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Interaction of neuromuscular blocking drugs with recombinant human m1–m5 muscarinic receptors expressed in Chinese hamster ovary cells

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1 Neuromuscular blocking drugs (NMBD's) are known to produce cardiovascular side effects manifesting as brady/tachycardias. In this study we have examined the interaction of a range of steroidal NMBD's with recombinant human m1–m5 muscarinic receptors expressed in Chinese hamster ovary cells. Our main hypothesis is that NMBD's may interact with m2 (cardiac) muscarinic receptors.

2 All binding studies were performed with cell membranes prepared from CHO m1–m5 cells in 1 ml volumes of 20 mM HEPES, 1 mM $MgCl_2$ at pH 7.4 for 1 h. Muscarinic receptors were labelled with [^{3}H]-NMS and displacement studies were performed with pancuronium, vecuronium, pipecuronium, rocuronium and gallamine. In addition a range of muscarinic receptor subtype selective reference compounds were included. In order to determine the nature of any interaction the effects of pancuronium, rocuronium and vecuronium on methacholine inhibition of forskolin stimulated cyclic AMP formation in CHO m2 cells was examined. Cyclic AMP formation was assessed in whole cells using a radioreceptor assay. All data are mean ± s.e.mean ($n \geqslant 5$).

3 The binding of [^{3}H]-NMS was dose-dependent and saturable in all cells tested. B_{max} and K_d values in m1–m5 cells were 2242 ± 75, 165 ± 13, 1877 ± 33, 458 ± 30, 127 ± 2 fmol mg^{-1} protein and 0.11 ± 0.02, 0.15 ± 0.01, 0.12 ± 0.01, 0.12 ± 0.01, 0.22 ± 0.01 nM respectively.

4 The binding of [^{3}H]-NMS was displaced dose dependently (pK_{50}) by pirenzepine in CHO m1 membranes (7.97 ± 0.04), methoctramine in CHO m2 membranes (8.55 ± 0.1), 4-diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP) in CHO m3 membranes (9.38 ± 0.03), tropicamide in CHO m4 membranes (6.98 ± 0.01). 4-DAMP, pirenzepine, tropicamide and methoctramine displaced [^{3}H]NMS in CHO m5 membranes with pK_{50} values of 9.20 ± 0.14, 6.59 ± 0.04, 6.89 ± 0.05 and 7.22 ± 0.01 respectively. These data confirm homogenous subtype expression in CHO m1–m5 cells.

5 [^{3}H]NMS binding was displaced dose-dependently (pK_{50}) by pancuronium (m1, 6.43 ± 0.12; m2, 7.68 ± 0.02; m3, 6.53 ± 0.06; m4, 6.56 ± 0.03; m5, 5.79 ± 0.10), vecuronium (m1, 6.14 ± 0.04; m2, 6.90 ± 0.05; m3, 6.17 ± 0.04; m4, 7.31 ± 0.02; m5, 6.20 ± 0.07), pipecuronium (m1, 6.34 ± 0.11; m2, 6.58 ± 0.03; m3, 5.94 ± 0.01; m4, 6.60 ± 0.06; m5, 4.80 ± 0.03), rocuronium (m1, 5.42 ± 0.01; m2, 5.40 ± 0.02; m3, 4.34 ± 0.02; m4, 5.02 ± 0.04; m5, 5.10 ± 0.03) and gallamine (m1, 6.83 ± 0.05; m2, 7.67 ± 0.04; m3, 6.06 ± 0.06; m4, 6.20 ± 0.03; m5, 5.34 ± 0.03).

6 Cyclic AMP formation was inhibited dose dependently by methacholine in CHO m2 cells pEC_{50} for control and pancuronium (300 nM) treated cells were 6.18 ± 0.34 and 3.57 ± 0.36 respectively. Methacholine dose-response curves in the absence and presence of rocuronium (1 μM) and vecuronium (1 μM) did not differ significantly. Pancuronium, vecuronium and rocuronium did not inhibit cyclic AMP formation alone indicating no agonist activity.

7 With the exception of rocuronium there was a significant interaction with m2 muscarinic receptors with all NMBD's at clinically achievable concentrations suggesting that the brady/tachycardias associated with these agents may result from an interaction with cardiac muscarinic receptors. Furthermore pancuronium at clinically achievable concentrations antagonised methacholine inhibition of cyclic AMP formation in CHO m2 cells further suggesting that the tachycardia produced by this agent results from muscarinic antagonism. The mechanism of the bradycardia produced by vecuronium is unclear.

Keywords: Muscarinic receptors; radioligand binding; neuromuscular blocking drugs

Introduction

Muscular paralysis produced by neuromuscular blocking drugs results from a direct block of nicotinic receptors at the neuromuscular junction. (Aglan & Pollard, 1995). Steroidal neuromuscular blocking drugs (NMBD e.g. pancuronium, vecuronium, pipecuronium and rocuronium) are generally free of the troublesome side effects associated with non-steroidal neuromuscular relaxants (e.g. histamine release (Basta *et al*., 1983) and malignant hyperthermia (Magee *et al*., 1987)). However, pancuronium (Parmentier *et al*., 1979) and rocuronium (Motsch *et al*., 1995) have been associated with episodes of tachycardia in patients undergoing surgery. In addition, vecuronium may produce bradycardia (Lema *et al*., 1992) and cardiac arrest (Milligan & Beers, 1985). Rocuronium may produce a small tachycardia but only in high doses (Stevens *et al*., 1997). The bradycardic effects of vecuronium have been attributed to the combined use of large doses of opioids (Couture *et al*., 1996). However in a recent study Stevens *et al*. (1997) reported a bradycardia that was not attributed to concurrent administration of opiates.

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The underlying basis of these cardiovascular effects are largely unknown but vagolysis (Fitzal *et al*., 1983; Lee Son *et al*., 1981; Saxena & Bonta, 1970) and monoamine uptake inhibition (Salt *et al*., 1980; Docherty & McGrath, 1978) have been suggested. It has also been suggested that the bradycardia produced by vecuronium may result from increased vagal tone secondary to surgical manipulation (Miller & Savarese, 1990).

We hypothesize that NMBD's which produce bradycardias may act as muscarinic agonists and NMBD's that produce tachycardia may act as muscarinic antagonists. We have therefore performed a detailed examination of the binding of five NMBD's to m1–m5 recombinant muscarinic receptors stably expressed in CHO cells. In addition, the effects of pancuronium (producing a tachycardia), rocuronium (producing a slight tachycardia) and vecuronium (producing a bradycardia) on methacholine inhibition, of cyclic AMP formation in CHO m2 cells were examined in order to study the functional consequences of any m2 interaction.

Methods

Cell culture

Chinese hamster ovary cells (CHO) expressing recombinant human m1–m5 muscarinic receptors were provided by Dr N.J.M. Buckley (UCL, London). Cells were maintained in alpha Minimal Essential Medium supplemented with 10% newborn calf serum, 100 i.u. penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} fungizone. Stock cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2/Air. Stock cultures were fed twice weekly and passaged when confluent.

Membrane preparation

All binding studies were performed using membranes prepared on the day of use. Confluent monolayers of cells were washed with and harvested into homogenization buffer (10 mM HEPES, 0.05% EDTA, 0.9% NaCl at pH 7.4). Cells were homogenized using an Ultra-Turrax (T25) and pelleted at 20,000 g. The membranes were then re-suspended in assay buffer (20 mM HEPES, 1 mM $MgCl_2$ at pH 7.4), re-homogenized and re-pelleted twice more. Membranes were re-suspended at approximately 0.3 mg ml^{-1} and diluted further as appropriate for use in saturation and displacement studies. Protein concentrations were determined according to Lowry *et al*. (1957).

[^{3}H]-NMS binding assay

All binding assays were performed in 1 ml volumes of assay buffer for 1 h at 37°C using cell membranes (approximately 80–100 μg of protein). Saturation analyses to determine the equilibrium dissociation constant (K_d) and the maximal binding capacity (B_{max}) were performed using increasing concentrations of [^{3}H]-NMS (0.008–3.23 nM). Non-specific binding was defined in the presence of excess (10 μM) atropine. Following incubation, each sample was filtered under vacuum through Whatman GF/B filters using a Brandel cell harvester to separate bound and free radioactivity. Filter retained (bound) radioactivity was extracted for at least 8 h in 4 ml of scintillation fluid prior to estimation using a β-scintillation counter. In displacement studies a fixed concentration of [^{3}H]-NMS ($\sim$0.3 nM) was displaced by a range of NMBD's and muscarinic receptor subtype selective reference compounds. All drugs and radioligands were dissolved in assay buffer.

Measurement of cyclic AMP formation

Cyclic AMP studies were performed using whole cells prepared on the day of use. Confluent monolayers of cells were washed and harvested as above. Cells were pelleted at 1500 r.p.m. and resuspended in Krebs/HEPES buffer (in mM: 143 NaCl, 47 KCl, 1.2 KH_2PO, 1.2 $MgSO_4$ $7H_2O$, 11.7 glucose, 10 HEPES, 2.6 $CaCl_2$ $2H_2O$ at pH 7.4), washed and re-pelleted thrice more. The whole cells were re-suspended at approximately 1.0 mg ml^{-1} and diluted further as appropriate for use in cyclic AMP measurement studies. Protein concentrations were determined as above.

All cyclic AMP studies were performed in 0.3 ml volumes of Krebs/HEPES buffer at 37°C for 15 min using whole cells (approximately 150–170 μg of protein) incubated with methacholine (10^{-8}–10^{-2} M), forskolin (1 μM), 3-isobutyl-1-methylxanthine (1 mM), pancuronium (300 nM), vecuronium (1 μM) and rocuronium (1 μM) in various combinations. NMBD concentrations were chosen from binding studies. Reactions were terminated with 10 M HCl, neutralized with 10 M NaOH and buffered with 1 M Tris, pH 7.4. Samples were then centrifuged at 13,500 r.p.m. for 2 min and supernatent was removed and incubated with [^{3}H]-cyclic AMP and binding protein (a differential centrifugate of bovine adrenal glands) made up in cyclic AMP assay buffer (50 mM Tris, 4 mM EDTA, pH 7.4) for at least 2 h at 4°C. Unbound [^{3}H]-cyclic AMP was removed using a 1% activated charcoal, 0.4% BSA mix in cyclic AMP assay buffer, incubated for 1 min at 20°C and then centrifuged for 1 min at 13,500 r.p.m. The supernatant from each sample was removed and added to 1 ml of scintillation fluid. Samples were left for at least 8 h and cyclic AMP levels were estimated on a β-counter using a RIASMART program. Cyclic AMP standards ranged from 0.5–10 pmol (Brown *et al*., 1971; Hirst *et al*., 1995).

Sources of reagents

All tissue culture media and plastic ware were from Life Technologies (Paisley, U.K.). [^{3}H]-NMS (68–85 Ci mmol^{-1}) was from Amersham (Little Chalfont, U.K.). [^{3}H]-cyclic AMP (0.250 Ci mmol^{-1}) was from NEN Life Science Products (London, U.K.). Pirenzepine (PZP), methoctramine and 4-diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP) were from RBI (Poole, U.K.). Tropicamide, gallamine, pancuronium, atropine, activated charcoal, bovine serum albumin (BSA), forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma (Poole, U.K.). Vecuronium, rocuronium and pipecuronium were kind gifts from Organon Teknika (Newhouse, U.K.). All other reagents were of analytical grade.

Data analysis

B_{max} and K_d were obtained from Scatchard (1949) transformation of the specific binding curves. The Log molar concentration of drug producing 50% displacement of specific binding (pIC_{50}) and the log molar concentration of drug that produced 50% inhibition of the maximum cyclic AMP response (pEC_{50}) were obtained by computer assisted non-linear regression curve analysis (Graphpad-Prism). pIC_{50} values were corrected for the competing mass of [^{3}H]-NMS according to Cheng & Prusoff (1973) to yield pK_{50}. All data are expressed as mean $\pm$ s.e.mean ($n \geqslant 4$). pK_{50} and pEC_{50} values were compared

using an unpaired Student's *t*-test and considered significant when $P<0.05$. In cyclic AMP experiments methacholine curves in the absence and presence of NMBD's were compared by two way ANOVA and the curves were different when $P<0.05$.

Results

The binding of [^{3}H]-NMS was dose dependent and saturable in all cells with B_{max} and K_d values of 2242 ± 75, 165 ± 13, 1877 ± 33, 458 ± 30, 127 ± 2 fmol mg^{-1} protein and 0.11 ± 0.02, 0.15 ± 0.01, 0.12 ± 0.01, 0.12 ± 0.01, 0.22 ± 0.01 nM in CHO m1–m5 cells respectively.

The binding of [^{3}H]-NMS was displaced dose dependently (pK_{50}, slope factor) by pirenzepine (PZP) in CHO m1 membranes (7.97 ± 0.04, 0.90 ± 0.01), methoctramine in CHO m2 membranes (8.55 ± 0.1, 1.41 ± 0.14), 4-diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP) in CHO m3 membranes (9.38 ± 0.03, 0.86 ± 0.02), tropicamide in CHO m4 membranes (6.98 ± 0.01, 0.91 ± 0.03) and 4-DAMP (9.20 ± 0.14, 0.77 ± 0.03) PZP (6.59 ± 0.04, 0.95 ± 0.01), tropicamide (6.89 ± 0.05, 0.76 ± 0.02) and methoctramine (7.22 ± 0.01, 1.16 ± 0.03) in CHO m5 membranes.

In addition [^{3}H]-NMS binding was displaced dose dependently by a range of NMBD's in CHO m1 membranes (Figure 1), CHO m2 membranes (Figure 2), CHO m3 membranes (Figure 3), CHO m4 membranes (Figure 4) and CHO m5 membranes (Figure 5) with pK_{50} values and slope factors as shown in Table 1. Rank order pK_{50} values for m1–m5 receptor subtypes is shown in Table 2. There was no correlation between the dose required for tracheal intubation and K_{50} m1–m5 (Linear regression; r^2 0.01–0.04, $P>0.05$) and there is insufficient data in the literature to compare pK_{50} m1–m5 with doses producing unstable cardiovascular responses.

Formation of cyclic AMP was inhibited dose dependently by methacholine (Figure 6). In 15 control experiments the pEC_{50} value and maximum inhibition was 6.32 ± 0.19 and 39.1 ± 3.6% respectively. As a positive control the response to 100 μM methacholine was fully reversed by 1 nM atropine. Pancuronium (300 nM, Figure 6) produced a significant parallel rightward shift in the methacholine response ($pEC_{50-pancuronium}$, 6.18 ± 0.34; $pEC_{50+pancuronium}$, 3.57 ± 0.36, $P<0.05$). There was no difference between the whole curves for methacholine in the absence and presence of 1 μM vecuronium and 1 μM rocuronium ($P>0.05$ two-way ANOVA, data not shown). Pancuronium, rocuronium and vecuronium alone did not affect cyclic AMP formation (data not shown).

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. This interaction with the cardiac m2 receptor may explain the effects of these agents observed on the heart.

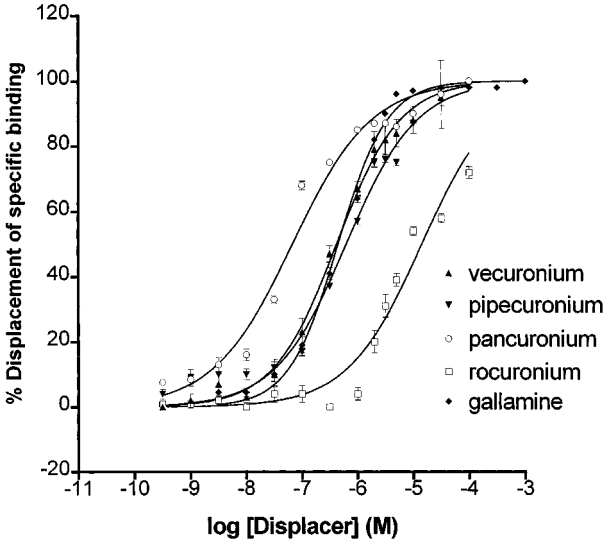

Figure 2 Displacement of [^{3}H]-NMS by neuromuscular blocking drugs in CHO m2 cells. Data$\pm$s.e.mean ($n\geqslant5$).

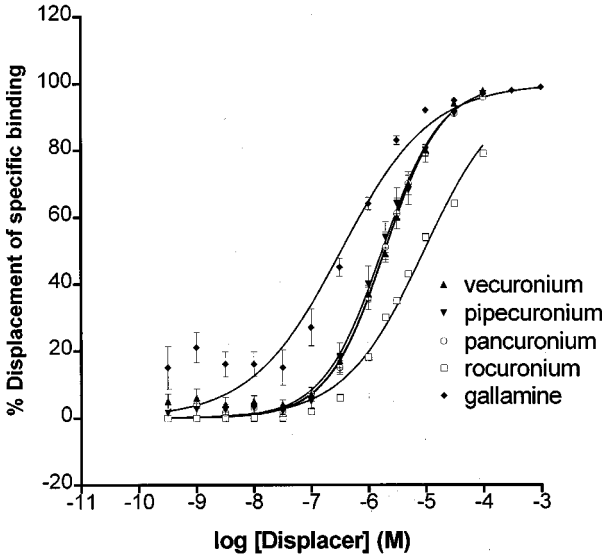

Figure 1 Displacement of [^{3}H]-NMS by neuromuscular blocking drugs in CHO m1 cells. Data$\pm$s.e.mean ($n\geqslant5$).

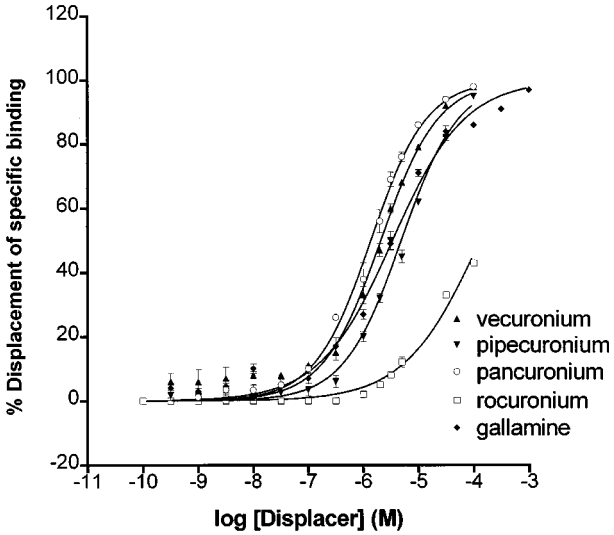

Figure 3 Displacement of [^{3}H]-NMS by neuromuscular blocking drugs in CHO m3 cells. Data$\pm$s.e.mean ($n\geqslant5$).

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 in rat heart (Appadu & Lambert, 1994), in this study we have used the human isoform and can therefore eliminate any interpretation problems related to species differences. In CHO m1–m3 we included pirenzepine, methoctramine and 4-DAMP as high affinity subtype selective reference compounds. The K_{50} values obtained are consistent with values reported in the literature (Waelbroeck *et al*., 1992; Buckley *et al*., 1989) and confirm the subtype expressed. In CHO m4 cells we utilized tropicamide which exhibits modest m4 selectivity and K_{50} of around 100 nM. The rank order K_{50} of 4-DAMP > PZP > tropicamide > methoctramine in CHO m5 cells (Caulfield, 1993) is also consistent with the literature although a value for tropicamide could not be found.

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 adenylyl cyclase and activation lowers intracellular cyclic AMP levels. In addition m2/4 receptors also activate an inwardly rectifying K^+ channel to produce hyperpolarization (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 $Ins(1,4,5)P_3$ and diacylglycerol formation and subsequently increase intracellular Ca^{2+} (Caulfield, 1993; Bonner, 1989). There is some evidence that phospholipase C coupled muscarinic receptors may increase cyclic AMP formation but

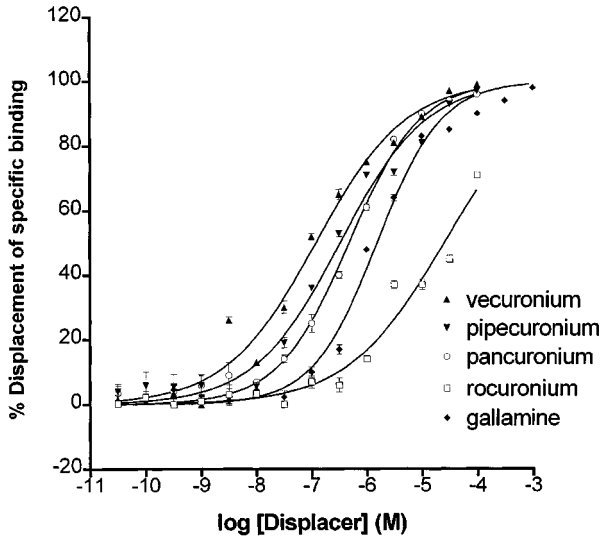

Figure 4 Displacement of [^{3}H]-NMS by neuromuscular blocking drugs in CHO m4 cells. Data ± s.e.mean ($n \geqslant 5$).

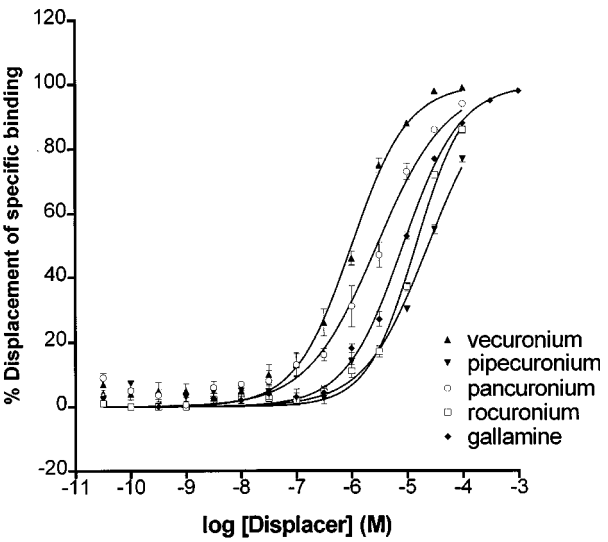

Figure 5 Displacement of [^{3}H]-NMS by neuromuscular blocking drugs in CHO m5 cells. Data ± s.e.mean ($n \geqslant 5$).

Table 1 pK_{50} (K_{50}) values of NMBDs in CHO cells expressing recombinant m1–m5 muscarinic receptors

	CHO m1	*CHO m2*	*CHO m3*	*CHO m4*	*CHO m5*
Pancuronium	6.43 ± 0.12 (371 nM)	7.68 ± 0.02 (21 nM)	6.53 ± 0.06 (295 nM)	6.56 ± 0.03 (275 nM)	5.79 ± 0.10 (1622 nM)
K_{50} 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 (724 nM)	6.90 ± 0.05 (126 nM)	6.17 ± 0.04 (676 nM)	7.31 ± 0.02 (49 nM)	6.20 ± 0.07 (631 nM)
K_{50} 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 (457 nM)	6.58 ± 0.03 (263 nM)	5.94 ± 0.01 (1148 nM)	6.60 ± 0.06 (251 nM)	4.80 ± 0.03 (15849 nM)
K_{50} 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 (3801 nM)	5.40 ± 0.02 (3981 nM)	4.34 ± 0.02 (45709 nM)	5.02 ± 0.04 (9550 nM)	5.10 ± 0.03 (7943 nM)
K_{50} 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 (148 nM)	7.67 ± 0.04 (21 nM)	6.06 ± 0.06 (871 nM)	6.20 ± 0.03 (631 nM)	5.34 ± 0.03 (4571 nM)
K_{50} 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 means ± s.e.mean ($n \geqslant 5$). *Expressed as a ratio of K_{50} m2 (e.g. for pancuronium at m1 371 nM/21 nM = 17.6). With the exception of vecuronium at m4 (#$P < 0.05$) receptors all NMBD's displaced with equal or higher affinity at m2 subtype.

Table 2 Rank order pK_{50} at m1–m5 muscarinic receptor subtypes for gallamine (Ga), pancuronium (Pa), pipecuronium (Pi), vecuronium (Ve) and rocuronium (Ro)

Subtype	*Rank order pK_{50}*
m1	Ga > Pa ≥ Pi > Ve > Ro
m2	Pa = Ga > Ve > Pi > Ro
m3	Pa > Ve > Ga > Pi > Ro
m4	Ve > Pi ≥ Pa > Ga > Ro
m5	Ve > Pa > Ga > Ro > Pi

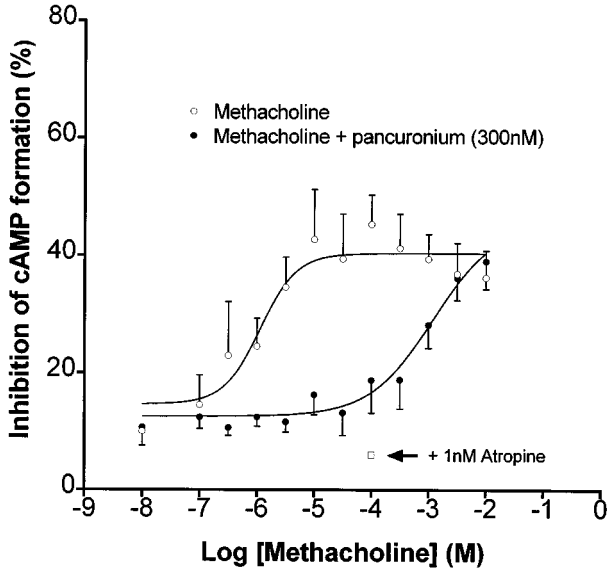

Figure 6 Effect of Pancuronium (300 nM) on methacholine inhibition of forskolin stimulated cyclic AMP formation in CHO m2 cells. Data ± s.e.mean ($n = 7$). As a positive control the effects of 1 nM atropine on 100 μM methacholine response are included.

this is most likely due to a Ca^{2+} dependent activation of adenylyl cyclase (Caulfield, 1993; Hulme *et al*., 1990).

Neuromuscular blocking drugs are used clinically to produce muscle relaxation suitable to facilitate tracheal intubation, ventilation of the lungs and subsequent surgery (Miller & Savarese, 1990). These agents are either depolarizing (e.g. succinylcholine, Puhringer *et al*., 1992) or non-depolarizing and whilst their precise mode of action differs slightly the net result is reduced neuromuscular transmission. Non-depolarizing neuromuscular blockers are potent inhibitors of the muscle type nicotininc receptor (Hunter, 1995).

The cardiovascular 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 & Savarese, 1990) in short, the observed effects result from an interaction with the autonomic nervous system. As demonstrated in this study all the muscle relaxants used interact with the muscarinic receptors at differing concentrations. Typical plasma concentrations producing 50% depression of muscle twitch tension seen in humans are illustrated in Table 3.

Muscarinic blockade of adrenergic neurons reduces the negative feedback regulation on acetylcholine release leading to an increase in heart rate (Vercruysse *et al*., 1978). This effect has been used to explain how pancuronium produces tachycardia, but direct block of sinus node muscarinic receptors (Gardier *et al*., 1978) and direct release of noradrenaline from the nerve terminals (Domenech *et al*., 1976) have also been suggested. The vagolytic effect of pancuronium increases heart rate and hence blood pressure resulting in a baroreceptor mediated decrease in sympathetic tone (Roizen *et al*., 1979). In this study pancuronium has the highest K_{50} for the m2 receptor and an interaction at clinically relevant concentrations could occur at m1, m3 and m4. It is unclear whether pancuronium produces an effect on glandular or GI tract tissue that can be attributed to muscarinic and not nicotininc action. Effects at central muscarinic receptors are unlikely as these agents pass the blood brain barrier extremely poorly (Fahey *et al*., 1989).

Table 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*	$Cp_{50(total)}$ $\mu g\ ml^{-1}$	$Cp_{50(free)}$
Gallamine	0.30	5.82	6.53 μM
Pancuronium	0.93	0.193	245 nM
Pipecuronium	0.75	0.067	68 nM
Vecuronium	0.43	0.09	61 nM
Rocuronium	0.75	1.22	1.5 μM

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

Vecuronium is known to produce bradycardia under 'balanced' anaesthesia (Stevens *et al*., 1997) and may even produce cardiac arrest (Milligan & Beers, 1985). The mechanism underlying these effects are poorly understood. In dog hearts vecuronium increases myocardial contractile force with only a small change in sinus rate. In addition, adrenergic and non-adrenergic cardiotonic properties, inhibition of both postsynaptic muscarinic and neuronal nicotinic receptor mediated responses and a potentiation of the positive chronotropic and inotropic responses to sympathomimetics have also been reported (Narita *et al*., 1992). Vecuronium inhibits the negative cardiac responses to parasympathetic stimulation and carbachol suggesting that vecuronium blocks postsynaptic muscarinic receptors in the dog heart (Narita *et al*., 1992). Our data support an interaction with cardiac m2 (and m4) receptors at clinically achievable concentrations.

Pipecuronium has the most favourable side effect profile with respect to the cardiovascular system. Indeed this is the drug of choice for surgery where cardiovascular stability is particularly important (Foldes *et al*., 1990; Larijani *et al*., 1989). Rocuronium produces minimal change in the cardiovascular system. However a small pancuronium like tachycardia has been observed at high doses in the presence and absence of opioids (Stevens *et al*., 1997; McCoy *et al*., 1993). Consistent with this *in vivo* data we report that at clinically achievable concentrations pipecuronium and rocuronium are unlikely to interact with muscarinic receptors.

Gallamine is a well characterized antagonist at m2 receptors (Ellis *et al*., 1991; Dunlap *et al*., 1983) with a reported K_i of 41.7 nM in binding studies (Leppik *et al*., 1994) and was included in these studies as a reference compound. Clinically gallamine produces an anticipated tachycardia probably resulting from an atropine like block of m2 receptors (Eisle *et al*., 1971; Brown & Crout, 1970).

The clinical relevance of these data clearly resides in the observed interaction with the m2 muscarinic receptor. This subtype is located on the presynaptic terminal of the vagus nerve and postsynaptically on the heart. An agonist-type

interaction on the heart could produce a bradycardia and an antagonist-type interaction could produce a tachycardia. Based on the *in vivo* data described above we would predict that pancuronium would be a muscarinic antagonist and produce atropine like effects and that vecuronium would be an agonist and produce acetylcholine like effects. Cyclic AMP has been shown in many studies to be intimately linked with changes in cardiac activity, as cyclic AMP affects cardiac contractility by altering intracellular Ca^{2+} movement and other cardiac muscle Ca^{2+} related events (Weishaar *et al*., 1988). In an attempt to probe the nature of the interaction with the m2 receptor we examined the effects of pancuronium (an agent that produces a tachycardia), rocuronium (an agent that may produce a weak tachycardia) and vecuronium (an agent that produces a bradycardia). We would predict that pancuronium and to a much lesser extent rocuronium would have antagonist action at m2 receptors and reverse methacholine inhibition of cyclic AMP formation. Vecuronium, on the other hand, would produce an inhibition of cyclic AMP formation in its own right, i.e. possess direct agonist activity. We have clearly shown that pancuronium has antagonist activity that fits with the side effect profile seen with this agent. The lack of effect of rocuronium, an agent that produces a small tachycardia (at higher doses) may be due to the concentration used (1 μM). However, increasing the concentration further would exceed those seen clinically. It is likely that the tachycardia produced by rocuronium does not result from muscarinic antagonism. The lack of antagonist action with vecuronium is again consistent with the clinical profile of this NMBD. However, the lack of direct agonist activity is difficult to explain. In the study of Stevens *et al*. (1997) the bradycardia produced by vecuronium was smaller than the tachycardia produced by pancuronium and was slower to develop. These data may indicate different modes of action, although there is no mechanistic evidence to support this supposition. What is clear from our data is that in CHO m2 cells, when assaying cyclic AMP formation, vecuronium does not posses any agonist activity.

An interaction with the m2 muscarinic receptor alone is unlikely to explain the effects of all neuromuscular blocking drugs on the cardiovascular system. The relative contribution of monoamine uptake inhibition is an important issue that will need to be addressed.

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