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Competitive and non-competitive antagonism exhibited by 'selective' antagonists at atrial and ileal muscarinic receptor subtypes

R.M. Eglen[1] & R.L. Whiting

Institute of Pharmacology, Syntex Research, 3401 Hillview Avenue, Palo Alto, California 94303, U.S.A.

1 The affinity of a number of 'selective' agonists and antagonists has been assessed at atrial or ileal muscarinic receptors by use of *in vitro* functional analysis.

2 The most selective compound for ileal muscarinic receptors was silabenzhexol (approx. 50 fold), and to a lesser extent benzhexol (approx. 5 fold). Conversely, the most selective compound for the atrial muscarinic receptors was AF-DX 116 (approx. 6 fold).

3 The novel M_1-receptor antagonist, telenzepine and other antagonists such as propantheline and isopropamide did not distinguish between atrial and ileal receptors.

4 Dicyclomine, adiphenine, hexahydroadiphenine and oxyphenonium exhibited competitive antagonism at atrial receptors but non-competitive antagonism at ileal receptors. No conclusions could, therefore, be drawn with regard to their selectivity.

5 The agonists, arecaidine propargyl ester (APE), ethoxyethyltriethylammonium (EOE) and carbachol, exhibited some selectivity in potency but little difference in affinity.

6 It is concluded that the study supports the existence of ileal and atrial muscarinic receptor subtypes. However, the use of dicyclomine and related compounds in receptor classification is limited.

Introduction

Muscarinic receptors are currently classified according to their affinity towards pirenzepine. M_1-receptors exhibit a high affinity, whilst M_2-receptors exhibit a low affinity (Hammer & Giachetti, 1982). Atrial and ileal receptors are currently classified as M_2 (Barlow *et al.*, 1981; Clague *et al.*, 1985).

A number of groups have proposed that ileal and atrial receptors may be distinguished pharmacologically (Barlow *et al.*, 1976; Barlow *et al.*, 1980; Barlow & Shepherd, 1985; Fuder *et al.*, 1985; Clague *et al.*, 1985; Giachetti *et al.*, 1986). Antagonists such as 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, Barlow *et al.*, 1976), its pentamethylene derivative (Barlow & Shepherd, 1985), and silicon derivatives of diphenidol and procyclidine (Lambrecht *et al.*, 1984; Mutschler & Lambrecht, 1984; Fuder *et al.*, 1985; Lambrecht *et al.*, 1986) exhibit varying degrees of selectivity towards ileal muscarinic receptors. The most selective of these, hexahydrosiladiphenidol (Fuder *et al.*, 1985) exhibited 27 fold selectivity towards the ileal muscarinic receptor, an order of selectivity comparable with that of pirenzepine for the putative M_1-receptor in ganglia in comparison to the putative M_2-receptor in the ileum (Brown *et al.*, 1980). Differences between ileal and atrial muscarinic receptors have also been apparent in a limited number of ligand binding studies (Choo & Mitchelson, 1985).

Antagonists exhibiting the converse atrial selectivity have, until recently, been limited to the neuromuscular relaxants such as gallamine or pancuronium (see Mitchelson, 1984, for review). The aforementioned compounds are limited in their use in receptor classification, because of their allosteric interactions, resulting in non-linear Schild plots with slopes of less than unity (Mitchelson, 1984; Clague *et al.*, 1985). However, the novel antagonist, AF-DX 116 (11-(2-[(diethylamino) methyl]-1-piperidinyl acetyl)-5, -11-dihydro-6H-pyrido (2.3-b) (1.4)-benzodiazepin-6-one) has been shown, on the basis of ligand binding studies, to exhibit selectivity for M_2-, low pirenzepine affinity sites present in the myocardium (Hammer *et al.*, 1986) and has been proposed as an M_2- selective antagonist (Hammer *et al.*, 1986). In addition, the compound has been reported to exhibit a degree of

[1] Author for correspondence.

selectivity at atrial muscarinic receptors, in comparison to those in smooth muscle (Giachetti *et al.*, 1986). It has also been reported (Anwar-ul *et al.*, 1986) that the plant alkaloid, himbacine, exhibits approx. 10 fold selectivity for atrial muscarinic receptors, in comparison to those on the ileum.

There have been proposed (Mutschler & Hultzsch, 1973; Mutschler & Lambrecht, 1984; Barlow & Weston-Smith, 1985) agonists which exhibit atrial selectivity, i.e. arecaidine propargyl ester (APE), and ileal selectivity, i.e. ethoxyethyltriethylammonium (EOE). However, no data are available on the affinity of these agonists for these receptors.

The aim of the present study was therefore to determine the affinity of a series of muscarinic antagonists and agonists, in order to compare their selectivity. In addition, the selectivity of antagonists recently proposed to exhibit selectivity for the putative M_1-receptor have also been studied. These include, telenzepine, a derivative of pirenzepine, which has been reported to possess a similar *in vivo* selectivity profile (Eltze *et al.*, 1985) and dicyclomine. Dicyclomine has been reported to bind selectively to cerebral cortical membranes, in comparison to those in the myocardium (Kenny *et al.*, 1985: Freedman *et al.*, 1986). It has also been reported that dicyclomine exhibits a higher affinity towards the parotid gland binding sites than to those present in the ileum or urinary bladder (Nilvebrandt & Sparf, 1983). Schiavone *et al.* (1985) found that dicyclomine selectively inhibits gastric secretion. Marchi & Raiteri (1985) reported that dicyclomine antagonized muscarinic receptors that inhibit dopamine release, rather than receptors that inhibit acetylcholine release. These functional properties have been ascribed to its M_1-receptor antagonism. The affinity of telenzepine and dicyclomine (and related compounds such as adiphenine and hexahydroadiphenine) has therefore been assessed in this study at atrial and smooth muscle muscarinic receptors.

Preliminary accounts of this work have recently been communicated to the British Pharmacological Society (Eglen *et al.*, 1985; Eglen & Whiting, 1986).

Methods

The methods used have been described by Clague *et al.* (1985). All experiments were conducted at pH 7.4, 30°C, on tissue from Dunkin-Hartley guinea-pigs (female, 250–300 g body wt). Carbachol, except where indicated, was used as the agonist.

Hexamethonium was absent from the buffer solution, since previous studies (Barlow *et al.*, 1976; Clague *et al.*, 1985) have indicated that nicotinic interactions were without effect on the $-\log K_d$ estimations at the agonist concentrations employed.

Ileum

Portions of proximal ileum (1.5 cm) were isolated, washed and resuspended under 1.0 g tension in modified Tyrode buffer. After 1 h equilibration, agonists were added for 30 s in a 5 min dose cycle. The responsiveness of each tissue was assessed by repeating the carbachol concentration-response curve until reproducible responses were obtained.

Atria

Paired atria were suspended under 1.0 g tension in modified Krebs-bicarbonate buffer. After 1 h equilibration, non-cumulative concentration-response curves were constructed to the agonist. Agonists were added for 3 min, a 5 min dose-cycle was used, and tissue responses were measured over the last 15 s of each agonist exposure period.

Trachea

Helical spirals of trachea were suspended under 1.0 g tension, in similar modified Krebs bicarbonate buffer to that used for the atria. The tissues were allowed 1 h to equilibrate, and cumulative concentration-response curves to each agonist were constructed. Incremental concentrations of each agonist were added either when the previous response had attained a plateau, or after 5 min had elapsed. Concentration-response curves were repeated until reproducible responses were attained.

Bladder

The urinary bladder was isolated distally, and cut tangentally to form an open sheet. This was suspended under 1.0 g tension, and experiments performed as described for the trachea, above.

Measurement of responses

Changes in isometric tension were measured in the ileum, trachea and bladder. In the latter preparation, muscarinic agonists induced increases in both the rate of spontaneous phasic contraction and changes in baseline tension. Only these latter responses were used. Atrial responses were determined as changes in the rate of spontaneous beating.

Antagonist affinity ($-\log K_d$)

Concentration-response curves were repeated in the presence of at least three concentrations of antagonist, allowing 45 min equilibration at each concentration. The dose-ratios were calculated, and the $-\log K_d$ value and Schild slope were derived by the method of

Arunlakshana & Schild (1959). The dose-ratios for each tissue were pooled for each concentration of antagonist and a line of best fit drawn through the data using linear regression by the method of least squares. The intercept with the abscissae and slope of the resulting line were then calculated.

Agonist potency ($-\log EC_{50}$)

The potency of the agonists used was derived by a non-linear iterative curve-fitting procedure (Michel & Whiting, 1985).

Agonist affinity ($-\log K_A$)

The method used was that of Furchgott & Bursztyn (1967). Concentration-response curves to the agonist were constructed prior to and after inactivation of a portion of the receptor population, by incubation of the tissues with phenoxybenzamine (3 μM) for 20 min. After this period, the tissues were washed twice, and the concentration-response curve repeated. These procedures resulted in a dextral shift in the concentration-response curve and a depression in the maximum response of between 20 and 80%. Preliminary experiments had shown that phenoxybenzamine, with this regime, inactivated only the muscarinic receptors, since incubation of the tissues with atropine (0.1 μM) completely protected against inactivation.

Several equiactive concentrations of each agonist before, [A] and after, [A^1] inactivation were determined; 1/[A] was then plotted against 1/[A^1] and a straight line fitted through the data by linear regression analysis; from the slope and the intercept on the ordinate scale, the dissociation constant (K_A) of the agonist-receptor complex was calculated.

Agonist relative efficacy (e_r)

This was determined according to the method of Ringdahl & Jenden (1983), using the relationship:

$$e_r = \frac{e_1}{e_2} = \frac{\dfrac{A_2}{K_{A2} + A_2}}{\dfrac{A_1}{K_{A1} + A_1}}$$

where e_r = relative efficacy; e_1 = efficacy of carbachol; e_2 = efficacy of test agonist; A_1 and A_2 are concentrations of carbachol and the test agonist which elicits 50% response (i.e. EC_{50}); K_{A1} and K_{A2} are dissociation constants of carbachol and the test agonist.

Statistical analysis

This was performed using Student's t test, accepting $P < 0.05$ as being significant.

Physiological salt solutions

The composition was as follows (mM), for the ileum: 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; for the atria, trachea and bladder: 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.

All solutions were gassed with 5% CO_2/95% O_2.

Drugs used

The following were obtained from Sigma: adiphenine, carbachol, isopropamide, oxyphenonium, benzhexol (trihexyphenidyl). AF-DX 116, APE and silabenzhexol were synthesized by Dr R. Clark, Syntex, Palo Alto. The following were generously donated: dicyclomine (Dow-Merrill), EOE (Dr R.B. Barlow, University of Bristol), hexahydroadiphenine (Ciba-Geigy), phenoxybenzamine (S.K and F) and telenzepine (Byk-Guilden).

Results

Antagonists

The $-\log K_d$ values and corresponding Schild slopes are shown in Table 1. All antagonists acted competitively at atrial muscarinic receptors, since the Schild slopes were not significantly different ($P > 0.05$) from unity. Telenzepine, benzhexol, silabenzhexol, propantheline, isopropamide and AF-DX 116 exhibited similar behaviour at ileal muscarinic receptors. In contrast, dicyclomine, adiphenine, hexahydroadiphenine and oxyphenonium acted non-competitively at ileal muscarinic receptors, since the Schild slopes were significantly ($P < 0.05$) less than unity. In all experiments, however, the Schild plots were linear through the concentration range tested for each antagonist.

There were no significant differences ($P > 0.05$) observed between ileal and atrial $-\log K_d$ values obtained with telenzepine, dicyclomine, adiphenine, hexahydroadiphenine, oxyphenonium, propantheline and isopropamide. In contrast, benzhexol and silabenzhexol exhibited significantly ($P < 0.05$) greater $-\log K_d$ values at ileal muscarinic receptors, in comparison to the atria. The converse was true for AF-DX 116.

Dicyclomine, adiphenine, hexahydroadiphenine and oxyphenonium were further studied at muscarinic

Table 1 Antagonist affinities at ileal and atrial receptors

	Ileum		*Atria*	
	$-\log K_d$	Slope	$-\log K_d$	Slope
Telenzepine	7.81 ± 0.03	0.98 ± 0.03	7.91 ± 0.05	1.00 ± 0.08
Benzhexol	8.00[a] ± 0.02	0.99 ± 0.05	7.54 ± 0.01	1.04 ± 0.09
Silabenzhexol	8.83[a] ± 0.05	1.02 ± 0.08	7.48 ± 0.03	1.03 ± 0.03
AF-DX 116	5.72[a] ± 0.03	1.00 ± 0.03	6.49 ± 0.02	0.96 ± 0.05
Propantheline	9.41 ± 0.03	1.04 ± 0.03	9.24 ± 0.08	0.91 ± 0.05
Ispropamide	8.93 ± 0.06	0.96 ± 0.07	8.67 ± 0.06	1.02 ± 0.10
Dicyclomine	7.34[a] ± 0.02	0.65[b] ± 0.09	6.83 ± 0.06	0.97 ± 0.09
Adiphenine	7.01[a] ± 0.01	0.63[b] ± 0.10	6.51 ± 0.03	0.97 ± 0.11
Hexahydroadiphenine	7.97[a] ± 0.05	0.58[b] ± 0.11	7.32 ± 0.02	1.00 ± 0.08
Oxyphenonium	9.95[a] ± 0.09	0.51[b] ± 0.12	9.75 ± 0.05	1.01 ± 0.10

$-\log K_d$ values are mean ± s.e.mean, derived from 4–6 preparations. The slope was calculated as described by Arunlakshana & Schild (1959).
[a]Indicates significantly ($P < 0.05$) different from atrial values. [b]Indicates significantly ($P < 0.05$) different from unity.

receptors present on the trachea and bladder. Similar behaviour was observed at these receptors as was observed at ileal muscarinic receptors. These data are shown in Table 2. The $-\log K_d$ values and Schild slopes observed were not significantly different ($P > 0.05$) from those observed previously in the ileum and in addition the Schild slopes were again significantly ($P < 0.05$) less than unity.

Agonists

The potency ($-\log EC_{50}$) and affinity ($-\log K_A$) for carbachol, APE and EOE are shown in Table 3. In addition, the efficacy (e_r) relative to carbachol is shown in Table 3.

Carbachol and APE exhibited no significant difference ($P > 0.05$) between ileal and atrial muscarinic

Table 2 Antagonist affinities at tracheal and bladder muscarinic receptors

	Trachea		*Bladder*	
	$-\log K_d$	Slope	$-\log K_d$	Slope
Dicyclomine	7.21 ± 0.05	0.62 ± 0.08	7.29 ± 0.06	0.67 ± 0.09
Adiphenine	6.83 ± 0.06	0.70 ± 0.09	6.93 ± 0.08	0.65 ± 0.05
Hexahydroadiphenine	7.92 ± 0.03	0.55 ± 0.10	7.96 ± 0.08	0.51 ± 0.09
Oxyphenonium	9.87 ± 0.08	0.53 ± 0.11	9.88 ± 0.05	0.56 ± 0.11

$-\log K_d$ values are mean ± s.e.mean, derived from 4–6 preparations. All slopes (calculated as described by Arunlakshana & Schild, 1959) significantly ($P < 0.05$) less than unity.

Table 3 Agonist potencies ($-\log EC_{50}$) and affinities ($-\log K_A$) at ileal and atrial receptor

	$-log\ EC_{50}$	$-log\ K_A$	e_r
Ileum			
Carbachol	6.77 ± 0.03	5.09 ± 0.08*	1.00
APE	7.70 ± 0.05	6.74 ± 0.05	4.95
EOE	6.01 ± 0.03*	4.71 ± 0.07*	2.39
Atria			
Carbachol	6.72 ± 0.05	4.76 ± 0.04	1.00
APE	7.85 ± 0.07	6.71 ± 0.05	6.13
EOE	5.00 ± 0.05	4.25 ± 0.07	13.64

$-\log EC_{50}$ and $-\log K_d$ values are mean ± s.e.mean, $n = 4-6$.
*Indicates significantly different ($P < 0.05$) from atrial value.
The greater the relative efficacy value (e_r), the lower the agonist efficacy.

receptors in terms of potency. However, EOE exhibited a significant ($P < 0.05$) 10 fold degree of selectivity for the ileal muscarinic receptor.

Carbachol and EOE exhibited slight, but significant differences ($P < 0.05$), in affinity constants between the two tissues whilst this was not observed for APE. EOE and APE exhibited lower relative efficacy values than carbachol and this was reflected in the fact that, following receptor inactivation, the maximum of the concentration-response curves were depressed to a greater extent with these agonists than with carbachol. However, both compounds were full agonists in these preparations.

Discussion

This study has investigated the differences in affinity of either antagonists ($-\log K_d$) or agonists ($-\log K_A$) at ileal or atrial muscarinic receptors. As in a similar study by our group (Clague *et al.*, 1985), the criteria proposed by Furchgott (1972) and Kenakin (1984) have been used in the interpretation of the data.

The differences in $-\log K_d$ values exhibited by silabenzhexol and to a lesser extent by benzhexol lend support to the hypothesis of ileal and atrial muscarinic subtypes. The difference, particularly with silabenzhexol, was greater than the minimum 0.5 $-\log K_d$ units described by Furchgott (1972) as evidence for receptor heterogeneity. In addition, the Schild slopes were linear and not significantly different from unity, indicating that the $-\log K_d$ value was a good estimate of the affinity constant (Kenakin, 1984).

However, it should be noted that whilst in this study, the replacement of the carbon by silicon increased the ileal affinity of benzhexol, the order of selectivity is not the same as was observed by Mutschler & Lambrecht (1984). The most selective compound identified by this group was hexahydrosiladiphenidol. The reason for this disparity in orders of selectivity between their results and ours is unknown. However, since $-\log K_d$ values for silabenzhexol at muscarinic receptors present on the trachea, bladder or taenia caeci have been found to be about 8.5 (Eglen, unpublished observation) it is possible that the value obtained at ileal muscarinic receptors in the present study, may be an overestimation.

AF-DX 116 exhibited selectivity for the atrial muscarinic receptors. This compound, in contrast to compounds such as gallamine (see Mitchelson, 1984), also acted competitively. These data confirm the myocardial selectivity previously reported by Hammer *et al.* (1986) and Giachetti *et al.* (1986). However, the absolute values obtained in the present study differ from those previously reported (Giachetti *et al.*, 1986: ileum $-\log K_d = 6.4$, atria (force) = 7.3). It is interesting to note that the compound has been reported to exhibit a high degree of selectivity between muscarinic binding sites in the myocardium and lacryimal gland (Hammer *et al.*, 1986), which may further indicate heterogeneity of muscarinic receptors.

These two antagonists, AF-DX 116 and derivatives of benzhexol, appear to be useful agents, in addition to 4-DAMP (Barlow & Shepherd, 1985) and hexahydrosilaldiphenidol (Fuder *et al.*, 1985) in the classification of muscarinic receptors. However, this may not be true for the agonists examined. Carbachol, in agreement with previous data (Clague *et al.*, 1985) was not selective in terms of potency and only marginally so in terms of affinity. This was also observed for APE and in this respect the data differ from those of other workers (Mutschler & Hulztch, 1973; Mutschler & Lambrecht, 1984; Barlow & Shepherd, 1985) who found approximately a 5 fold selectivity for atrial receptors. EOE in terms of potency was found to be 10 fold selective for ileal receptors, in agreement with Barlow & Weston-Smith (1985). However, its selectivity in terms of affinity was less evident and was similar to carbachol. This may indicate other reasons for its selective potency, such as effective receptor reserve.

The putative M_1-antagonists, telenzepine and dicyclomine (Eltze *et al.*, 1985; Schiavone *et al.*, 1985) did not distinguish between ileal and atrial receptors. Telenzepine exhibited a higher $-\log K_d$ for both receptors than was observed with pirenzepine (6·8, Clague *et al.*, 1985) and the atrial value is in good agreement with that of Eltze *et al.* (1985). No conclusion can be arrived at with regard to its M_1-selectivity from the present study.

Dicyclomine, adiphenine, hexahydroadiphenine and oxyphenonium exhibit higher affinities for muscarinic binding sites in the rat cerebral cortex, in

comparison to those in the myocardium (Michel, unpublished observations). All four compounds, however, appeared to possess properties other than muscarinic antagonism since the antagonism was non-competitive at smooth muscle muscarinic receptors. The non-competitive nature of antagonism by dicyclomine observed in this study is in good agreement with observations of other workers (Brown *et al.*, 1950; McGrath *et al.*, 1964; Downie *et al.*, 1977). The compound has been shown (see above references) to possess non-specific muscle relaxant properties and antagonize the contractile responses to histamine and potassium, in addition to muscarinic agonists. These properties appear to limit their use in muscarinic receptor classification using functional assays. The selectivity reported by Schiavone *et al.* (1985) against gastric acid secretion in comparison to motility may be due to its histamine antagonism (Downie *et al.*, 1977). In addition, the disparity between the $-\log K_d$ values obtained by Marchi & Raiteri (1985) at presynaptic muscarinic receptors inhibiting dopamine and acetylcholine release using dicyclomine may also be due to its non-specific actions. One may conclude, therefore, that dicyclomine is not suitable as an M_1-antagonist for functional reasons, in addition to its dissimilar binding characteristics when compared to pirenzepine (Kenny *et al.*, 1985; Freedman *et al.*, 1986), i.e. the Hill slope is unity for rat cortical binding sites.

In conclusion, the data support the concept of ileal and atrial muscarinic receptor subtypes. The use of dicyclomine and related compounds appears to be limited in muscarinic classification, since they exhibit properties unrelated to muscarinic antagonism. The order of selectivity of some ileal and atrial antagonists is now approaching that of pirenzepine, upon which the M_1/M_2 classification is based (Hammer & Giachetti, 1982). Taken together, there are at least two compounds that are selective for muscarinic receptors in the smooth muscle (4-DAMP and hexahydrosiladiphenidol; Barlow & Shepherd, 1985; Fuder *et al.*, 1985) and conversely, two compounds that are selective for the atrial receptors (AF-DX 116 and himbacine; Giachetti *et al.*, 1986 and Anwar-ul *et al.*, 1986). However, it should be noted that there is still a lack of selective agonists and the selectivities observed with the antagonists are still small.

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