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Direct binding studies on ileal and cardiac muscarinic receptors

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1 Functional studies have indicated that muscarinic receptors in cardiac tissue differ from those in the ileum. In the present study ileal and cardiac muscarinic receptors identified by [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) were characterized and the selectivity of currently available ileal and atrial selective antagonists determined.

2 In terms of the current functional classification of muscarinic receptors both ileal and cardiac muscarinic receptors were of the M_2 subtype based upon their low affinity for pirenzepine.

3 Cyclohexylphenyl(2-piperidinoethyl)silanol (CPPS), a highly ileal selective antagonist in functional studies, was unable to distinguish between ileal and atrial muscarinic receptors identified in binding studies. Furthermore, although AF-DX 116 and dicyclomine were able to differentiate atrial and ileal muscarinic receptors, neither compound was more than 2 fold selective. These data indicate that it is not possible to subclassify ileal and atrial muscarinic receptors using direct ligand binding studies with these antagonists.

4 In circular ileal smooth muscle, apparent heterogeneity of the M_2 muscarinic receptor population was observed. Thus AF-DX 116 identified two populations of sites with affinities differing by 30 fold. These two populations of M_2 muscarinic receptors may represent the typical M_2 muscarinic receptors identified in cardiac tissue and the more recently discovered 'gland type' M_2 muscarinic receptors.

5 The circular ileal smooth muscle tissue homogenate was able to decrease dramatically the apparent affinity of adiphenine. This activity, which appeared to result from a phenylmethylsulphonylfluoride (PMSF) sensitive protease effect, should be considered when conducting studies using this tissue preparation and compounds of similar structure to adiphenine.

Introduction

Within the past decade data obtained in both functional and ligand binding studies have indicated that several antagonist ligands bind with differential affinities to muscarinic receptors obtained from different tissues and that these findings are not consistent with the presence of a homogeneous population of muscarinic receptors (Burgen, 1984). In order to account for these paradoxical findings it has been suggested that muscarinic receptor subtypes exist. Currently two classification schemes are used as working hypotheses to characterize the interaction of ligands with muscarinic receptors.

The first, and most widely used, of these classification schemes 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 anti-ulcer drug pirenzepine (Hammer *et al.* 1980). This classification, which relies heavily upon data obtained in radioligand binding studies, defines those muscarinic receptors that display high affinity for pirenzepine as M_1 and those with low affinity as M_2 (Hammer & Giachetti, 1982).

Functional studies, although providing supportive evidence for the concept of M_1 and M_2 muscarinic receptor subtypes (Goyal & Rattan, 1978; Brown *et al.*, 1980), have contributed evidence for an alternative subclassification of muscarinic receptors. Thus, based on data obtained with gallamine (Riker & Wescoe, 1951; Clark & Mitchelson, 1976) and 4-DAMP (Barlow *et al.*, 1980), the muscarinic receptors in the atria appear to differ from those in the ileum. Recent studies have provided further supportive evidence for differences in ileal and atrial muscarinic receptors in that hexahydrosiladifenidol (Mutschler & Lambrecht, 1984), cyclohexylphenyl(2-piperidinoethyl)silanol (Eglen & Whiting, 1986), himbacine (Anwar-ul *et al.*,

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1986) and AF-DX 116 (Giachetti *et al.*, 1986) have been shown to differentiate ileal and atrial muscarinic receptors.

To date only Choo & Mitchelson (1985) and Choo *et al.* (1985) have investigated the potential differences between ileal and atrial muscarinic receptors using ligand binding studies. The purpose of the present study was therefore to characterize the ileal and cardiac muscarinic receptor binding sites and to determine the affinity of the currently available ileal and atrial selective antagonists cyclohexylphenyl(2-piperidinoethyl)silanol (CPPS) and AF-DX 116 for these receptors.

Methods

Membrane preparation

In all studies EDTA washed membrane preparations (Cheung *et al.*, 1982) were utilized. The heart and entire ileum were removed from male Dunkin-Hartley guinea-pigs (300–450 g; Bantam and Kingman). In the present study tissues from 30 animals were utilized. The preceding and all subsequent membrane preparation procedures were conducted at 4°C. Atria and ventricles were dissected from each other. The ileal contents were washed with ice-cold Tris-EDTA homogenizing buffer (50 mM Tris; 5 mM disodium EDTA; pH 7.4 at 4°C). Longitudinal ileal smooth muscle was separated from the underlying circular muscle by the method of Rang (1964). The remaining ileal tissue was everted and the circular muscle separated from the mucosal and serosal contents by repeatedly passing strips of muscle through serrated forceps until an opaque muscle layer remained.

Tissues were separately homogenized (1 g wet weight tissue to 30 ml buffer; Polytron P10: setting 10: 2 × 20 s bursts) in homogenizing buffer, passed through a double layer of cheesecloth and centrifuged at 500 *g* for 10 min. The supernatant was reserved and the pellet rehomogenized in buffer, filtered and centrifuged as described above. The supernatant from this centrifugation was combined with the original supernatant and centrifuged at 30,000 *g* in an RC5B refrigerated centrifuge (SS34 head) for 15 min. The supernatant from this step was discarded and the pellet washed superficially with 2 × 40 ml aliquots of homogenizing buffer before resuspending (Polytron P10; 1 × 5 s burst; setting 5) in homogenizing buffer and centrifuging at 30,000 *g*. The membrane pellet so obtained was washed a further two times using the same protocol but substituting ice-cold Tris-EDTA-magnesium (TEM) assay buffer (composition:- 50mM Tris; 0.5 mM EDTA; 5 mM $MgCl_2$; pH 7.4 at 4°C) for homogenizing buffer. The final membrane pellet obtained was resuspended in assay buffer (pH 7.4 at 32°C) and stored under liquid nitrogen until required. It should be noted that similar results to those described in this paper were obtained when using atrial and longitudinal ileal smooth muscle membranes that had been prepared and assayed in the presence of 1 mM phenylmethylsulphonylfluoride (PMSF) and 5 mM EDTA and in the absence of divalent cations.

Binding assays

In some experiments TEM assay buffer was used. The majority of experiments were performed using a modified Tris-Krebs solution 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: pH 7.4 at 32°C. This buffer was utilized in preference to a modified Krebs-bicarbonate buffer (composition), mM: NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.1, D-glucose 10, which was used in initial studies, but which proved impractical due to the requirement to gas assay tubes in order to maintain adequate pH control. Similar K_D values for [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) were obtained with the latter two buffer systems.

Saturation experiments

In saturation studies, [^{3}H]-NMS was incubated to equilibrium with membranes (4–20 pM with respect to [^{3}H]-NMS binding sites) in polypropylene tubes containing the appropriate assay buffer at 32°C (preliminary kinetic experiments indicated that 3 h was sufficient for equilibration of the lowest concentrations of [^{3}H]-NMS (5 pM) used in these studies).

The assay volume was 3 ml. In all studies 10–13 concentrations of radioligand, ranging from at least 10 fold lower to 10 fold greater than the [^{3}H]-NMS K_D values, were employed. At each concentration of radioligand total binding was determined in triplicate and non-specific binding (NSB), defined as radioligand binding in the presence of 1 μM atropine, was determined in duplicate. Extensive studies established that this concentration of atropine adequately defined NSB of [^{3}H]-NMS at concentrations from 5×10^{-12} to 4×10^{-9} mol l^{-1}.

At equilibrium, bound ligand was separated from free ligand by vacuum (22 mmHg) filtration over double SKATRON receptor binding filtermats using a SKATRON cell harvester. After filtration the filtermats were washed for 10 s with 5 ml of room temperature distilled water. The filtermats were pretreated with polyethyleneimine, by soaking in a 0.1% solution 1–2 h before use, in order to reduce filter binding of [^{3}H]-NMS.

Competition experiments

In these studies a fixed concentration of [^{3}H]-NMS (80–150 pM) was incubated with membranes (4–

10 pM with respect to [^{3}H]-NMS binding sites) in a final volume of 3 ml of the appropriate assay buffer for 3 h at 32°C in the presence or absence of the competing compounds. For all compounds studied at least 15 concentrations (ranging from 1000 times below to 1000 times greater than the IC_{50} value for the compound under study) were employed.

Bound from free ligand was separated by vacuum filtration as previously described.

Data analysis

All binding data were analysed by use of iterative curve fitting procedures. Saturation and competition binding data were analysed by use of LIGAND (Munson & Rodbard, 1980). Competition binding data were also analysed by use of an iterative curve fitting program (Michel & Whiting, 1984) to provide affinity estimates and Hill coefficients (n_H) for competing compounds. When data were analysed by the latter method, the IC_{50} values obtained were corrected for the presence of radioligand according to the method of Cheng & Prusoff (1973). Unless otherwise stated, all values are the geometric mean, and s.e.mean, derived from between 4 and 6 experiments. Statistical evaluations were performed by means of Student's unpaired *t* test.

Protein assays

Protein was assayed by the dye binding method (Biorad) using bovine serum albumin as standard.

Drugs and radiochemicals

[^{3}H]-NMS (specific activity 72 Ci mmol^{-1}) was obtained from Amersham. Pirenzepine hydrochloride was obtained from Boeringer Ingelheim. Adiphenine, atropine sulphate, N-methyl atropine chloride (NMA), (−)-scopolamine, (−)-N-methyl scopolamine chloride (NMS), physostigmine, phenylmethylsulphonyl-fluoride (PMSF), bacitracin, aprotonin, pepstatin A and gallamine triethiodide were purchased from Sigma Chemical Company as were all chemicals and reagents used. AF-DX116 (11-[[2-(diethylamino)methyl]-1-piperidinyl] acetyl-5, 11-dihydro-6H-pyrido[2,3-b] [1,4] benzodiazepine-6-one) CPPS and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) were synthesized by Dr R. Clark and Dr D. Repke (IOC, Syntex, Palo Alto).

Results

Saturation binding of [^{3}H]-NMS in guinea-pig tissue homogenates

Saturation binding properties of [^{3}H]-NMS in TEM buffer In TEM assay buffer, [^{3}H]-NMS bound reversibly (data not shown) and with high affinity in all tissues studied (Table 1). In all four tissues good ratios (75–96%) of specific to total binding were obtained when using concentrations of [^{3}H]-NMS up to the K_D value (data not shown).

Scatchard plots (Scatchard, 1949) of specific binding data obtained in the TEM assay buffer displayed marked signs of upward concave curvature (Figure 1),

Table 1 Binding parameters [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) in guinea-pig membranes assayed in TEM buffer

		Site 1		*Site 2*	
Tissue	*Data type*	K_D[a]	B_{max}[b]	K_D[a]	B_{max}[b]
Longitudinal	Specific	0.82 (0.07)	760 (72.09)	17.0 (2.22)	1140 (99.31)
ileum	Total	0.71 (0.08)	820 (67.21)	13.1 (3.32)	1202 (97.28)
Circular	Specific	1.39 (0.41)	99 (17.23)	25.3 (6.12)	185 (33.07)
ileum	Total	1.22 (0.31)	101 (18.18)	19.1 (4.32)	172 (15.09)
Atria	Specific	0.78 (0.09)	107 (11.19)	18.2 (1.92)	201 (24.24)
	Total	0.59 (0.09)	115 (10.06)	21.1 (2.52)	220 (18.66)
Ventricle	Specific	0.85 (0.11)	147 (13.30)	14.0 (1.39)	132 (14.60)
	Total	0.67 (0.10)	122 (11.32)	11.6 (2.12)	153 (16.11)

Values shown are the best fit parameters obtained after fitting specific and total binding data either to models describing the binding of [^{3}H]-NMS to one or more specific binding sites or to a model which assumed binding in a cooperative manner. In addition specific binding data were fitted to a model which assumed the presence of single or multiple populations of [^{3}H]-NMS binding sites together with a non-specific component of binding. In all cases the best fit to the data was obtained using a model which assumed two saturable populations of [^{3}H]-NMS binding sites.

Values in parentheses represent the s.e.mean. In all cases the number of experiments was 4. In each experiment the number of data points was 12.

[a] K_D values are in units of mol l^{-1} × 10^{-10}

[b] B_{max} values are expressed in terms of fmol mg^{-1} protein

TEM = Tris-EDTA-magnesium buffer (for composition see Methods).

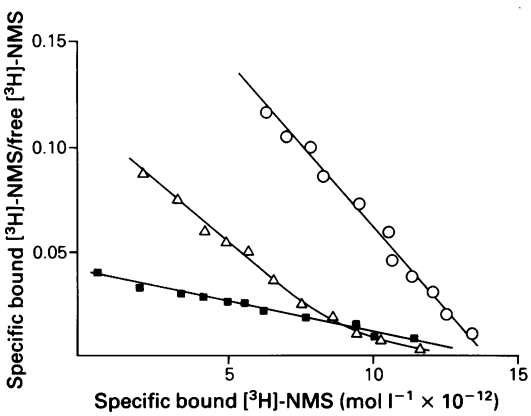

Figure 1 Scatchard plot of specific [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding to rat, EDTA washed, atrial membranes assayed inTris-Krebs (■) and in TEM buffer in the absence (Δ) and presence (O) of 10^{-4} mol l^{-1} GTP. Data shown are from a representative experiment using the 3 buffer systems. Membrane protein was 126 μg.

The following binding parameters were obtained: TEM buffer: 2 site fit; K_D site 1 = 8.2×10^{-11} mol l^{-1}, B_{max} site 1 = 96 fmol mg^{-1} protein; K_D site 2 = 1.6×10^{-9} mol l^{-1}, B_{max} site 2 = 212 fmol mg_2^{-1} protein. Tris-Krebs: 1 site fit; $K_D = 3.4 \times 10^{-10}$ mol l^{-1}, B_{max} = 330 fmol mg^{-1} protein TEM in the presence of GTP: 1 site fit; $K_D = 8 \times 10^{-11}$ mol l^{-1}, B_{max} = 336 fmol mg^{-1} protein.

indicating either cooperativity in binding, heterogeneity of binding sites or displacement by atropine of [^{3}H]-NMS NSB.

Computer aided analysis of the data failed to provide any evidence for cooperative interactions as did separate kinetic experiments (data not shown).

Although specific [^{3}H]-NMS binding data could be interpreted as comprising both a high affinity low capacity interaction and a non-specific component, possibly representing a small portion of displaced [^{3}H]-NMS NSB (data not shown), the best fit to the data was obtained assuming the presence of two saturable populations of [^{3}H]-NMS binding sites (Table 1). This was also borne out by a direct analysis of total [^{3}H]-NMS binding isotherms in which NSB was estimated together with the K_D and B_{max} (Table 1). Using this approach the data were better described in terms of [^{3}H]-NMS binding to two saturable populations of sites. Furthermore, the binding parameters arrived at using this method were in close agreement with those obtained when specific binding data were analysed assuming a two site model of binding (Table 1).

It should be stressed that although binding parameters for the low affinity component of [^{3}H]-NMS binding have been included in Table 1, these values must be considered only as approximations since the highest concentration of radioligand employed in these studies ($6-8 \times 10^{-9}$ mol l^{-1}) was only 2–4 fold greater than the apparent K_D of the low affinity [^{3}H]-NMS binding site.

Table 2 Parameters for the binding of [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) to guinea-pig membranes in TEM supplemented with GTP and in modified Tris-Krebs solution

Tissue	*Buffer*	K_D[a]	B_{max}[b]
Longitudinal	TEM + GTP (0.1 mM)	0.70 (0.06)	1762 (131.2)
ileum	Modified Tris-Krebs	4.84 (0.33)	1629 (177.1)
Circular	TEM + GTP (0.1 mM)	—	—
ileum	Modified Tris-Krebs	3.44 (0.29)	262 (25.3)
Atria	TEM + GTP (0.1 mM)	0.62 (0.11)	312 (28.2)
	Modified Tris-Krebs	3.14 (0.22)	298 (18.2)
Ventricle	TEM + GTP (0.1 mM)	0.59 (0.10)	290 (23.2)
	Modified Tris-Krebs	4.16 (0.41)	272 (28.2)

Values shown are the best fit parameters obtained after fitting specific binding data either to models describing the binding of [^{3}H]-NMS to one and two specific binding sites or to a model assuming cooperativity in binding. In addition the data were fitted to a model which assumed the presence of single or multiple populations of [^{3}H]-NMS binding sites together with a non-specific component of binding. With the exception of experiments in circular ileum using TEM buffer with 0.1 mM GTP, the data could be best described by assuming [^{3}H]-NMS to identify a homogeneous population of binding sites.

In circular ileum the data were best described by a model which assumed two components of [^{3}H]-NMS binding: site 1 $K_D = 0.61$ (0.07) $\times 10^{-10}$ mol l^{-1}, B_{max} = 80 (18.2) fmol mg^{-1} protein. Site 2, $K_D = 21.2$ (4.8) $\times 10^{-10}$ mol l^{-1}, B_{max} = 191 (17.4) fmol mg^{-1} protein.

Values in parentheses represent the s.e.mean. In all cases the number of experiments was 4. In each experiment the number of data points was 12.

[a] K_D values are in units of mol $l^{-1} \times 10^{-10}$.

[b] B_{max} is expressed in terms of fmol mg^{-1} protein.

Guanine nucleotide effects on [^{3}H]-NMS binding in TEM buffer The apparent heterogeneity of binding sites identified by [^{3}H]-NMS in the TEM buffer made characterization of these receptors complex, since it was uncertain which of the two [^{3}H]-NMS binding sites represented the muscarinic receptor identified in functional studies. Competition studies utilizing the range of muscarinic antagonists in the present experiments failed to resolve this issue since, with the exception of AF-DX 116 in circular ileal smooth muscle, all compounds appeared to produce mass action competition curves with Hill coefficients close to unity (data not shown). Consequently, attempts were made to employ conditions under which [^{3}H]-NMS bound to a homogeneous population of binding sites.

Since guanine nucleotides have been found to abolish the heterogeneity of ^{3}H-muscarinic antagonist binding in cardiac tissue (Hulme *et al.*, 1981); Burgisser *et al.*, 1982), their effects were examined. With the exception of experiments using circular ileal smooth muscle, the inclusion of guanosine triphosphate (GTP, 1×10^{-4} mol l^{-1}) in the TEM buffer abolished the heterogeneity of [^{3}H]-NMS binding, and resulted in [^{3}H]-NMS binding to a homogeneous population of high affinity sites (Table 2; Figure 1). [^{3}H]-NMS identified approximately the same total number of binding sites in the presence and absence of GTP (cf. Tables 1 and 2; Figure 1). While the major effect of GTP was to abolish low affinity [^{3}H]-NMS binding in TEM buffer, it should be noted that the K_D value of [^{3}H]-NMS in the presence of GTP was somewhat lower than the K_D of the high affinity component of [^{3}H]-NMS binding in the absence of GTP. The effects observed were guanine nucleotide specific since adenosine triphosphate and inosine triphosphate, both at a concentration of 1×10^{-4} mol l^{-1}, did not modify the binding of [^{3}H]-NMS in TEM assay buffer (data not shown).

Saturation binding properties in physiological assay buffer While the use of the GTP resulted in homogeneity of [^{3}H]-NMS binding, the relevance of the sites identified to those observed in functional studies was uncertain. Furthermore, GTP did not affect [^{3}H]-NMS binding site heterogeneity in circular ileal smooth muscle. Previous studies by Hulme *et al.* (1981) using rat cardiac membranes have also demonstrated heterogeneity of [^{3}H]-NMS binding in low ionic strength buffers and have indicated that such heterogenous interactions are abolished in high ionic strength buffer. Consequently, the remainder of the studies presented here were performed using a modified Tris-Krebs buffer, which not only provided higher ionic strength but also enabled a direct comparison of the present data with results obtained in functional studies.

In modified Tris-Krebs assay buffer there was no evidence for heterogeneity of [^{3}H]-NMS binding in the 4 tissue preparations studied. Instead, [^{3}H]-NMS bound to a homogeneous population of saturable binding sites. The modest decrease in the number of [^{3}H]-NMS binding sites identified in Tris-Krebs buffer compared to that in the TEM buffer systems (cf. Tables 1 and 2) was not significant ($P > 0.05$). There were no similarities between the K_D values for NMS in modified Tris-Krebs buffer and either of the affinity estimates obtained in TEM buffer or TEM buffer supplemented with 1×10^{-4} mol l^{-1} GTP (cf. Table 1 *vs* Table 2). Thus, in general, the K_D values for [^{3}H]-NMS in modified Tris-Krebs buffer were intermediate between the high affinity K_D values obtained in the presence or absence of GTP in TEM buffer and the low affinity K_D values obtained in the absence of GTP in TEM buffer.

Distribution of muscarinic receptors Since partially purified membrane preparations were utilized in all studies, comparisons of receptor densities are strictly not appropriate. Nevertheless, it was evident that the density of [^{3}H]-NMS binding sites in longitudinal ileal smooth muscle far exceeded those in either circular smooth muscle or in cardiac tissue (Table 2). In terms of cardiac tissue there was no marked difference between the densities of [^{3}H]-NMS binding sites in the atria and in the ventricles. The K_D for [^{3}H]-NMS was similar in all four tissues studied.

Pharmacology of [^{3}H]-NMS binding sites

General pharmacology The affinity estimates of a series of compounds for the sites labelled by [^{3}H]-NMS in guinea-pig longitudinal and circular ileal smooth muscle and in atrial and ventricular tissue are shown in Table 3.

The pharmacology of the [^{3}H]-NMS binding sites was consistent with that of the muscarinic receptor. The affinity values obtained in the direct binding studies in cardiac ileal membranes were similar to those obtained at cardiac muscarinic receptors in functional studies. Thus, atropine, 4-DAMP and pirenzepine exhibit pA_2 values of 8.87, 7.90 and 6.60, respectively in functional studies on cardiac muscarinic receptors (Clague *et al.*, 1985).

Based upon the low affinity of pirenzepine for the muscarinic receptors in each of the tissues studied (pK_i values 6.4–6.7; Table 3) the muscarinic receptors identified could be classified as belonging to the M_2 subtype. Pirenzepine – [^{3}H]-NMS competition curves displayed Hill coefficients close to unity indicating the absence of M_1 muscarinic receptors. Furthermore, direct binding studies using [^{3}H]-pirenzepine also failed to find any evidence for even a small subpopulation of M_1 muscarinic receptors (data not shown). In

Table 3 Affinity of competing compounds for [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding sites in guinea-pig membranes assayed in modified Tris-Krebs buffer

Ligand	*Atria* pK_i, n_H	*Ventricle* pK_i, n_H	*Longitudinal ileum* pKi_i, n_H	*Circular ileum* pKi_i, n_H
Adiphenine	6.68 (0.06)	6.68 (0.10)	6.78 (0.08)	4.96 (0.04)
	0.89 (0.05)	0.93 (0.05)	0.97 (0.11)	3.21 (0.22)*
AF-DX 116	7.14 (0.06)	7.18 (0.06)	6.81 (0.05)	6.58 (0.04)
	1.02 (0.06)	1.08 (0.05)	0.97 (0.07)	0.77 (0.05)*
Atropine	8.61 (0.07)	8.78 (0.08)	8.67 (0.06)	8.67 (0.07)
	1.26 (0.09)*	0.96 (0.04)	1.08 (0.06)	1.08 (0.05)
CPPS	7.69 (0.06)	7.65 (0.07)	7.81 (0.06)	7.79 (0.08)
	0.90 (0.05)	1.03 (0.04)	0.95 (0.06)	1.03 (0.04)
4-DAMP	8.15 (0.07)	8.03 (0.05)	8.27 (0.05)	8.53 (0.05)
	0.90 (0.05)	0.96 (0.04)	0.90 (0.05)	0.90 (0.06)
Dicyclomine	7.35 (0.05)	7.34 (0.05)	7.60 (0.04)	7.77 (0.06)
	0.96 (0.03)	0.98 (0.05)	0.89 (0.06)	0.92 (0.05)
N-methyl	8.85 (0.05)	8.90 (0.04)	8.97 (0.07)	9.10 (0.05)
atropine	1.22 (0.07)*	1.23 (0.10)*	1.03 (0.04)	0.89 (0.07)
(−)-N-methyl	9.57 (0.06)	9.54 (0.07)	9.50 (0.08)	9.60 (0.05)
scopolamine	0.93 (0.04)	1.00 (0.06)	0.92 (0.06)	0.97 (0.05)
Pirenzepine	6.47 (0.04)	6.52 (0.06)	6.53 (0.05)	6.65 (0.05)
	0.92 (0.06)	0.94 (0.06)	0.99 (0.04)	0.98 (0.07)
(−)Scopolamine	8.65 (0.07)	8.74 (0.06)	8.72 (0.04)	9.06 (0.05)
	1.02 (0.04)	0.97 (0.06)	0.92 (0.05)	0.91 (0.05)

All affinity values are expressed as the negative log of the inhibitory affinity constant (K_i) and are in mol l^{-1}. Values in parentheses are the s.e.mean. The number of experiments was 4.
*Represents a n_H significantly different ($P < 0.05$) from unity.

longitudinal ileal smooth muscle and in atrial and ventricular tissue the affinity values for pirenzepine were essentially identical. A marginally, but significantly (Table 3; Figure 3), higher pirenzepine affinity was obtained at circular ileal smooth muscle muscarinic receptors than at atrial muscarinic receptors.

In the competition binding studies nearly all of the compounds examined displayed Hill coefficients close to unity and could only be analysed assuming competition with a homogeneous population of [^{3}H]-NMS binding sites. The only marked exceptions were adiphenine and AF-DX 116 in circular ileal smooth muscle, N-methyl atropine in cardiac tissue and atropine in atria. The n_H of the NMS and atropine competition curves in both the atria and ventricle were significantly greater than unity. This was also observed in the presence of 1 mM PMSF (protease inhibitor) and also when a longer (4 h) incubation period was used (data not shown).

The n_H of the adiphenine – [^{3}H]-NMS competition curve obtained in circular ileal smooth muscle greatly exceeded unity and the compound displayed a markedly lower affinity in this preparation than in the other tissues studied (Table 3). These findings, however, appeared to be due to the metabolism or degradation of adiphenine by the ileal circular smooth muscle homogenate. Thus, 1 mM PMSF or 5 mM EDTA (but not pepstatin, bacitracin or physostigmine; Table 4) increased the affinity of adiphenine for circular ileal smooth muscle muscarinic receptors and decreased the n_H of the competition curves towards unity. This 'degradative action' appeared to be exerted only against adiphenine and its more potent analogue hexahydroadiphenine (data not shown), since PMSF did not affect the [^{3}H]-NMS competition curves for any of the other ligands studied (data not shown). The inclusion of 1 mM PMSF in assays using longitudinal ileal smooth muscle and atrial membranes did not significantly modify either the affinity or the selectivity of adiphenine, AF-DX 116, CPPS, 4-DAMP or pirenzepine for ileal or atrial muscarinic receptors.

Pharmacology of cardiac muscarinic receptors A comparison of the ligand affinities for muscarinic receptors in atrial and ventricular tissue (Table 3) indicated no significant differences between the muscarinic receptors studied.

Comparison of longitudinal ileal smooth muscle and atrial muscarinic receptors In Figure 2 a comparison between the ligand affinities determined in atrial and ventricular tissue with those obtained in the long-

Table 4 The effect of protease inhibitors and physostigmine on the adiphenine – [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) competition curves obtained in guinea-pig circular ileal smooth muscle membranes

Treatment	pK_i	n_H
Control	4.96 (0.06)	3.21 (0.22)*
Bacitracin (0.1%)	4.83 (0.07)	3.94 (0.32)*
Pepstatin A (100 μg ml^{-1})	4.84 (0.04)	3.53 (0.56)*
Aprotinin (100 μg ml^{-1})	4.76 (0.07)	3.85 (0.24)*
Physostigmine (1 μM)	4.82 (0.08)	3.90 (0.22)*
EDTA (5 mM)	5.62 (0.07)†	1.63 (0.11)*†
PMSF (0.1 mM)	6.12 (0.07)†	1.32 (0.07)*†
PMSF (1 mM)	6.82 (0.06)†	1.21 (0.07)*†

In these studies the protease inhibitors or physostigmine, when examined were preincubated with the tissue homogenate for 15 min before use and were present throughout the competition experiment.
All affinity values are expressed as the negative log of the affinity constant and are in mol l^{-1}.
Values in parentheses are the s.e.mean. The number of experiments was 4.
*Represents a n_H significantly different ($P < 0.05$) from unity.
†Value significantly different ($P < 0.05$) from that obtained in the control group.

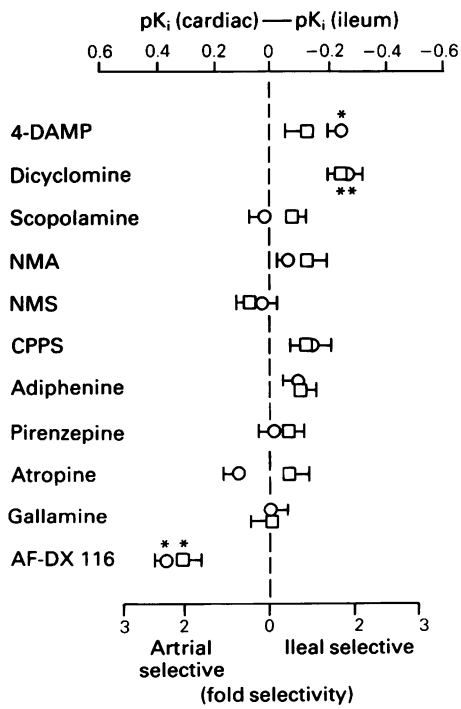

Figure 2 Comparison of the affinity of a series of ligands for cardiac (atrial (O) or ventricular (□)) and longitudinal ileal smooth muscle muscarinic receptors. *Significant ($P < 0.05$) difference in affinity between cardiac and longitudinal ileal smooth muscle muscarinic receptors.

itudinal ileal smooth muscle are presented. The data are not presented as a conventional correlation plot format. Instead, a selectivity profile has been presented since this format is more useful in graphically depicting the generally small differences in the affinity of muscarinic ligands for putative muscarinic receptor subtypes.

Surprisingly there were no marked differences in the affinity estimates for any of the compounds examined. Thus, only two compounds, AF-DX 116 and dicyclomine, showed significantly different affinities for muscarinic receptors in longitudinal ileal smooth muscle and cardiac tissue. The most selective of these agents was AF-DX 116 which showed only a two fold selectivity for longitudinal ileal smooth muscle muscarinic receptors. Dicyclomine was marginally (less than two fold) selective for longitudinal ileal smooth muscle muscarinic receptors. Both 4-DAMP and CPPS were non-selective antagonists.

While there was no obvious difference between atrial and ventricular muscarinic receptors, it should be noted that, in general, the differences between muscarinic receptors of longitudinal ileal smooth muscle and cardiac tissue were marginally greater when affinity values determined in ventricular, as opposed to atrial tissue, were utilized (Figure 2). Indeed, using this comparison, 4-DAMP, in addition to dicyclomine and AF-DX 116, showed a significant difference in affinity at ileal and cardiac muscarinic receptors.

Rather surprisingly, in a comparison of ligand affinities between the four tissues studied, the greatest

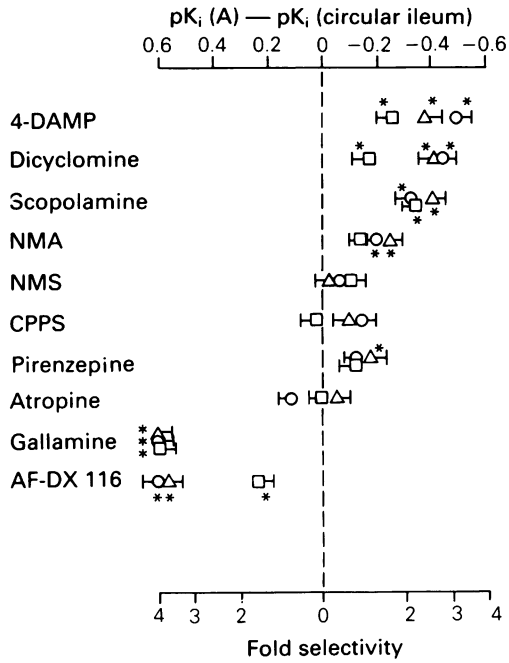

Figure 3 Comparison of the affinity of a series of ligands for circular ileal smooth muscle muscarinic receptors with their affinity for longitudinal ileal smooth muscle (□), atrial (△) or ventricular (○) muscarinic receptors. *Significant ($P<0.05$) difference in affinity between muscarinic receptors in the tissues compared. 'A' represents data obtained in either longitudinal ileal smooth muscle (□), atrial (△) or ventricular (○) tissue.

differences between ligand affinities were observed between circular ileal smooth muscle and ventricular muscarinic receptors (Figure 3). Thus, based on such a comparison, AF-DX 116 was 4 fold selective for ventricular receptors, while dicyclomine, scopolamine and 4-DAMP were 3 fold selective for circular ileal smooth muscle muscarinic receptors.

Similar, although not so marked, indications that circular ileal smooth muscle muscarinic receptors differed from those present in the other tissues examined were also obtained when ligand affinities at circular ileal smooth muscle muscarinic receptors were compared with those obtained at longitudinal ileum smooth muscle, or atrial muscarinic receptors (Figure 3).

Of particular interest in these studies was the observation that in circular ileal smooth muscle AF-DX 116 – [^{3}H]-NMS competition curves were characterized by a low Hill coefficient. A detailed analysis of these data indicated that AF-DX 116 was able to distinguish two populations of [^{3}H]-NMS binding sites with pK$_i$ values of 7.02 and 5.81 (Table 5), thereby providing evidence that the muscarinic receptors of circular ileal smooth muscle were possibly heterogeneous. The high affinity component of AF-DX 116 binding to muscarinic receptors of circular ileal smooth muscle, which comprised 65% of [^{3}H]-NMS binding, was close to that obtained at muscarinic receptors in longitudinal ileum and cardiac tissue (cf Table 3 and Table 5). The low affinity component of AF-DX 116 binding (pK$_i$ = 5.81) differed from the affinity of this compound for muscarinic M$_1$ receptors in guinea-pig cortex (pK$_i$ = 6.36, unpublished observations) but was similar to the affinity value (pK$_i$ = 5.76; unpublished observations) obtained in submaxillary glands.

The effects of gallamine on [^{3}H]-NMS binding The interaction of gallamine with muscarinic receptors in the four tissues studied deserves consideration since this compound has been demonstrated to be an atrial selective agent (Riker & Wescoe, 1951; Clark & Mitchelson, 1976). While Stockton *et al.* (1983) have shown gallamine to be an allosteric regulator of muscarinic receptors, they have demonstrated that the affinity of this compound for its allosteric site can be calculated to a first approximation from competition studies employing low concentrations of radioligand.

The results of experiments which examined the

Table 5 Detailed analysis of AF-DX 116 – [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) competition data in circular ileal smooth muscle membranes

Hill model		*Two site model*				
		Site 1		*Site 2*		
pIC$_{50}$corr	n$_H$	*pK$_i$*	*% specific binding*	*pK$_i$*	*% specific binding*
6.58	0.77	7.02	65.3	5.81	34.7
	(0.05)	(0.10)	(4.70)	(0.14)	(4.70)

Values in parentheses are the s.e.mean. The number of experiments was 4. All affinity estimates are expressed as the negative log of the constant or the pIC$_{50}$ corr value and are in mol l^{-1}.

Table 6 The effect of gallamine on [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding in guinea-pig cardiac and ileal smooth muscle membranes

	Binding parameters calculated according to:				
	Specific [^{3}H]-NMS binding			*Gallamine displaceable binding*	
Tissue	pIC_{50}	n_H	*% max inhibition*	pIC_{50}	n_H
Atria	5.74	0.76*	93.1	5.90	0.92
	(0.06)	(0.05)	(1.35)	(0.05)	(0.06)
Ventricle	5.75	0.81*	90.2	5.90	1.04
	(0.04)	(0.04)	(1.12)	(0.06)	(0.04)
Longitudinal	5.39	0.45*	79.3	5.91	0.90
ileum	(0.06)	(0.06)	(2.12)	(0.05)	(0.07)
Circular	4.68	0.46*	74.6	5.30	0.75*
ileum	(0.03)	(0.04)	(1.86)	(0.04)	(0.05)

Values in parentheses are the s.e.mean. The number of experiments was 4.
All affinity estimates are expressed as the negative log of the affinity constant or pIC_{50} value are in in mol l^{-1}.
*Represents a n_H significantly less than unity ($P < 0.05$).
All % maximum inhibition values were significantly different ($P < 0.05$) from the expected value of 100%.

ability of gallamine to inhibit the binding of a low concentration (5×10^{-11} mol l^{-1}) of [^{3}H]-NMS are presented in Table 6. The gallamine – [^{3}H]-NMS competition curves displayed low Hill coefficients in the four tissues studied and were characterized by an inability of gallamine to inhibit completely [^{3}H]-NMS specific binding. Thus, in ileal tissue only 74–79% of specific binding was inhibited while in cardiac tissue the value was between 90 and 93%. Because of the incomplete inhibition produced by gallamine the Hill coefficients may be of little meaning.

To account for the inability of gallamine to displace all [^{3}H]-NMS specific binding, affinity values and Hill coefficients have also been calculated on the basis of gallamine displaceable binding (Table 6). While the affinity of gallamine for longitudinal ileal smooth muscle and cardiac tissue obtained using this approach did not differ, the affinity estimate obtained in circular ileal smooth muscle was 4 fold lower than in the other three tissues. The affinity estimate for gallamine in circular ileal smooth muscle was intermediate between the affinity obtained in cardiac tissue ($pIC_{50} = 5.90$) and that obtained at cortical M_1 muscarinic receptors labelled using [^{3}H]-NMS ($pIC_{50} = 4.89$; unpublished observation) and at muscarinic receptors of submaxillary gland ($pIC_{50} = 4.93$; unpublished observation). It should also be noted that gallamine – [^{3}H]-NMS interaction curves obtained in circular ileal smooth muscle displayed a low Hill coefficient, whereas at the other tissues examined Hill coefficients close to unity were observed (Table 6, 'Gallamine displaceable binding'). Despite this low Hill coefficient for gallamine in the circular ileum it was not possible to fit the data to a model assuming 2 populations of binding sites.

Discussion

The major aims of the present study were to characterize the binding of [^{3}H]-NMS to muscarinic receptors present in guinea-pig ileal and cardiac tissues and to compare the affinities of ileal and atrial selective ligands at these sites.

In terms of characterizing the receptors in the tissues examined, the data indicated that in all tissues studied muscarinic receptors were exclusively of the M_2 subtype as adjudged by their low affinity ($K_i = 2–4 \times 10^{-7}$ mol l^{-1}) for pirenzepine. These findings were entirely in agreement with previous studies using guinea-pig cardiac (Nilvebrandt & Sparf, 1986) and ileal (Nilvebrandt & Sparf, 1983) tissues.

In the low ionic strength TEM buffer system used in initial studies, [^{3}H]-NMS binding was heterogeneous. Because of differences in assay buffers, radioligands utilized and assay conditions employed comparisons with previous studies are complex. Nevertheless, heterogeneity of [^{3}H]-NMS binding has been described in rat (Hulme *et al.*, 1981), and in frog (Burgisser *et al.*, 1982) cardiac tissue as well as in guinea-pig longitudinal ileal smooth muscle (Choo *et al.*, 1985) when low ionic strength assay buffers have been utilized. Based upon the ability of guanine nucleotides to induce a single affinity state of the muscarinic receptor identified by antagonists (Hulme *et al.*, 1981), Bugisser *et al.* (1982) have attributed the heterogeneity of ^{3}H-muscarinic antagonist binding to the existence of multiple, guanine nucleotide interconvertible, affinity states of the muscarinic receptor.

In the present study, using guinea-pig tissue, we were able to confirm previous observations made in other species (Hulme *et al.*, 1981; Burgisser *et al.*,

1982), that guanine nucleotides could abolish the apparent heterogeneity or radioligand binding in cardiac membranes. Furthermore, similar effects of guanine nucleotides were demonstrated in longitudinal ileal smooth muscle.

GTP did not modify [^{3}H]-NMS binding in circular ileal smooth muscle preparations. With regard to this lack of effect of GTP, it should be noted that the ileal smooth muscle homogenates exhibit a considerable degree of proteolytic activity (Birdsall *et al.*, 1979) and that the circular ileal smooth muscle homogenate used in the present study was able to degrade extensively adiphenine and hexahydroadiphenine. It is therefore possible that the failure of GTP to modify binding of [^{3}H]-NMS to circular ileal smooth muscle muscarinic receptors may have been due to degradation of GTP by some enzymatic activity of the tissue preparation. Clearly such an explanation is speculative and further experiments employing a GTP regenerating system, stable GTP analogues or using the protease inhibitor PMSF (which inhibited the degradation of adiphenine) are necessary to verify this hypothesis.

It should be noted that all of the unlabelled antagonists used in the present study, with the exception of AF-DX 116, produced inhibition curves with n_H values close to unity when assayed in TEM buffer. This suggested either that all of the compounds studied displayed the same differential affinity as [^{3}H]-NMS for the putative different states of the muscarinic receptor, or that they could not discriminate between the affinity states. These findings are in contrast to those from previous studies using rat cardiac tissue (Hulme *et al.*, 1981; Christopher *et al.*, 1986). The reason for this discrepancy is uncertain, but may reflect differences in species used or methodology adopted, or may be due to the fact that when using a low concentration of radioligand (0.1 nM) more than 90% of binding would have been to the high affinity component of [^{3}H]-NMS binding. Under such conditions identification of a second site in competition studies would have been difficult.

In view of the complex binding of [^{3}H]-NMS in TEM buffer an alternative Krebs-based buffer was selected. This also enabled a more realistic comparison to be made between the present binding studies and functional studies in which Krebs-based buffers are predominantly used. In the modified Tris-Krebs buffer [^{3}H]-NMS labelled homogeneous populations of binding sites in all four tissue preparations studied. The maximal binding capacity obtained in modified Tris-Krebs buffer was not markedly different from the total number of muscarinic receptors identified in TEM buffer either in the presence or absence of GTP.

In longitudinal ileal smooth muscle and cardiac tissue, although [^{3}H]-NMS identified homogeneous populations of binding sites in modified Tris-Krebs assay buffer and in TEM buffer supplemented with GTP, the K_D values obtained were higher in the former buffer system. Similar results have previously been obtained in rat cardiac tissue (Hulme *et al.*, 1981). The reasons for the difference in the K_D values obtained in the two buffer systems is uncertain, although Burgen (1986) has shown that increasing ionic strength can decrease the affinity of antagonists for rat cardiac muscarinic receptors. However, it should also be noted that Hosey (1982) has demonstrated an opposite effect of increasing ionic concentration at chick cardiac muscarinic receptors.

In the present study muscarinic receptor density in atria and ventricle did not differ markedly. This is in good agreement with the results of previous studies in the guinea-pig by Wei & Sulakhe (1978). In ileal tissue, however, a much higher density of muscarinic receptor binding sites was observed in the longitudinal ileal smooth layer than in the circular smooth muscle. Indeed, in the present study the longitudinal ileal smooth muscle of guinea-pig possessed the greatest density of muscarinic receptors. These results are in good agreement with those obtained by Yamamura & Snyder (1974) using [^{3}H]-quinuclidinyl benzilate. The longitudinal and circular ileal smooth muscle homogenates differed somewhat, in that the circular ileal smooth muscle homogenate was able to decrease markedly the affinity of adiphenine and hexahydroadiphenine for the muscarinic receptor. This action, which appeared to be due to the presence of a PMSF-sensitive protease activity that was able to degrade both adiphenine and hexahydroadiphenine, should therefore be considered when estimating the affinity of compounds with similar structures. The reasons for the failure of the other protease inhibitors to affect the degradation of adephenine is unknown at present. None of the other antagonists examined appeared to be degraded by the circular ileal smooth muscle homogenate.

To characterize pharmacologically the muscarinic receptors present in the four tissues studied, 11 compounds were examined for their ability to compete with [^{3}H]-NMS for its binding sites. As previously mentioned, functional studies have provided considerable evidence that atrial muscarinic receptors that modulate both the force of contraction and rate of beating differ from the receptors responsible for the contractile response to muscarinic agonists in ileum (Barlow *et al.*, 1980). However, the present study was unable to detect any marked pharmacological differences between the muscarinic receptors present in longitudinal ileal smooth muscle and atrial tissue. Thus, only AF-DX 116 and dicyclomine displayed significantly differences in affinity between atrial and longitudinal ileal smooth muscle muscarinic receptors. These differences were marginal with the two fold selectivity of AF-DX 116 for atrial receptors being less than the 5 fold difference found in functional studies

(De Jonge *et al.*, 1986). Furthermore, CPPS, which has been reported to display a 25 fold selectivity for ileal receptors (Eglen & Whiting, 1986), was found to be a non-selective ligand. In addition 4-DAMP, which has been shown to display between a 4 fold (Choo & Mitchelson, 1985) and 11 fold selectivity (Barlow *et al.*, 1980) for ileal muscarinic receptors in functional studies, displayed little selectivity for the longitudinal ileal smooth muscle muscarinic receptors identified in the present study. The results with respect to 4-DAMP were similar to those presented by Choo & Mitchelson (1985), who observed only a 2.6 fold ileal selectivity for this compound; although, in their binding studies this difference achieved statistical significance. In this respect it is perhaps worth noting that when a comparison of antagonist affinities at ventricular and longitudinal ileal smooth muscle muscarinic receptors was performed, 4-DAMP was significantly (1.6 fold) ileal selective.

Experiments using the atrial selective ligand gallamine (Clark & Mitchelson, 1976) produced some interesting results. Firstly, and as previously found in binding studies by Choo *et al.* (1985), the ligand was non-selective between guinea-pig atrial and longitudinal ileal smooth muscle muscarinic receptors. This is in marked contrast to binding results obtained in rat tissue where gallamine displayed a 16 fold selectivity for cardiac as opposed to ileal receptors (Stockton *et al.*, 1983). In the present study, however, it was noticeable that the maximal inhibition of [^{3}H]-NMS binding was greater in cardiac than in ileal preparations, possibly suggesting a greater coupling of the gallamine binding site to the [^{3}H]-NMS binding site in cardiac tissue. Perhaps the ability of gallamine to inhibit selectively muscarinic receptor function in guinea-pig atria resides in these differences between the coupling of the gallamine binding site to muscarinic receptors in the two tissues.

While the competition data obtained in longitudinal ileal and cardiac tissues indicate a trend towards ileal–artrial muscarinic receptor differences, the selectivity of the compounds studied was less than 3 fold. This selectivity was even lower than the 3 fold selectivity suggested by Furchgott (1972) as a criteria for defining different receptors. The reason for the failure to confirm the functional subclassification of ileal and atrial muscarinic receptors in the present binding study is unknown. Perhaps cellular integrity is essential to demonstrate ileal and atrial muscarinic receptor subtypes. Certainly, binding studies by Birdsall *et al.* (1986) have demonstrated that the environment of the cardiac muscarinic receptor exerts a profound influence on the affinity of pirenzepine in this tissue. Experiments utilizing tissue slices may help to resolve this issue. Alternatively it is possible that [^{3}H]-NMS may not have been labelling functionally relevant sites in the present study. Thus, in many functional studies in which the affinity of antagonists for cardiac muscarinic receptors have been determined, agonist effects upon the rate of contraction have been studied. Clearly since these muscarinic receptors are located around the pacemaker region of the sino-atrial node they would not be detected in binding studies. If these receptors therefore differed from the receptors present in the remainder of the atria then they would not have been identified in the present study. It should be stressed, however, that this appears unlikely since it has been difficult to demonstrate a difference between muscarinic receptors mediating rate and the more widely distributed muscarinic receptors mediating force of contraction (Clague *et al.*, 1985).

It should also be considered that proteolytic activity was occurring in the ileal membranes as has been described by Birdsall *et al.* (1979). However, the inclusion of proteolytic inhibitors in the assay buffers used to prepare and assay the atrial and longitudinal ileal membranes did not affect the selectivity of adiphenine, AF-DX 116, CPPS, 4-DAMP or pirenzepine. Whatever the reason for the discrepancy between the present binding studies and functional studies, it is clear that until more selective muscarinic receptor antagonists are available the subclassification of ileal and atrial muscarinic receptors is only possible from a functional standpoint.

While the present study was unable to find any marked differences between atrial and longitudinal ileal smooth muscle muscarinic receptors, the data did indicate that circular ileal smooth muscle muscarinic receptors differed from those present in the other three tissues studied. Thus 4-DAMP, dicyclomine, scopolamine and pirenzepine could be classified as selective for muscarinic receptors present in circular ileal smooth muscle, while AF-DX 116 displayed greater affinity at cardiac and longitudinal ileal smooth muscle muscarinic receptors. These differences were at most 3–5 fold when comparing circular ileal smooth muscle and ventricular receptors and were less marked when comparing circular ileal smooth muscle muscarinic receptors with those present in the atria or on longitudinal ileal smooth muscle.

Of particular interest in these studies was the observation that the AF-DX 116–[^{3}H]-NMS competition curve in circular ileal smooth muscle was shallow and that AF-DX 116 was able to distinguish two populations of [^{3}H]-NMS binding sites. The high affinity component of AF-DX 116 binding displayed a similar affinity to that of AF-DX 116 at cardiac and longitudinal ileal smooth muscle muscarinic receptors and appeared to represent the same or similar receptors to those identified in these tissues. The exact nature of the low affinity component of AF-DX 116 binding was uncertain. It did not appear to represent the presence of M_1 muscarinic receptors, since studies

using pirenzepine indicated the absence of these receptors in this preparation. Furthermore, the low affinity K_i value of AF-DX 116 in circular ileal smooth muscle differed from that obtained at the M_1 receptor. Recently, evidence has been presented which indicates that the M_2 receptors in gland tissue differ from those in the heart (De Jonge *et al.*, 1986). Indeed, initial studies with AF-DX 116 indicated that this compound displayed considerably lower affinity for muscarinic receptors of the gland than for those in the heart (Hammer *et al.*, 1986). The finding that the low affinity AF-DX 116 binding component at circular ileal smooth muscle muscarinic receptors was similar to that obtained in gland tissue, indicated that the circular ileal smooth muscle muscarinic receptors were heterogeneous and may have contained 'gland-like' muscarinic receptors. The presence of such a heterogeneous population of M_2 muscarinic receptors in the circular ileum could also explain the differential affinity of 4-DAMP and dicyclomine for circular ileal smooth muscle muscarinic receptors, since these compounds are selective antagonists of 'gland-like' muscarinic receptors (De Jonge *et al.*, 1986).

The data obtained using gallamine also indicated possible differences between circular ileal smooth muscle muscarinic receptors and those present in both cardiac tissue and longitudinal ileal smooth muscle. Thus, the affinity estimate for gallamine in circular ileal smooth muscle was lower than that obtained at both cardiac and longitudinal ileal smooth muscle muscarinic receptors. Furthermore, the value obtained in circular ileal smooth muscle was close to the affinity of this compound for the 'gland type' M_2 muscarinic receptor. It should be stressed, however, that because gallamine probably exerts its effects at an allosteric binding site coupled to the muscarinic receptor (Stockton *et al.*, 1983), it is possible that the ability of gallamine to distinguish between muscarinic receptors in different tissues may arise more from differences in the affinity of gallamine for its site or in the coupling of the gallamine binding site to the muscarinic receptor, rather than from the presence of muscarinic receptor subtypes.

Because of the crude method employed in obtaining the circular ileal smooth muscle preparation, it is not possible at present to determine whether the 'gland like' muscarinic receptors in the circular ileal smooth muscle homogenate originate from the smooth muscle layer, or arise as a result of contamination with residual mucosal and epithelial cells.

In conclusion, the results of the present study demonstrate M_2 muscarinic receptors in guinea-pig cardiac tissue and ileum. Although there are some differences in affinity estimates at atrial and longitudinal ileal smooth muscle muscarinic receptors, these are of insufficient magnitude to allow definitive subclassification of ileal and atrial muscarinic receptors. Consequently, confirmation of ileal-atrial muscarinic receptor heterogeneity from a binding standpoint must be left as unresolved until more selective ligands are available. In contrast to the comparison between longitudinal ileal smooth muscle and atrial M_2 muscarinic receptors, the results obtained indicated that circular ileal smooth muscle muscarinic receptors were possibly heterogeneous and contained two subpopulations of M_2 muscarinic receptors. The major subpopulation of M_2 receptors in this preparation was identical to that found in cardiac tissue in longitudinal ileal smooth muscle. The other population of M_2 receptors shared some pharmacological similarities with the subtype of muscarinic receptor that is found predominantly in glandular tissue (De Jonge *et al.*, 1986).

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