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Characterisation of muscarinic receptor subtypes in avian smooth muscle

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Abstract

The identity of the muscarinic receptor subtype in the chick ileum was investigated in functional and binding studies. Preliminary studies [Choo, L.-K., Mitchelson, F., Napier, P. 1988. J. Auton. Pharmacol. 8, 259–266] suggested apparent avian and mammalian family differences in the muscarinic receptor profile of ileal smooth muscle. In the current study, further characterisation was undertaken using a greater range of antagonists exhibiting high affinity for specific muscarinic receptor subtypes. Dissociation constants from functional and binding experiments were compared with published values for antagonists at each of the five muscarinic receptor subtypes. Linear regression and correlation analyses revealed the receptor initiating the contractile response was most likely of the muscarinic M_3 receptor subtype as the slope of the linear regression was 1.01 ± 0.14 and the corresponding correlation coefficient (r) was 0.95. The mammalian muscarinic M_5 receptor subtype also showed a high correlation with the data giving a slope of 0.89 ± 0.27 and r value of 0.76. These findings were in direct contrast to those from binding experiments in which the single binding site detected was of the muscarinic M_2 receptor subtype. The slope of the linear regression was 1.14 ± 0.24 with an r value of 0.87. Thus, these results suggest that there exists a high proportion of the muscarinic M_2 receptor subtype within the tissue that does not contribute to the functional response.

Keywords: Muscarinic receptor subtype; Ileum, avian; Binding affinity; Functional affinity

1. Introduction

The existence of a functional muscarinic M_3 receptor in mammalian gut has been well established. In guinea-pig ileum, the receptor eliciting a contractile response exhibits high affinity for the muscarinic M_3 cholinoceptor-selective antagonist HHSiD (Waelbroeck et al., 1989). Conversely, this receptor exhibits low affinity for both the muscarinic M_2 receptor-selective antagonist AF-DX 116 (Hammer et al., 1986) and the muscarinic M_1 receptor-selective antagonist pirenzepine (Eglen and Whiting, 1987). Similar findings have been reported in rat ileum (Lambrecht et al., 1989) and human colon (Kerr et al., 1995).

Preliminary studies in this laboratory suggested apparent differences in the functional muscarinic receptor profile of ileal smooth muscle in the guinea-pig and chick. Choo et al. (1988) studied a muscarinic receptor subtype in chick ileum that elicited a contractile response to carbachol and was antagonised by atropine. However, the chick receptor exhibited fivefold higher affinity for pirenzepine and the muscarinic M_2 receptor-selective antagonist gallamine than guinea-pig ileal receptors. Conversely, guinea-pig ileal receptors exhibited 13-fold higher affinity for the muscarinic M_2/M_4 receptor-selective antagonist, himbacine. The himbacine analogue himandravine also has a twofold higher affinity for chick ileal receptors as compared to guinea-pig ileal receptors (Darroch et al., 1990).

In the mammalian gut, the binding affinity profiles of a number of antagonists were found to be consistent with the existence of both muscarinic M_2 and M_3 receptors (Giraldo et al., 1988; Lazareno and Roberts, 1989), with approximately 70% of the receptor population being of the muscarinic M_2 receptor subtype and 30% of the muscarinic M_3 receptor subtype.

A further study was initiated to elucidate the muscarinic receptor subtype in chick ileal smooth muscle using both functional and binding experiments. More selective antag-

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Table 1
The range of pK_i and pK_B values reported for the antagonists and the associated mean values (in brackets) at various muscarinic receptor subtypes in binding or functional experiments on native muscarinic M_1–M_4 receptors and in binding studies on cloned muscarinic m4 and m5 receptors

	M_1	M_2	M_3	M_4	M_5
Pirenzepine	7.8–8.3[1,2] (8.1)	6.4–6.8[1,3] (6.6)	6.8–7.1[1,3] (6.9)	7.0–7.7[2,4] (7.4)	5.9–7.1[5,6] (6.5)
Benzhexol	8.7–9.2[7,8] (9.0)	7.5–7.9[3,9] (7.7)	7.8–8.5[3,9] (8.2)	8.3–8.7[10] (8.5)	7.8–8.3[5,11] (8.1)
Himbacine	7.1–8.2[1,3] (7.7)	8.0–8.4[1,12] (8.2)	7.0–7.6[2,3] (7.3)	7.9–8.8[4,13] (8.4)	6.3[6,14] (6.3)
AF-DX 116	6.1–7.1[1,15] (6.6)	6.7–7.5[9,16] (7.1)	5.6–6.5[4,15] (5.9)	6.5–7.0[17,18] (6.8)	5.5–7.5[19,20] (6.5)
AF-DX 384	7.0–7.6[6,21] (7.3)	8.2–9.0[22,23] (8.6)	6.8–7.6[21,24] (7.1)	7.9–8.2[4,25] (8.1)	6.3[6] (6.3)
AQ-RA 741	7.0–7.8[26,27] (7.4)	7.8–8.6[10,28] (8.2)	6.6–6.9[24,29] (6.8)	8.0–8.2[4,20] (8.1)	6.1[6,29] (6.1)
4-DAMP	9.0–9.4[1,3] (9.2)	7.6–8.4[6,30] (8.1)	8.0–9.3[3,30] (8.7)	8.5–9.2[26,27] (9.0)	9.0[6] (9.0)
Tripitramine	7.6[31] (7.6)	9.2–9.9[8,31] (9.5)	6.2–6.8[8,31] (6.5)	7.9–8.2[8,31] (8.1)	7.5[32] (7.5)
p-F-HHSiD	7.2–7.8[2,33] (7.5)	6.5[33] (6.5)	7.9–8.0[2,33] (7.9)	7.5–7.8[6,34] (7.7)	6.9–7.0[5,6] (7.0)
HHD	7.8–8.2[7,35] (8.0)	6.8–7.0[7,35] (6.9)	7.9–8.0[35,36] (8.0)	7.9[7] (7.9)	7.1[20] (7.1)

[1] Dorje et al. (1990).
[2] Eglen et al. (1994).
[3] Lazareno et al. (1990).
[4] Doods et al. (1994).
[5] Guo et al. (1995).
[6] Dorje et al. (1991).
[7] Waelbroeck et al. (1992).
[8] Chiarini et al. (1995).
[9] Eglen and Whiting (1987).
[10] Hey et al. (1994).
[11] Bolden et al. (1992).
[12] Darroch et al. (1990).
[13] Caulfield and Brown (1991).
[14] Miller et al. (1992).
[15] Hammer et al. (1986).
[16] Giraldo et al. (1988).
[17] Esqueda et al. (1996).
[18] Waelbroeck et al. (1990).
[19] Bonner et al. (1988).
[20] Buckley et al. (1989).
[21] Mayer (1989).
[22] Entzeroth and Mayer (1991).
[23] Miller et al. (1991).
[24] Bungardt et al. (1992).
[25] Eltze et al. (1997).
[26] Melchiorre et al. (1995).
[27] Liebmann et al. (1992)
[28] Eltze et al. (1993).
[29] Doods et al. (1993).
[30] Gardner et al. (1988).
[31] Melchiorre et al. (1993).
[32] Maggio et al. (1994).
[33] D'Agostino et al. (1994).
[34] Waelbroeck et al. (1991).
[35] Lambrecht et al. (1989).
[36] Waelbroeck et al. (1989).

onists exhibiting high affinity for particular muscarinic M_1 to M_4 receptor subtypes (Caulfield, 1993; Eglen and Watson, 1996) have become available since the initial study and these were used to assess the receptor(s) in chick gut. To date, no binding analyses for smooth muscle of the chick ileum have been published and this study represents the first investigation for this region of the chick alimentary tract. Since there is no single antagonist that exhibits adequate subtype specificity (see Table 1), the pharmacological characterisation of an unknown subtype must be based on the rank order of affinities of a range of selective antagonists. In the present study, 10 antagonists with different selectivity profiles were used.

2. Materials and methods

2.1. Chick sources and isolated tissue preparation

Male White Leghorn-cross chicks were obtained at age 1 day (Research Poultry Farm, Victoria, Australia) and were sacrificed by decapitation at 7–18 days of age. Lengths of ileum (2–3 cm) were isolated from a region of intestine 10 cm from the ileo-caecal junction. The ileum was flushed with ice-cold chick physiological solution (Bolton, 1967), the composition of which is as follows: 118.4 mM NaCl; 4.6 mM KCl; 2.0 mM $CaCl_2$; 0.5 mM $MgCl_2$; 1.2 mM KH_2PO_4; 25 mM $NaHCO_3$; 11.1 mM glucose; and 13.2 mM sucrose. For functional studies, the ileum was then placed in a 10 ml organ bath containing chick physiological solution at 37°C and gassed with a mixture of 95% O_2–5% CO_2. The muscle was mounted under 1 g resting tension and allowed to equilibrate for 30 min before commencing the experiment. The contractile responses to the agonist were recorded isometrically via a Grass force displacement transducer FT.03 C and a Grass polygraph Model 79 D trace recorder.

2.2. Concentration–response curves

Cumulative concentration–response curves were obtained by addition of successively increasing concentrations of carbachol with 3, 10, 30, 100-fold, etc., increases in the initial concentration until a maximal response was obtained. Cumulative concentration–response curves were duplicated at 30 min intervals and the procedure was repeated until constant responses were established. The tissue was exposed to the initial antagonist concentration for 60 min with washout and re-addition of antagonist at 30 min. The cumulative concentration–response curve to carbachol was then re-established in duplicate in the presence of the antagonist. These concentration–response curves were then repeated in the presence of at least two other concentrations of antagonist, the tissue having been incubated with the subsequent concentrations for 30 min periods.

2.3. Analysis of functional responses

Contractile responses to carbachol in the chick ileum were converted to a percentage of the maximum control contractile response and plotted against the concentration of agonist used. Estimates of EC_{50} values and slopes of the concentration–response curves were determined by non-linear regression analysis using the program Graph Pad Prism™ 2.0 (Graphpad Software, San Diego, Ca, USA).

Concentration ratios for antagonists were determined by dividing the EC_{50} of the agonist in the presence of the antagonist by the EC_{50} value in the absence of the antagonist. Arunlakshana–Schild plots of log (concentration ratio -1) versus log antagonist concentration (Arunlakshana and Schild, 1959) were constructed using data obtained from experiments encompassing at least a 10-fold antagonist concentration span. The pA_2 values were determined from linear regression analysis and when the slope of the line did not differ significantly from unity ($P > 0.05$), regressions with the slope constrained to unity were also fitted through the points on the plot to obtain pK_B values.

2.4. Tissue homogenate

Sections of whole ileum were isolated as above for the functional experiments and rapidly dissected, chilled on ice and kept moist with ice-cold phosphate buffer (50 mM Na_2HPO_4; pH 7.4). The tissues were blotted dry, weighed, minced finely with scissors and 10 ml of ice-cold phosphate buffer per gram of tissue wet weight was added. Tissues were then homogenised with an Ultra-Turrax (Janke and Kunkel) set at 0.75 × maximum speed for three 30 s bursts with 15 s periods of cooling on ice between homogenisations. The homogenate was centrifuged at 4°C for 12 min at 40,000 × *g*. The pellet was resuspended in phosphate buffer (4 ml of buffer per gram of original wet weight) with homogenisation by 10 strokes of a loose-fitting Potter–Elvejhem homogeniser. Protein concentration was determined by the method of Bradford (1976) using γ globulin (IgG) as the standard.

2.5. Saturation and displacement studies

The equilibrium dissociation constant (K_d) of the non-selective muscarinic receptor ligand [^{3}H]quinuclidinyl benzilate was determined in saturation studies according to the method of Choo et al., (1985). Radioligand binding assays were performed in duplicate at 37°C. Tissue homogenates (110 μg protein per 100 μl) were pre-incubated for 10 min in a shaking water bath, prior to addition of the radioligand. Ten concentrations of [^{3}H]quinuclidinyl benzilate (1 pM–5 nM) were added to the incubation medium and incubated for a further 60 min. Non-specific binding was determined in the presence of 10 μM atropine. At the end of the incubation period, tubes were rapidly cooled in an ice–water slurry then 2 ml of ice-cold phosphate buffer was added before filtration through Whatman GF/B filters positioned on a Brandell Receptor Binding Cell Harvester. All filters were pre-soaked for several hours in 0.05% v/v polyethyleneimine and 20 μM atropine to saturate specific binding sites that may be present on the filters. The filters were washed three times with 5 ml of ice-cold phosphate buffer and then placed in a plastic vial with 5 ml of

Table 2
Slope factors and pK_B values obtained from functional studies in chick ileum with carbachol as agonist versus a range of antagonists

Antagonist	Concentration range (μM)	Slope ± S.E.M.[a]	n[b]	pK_B ± S.E.M.
Pirenzepine	0.5–10	1.07 ± 0.17	10	6.61 ± 0.07
Benzhexol	0.05–2	1.01 ± 0.01	11	7.97 ± 0.01
AF-DX 116	1–100	0.78 ± 0.19	20	5.87 ± 0.14
Himbacine	0.1–10	0.74 ± 0.14	12	6.7 ± 0.02
AQ-RA 741	5–50	1.01 ± 0.11	8	6.18 ± 0.04
AF-DX 384	0.1–10	0.89 ± 0.19	24	7.28 ± 0.12
Tripitramine	1–10	1.20 ± 0.02	12	6.37 ± 0.06
4-DAMP	0.05–0.5	0.99 ± 0.13	16	8.36 ± 0.04
HHD	0.1–5	0.85 ± 0.30	15	7.22 ± 0.15
p-F-HHSiD	0.1–5	1.02 ± 0.21	19	7.37 ± 0.07

[a] Slope of the Arunlakshana–Schild plot ± S.E.M.
[b] Number of data points used in the Arunlakshana–Schild regression.

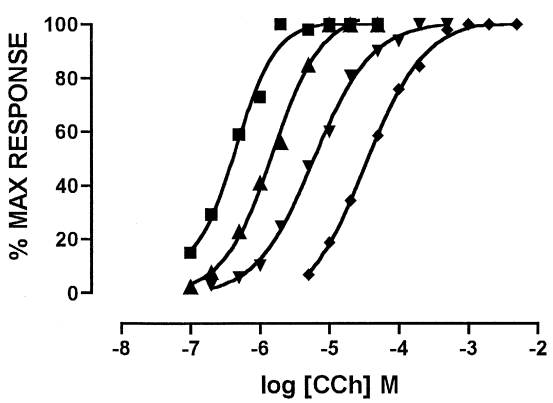

Fig. 1. Representative diagram of the displacement of the agonist concentration response curves by increasing concentrations of the antagonist, *p*-F-HHSiD. Plots are expressed as the percent of the maximal response to carbachol (*y*-axis) versus the log agonist concentration (*x*-axis). Responses to carbachol are shown in the absence (■) and presence of *p*-F-HHSiD, 1×10^{-7} M (▲), 5×10^{-7} M (▼), 1×10^{-6} M (◆).

Table 3
Binding constants (pK_i; nM) and slope factors obtained from competition binding studies with [^{3}H]quinuclidinyl benzilate versus a range of antagonists

Antagonist	pK_i[a]	n_H[b]	n[c]
Pirenzepine	6.8 (6.6–7.0)	0.94 (0.63–1.45)	4
Benzhexol	7.8 (7.7–8.2)	0.86 (0.71–0.98)	4
AF-DX 116	6.6 (6.4–6.8)	0.88 (0.84–0.97)	3
Himbacine	7.5 (7.2–7.8)	0.87 (0.43–1.77)	4
AQ-RA 741	8.1 (7.9–8.3)	0.86 (0.63–0.95)	4
AF-DX 384	8.3 (7.9–8.7)	0.88 (0.72–0.94)	4
Tripitramine	8.2 (8.0–8.3)	0.85 (0.62–1.16)	5
4-DAMP	8.0 (7.7–8.2)	0.87 (0.70–1.10)	4
HHD	7.3 (7.3–7.4)	0.84 (0.55–0.97)	5
p-F-HHSiD	6.2 (6.1–6.3)	1.03 (0.81–1.30)	6

[a] −log dissociation constant used in correlation analysis and corresponding (−log 95% confidence intervals).
[b] Average slope factor for *n* experiments (95% confidence intervals) determined using EBDA.
[c] Number of experiments.

Packard Filter Count™. The vials were agitated and radioactivity was counted by a liquid scintillation analyser (Packard Tricarb 2000CA). For competition binding studies, duplicate homogenates were pre-incubated in the presence of up to 17 concentrations of the unlabelled antagonists for 10 min at 37°C, then, 0.1 nM [^{3}H]quinuclidinyl benzilate was added and incubated for a further 60 min. Non-specific binding was determined in the presence of 10 μM atropine. The reaction was terminated and filtration undertaken as described above.

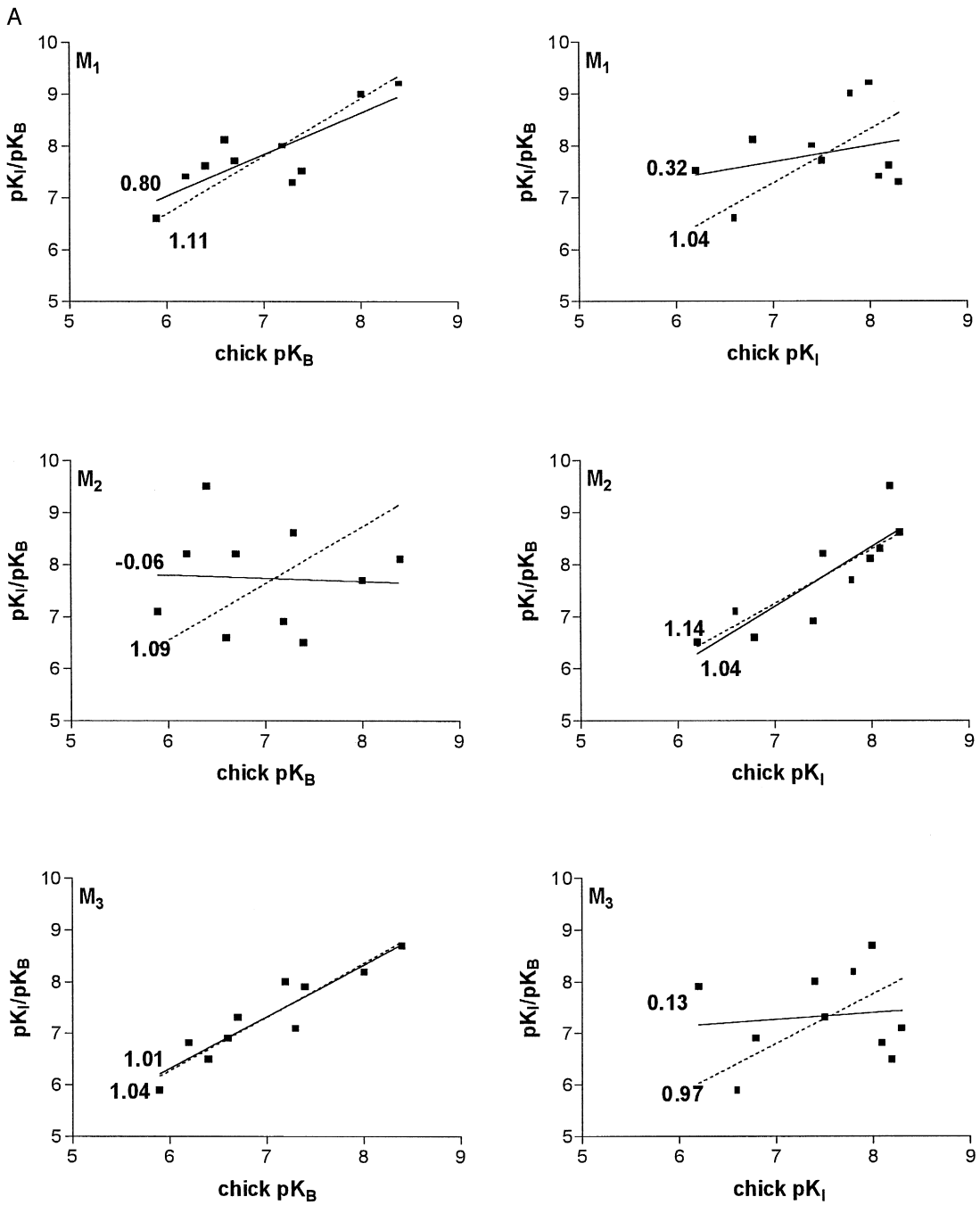

Fig. (2A and B). Comparison of antagonist affinities for receptor subtypes. The *x*-axis represents the dissociation constants (pK_B or pK_i) obtained in either the functional or the binding studies on chick ileum (Tables 2 and 3). The *y*-axis represents mean values from the range of published pK_i or pK_B values for each of the antagonists, respectively, with the specific receptor subtypes (Table 1). The line (—) represents the best unconstrained regression through the points. The line (· · · · ·) represents the regression forced through the origin (0,0). The slopes of the respective lines are indicated. Where there is good correlation of experimental data with the published values, a slope close to unity would be expected for the constrained correlation.

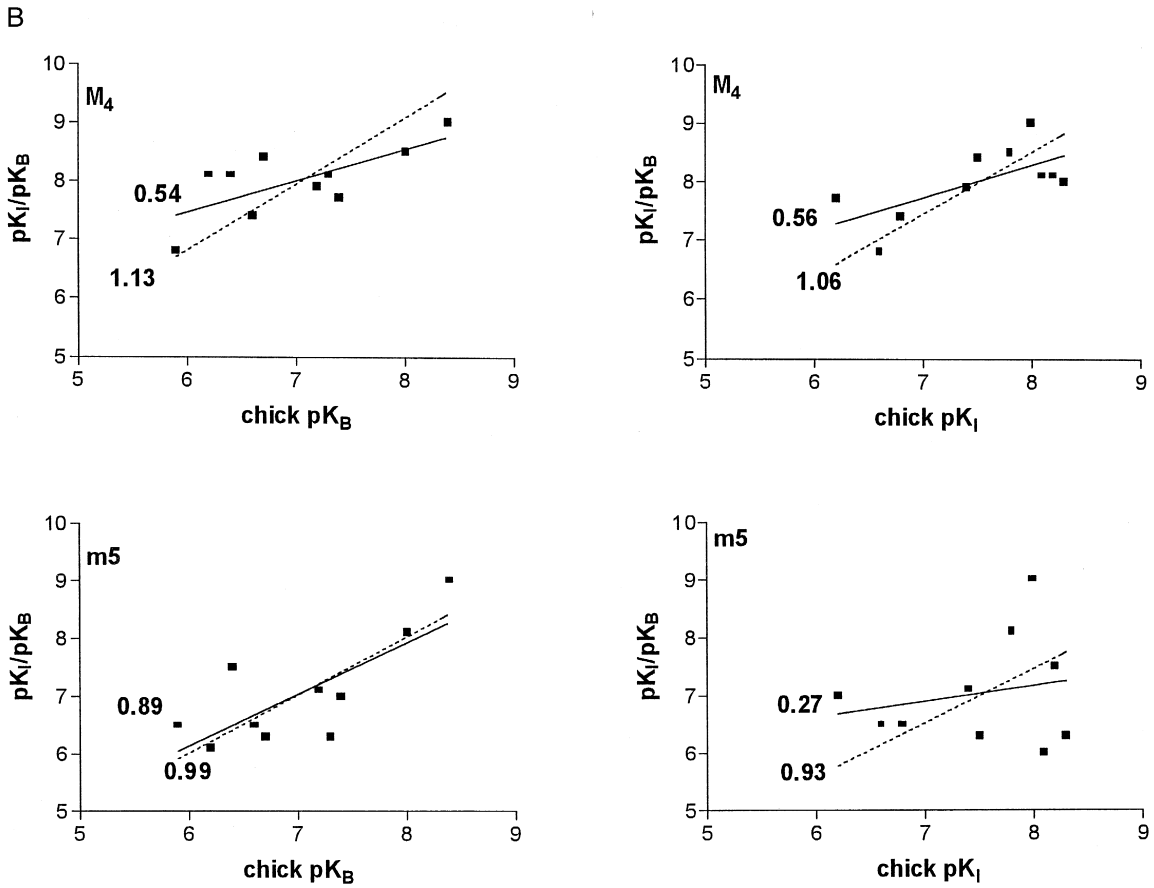

Fig. 2 (*continued*).

2.6. Drug sources

11[[2-[(diethylamino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*]-[1,4]benzodiazepin-6-one (AF-DX 116), 5,11-dihydro-11-[[[2-[2-[(dipropylamino)-methyl]-1-piperidinyl]ethyl]amino]carbonyl]-6*H*-pyrido-[2,3-*b*][1,4]-benzodiazepin-6-one (AF-DX 384 methane sulphonate) and 11-[[4-[4-(diethylamino)-butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*] [1,4]benzodiazepin-6-one (AQ-RA 741 hydrochloride) (Thomae, Biberach an der Riss, Germany); atropine sulphate (Sigma, St. Louis, MO, USA); 4-DAMP (4 diphenylacetoxy-N-methylpiperidine methiodide) (gift, Prof. R. Barlow, Bristol, England); (±)-benzhexol hydrochloride (Lederle, London, UK); carbamoylcholine chloride (carbachol) (Sigma); (±)-hexahydrodifenidol (HHD) (gift, Profs. E. Mutschler and G. Lambrecht, University of Frankfurt, Germany); himbacine hydrochloride (gift, Prof. W. Taylor, Sydney, Australia); pirenzepine hydrochloride (Boehringer Ingelheim, Sydney, Australia); *para*-fluoro-hexa-hydrosiladifenidol (*p*-F-HHSiD) (gift, Profs. E. Mutschler and G. Lambrecht, University of Frankfurt, Germany); tripitramine (gift, Prof C. Melchiorre, Bologna, Italy); [^{3}H](−)-quinuclidinyl benzilate (specific activity 49 Ci/mmol; Du Pont, NEN, USA).

2.7. Analysis of binding data

Results from saturation and competition studies were analysed via the computer program Equilibrium Binding Data Analysis (EBDA; Macpherson, 1983) followed by the computer program 'LIGAND' (Munson and Rodbard, 1980).

2.8. Correlation and linear regression analyses

The receptor subtypes detected in functional and binding studies were characterised by determination of the strength of the linear relationship between dissociation constants obtained in chick ileal functional studies (pK_B) or binding studies (pK_i) and mean published values (pK_i and pK_B) for particular subtypes. This was calculated using the Pearson product–moment correlation coefficient (r) and the coefficient of determination (r^2) and linear regression analysis. Both correlation and linear regression analyses were performed on the data using GraphPad Prism 2.0. The ranges of published dissociation constants and their corresponding mean values used for each antagonist at the five muscarinic receptor subtypes are shown in Table 1. Additionally, for each comparison of dissociation constants at specific subtypes, the linear regression was

Table 4
Results of correlation and linear regression analysis for functional studies indicating slopes of unconstrained regressions and their corresponding r and r^2 values and slopes of the constrained regression lines

	M_1	M_2	M_3	M_4	M_5
Unconstrained slope[a]	0.80 ± 0.20	−0.06 ± 0.45	1.01 ± 0.14	0.54 ± 0.19	0.89 ± 0.27
r[b]	0.81	−0.05	0.95	0.70	0.76
r^2[c]	0.65	0.002	0.87	0.50	0.59
Constrained slope[d]	1.11 ± 0.02	1.09 ± 0.06	1.04 ± 0.01	1.13 ± 0.03	0.99 ± 0.02

[a] Slope of the regression ± S.E.M.
[b] Pearson product–moment correlation coefficient.
[c] Coefficient of determination.
[d] Slope of the regression line constrained to pass through the origin ± S.E.M.

constrained to pass through the origin (0,0). A slope value and regression coefficient were obtained and compared to a value of unity, the theoretical value expected for a line based on an ideal correlation with the receptor subtype with which comparison was made.

3. Results

3.1. Functional studies

The $-\log EC_{50}$ value (± S.E.M.) obtained from control concentration–response curves to carbachol was 5.45 ± 0.08 ($n = 15$). All antagonists employed in this study produced parallel shifts of the concentration–response curve to carbachol (Fig. 1). From the analysis of the Arunlakshana–Schild plot, the resulting pA_2 and pK_B values were obtained and pK_B values are shown in Table 2. None of the antagonists produced Arunlakshana–Schild plots with a slope significantly different from unity ($P > 0.05$).

3.2. Saturation and displacement binding studies with [^{3}H]quinuclidinyl benzilate

Specific binding was a saturable process, which reached a limiting value with increasing concentrations of [^{3}H]quinuclidinyl benzilate. Analysis of the saturation binding curves revealed that the mean K_d value (± S.E.M.) was 158 ± 63 pmol ($n = 4$). The B_{max} value (± S.E.M.) was 419 ± 40 fmol/mg of protein. Data from analysis of competition binding curves are noted in Table 3. Several of the mean slope values from individual binding experiments were significantly different from unity although combined analysis of the data did not reveal two-site binding.

3.3. Statistical analysis

As noted, the pK_B values obtained in functional studies and the pK_i values obtained in binding studies on chick ileum were compared with the mean published pK_i or pK_B values of the antagonists for specific mammalian receptor subtypes, using linear regression analysis. The results are presented in Fig. (2A and B). The data were further analysed by correlation analysis and are presented in Tables 4 and 5. For functional studies, the muscarinic M_3 and M_5 receptor plots show good correlations, whereas the muscarinic M_2 receptor plot best fits the binding data.

4. Discussion

In general, the affinities obtained in the functional studies exhibited good agreement with the existence of the muscarinic M_3 receptor subtype, and to a lesser extent,

Table 5
Results of correlation and linear regression analysis for binding studies indicating slopes of unconstrained regression lines and their corresponding r and r^2 values and slopes of the constrained regression lines

	M_1	M_2	M_3	M_4	M_5
Unconstrained slope[a]	0.32 ± 0.36	1.14 ± 0.24	0.13 ± 0.41	0.56 ± 0.22	0.27 ± 0.44
r[b]	0.29	0.87	0.10	0.67	0.22
r^2[c]	0.09	0.74	0.01	0.45	0.04
Constrained slope[d]	1.04 ± 0.04	1.04 ± 0.02	0.97 ± 0.04	1.06 ± 0.02	0.93 ± 0.04

[a] Slope of the regression ± S.E.M.
[b] Pearson product–moment correlation coefficient.
[c] Coefficient of determination.
[d] Slope of the regression line constrained to pass through the origin ± S.E.M.

with the mammalian muscarinic M_5 receptor gene product. These findings, however, are in direct contrast to the binding experiments, in which the single binding site appeared to be the muscarinic M_2 receptor subtype.

The r and r^2 values for the correlation analysis based on functional studies (Table 4) revealed that the best correlation was with the muscarinic M_3 receptor subtype, followed by successively poorer correlations with muscarinic M_1, M_5, M_4 and M_2 receptor subtypes. For binding studies, there was high correlation with the muscarinic M_2 receptor subtype, and again, successively poorer correlation with the muscarinic M_4, M_1, M_5 and the M_3 receptor subtypes (Table 5). The slope of the regression constrained to pass through the origin was also used as a measure of the relationship between dissociation constants in the chick ileum and published values. A line with a slope closest to a value of unity would represent an ideal correlation. For functional studies, the best agreement was found to be with the muscarinic M_3 and M_5 subtypes (Table 4 and Fig. 2). For binding studies, inspection of the data in Fig. 2 showed the best agreement to be with the muscarinic M_2 receptor subtype.

Overall, these analyses indicated that the receptor responsible for the contractile response was most likely the muscarinic M_3 receptor subtype. In contrast, analysis of competition binding curves suggested the presence of a uniform population of the muscarinic M_2 receptor subtype. This is different from several published studies in mammalian smooth muscle, where a heterogenous population of binding sites was detected in which up to 30% appear to be of the muscarinic M_3 receptor subtype and the majority ($> 70\%$) are the muscarinic M_2 receptor subtype (Doods et al., 1994; Eglen et al., 1994; Giraldo et al., 1988; Lazareno and Roberts, 1989) with the muscarinic M_3 receptor population being responsible for the contractile response (Waelbroeck et al., 1989; Lambrecht et al., 1989). The muscarinic M_2 receptor may be coupled via G_i to activate opening of a cation channel (Zholos and Bolton, 1997) or to inhibit adenylyl cyclase and counter relaxation produced by β-adrenoceptor activation (Ehlert et al., 1999).

The differences between binding studies in chick ileum as compared to mammalian tissue and the discrepancy between the functional and binding data could be partially explained on the basis of some limitations of computer analysis of competition binding curves for assessing heterogenous populations of receptors (De Lean et al., 1981; Limbird, 1996). Typically, 15–18 data points are required in competition displacement curves to resolve one-site from two-site fits in a statistically significant manner (Limbird, 1996). In experiments on chick ileal, homogenates up to 17 data points were used and, hence, one would expect statistically significant resolution. However, one factor that may contribute to lack of significant discrimination is the low selectivity of muscarinic receptor antagonists. None of the antagonists used exhibits greater than 30-fold higher affinity for one subtype over all others (see Table 1). De Lean et al. (1981) noted that when the total binding site population comprises a 90%:10% mix of receptor subtypes, a competitive antagonist needed to exhibit an affinity from 70- to 200-fold greater at one receptor subtype than another for statistical analysis to be able to resolve the data into a two-site rather than a one-site fit. Caulfield (1993) cautioned that identification of muscarinic receptor subtypes may be hindered by the limited subtype selectivity exhibited by antagonists and the current findings exemplify this difficulty. It may be possible that the receptor identified in functional studies on chick ileum represents a small percentage ($< 10\%$) of the total receptor population that was not able to be discriminated in this binding analysis.

As noted, correlation coefficients obtained in comparison of functional data with published affinities indicated the existence of a muscarinic M_3 receptor. However, individual comparisons of dissociation constants in the chick functional studies with published values for mammalian receptors reveal several discrepancies and, therefore, the definite characterisation of a muscarinic M_3 receptor subtype is limited. The values for antagonists such as AF-DX 116, AF-DX 384, 4-DAMP and tripitramine are close to, or within the range for the muscarinic M_3 receptor subtype. However, the dissociation constants for himbacine, AQ-RA 741, *p*-F-HHSiD and HHD exhibit some discrepancies. Moreover, there are similarities in affinities for the muscarinic M_5 receptor subtype and dissociation

Table 6
Range of pK_i or pK_B values and available mean values (in brackets) reported for the antagonists at native (capital) and cloned (lower case) chick receptors and binding and functional affinities obtained in studies with chick ileum

Antagonist	M_2/m2	M_3/m3	M_4/m4	m5	Chick ileum	
					pK_B	pK_i
Pirenzepine	7.2–7.9[a,b,c,d,e] (7.6)	6.4[f]	7.3–10[a,c,d,g] (8.7)	5.5[h]	6.6	6.8
Benzhexol	8.7[a]	–	–	–	7.9	7.8
Himbacine	7.4–8.2[a,b] (7.8)	–	8.2[a]	–	6.7	7.5
AF-DX 116	6.6–7.7[c,d] (7.2)	–	5.0–7.1[c,d] (6.1)	5.8[h]	5.9	6.6

[a] Lazareno et al. (1990).
[b] Choo et al. (1988).
[c] Tietje and Nathanson (1991).
[d] Jakubik and Tucek (1994).
[e] Jeck et al. (1988).
[f] Gadbut and Galper (1994).
[g] Tietje et al. (1990).
[h] Creason et al. (2000).

constants for several antagonists, such as benzhexol, AQ-RA 741 and HHD.

The individual differences reported between dissociation constants in chick as compared to mammalian receptors in this study and as reported by Choo et al. (1988) suggest the possibility of an avian correlate of a muscarinic receptor subtype. Some avian and mammalian family differences occur in amino acid sequences of muscarinic receptor subtypes. Avian correlates have been reported in atria (chick muscarinic M_2 receptor; Tietje and Nathanson, 1991), heart (chick muscarinic M_4 receptor; Lazareno et al., 1990) and brain (chick muscarinic M_3 receptor; Gadbut and Galper, 1994) and a recent reverse transcriptase polymerase chain reaction study has revealed the existence of a chick muscarinic M_5 receptor subtype in embryonic heart and brain (Creason et al., 2000). Dissociation constants of a limited range of selective muscarinic receptor antagonists, such as benzhexol, pirenzepine, himbacine and AF-DX 116, have been published for chick receptors (Table 6). Comparison of the dissociation constants obtained in the present binding studies for the chick ileum show some agreement with the published values for the chick muscarinic M_2/m2 receptors (Table 6). The values in the functional experiments for pirenzepine now suggest the presence of that the muscarinic M_3 receptor subtype, rather than muscarinic M_5 receptor subtype, given the finding of a low affinity for pirenzepine at the chick muscarinic m5 receptor (Creason et al., 2000).

Thus, in conclusion, it appears that the receptor in the chick ileum responsible for the contractile response most closely resembles the muscarinic M_3 receptor subtype. The receptor responsible for the functional response could not be the same as that detected in binding studies, which suggests that it exists only as a small proportion of the total binding population. The characterisation of the binding site revealed it to be most consistent with the muscarinic M_2 receptor subtype, although this population does not appear to contribute to the functional response. The absolute values for the antagonist dissociation constants limit the characterisation of the receptors so that their final identity remains to be elucidated.

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