A., HARSING L.G. JR., FOLDES F.F., GOLDINER P.L., VIZI E.S. (1987). Direct evidence that pancuronium and gallamine enhance the release of norepinephrine from the atrial sympathetic nerve by inhibiting prejunctional muscarinic receptors. *Jnl. Autonomic Sys.*, 18(1), 55-60.

LAMBERT D.G., APPADU B.L. (1995). Muscarinic receptor subtypes: do they have a place in clinical anaesthesia? *Brit. J. Anaes.*, **74(5)**: 497-498.

LAMBERT D.G., NAHORSKI S.R. (1990). Second messenger responses associated with stimulation of neuronal muscarinic receptors expressed by a human neuroblastoma SH-SY5Y. *Progress in Brain Research.* **84**, 31-42.

LARIJANI, G.E., BARTKOWSKI, R.R., AZAD, S.S., SELTZER, J.L., WEINBERGER, M.J., BEACH, C.A., GOLDBERG, M.E. (1989). Clinical pharmacology of pipecuronium bromide. *Anesth. Analg.*, **68**, 734-739.

LAVERY G.G., MIRAKHUR R.K., CLARK R.S., GIBSON F.M. (1986). The effects of atracurium, vecuronium and pancuronium on heart rate and arterial pressure in normal individuals. *Eur. J. Anaesthesiol.*, 3(6), 459-467.

LEE C.Y. (1972). Chemistry and pharmacology of polypeptide toxins in snake venoms. *Ann. Rev. Pharmacol.*, **12**, 265-286.

LEE SON, S., WAUD, B.E., WAUD, D.R.. (1981). A comparison of the neuromuscular blocking and vagolytic effects of ORG NC 45 and pancuronium. *Anesthesiology*, **55**, 12-18.

LEPPIK, R.A., MILLER, R.C., ECK, M., PAQUET, J-L. (1994). Role of acidic amino acids in the allosteric modulation by gallamine of antagonist binding at the m2 muscarinic acetylcholine receptor. *Mol. Pharmacol.*, **45** (5), 983-990.

LIENHART A, GUGGIARI M., TAUVENT A., MANEGLIA R., COUSIN M.T., VIARS P. (1983). Haemodynamic effects of vecuronium in man. *Ann. Fr. Anesth. Reanim.* 2(1), 7-16.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., RANDAL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.

MACKENNA B.R., CALLANDER R. (1990). *Illustrated Physiology.* **5th** edition, Churchill Livingstone.

MAGEE, D.A., ROBINSON, R.J.S. (1987). Effect of stretch excersises on suxamethonium induced fasciculations and myalgia. *Brit. J. Anaes.*, **59**, 596-601.

MANABE N. (1997). The effect of non-depolarising neuromuscular blocking agents on the release of acetylcholine from the right atrium of the guinea pig. *Acta Med. Okayama*, 51(1), 13-18.

MANABE N., FOLDES F.F., TOROCSIK A., NAGASHIMA H., GOLDINER P.L., VIZI E.S. (1991). Presynaptic interaction between vagal and sympathetic innervation in the heart: modulation of acetylcholine and noradrenaline release. *Jnl. Autonomic Nerv. Sys.*, 32, 233-242.

MARSHALL R.J., MCGRATH J.C., MILLER R.D., DOCHERTY J.R., LAMAR J.C., (1980). Comparison of the cardiovascular actions of ORG NC45 with those producing any other non-depolarising neuromuscular blocking agents in experimental animals. *Brit. J. Anaesth.*, 52, 21S.

MCCOY, E.P., MADDINENI, V.R., ELLIOTT, P., MIRAKHUR, R.K., CARSON, I.W., COOPER, R.A.. (1993). Haemodynamic effects of rocuronium during fentanyl anaesthesia: Comparison with vecuronium. *Can. J. Anaesth.*, **40**, 703-708.

MERETOJA O.A., TAIVANINEN T., WIRTAVUORI K. (1995). Pharmacodynamic effects of 51W89, an isomer of atracurium, in children under halothane anaesthesia. *Brit. J. Anaesth.*, **74**, 6-11.

MILLER, R.D., SAVARESE, J.J. (1990), Pharmacology of muscle relaxants and their antagonists. *Anesthesia*, **1** (3rd edition), 389-504.

MILLER R.D., AGOSTON S., BOOIJ L.D.H.J. (1978). The comparitive potency and pharmacokinetics of pancuronium and its metabolites in anesthestized man. *Anesthesiology*, **207**, 539-542.

MILLIGAN, K.R., BEERS, H.T. (1985). Vecuronium associated cardiac arrest. *Anaesthesia*, **40 (4)**, 385.

MOHR K., STASCHEN C-M., ZIEGENHAGEN M. (1992). Equipotent allosteric effect of W84 on [^{3}H]NMS-binding to cardiac muscarinic receptors from guinea-pig, rat and pig. *Pharm. Tox.* **70**, 198-200.

MONOD J., WYMAN J., CHANGEAUX J-P. (1965). On the nature of allosteric transitions: a plausible model. *J. Miol. Biol.* **12**, 88-118.

MOTSCH, J., LEUWER, M., BITTIGER, B.W., BACH, A., SCHONSTEDT, R., MARTIN, R. (1995) Dose response, time course and recovery of rocuronium bromide in children during halothane anaesthesia. *Eur. J. Anaesthesiol.*, **12 (suppl 11)**, 73-78.

MUIR A.W., HOUSTON J., GREEN K.L., MARSHALL R.J., BOWMAN W.C., MARSHALL I.G. (1989). Effects of a new neuromuscular blocking agent (ORG 9426) in anaesthetized cats and pigs in isolated nerve-muscle preparations. *Brit. J. Anaesth.*, 63, 400-410.

MUSCHOLL, E. (1980). Peripheral muscarinic control of noradrenaline release in the cardiovascular system. *Am. J. Physiol.*, **239**, H713-H720.

NAGUIB M., SAMARKANDI A.H., BAKHAMEES H.S., MAGBOUL M.A., EL-BAKRY A.K. (1995). Comparative potency of steroidal neuromuscular blocking drugs and isobolographic analysis of the interaction between rocuronium and other aminosteroids. *Brit. J. Anaesth.*, **52**, 37-42.

NANA, A., CARDAN, E., DOMOKOS, M. (1973). Blood catecholamine changes after pancuronium. *Acta. Anaesthesiol. Scand.*, **17**, 83-87.

NARITA, M., FURUKAWA, Y., REN, L.M., KARASAWA, Y., TAKEI, M., MURAKAMI, M., TAKAYAMA, S., CHIBA, S. (1992). Cardiac effects of vecuronium and its interaction with autonomic nervous system and in isolated perfused canine hearts. *J. Cardiovascular Pharmacology,* **19**, 1000-1008.

NEUBIG R.R., COHEN J.B. (1979). Equilibrium binding of [^{3}H]-tubocurarine and [^{3}H]-acetylcholine by Torpedo post-synaptic membranes; stoichiometry and ligand interactions. *Biochemistry*, **18**, 5464-5475.

NIEDHART P.P., CHAMPION P., VOGEL J., ZSIGMOND E.K., TASSANYIE. (1994). A comparison of pipecuronium with pancuronium on haemodynamic variables and plasma catecholamines in coronary artery bypass patients. *Can. J. Anaes.*, 41(6), 469-474.

OKANLAMI O.A., FRYER A.D., HIRSHMAN C. (1996). Interaction of nondepolarising muscle relaxants with m2 and m3 muscarinic receptors in guinea pig lung and heart. *Anaesthesiology*. 84, 155-161.

ORNSTEIN, E., MATTEO, R., SCHWARTZ, A.E., JAMBDAR, S., DIAZ, J. (1992). Pharmacokinetics and pharmacodynamics of Pipecuronium bromide (Arduan) in elderly surgical patients. *Anesth. Analg.,* **74 (6)**, 841-844.

OSMER C., WULF K., VÖGELE C., ZICKMANN B., HEMPELMANN G. (1998). Cardiovascular effects of Org 9487 under isoflurane anaesthesia in man. *Eur. J. Anaesthsiol.,* 15, 585-589.

PARMENTIER, P., DAGNELIE, P. (1979). Dose related tachycardia induced by pancuronium during balanced anaesthesia with and without droperidol. *Brit. J. Anaes.*, **51**, 157-160.

PRAKASH P.V., GOWRAGANAHALLI J., NGOC T.P. (1990). Two distinct, divalent cation-sensitive, antagonist binding states of heart muscarinic receptors: differential modulation by guanine nucleotide. *Gen. Pharmac.* **21 (2)**, 211-218.

PROSKA J., TUCEK S. (1993). Mechanisms of steric and cooperative actions of alcuronium on cardiac muscarinic acetylcholine receptors. *Mol. Pharmacol.* **45**, 709-717.

PUHRINGER,F.K., KHUENL-BRADY, K.S., KOLLER, J., MITTERSCHIFFHALER, G. (1992). Evaluation of the endotracheal intubating conditions of rocuronium (ORG 9426) and succinylcholine in out-patient surgery. *Anesth. Analg.*, **75**, 37-40.

RAMZAN M.I., TRIGGS E.J., SHANKS C.A. (1981). Gallamine disposition in surgical patients with chronic renal failure. *Brit. J. Clin. Pharmacol.*, **12**, 141-147.

RANG H.P., DALE M.M., RITTER J.M. (1995). *Pharmacology.* **3rd** edition, Churchill Livingstone.

RHEE S.G., CHOI K.D. (1992). Regulation of inositol phospholipid specific phospholipase C isoenzymes. *J. Biol. Chem.* **267**, 12393-12396.

ROBERTSON E.N., HULL J.M., VERBEEK A.M., BOOIJ L.H. (1994). A comparison of rocuronium and vecuronium: the pharmacodynamic, cardiovascular and intra-ocular effects. *Eur. J. Anaesthesiol.* 11(9), 116-121.

ROESKE W.R., VENTER J.C. (1984). The differential binding of [^{3}H]-pirenzepine vs [^{3}H]-quinuclidinylbenzilate binding to soluble rat-brain muscarinic receptors indicates that pirenzepine binds to an allosteric state of the muscarinic receptor. *Biochem. Biophys. Res. Com.*, **118**(3), 950-957.

ROIZEN, M.F., FORBES, A.R., MILLER, R.D., LAKE, R., STANSKI, D. (1979). Similarity between effects of pancuronium and atropine on plasma norepinephrine levels in man. *J. Pharmacol. Exp. Ther.*, **211**, 419-422.

SALT, P.J., BARNES, P.K., CONWAY, C.M.. (1980). Inhibition of neuronal uptake of noradrenaline in the isolated perfused rat heart by pancuronium and its homologues ORG 6368, ORG 7268, and NC 45. *Brit. J. Anaes.*, **52**, 313-317.

SANTOLI F, SCEBBA L, AZZOLINA G. (1975). L-tubocurarine and pancuronium bromide in open-heart surgery. Arrthymia inducing action of pancuronium in patients under halothane. *Minerva Anestesiol.*, 41(1), 1-6.

SAVARESE, J.J., MILLER, R.D., LIEN, C.A., CALDWELL, J.E. (1990). Pharmacology of muscle relaxants and their antagonists. *Anesthesia*, **14**, 405-487.

SAVARESE J.J., ALI H.H., BASTA S.J., EMBREE P.B., SCOTT R.P.F., SUNDER N., WEAKLY J.N., WASILA W.B., EL-SAYAD H.A. (1988). The clinical neuromuscular pharmacology of Mivacurium chloride (BW B1090U). *Anesthesiology*, **68**, 723-732.

SAXENA, P.R., BONTA, I.L. (1970). Mechanism of selective cardiac vagolytic action of pancuronium bromide. Specific blockade of cardiac muscarinic receptors. . *E. J. Pharmc.*, **11**, 332-341.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci. USA*, **52**, 660-672.

SEARLE N.R., SAHAB P., BLAIN R., TAILLEFER J., TREMBLAY N., HARDY J.F., ROY M., GAGNON L., BELISLE S. (1994). Haemodynamic and pharmacodynamic comparison of doxacurium and high dose vecuronium during coronary artery bypass surgery: a cost-benefit study. *Cardiothoracic Vasc. Anaesth.*, 8(5), 490-494.

SKIVINGTON, M.A.. (1972). Protein binding of three tritiated muscle relaxants. *Brit. J. Anaes.*, **44**, 1030-1034.

STANDAERT F.G. (1986). Basic physiology and pharmacology of the neuromuscular junction. *Anesthesia*, **2nd edition**, p 835-870.

STARR N.J., SETHNA D.H., ESTAFANOUS F.G. (1986). Bradycardia and asystole following the rapid administration of sufentanil with vecuronium. *Anesthesiol.*, 64, 521-523.

STEVENS, J.B., HECKER, R.B., TALBOT, J.C., WALKER, S.C. (1997). The haemodynamic effects of rocuronium and vecuronium are different under balanced anaesthesia. *Acta Anaesthesiol. Scanda.*, **41**, 502-505.

STOCKTON J.M., BIRDSALL N.J.M., BURGEN A.S.V., HULME E.C. (1983). Modification of the binding properties of muscarinic receptors by gallamine. *Mol. Pharmacol.*, **23**, 551-557.

STOOPS C.M., CURTIS C.A., KOVACH D.A., MCCAMMON R.L., STOELTING R.K., WARREN T.M., MILLER D., BOPP S.K., JUGOVIC D.J., ABOU-DONIA M.M. (1989). Hemodynamic effects of Mivacurium chloride administered to patients during oxygen-sufentanil anesthesia for coronary artery bypass grafting of valve replacement. *Anesth. Analg.*, **68**, 333-339.

TASSONYI, E., SZABO, G., VERECZKEY, L. (1981).. Pharmacokinetics of pipecurium bromide, a new non-depolarising neuromuscular blocking agent, in humans. *Arzneimittel-Forschung*, **31 (10)**, 1754-1756.

TSIEN, R.W., BEAN, B.P., HESS, P., LANSMAN, J.B., NILIUS, B., NOWYCKY, M.C. (1986). Mechanisms of calcium channel modulation by b-adrenergic agents and dihydropiridine calcium agonists. *J. Mol. Cell. Cardiol.*, **18**, 691-710.

TUCEK S., PROSKA J. (1995). Allosteric modulation of muscarinic acetylcholine receptors. *TiPS.* **16**, 205-212.

VAN DEN BROEK L., WIERDA J.M.K.H., SMEULERS N.J., PROOST J.H. (1994). Pharmacodynamics and pharmacokinetics of an infusion of ORG 9487, a new short-acting steroidal muscle relaxant. *Anesth. Analg.*, **77**, 579-584.

VETTERMAN J., BECK K.C., LINDAHL S.G., BRICHANT J.F., REHDER K. (1988). Actions of enflurane, isoflurane, vecuronium, atracurium and pancuronium to pulmonary vascular resistance in dogs. *Anesthesiology*, 69(5), 688-695.

VIBY-MORGENSEN J. (1980). Correlation of succinylcholine duration of action with plasma cholinesterase activity in subjects with the genotypically normal enzyme. *Anesthesia.* **53**, 517-520.

VIZI E.S. (1995). Effects other than relaxation of non-depolarizing muscle relaxants. In: Muscle relaxants, p67-81, FUKUSHIMA K., OCHIAI R., (eds.), Springer Verlag, Tokyo.

VIZI E.S., LENDVAI B. (1997). Side effects of nondepolarising muscle relaxants: relationships to their antinicotinic and antimuscarinic actions. *Pharmacol. Ther.*, **73**(2), 75-89.

WAELBROECK, M., CAMUS, J., TASTENOY, M., CHRISTOPHE, J. (1992). Binding properties of nine 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) analogues to M_1, M_2, M_3 and putative M_4 muscarinic receptor subtypes. *Br. J. Pharmac.*, **105**, 97-102.

WALDMANN C.S., WARK K.J., SEBEL P.S., FENECK R.O. (1986). Haemodynamic effects of atracurium, vecuronium and pancuronium during sufentanil anaesthesia for coronary artery bypass. *Acta Anaesthesiol. Scand.* 30(5), 351-356.

WATLING K.J. (1998). *The RBI Handbook of Receptor Classification and Signal Transduction.* 3rd edition, RBI publishing.

WEISHAAR, R.E., KOBYLARZ-SINGER, D.C., QUADE, M.M., KAPLAN, H.R. (1988). Role of cyclic AMP in regulating cardiac muscle contractility: Novel pharmacological approaches to modulating cyclic AMP degradation by phosphodiesterase. *Drug Development Research*, **12**, 119-129.

WIERDA, J.M.K.H., PROOST, J.H. (1995). Structure-pharmacodynamic-pharmacokinetic relationships of steroidal neuromuscular blocking agents. *E. J. Anaes.*, **12 (suppl. 11)**, 45-54.

WIERDA J.M., SCHURINGA M., VAN DEN BROEK L. (1997). Cardiovascular effects of an intubating dose of rocuronium 0.6mg kg^{-1} in anaesthetized patients, paralysed with vecuronium. *Brit. J. Anaesth.*, 78(5), 586-587.

WINTERSTEINER O., DUTCHER J.D. (1943). Curare alkaloids from *Chondodendron tomentosum. Science*, **97**, 467-470.

WOOD, M., STONE, W.J., WOOD, A.J.J.., (1983). Plasma binding of pancuronium: Effects of age, sex and disease. *Anesth. Analg.*, **62 (1)**, 29-32.

YAJIMA, C., SUGAI, N., HANAOKA, K., NUMATA, K., YAMAMOTO, K., KUSHIDA, Y., MURAKAMI, S., YAMAMURA, H, (1990). Comparitive pharmacokinetics of pipecuronium bromide, pancuronium bromide and vecuronium bromide in anesthetized man. . *Masui-Japanese Journal of Anesthesiology*, **37 (7)**, p882-888.

Publications arising from this thesis.

Full papers.

1. CEMBALA T.M., SHERWIN J.D., TIDMARSH M.D., APPADU B.L., LAMBERT D.G. (1998). The interaction of steroidal neuromuscular blocking drugs with recombinant human m1-m5 muscarinic receptors expressed in Chinese hamster ovary cells. *Brit. J. Pharmacol.*, **125**, 1088-1094.

2. CEMBALA T.M., FORDE S., and LAMBERT D.G. Allosteric effects of neuromuscular blocking drugs at recombinant m2 muscarinic receptors. In preparation for *Eur. J. Pharmacol.*

Editorial.

1. CEMBALA T.M., SHERWIN J.D., and LAMBERT D.G. Cardiovascular effects of the amino steroid neuromuscular blocking drugs. *Br. J. Anaesthesia.* (Under revision)

Abstracts (Posters)

1. CEMBALA T.M., APPADU B., and LAMBERT D.G. (1996) Interaction of vecuronium with recombinant human m2 muscarinic receptors. *Br. J. Anaesthesia.* **77**, 291P.

2. CEMBALA T.M., TIDMARSH M.D., APPADU B.L., and LAMBERT D.G. (1997) Interaction of neuromuscular blocking drugs with recombinant human m1-m3 muscarinic receptors. *Br. J. Pharmacol.* **120**, 183P.

3. CEMBALA T.M., APPADU B.L., LAMBERT D.G. (1998). The effect of steroidal neuromuscular blocking drugs on norepinephrine uptake in SH-SY5Y cells. *Brit. J. Anaesth.* **81**, 641P-642P.

4. CEMBALA T.M., APPADU B.L., LAMBERT D.G. (1998). Effects of steroidal neuromuscular blocking drugs on [^{3}H]-noradrenaline release from SH-SY5Y cells. *Biochem. Soc. Trans.* University of Leicester Meeting 21-23 September 1998.

5. SHERWIN, J.D., CEMBALA, T.M., APPADU, B.L., LAMBERT, D.G. (1998). Pancuronium reverses methacholine inhibition of cAMP formation in Chinese hamster ovary cells expressing recombinant human muscarinic m2 receptors (CHOm2). *Brit. J. Anaesthesia.* **81,** 292P-293P.

6. FORDE S., CEMBALA T.M., LAMBERT D.G. (1999). Further studies on the interaction of pancuronium and vecuronium with recombinant m2 muscarinic receptors. *Brit. J. Anaesthesia.* **83,** 189-190P.