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Functional and binding studies with muscarinic M_2-subtype selective antagonists

S. Lazareno & F.F. Roberts

Department of Neuropharmacology, Glaxo Group Research Ltd., Ware, Herts., SG12 ODP

1 The potency of a series of selective muscarinic antagonists has been measured on two functional isolated tissue preparations (rat ileum and atria) and these compared with their potency on a range of binding preparations in order to determine whether the subtypes of M_2 receptor measured functionally are the same as those measured in binding studies.

2 On the functional preparations pirenzepine, hexahydrosiladiphenidol (HSD) and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) were more potent on the ileum than on the atrium (3 fold, 29 fold and 5 fold respectively), whereas himbacine, AF-DX 116 and methoctramine showed the opposite selectivity (5 fold, 3 fold and 56 fold respectively). Atropine had a similar potency on the ileum and atrium.

3 [^{3}H]-N-methyl scopolamine was used to study M_2 binding sites on membranes from rat heart and rat submandibular gland. Each preparation appeared to contain a homogeneous binding site population. The potencies of the five M_2 selective antagonists (and pirenzepine) in binding studies to heart membranes were very similar to those observed in functional studies of rat atria (correlation coefficient = 0.98). Similarly the binding to submandibular gland membranes was very similar to that observed in functional studies on rat ileum (correlation coefficient = 0.97).

4 [^{3}H]-pirenzepine was used to examine the binding of these antagonists to M_1 binding sites on membranes from rat cerebral cortex. The affinities of 4-DAMP, HSD, AF-DX116 and himbacine at M_1 sites were similar to their affinities on the gland. Only pirenzepine and methoctramine had higher affinity on M_1 sites than on the gland.

5 Himbacine had a 20 fold lower affinity at M_1 binding sites than at heart sites, and it should therefore be an important tool in identifying M_1 sites.

6 Inhibition of [^{3}H]-N-methyl scopolamine binding to rat ileum and rat brainstem by M_2-selective antagonists was best described by a two-site model. In both cases the major population of sites (70—90%) appeared to be similar to sites found on the heart (correlation coefficients = 0.95 and 0.97). The other site appeared to be similar to that on the submandibular gland (correlation coefficients = 0.96 and 1.00).

7 The correlations observed in these studies in which a range of selective muscarinic antagonists was used lend weight to previous studies indicating the presence of three functionally important muscarinic receptor subtypes, typified by the binding sites studied in the cerebral cortex, submandibular gland and heart.

8 We propose that the sub-classification of the M_2 muscarinic receptor into M_2 and M_3 subtypes on the basis of ligand binding studies should be extended to cover functionally-defined receptors as well.

Introduction

Although it is generally agreed on the basis of pharmacological studies that muscarinic receptors exist in a variety of subtypes (Mitchelson, 1988) it is not clear yet how many there are and in which tissues they occur. Traditionally receptors with a high affinity for pirenzepine, such as those in the cerebral cortex, are referred to as M_1 and those with a lower affinity for pirenzepine are referred to as M_2 (Goyal & Rattan, 1978). M_2-type receptors have been described in functional preparations such as the guinea-pig isolated atrium and ileum.

Early attempts to distinguish between M_2 receptors used such drugs as 4-diphenyl-acetoxy-N-methylpiperidine (4-DAMP) (Barlow *et al.*, 1976)

and hexahydrosiladiphenidol (HSD) (Mutschler & Lambrecht, 1984). Both these antagonists were more potent on ileum than on atrium. Only gallamine had the reverse selectivity but results obtained with this compound are difficult to interpret due to its allosteric activity (Stockton *et al.*, 1983). Recently three apparently competitive heart selective antagonists have been described: AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]-benzodiazepine-6-one) (Giachetti *et al.*, 1986), methoctramine (N,N'-bis[6-[(2-methoxybenzyl)amino]-hexyl]-1,8-octane-diamine tetrahydrochloride) (Melchiorre *et al.*, 1987) and himbacine (Anwar-ul *et al.*, 1986).

In the present study we have used all of the five selective antagonists mentioned above, together with pirenzepine and the non selective antagonist atropine, in binding and functional studies to a range of tissues. This number of antagonists has enabled quantitative rather than qualitative comparisons between binding and functional studies in a range of preparations and clarifies some of the discrepancies previously described.

Methods

In all experiments tissue from male Hooded Lister rats weighing 150–250 g was used.

Functional studies

Isolated tissue preparations Rat ileum or rat atria preparations were set up in 5 ml organ baths as described by Edinburgh Staff (1970) in a bicarbonate buffered solution at 37°C containing (mM): NaCl 120, KCl 4.7, $MgSO_4H_2O$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 2.5, $CaCl_2$ 2.5, glucose 10, and gassed with 95% O_2 and 5% CO_2. Concentration-effect curves to bethanechol were obtained every hour by use of either serial dosing (atria) or cumulative dosing (ileum). Responses were recorded either with a T3 Bioscience isotonic transducer (ileum contractions) or a Grass FT03 isometric transducer (atria rate of beating).

Experimental design Each antagonist was examined over at least a 30 fold concentration-range, the highest concentration producing a concentration-ratio greater than 30. Each concentration was examined at least in triplicate in independent experiments as described below.

In a typical experiment on rat ileum four preparations were examined in parallel. Two control concentration-effect curves to bethanechol were obtained on each and then each was equilibrated with a different concentration of antagonist. Two concentration-effect curves were then obtained in the presence of the antagonist. Data from preparations in which the latter two curves, or the two control curves, were not superimposable were excluded from the analysis. Each antagonist was examined in at least three experiments.

A somewhat different experimental design was used with atria as only one preparation can be obtained from each animal. Again four preparations were examined on each day, but with this preparation a different antagonist was examined on each. Two control concentration-effect curves to bethanechol were obtained on each before the preparation was exposed to the first concentration of the antagonist. A further concentration-effect curve to bethanechol was then obtained (i.e. corresponding to approximately 40 min of equilibration) before exposure to the next concentration of the antagonist. Three concentrations of antagonist could be examined on each preparation in this way. Preparations in which the atria were arrhythmic or the two control curves were not superimposable were discarded from the analysis. Control experiments established that there was no consistent sensitivity change of such preparations over the normal experiment duration.

Data analysis In order to estimate the concentration-ratio produced by each concentration of antagonist, on ileum preparations the peak contractions during exposure to bethanechol were recorded and plotted against the concentration of bethanechol. Such preparations show a slight reduction of basal tone with time and so strict concentration-ratios were measured corresponding to the effect produced by the EC_{50} concentration of the control curve. With atrial preparations the peak rates of beating during exposure to each concentration of bethanechol were recorded and plotted against the concentration of bethanechol. With atrial preparations no consistent changes in basal rate were seen and EC_{50} values were recorded for the control and subsequent curves. All antagonists appeared to produce parallel shifts of the log concentration-effect curve for bethanechol and none of the slopes of the Schild plots were significantly different from unity. pK_B values for each concentration-ratio were therefore obtained using the Gaddum equation (Schild, 1949):

$$(r - 1) = [B]/K_B$$

where r is the concentration ratio, [B] is the concentration of antagonist and K_B is its apparent dissociation constant, assuming that it is acting competitively. $pK_B = -\log K_B$. The arithmetic mean of these values and their standard errors were calculated.

Binding

Membrane preparations Rats were stunned and decapitated. The cerebral cortices and brainstems, including the colliculi, pons and medulla, were dissected on ice. Hearts and submandibular glands were cleared of fat and cut into small pieces. Ileum longitudinal muscle was obtained as described by Paton & Vizi (1969). Tissues were homogenized in 20 volumes of homogenizing buffer (20 mM NaHEPES plus 10 mM Na_2 EDTA, pH 7.2) with an Ultraturrax homogenizer (three or four 5 s bursts) and incubated on ice for 30 min. The homogenates of peripheral tissues were strained through two layers of gauze. The homogenates were spun at 40,000 *g* for 10 min, resuspended in assay buffer (20 mM NaHEPES + 100 mM NaCl + 0.5 mM Na_2 EDTA, pH 7.4) in a concentration of 200 mg wet weight per ml and either used fresh or stored in 1 ml aliquots at −70°C.

Assay conditions Fresh or thawed homogenate was diluted in assay buffer (5 mg ml^{-1} for cortex, ileum and heart, 3 mg ml^{-1} for submandibular gland or 2 mg ml^{-1} for brainstem) and incubated in duplicate (heart, gland, ileum and brainstem) or triplicate (cortex) with radioligand and competing agents in a final volume of 1.02 ml in polypropylene microfuge tubes at 23°C for 90 min, preliminary studies having shown that equilibrium was attained by this time. The assay was terminated by centrifugation for 2 min at 13,000 *g* in a Burkard Koolspin centrifuge. The pellets were washed twice with iced 0.9% saline, solubilised with 50 μl 1 N NaOH, neutralised and counted for radioactivity at an efficiency of 30–40%. Some assays were terminated by filtration with a Brandel cell harvester and Whatman GF/B filter strips previously soaked in 0.1% polyethylenimine and dried before use: in these cases 5 ml test tubes were used for the assay. The use of filtration had no effect on any pharmacological parameters of test drugs, but it did lead to an increase in the apparent B_{max} of submandibular gland, suggesting that the viscosity of homogenates of this tissue led to incomplete recovery of binding sites in the centrifugation assay.

Experimental design Each assay used a single type of tissue. In assays with [^{3}H]-pirenzepine a fixed concentration of 0.5–0.7 nM was used, and 5–9 concentrations of competing drug. Assays with [^{3}H]-NMS contained a saturation curve with 22 radioligand concentrations between 15 pM and 3 nM, and 5 or 6 inhibition curves against a fixed concentration of 0.2–0.3 nM [^{3}H]-NMS, each consisting usually of 13 or 16 concentrations spanning 4 or 5 log units. Non-specific binding of the fixed radioligand concentration was measured in the presence of 1 μM atropine. No more than 10% of the fixed radioligand concentration was bound to the membranes.

Data analysis Saturation curves were analysed with the multi-site curve fitting programme LIGAND (Munson & Rodbard, 1980). Non-specific binding was estimated by the programme, preliminary experiments having shown that the estimates agreed well with binding measured in the presence of 1 μM atropine. Saturation curves were always best fitted to a one-site model.

The inhibition curves from a single experiment were analysed simultaneously with the logistic curve fitting programme ALLFIT (de Lean *et al.*, 1978). The curves with cortex, heart and submandibular gland had slope factors close to 1 and were fitted adequately with a one-site model using LIGAND. IC_{50} values obtained with [^{3}H]-NMS in heart and submandibular gland were converted to K_i values using the K_d of [^{3}H]-NMS measured in the same experiment and the equation of Cheng & Prusoff (1973); the IC_{50} values obtained with [^{3}H]-PZ in cortex are equivalent to K_i values since the fixed concentration of [^{3}H]-PZ was much lower than its K_d.

Experiments in ileum muscle and brainstem were further analysed by use of LIGAND. Initially a 1-site model was simulated by selecting a 2-site model with all drugs constrained to be non-selective. Then the two K (affinity) values of the flattest curve (usually methoctramine) were allowed to float, and the fit was always significantly improved, indicating that the data were always better fitted to a 2-site model. In subsequent fits the K value of the flattest remaining constrained curve was allowed to float. [^{3}H]-NMS was always constrained to be non-selective since its K values influence the K values of the displacers and any selectivity of [^{3}H]-NMS was too small to measure accurately. If [^{3}H]-NMS did have different affinities for the two sites, constraining them to be the same would result in small systematic errors in the K values of the displacers. Each experiment therefore yielded a single K_d and B_{max} for [^{3}H]-NMS, the proportion of each of the two sites, and K values for each antagonist at each site.

The values reported are the mean and s.e.mean of pK_i, pK_d or B_{max} from 3–6 independent experiments.

Materials

[^{3}H]-N-methyl scopolamine (specific activity 72 Ci $mmol^{-1}$) was obtained from Amersham International. [^{3}H]-pirenzepine (specific activity 87 Ci $mmol^{-1}$) was obtained from New England Nuclear. Atropine sulphate and bethanechol chloride were from Sigma. The following compounds were generous gifts: 4-DAMP (Dr R.B. Barlow, Bristol),

Table 1 pK_B and Schild slope values for inhibiting bethanechol-stimulated responses in atria and ileum

	Atria		*Ileum*	
	pK_B	*Slope* (*n*)	pK_B	*Slope* (*n*)
Atropine	9.16 ± 0.04	1.07 ± 0.08 (9)	9.25 ± 0.05	0.94 ± 0.09 (14)
Pirenzepine	6.31 ± 0.04	0.98 ± 0.10 (15)	6.73 ± 0.05	1.02 ± 0.10 (13)
4-DAMP	8.20 ± 0.02	1.02 ± 0.08 (12)	8.92 ± 0.05	1.05 ± 0.09 (12)
HSD	6.76 ± 0.09	1.36 ± 0.22 (11)	8.22 ± 0.04	0.93 ± 0.10 (9)
AF-DX 116	7.07 ± 0.03	0.98 ± 0.05 (5)	6.59 ± 0.07	0.91 ± 0.06 (3)
Himbacine	8.13 ± 0.05	0.95 ± 0.12 (9)	7.41 ± 0.09	1.03 ± 0.13 (10)
Methoctramine	8.00 ± 0.02	1.01 ± 0.04 (8)	6.25 ± 0.05	1.05 ± 0.10 (12)

The slopes were calculated by linear regression of log (concentration-ratio −1) against the log of the antagonist concentration, and the standard error of the slope was derived from the linear regression analysis. pK_B values were calculated from each concentration-ratio by the Gaddum Equation. Mean ± s.e.mean values are shown. *n* is the number of values. These were obtained from >3 independent experiments except in the case of AF-DX 116 where there was only sufficient drug for 1–2 independent experiments.
For abbreviations see text.

pirenzepine (Dr A. Donetti, de Angeli), hexahydrosiladiphenidol (Dr R. Tacke, Braunschweig), himbacine (Prof. W.C. Taylor, Sydney), methoctramine (Prof. C. Melchiorre, Camerino).

Results

Functional studies

Antagonist potencies at muscarinic receptors in atria and ileum are shown in Table 1. The most selective compounds were HSD (29 fold ileum selective) and methoctramine (56 fold atria selective). 4-DAMP, AF-DX 116 and himbacine had selectivities of 5 fold or less.

Binding studies

Saturation analysis [^{3}H]-NMS bound to apparently homogeneous populations of sites in all four tissues. The saturation parameters are shown in the legends to Tables 2 and 3.

Single site analysis The potencies of drugs for inhibition of [^{3}H]-PZ binding in cortex (M_1 sites) and [^{3}H]-NMS binding in submandibular gland and heart are shown in Table 2. In agreement with previous reports, pirenzepine was most potent in cortex, 4-DAMP and HSD were about 10 fold more potent in gland than in heart, AF-DX 116 was weakly heart-selective, and himbacine and methoctramine were 20 to 30 fold more potent in heart than in gland. Methoctramine had an intermediate potency

Table 2 pK_i and slope factor values for inhibiting [^{3}H]-pirenzepine in cortex (M_1) or [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) in heart and submandibular gland

	Cortex		*Heart*		*Gland*	
	pK_i	*Slope*	pK_i	*Slope*	pK_i	*Slope*
Atropine	9.17 ± 0.05	1.29 ± 0.03	8.99 ± 0.03	0.98 ± 0.01	9.40 ± 0.02	1.20 ± 0.06
Pirenzepine	7.97 ± 0.02	0.91 ± 0.03	6.42 ± 0.02	0.90 ± 0.03	6.99 ± 0.03	0.85 ± 0.06
4-DAMP	8.85 ± 0.11	0.96 ± 0.07	8.19 ± 0.04	0.99 ± 0.03	9.27 ± 0.03	0.97 ± 0.02
HSD	7.88 ± 0.06	1.10 ± 0.05	6.84 ± 0.09	0.91 ± 0.02	8.30 ± 0.02	0.91 ± 0.08
AF-DX 116	6.73 ± 0.06	0.92 ± 0.04	7.15 ± 0.15	0.98 ± 0.12	6.48 ± 0.04	0.94 ± 0.04
Himbacine	7.06 ± 0.05	0.84 ± 0.02	8.43 ± 0.07	0.85 ± 0.06	7.07 ± 0.03	0.88 ± 0.02
Methoctramine	7.30 ± 0.04	0.89 ± 0.05	8.07 ± 0.04	1.07 ± 0.04	6.56 ± 0.02	0.84 ± 0.03

IC_{50} values were estimated with the logistic curve fitting programme ALLFIT, and those obtained with [^{3}H]-NMS were converted to K_i values using the K_d value for [^{3}H]-NMS measured in the same experiment. IC_{50} values obtained with [^{3}H]-PZ were assumed to be equivalent to K_i values. Values are the mean and standard error of at least three observations. K_d and B_{max} values for [^{3}H]-NMS were: heart, 264 ± 22 pM and 9.6 ± 0.6 pmol g^{-1} tissue; submandibular gland, 120 ± 16 pM and 6.7 ± 1.5 pmol g^{-1} tissue ($n \geq 4$).
For abbreviations see text.

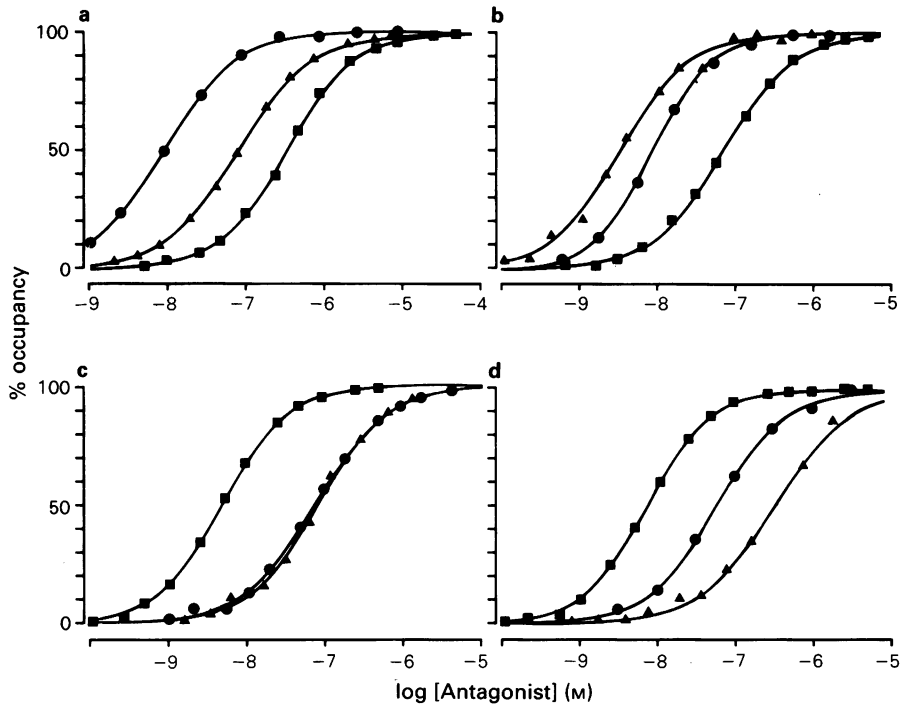

Figure 1 Occupancy curves for four antagonists (a) pirenzepine, (b) hexahydrosiladiphenidol, (c) himbacine, (d) methoctramine at three populations of sites: inhibition curves were constructed against [^{3}H]-pirenzepine ([^{3}H]-PZ) in cortex (●) and [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) in heart (■) and submandibular gland (▲) and analysed with ALLFIT as described in Methods. In each experiment with [^{3}H]-NMS its K_d was measured and used to adjust the concentrations and IC_{50} of competing drugs according to the formula: Adjusted concentration = true concentration/([^{3}H]-NMS]/K_d + 1). Total and non-specific binding were estimated by ALLFIT and used to calculate % inhibition values. These values and the computer derived fits are plotted against the adjusted concentration of competing drug to yield % occupancy curves, i.e. they indicate the predicted occupancy of muscarinic sites by the unlabelled drug had [^{3}H]-NMS not been present. The inhibition curves obtained with [^{3}H]-PZ are equivalent to occupancy curves because the fixed concentration of [^{3}H]-PZ was less than a tenth its K_d. Each curve represents data obtained in a single experiment.

Table 3 Antagonist pK_i at muscarinic receptor subtypes in brainstem and ileum

	Brainstem		*Ileum*	
	R_1	R_2	R_1	R_2
% total sites	84 ± 2		69 ± 9	
4-DAMP	8.4 ± 0.1	9.2 ± 0.1	8.1 ± 0.2	8.8 ± 0.1
HSD	7.1 ± 0.1	8.2 ± 0.1	6.6 ± 0.2	7.7 ± 0.3
AF-DX 116	7.4 ± 0.1	6.3 ± 0.1	7.5 ± 0.2	6.4 ± 0.2
Himbacine	8.3 ± 0.1	6.6 ± 0.1	8.6 ± 0.1	7.3 ± 0.2
Methoctramine	8.4 ± 0.1	6.4 ± 0.1	8.4 ± 0.1	6.8 ± 0.1

Each experiment contained a saturation curve for [^{3}H]-NMS and inhibition curves with four or five of the above drugs. The curves were analysed simultaneously by use of the 2-site curve-fitting programme LIGAND as described in Methods. The most numerous population of sites was designated R_1. Results are the mean and standard error of at least three observations. K_d and B_{max} values for [^{3}H]-NMS were: brainstem, 171 ± 21 pM and 20.1 ± 1.8 pmol g^{-1} tissue; ileum muscle, 240 ± 40 pM and 8.5 ± 1.6 pmol g^{-1} tissue ($n \geqslant 4$).
Abbreviations as in text.

at M_1 sites, while himbacine had a similar low potency in cortex and gland. Drug potencies in heart (in the presence of Mg^{2+}) were not modified by 0.1 mM GTP (data not shown). Occupancy curves for the four most selective compounds are shown in Figure 1.

Two-site analysis No high affinity pirenzepine binding could be detected in ileum or brainstem. The potencies of the five 'M_2-selective' antagonists were determined for a 2-site model as described in Methods, and the values are shown in Table 3. The major site, designated R_1, comprised 84 ± 2% of binding in brainstem and 69 ± 9% in ileum muscle. In both tissues this site was 'heart-like'. Methoctramine was particularly discriminating in these assays (Figure 2).

Correlations

In Figure 3 the potencies of the five 'M_2-selective' antagonists at heart and gland binding sites are plotted against their functional potencies in atria and ileum, and against the two sites detected in brainstem and ileum muscle. Binding potencies in heart were found to be highly correlated ($P < 0.001$) with functional potencies in atria ($r = 0.98$) and with binding potencies at R_1 in ileum muscle ($r = 0.95$) and brainstem ($r = 0.97$), but uncorrelated with functional potencies in ileum ($r = -0.01$) and binding potencies at R_2 ($r = 0.29$ and -0.02). In a complementary way binding potencies in submandibular gland were highly correlated with functional potencies in ileum ($r = 0.97$) and with binding potencies at R_2 in ileum muscle ($r = 0.96$) and brainstem ($r = 1.00$), but uncorrelated with functional potencies in atria ($r = 0.12$) and binding potencies at

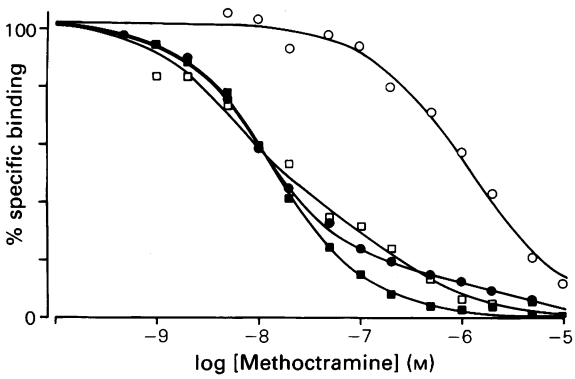

Figure 2 Representative inhibition curves obtained with methoctramine against [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) in heart (■), submandibular gland (○), brainstem (●) and ileum muscle (□). The K_d of [^{3}H]-NMS was measured in each experiment and used to calculate K_i values as described in Methods. The curves in heart and gland were analysed with ALLFIT and yielded pK_is of 8.1 and 6.6 respectively, and slope factors of 0.96 and 0.87 respectively. The curves in brainstem and ileum were analysed with LIGAND to yield the following values: in brainstem pK_1 8.4, pK_2 6.1, R_1 85%; in ileum pK_1 8.6, pK_2 6.9, R_1 64%. The lines were drawn according to these parameters.

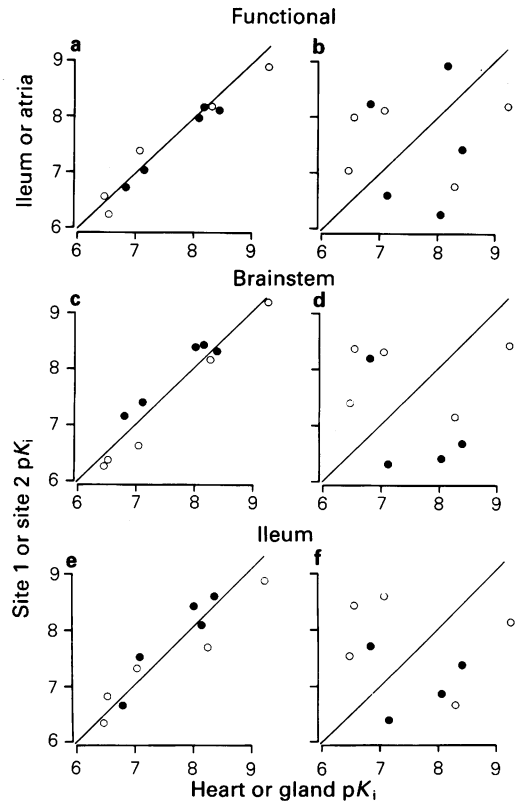

Figure 3 Relationship of mean −log dissociation constants (pK_B or pK_i) from Tables 1, 2 and 3 of 4-diphenyl-acetoxy-N-methylpiperidine, hexahydrosiladiphenidol, AF-DX 116, himbacine and methoctramine measured in functional studies (using atria and ileum) (graphs a and b), and at the two populations of sites found in both brainstem (graphs c and d) and ileum (graphs e and f) (the proportionally larger population of sites in each tissue is designated site 1) and the mean pK_i values found in binding studies in heart (●) and submandibular gland (○). In the left hand graphs (a, c and e) the drug potencies in the atria and at site 1 are plotted against their binding potency in heart (●), and the potencies in ileum and at site 2 are plotted against their binding potency in gland (○). The reverse relationships are plotted in the right hand graphs (b, d and f). The diagonal is the line of unity.

R_1 ($r = -0.28$ and 0.07). Linear regression analysis of the highly correlated measures revealed slopes not significantly different from unity and intercepts not significantly different from zero (results not shown).

Discussion

There are at least four structurally distinct muscarinic receptors expressed in mammalian tissues (Bonner *et al.*, 1987; Peralta *et al.*, 1987). A fifth muscarinic receptor gene (Bonner *et al.*, 1988) has not so far been found to be expressed. A number of recent pharmacological classifications have described three subtypes of muscarinic receptor, but it is not clear whether the classifications refer to the same three subtypes and, in particular, whether the binding site in glands is the same as the functional receptor in smooth muscle (Mitchelson, 1988).

In confirmation of previous reports (Barlow *et al.*, 1976; Mutschler & Lambrecht, 1984; Giachetti *et al.*, 1986; Hammer *et al.*, 1986; de Jonge *et al.*, 1986; Anwar-ul *et al.*, 1986; Melchiorre *et al.*, 1987; Michel & Whiting, 1988a; Wang *et al.*, 1988), we found 4-DAMP, HSD, AF-DX 116, himbacine and methoctramine to discriminate between functional muscarinic responses in atria and ileum, and between apparently homogeneous populations of [^{3}H]-NMS binding sites in heart and submandibular gland. The correlations we have found between these measures support suggestions that the binding sites in heart (mainly ventricle) are equivalent to the receptors studied in the functional atrial preparation, and that the binding sites in gland are equivalent to the receptor subtype mediating the functional response recorded from ileum. We propose that the 'M_1 M_2 M_3' classification (de Jonge *et al.*, 1986; Doods *et al.*, 1987), where M_2 receptors predominate in heart, be applied to muscarinic receptor subtypes studied in both binding and functional studies. These receptors probably correspond to the m1, m2 and m3 genes of Bonner *et al.* (1987) (HM_1, HM_2 and HM_4 genes of Peralta *et al.*, 1987).

In our binding studies, himbacine and methoctramine were 20 to 30 fold selective for heart over gland sites. Methoctramine had intermediate potency for M_1 sites, while himbacine had a similar low potency at M_1 and gland sites. This result suggests that himbacine will be an important tool in the classification of 'M_1' binding sites and receptors, which have hitherto been defined exclusively with the use of pirenzepine. In our functional studies with atria and ileum, however, himbacine was less selective than methoctramine.

The inhibition constants we obtained for methoctramine and AF-DX116 are consistent with the literature, but a disconcertingly wide range of values has been reported for both compounds (e.g. Micheletti *et al.*, 1987; Eglen & Whiting, 1987; Michel & Whiting, 1988a,b; Giraldo *et al.*, 1988). Batink *et al.* (1987) obtained values for AF-DX 116 similar to ours using functional ileum and atria preparations and binding to heart, but a much weaker value with binding to submandibular gland, leading the authors to propose four subtypes of muscarinic receptor. The reason for these discrepancies is unclear.

The inhibition curves we obtained in binding studies with brainstem and ileum muscle were more complex than those obtained in heart and submandibular gland, and were consistent with the presence of two populations of sites. The correlations we obtained, between drug potencies at these two populations of sites and at sites in heart and gland, indicate that in both tissues the major population of sites had a heart-like pharmacology, while the minor population was gland-like.

Heterogeneity of non-M_1 sites in brain has been elegantly demonstrated by Waelbroeck *et al.* (1986; 1987), who found that [^{3}H]-NMS had different kinetics at the subtypes, and took advantage of this fact to restrict heterogeneity in the populations of sites studied with selective antagonists. Flat inhibition curves with AF-DX 116 in various brain regions, suggesting heterogeneity, were also found by Giraldo *et al.* (1987a). In previous studies, binding sites in smooth muscle studied with gland-selective drugs appeared similar to sites in heart (Choo & Mitchelson, 1985; Nilvebrant, 1986). Recently a number of workers have detected heterogeneity in binding to smooth muscle membranes with AF-DX116 (Roffel *et al.*, 1987; Giraldo *et al.*, 1987b; Michel & Whiting, 1987; Monferini *et al.*, 1988; Ladinski *et al.*, 1988) and methoctramine (Michel & Whiting, 1988b). The inability of gland-selective drugs to reveal heterogeneity in smooth muscle membranes may be explained by the small selectivity of available compounds and the fact that the radioligand often used, [^{3}H]-NMS, itself has some selectivity for 'gland-like' sites which will reduce the apparent selectivity of gland-selective drugs (Waelbroeck *et al.*, 1986; 1987). We are able to estimate affinities for gland-selective compounds at the two sites in brainstem and ileum muscle because each experiment, containing both heart- and gland-selective drugs, was analysed as a whole. Our method of analysis caused the most discriminating drugs, methoctramine and himbacine, to constrain the relative proportions of sites in a particular experiment, allowing two-site analysis of 4-DAMP and HSD, which by themselves were not selective enough to define the minor populations of sites.

It is surprising that the 'gland-like' subtype corresponding to functional effects in ileum should comprise only a small fraction of the muscarinic sites.

Our estimates do not take account of any selectivity of [^{3}H]-NMS itself, since [^{3}H]-NMS was always constrained to be non-selective (see Methods). If the correlations obtained with the selective drugs extend to [^{3}H]-NMS, its proportional occupancy at 0.2–0.3 nM will be greater at R_2 than at R_1 sites, and our values for the sizes of R_2, the 'gland-like' component, will be overestimates. It is unlikely that the 'heart-like' sites derive by proteolysis from the 'gland-like' sites since inclusion of protease inhibitors in the membrane preparation and assay did not affect the relative proportions of sites (Michel & Whiting, 1988b; Lazareno, unpublished observations). The recent report that smooth muscle expresses m2 RNA together with m3 RNA (Maeda *et al.*, 1988) supports the suggestion that ileum muscle contains both 'heart-like' (M_2) and 'gland-like' (M_3) receptors. The functional significance of the 'heart-like' sites in smooth muscle is at present unclear.

The 'M_1, M_2, M_3' pharmacological classification of muscarinic receptors is probably incomplete: a fourth muscarinic receptor gene (m4) is expressed in rat brain (Buckley *et al.*, 1988), gene products may undergo post-transcriptional modification, and the pharmacology of a receptor protein may be modified by its membrane environment and effector mechanisms. If further subtypes are contained among the populations we have studied, their presence may be hinted at by some of the anomalies in our data. For example, the selectivity of himbacine was at least four times greater in binding than in functional studies, and its slope factors in 'single site' assays were slightly shallow. These and other anomalies are small and may be related to allosteric effects shown by himbacine (and methoctramine) at concentrations greater than 1 μM (Giraldo *et al.*, 1988; Lazareno, unpublished observations).

In conclusion, our results with both functional and binding studies are consistent with the pharmacological subdivision of muscarinic receptors into M_1, M_2 and M_3 subtypes. M_2 receptors occur in heart, ileum muscle and brainstem, and mediate the negative chronotrophic effect in atria; M_3 receptors occur in submandibular gland, ileum muscle and brainstem and mediate the contractile effect in ileum. Our results also indicate that the cardioselective compounds himbacine and methoctramine are useful new tools in the study of muscarinic mechanisms and receptor subtyes.

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