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The interaction of hexamethonium with muscarinic receptor subtypes *in vitro*

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1 The action of hexamethonium has been studied at a range of muscarinic receptors *in vitro* by use of both functional and radioligand binding studies.

2 In functional studies, hexamethonium exhibited little or no significant ($P < 0.05$) antagonism of contractile responses to carbachol at muscarinic receptors in the guinea-pig ileum, oesophageal muscularis mucosae, urinary bladder and trachea. However, antagonism was observed at muscarinic receptors in the guinea-pig left atria mediating negative inotropic responses and the calculated pK_B value was 3.80. Hexamethonium also antagonized contractile responses to carbachol in the canine saphenous vein. The pK_B value at these receptors was 3.75.

3 In the presence of 3.2 mM hexamethonium, the pA_2 value for methoctramine at atrial muscarinic receptors was reduced by approximately 10 fold (control pA_2 value was 7.81 ± 0.05; pA_2 value in hexamethonium was 6.73 ± 0.04). In contrast at tracheal muscarinic receptors, the pA_2 values for methoctramine were unaffected in the presence of 3.2 mM hexamethonium (control $pA_2 = 5.58 \pm 0.07$; pA_2 value in hexamethonium was 5.63 ± 0.12). All values quoted are mean ± s.e.mean, $n = 8$.

4 In competition radioligand binding studies, hexamethonium exhibited a higher affinity for cardiac M_2 receptors ($pK_i = 3.68$) than for cerebrocortical M_1 receptors ($pK_i = 3.28$) or for submaxillary gland M_3 receptors ($pK_i = 2.61$). At M_2 receptors hexamethonium at concentrations of 0.1–10 mM, increased the half life of the dissociation rate of [^{3}H]-N-methylscopolamine 1.6–4.3 fold. This was observed at M_3 receptors only at 10 mM, when the half life was increased 1.7 fold.

5 We conclude that hexamethonium, in addition to its well characterized nicotinic antagonist properties, can act as a weak muscarinic antagonist and differentiates between cardiac M_2 receptors and glandular/smooth muscle M_3 receptors. However, hexamethonium differentiates less clearly between M_1 and M_2 receptors. The selectivity between M_2 and M_3 receptors observed in the present study with hexamethonium is comparable to other M_2 selective antagonists such as AF-DX 116 and himbacine.

6 Caution should be exercised with regard to the inclusion of hexamethonium in functional studies of M_2 muscarinic receptor subtypes at concentrations of 0.1 mM and above.

Introduction

Hexamethonium has been shown to act as a nicotinic receptor antagonist and is frequently used to exclude potential nicotinic receptor interactions of muscarinic agonists such as carbachol (e.g. Barlow *et al.*, 1972). A number of workers have also suggested that hexamethonium may act directly at the muscarinic receptor in the ileum (Geddes *et al.*, 1974), ganglia (Brown *et al.*, 1980) and cardiac tissue (Rand & Stafford, 1967; Lüllman *et al.*, 1969; Leung & Mitchelson, 1982; Zonta *et al.*, 1987). It has been shown (Zonta *et al.*, 1987) in functional studies that hexamethonium exhibits a pA_2 value of 4 at atrial muscarinic receptors. Dunlap & Brown (1983) reported an IC_{50} value of 990 μM in competition radioligand binding experiments using atrial membranes. In contrast, hexamethonium, at concentrations of 0.28 mM and above exerted little antagonism at muscarinic receptors in the bronchi or ileum (Barlow *et al.*, 1972; Clague *et al.*, 1985). These data suggest that hexamethonium acts as a selective muscarinic antagonist at receptors mediating nega-

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tive inotropic responses, in comparison to those mediating smooth muscle contraction. In agreement with this hypothesis, it has been shown (Leung & Mitchelson, 1982) that in the presence of hexamethonium, pA_2 values for homatropine and pancuronium at atrial receptors were reduced, whilst no effect was observed on pA_2 values for these antagonists at ileal muscarinic receptors. Similar findings were also observed recently in studies with methoctramine (Eglen *et al.*, 1988a).

The ability of hexamethonium to discriminate between muscarinic receptors in the atria and smooth muscle is consistent with the hypothesis that muscarinic receptors in the atria differ from those in smooth muscle (Barlow *et al.*, 1980). This hypothesis is based upon the differential affinities of a number of antagonists. Those selective for muscarinic receptors in the atria include AF-DX 116, himbacine, methoctramine, gallamine and pancuronium. In addition, those selective for muscarinic receptors in smooth muscle include 4-DAMP (4-diphenylacetoxy-N-methyl piperidine methiodide), hexahydrosiladiphenidol, silahexocylium and silabenzhexol (see Eglen & Whiting, 1986; Mihm & Wetzel, 1987 for reviews).

In the present study we have characterized the action of hexamethonium at muscarinic receptors using both functional and radioligand binding assays. It should be noted that the nomenclature used in this manuscript to describe muscarinic receptor subtypes is based upon proposals by Doods *et al.* (1987) and Hammer *et al.* (1987). Consequently M_1 receptors are characterized by a high affinity for pirenzepine; M_2 receptors exhibit a high affinity for methoctramine, himbacine and AF DX 116 while M_3 receptors exhibit a high affinity for 4-DAMP and hexahydrosiladiphenidol. However, although this classification has yet to encompass muscarinic receptors present on smooth muscle, a number of workers (e.g. Michel & Whiting 1988; Giraldo *et al.*, 1988) have shown similarities between the muscarinic receptor subtype present in glandular and smooth muscle tissue. In the present study, the muscarinic receptors present in smooth muscle are, therefore, referred to as M_3 receptors for the purposes of clarity.

In order to correlate the radioligand binding studies at M_1 receptors, a secondary aim of the study was to examine a functional M_1 receptor assay. O'Rourke & Vanhoutte (1987) have shown that muscarinic receptors mediating contractions of the canine saphenous vein were antagonized by pirenzepine with a high affinity ($pA_2 = 8.1$) and by gallamine with a low affinity ($pA_2 = 4.7$). These data were considered to indicate that M_1 muscarinic receptors mediate contractile responses in this tissue. We have therefore characterized this tissue using pirenzepine, methoctramine and hexamethonium. A preliminary account of this work has been presented at the Dublin meeting of the British Pharmacology Society (Eglen *et al.*, 1988b).

Methods

Functional studies

The following preparations were studied *in vitro*: guinea-pig (Dunkin-Hartley, 300–350 g) ileum, oesophageal muscularis mucosae, trachea, urinary bladder, left atria, and canine saphenous vein. The ileum, oesophageal muscularis mucosae and urinary bladder were placed in Tyrode physiological salt solution (pH 7.4, 37°C); the trachea, left atria, and saphenous were placed in Krebs physiological salt solution (ph 7.4, 37°C). The composition of these salt solutions is given below.

The proximal ileum was prepared according to the method described by Clague *et al.* (1985). Oeso-

Table 1 Muscarinic receptor profiles of preparations used in this study

Functional studies	*Muscarinic receptor subtype*	*Reference*
Canine saphenous vein	M_1	O'Rourke & Vanhoutte (1987)
Guinea-pig left atria	M_2	Clague *et al.* (1985)
Guinea-pig ileum	M_3	Clague *et al.* (1985)
Guinea-pig OMM[a]	M_3	Eglen & Whiting (1988)
Guinea-pig trachea	M_3	Eglen & Whiting (1988)
Guinea-pig urinary bladder	M_3	Eglen & Whiting (1987)
Radioligand binding studies		
Rat cerebral cortex	M_1	Delmendo *et al.* (1989)
Rat cardiac tissue	M_2	Delmendo *et al.* (1989)
Rat submaxillary gland	M_3	Delmendo *et al.* (1989)

[a] OMM = oesophageal muscularis mucosae

phageal muscularis mucosal tissue was prepared according to the method of Kamikawa *et al.* (1985). Zig-zag strips of trachea were prepared according to the method of Eglen *et al.* (1988a). Sheets of urinary bladder tissue were prepared according to methods described by Eglen & Whiting (1987). Two mm rings of canine saphenous vein were prepared according to the method of O'Rourke & Vanhoutte (1987). Left atria were electrically paced (2 Hz, threshold voltage + 20%, 5 ms duration) according to methods described by Blinks (1966). All preparations were suspended under 1.0 g tension with the exception of the left atria which was suspended under 0.5 g tension. In all studies, the preparations were allowed 60 min to equilibrate, during which time the bathing solution was replaced every 15 min.

The experimental protocol was as follows: at the end of the 60 min equilibration period, all smooth muscle preparations were exposed to 50 mM KCl for a period of 30 s or until an equilibrium response was obtained. Preliminary experiments had shown that without prior exposure to KCl, the slope of a second concentration-response curve to carbachol was significantly steeper than the initial curve. The contractions to KCl also provided a measure of the maximal response of the preparation. Thirty min after exposure to KCl, concentration-response curves to carbachol were established. Cumulative curves were constructed in all preparations with the exception of studies using the ileum, in which curves were constructed in a non-cumulative fashion (30 s exposure on a 5 min dose-cycle; Clague *et al.*, 1985). When a 3 fold increase in concentration produced no further increase in response, the tissue was washed at 10 min intervals until the tissue relaxed and baseline tension was re-established.

In experiments in which the effects of antagonists were studied, an equilibration of 60 min was allowed prior to construction of a second concentration-response curve in the presence of antagonist. In experiments studying the effects of hexamethonium alone, 30 min were allowed for equilibration. In all experiments, control tissues were run in which no antagonist was added, in order to check for any change in the sensitivity of the tissue for the agonist.

All responses were determined as changes in isometric tension (mg). The smooth muscle responses were measured with a Hugo Sachs K30 force transducer and displayed on a Graphtec Watanabe Linearecorder. Atrial responses were measured with a Grass FT03 force transducer and displayed on a Grass polygraph.

Radioligand binding studies

Membrane preparations Membranes were prepared as described previously (Michel & Whiting, 1987). EDTA washed cerebrocortical, cardiac and submaxillary gland membranes were prepared from 200–300 g male Sprague-Dawley rats. Tissues were homogenized in 50 mM Tris, 5 mM Na_2EDTA buffer (pH 7.4 at 4°C) in a Polytron P10 tissue disrupter (setting 10; 2 × 10 s bursts). The homogenate was centrifuged at 48,000 *g* for 15 min. The pellet obtained was washed by resuspension and centrifugation, once in homogenizing buffer and twice in 50 mM EDTA buffer (pH 7.4 at 4°C). Membranes were stored under liquid nitrogen until required.

Binding assays Muscarinic receptors in cardiac and submaxillary gland membranes were labelled with [^{3}H]-N-methylscopolamine ([^{3}-H]-NMS) while muscarinic receptors present in rat cerebral cortex were labelled with [^{3}H]-pirenzepine. All binding assays were conducted at 37°C in a final volume of 3 ml of Tris-Krebs assay buffer the composition of which is given below at pH 7.4 at 37°C. In all studies atropine (1 μM) was used to define non-specific radioligand binding (NSB). [^{3}H]-NMS was present at a fixed concentration of 0.1 nM while [^{3}H]-prienzepine was present at a concentration of 0.5 nM. Incubations were for 2 h at 37°C and were terminated by vacuum filtration over Whatman GF/B glass fibre filters using a Brandel 48 well cell harvester. After filtration the filters were washed with 15 ml of ice cold water. The filters were pretreated with 0.1% polyethyleneimine 18 h before use in order to reduce filter binding of the radioligands. Radioactivity retained on the filters was determined by liquid scintillation counting.

In kinetic studies to examine the effect of hexamethonium on the dissociation of [^{3}H]-NMS from muscarinic receptors, 0.2 ml aliquots of a pre-equilibrated solution of muscarinic receptor and [^{3}H]-NMS were added to 20 μl of atropine in the presence and absence of hexamethonium and the dissociation reaction was allowed to proceed for various times before separating bound ligand from free ligand as described above. In all studies the final assay concentration of atropine during dissociation was 1 μM.

Analysis of results

In all functional studies, the data were fitted to a non-linear iterative curve fitting procedure (Parker & Waud, 1971) using RS1 software (BBN corporation). This enabled calculation of the potency (EC_{50}), maximum response and slope of the concentration-response curve. The antagonist affinities were calculated, using at least 4 antagonist concentrations, by the method of Arunlakshana & Schild (1959).

Saturation binding data were analysed using

LIGAND (Munson & Rodbard, 1980) while competition binding data were analysed by a curve fitting programme (Michel & Whiting, 1984) based on the method of Parker & Waud (1971). In the latter case IC_{50} values were converted to K_i values by the Cheng-Prusoff approximation (1973).

Drugs used

The following were used: carbachol (Sigma Chemical Co. Ltd), hexamethonium (Sigma), methoctramine (Research Biochemicals Inc.), [^{3}H]-pirenzepine (NEN), [^{3}H]-NMS (NEN), pirenzepine (Boots plc), AF-DX 116 (Research Biochemicals Inc.) and gallamine (Sigma).

Physiological salt solutions

The compositions of the physiological salt solutions used in the study are given below (mM).

Tyrode solution: NaCl 136.9, KCl 2.7, $MgCl_2 \cdot 6H_2O$ 1.1, $NaH_2PO_4 \cdot 2H_2O$ 0.4, glucose 5.6, $NaHCO_3$ 11.8 and $CaCl_2 \cdot 6H_2O$ 1.8.

Krebs solution: NaCl 118.4, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, glucose 10.0, $NaHCO_3$ 25.0 and $CaCl_2 \cdot 6H_2O$ 2.5.

Tris-Krebs solution: NaCl 118.4, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, glucose 10.0, $NaHCO_3$ 25.0 $CaCl_2 \cdot 6H_2O$ 2.5 and Tris-HCl 10.0.

All solutions were gassed with 5% CO_2 in oxygen.

Results

Functional studies

Hexamethonium (10 μM–3.2 mM) did not significantly antagonize responses to carbachol in the guinea-pig ileum and oesophageal muscularis mucosae. The $-\log EC_{50}$ values in the presence and absence of hexamethonium are shown in Table 2. The pK_B values for hexamethonium at muscarinic receptors in these tissues are therefore less than 2.5. It was, however, observed that a small but significant ($P < 0.05$) dextral shift in the concentration-response curve to carbachol in the presence of 3.2 mM hexamethonium occurred at urinary bladder and tracheal receptors. This was not observed in the presence of lower concentrations of hexamethonium (10 μM–1 mM) in these preparations.

The responses to carbachol in the left atria were significantly ($P < 0.05$) shifted to the right in the presence of hexamethonium. The pA_2 value was 3.75 ± 0.13 and the slope of the Schild plot was 0.57 ± 0.04 (mean $\pm$ s.e.mean, $n = 8$). These data are shown in Table 3 and Figure 1 together with reference pA_2 values and Schild slopes for a range of muscarinic receptor antagonists previously reported by our group (Eglen & Whiting, 1986; Eglen *et al.*, 1988b). Hexamethonium displaced the concentration-response curve to carbachol to the right in a parallel fashion, with no diminution in the maximum responses.

Table 2 Effect of hexamethonium on the potency of carbachol in smooth muscles

Preparation	*Control*	$-\log EC_{50}$ + *Hexamethonium* (3.2 mM)
Ileum	6.83 ± 0.03	6.78 ± 0.08
OMM	6.72 ± 0.05	6.83 ± 0.02
Urinary bladder	6.92 ± 0.03	6.53 ± 0.02*
Trachea	6.13 ± 0.08	5.48 ± 0.12*

Values are mean $\pm$ s.e.mean, $n = 8$.
OMM = oesophageal muscularis mucosae.
* Significantly different from control value ($P < 0.05$; Student's t test).

The pA_2 values for methoctramine were also estimated in the presence and absence of hexamethonium (3.2 mM) at muscarinic receptors present in the left atria and trachea. It was observed that the pA_2 values for methoctramine were significantly ($P < 0.05$) reduced at muscarinic receptors in the left atria but not in the trachea. Control pA_2 values with Schild slopes in parentheses, were 7.81 ± 0.05 (0.83 ± 0.08) and 5.58 ± 0.07 (0.98 ± 0.04); pA_2 values in the presence of 3.2 mM hexamethonium

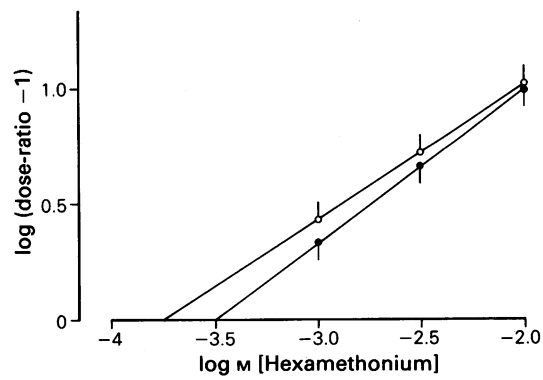

Figure 1 Schild analysis of antagonism of muscarinic responses by hexamethonium in guinea-pig electrically paced atria (○) and canine saphenous vein (●). Values are mean ($n = 4$–8) and vertical lines indicate s.e.mean.

Table 3 Affinity of muscarinic antagonists for muscarinic receptor subtypes in functional studies

	(M_1) *Canine saphenous vein*		(M_2) *Guinea-pig atria*		(M_3) *Guinea-pig ileum*	
Antagonist	pA_2	*Slope*	pA_2	*Slope*	pA_2	*Slope*
AF-DX 116	ND		6.49 ± 0.02	0.96 ± 0.05	5.72 ± 0.03	1.00 ± 0.03
Hexamethonium	3.52 ± 0.13	0.57 ± 0.04*	3.75 ± 0.13	0.57 ± 0.04*	<2.5	—
Himbacine	ND		8.52 ± 0.06	1.00 ± 0.01	7.52 ± 0.08	0.98 ± 0.05
Methoctramine	6.43 ± 0.12	0.81 ± 0.07*	7.81 ± 0.05	0.83 ± 0.08*	5.82 ± 0.07	1.03 ± 0.08
Pirenzepine	8.21 ± 0.04	0.80 ± 0.03*	6.81 ± 0.03	0.93 ± 0.03	6.77 ± 0.05	0.90 ± 0.03

Values are mean (± s.e.mean) from 4–8. The slope of the Schild plot is given in parentheses. Slopes marked with an asterisk indicate significantly different ($P < 0.05$) from unity.
ND indicates data not determined.
Values shown for AF-DX 116 and pirenzepine at atrial and ileal receptors are previously reported by Eglen & Whiting (1986); values for himbacine and methoctramine at these receptors are previously reported by Eglen *et al.* (1988b).

were 6.73 ± 0.04 (1.06 ± 0.13) and 5.63 ± 0.12 (0.91 ± 0.05), respectively; mean ± s.e.mean, $n = 4$–8.

The pA_2 value for pirenzepine, methoctramine and hexamethonium at muscarinic receptors mediating contractions of the canine saphenous vein are shown in Table 3 and Figure 1. The concentration-response curves to carbachol were shifted to the right in a parallel fashion in the presence of hexamethonium. The slope of the Schild plot for hexamethonium, and to a lesser extent, pirenzepine and methoctramine were significantly ($P < 0.05$) less than unity, which indicates deviation from competitive antagonism.

Radioligand binding studies

In ligand binding studies (Figure 2), hexamethonium ($pK_I = 3.68 \pm 0.05$; nH = 0.87 ± 0.04; $n = 3$) displayed higher affinity for the cardiac M_2 muscarinic receptor and the cerebrocortical M_1 ($pK_I = 3.38 \pm 0.12$; nH = 0.83 ± 0.05; $n = 4$) than for the submaxillary gland M_3 receptors ($pK_I = 2.61 \pm 0.05$; nH = 0.63 ± 0.10; $n = 3$). The 11.7 fold selectivity of hexamethonium for M_2 over M_3 receptors was comparable to the 10 fold selectivity of AF-DX 116 for these two receptor subtypes (data not shown).

In these experiments the Hill coefficients for the hexamethonium displacement curves were significantly less than unity. The effect of hexamethonium on radioligand dissociation was also examined for the M_2 and the M_3 receptor. At the cardiac M_2 receptor, hexamethonium at concentrations of 0.1 mM and greater significantly decreased the dissociation rate of [^{3}H]-NMS (Figure 3). The half life of the M_2 receptor-[^{3}H]-NMS complex was increased 1.6, 2.2 and 4.3 fold, respectively, at hexamethonium concentrations of 0.1 mM, 1 mM and 10 mM. Hexamethonium decreased the dissociation rate of [^{3}H]-NMS from the M_3 muscarinic receptor of the rat submaxillary gland 1.7 fold at a concentration of 10 mM. Concentrations of 1 and 0.1 mM did not modify the dissociation rate of [^{3}H]-NMS at the M_3 receptor.

Discussion

In the present study, the interaction of hexamethonium with three subtypes of the muscarinic receptor has been characterized. Hexamethonium has been frequently employed as a tool to exclude

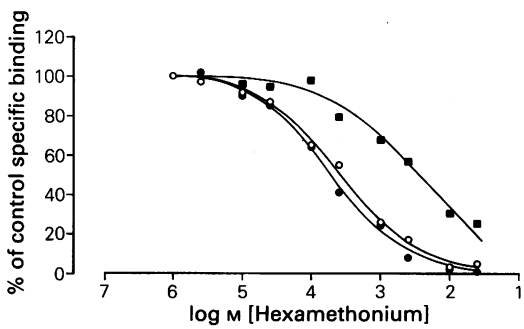

Figure 2 Effect of hexamethonium on the binding of [^{3}H]-pirenzepine to rat cortical muscarinic receptors (○) and [^{3}H]-N-methylscopolamine to rat cardiac (●) and rat submaxillary gland (■) muscarinic receptors. The radioligand and membrane preparation were incubated in the presence or absence of hexamethonium for 2 h prior to separating bound ligand from free ligand. The graphs are from a representative experiment and show the inhibition of control specific binding produced by various concentrations of hexamethonium in the three preparations examined.

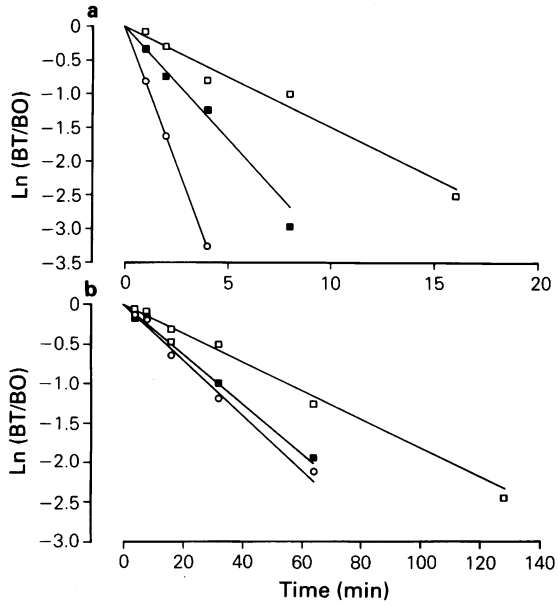

Figure 3 Effect of hexamethonium on the dissociation of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) from muscarinic receptors present in rat cardiac membranes (a) or in rat submaxillary gland membranes (b). Specific binding of [^{3}H]-NMS was measured at various times (BT) after initiating dissociation of equilibrated receptor-[^{3}H]-NMS complexes by adding atropine to a final concentration of 1 μM and is expressed relative to the level of binding immediately before initiating dissociation (BO). Parallel experiments were conducted in the absence (○) and the presence of 1 mM (■) or 10 mM (□) hexamethonium.

nicotinic effects of muscarinic agonists such as carbachol. However, as shown by Leung & Mitchelson (1982), its inclusion can modify the interaction of antagonists with muscarinic receptors. This was demonstrated with pancuronium (Leung & Mitchelson, 1982) and, in the present study, with methoctramine. The effect of hexamethonium appeared to be specific for cardiac M_2 receptors, since the pA_2 values for methoctramine were not significantly affected by inclusion of hexamethonium at tracheal M_3 receptors. These data indicate that hexamethonium may discriminate between cardiac M_2 receptors and M_3 receptors mediating smooth muscle contraction.

This hypothesis is in accordance with studies in which the effects of hexamethonium were studied alone. Antagonism was observed at cardiac (M_2) receptors, but not at M_3 receptors present in the ileum, oesophageal muscularis mucosae and urinary bladder. The reason for the small but significant shift observed at urinary bladder and tracheal M_3 receptors is at present unknown, and is in contrast to data reported by Barlow *et al.* (1972) using bronchial preparations. The lack of effect of hexamethonium at ileal muscarinic receptors is in agreement with previous studies (Barlow *et al.*, 1972; Leung & Mitchelson, 1982).

The interaction of hexamethonium at cardiac M_2 receptors deviated from competitive antagonism, since the slope of the Schild plot was significantly different from unity. This is in contrast to work by Zonta *et al.* (1982) in which a Schild slope of 1.02 was observed. Leung & Mitchelson (1982), using combination dose-ratio studies with hexamethonium and mecamylamine, observed competitive antagonism at all hexamethonium concentrations except 0.2 mM. Geddes *et al.* (1974), suggested that hexamethonium may act allosterically at ileal receptors, a finding further studied in the radioligand binding studies which are discussed below. The estimates of the affinity of hexamethonium for the M_2 receptor, whilst complicated by the lack of competitive antagonism, were slightly less than the pA_2 values obtained by Zonta *et al.* (1987), and by Lüllman *et al.* (1969), i.e. 4.0 and 3.9 respectively.

Hexamethonium, therefore, in agreement with previous studies (see Introduction), appears to act selectively to antagonize M_2 muscarinic receptors, although the precise nature of the antagonism could not be ascertained from the functional studies.

Hexamethonium also appeared to act as an antagonist of M_1 receptors mediating contractions of the canine saphenous vein. The preparation appears to possess functional M_1 receptors since it exhibited a high pirenzepine pA_2 value in agreement with previous reports by O'Rourke & Vanhoutte (1987). The data obtained with methoctramine and hexamethonium further substantiate the M_1 nature of this receptor since the pA_2 value for methoctramine, in this preparation, was intermediate between its affinity at the M_2 and M_3 receptors. This finding parallels the results obtained in the radioligand binding studies. The pK_i value for hexamethonium at the M_1 receptor in the cerebral cortex, was also similar to that observed in the dog saphenous vein. On the basis of the functional data it would appear that while hexamethonium can differentiate between M_2 and M_3 receptors, it cannot distinguish between M_1 and M_2 receptors. In both the radioligand binding and functional studies only a small difference was observed between the affinity of hexamethonium for the M_1 and M_2 receptors. However, again in good agreement with the functional studies, a lower pK_i value was observed at M_3 glandular receptors. While a pA_2 value could not be determined in the functional studies a pK_i value of 2.7 was obtained in the binding studies.

These data suggest that the selectivity of hexamethonium for M_2 receptors, in comparison to M_3 receptors is approximately 12 fold. As can be seen by inspection of either the pA_2 values (Table 3) or pK_i values this order of selectivity is similar to that reported for AF-DX 116 or himbacine (approx. 10 fold). Whilst the absolute affinity of hexamethonium was very low, only methoctramine has been reported to date to exhibit a greater selectivity (approx. 50-fold; Eglen *et al.*, 1988a).

The nature of the antagonism by hexamethonium at M_1 receptors in the canine saphenous vein resembled that observed at cardiac M_2 receptors, in that it also deviated from competitive antagonism. The functional data suggested that hexamethonium exhibits allosteric properties, since the Schild slopes were less than unity. In the binding studies, the low Hill coefficients also indicated possible allosteric interactions. Although, hexamethonium decreased the dissociation rate of [^{3}H]-NMS at both the M_2 and to a lesser extent the M_3 receptor, previous studies by Freedman *et al.* (1986) have demonstrated that a wide range of compounds also interact with the allosteric site on the muscarinic receptor at concentrations of 0.1 to 1 mM.

In conclusion, hexamethonium acts as muscarinic antagonist which discriminates both the M_1 and M_2 receptor from the M_3 receptor. Caution should therefore be used in the inclusion of hexamethonium at concentrations of 0.1 mM and above in studies designed to study the interaction of antagonists with muscarinic receptor subtypes.

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