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Characterization of an atypical muscarinic cholinoceptor mediating contraction of the guinea-pig isolated uterus

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1 In many smooth muscle tissues a minor M_3-muscarinic acetylcholine (mACh) receptor population mediates contraction, despite the presence of a larger M_2-mACh receptor population. However, this is not the case for guinea-pig uterus where radioligand binding and functional studies exclude a dominant role for M_3-mACh receptors.

2 Using tissue from animals pre-treated with diethylstilboestrol, estimates of antagonist affinity were made before and after selective alkylation procedures, together with estimates of agonist affinity to characterise the mACh receptor population mediating carbachol-induced contraction of guinea-pig isolated uterus.

3 Antagonist affinity estimates made at 'protected' receptors were not significantly different from those made in untreated tissues. However all estimations were significantly different from those reported in guinea-pig ileum and atria. The rank order of affinities were atropine > zamifenacin = tripitramine > methoctramine. Carbachol-induced contractions were insensitive to the M_4-selective muscarinic toxin MTx-3, or PD102807 (0.1 μM) ruling out a role for M_4-mACh receptors.

4 The agonist affinity value for L-660,863, a putative 'M_2-selective' agonist of 5.44 ± 0.30 ($n=6$) was significantly different from that reported in guinea-pig atria. In contrast, the pK_A value for carbachol (4.22 ± 0.17; $n=8$) agrees with that reported for guinea-pig ileum.

5 Carbachol-induced contractions were insensitive to pertussis toxin although carbachol-induced inhibition of forskolin-stimulated cyclic AMP production was attenuated, ruling out the involvement of G_i-proteins in contraction.

6 Radioligand binding studies revealed a K_D for N-[^{3}H]-methylscopolamine of 0.12 ± 0.05 nM and a B_{max} of 147 ± 18 fmol mg protein^{-1}. Antagonist affinity estimates made using competition binding studies supported previous data suggesting the presence of a homogenous population of M_2-mACh receptors.

7 These data suggest a small population of mACh receptors with an atypical operational profile which can not be distinguished using radioligand binding studies may mediate carbachol-induced contraction of guinea-pig isolated uterus.

Keywords: Muscarinic acetylcholine receptors; tripitramine; zamifenacin; L-660,863; selective alkylation; smooth muscle contraction; guinea-pig (uterus)

Introduction

Uterine muscle contracts rhythmically both *in vivo* and *in vitro*, and these contractions are intrinsic to the tissue, requiring no hormonal or neural inputs to maintain them (Wray, 1993). Nevertheless, smooth muscle contractility can also be modulated by a variety of neurotransmitters and hormones. The uterine body of many species including human, is extensively innervated by cholinergic neurones of the autonomic nervous system (Traurig & Papka, 1993; Tetsuro *et al*., 1994) and this probably allows co-ordinate control of contraction and blood flow (Sato *et al*., 1996). Evidence has been presented to show that the populations of muscarinic acetylcholine (mACh) receptors and adrenoceptors responding to post-ganglionic neurotransmitter release may change in response to hormonal changes, particularly in oestrogen levels, during the oestrus cycle (Riemer *et al*., 1987; Varol *et al*, 1989; Arkinstall & Jones, 1990; Matucci *et al*., 1996). Although it can be shown that mACh receptors which couple to phosphoinositide responses and Ca^{2+} mobilization are present in this tissue, a role for this family of receptors in uterine physiology has not been firmly established (Eglen *et al*., 1994; Challiss & Blank, 1997).

In most smooth muscle tissues, M_2- and M_3-mACh receptor subtypes form the postjunctional mACh receptor population, and in the majority of these tissues it can be shown that M_3-mACh receptors initiate contraction, presumably *via* their coupling to the phosphoinositide cycle (Roffel *et al*., 1990; Yang *et al*., 1991; Mahesh *et al*., 1992; Nahorski *et al*., 1994). However, this may not be the case for uterine smooth muscle. In contrast to most smooth muscles, myometrial contraction may be mediated by a subtype other than the M_3-mACh receptor. Initial studies in isolated guinea-pig uterus suggesting that M_2-mACh receptors are responsible for contraction (Eglen *et al*., 1989) were supported by subsequent functional studies using an extensive series of mACh receptor antagonists (Bognar *et al*., 1992; Doods *et al*., 1993). Alternative suggestions that M_2- and M_3-mACh receptors may be jointly responsible (Leiber *et al*., 1990), or M_4-mACh receptors may mediate this response (Dörje *et al*., 1990) have also been proposed. Although immunological methods have demonstrated the presence of M_2- and M_4-mACh receptors in rabbit

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uterus (Dörje *et al*., 1991) and Northern blot analysis of guinea-pig uterine tissue revealed evidence for m2 and m4 mRNA transcripts (Eglen *et al*., 1992) it is unclear whether such receptors can mediate a contractile response in myometrial tissue. Inhibition of adenylyl cyclase and stimulation of phosphoinositide-specific phospholipase C by mACh receptor agonists both can occur in guinea-pig isolated uterus, with the former being attributed to M_2- and the latter to M_3-mACh receptor activation (Marc *et al*., 1988; Leiber *et al*., 1990).

Due to the low receptor subtype selectivity of many of the agents used to characterize the uterine mACh receptor(s) in previous studies, and a lack of any clear conclusions, we have carried out an extensive characterization of the mACh receptor subtype(s) responsible for contraction in guinea-pig isolated uterine smooth muscle. We have used guinea-pigs pre-treated with a synthetic oestrogen in order to synchronize all animals to the oestrogen-dominant phase of the reproductive cycle. The possibility was addressed that the contraction of uterus is mediated by M_2- and M_3-mACh receptors, either alone or in a synergistic fashion, by using M_2/M_3-selective antagonists in conjunction with selective alkylation procedures, *in vitro*. A preliminary account of these data has been presented previously (Boxall *et al*., 1997).

Methods

Animals and tissue preparation

Adult, female Dunkin Hartley guinea pigs were treated with diethylstilboestrol 0.1 mg kg^{-1} i.p. and killed by CO_2 asphyxiation 24 h later, or 48 h later if also treated with pertussis toxin (see below). Uterine horns were each cut into four longitudinal strips and mounted for contractile studies, or membranes were prepared for radioligand binding studies as described below.

Contractile studies

Uterine strips were suspended in 10 ml organ baths at an initial tension of 1 g in a modified Sund's solution (pH 7.4) containing 154 mM NaCl, 5.63 mM KCl 0.98 mM $MgCl_2$, 5.95 mM $NaHCO_3$, 0.48 mM $CaCl_2$ 2.78 mM glucose, 3 μM indomethacin, 1 μM tetrodotoxin, 30 μM cocaine and 30 μM corticosterone, at 32°C. This solution was constantly aerated with 95% O_2/5% CO_2, and contractile activity measured using an isometric force transducer. The low temperature and extracellular Ca^{2+} concentration served to reduce spontaneous contractility. The addition of tetrodotoxin prevented neuronal release of acetylcholine from intact nerve terminals thus excluding the involvement of neuronal nicotinic and muscarinic acetylcholine receptors. The use of indomethacin reduces cyclooxygenase activity and therefore synthesis of contractile eicosanoids.

Agonist additions were cumulative (0.5 log molar concns) and antagonist affinity estimates were obtained by Schild regression analysis (Arunlakshana & Schild, 1959) of carbachol-induced contractions. At least five concentrations of antagonist were used, which were equilibrated with the tissue for 1 h. This period was sufficient in each case to allow equilibration of the antagonist, as no further antagonism was seen when the incubation was increased to 2 h for each antagonist. Each uterine strip was exposed only to a single concentration of the antagonist.

Selective alkylation procedures were carried out in a similar manner to Thomas *et al*. (1993). Tissues were treated with 3 μM phenoxybenzamine for 20 min with or without prior 60 min equilibration with 0.1 μM methoctramine. The tissues were washed every 20 min in Sund's solution and antagonists replaced where necessary. Those tissues not treated with methoctramine acted as controls to observe alkylation of 'unprotected' receptors. Tissues were then washed every 5 min for 90 min to ensure that all antagonist was washed from the tissues. Responses to carbachol were not significantly different after incubation with methoctramine alone followed by this washout protocol from time-matched controls (results not shown). After washout of phenoxybenzamine and methoctramine a concentration curve to carbachol was constructed which was further characterized using a single concentration of antagonist after a period of 60 min, and pA_2 values calculated at these 'protected' receptors.

Partial receptor inactivation using alkylation procedures was used to estimate agonist affinities in this tissue. Agonist concentration-response curves were constructed, followed by incubation with 0.1 μM phenoxybenzamine for 20 min. Concentration-response curves were repeated and agonist affinities estimated.

To assess the effect of pertussis toxin pre-treatment, 24 h after diethylstilboestrol was injected, pertussis toxin (50 μg kg^{-1}) or vehicle was injected via the external jugular vein of the guinea pig. The venous cut down, injection and suture closure were carried out under ether anaesthesia and the guinea pigs allowed to recover under supervision. There were no gross adverse effects of pertussis toxin pre-treatment on the guinea pigs during the time between administration, recovery and sacrifice. The guinea pigs were killed in the same manner as before, 24 h after pertussis toxin or vehicle administration. Some uterine tissue was used for biochemical studies to measure the effects of pertussis toxin on inhibition of adenylyl cyclase (see below) while the rest was used for contractile studies as discussed above.

Measurement of adenylyl cyclase activity

Uteri from animals pretreated with diethylstilboestrol were dissected into inositol-free Ham's F12 medium at 37°C and strips were chopped using a McIlwain chopper into cubes. The cubes were washed three times with fresh medium and spun at 500 *g* for 5 min. The uterus tissue was then incubated with collagenase (1 mg ml^{-1}) at 37°C for 1 h with occasional shaking. The treated preparation was then passed through a cell sieve, washed thoroughly with medium and centrifuged as before. The medium was removed and the cells resuspended carefully in a known volume of fresh medium. The ATP pools of the cells were then labelled with 5 μCi ml^{-1} [^{3}H]-adenine for 1 h at 37°C. Cells were then washed and centrifuged as before. Antagonist or buffer was preincubated with 200 μl of [^{3}H]-adenine-labelled cell suspension (approx. 25 μg protein) for 20 min at 37°C and the following were then added simultaneously: carbachol (at various concentrations) or buffer, forskolin (10 μM) or buffer and 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM). After 30 min incubation at 37°C the reaction was stopped by the addition of 2.2 M HCl, followed by mixing and placing in an ice-water bath. [^{3}H]-cyclic AMP was separated by column chromatographic methods as described by Daniels & Alvarez, (1992).

Measurement of inositol phosphate accumulation

Uteri from animals pretreated with diethylstilboestrol were dissected into Sund's solution at 37°C and roughly chopped using scissors. The tissue was cross-chopped (300 × 300 μM)

using a McIlwain chopper and then shaken in Sund's solution in a waterbath at 37°C. The tissue was washed with eight changes of buffer by allowing the slices to sediment and removing buffer and cell debris by suction. Slices were packed under gravity into a repeating pipette tip and 50 μl of slices were dispensed into insert vials. Throughout the experiment the vials were purged with O_2/CO_2 every 15 min and capped. Phospholipid pools were labelled using 5 μCi ml^{-1} [^{3}H]-inositol for 3 h at 37°C. Accumulation of label into the phospholipid pool was linear with time over 3 h and could be stimulated by agonist (results not shown). Lithium chloride (10 mM) was added to the slices 15 min prior to stimulation for 30 min with varying concentrations of carbachol. The reactions were stopped with trichloroacetic acid (1 M) and the vials were left on ice for 20 min before centrifugation at 1000 *g* for 10 min. The supernatant was extracted with 3 × 5 ml water-saturated diethylether. Total [^{3}H]-inositol phosphates were separated using column chromatographic methods using Dowex (Cl^--forms) columns as described previously (Challiss *et al*., 1992).

Preparation of uterus membranes

For membrane preparations, guinea pigs were pre-treated with diethylstilboestrol and killed as described above, the uterine horns removed and roughly chopped with scissors into 20 ml ice-cold 10 mM HEPES, 10 mM EDTA, pH 7.4 and homogenized using a Polytron homogenizer for 10 bursts of 3 s at maximal speed on ice. The membranes were then centrifuged at 200 *g* for 5 min to remove large tissue fragments. The supernatant was spun at 40 000 *g* for 15 min at 4°C. The pellet was resuspended in a HEPES buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4) and homogenized and spun as before. The final membrane preparation was resuspended in HEPES buffer with 0.1 mM EDTA, protein concentration measured using the method of Lowry *et al*. (1951) and diluted to 1 mg ml^{-1}. Aliquots of membranes were snap frozen in liquid N_2 and stored at −80°C for later use.

Radioligand binding studies

For binding assays the buffer used contained 10 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4 (buffer A). For saturation binding, 25 μg of uterus membranes were incubated with increasing concentrations of N-[^{3}H]-methylscopolamine ([^{3}H]-NMS) for 90 min at 37°C in 200 μl final volume of buffer. Incubations were performed in duplicate and the non-specific binding described using 1 μM atropine. The reactions were stopped by diluting with ice-cold buffer and vacuum filtering over GF/B Whatman glass filters using a Brandell cell harvester. Filters were washed with 3 × 5 ml buffer A and then air-dried and counted using liquid scintillation spectrophotometry. For displacement binding experiments, approximately 0.5 nM [^{3}H]-NMS was incubated at 37°C for 90 min with 25 μg uterine membranes and increasing concentrations of antagonists, to a final volume in buffer as before of 200 μl. At least 12 concentrations of each antagonist were used over six orders of magnitude. Non-specific binding was described as for saturation binding, and incubations were performed in duplicate. The incubation was terminated by rapid vacuum filtration and filters washed as previously described.

Data analysis

All data are presented as means ± s.e.mean for the number of experiments indicated. Where Schild slopes were not significantly different from one, slopes were constrained to one to calculate pK_B values. Agonist affinity values were calculated using simultaneous operational curve-fitting methods using SigmaPlot (Jandel Scientific, Corte Madera, CA, U.S.A.) as described by Wiener & Thalody (1993). In radioligand binding experiments, the equilibrium constant (K_D) for [^{3}H]-NMS and the B_{max} were estimated from curves generated by non-linear regression of saturation isotherms using GraphPad Prism (San Diego, CA, U.S.A.). Competition curves from three independent experiments for each antagonist were analysed separately using GraphPad Prism and curves for one and two binding sites were fitted to the data. The curve of best fit was used to estimate the IC_{50} for the antagonist (from which the association constant (K_i) could be derived according to Cheng & Prusoff, (1973) and the Hill coefficient (Dahlquist, 1978). Statistical significance was assessed by Student's *t*-test for unpaired observations.

Materials

[^{3}H]-adenine and N-[^{3}H]-methylscopolamine were purchased from Amersham Life Sciences (Little Chalfont, U.K.). Carbachol, diethylstilboestrol, collagenase, tetrodotoxin and pertussis toxin were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Cocaine, corticosterone, indomethacin, phenoxybenzamine, methoctramine, atropine and zamifenacin were prepared in-house at Roche Bioscience (Palo Alto, CA, U.S.A.). Tripitramine was a kind gift from Dr C. Melchiorre (University of Bologna, Italy) or was purchased from Research Biochemicals International (Natick, MA., U.S.A.). Muscarinic toxin 3 (Mtx-3) isolated from venom of the green mamba (*Dendroaspis augusticeps*) was a kind gift from Dr E. Karlsson (Biomedical Centre, Uppsala, Sweden). PD102807 was generously provided by Dr R. Schwarz (Parke Davis).

Results

Diethylstilboestrol pre-treatment

Using histological preparations of uterus and vagina from diethylstilboestrol pre-treated animals, evidence of the oestrogen-dominant phase of the reproductive cycle could be observed. Endometrial proliferation and glandular activity of the uterus preparations and vaginal keratinization was evident, indicating that diethylstilboestrol pre-treatment leads to obvious morphological changes associated with oestrogen dominance (results not shown). This demonstrated that the reproductive cycles of the guinea-pigs were successfully synchronized.

Pharmacological characterization of contractile response

Carbachol produced a concentration-dependent increase in tension of uterine smooth muscle with a pEC_{50} value of 5.79 ± 0.05 (n = 12), and a maximal tension of 2–5 g in these tissues. This response was sensitive to muscarinic antagonists causing dextral shifts of the concentration-response curve in a parallel manner with no decrease in maximal tension for the concentrations of antagonist used, indicating that this antagonist was competitive (Figure 1). The Schild slopes were not significantly different from one ($P > 0.05$) and were thus constrained to one to estimate affinity values (pK_B) (Table 1). Contractions to carbachol were abolished by phenoxybenzamine (in the absence of methoctramine), but the response could be protected by prior equilibration with 0.1 μM methoctramine

followed by extensive washing (Figure 2). Under these conditions a rightward shift and small decrease (10±2%) in maximum response was observed. Antagonist affinities estimated at the 'protected' receptors using a single concentration of antagonist were not significantly different from estimations made in untreated tissues ($P>0.05$) (Table 1).

It was noted that for all antagonists, the affinities in both untreated tissues and at 'protected' receptors were significantly different from those estimations previously reported at M_2- and M_3-mACh receptors ($P<0.05$) (Table 1) (Caulfield, 1993; Chiarini *et al*., 1995). The M_4-selective muscarinic toxin 3 (Mtx-3; Jolkkonen *et al*., 1994) failed to have any effect on carbachol-induced contraction ($n=5$) suggesting that M_4-mACh receptors were not involved (Figure 3). Similar findings were obtained with PD102807 (Figure 4) and the small dextral shift observed (dose ratio = 5.46±0.87) was not significantly different to that seen with vehicle alone in control tissues (dose ratio = 3.56±0.27). Attempts to evoke a greater shift using PD102807 (1 μM) were unsuccessful due to the poor solubility of the compound and the consequent vehicle (DMSO) effects on the uterus preparations. Also, the

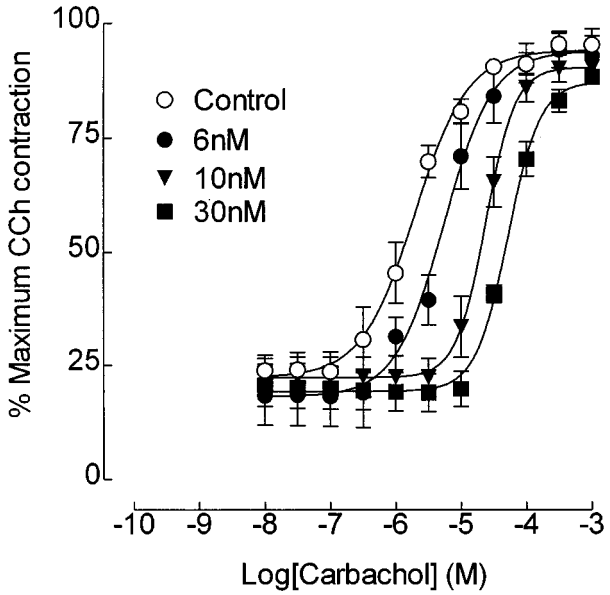

Figure 1 Antagonism of carbachol-induced contraction of guinea-pig isolated uterus strips by zamifenacin. Concentration-response curves to carbachol were constructed in the absence or presence of zamifenacin (6, 10, 30 nM), which was equilibrated in the tissue for 1 h prior to agonist stimulation. Data are shown as % maximum carbachol-induced contraction prior to incubation with antagonist and are expressed as means±s.e.mean of at least three independent experiments. The effect of three out of five concentrations of zamifenacin used have been illustrated for clarity. Similar effects were seen with the other antagonists used.

Table 1 Antagonist affinity estimates (pK_B/pA_2) at muscarinic acetylcholine receptors mediating carbachol-induced contraction of guinea-pig isolated uterus smooth muscle

	Untreated (pK_B)	*'Protected'* (pA_2)	*M_2 atria*	*M_3 ileum*
Atropine	9.3±0.1	ND	9.1[a]	9.3[a]
Methoctramine	7.1±0.1	7.1±0.1	7.9[a]	6.0[a]
Zamifenacin	8.3±0.1	8.6±0.1	6.6[a]	9.3[a]
Tripitramine	8.1±0.1	8.3±0.1	9.7[b]	6.5[b]
p-F-HHSiD	7.5±0.1	ND	6.0[c]	7.9[c]
4-DAMP	8.9±0.1	ND	7.8[c]	9.3[c]
Pirenzepine	6.8±0.1	ND	6.8[c]	6.9[c]
PD102807	<7.0	ND	ND	ND
MTx-3	<6.0	ND	ND	ND

Values shown are means±s.e.mean, n≥7. ND = not determined. [a]Caulfield (1993); [b]Chiarini *et al*. (1995); [c]Eglen *et al*. (1994).

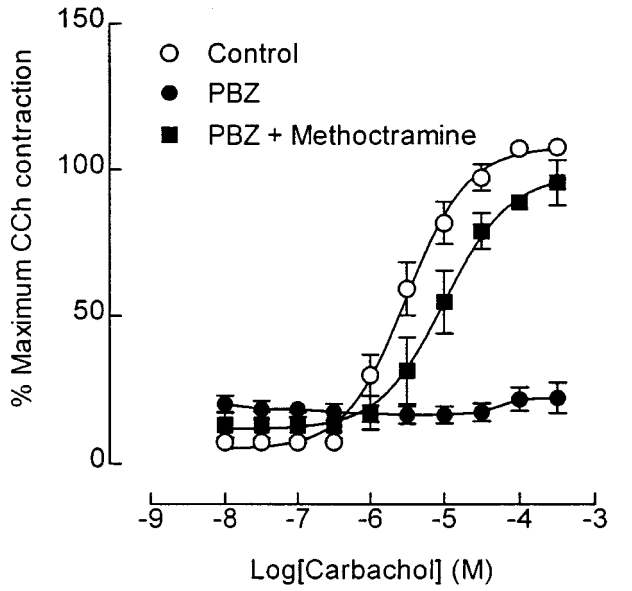

Figure 2 Alkylation and protection of mACh receptors mediating carbachol-induced (CCh) contraction of guinea-pig uterus strips. Phenoxybenzamine (PBZ; 3 μM) was incubated for 20 min alone, or in the presence of previously equilibrated methoctramine (0.1 μM; 1 h). Antagonists were then completely washed from the tissues before construction of the above concentration-response curves. Contraction is shown as a % of the maximum carbachol response prior to incubation with the antagonists, and values are expressed as means±s.e.mean for six to eight separate experiments.

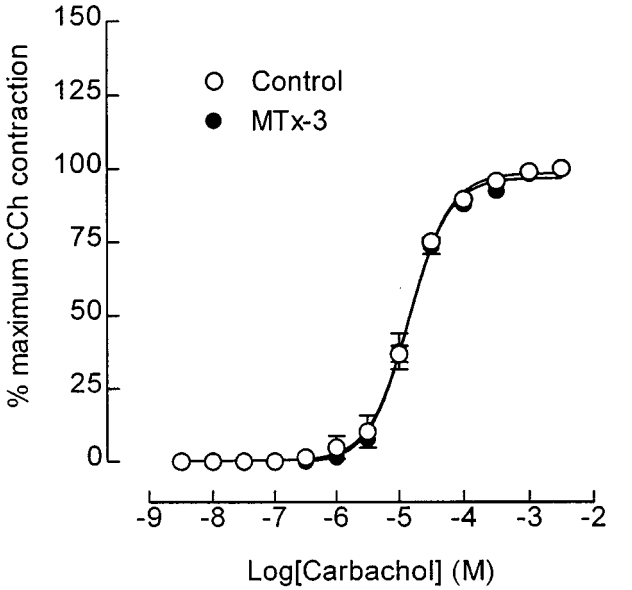

Figure 3 Effect of MTx-3 (muscarinic toxin 3) on carbachol-induced (CCh) contraction of guinea-pig uterus strips. Mtx-3 was equilibrated with the tissue for 1 h prior to agonist stimulation. Data are shown as % maximum carbachol-induced contraction prior to incubation with toxin and are expressed as means±s.e.mean of at least three independent experiments.

low affinity value for pirenzepine in untreated preparations indicated that M_4-mACh receptors were not responsible for contraction of this tissue (pK_B at M_4-mACh receptors = 7.7; Eglen *et al.*, 1997).

The affinity value (pK_A) calculated for the putative 'M_2-selective' agonist L-660,863 (Harris *et al.*, 1991) of 5.44 ± 0.03 ($n = 6$) (Figure 5) was significantly different from the value previously reported for its affinity at M_2-mACh receptor (7.6 ± 0.05). In contrast, the pK_A value estimated for carbachol, which does not discriminate between muscarinic receptor subtypes, of 4.22 ± 0.17 ($n = 8$) agrees with that previously reported in guinea pig ileum (4.7 ± 0.5; Ford *et al.*, 1991).

Effect of pertussis toxin pre-treatment

Pertussis toxin pre-treatment had no significant effect on carbachol-induced contraction when compared with tissues from vehicle-injected controls ($P > 0.05$) (Figure 6). This contrasted with effect on adenylyl cyclase activity in collagenase-treated uterus cell preparations. Thus, carbachol caused a $35 \pm 3\%$ inhibition of forskolin-stimulated cyclic AMP accumulation; an effect almost entirely attenuated by pertussis toxin pre-treatment (Figure 7).

Inositol phosphate accumulation

Stimulation with a maximal carbachol concentration for 30 min produced a $31 \pm 7\%$ increase in total [^{3}H]-inositol phosphate accumulation over basal levels. Although this increase was reproducible and statistically significant ($P < 0.01$), the size of the response precluded pharmacological characterization.

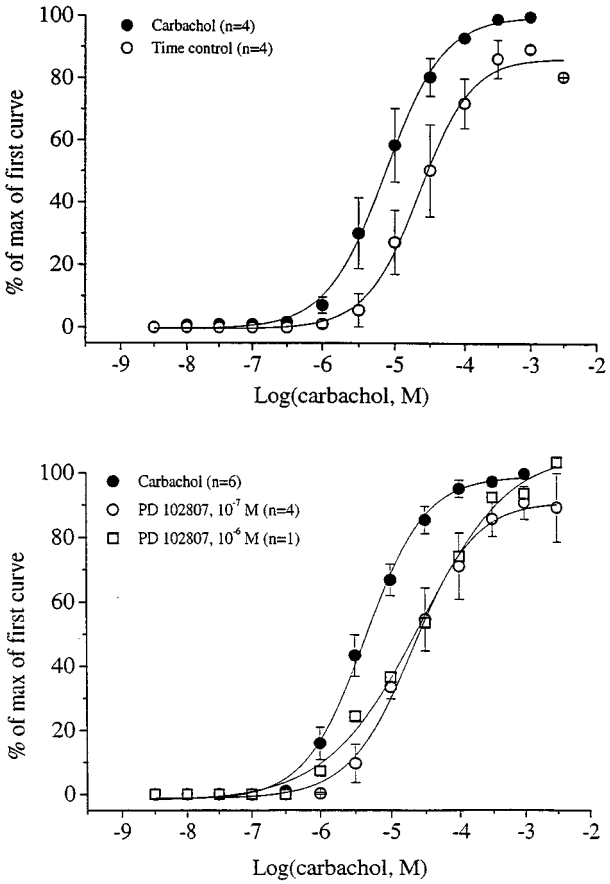

Figure 4 Effect of PD 102807 on carbachol-induced contraction of guinea-pig uterus strips. The upper panel shows time-control data. Data are shown as % maximum carbachol-induced contraction prior to incubation with antagonist and are expressed as means ± s.e.mean for number of experiments indicated in the Figure keys.

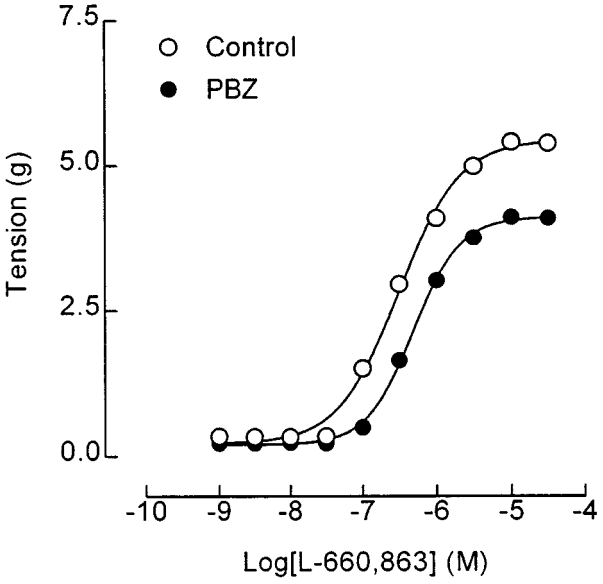

Figure 5 Partial inactivation of contractile response to L-660,863, a 'M_2-selective' agonist, by phenoxybenzamine in guinea-pig uterus strips. Phenoxybenzamine (PBZ; 0.1 μM) was incubated for 20 min before construction of a second concentration-response curve to the agonist. Data are expressed as absolute tension and are from a single experiment representative of six independent experiments. K_A values were calculated separately for each experiment and then means and s.e.mean were derived.

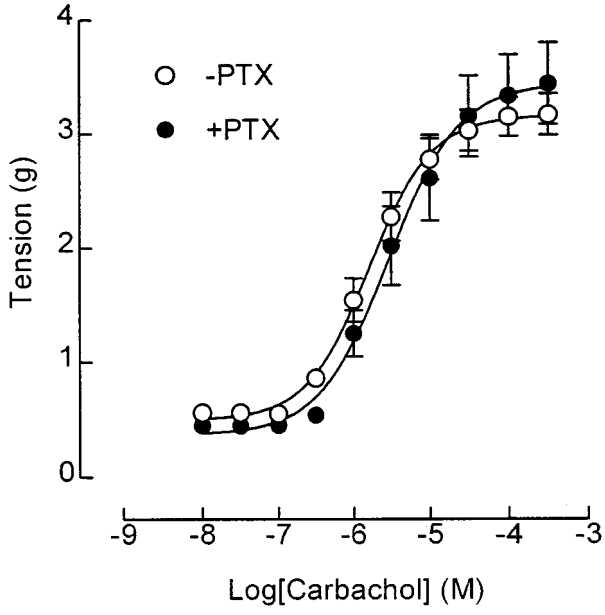

Figure 6 Effect of pertussis toxin pre-treatment on carbachol-induced contraction of guinea-pig uterus strips. Animals were pre-treated with pertussis toxin (PTx; 50 μg kg^{-1}) or vehicle via the external jugular vein 24 h prior to sacrifice. Single concentration-response curves to carbachol were constructed after tissue equilibration. Contraction is expressed as absolute tension and presented as means ± s.e.mean for three separate experiments.

Pharmacological characterization of [^{3}H]-NMS binding

An equilibrium dissociation constant (K_D) of 0.12 ± 0.05 nM for [^{3}H]-NMS was obtained from saturation binding experiments ($n=3$) which were also used to calculate a B_{max} of 147 ± 18 fmol mg protein^{-1} for mACh receptors in this membrane preparation. The affinity values for subtype-selective antagonists were estimated using competition radioligand binding techniques in uterus membrane preparations (Table 2). These affinity values were not significantly different from those expected for M_2-mACh receptors and supported previous ligand binding studies in this tissue (Eglen *et al.*, 1989; Doods *et al.*, 1993). The Hill slopes of the displacement isotherms were not significantly different from one, indicating that only one population of mACh receptors can be detected by this method.

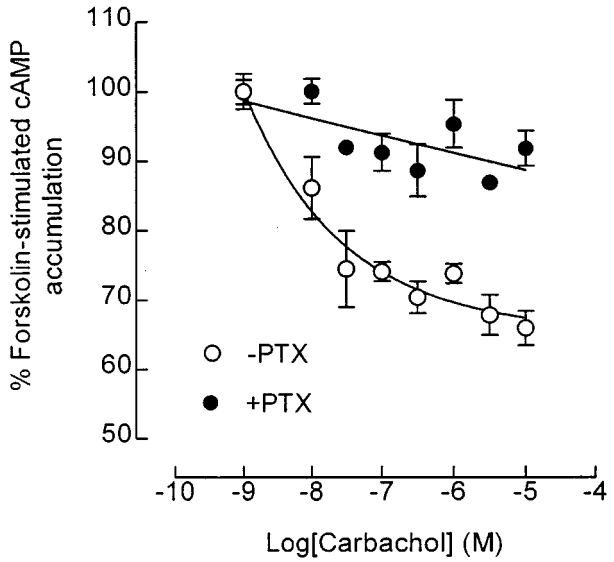

Figure 7 Effect of pertussis toxin pre-treatment on carbachol-induced inhibition of forskolin-stimulated cyclic AMP accumulation in dissociated guinea-pig uterus smooth muscle cells. Animals were treated with pertussis toxin (PTx; 50 μg kg^{-1}) or vehicle *via* the external jugular vein 24 h prior to sacrifice. Cells were dissociated using collagenase (1 mg ml^{-1}) (see Methods). Data are expressed as percentage of forskolin-stimulated cyclic AMP accumulation in the absence of carbachol, and presented as means $\pm$ s.e.mean for three separate experiments.

Table 2 Antagonist affinity estimates (pK_i) determined by competition radioligand binding studies in EDTA-washed uterus membrane preparations

	pK_i (logM)	n_H	M_1	M_2	M_3	M_4
Atropine	8.9 ± 0.3	1.1 ± 0.2				
Methoctramine	8.1 ± 0.1	1.2 ± 0.2	7.3	7.9	6.7	7.5
Zamifenacin	7.6 ± 0.2	1.0 ± 0.2	7.4	7.6	8.0	7.6
Tripitramine	9.3 ± 0.2	0.9 ± 0.1	8.8	9.6	7.4	7.9

Values shown are means $\pm$ s.e.mean, $n=3$. Hill slopes are not significantly different from 1 ($P<0.05$). Values in columns 4–7 have been determined in ligand binding studies at cloned muscarinic receptors expressed in CHO cells (Eglen *et al.*, 1997).

Discussion

The characterization of mACh receptor-mediated contraction in uterine tissue has been hampered both by the inherent variability of the contractile responses observed and the lack of subtype-specific mACh receptor ligands. Moreover, several studies of this tissue fail to account for potential changes in receptor populations due to fluctuating steroid hormone levels, despite studies showing this to be a critical factor (Riemer *et al.*, 1987; Varol *et al.*, 1989; Arkinstall & Jones, 1990; Matucci *et al.*, 1996). In the present study pre-treatment with diethylstilboestrol induced all guinea-pigs to enter an oestrogen-dominant phase of the reproductive cycle, thus normalizing the steroid hormone levels as much as practically possible, and implying the mACh receptor populations are under a constant hormonal influence.

Although only a limited number of antagonists were used to characterize the muscarinic response of this tissue those chosen comprise some of the most 'M_2/M_3 selective' antagonists available. Surprisingly these have not been used before despite the large number of antagonists used previously (Dörje *et al.*, 1990; Doods *et al.*, 1993). Moreover, these M_2/M_3-mACh receptor-selective compounds are of interest given the coexpression of M_2- and M_3-mACh receptors in most smooth muscles, and the apparent absence of M_3-mACh receptors in guinea-pig uterus.

In the present study, the affinity estimates made for these 'M_2/M_3 selective' antagonists did not correlate with those made at M_2- or M_3-mACh receptors (Caulfield, 1993; Chiarini, *et al.*, 1995). This excludes the possibility that carbachol-induced contraction of guinea-pig uterus is mediated by a homogeneous population of M_2- or M_3-mACh receptors alone, and these data *per se* may suggest that M_2- and M_3-mACh receptors are synergistically activated to produce atypical affinity estimates. Selective alkylation procedures were employed to clarify this point, and in fact disprove this hypothesis. Alternatively the mACh receptor population responsible for carbachol-induced contraction is atypical and remains to be classified.

Given that MTx-3, a selective M_1/M_4-mACh receptor toxin (Jolkkonen *et al.*, 1994), and PD102807 (Schwarz *et al.*, 1997) had no effect on contraction, the possibility of involvement of M_4-mACh receptor can be excluded. These data contradict the conclusion of Dörje *et al.* (1990) in which activation of a M_4-mACh receptor was invoked. Two caveats need to be applied when considering the use of MTx-3 or PD102807 as ligands to determine involvement of M_4-mACh receptors. First, functional data on these compounds are either absent in the case of MTx-3, or extremely restricted in the case of PD102807 (Gross *et al.*, 1997a). Nonetheless, radioligand binding data (Schwarz *et al.*, 1997) and unpublished data from our laboratory support the literature in terms of affinity profiles at recombinant mACh receptors. Second, a functional confirmation of the selectivity of these compounds has proven problematic given the paucity of robust and accepted bioassays exhibiting M_4-mACh receptor pharmacology, however the recent report that the isolated rabbit anococcygeus muscle may serve as a functional M_4-mACh receptor model may soon remedy this shortcoming (Gross *et al.*, 1997b).

In tissues where a heterogeneous receptor population exists, selective alkylation procedures have been used to isolate single subtype populations (Eglen & Harris, 1993; Thomas *et al.*, 1993). This technique, using a reversible selective antagonist, enables a given receptor type to be 'protected' from inactivation by non-selective alkylating agents such as phenozybenzamine. In the absence of a protecting ligand,

when the tissues were exposed to 3 μM phenoxybenzamine and extensively washed, the responses to carbachol were abolished. In contrast, prior equilibration with methoctramine (0.1 μM) almost completely attenuated this effect of phenoxybenzamine, such that only a small dextral shift and slight depression of the maximum response was seen. Methoctramine at 0.1 μM would be expected to occupy more than 85% of M_2-mACh receptors and less than 10% of M_3-mACh receptors. Therefore it would be expected that any M_3-mACh receptors in this tissue would have been largely inactivated by phenoxybenzamine, whilst the M_2-mACh receptors would have been protected from such inactivation. However, characterization of antagonist affinities at these 'protected' receptors showed no differences between these and affinity estimates made in untreated tissues. This suggests that the dextral shift and slight depression of maximum was caused by removal of part of a single population of receptors as opposed to the removal of a single subtype of receptors from a heterogeneous population. Therefore it is extremely unlikely that contraction is caused by concurrent activation of M_2- and M_3-mACh receptors.

Scepticism abounds as to the usefulness of agonist affinity estimates in characterization of receptors. However, these data may provide strong supporting evidence for other methods of pharmacological characterization of receptors (Keen, 1991; Leff, 1995). Hence, our finding that the agonist affinity value for the putative 'M_2-selective' agonist L-660,863 differed from that expected at a M_2-mACh receptor is further evidence to support conclusions made based upon atypical antagonist affinity profiles. Since the affinity estimates for carbachol (which is not selective between muscarinic receptor subtypes) agrees with that previously reported at M_3-mACh receptors in guinea pig ileum (Ford *et al*., 1991), collectively the contractile data of this study would suggest that contractions to carbachol in guinea-pig isolated uterus are mediated not by M_2-, M_3- or M_4-mACh receptors as previously reported, but by a single homogeneous population of mACh receptor with an atypical operational profile.

Estimates of antagonist affinities in radioligand binding studies were not in agreement with those estimated in functional studies (*cf*. Tables 1 and 2). The affinity profile of the chosen 'M_2/M_3 selective' antagonists was consistent with the presence of a homogeneous population of M_2-mACh receptors. The Hill slopes of competition curves of antagonists to [^{3}H]-NMS binding would suggest that only one muscarinic receptor subtype is expressed in this tissue. However, it has been shown by immunological methods and Northern blotting techniques that both M_2- and M_4-mACh receptors can coexist in uterine tissues (Dörje *et al*., 1991; Eglen *et al*., 1992), so it may be that these antagonists are still not sufficiently selective between mACh receptor subtypes to detect coexpression, particularly if expression levels of one receptor subtype are low. These data however support previous evidence from Eglen *et al*. (1989) and Doods *et al*. (1993), in which radioligand binding and functional data suggested that M_2-mACh receptors alone were responsible for contraction in this tissue. Thus, it would seem that whilst there are conflicting functional data regarding the nature of the response, all radioligand studies carried out on guinea-pig uterus concur that M_2-mACh receptors only can be detected.

This is interesting given the insensitivity of the contractile response to prior systemic administration of pertussis toxin which abolishes M_2-mACh receptor-mediated responses. This supports data produced by *in vitro* pre-treatment of guinea-pig uterus with pertussis toxin carried out by Marc *et al*. (1988). In the present study we have shown that pertussis toxin abolishes the G_i-protein-mediated inhibitory effect of carbachol on forskolin-stimulated cyclic AMP accumulation, giving confidence that systemic administration allows effective delivery of toxin to the uterus and consequent ADP-ribosylation of sensitive G proteins. The insensitivity of the contractile response to pertussis toxin allows us to conclude that it is not mediated via G_i-proteins.

The results of the present study do not exclude the possibility that M_2-mACh receptors mediate the carbachol-induced contraction via a G protein which is pertussis toxin-insensitive. Thus, it is conceivable that phosphoinositide cycle activation might occur via an atypical M_2-mACh receptor-$G_{q/11}$ protein linkage, with such an interaction affecting the measured functional affinities, but not the binding affinities of the antagonists. In the absence of further studies and analysis of the possible outcome(s) of such promiscuous coupling of the receptor on measured affinities of ligands, this conclusion remains speculative. Furthermore, we have been unable to test this hypothesis by conducting a pharmacological characterization of the phosphoinositide response due to the small agonist-induced increase in [^{3}H]-inositol phosphate accumulation. Although others have assessed phosphoinositide turnover in this tissue (Leiber *et al*., 1990), total inositol phosphate accumulations were normalized and the magnitude of response not stated.

In conclusion, we have found that mACh receptors in oestrogen-dominant guinea-pig uterus display an atypical pharmacological profile when examined functionally, but display classical M_2-mACh receptor pharmacology when characterized using a radioligand binding approach. This could be due to the existence of a small population of atypical mACh receptors that mediate carbachol-induced contraction, but which cannot be distinguished using competitive radioligand binding studies. Our findings are consistent with those recently reported by Munns & Pennefather (1998) using uterus from oestrogen-primed rats, and suggest that the mACh receptor population linked to uterine contraction in guinea-pigs, rat and perhaps other species may differ from that seen in the majority of smooth muscle types.

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