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Characterization of muscarinic receptors mediating contractions of circular and longitudinal muscle of human isolated colon

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1 The effects of seven muscarinic receptor antagonists were used to characterize the receptors which mediate carbachol-evoked contractions of intertaenial circular and taenial longitudinal muscle in human isolated colon. The effects of these antagonists were studied upon colon contractions induced by cumulatively added carbachol which had mean EC_{50} values of 11.7 ± 2.3 μM ($n=8$) and 12.6 ± 2.3 μM ($n=8$) respectively upon circular and longitudinal smooth muscle.

2 All antagonists displaced concentration-response curves to carbachol to the right in a parallel manner. The maximum concentration of each antagonist added (30 nM–10 μM) did not significantly suppress the maximum response.

3 In circular muscle, the M_3 muscarinic receptor antagonists, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), hexahydrosiladiphenidol (HHSiD) and para-fluoro-hexahydrosiladiphenidol (*p*-F-HHSiD) inhibited responses with pA_2 values of 9.41 ± 0.23, 7.17 ± 0.07, 6.94 ± 0.18 respectively. The M_2 muscarinic receptor antagonist, AF-DX 116, the M_2/M_4 muscarinic receptor antagonist, himbacine, and the M_1 muscarinic receptor antagonist, pirenzepine, yielded pA_2 values of 7.36 ± 0.43, 7.47 ± 0.14 and 7.23 ± 0.48 respectively. The non-selective antagonist, atropine, had a pA_2 of 8.72 ± 0.28.

4 In longitudinal muscle 4-DAMP, HHSiD, p-F-HHSiD, AF-DX 116, himbacine and pirenzepine gave pA_2 values of 9.09 ± 0.16, 7.45 ± 0.43, 7.44 ± 0.21, 6.44 ± 0.1, 7.54 ± 0.40, 6.87 ± 0.38 respectively. Atropine yielded a pA_2 value of 8.60 ± 0.08.

5 The pharmacological profile of antagonist affinities at the muscarinic receptor population responding to muscarinic agonist-evoked contraction is similar to that widely accepted as characterizing the activation of an M_3 muscarinic receptor subtype, although pA_2 values of some antagonists are lower than that seen in other investigations.

Keywords: Muscarinic receptors; human colon; gastrointestinal smooth muscle

Introduction

The parasympathetic nervous system transmitter, acetylcholine, has a major role in determining gastrointestinal motility (Gonella *et al.*, 1987). Four subtypes of muscarinic acetylcholine receptors (M_1–M_4) have been identified by structural and pharmacological techniques and a cloned fifth gene suggests an M_5 receptor may exist (Dorje *et al.*, 1991; Caulfield, 1993; Eglen *et al.*, 1994). The pharmacological characterization of a muscarinic receptor population must be based upon the rank order of relative affinities of a spectrum of muscarinic antagonists; this is necessary as the absolute selectivity of currently available muscarinic receptor antagonists for any one muscarinic receptor subtype is poor (Grimm *et al.*, 1994). In the published literature, atropine has been used as a non-selective high affinity antagonist at muscarinic receptors. Pirenzepine is a relatively high affinity antagonist for M_1 muscarinic receptors with intermediate affinity for M_4 muscarinic receptors and lower affinity for the M_2 and M_3 muscarinic receptors (Lambrecht *et al.*, 1989; Caulfield & Brown, 1991; Dorje *et al.*, 1991; Grimm *et al.*, 1994). AF-DX 116 (11-[[[2-(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyridol[2,3,-*b*][1,4]benzodiazepine-6-one) has high affinity for the M_2 muscarinic receptor, but also has affinity for the M_1 muscarinic receptor with lower affinity for the M_3 and M_4 muscarinic receptors (Birdsall & Hulme, 1983; Giachetti *et al.*, 1986; Hammer *et al.*, 1986). 4-DAMP has a relatively high affinity for M_3 muscarinic receptors but also has affinity at M_1 and M_4 muscarinic receptor subtypes (Dodds *et al.*, 1987; Eltze & Figala, 1988; Eltze *et al.*, 1993; Grimm *et al.*, 1994). HHSiD and *p*-F-HHSiD have a relatively higher affinity for M_3 muscarinic receptors than M_2 muscarinic receptors but do have affinity for M_1 and M_4 muscarinic receptors (Lambrecht *et al.*, 1989; Waelbroeck *et al.*, 1990). Himbacine has relatively high affinity for M_2 and M_4 muscarinic receptors with lower affinity for M_1 and M_3 muscarinic receptors (Eglen *et al.*, 1988; Caulfield & Brown, 1991; Miller *et al.*, 1992; Russo *et al.*, 1993).

Investigations into muscarinic receptor agonist-induced contractions in guinea-pig ileum (Clague *et al.*, 1985; Eglen *et al.*, 1990b; Eglen & Harris, 1993) and rat ileum (Lambrecht *et al.*, 1989) suggested that stimulation of the M_3 receptor subtype predominantly accounted for the contractile response. Investigations using radioligand binding, functional studies and examination of the receptor effector transduction system in guinea-pig ileum have also indicated that a significant percentage of the muscarinic receptor population is an M_2 subtype and that this has an adenosine 3′:5′-cyclic monophosphate (cyclic AMP) inhibitory effect that may also effect contractility (Candell *et al.*, 1990; Griffin & Ehlert, 1992; Thomas *et al.*, 1993; Thomas & Ehlert, 1994).

In human colon smooth muscle, Gomez *et al.* (1992) showed using radioligand binding techniques that the muscarinic receptor population is heterogeneous with both M_2 and M_3 muscarinic receptors present. Functional characterization of the muscarinic receptor population has not been performed, however, and this paper reports for the first time investigations into the effects of seven muscarinic receptor antagonists on the contractile responses of the longitudinal and circular muscle of human colon to carbachol. A preliminary account of part of this work has been published in abstract form (Kerr *et al.*, 1994).

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Methods

Segments of macroscopically normal ascending, descending or sigmoid colon were obtained from sites away from the tumour at resections for carcinoma. The tissue was transferred into ice-cold oxygenated (95% O_2 and 5% CO_2) Krebs buffer (mM:NaCl 121.5, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.0, pH 7.4). The adhering fat and connective tissue, mucosa and submucosa were separated from the muscles by blunt dissection. The specimens were either used fresh or after overnight storage at 4°C. The contractile response to carbachol did not differ between fresh and stored tissues or between tissue from different regions of the colon. Data were, therefore, pooled. In longitudinal muscle (LM) preparations, full thickness smooth muscle strips 10 mm long and 2–3 mm wide were cut in a longitudinal orientation from the readily identified bands of taenia coli and in a circular orientation from the muscle coats between the taenia coli for circular muscle (CM) preparations (Tam *et al.*, 1994).

Each of the strips was placed in Krebs buffer in 10 ml organ baths and bubbled with 95% O_2 and 5% CO_2 at 37°C. Measurement of isometric muscle tension was recorded via a Maclab data acquisition system interfaced with a Macintosh computer. The sampling rate of the Maclab was set at 20 samples min^{-1} without filtering. The tissues were set up under a tension of 15 mN and allowed to equilibrate for 60 min without further adjustment to tension and with a change of buffer every 15 min. On setting-up the tissues and equilibrating, circular muscle preparations developed only a low basal tone and variable degrees of spontaneous activity (Tam *et al.*, 1994). Longitudinal muscle developed a greater tone ($\cong$17mN) but less spontaneous activity. During the progressively increasing contractions seen with cumulatively added increasing concentrations of carbachol, irregular spontaneous contractions that would interfere with data collection were only occasionally seen. However, if carbachol was left in the bath without washing the maximum developed contraction gradually waned and frequent rapid spiking contractions were superimposed upon the falling tone.

Dose-response curves to carbachol were constructed within the range of 0.1 μM and 1 mM by cumulative additions with a contact time of 75 s for each dose. Each dose was 0.1 ml and administered in half log unit increments (total of 1.1 ml for full range). A 'priming' concentration-response curve was first constructed and washed out. The first operational response curve was carried out 15 min after the priming curve. Following wash out and after a further 30 min a second operational response curve was constructed. Carbachol concentration-response curves carried out in the absence and presence of 10 μM hexamethonium added for 30 min did not differ significantly ($n=3$ results not shown). Between the priming curve and the first operational curve a small rightward displacement of the curve was seen in both CM and LM. But, as shown in Figure 2, the first and second operational curves were superimposable. The first operational curve was, therefore, used as the control curve in studies with antagonists which were all added for 30 min before repeating the concentration-response curve to carbachol. Only one concentration of antagonist was added to each muscle strip. Each antagonist was tested at 3–4 different concentrations and each concentration was tested in tissues from at least 4 patients.

Expression of results

The effect of each cumulative concentration of carbachol was assessed by measuring the integrated area under the curve (AUC) above baseline during its 75 s contact time. The areas were measured by use of the Macintosh computer software Chart 3.3.4 (AD Instruments, U.K.). Each area was expressed as a percentage of AUC obtained on adding the maximum

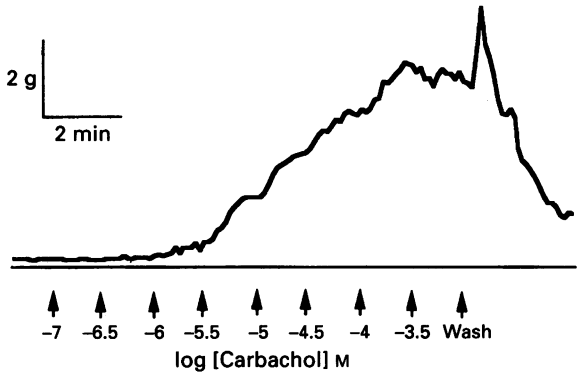

Figure 1 Representative tracing showing the effect of cumulative carbachol administration on the circular muscle of human colon *in vitro* with 75 s contact for each concentration.

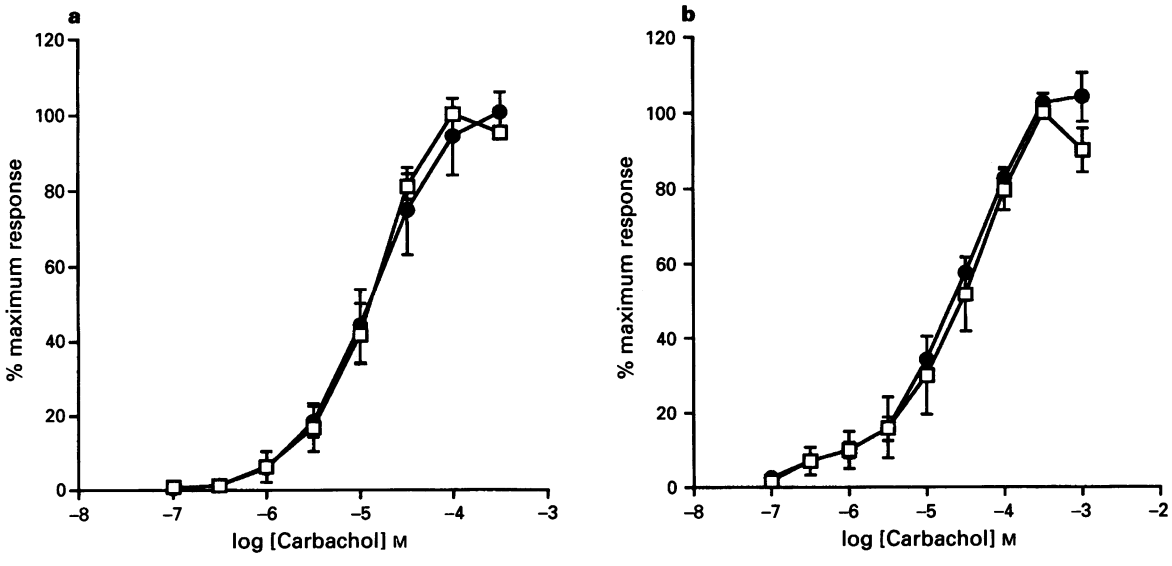

Figure 2 After an initial 'priming' response curve to carbachol two 'operational' response curves were constructed. The contractile effects of the first (□) and second (●) operational carbachol concentration-response curves in (a) circular muscle and (b) longitudinal muscle of human colon. The interval between the curves was 30 min. Mean ± s.e.mean of 8 separate experiments.

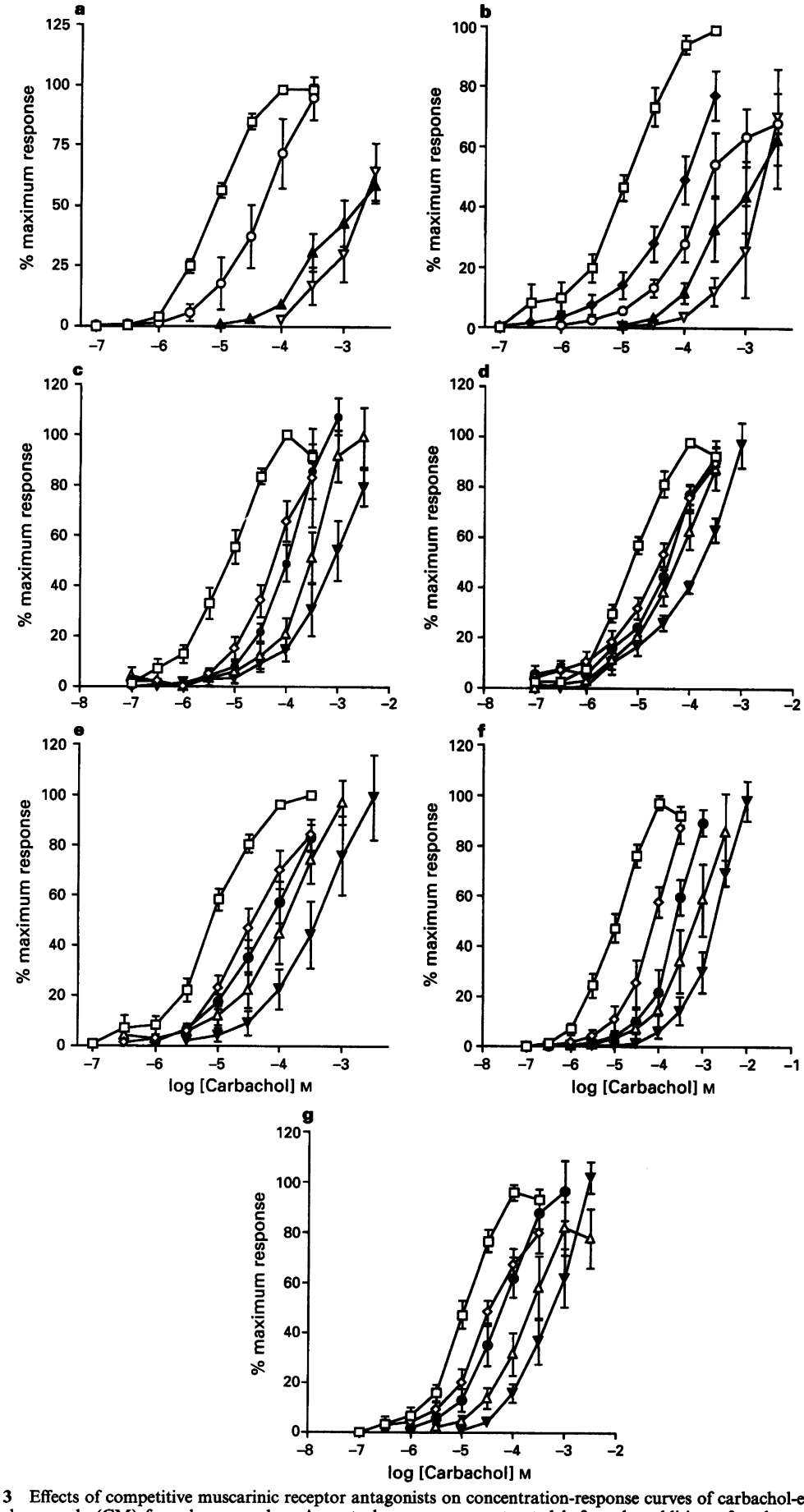

Figure 3 Effects of competitive muscarinic receptor antagonists on concentration-response curves of carbachol-evoked contractions of circular muscle (CM) from human colon. A control curve was constructed before the addition of each antagonist. Following 30 min incubation, the curve was repeated. Values are expressed as a percentage of the AUC of the maximum response obtained in the control response curve. Each antagonist concentration was tested on tissues from 4–6 patients: (a) atropine; (b) 4-DAMP; (c) HHSiD; (d) *p*-F-HHSiD; (e) AF-DX 116; (f) himbacine; (g) pirenzepine. Symbols indicate: (□) Control; (◆)3 nM; (○)10 nM; (▲)30 nM; (△)100 nM; (◇)0.3 μM; (●)1 μM; (△)3 μM; (▼)10 μM antagonist.

cumulative concentration of carbachol in the first operational response control curve. pA_2 values for antagonists were derived from Schild plots by the method of Arunlakshana & Schild (1959).

Drugs

The following drugs were used: carbachol, atropine sulphate, pirenzepine (Sigma Chemicals, UK), 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide; Cookson Chemicals, UK), HHSiD (hexahydrosiladiphenidol) and *p*-F-HHSiD (*para*-fluoro-hexahydrosiladiphenidol) from Research Biochemicals Incorporated (RBI). AF-DX 116 (11-[[[2-(diethylamino) methyl] - 1 - piperidinyl]acetyl] - 5,11 - dihydrol - 6H-pyridol[2,3,-*b*] [1,4] benzodiazepine-6-one) was a gift from Boehringer Ingelheim, Germany.

Himbacine was a gift from Professor W.C. Taylor, Department of Organic Chemistry, University of Syndey. Stock concentrations of drugs were dissolved in distilled water and diluted to working concentrations in Krebs buffer except for AF-DX 116 which was dissolved in 0.1 N HCl and then diluted in Krebs buffer.

Results

Effect of carbachol on colonic smooth muscle

Carbachol added cumulatively (0.1–300 μM) caused concentration-dependent contractions of isolated strips of CM and LM of human colon. The mean $-\log EC_{50}$ values were 5.06 ± 0.17 M ($n=8$) and 5.01 ± 0.15 M ($n=8$) for CM and LM respectively. A representative recording of the effects of carbachol on CM is shown in Figure 1. The effects of carbachol on LM were very similar. Figure 2 shows the first and second operational curves to carbachol in (a) CM and (b) LM separated by a time interval of 30 min and illustrates that these responses are superimposable.

The effects of the range of antagonists on circular muscle are shown in Figure 3a–3g. With increasing concentrations of all antagonists a progressive displacement of the carbachol concentration-response curve to the right was noted. The curves remained parallel and there was no suppression of the maximum response with the highest concentrations of antagonists utilised.

Figure 3a shows the effect of the nonselective antagonist, atropine, on carbachol-evoked contractions. The effects of the M_3 antagonists, 4-DAMP, HHSiD and p-F-HHSiD, are shown in Figure 3b, 3c and 3d respectively. Figures 3e and 3f illustrate the inhibitory effects of the M_2 and M_2/M_4 antagonists, AF-DX 116 and himbacine, respectively and Figure 3g shows the effect of pirenzepine, an M_1 antagonist, on contractions to carbachol.

The slopes of the Schild plots and pA_2 values estimated from these plots are shown in Table 1. None of the slopes differed significantly from unity.

Antagonist affinities on LM were determined by the same protocol as in CM. The effects of atropine and the three M_3 antagonists 4-DAMP, HHSiD and p-F-HHSiD on LM are shown in Figure 4a, b, c and d. Figure 4e, 4f and 4g illustrate the effects of AF-DX 116, himbacine and pirenzepine respectively on carbachol dose-response curves.

The antagonists used displaced the curves to the right without significantly affecting maximum concentrations. Table 1 presents the pA_2 values for these antagonists in LM. None of the slopes differed significantly from unity.

Discussion

Many studies in gastrointestinal smooth muscle preparations have demonstrated the co-existence of M_2 and M_3 muscarinic receptor subtypes. Evidence for this has been obtained from both radioligand binding studies in guinea-pig ileum (Giraldo *et al.*, 1987) and the effects upon muscle tension of muscarinic receptor agonists and antagonists including rat ileum (Lambrecht *et al.*, 1989; Candell *et al.*, 1990), guinea-pig ileum (Eglen & Harris, 1993; Honda *et al.*, 1993; Thomas *et al.*, 1993) and canine colon (Zhang *et al.*, 1991). In human colon, radioligand binding studies have demonstrated that approximately 80% of muscarinic receptors are of the M_2 subtype and 20% of the M_3 subtype (Gomez *et al.*, 1992). In animal tissues it is the M_3 muscarinic receptor that appears to mediate the major component of the evoked contractions induced by muscarinic receptor agonists by coupling to a phosphoinositide specific phospholipase C. The role of M_2 receptors in mediating contractility in different smooth muscle is still uncertain. Eglen & Harris (1993) demonstrated that in the absence of elevated cyclic AMP and with an intact M_3 receptor population, responses to (+)-*cis*-dioxolane in guinea-pig ileum did not involve M_2 receptors. However, under experimental conditions where intracellular cyclic AMP is elevated, M_2 receptor stimulation can elicit contractions or modulate adrenoceptor-induced relaxation by inhibition of adenylate cyclase in rat and guinea-pig ileum (Candell *et al.*, 1990; Thomas *et al.*, 1993; Reddy *et al.*, 1994). Additionally, in other tissues such as the guinea-pig uterus it is the M_2 muscarinic receptors through which a functional contractile response is mediated (Eglen *et al.*, 1989). Moreover, in the guinea-pig trachea, Watson & Eglen (1994) suggested a role for M_2 muscarinic receptors in

Table 1 pA_2 values of muscarinic antagonists against carbachol-induced contractions in circular (CM) and longitudinal muscle (LM) of human colon

	Circular muscle		*Longitudinal muscle*	
	pA_2	*Schild slope*	*pA_2*	*Schild slope*
Atropine	8.72 ± 0.28 (8.85)	1.28 ± 0.17	8.60 ± 0.08 (8.74)	1.20 ± 0.12
4-DAMP	9.41 ± 0.23 (9.35)	1.02 ± 0.1	9.09 ± 0.16 (8.91)	0.86 ± 0.07
HHSiD	7.17 ± 0.07 (7.12)	0.97 ± 0.07	7.45 ± 0.43 (7.36)	1.07 ± 0.14
p-F-HHSiD	6.94 ± 0.18 (6.80)	0.93 ± 0.11	7.44 ± 0.21 (7.27)	0.96 ± 0.17
AF-DX 116	7.36 ± 0.43 (7.10)	0.81 ± 0.16	6.44 ± 0.10 (6.39)	0.80 ± 0.12
Himbacine	7.47 ± 0.14 (7.30)	0.95 ± 0.03	7.54 ± 0.40 (7.29)	0.83 ± 0.10
Pirenzepine	7.23 ± 0.48 (7.06)	0.82 ± 0.17	6.87 ± 0.38 (6.69)	0.83 ± 0.17

Values are mean ± s.e.mean, $n=4-6$ and values in parentheses are mean pK_B values.

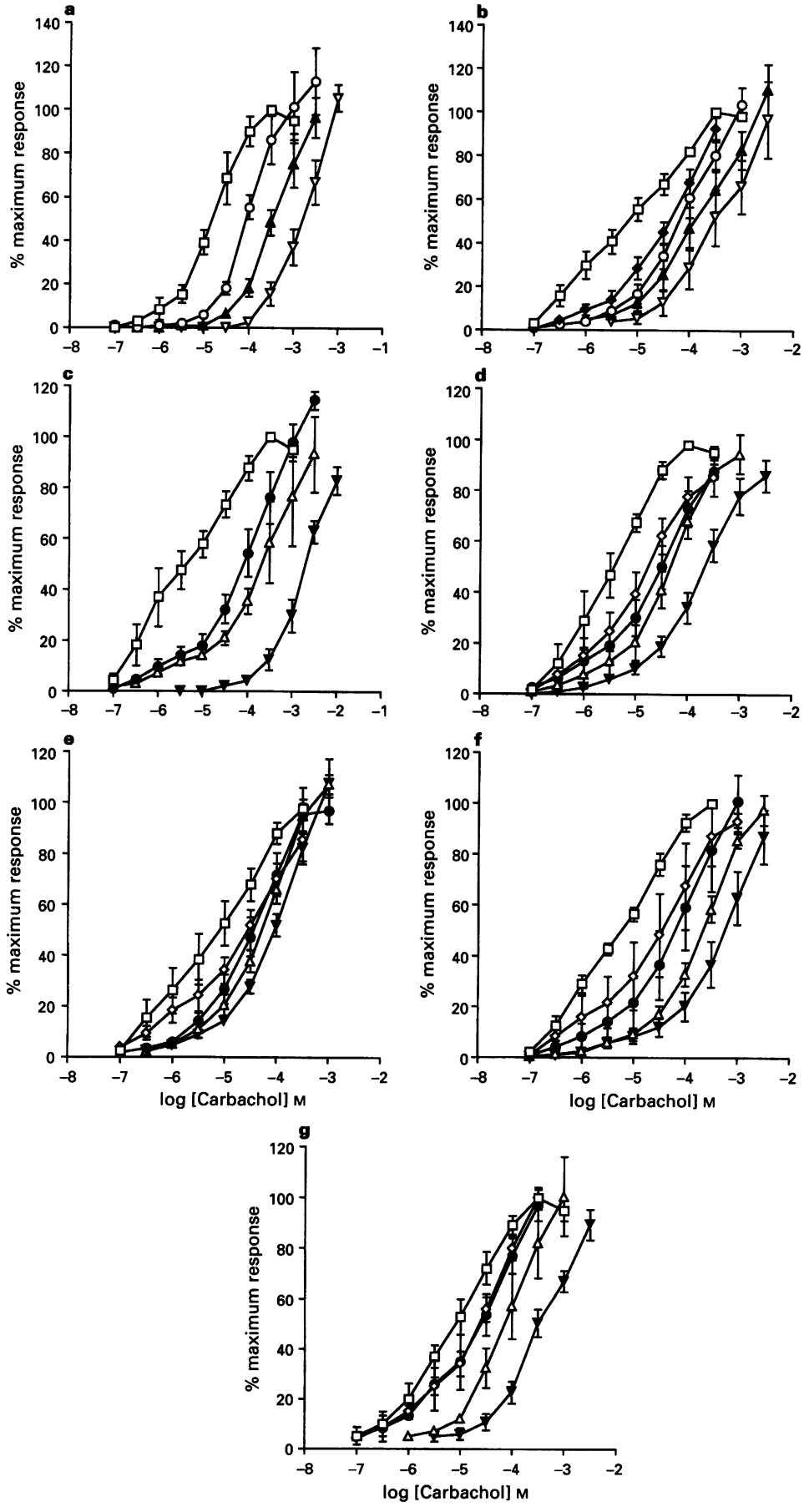

Figure 4 Effects of competitive muscarinic receptor antagonists on concentration-response curves of carbachol-evoked contractions of longitudinal muscle (LM) from human colon: (a) atropine; (b) 4-DAMP; (c) HHSiD; (d) *p*-F-HHSiD; (e) AF-DX 116; (f) himbacine; (g) pirenzepine. Symbols indicate: (□) control; (◆)3 nM; (○)10 nM; (▲)30 nM; (▽)100 nM; (◇)0.3 μM; (●)1 μM; (△)3 μM; (▼)10 μM antagonist.

modulating adrenoceptor-evoked relaxation but Roffel *et al.* (1994) did not subscribe to this suggesting that only the M_3 muscarinic receptor subtype is involved.

The conclusion that M_3 muscarinic receptors are involved in muscarinic receptor evoked contractile responses in many preparations is based on the relatively greater affinity of the M_3 receptors for 4-DAMP, HHSiD and p-F-HHSiD and lower affinities for pirenzepine, AF-DX 116 and himbacine. However, the selectivity of these antagonists for their respective receptors is relatively poor (Eglen *et al.*, 1994).

In an attempt to define the receptor subtype, the actions of atropine and six other muscarinic antagonists showing varying degrees of selectivity for different muscarinic receptor subtypes have been investigated. All the antagonists used displayed properties of competitive antagonism, displacing the concentration-response curve to carbachol to the right in a parallel manner and the maximum response to carbachol was not suppressed by any antagonists.

The pA_2 values for the non-selective antagonist, atropine (8.72 for CM and 8.60 for LM) approached the values obtained in several studies of tissues expressing M_3 muscarinic receptors (Grimm *et al.*, 1994) and the cloned hm_3 receptor (Bolden *et al.*, 1992).

4-DAMP has an affinity profile of $M_3 > M_1 \geqslant M_4 > M_2$ in functional studies (Eltze *et al.*, 1993; Eglen *et al.*, 1994). In our study, the pA_2 values for 4-DAMP were 9.41 (CM) and 9.09 (LM) which approaches the values obtained at the M_3 receptors identified in guinea-pig ileum (Eltze *et al.*, 1993) and murine airways (Garssen *et al.*, 1993) and the human cloned (hm_3) receptor (Dorje *et al.*, 1991).

AF-DX 116 has an affinity profile of $M_2 > M_1 \geqslant M_4 > M_3$ in functional pharmacological studies (Hammer *et al.*, 1986; Giachetti *et al.*, 1986; Giraldo *et al.*, 1987) and a similar profile in human cloned muscarinic receptors (Dorje *et al.*, 1991). In our study AF-DX 116 had at least a 100 fold lower affinity than 4-DAMP for muscarinic receptors in CM (7.36) and LM (6.44). The pA_2 value in LM is similar to the low affinity of AF-DX 116 for the muscarinic receptor subtype in the guinea-pig ileum (Giachetti *et al.*, 1986) but the higher value for CM is more similar to that observed in guinea-pig atrium (Giachetti *et al.*, 1986). It is unclear at this time why there is a difference for AF-DX 116 between the CM and LM muscle coats as this was not observed with the other muscarinic antagonists.

The antagonist profile for pirenzepine is $M_1 > M_4 > M_3 > M_2$ (Grimm *et al.*, 1994) with a similar ranking profile for human cloned muscarinic receptors (Dorje *et al.*, 1991). In our study the pA_2 values of 6.87 (LM) and 7.23 (CM) approached that observed at the M_3 receptor in guinea-pig ileum (Eltze & Figala, 1988). The affinity of the human colon smooth muscle receptors for the M_1 selective pirenzepine was 100 times less than for 4-DAMP, and the latter possesses relatively high M_3 and M_1 receptor antagonist activity. This suggests that activation of M_1 receptors do not contribute to contractions in the human colon.

HHSiD and *p*-F-HHSiD have antagonist profiles $M_3 > M_1 \geqslant M_4 > M_2$ at muscarinic receptors. In our study the pA_2 values for HHSiD were 7.17 (CM) and 7.45 (LM) compared with values for HHSiD in rat ileum of 7.77 (Lambrecht *et al.*, 1989) and guinea-pig ileum of 7.76 (Eltze *et al.*, 1993). With the antagonist *p*-F-HHSiD, pA_2 values in our study were 6.94 (CM) and 7.44 (LM). These values can be compared with smooth muscle preparations from other species which have been characterized as containing M_3 receptors. The pA_2 value of *p*-F-HHSiD in human colon longitudinal muscle is similar to that described in guinea-pig ileum 7.49 (Eltze *et al.*, 1992) and approaches that of rat ileum 7.88 (Lambrecht *et al.*, 1989) but is lower than that seen in guinea-pig muscularis mucosa (8.2) (Eglen *et al.*, 1990b). Moreover, in this study although the value obtained in circular muscle is low it is similar to that seen in guinea-pig trachea where a pA_2 value of 7.1 was described as indicative of an atypical receptor (Eglen *et al.*, 1990a; Watson & Eglen, 1994). The reasons for the more varied affinities for HHSiD and *p*-F-HHSiD are unclear but where the value observed was low, Eglen *et al.* (1990a) excluded a failure to reach equilibrium conditions and agonist-dependency as a cause. Moreover, in our studies the Schild plots were near unity and this is unlikely where equilibrium conditions do not obtain (Kenakin, 1984).

Himbacine exhibits an affinity profile $M_2 \geqslant M_4 > M_1 > M_3$ (Lazareno *et al.*, 1990; Caulfield & Brown, 1991; Russo *et al.*, 1993). In our study the pA_2 values for CM (7.47) and LM (7.54) were within the range (6.9–7.6) noted for its actions at a range of purported M_3 receptors (Caulfield, 1993; Eglen *et al.*, 1994). Moreover, our pA_2 values were less than that observed for himbacine at the M_4 muscarinic receptor of NG108–15 cells, 8.83 (Caulfield & Brown, 1991), the M_1 muscarinic receptor of the rat vas deferens, 8.64 (Eltze & Figala, 1988) and the M_2 muscarinic receptor of the rat heart, 8.34 (Lazareno *et al.*, 1990).

In conclusion, this study of the heterogeneous muscarinic receptors in human colon smooth muscle has identified a rank order of affinities for a range of muscarinic antagonists indicating that the activation of an M_3 muscarinic receptor subtype mediates carbachol-induced contractions. This is based upon the high affinity of the receptor for 4-DAMP and atropine and approximately 50–100 fold lower affinities for AF-DX 116, pirenzepine and himbacine. The hexahydrosiladiphenidol analogues also have affinities which approach those described in the literature at M_3 receptors. However, the contributions of receptors other than M_3 to the muscarinic-evoked contractile process is uncertain and the importance of these is under investigation. The reasons for the relatively low potency of carbachol in human colon smooth muscle *in vitro* is, as yet, unclear. We do not know if this is related to a low receptor reserve. Similar $-\log EC_{50}$ values were obtained whether areas under the curve or peak tension for each concentration of carbachol added were used to calculate data.

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