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Muscarinic receptor subtypes in the human colon: lack of evidence for atypical subtypes

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Abstract

Characteristics of muscarinic receptors were investigated in circular muscle from normal human colon. In saturation studies ($n = 18$), binding of [^{3}H]quinuclidinyl benzylate (QNB) was of high affinity (K_d 87.3 pM) and capacity (B_{max} 362 ± 27 fmol/mg protein), with no differences between ascending and sigmoid colon. Kinetic studies gave a K_d of 55 pM. Methoctramine and darifenacin displayed biphasic binding profiles, the high affinity components being compatible with a population of approximately 80 ± 5% M_2 and 13 ± 2% M_3 muscarinic receptors, respectively. Pirenzepine, mamba toxin 1 and mamba toxin 3 were very weak competitors, indicating negligible expression of muscarinic M_1 and M_4 receptors. Six other subtype-preferring antagonists exhibited K_i values typical of those reported at cloned human muscarinic M_2 receptors. In the presence of methoctramine, pre-treatment with alkylating agent 4-diphenylacetoxy-*N*-(2-chloroethyl)-piperidine hydrochloride (4-DAMP mustard) inhibited [^{3}H]quinuclidinyl benzylate binding to 26% of sites. Following alkylation of muscarinic M_3 receptors, darifenacin bound to a single low affinity site, indicating binding to muscarinic M_2 receptors.

Keywords: Muscarinic receptor; [^{3}H]Quinuclidinyl benzylate; Colon, human; Circular muscle; Radioligand binding; Alkylating agent

1. Introduction

Acetylcholine is the major excitatory transmitter in the gastrointestinal tract and initiates smooth muscle contraction by interacting with G protein-coupled muscarinic receptors. To date, five subtypes (M_1–M_5) of muscarinic receptors have been cloned and pharmacologically characterized (Caulfield and Birdsall, 1998), with evidence for multiple muscarinic receptor subtypes in smooth muscle-containing tissues, including the gastrointestinal tract (Eglen et al., 1996; Eglen, 2001).

In vitro, functional experiments have suggested that muscarinic M_3 receptors are predominant in mediating the contractile response in the gastrointestinal tract, since muscarinic M_3 receptor subtype-preferring antagonists can inhibit contractions produced by muscarinic agonists (Eglen et al., 1990; Eglen et al., 1994; Wallis, 1995). However, both radioligand binding and receptor antibody studies from a variety of smooth muscles have shown that the majority of muscarinic receptors present are the M_2 subtype, typically comprising ≥70% of the receptor population (Eglen et al., 1996).

In the colon, as in other regions of the gut, excitatory responses induced by activation of motor neurons can be inhibited by atropine, indicating the importance of cholinergic neurons in the normal regulation of physiological function in this tissue (Kunze and Furness, 1999). In guinea pig and dog colon, the muscarinic receptor subtype populations are similar to those seen in other gastrointestinal smooth muscle (Zhang et al., 1991; Sawyer and Ehlert, 1998). In the rat colon, lower proportions of muscarinic M_2 receptors, ranging from 39% to 55%, have been reported (Gomez et al., 1992; Zhang, 1996).

Although all five muscarinic receptor subtypes have been reported in human oesophageal muscularis (Preiksaitis et al., 2000), there is only limited information for the human colon. The sole binding study (Gomez et al., 1992) suggested that muscarinic M_2 receptors represented 76% of the total muscarinic receptor population, but this early study used only two subtype-preferring antagonists, pirenzepine

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and {[2-[(diethylamino)methyl]-1-piperidinyl]acetyl}5,11-dihydro-6*H*-pyrido[2,3*b*][1,4] benzodiazepine-6-one (AF-DX 116), to characterise the muscarinic receptor subtypes present. The dissociation constants (K_i values) obtained for each of these antagonists at the putative muscarinic M_2/M_3 receptors were from 4 to 8 fold higher than those generally reported for the corresponding cloned human muscarinic receptor subtypes, raising the possibility of atypical muscarinic receptors in the human colon. The one functional study on human colon (Kerr et al., 1995) concluded that contractions to carbachol were mediated by muscarinic M_3 receptors, as found in other gastrointestinal studies, but also found that some antagonists, including AF-DX 116, exhibited atypical dissociation constants.

The aim of this study was to use [^{3}H]quinuclidinyl benzilate receptor binding and a large range of subtype-preferring muscarinic receptor antagonists to characterise the receptors in human colon more thoroughly. Part of these studies used membranes alkylated with the irreversible ligand, 4-diphenylacetoxy-*N*-(2-chloroethyl)-piperidine hydrochloride (4-DAMP mustard). In solution, this forms an aziridinium ion which initially acts as a competitive antagonist but then forms a covalent bond with muscarinic receptors (Barlow et al., 1990). To define the contributions of receptor subtypes, we adopted a "receptor protection" approach, using a muscarinic M_2 subtype-preferring antagonist, followed by alkylation with 4-DAMP mustard, as previously described in muscle strip contractile studies (Ehlert and Griffin, 1998).

2. Materials and methods

2.1. Colon specimen collection

Specimens of 22 ascending, 4 descending and 32 sigmoid colon were collected from 33 male and 24 female patients (age range 33–86 years), undergoing colectomy for carcinoma, at the St. George Hospital, Sydney. Whole ring segments of macroscopically normal colon, 10–20 cm from the carcinoma, were placed immediately into ice-cold Krebs–Henseleit solution (composition in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and D-glucose 11.7), pregassed with carbogen (95% O_2, 5% CO_2). Specimens were immediately transported to the laboratory and transferred to fresh cold pregassed solution. Specimens were either refrigerated overnight before dissection or dissected immediately. The serosa, mucosa and submucosa were removed and taenia coli were cut away, leaving the circular muscle. This circular muscle (which also contained a layer of longitudinal muscle and the myenteric plexus) was cut into portions of approximately 500 mg, which were frozen in liquid nitrogen and then stored at − 70 °C until use. Specimens showing inflammation, of abnormal histological appearance, or from patients with obstruction or previous radiotherapy, were routinely discarded. This project was approved by the Human Ethics Committee of the University of New South Wales (97139).

2.2. Radioligand binding studies

2.2.1. Membrane preparation and incubation

Radioligand binding studies were carried out following the method described previously (Christopoulos et al., 1993). Approximately 500 mg circular muscle was finely minced in ice-cold sodium phosphate buffer (approximately 10 ml, 50 mM Na_2HPO_4, pH 7.4) and homogenized with a Polytron (setting 5, for 3 × 10 s). The suspension was then centrifuged at 1000 × *g* for 15 min. The pellet was discarded and the supernatant re-centrifuged at 40,000 × *g* for 20 min. The final pellet was resuspended in approximately 10 ml of 50 mM sodium phosphate buffer, pH 7.4.

Membranes were incubated with the non-selective radioligand [^{3}H]quinuclidinyl benzylate (QNB) in 50 mM phosphate buffer (pH 7.4) at 37 °C. Nonspecific binding was defined in replicate tubes using 10 μM atropine. The incubation was initiated by addition of membranes (final tissue concentration 2% wet weight) to each tube. Incubations were terminated by addition of 3 ml ice-cold 50 mM sodium phosphate buffer (pH 7.4). Membranes were filtered using a Brandel tissue harvester through GF/B filters (Whatman) presoaked overnight at 4 °C in sodium phosphate buffer containing 0.5% polyethyleneimine and 10 μM atropine. The filters were washed three times with 3 ml ice-cold buffer and then placed into scintillation vials containing 2 ml scintillant (Beckman Ready Safe). The vials were left overnight before the radioactivity was measured by liquid scintillation spectrometry.

2.2.2. Kinetic, competition and saturation studies

Association and dissociation studies were performed using four concentrations of [^{3}H]quinuclidinyl benzylate (50 pM to 2 nM). Association experiments were conducted with 5 time points over 180 min. In dissociation experiments, membranes were incubated with [^{3}H]quinuclidinyl benzylate for 2 h (determined as optimum incubation time). After 2 h, atropine (10 μM) was added and [^{3}H]quinuclidinyl benzylate allowed to dissociate from the membranes for up to 4 h, with membranes harvested at 10 time points.

In competition experiments, increasing concentrations of competitor were incubated in 50 mM sodium phosphate buffer (pH 7.4) with 200 pM [^{3}H]quinuclidinyl benzylate for 2 h before filtration and washing as above. For most compounds, 17 or more concentrations of competitor were used, but only 10 concentrations were used for quinuclidinyl benzylate and atropine.

In saturation experiments, eight concentrations of [^{3}H]quinuclidinyl benzylate (15 pM to 2 nM) were incubated with membranes for 2 h at 37 °C. Protein content was determined using established methods, employing bovine albumin as a standard.

2.2.3. Receptor alkylation studies

Since most subtype-preferring muscarinic ligands are not highly receptor-selective, we attempted to use selective alkylation by 4-DAMP mustard, with receptor protection, in order to further explore interactions of [^{3}H]quinuclidinyl benzylate with the muscarinic M_2 receptor in this preparation. An adaptation of the methods of Ehlert and Griffin (1998) and Waelbroeck et al. (1992) was used, with all procedures carried out at 37 °C. Although 4-DAMP mustard shows a ~ 10-fold higher affinity for muscarinic M_3 over muscarinic M_2 receptors (Ehlert et al., 1996), it is still necessary to "protect" muscarinic M_2 receptors with a reversible antagonist during the alkylation procedure, in order to selectively inactivate a high proportion of the muscarinic M_3 receptors (Thomas et al., 1992). This was achieved by the use of a protective concentration (200 nM) of methoctramine. On the basis of the K_i values obtained in the competition experiments, this concentration was predicted to occupy 95% of the muscarinic M_2 receptor subtypes but only 28% of the M_3 subtypes, in the absence of other antagonists.

Colon circular muscle membranes were homogenized in phosphate buffer as above and centrifuged for 15 min at 1000 × *g*. Part of the supernatant was then pre-incubated with 200 nM methoctramine for 30 min, to occupy and "protect" muscarinic M_2 receptors, followed by an additional 60 min incubation in the presence of 4-DAMP mustard (2.5 nM) to alkylate the remaining muscarinic receptors (hypothesized to be M_3). The membranes were centrifuged at 40,000 × *g* for 20 min and the pellet washed in sodium phosphate buffer for 30 min to remove methoctramine from the previously protected muscarinic M_2 receptors as well as unbound 4-DAMP mustard.

Membranes prepared by this method were then incubated in parallel with membranes from the same specimen, prepared without preincubation procedures, and used for saturation and for limited competition experiments.

2.3. Data analysis

For kinetic studies, the dissociation data were analysed using the global fit function of Graph Pad Prism (version 4, Graph Pad Software). For competition binding studies, all data were simultaneously fitted to a one or two site model using the non-linear regression analysis program of Graph Pad Prism (version 3). The affinities of competitors for [^{3}H]quinuclidinyl benzylate binding sites were expressed as IC_{50}. K_i was calculated according to the formula $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioligand that was used to estimate K_i (Cheng and Prusoff, 1973). The K_d value used was the kinetic K_d. Theoretical calculations based on the rate constants obtained in the kinetic studies (Motulsky and Mahan, 1984) indicate that equilibration would not have been reached during the time of incubation for low concentrations (< 80 pM) of [^{3}H]QNB so the kinetic K_d provides a better measure of the true K_d value. Since K_d and K_i are not normally distributed, these data are presented as geometric mean (95% confidence intervals {CI}), whereas all other data are shown as mean ± S.E.M.

Data from studies using preincubation with 4-DAMP mustard were compared using one-way analysis of variance (ANOVA) followed by the Bonferroni test (four groups) or two-way ANOVA (two groups).

2.4. Materials

[^{3}H]Quinuclidinyl benzylate was obtained from NEN (Boston, USA). Quinuclidinyl benzylate was obtained from ICN (Irvine, USA) and atropine, 4-diphenylacetoxy-*N*-methylpiperidine-methiodide (4-DAMP), 4-DAMP mustard, methoctramine, pirenzepine and the mamba toxins 1 and 3 were obtained from Sigma (St. Louis, USA). (11-[[4-[4-(Diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3*b*][1,4]benzodiazepine-6-one (AQ-RA 741), (6-chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)acetyl]-11H-dibenzo-[*b*,*e*][1,4] diazepine-11-one hydrochloride (UH-AH 37), AF-DX 116 (otenzepad) and (±)-5,11-dihydro-11-{[(2-{2-[(dipropylamino) methyl]-1-piperidinyl} ethyl) amino] carbonyl}-6*H*-pyrido [2,3*b*][1,4] benzodiazepine-6-one (AF-DX 384) were gifts from Dr. Karl Thomae

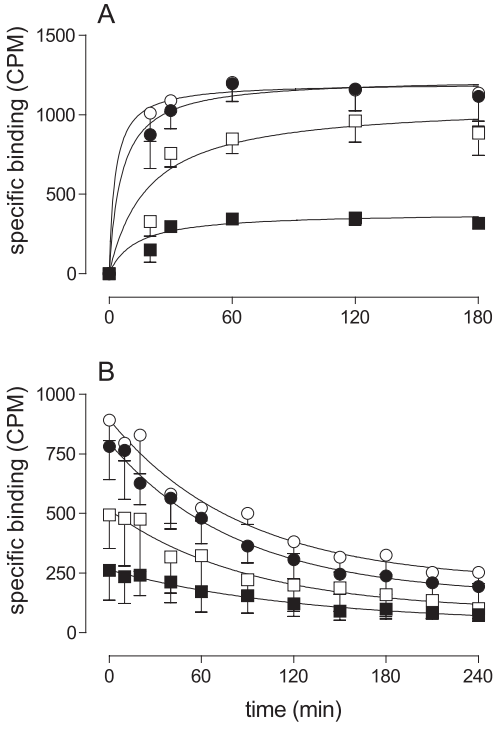

Fig. 1. Time course for [^{3}H]quinuclidinyl benzylate specific binding to human colon membranes, using four concentrations of radioligand: 2000 pM (○), 1000 pM (●), 200 pM (□), 50 pM (■). (A) Membranes were incubated with [^{3}H]quinuclidinyl benzylate for up to 3 h, with specific binding stable after 60–120 min (n = 3–5). (B) Dissociation of [^{3}H]quinuclidinyl benzylate from human colon membranes. After 2 h of incubation with [^{3}H]quinuclidinyl benzylate, atropine (10 μM) was added and the radioligand allowed to dissociate from the membranes over a 4-h period. Results are presented as percent of maximal binding plotted against time for dissociation (n = 2). Values are mean ± S.E.M.

GmbH (Biberach an der Riss, Germany). Darifenacin was a gift from Pfizer (Sandwich, UK). All other reagents were of analytical grade.

3. Results

3.1. Kinetic studies

In association studies, four concentrations of [^{3}H]quinuclidinyl benzylate were incubated with human colon circular muscle membranes for up to 3 h (Fig. 1A). Specific binding appeared stable at approximately 60–120 min, and an incubation time of 2 h was chosen for subsequent experiments. In dissociation experiments, [^{3}H]quinuclidinyl benzylate was incubated with membranes for 2 h, at which time 10 μM atropine was added and the [^{3}H]quinuclidinyl benzylate allowed to dissociate from the membranes over a 4-h period (Fig. 1B). Dissociation was mono-exponential (K_{off}, 0.012 ± 0.003 min^{-1}), and at the end of this time, approximately 25% of the [^{3}H]quinuclidinyl benzylate still remained bound to the receptor. The kinetic K_d was 55 pM (CI 28–115 pM).

3.2. Saturation studies

Membranes were incubated for 2 h in the presence of increasing concentrations of [^{3}H]quinuclidinyl benzylate

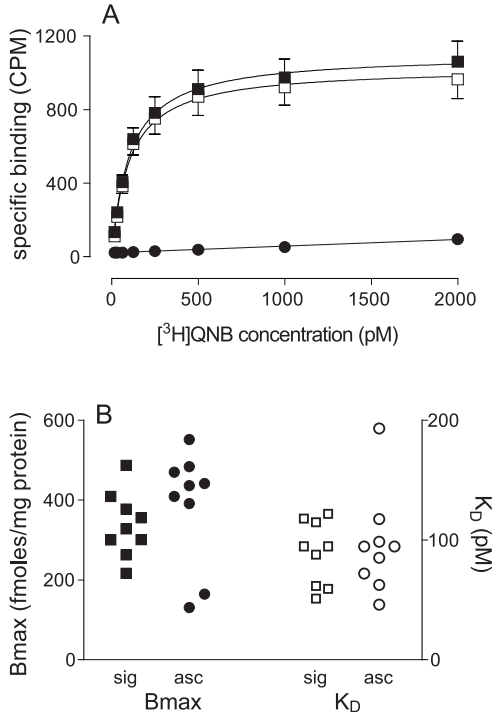

Fig. 2. (A) Saturation data obtained using increasing concentrations of [^{3}H]quinuclidinyl benzylate incubated with human colon circular muscle membranes. Total (■), specific (□) and nonspecific (●) binding (n = 10). Values are mean ± S.E.M. (B) Comparison of binding parameters in ascending colon (asc) and sigmoid colon (sig) circular muscle. There were no significant differences between regions.

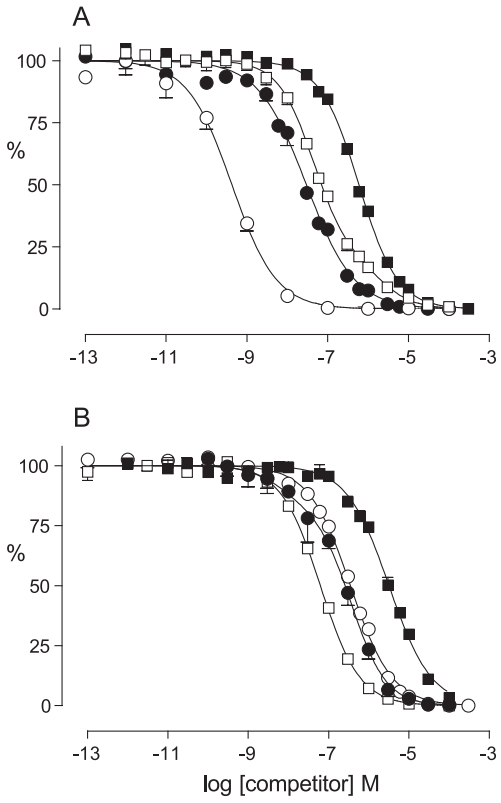

Fig. 3. Competition for [^{3}H]quinuclidinyl benzylate binding to human colon membranes by muscarinic receptor antagonists. (A) Quinuclidinyl benzylate (○); AQ-RA 741 (●); methoctramine (□); AF-DX 116 (■). (B) 4-DAMP (□); darifenacin (●); UH-AH 37 (○); pirenzepine (■).Values are mean ± S.E.M. (n = 3–7).

(Fig. 2A). Specific binding was saturable at approximately 500 pM [^{3}H]quinuclidinyl benzylate, with K_d 87.3 pM (CI 76–110 pM) and B_{max} 362 ± 27 fmol/mg protein (n = 18). Binding of [^{3}H]quinuclidinyl benzylate was to a single site (n_H 1.33 ± 0.5). There was no difference in receptor affinity or number between ascending (n = 9) or sigmoid (n = 9) colon (Fig. 2B), and no gender difference (data not shown). There was no correlation between patient age and either K_d or B_{max} (r^2 = 0.16 and 0.30, respectively).

3.3. Competition studies

A number of compounds were examined for their ability to compete with 200 pM [^{3}H]quinuclidinyl benzylate (Fig. 3; Table 1). The order of potency, based on K_i values, was quinuclidinyl benzylate≫atropine>AQ-RA 741>AF-DX 384>4-DAMP>methoctramine>darifenacin>UH-AH 37>AF-DX 116≫pirenzepine.

However, some compounds displayed slope factors less than unity, and a two-site analysis resulted in significantly better resolution of some of these data (Table 1). The high affinity (H) component of binding of the muscarinic M_2 receptor-preferring antagonist methoctramine represented 80 ± 5% of sites, with 47-fold difference in affinity between H and low (L) components. In contrast, the high affinity

Table 1
Potency of muscarinic receptor ligands as competitors for [^{3}H]QNB binding in human colon circular muscle membranes

Competitor	*n*	Slope factor	One-site K_i (nM) (CI)	Two-site K_i (nM)
QNB	3	0.79 ± 0.07	0.09 (0.07–0.12)	N/A
Atropine	5	0.94 ± 0.05	3.75 (3.29–4.28)	N/A
AQ-RA 741	7	0.73 ± 0.03	6.40 (5.62–7.29)	N/A
AF-DX 384	5	0.86 ± 0.06	11.9 (10.4–13.7)	N/A
4-DAMP	6	0.89 ± 0.03	13.5 (12.3–14.7)	N/A
Methoctramine	6	0.73 ± 0.03	17.6 (15.7–19.8)	80 ± 5% H: 9.42 (7.19–12.3) 20% L: 441 (130–1500)
Darifenacin	8	0.74 ± 0.04	58.9 (50.3–69.1)	13 ± 2% H: 0.24 (0.06–1.01) 87% L: 95.8 (78.8–117)
UH-AH 37	7	0.86 ± 0.03	76.5 (69.5–84.2)	N/A
AF-DX 116	7	0.91 ± 0.03	128 (118–139)	N/A
Pirenzepine	4	0.82 ± 0.03	687 (620–761)	N/A

N/A, two-site analysis not applicable.
H, high affinity component; L, low affinity component, with % of each component.

component of the muscarinic M_3 receptor-preferring darifenacin represented only 13 ± 2% of total binding sites, with 400-fold difference in affinity between H and L.

With regard to the muscarinic receptor ligands AF-DX 116 and AF-DX 384 (M_2 preferring), UH-AH 37 and pirenzepine (M_1 preferring), the data could be fitted to a two-site as well as to a one-site model, although the latter was statistically preferred. The high affinity components of the binding of these compounds represented 76%, 54%, 25% and 3%, respectively, of the total binding sites.

Mamba toxin 1 (M_1/M_4 receptor selective) and mamba toxin 3 (M_4 receptor selective), up to a concentration of 100 nM, were ineffective competitors of [^{3}H]quinuclidinyl benzylate binding in human colon circular muscle membranes (data not shown).

There was an excellent correlation (Fig. 4A, $r^2 = 0.923$) between the K_i values obtained for these antagonists (Table 1) and the corresponding K_i values for muscarinic M_2 receptors cited in the literature (Table 2), but the correlation was weaker (Fig. 4B, $r^2 = 0.487$) between the observed K_i values and those cited at muscarinic M_3 receptors (Table 2). For methoctramine and darifenacin, the H and L K_i values, respectively, were used in Fig. 4A, whereas the reverse is the case in Fig. 4B. There was no correlation between the observed K_i values and those cited at muscarinic M_1, M_4 or M_5 receptors ($r^2 = 0.12$, 0.059 and 0.139, respectively).

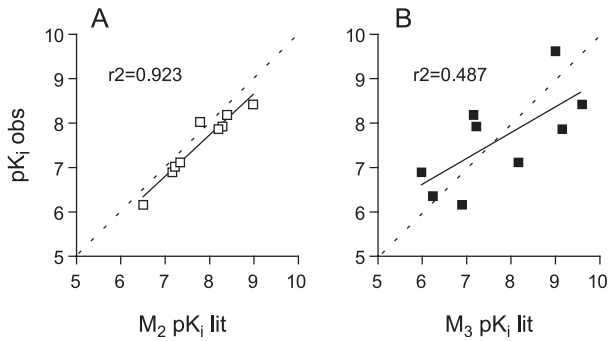

Fig. 4. Regression analysis comparing the calculated pK_i values (pK_i obs) obtained from Table 1 with the expected (pK_i lit) values cited in Table 2, at (A) muscarinic M_2 and (B) muscarinic M_3 receptors. Data are shown for all competitors excluding quinuclidinyl benzylate and the mamba toxins. Two site values for methoctramine and darifenacin are included. There was an excellent correlation ($r^2 = 0.923$) between expected K_i (see Table 2) and observed K_i for muscarinic M_2 receptors, whereas correlation ($r^2 = 0.487$) was weaker for K_i values at muscarinic M_3 receptors.

3.4. Further evaluation of the minor site

Comparison of the percentage of minor binding site found with either methoctramine or darifenacin showed no significant difference between the two estimates ($P < 0.05$) so a global fit of the data for these ligands was undertaken to determine a common percentage of the minor site. The value obtained was 14.2% ± 8. Fixing this percentage of minor site all the subtype-preferring ligands were re-evaluated for two site binding and the resulting pK_i values for the minor site were used in correlation plots versus pK_i estimated from mean literature values tabulated in Table 2 for the various receptor subtypes. The best correlation was with the muscarinic M_3 receptor ($r^2 = 0.548$), corresponding values for the muscarinic M_1, muscarinic M_4 and muscarinic M_5 receptors were 0.223, 0.381 and 0.268.

Table 2
Binding parameters (K_d[a] or K_i[b] values in nM) for muscarinic ligands in cell lines expressing recombinant human muscarinic receptor subtypes

Compound	Muscarinic receptor subtype				
	M_1	M_2	M_3	M_4	M_5
[^{3}H]Quinuclidinyl benzylate[a]	0.13	0.04	0.05	0.05	0.28
Atropine[b]	0.3	0.8–1.3	0.2–0.3	0.1–1.2	0.2–0.8
AF-DX 116[b]	420–740	**64–74**	790–1300	210–540	5100
AF-DX 384[b]	31	**4.4–6**	55–66	10–15	540–730
4-DAMP[b]	0.6–1.2	3.8–9	**0.4–1.0**	0.7–1.7	0.6–1.3
Darifenacin[b]	7.1–35	44–77	**0.75–1.25**	18–46	5.4–9.3
Methoctramine[b]	41–110	**13–20**	210–950	32–78	134–400
Pirenzepine[b]	**6.3–9.4**	220–410	75–180	17–48	66–160
UH-AH 37[b]	**1.8–2.4**	44–49	6.4–7.2	4–5	4.7–5.4
AQ-RA 741[b]	29–62	**3.7–4.4**	55–86	**1.5–15**	730–830

Values in bold indicate preferred receptor subtype.
[a] K_d values from Miller et al. (1992).
[b] Range of K_i values obtained from the following sources: Dörje et al. (1991); Gillberg et al. (1998); Gitler et al. (1992); Moriya et al. (1999); Wallis and Napier (1999); Watson et al. (1999); Wess et al. (1991).

3.5. Receptor alkylation studies

In preliminary experiments, saturation studies were performed using membranes alkylated with a range of concentrations (2.5–40 nM) of 4-DAMP mustard (data not shown). On the basis of these, a concentration of 2.5 nM was chosen for further saturation experiments, using membranes incubated with 4-DAMP mustard alone and in combination with 200 nM methoctramine (Fig. 5A). Preincubation with 4-DAMP mustard alone resulted in a variable decrease ($P < 0.001$, one-way ANOVA) in specific binding (33 ± 12%) of the specific binding in the absence of mustard at 2 nM [^{3}H]quinuclidinyl benzylate), equivalent to a B_{max} of 83 ± 10 fmol/mg protein. This suggests alkylation of 67% of the available muscarinic receptor population. When muscarinic M_2 receptors were "protected" with methoctramine, specific binding was reduced to 74 ± 6% of that in the absence of mustard (B_{max} 266 ± 17 fmol/mg protein), equivalent to alkylation of 26% of muscarinic receptors ($P < 0.05$ compared with 4-DAMP alone, one-way ANOVA; $P < 0.001$ compared with control, two-way ANOVA). The K_d values of [^{3}H]quinuclidinyl benzylate for control [91.2 pM (CI 75–107 pM)] and treated membranes [79.9 pM (CI 50–110 pM)] were unaltered. Preincubation with 200 nM methoctramine alone, followed by washing, did not significantly alter B_{max} (Fig. 5A).

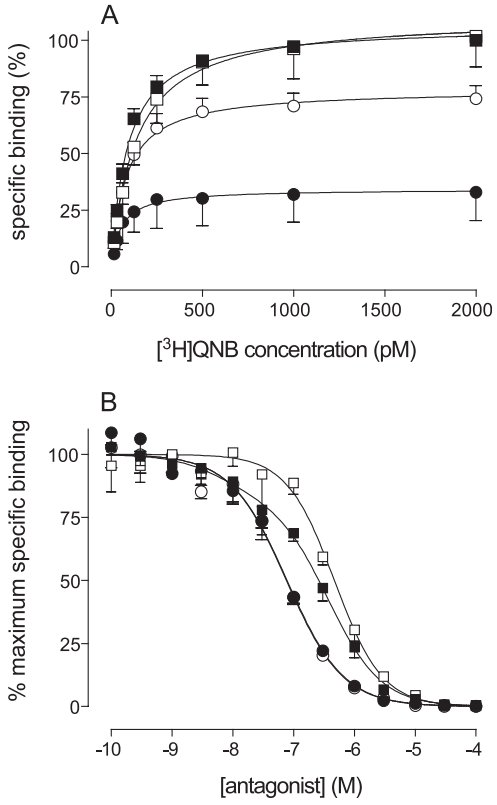

Fig. 5. Data from receptor alkylation experiments. (A) Saturation curves illustrating specific binding of [^{3}H]quinuclidinyl benzylate in untreated membranes (■, $n = 8$), in membranes incubated with 200 nM methoctramine alone (□, $n = 5$), and in membranes alkylated with 2.5 nM 4-DAMP mustard alone (●, $n = 4$) and in the presence of 200 nM methoctramine (○, $n = 8$). (B) Effect of membrane pretreatment (open symbols) with methoctramine and 4-DAMP mustard on competition curves to AF-DX 384 (●,○) and darifenacin (■,□). Experiments were conducted in parallel with untreated membranes from the same specimen (closed symbols). Note the steepening and the shift to the right of the curve to darifenacin, while that to AF-DX 384 is unaffected following receptor alkylation. Results are presented as mean ± S.E.M., $n = 4$; where error bars are not shown they lie within the dimensions of the symbol.

Competition curves for two antagonists were repeated using a paired design ($n = 4$) with methoctramine and 4-DAMP mustard pretreated membranes. Under these conditions where the majority of M_3 receptors can be presumed to be alkylated, the binding parameters for AF-DX 384 were unaltered (untreated membranes: slope 0.94, K_i 16.8 nM; alkylated membranes: slope 0.94, K_i 16.4 nM). The "untreated" curve for darifenacin was shallow (slope factor 0.78; K_i, H 1.40 nM, 16%; L 80.6 nM, 84%). However, after alkylation, the curve for darifenacin was steepened (slope factor 1.10) and shifted to the right, indicating binding to a single population of sites (Fig. 5B). The high affinity component was removed, with binding to a single site of K_i 101 nM, consistent with the affinity of darifenacin at muscarinic M_2 receptors (Table 2).

4. Discussion

The present study in human colon circular muscle membranes shows high affinity saturable and reversible binding of [^{3}H]quinuclidinyl benzylate to muscarinic receptors. The affinity determined in saturation studies was in agreement with that from kinetic and competition studies. Similar data were obtained from both ascending and sigmoid regions of the colon. It was noteworthy that there were no age-related changes in binding parameters, unlike a parallel study with [^{3}H]quinuclidinyl benzylate in human bladder detrusor muscle, where there was a negative correlation of B_{max} with age (Mansfield et al., 2003). The total number of binding sites (B_{max}, 362 fmol/mg protein) was comparable with that found with [^{3}H]quinuclidinyl benzylate in rat colon smooth muscle membranes (Zhang, 1996) and canine colonic smooth muscle cells (Zhang et al., 1991), but higher than that found in human oesophageal smooth muscle (Preiksaitis et al., 2000). However, this radioligand is not subtype selective (Miller et al., 1992; Table 2), and so the parameters obtained may represent binding to several of the five muscarinic receptor subtypes.

Studies investigating muscarinic receptors have been problematic due to the absence of selective agonists and the poor selectivity of most antagonists. In this study we used a number of muscarinic receptor antagonists with markedly different receptor subtype affinities, in order to determine which of the five muscarinic receptor subtypes were present in human colon membranes.

Methoctramine and darifenacin detected at least two binding sites in the colon. The high affinity site (K_i 9 nM)

of methoctramine corresponded to its reported affinity at muscarinic M_2 receptors and accounted for 80–86% of binding sites, with a lower affinity site (K_i 441 nM) corresponding to values reported at muscarinic M_3 receptors (Table 2). In contrast, the high affinity component of the binding of darifenacin represented only 13–16% of sites. Darifenacin has highest affinity for muscarinic M_3 receptors and the K_i value obtained (0.2 nM) is indicative of the presence of this receptor subtype, although its affinity for muscarinic M_5 receptors is also quite high (Table 2). The low affinity component (K_i 96 nM) corresponds to that reported at muscarinic M_2 receptors.

Evidence for [^{3}H]quinuclidinyl benzylate binding to muscarinic M_1, M_4 or M_5 receptors was weak and/or inconclusive, and the presence of very small populations cannot be excluded. The muscarinic M_1/M_4 receptor ligand, mamba toxin 1, was an ineffective competitor, and the muscarinic M_1 receptor-preferring ligand pirenzepine bound to a single population of low affinity binding sites, with a K_i value similar to that reported at muscarinic M_2 receptors (Table 2). There was no evidence of [^{3}H]quinuclidinyl benzylate binding to muscarinic M_4 or M_5 receptors. The highly selective muscarinic M_4 receptor ligand, mamba toxin 3, had no affinity for [^{3}H]quinuclidinyl benzylate binding sites, suggesting a lack of this receptor subtype. At present, there is no muscarinic receptor antagonist showing high affinity for muscarinic M_5 receptors in preference to other receptor subtypes, although AQ-RA 741 is notable for possessing a 10- to 200-fold lower affinity for the M_5 subtype than for other subtypes (Table 2). However, its K_i (6 nM) was suggestive of interaction with muscarinic M_2 and/or M_4 receptors (Table 2).

The general conclusion that muscarinic M_2 receptors predominate in human colon smooth muscle membranes is in accordance with one previous binding study in this tissue, which suggested muscarinic M_2 receptors to comprise 76% of sites (Gomez et al., 1992). However, that study used only AF-DX 116 and pirenzepine to delineate the subtypes present and found the two antagonists exhibited dissociation constants of 490 and 1740 nM, respectively, at the putative muscarinic M_2 receptor and AF-DX 116 exhibited a value of 8010 nM at the putative muscarinic M_3 receptor. These values indicate a much lower affinity than is generally exhibited by these antagonists at the corresponding cloned human muscarinic receptors (see Table 2), and originally suggested interaction with atypical muscarinic receptors. In contrast, our findings showed typical affinity profiles for all the antagonists tested with firm evidence for muscarinic M_2 and M_3 receptors representing the major and minor binding sites, respectively. The total number of binding sites with [^{3}H]quinuclidinyl benzylate is higher than that previously reported (30 fmol/mg protein) with [^{3}H]*N*-methyl scopolamine (Gomez et al., 1992). The difference probably relates to a difference in membrane preparation and/or the radioligand used: [^{3}H]quinuclidinyl benzylate typically binds to a larger population of sites than the quaternary amine, [^{3}H]*N*-methyl scopolamine, whenever the two compounds are compared in the one tissue (Fang et al., 1993; Lee and El-Fakahany, 1985).

In many regions of the gastrointestinal tract, there is a mixed population of muscarinic M_2 and M_3 receptors with a predominance of M_2 (Eglen et al., 1996; Ehlert et al., 1999; Eglen, 2001) and this situation also appears to be the case in the human colon. In order to distinguish two site binding where there is $\leq$20% of one site, it is necessary for the competing antagonist to have 30–100-fold difference in affinity for the two sites (De Lean et al., 1982). While this was the case with methoctramine and darifenacin, most available antagonists, including AF-DX 116, possess only ~10-fold difference in affinity between muscarinic M_2 and M_3 receptors (Table 2; Caulfield and Birdsall, 1998) and were found to exhibit single site binding. In the human colon study by Gomez et al. (1992), using [^{3}H]*N*-methyl scopolamine, binding of AF-DX 116 could be resolved into two sites and similar data were reported for the same radioligand in human gastric smooth muscle, where AF-DX 116 delineated 79% of high affinity sites (Bellido et al., 1995). Other studies using [^{3}H]quinuclidinyl benzylate have reported only one site binding with AF-DX 116, in colon circular muscle from rat (Zhang, 1996) and dog (Zhang et al., 1991).

Therefore, such competition experiments are rather unsatisfactory in precise delineation of proportions and affinities of receptor subtypes. To characterise further the two putative muscarinic receptor subtypes, selective alkylation of the muscarinic M_3 receptor was undertaken using 4-DAMP mustard (Barlow et al., 1990), with "protection" of the muscarinic M_2 receptors by methoctramine. This approach appeared successful. In saturation studies, pretreatment with both methoctramine and 4-DAMP mustard led to a 26% decrease in B_{max} for [^{3}H]quinuclidinyl benzylate but no change in K_d. When "protection" with methoctramine was omitted, the same concentration of 4-DAMP mustard resulted in a loss of ~ 70% of [^{3}H]quinuclidinyl benzylate binding sites. The selective alkylation of muscarinic M_3 receptors was confirmed in competition studies, where the original, rather shallow, two-site competition curve for darifenacin was converted to a curve supportive of binding to a single site of low affinity, demonstrating interaction at muscarinic M_2 receptors only. No change was observed in the binding curve for AF-DX 384 after treatment with 4-DAMP mustard. Presumably, this was due to the finding in control experiments that AF-DX 384 exhibited only one site competitive inhibition of [^{3}H]quinuclidinyl benzylate binding (slope factor 0.94) and the elimination of the muscarinic M_3 sites did not significantly alter this situation.

There is a major discrepancy between conclusions arising from binding and functional data. Results from a wide range of functional studies indicate that contraction to cholinomimetics is mediated by muscarinic M_3 receptors in gastrointestinal smooth muscle (Eglen et al., 1996; Ehlert et al., 1999; Eglen 2001), including human colon (Kerr et al., 1995). While the muscarinic M_2 receptor subtype represents

the majority of the muscarinic receptors in human colon smooth muscle, the role of these muscarinic M_2 receptors is unclear. Studies in muscarinic M_2 receptor knockout mice have shown only a small decrease in muscarinic agonist potency for contracting the gut (Stengel et al., 2000; Bymaster et al., 2001). Muscarinic M_2 receptors have been reported to cause opening of a non-specific cation channel (Zholos and Bolton, 1995) and activation of ERK1/2 leading to phosphorylation of caldesmon (Cook et al., 2000). Thus, activation of muscarinic M_2 receptors may facilitate the contraction produced by muscarinic M_3 receptor activation (Zhang et al 1991; Ehlert et al., 1999), perhaps by reducing cyclic AMP-dependent processes, thereby facilitating membrane depolarization (Carl et al., 1991).

In conclusion, most muscarinic receptor antagonists displayed rather poor selectivity for individual receptor subtypes, limiting our ability to fully characterise the muscarinic receptor population in the human colon. The technique of receptor protection followed by receptor alkylation with 4-DAMP mustard, which has previously only been used for in vitro functional experiments, can also be used effectively for binding experiments to elucidate the receptor populations present. However, no evidence was found that the muscarinic receptors present in the human colon exhibited atypical dissociation constants for the various antagonists tested. The binding profile of the most subtype-selective ligands used was consistent with binding of [^{3}H]quinuclidinyl benzylate to a majority (86%) of muscarinic M_2 and a minority (14%) of M_3 receptors. However, we cannot exclude the possibility that minor populations of muscarinic M_1, M_4 or M_5 receptors also exist, particularly since, using molecular techniques, we have preliminary evidence for the presence of M_5 and M_1, as well as M_2 and M_3, in colon circular muscle samples (Burcher et al., 2003).

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