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Functional and direct binding studies using subtype selective muscarinic receptor antagonists

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1 Muscarinic receptor antagonists were examined in direct binding studies on guinea-pig cardiac and cortical muscarinic receptors. Pirenzepine, dicyclomine and hexahydroadiphenine were shown to be selective ligands for the putative M_1-muscarinic receptor.

2 Functional affinity estimates of the muscarinic ligands studied was determined from their ability to inhibit carbachol-stimulated inositol phosphate (IP) accumulation in guinea-pig cortical slices.

3 The affinity estimates for the inhibition of muscarinic agonist-stimulated IP accumulation were better correlated with affinity estimates obtained from binding studies on the M_1 than the M_2 muscarinic receptor.

4 These data provide additional evidence, both from direct binding and functional studies, for the presence of M_1 and M_2 muscarinic receptor subtypes.

Introduction

Muscarinic receptors are no longer considered to exist as a homogeneous entity, rather, it has been proposed that subtypes of the receptor exist (Burgen, 1984). Currently two classification schemes are used as working hypotheses to characterise the interaction of ligands with muscarinic receptors.

Functional studies have provided evidence that ileal and atrial muscarinic receptors differ since gallamine (Clark & Mitchelson, 1976), 4-DAMP (4-diphenylacetoxy-N-methylpiperidine; Barlow *et al.*, 1980), hexahydrosiladiphenidol (Mutschler & Lambrecht, 1984), CPPS (cyclohexylphenyl [2-piperidinoethyl] silanol; Eglen & Whiting, 1986), himbacine (Anwar-ul *et al.*, 1986) and AF-DX 116 (Giachetti *et al.*, 1986) have been shown to exhibit different affinities at muscarinic receptors in ileal and atrial tissue.

An alternative scheme proposes the presence of M_1 and M_2 receptor subtypes. This classification, originally proposed by Goyal & Rattan (1978) to account for the paradoxical differences in muscarinic receptors of the opposum gastrointestinal tract, has been extended to account for the apparent heterogeneity of muscarinic binding sites identified by the atypical antiulcer drug pirenzepine (Hammer *et al.*, 1980). This classification, which relies heavily upon data obtained from radioligand binding studies, defines those muscarinic receptors that display high affinity for pirenzepine as M_1 and those with low affinity for pirenzepine as M_2 (Hammer & Giachetti, 1982).

Although muscarinic receptors with high affinity for pirenzepine have been identified in functional studies (Goyal & Rattan, 1978, Brown *et al.*, 1980; Gil & Wolfe, 1985), a dual criticism of the M_1/M_2 muscarinic receptor subclassification has been its heavy reliance upon binding data and the restriction of functional studies to those using pirenzepine (Eglen & Whiting, 1985).

Recently, dicyclomine (Luber-Narod & Potter, 1983), AF-DX 116 (Hammer *et al.*, 1986) and hexahydroadiphenine (Michel & Whiting, 1986) have been identified in direct binding studies as additional ligands that can differentiate between M_1 and M_2 muscarinic receptors. However, with the exception of dicyclomine, these compounds have not been examined for M_1-muscarinic receptor affinity in quantitative functional tests, and although dicyclomine has been shown to display high potency for certain muscarinic receptors, these did not appear to relate to any of the conventional pirenzepine binding sites (Marchi & Raiteri, 1985).

In terms of functional studies on the M_1 muscarinic receptor, Gil & Wolfe (1985) have demonstrated that pirenzepine is able to inhibit muscarinic agonist-stimulated inositol phosphate (IP) accumulation with high affinity, suggesting that this response may provide a functional correlate of the M_1 muscarinic receptor identified in direct binding studies.

Therefore, the aims of the present study were firstly to examine, by use of quantitative direct binding studies, the ligands that differentiate between the

putative M_1 and M_2 muscarinic receptors and secondly to determine the affinity of these compounds in inhibiting muscarinic agonist-stimulated IP accumulation.

Methods

Agonist-stimulated inositol phosphate accumulation

The methods used for pre-labelling polyphosphoinositides (PPI) and stimulating IP accumulation were as previously described by Brown *et al.* (1984) with minor modifications. Briefly, 350 × 350 μm, cross chopped, cortical slices were prepared from the cortices of 200–350 g male Dunkin-Hartley guinea-pigs. The slices were washed 3 times with 200 ml of oxygenated Krebs-bicarbonate solution (37°C) during a 30 min period before use. Subsequently, the PPI in 50 μl aliquots of a packed slice preparation were radiolabelled by incubation for 45 min at 37°C in 990 μl of lithium (5 mM) containing Krebs-bicarbonate supplemented with 1 μCi of [^{3}H]-myo-inositol.

At the end of the labelling period, carbachol, the muscarinic agonist used in these studies, was added in a final volume of 10 μl. All experiments were performed in flat bottomed 20 ml glass scintillation vials, rather than in narrow test tubes since greater levels of carbachol-stimulated IP accumulation were obtained when using the scintillation vials. This was presumably due to the greater surface area for transfer of oxygen into the Krebs buffer when using wide bottomed vials as opposed to narrow test tubes. Agonist-stimulated IP accumulation was terminated after a 45 min incubation at 37°C by addition of 2 ml of methanol/chloroform (1:2 vol/vol). IPs formed were extracted into the aqueous phase by sonication (1 min) followed by shaking for 20 min and subsequently separated on Dowex columns (in the formate form) as described by Minneman & Johnson (1984). Radioactivity present in the IP fraction was determined by liquid scintillation counting.

Antagonists when present were added along with the [^{3}H]-myo-inositol, thereby enabling a minimum 45 min antagonist equilibration period.

Data were analysed by iterative curve fitting techniques (Michel & Whiting, 1984) to determine agonist potencies, maximal stimulation of IP accumulation and the slope of the agonist concentration-response curve (CRC).

Ligand binding studies

Binding data were obtained using EDTA washed (Cheung *et al.*, 1982) guinea-pig cerebral cortex and cardiac membranes. Assays were conducted in a final volume of 3 ml of Tris-Krebs buffer system of the following composition (mM): NaCl 144, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.1, D-glucose 10, Tris 10 with a pH of 7.4 at 32°C. Cortical or muscarinic membranes (to provide 5–20 pM final assay concentration of muscarinic receptor binding sites) were incubated with 0.1 nM [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) and competing ligands for 3 h at 32°C. In some studies M_1-muscarinic receptors of cortical membranes were labelled with [^{3}H]-pirenzepine ([^{3}H]-Pir). Bound ligand was separated from free ligand by vacuum filtration with a SKATRON cell harvester. The SKATRON glass fibre filtermats were soaked in 0.1% polyethyleneimine solution 24 h before use in order to reduce filter binding of the radioligands. Non specific binding (NSB) was determined by use of 1 μM atropine. Data were analysed by use of iterative curve fitting techniques (Munson & Rodbard, 1980: Michel & Whiting, 1984).

Materials

[^{3}H]-NMS (specific activity 72 Ci mmol^{-1}), [^{3}H]-Pir (specific activity 76 Ci mmol^{-1}) and [^{3}H]-myo-inositol (specific activity 16 Ci mmol^{-1}) were obtained from Amersham. The [^{3}H]-myo-inositol was purified by addition of several mg of Dowex resin (in the formate form) before use to remove radiolysis products. Hexahydroadiphenine and AF-DX 116 (11-[(2-(diethylamino) methyl-1-piperidinyl)-acetyl]-5, 11-dihydro-6H-pyrido (2, 3-b) (1, 4)-benzodiazepine-6-one) were obtained from Ciba-Geigy. Dicyclomine was synthesized by Dr R. Clark. All other compounds and chemicals used were obtained from Sigma Chemical Company.

Results

Direct binding studies

Saturation binding properties The binding parameters for the radioligands used in the present study are shown in Table 1. In all saturation studies the radioligands identified apparently homogeneous populations of binding sites. Under our assay conditions the cardiac muscarinic receptors identified by [^{3}H]-NMS were exclusively of the M_2 subtype on the basis of the low affinity of pirenzepine for these sites (Table 2) and by the failure to observe even a small component of high affinity pirenzepine binding, both in competition and direct binding studies, in this tissue (data not shown). Cerebrocortical muscarinic receptors labelled with [^{3}H]-NMS appeared to comprise both M_1 and M_2 muscarinic receptors since there were both high and low affinity components to the pirenzepine – [^{3}H]NMS competition curves (Table 2). [^{3}H]-Pir on the other hand apparently bound to a homogeneous population of M_1 muscarinic receptors in cortical membranes since the saturation data with this radioligand indicated the presence of only a single

Table 1 Parameters for the binding of muscarinic receptor ligands to guinea-pig tissue homogenates in modified Tris Krebs buffer

Membranes	*Radioligand*	K_d (mol litre^{-1})	B_{max} (fmol mg protein^{-1})
Cardiac	[^{3}H]-NMS	$3.01 \pm 0.27 \times 10^{-10}$	325 ± 23
Cerebrocortical	[^{3}H]-NMS	$2.24 \pm 0.41 \times 10^{-10}$	2400 ± 283
Cerebrocortical	[^{3}H]-Pir	$2.61 \pm 0.62 \times 10^{-8}$	1207 ± 398

For each radioligand the data represent the affinity estimates obtained from simultaneously fitting data from 4–6 experiments (44–70 data points total) to models assuming the presence of single or multiple populations of interacting or non-interacting sites. In each case the best fit to the data, as adjudged using the extra sum of the squares principle (Munson & Rodbard, 1980), was obtained by assuming the presence of a single population of saturable non-interacting binding sites. Values shown for each parameter represent the mean ± s.e.
[^{3}H]-NMS = [^{3}H]-N-methyl-scopolamine; [^{3}H]-Pir = [^{3}H]-pirenzepine.

population of high affinity binding sites. Furthermore, all of the compounds examined in the present study produced mass action competition curves when using [^{3}H]-Pir as the radioligand in cortical membranes (Table 2).

Pharmacology of ligand binding sites The affinity estimates of the ligands examined at muscarinic receptors of cardiac and cerebrocortical membranes are shown in Table 2. As can be seen dicyclomine, hexahydroadiphenine and pirenzepine were more than 10 fold selective for the M_1 receptors of guinea-pig cerebral cortex labelled with [^{3}H]-Pir than for M_2 muscarinic receptors of cardiac membranes labelled with [^{3}H]-NMS. AF-DX 116 displayed the converse selectivity and was some 1.7 fold M_2 selective. Representative displacement curves for these compounds are shown in Figure 1.

When [^{3}H]-NMS was used to label cerebrocortical membranes a similar selectivity profile was obtained. Thus dicyclomine, pirenzepine and hexahydroadiphenine could be classified as cortical or M_1 muscarinic receptor selective ligands while AF-DX 116 was cardiac or M_2 muscarinic receptor selective.

It should be noted that the overall selectivity of the ligands for cortical muscarinic receptor was somewhat lower when [^{3}H]-NMS rather than [^{3}H]-Pir was used to label these sites (Table 2). In addition it should also be noted that although the pirenzepine – [^{3}H]-NMS competition data indicated the presence of both M_1 and M_2 receptors in the cerebrocortical membranes neither dicyclomine nor hexahydroadiphenine identified this heterogeneity even though they were of comparable M_1 selectivity to pirenzepine.

Inositol phospholipid turnover

Determination of antagonist affinities Functional affinity estimates for antagonists were determined against the carbachol-stimulated accumulation of IP in guinea-pig cortical slices. Carbachol stimulated the accumulation of IP in a concentration-dependent manner ($ED_{50} = 3.2 \times 10^{-5}$ mol litre^{-1}; nH = 0.91) and was a full agonist in guinea-pig cortical slices causing an approximately 5–15 fold (mean value = 8.3) stimulation of IP accumulation over basal levels.

Antagonist affinities in inhibiting carbachol-stimulated IP accumulation are shown in Table 3. The concentration of carbachol that was selected (1 mM) produced appreciable stimulation of IP accumulation but still retained specificity for muscarinic receptors as shown by the failure of hexamethonium (1×10^{-4} mol litre^{-1}), phentolamine (1×10^{-7} mol litre^{-1}), propranolol (1×10^{-7} mol litre^{-1}), physostigmine (1×10^{-6} mol litre^{-1}) or pargyline (1×10^{-4} mol litre^{-1}) to affect the response of the tissue slices to 1 mM carbachol (data not shown).

With the exception of pirenzepine, the Hill slopes of the antagonist-carbachol competition curves were close to unity. Iterative curve fitting of the data indicated that pirenzepine identified two components of carbachol-stimulated IP accumulation (Table 3). Upon converting the IC_{50} values for these components to K_i values by use of the Cheng-Prusoff approximation (1973) it was apparent that pirenzepine inhibited 78% of the carbachol response with a pKi of 8.18 (Table 3). This value was in reasonable agreement with the affinity estimate (8.0) for the M_1 receptor identified in direct binding studies on cortical membranes (Table 2). The low pirenzepine affinity component of carbachol-stimulated IP accumulation appeared to represent an interaction with an M_2 muscarinic receptor subtype since the pKi of pirenzepine (6.23) was similar to the pKi value of 6.51 obtained in direct binding studies on the M_2 muscarinic receptor (Table 2).

Table 2 Affinity estimates for competing ligands at muscarinic receptors labelled by [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) and [^{3}H]-pirenzepine ([^{3}H]-Pir) in guinea-pig cardiac and cerebrocortical membranes

	Cortex (M_1)				*Heart* (M_2)		*Selectivity Ratio*	
	[^{3}H]-Pir		[^{3}H]-NMS		[^{3}H]-NMS			
Compound	pKi	nH	pKi	nH	pKi	nH	A^+	B^+
AF-DX 116	6.95	0.87	6.99	0.98	7.19	1.02	0.6	0.6
	(0.10)	(0.06)	(0.08)	(0.06)	(0.07)	(0.04)		
Atropine	9.20	1.03	9.10	1.00	8.60	1.06	4.4	3.2
	(0.08)	(0.05)	(0.04)	(0.04)	(0.08)	(0.04)		
Dicyclomine	8.70	1.06	8.51	0.91	7.35	1.11	22.4	14.5
	(0.06)	(0.05)	(0.06)	(0.08)	(0.09)	(0.08)		
Gallamine	5.57	0.94	5.01	NC	6.18	NC	0.3	0.1
	(0.06)	(0.05)	(0.06)		(0.09)			
Hexahydroadiphene	8.82	1.23	8.68	0.95	7.34	0.91	30.2	21.9
	(0.05)	(0.11)	(0.09)	(0.06)	(0.06)	(0.04)		
Pirenzepine	8.00	1.00	7.35	0.68*	6.51	0.93	30.9	16.2[a]
	(0.05)	(0.06)	(0.07)	(0.04)	(0.04)	(0.04)		
Secoverine	8.60	1.09	8.55	1.03	8.35	0.98	1.8	1.6
	(0.06)	(0.08)	(0.08)	(0.05)	(0.05)	(0.06)		

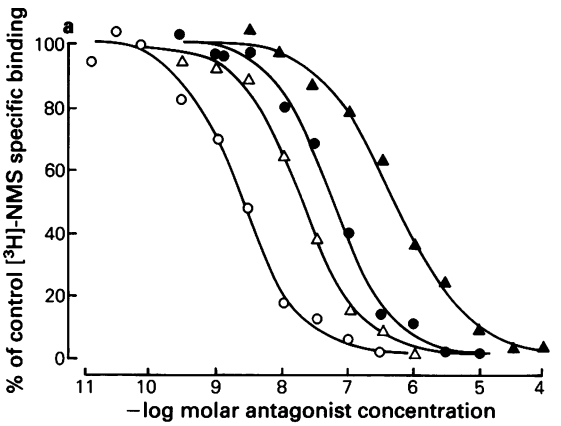
Each value is the mean of 4–6 experiments. Values in parentheses represent s.e.mean.

* denotes a nH value significantly ($P < 0.05$) less than unity. Data better described by assuming the presence of two populations of binding sites. A high affinity site (pKi = 7.72) comprised 52% of the receptors while the remaining 48% of receptors were of low affinity (pKi = 6.8).

NC indicates nH not calculated since gallamine only inhibited 85–90% of specific binding. For these experiments NSB was defined as gallamine displaceable binding and the data were analyzed assuming an interaction with a single population of binding sites.

+ denotes the selectivity ratio. A is Ki vs [^{3}H]-NMS heart/Ki vs [^{3}H]-Pir cortex. B is Ki vs [^{3}H]-NMS heart/Ki vs [^{3}H]-NMS cortex.

[a]For pirenzepine the ratio has been calculated using Ki value for the high pirenzepine affinity component of [^{3}H]-NMS binding.



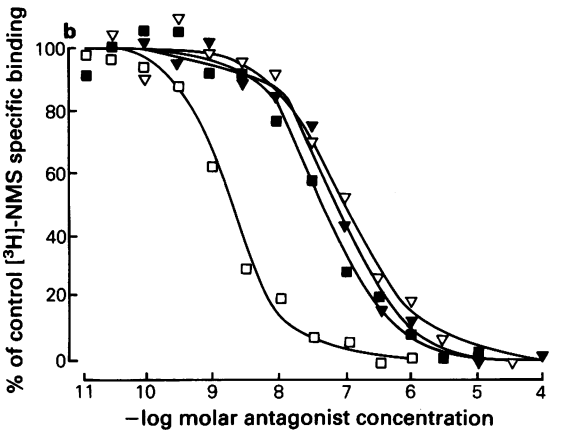


Figure 1 (a) Displacement of specific [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) binding by dicyclomine (○,●) and pirenzepine (△,▲) in either cerebrocortical membranes (open symbols) or cardiac membranes (closed symbols). Data are from a single representative experiment. In cerebrocortical membranes total binding was 1900 d.p.m. and NSB was 95 d.p.m. In the cardiac membranes total binding was 650 d.p.m. and NSB was 70 d.p.m. (b) Displacement of specific [^{3}H]-NMS binding by AF-DX 116 (▽,▼) and hexahydroadiphenine (□,■) in either cerebrocortial membranes (open symbols) or cardiac membranes (closed symbols). Data are from a single representative experiment. In cerebrocortical membranes total binding was 1900 d.p.m and NSB was 95 d.p.m. In the cardiac membranes total binding was 650 d.p.m. and NSB was 70 d.p.m.

Correlation between functional and direct binding data

In Figures 2, 3 and 4 a correlation between the affinity estimates obtained in the direct binding and functional studies have been presented.

The affinity estimates obtained for inhibition of carbachol-stimulated IP accumulation were poorly correlated ($r = 0.724$) with the pKi values obtained at the M_2 muscarinic receptor of cardiac membranes (Figure 2). More importantly, with the exception of AF-DX 116 and atropine, the affinity estimates of the compounds studied for inhibiting IP accumulation and M_2-muscarinic receptor binding were far removed from the line of identity. In contrast, a better correlation ($r = 0.989$) was obtained when affinity estimates obtained in functional studies were compared with inhibition constants determined against [^{3}H]-Pir in cortical membranes (Figure 3). Furthermore, for all compounds studied the data, when plotted as in Figure 3, lay close to the line of identity. On the basis of the pirenzepine displacement isotherm [^{3}H]-NMS only labelled 52% of muscarinic receptors in cerebral guinea-pig cortex with high affinity. Despite this fact, there was a reasonable correlation ($r = 0.977$) between the ability of compounds to inhibit IP accumulation and their affinity for the [^{3}H]-NMS binding sites of guinea-pig cerebral cortex membranes (Figure 4).

Table 3 Muscarinic antagonist affinities in inhibiting carbachol-stimulated inositol phosphate (IP) accumulation in guinea-pig cerebrocortical slices

Antagonist	n	*pKi*	*Hill slope*
AF-DX 116	4	6.71 (0.17)	1.13 (0.07)
Atropine	4	8.93 (0.15)	1.04 (0.03)
Dicyclomine	4	7.92 (0.09)	0.93 (0.05)
Gallamine	3	5.30 (0.16)	0.87 (0.13)
Hexahydroadiphenine	4	8.57 (0.15)	0.87 (0.12)
Pirenzepine	6	7.74 (0.13)	0.67 (0.05)[+]

[+] Data better described by assuming pirenzepine to distinguish two components of carbachol stimulated IP accumulation in guinea-pig cortical slices. The high affinity (pKi = 8.18) component comprised 78% of the stimulation while the low affinity (pKi = 6.23) component comprised the remaining 22%.

Values in parentheses after pKi or Hill slope value represent the s.e. associated with these parameter estimates.

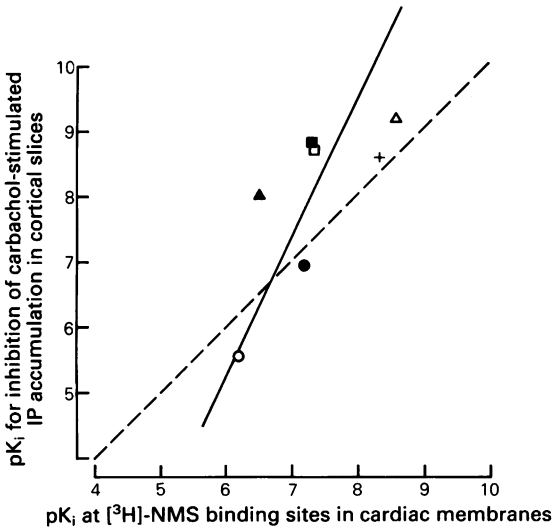


Figure 2 Comparison of antagonist affinities at guinea-pig cardiac [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) binding sites and muscarinic receptors mediating carbachol-stimulated inositol phosphate (IP) accumulation in guinea-pig cortical slices. The dotted line is the line of identity while the solid line is the best fit regression line. Data are for:- (●) AF-DX 116; (△) atropine; (□) dicyclomine; (○) gallamine; (■) hexahydroadiphenine; (▲) pirenzepine; (+) secoverine. Regression coefficient = 0.724.

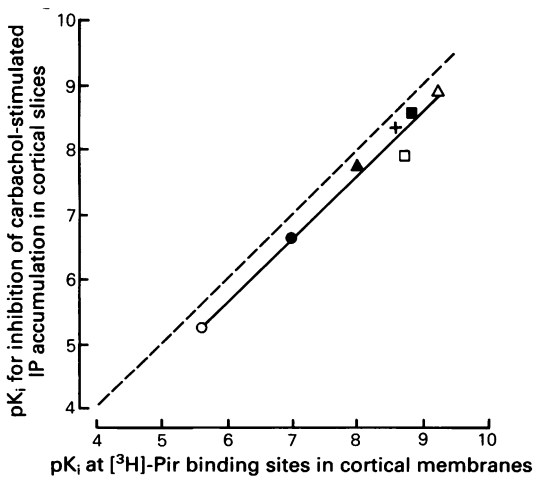


Figure 3 Comparison of antagonist affinities at guinea-pig cerebrocortical [^{3}H]-pirenzepine ([^{3}H]-Pir) binding sites and muscarinic receptors mediating carbachol-stimulated inositol phosphate (IP) accumulation in guinea-pig cortical slices. The dotted line is the line of identity while the solid line is the best fit regression line. Data are for:- (●) AF-DX 116; (△) atropine; (□) dicyclomine; (○) gallamine; (■) hexahydroadiphenine; (▲) pirenzepine; (+) secoverine. Regression coefficient = 0.989.

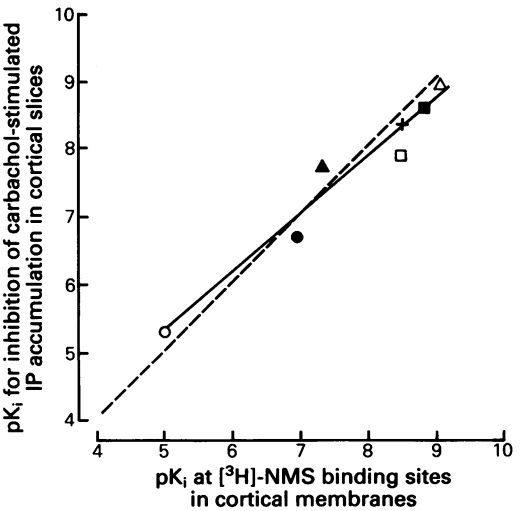


Figure 4 Comparison of antagonist affinities at guinea-pig cerebrocortical [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) binding sites and muscarinic receptors mediating carbachol-stimulated inositol phosphate (IP) accumulation in guinea-pig cortical slices. The dotted line is the line of identity while the solid line is the best fit regression line. Data are for:- (●) AF-DX 116; (△) atropine; (□) dicyclomine; (○) gallamine; (■) hexahydroadiphenine; (▲) pirenzepine; (+) secoverine. Regression coefficient = 0.977.

Determination of antagonist pA_2 values Since the functional data were calculated using the Cheng-Prusoff approximation which might not be applicable in systems in which a receptor reserve exists or where response is not directly coupled to receptor occupancy (Eglen *et al.*, 1987), the affinity estimates of several of the compounds studied were also calculated by the method of Arunlakshana & Schild (1959). The data are presented in Table 4. A representative experiment using pirenzepine is shown in Figure 5a. The Schild plot for pirenzepine is shown in Figure 5b.

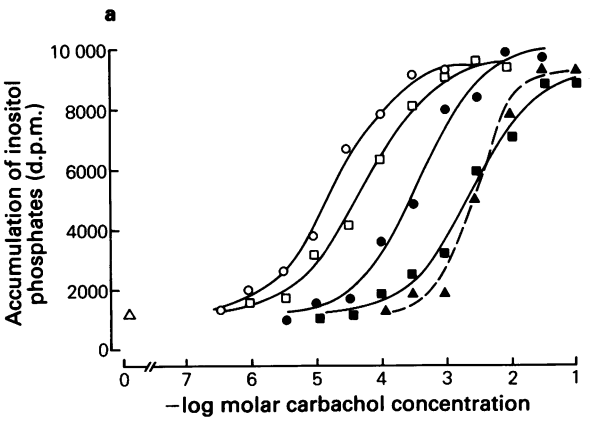
At low concentrations (i.e. those producing a less than 100 fold shift in the carbachol EC_{50}) each of the 6 antagonists studied behaved as a competitive inhibitor of carbachol-stimulated IP accumulation as judged by the approximately parallel rightward shifts in the carbachol concentration-response curve (CRC) produced with no depression in the maximal response. Arunlakshana-Schild (AS) analysis of the data also indicated a competitive mechanism of action for these compounds since AS plots were linear and possessed slopes close to unity. The only exception was dicyclomine for which the slope was marginally lower than unity.

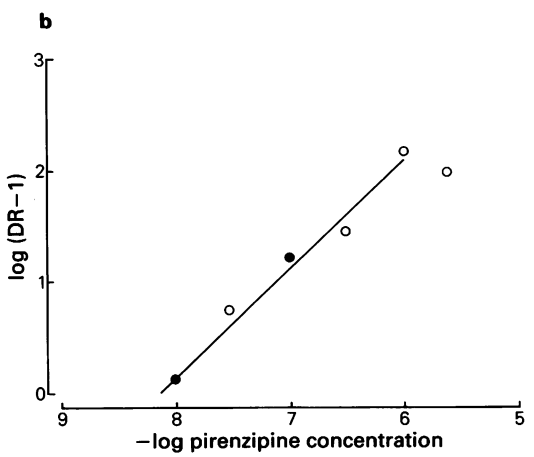
At high concentrations all of the compounds

Table 4 Muscarinic antagonist pA_2 values for inhibiting carbachol-stimulated inositol phosphate (IP) accumulation

Antagonist		*pA_2*	*AS slope*[+]	*pA_2**	*Concentrations tested* (nM)
AF-DX 116	(12)	6.29 (0.13)	0.89 (0.07)	6.19 (0.15)	1000, 3000, 10000, 30000
Atropine	(12)	9.25 (0.09)	0.97 (0.03)	9.23 (0.10)	3, 10, 30, 100
Hexahydroadiphenine	(9)	8.15 (0.10)	1.09 (0.08)	8.21 (0.12)	10, 30, 100
Dicyclomine	(12)	7.88 (0.09)	0.87 (0.09)	7.80 (0.11)	30, 100, 300, 1000
Pirenzepine	(12)	8.15 (0.12)	0.98 (0.05)	8.13 (0.14)	10, 30, 100, 300, 1000
Secoverine	(12)	8.35 (0.10)	1.10 (0.07)	8.42 (0.13)	10, 30, 100, 300

Values in parentheses after compound name represent the number of data points upon which the Arunlakshana-Schild (AS) analysis was based.
Values in parentheses after pA_2 or AS slope values represent the s.e. associated with these parameter estimates.
[+] All AS slope values shown are not significantly different from unity.
*pA_2 with AS slope constrained to unity.





studied displayed signs of non-competitive inhibition of carbachol-stimulated IP accumulation. Thus dicyclomine (3 μM), pirenzepine (3 μM), atropine (1 μM), AF-DX 116 (100 μM) and hexahydroadiphenine (1 μM) produced a marked increase in the slope of the carbachol CRC (Figure 5a: data for pirenzepine). Furthermore, when these data were included in the AS analysis, the AS plots obtained deviated from linearity and displayed slopes lower than unity (data not shown).

For these reasons the pA_2 values were only calculated using the low concentrations of antagonist indicated in Table 4. For atropine, dicyclomine and AF-DX 116 the pA_2 values calculated were in close agreement with the pKi values for inhibition of carbachol-stimulated IP accumulation. For pirenzepine the pA_2 value of 8.15 was almost identical to the high affinity site ($pK_i = 8.18$) associated with carbachol-stimulated IP accumulation.

Figure 5 (a) Inhibition of carbachol-stimulated inositol phosphate (IP) accumulation by pirenzepine. Shown are carbachol-induced increases in the basal level of IP accumulation (△) in the absence (○) or presence of pirenzepine at concentrations of 1×10^{-8} M (□) 1×10^{-7} M (●), 1×10^{-6} M (■) and 3×10^{-6} M (▲). Note that in the presence of 3×10^{-6} M pirenzepine the slope of the carbachol concentration-response curve is significantly greater ($P < 0.05$) than in the absence of pirenzepine. (b) Arunlakshana-Schild (AS) plot for the pirenzepine inhibition of carbachol-induced IP accumulation in guinea-pig cortical slices. The data points represent either the mean of two values (○) or are the mean of 3 values (●). The plot yielded a pA_2 value of 8.15 and an AS slope of 0.98. Note that the data obtained using a pirenzepine concentration of 3×10^{-6} M were omitted from these calculations since this concentration of pirenzepine significantly increased the slope of the carbachol concentration-response curve.

Discussion

The major aim of the present study was to determine the affinity of selective M_1 receptor antagonists in a functional assay and to compare the affinity estimates with those obtained in direct binding studies.

In direct binding studies the three selective M_1 muscarinic receptor antagonists chosen for study, dicyclomine, pirenzepine and hexahydroadiphenine displayed similar M_1 muscarinic receptor selectivity in guinea-pig cortical homogenates assayed in Tris-Krebs buffer as in rat cortical membranes assayed in low ionic strength assay buffer. Thus, in agreement with previous studies using lower ionic strength buffers, pirenzepine (Hammer *et al.*, 1980), dicyclomine (Luber-Narod & Potter, 1984) and hexahydroadiphenine (Michel & Whiting, 1987) were 10–30 fold more potent at the cortical M_1 muscarinic receptor labelled with [^{3}H]-Pir than at the cardiac M_2 muscarinic receptor when assayed in a Tris-Krebs buffer.

Pirenzepine identified heterogeneity in the binding of the non-selective radioligand [^{3}H]-NMS to guinea-pig cortical membranes. The high and low affinity components of pirenzepine binding were consistent with the current definition of M_1 and M_2 muscarinic receptors and confirmed the presence of at least two subtypes of muscarinic receptor in guinea-pig cortex.

It was noticeable that for dicyclomine and hexahydroadiphenine no evidence for similar heterogeneity of [^{3}H]-NMS binding in cortical membranes could be obtained. This was despite the fact that these ligands were comparable to pirenzepine in terms of their selectivity for M_1 muscarinic receptors of cortex over M_2 muscarinic receptors of cardiac tissue. The reason for this discrepancy is uncertain, but may indicate that the M_2 component of [^{3}H]-NMS binding in guinea-pig cortical membranes identified by pirenzepine does not represent binding to the same M_2 receptor as is present in cardiac tissue.

To determine whether these affinity estimates obtained in tissue homogenates were also observed in functional studies, antagonist affinity estimates were determined from the ability of the compounds studied to inhibit muscarinic receptor-mediated stimulation of IP accumulation in cortical slices. Previous studies on muscarinic agonist-stimulated IP accumulation in cortical slices (Gil & Wolfe, 1985; Fisher & Bartus, 1985) have shown this response to be inhibited by low concentrations of pirenzepine suggesting that it is mediated through M_1 muscarinic receptors. For these studies, the ability of the antagonists studied to inhibit the IP accumulation produced by a fixed concentration of carbachol was determined. The concentration of carbachol that was selected (1 mM) was one that produced appreciable stimulation of IP accumulation but which still retained specificity for muscarinic receptors, thereby minimizing the problems of non-specificity of carbachol action.

The use of carbachol at a concentration of 1 mM also helped to reduce the potential problems of ligand depletion arising from the high concentration of muscarinic receptors (0.3 nM; final assay concentration) present in this preparation. Thus based upon an ED_{50} of 3×10^{-5} mol litre^{-1} for carbachol, and using the Cheng-Prusoff approximation, the observed IC_{50} values for the antagonists studied were increased 33 fold. For atropine, the most potent antagonist studied, and consequently the compound for which the potential for ligand depletion was greatest, this IC_{50} value was 2×10^{-8} mol litre^{-1} some 67 fold greater than the concentration of muscarinic receptor binding sites in these preparations. Under these conditions the potential problems of ligand depletion were minimal. The only potential drawback to this method was the need to convert IC_{50} values to affinity estimates using the Cheng-Prusoff approximation (1973). In systems with a high degree of receptor reserve the use of the Cheng-Prusoff approximation may result in an overestimation of antagonist affinity (Eglen *et al.*, 1987). However, since the receptor reserve in cortical slices is low or non-existent (Brown *et al.*, 1984), the approximations made in the present work were valid.

In experiments in which the pirenzepine inhibition of IP accumulation stimulated by a fixed concentration of carbachol was examined, the competition data were characterized by a low Hill coefficient. A detailed analysis of the data indicated that pirenzepine was able to differentiate two components of carbachol stimulated IP accumulation. The major component of IP accumulation displayed a similarly high affinity for pirenzepine as was observed in binding studies on the M_1 muscarinic receptor of cortex labelled with [^{3}H]-Pir and thus appeared to represent an interaction with this receptor. The minor component displayed a pirenzepine affinity that was similar to that obtained in both binding and functional studies on the M_2 muscarinic receptor. Several other groups have also demonstrated the ability of pirenzepine to identify heterogeneity of muscarinic receptors involved in IP accumulation (Fisher & Bartus, 1985: Lazareno *et al.*, 1985).

On the basis of these findings it would appear that pirenzepine identifies at least two subtypes of muscarinic receptor responsible for stimulating IP accumulation in guinea-pig cortex. However, for the other compounds studied, with the exception of gallamine, the Hill coefficients were close to unity indicating that these ligands, unlike pirenzepine, could not identify heterogeneity of muscarinic receptors involved in stimulating IP accumulation. Nevertheless, the affinity estimates obtained for these compounds added credence to the idea that M_1 muscarinic receptors were mediating IP accumulation in guinea-pig cortical slices. Thus, a comparison of affinity estimates for inhibiting carbachol-stimulated IP

accumulation with those obtained in binding studies showed IP accumulation to be better correlated with M_1 muscarinic receptor affinity than with M_2 muscarinic receptor affinity. In particular, both dicyclomine and hexahydroadiphenine displayed a higher affinity in inhibiting IP accumulation and [^{3}H]-Pir binding in cortex whereas gallamine showed low affinity for inhibition of [^{3}H]-Pir binding and carbachol-stimulated IP accumulation.

It should be noted that gallamine has been reported to function as both an allosteric modulator of muscarinic receptors (Stockton *et al.*, 1983; Dunlap & Brown, 1983) and, at low concentrations, as a competitive muscarinic receptor antagonist (Burke, 1986). Detailed mechanistic experiments were not conducted in the present study, although against [^{3}H]Pir binding gallamine appeared to function as a competitive antagonist as judged by the mass action competition curve obtained.

In order to verify the indirect affinity estimates a second approach to determining antagonist affinity, namely that of Arunlakshana & Schild (1959) was employed. With this method, pA_2 values could only be determined with a relatively narrow range of antagonist concentrations. Thus, for the four compounds under study, the use of concentrations of antagonist that produced more than a 100 fold increase in the carbachol EC_{50} also produced evidence for a non-competitive inhibition of carbachol-stimulated IP accumulation. Whether this resulted from non-specificity of agonist action arising from the use of excessively high concentrations of carbachol or was due to the possible non-specific effects of some of the antagonists, as has been demonstrated for dicyclomine (Downie *et al.*, 1977), remains to be determined.

When data obtained with concentrations of antagonist that produced less than a 100 fold increase in the carbachol EC_{50} were analysed, apparently competitive antagonism was obtained as indicated by linear AS plots with slopes close to unity. For dicyclomine, hexahydroadiphenine, AF-DX 116 and atropine the pA_2 values calculated in this manner were in close agreement with the pK_i values obtained against carbachol-stimulated IP accumulation. As previously mentioned for pirenzepine, apparent heterogeneity of muscarinic receptors involved in IP accumulation was observed, and while the overall pKi value differed somewhat from the pA_2, the high affinity pKi was in excellent agreement with the pA_2 value.

Whether the receptors mediating IP accumulation are homogeneous or heterogeneous is still uncertain. While pirenzepine was able to discriminate two populations of binding sites in both functional and binding studies, hexahydroadiphenine and dicyclomine, although displaying similar selectivity as pirenzepine for cortical M_1 as opposed to cardiac M_2 muscarinic receptors, only identified a single population of sites in both binding and functional studies.

Of interest in this regard is the recent demonstration that M_2 muscarinic receptors, as defined by low affinity for pirenzepine, are not homogeneous and that M_2 receptors of cardiac tissue and exocrine glands differ (Hammer *et al.*, 1986). This finding coupled with the observation that in direct binding studies both dicyclomine and hexahydroadiphenine are only 3–5 fold more potent at gland M_2 receptors than at cortical M_1 muscarinic receptors, whereas pirenzepine displays an almost 10 fold lower affinity for gland muscarinic receptors than for cortical M_1 muscarinic receptors (A.D. Michel, unpublished observations), may indicate that the M_2 muscarinic receptors present in cortex more closely resemble those present in the gland than those present in the heart. Clearly such an explanation is speculative at present and further work with more selective ligands for the proposed M_2 muscarinic receptor subtypes is required to confirm or refute such a suggestion.

In conclusion, the present work demonstrates that the muscarinic receptors present in guinea-pig cortex are different from those present in cardiac tissue. This conclusion is derived from both functional and direct binding studies and is not based solely on differences in affinity estimates for pirenzepine but is extended to include two additional compounds of differing chemical structure.

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