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Functional role of M_2 and M_3 muscarinic receptors in the urinary bladder of rats *in vitro* and *in vivo*

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1 Urinary bladder smooth muscle is enriched with muscarinic receptors, the majority of which are of the M_2 subtype whereas the remaining minority belong to the M_3 subtype. The objective of the present study was to assess the functional role of M_2 and M_3 receptors in the urinary bladder of rat *in vitro* and *in vivo* by use of key discriminatory antagonists.

2 In the isolated bladder of rat, (+)-*cis*-dioxolane produced concentration-dependent contractions ($pEC_{50} = 6.3$) which were unaffected by tetrodotoxin (0.1 μM). These contractions were antagonized by muscarinic antagonists with the following rank order of affinity (pA_2) estimates: atropine (9.1) > 4-diphenyl acetoxy-methyl piperidine methiodide (4-DAMP) (8.9) > darifenacin (8.5) > para fluoro hexahydrosiladifenidol (p-F-HHSiD) (7.4) > pirenzepine (6.8) > methoctramine (5.9). These pA_2 estimates correlated most favourably ($r = 0.99$, $P < 0.001$) with the binding affinity (pK_i) estimates of these compounds at human recombinant muscarinic m_3 receptors expressed in Chinese hamster ovary cells, suggesting that the receptor mediating the direct contractile responses to (+)-*cis*-dioxolane equates with the pharmacologically defined M_3 receptor.

3 As M_2 receptors in smooth muscle are negatively coupled to adenylyl cyclase, we sought to determine whether a functional role of M_2 receptors could be unmasked under conditions of elevated adenylyl cyclase activity (i.e., isoprenaline-induced relaxation of KCl pre-contracted tissues). Muscarinic M_3 receptors were preferentially alkylated by exposing tissues to 4-DAMP mustard (40 nM, 1 h) in the presence of methoctramine (0.3 μM) to protect M_2 receptors. Under these conditions, (+)-*cis*-dioxolane produced concentration-dependent reversal (re-contraction) of isoprenaline-induced relaxation ($pEC_{50} = 5.8$) but had marginal effects on pinacidil-induced, adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-independent, relaxation. The re-contractions were antagonized by methoctramine and darifenacin, yielding pA_2 estimates of 6.8 and 7.6, respectively. These values are intermediate between those expected for these compounds at M_2 and M_3 receptors and were consistent with the involvement of both of these subtypes.

4 In urethane-anaesthetized rats, the cholinergic component (~55%) of volume-induced bladder contractions was inhibited by muscarinic antagonists with the following rank order of potency ($ID_{35\%inh}$, nmol kg^{-1}, i.v.): 4-DAMP (8.1) > atropine (20.7) > methoctramine (119.9) > darifenacin (283.3) > pirenzepine (369.1) > p-F-HHSiD (1053.8). These potency estimates correlated most favourably ($r = 0.89$, $P = 0.04$) with the pK_i estimates of these compounds at human recombinant muscarinic m_2 receptors. This is consistent with a major contribution of M_2 receptors in the generation of volume-induced bladder contractions, although the modest potency of darifenacin does not exclude a role of M_3 receptors. Pretreatment with propranolol (1 mg kg^{-1}, i.v.) increased the $ID_{35\%inh}$ of methoctramine significantly from 95.9 to 404.5 nmol kg^{-1} but had no significant effects on the inhibitory responses to darifenacin. These data suggest an obligatory role of β-adrenoceptors in M_2 receptor-mediated bladder contractions *in vivo*.

5 The findings of the present study suggest that both M_2 and M_3 receptors can cause contraction of the rat bladder *in vitro* and may also mediate reflex bladder contractions *in vivo*. It is proposed that muscarinic M_3 receptor activation primarily causes direct contraction of the detrusor whereas M_2 receptor activation can contract the bladder indirectly by reversing sympathetically (i.e. β-adrenoceptor)-mediated relaxation. This dual mechanism may allow the parasympathetic nervous system, which is activated during voiding, to cause more efficient and complete emptying of the bladder.

Keywords: Muscarinic receptor; M_2-receptor; M_3-receptor; darifenacin; methoctramine; detrusor; urinary bladder; voiding; micturition

Introduction

The parasympathetic nervous system is the principal excitatory innervation to the detrusor smooth muscle of urinary bladder (de Groat *et al*., 1993). Acetylcholine, released from post-ganglionic cholinergic nerves, activates postjunctional muscarinic receptors in the detrusor causing it to contract which, when accompanied by outlet relaxation, leads to voiding of urine (de Groat *et al*., 1993). Muscarinic receptors are pharmacologically classified into four subtypes, denoted as M_1, M_2, M_3 and M_4, based on the affinities of selective ligands such as pirenzepine ($M_1 > M_4 = M_2 = M_3$), methoctramine ($M_2 > M_4 > M_1 = M_3$), darifenacin ($M_3 >> M_1 = M_4 > M_2$), para fluoro hexadrosiladifenidol (p-F-HHSiD) ($M_3 = M_1 = M_4 > M_2$) and 4-diphenyl acetoxy-methyl piperidine methiodide (4-DAMP) ($M_3 = M_1 > M_2 = M_4$) (see Caulfield, 1993; Eglen *et al*., 1996a for reviews). A fifth receptor, denoted as m_5, has also been cloned but an unambiguous functional correlate for this site has yet to be demonstrated.

Most tissues of the lower genitourinary tract, including the urinary bladder, are enriched with muscarinic receptors (Eglen & Hegde, 1997). Radioligand binding studies with [^{3}H]-QNB,

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[^{3}H]-NMS or [^{3}H]-4-DAMP have identified a high density of muscarinic receptors in rat (Monferini *et al*., 1988), rabbit (Lepor & Kuhar, 1984; Batra, 1987; Levin *et al*., 1988; Ruggieri & Luthin, 1990), guinea-pig (Nilvebrant & Sparf, 1983) and human (Batra, 1987; Levin *et al*., 1988; Lepor *et al*., 1989; Ruggieri & Luthin, 1990; Kondo *et al*., 1993; 1995) bladder. Several of these studies have obtained shallow Hill slopes with certain displacing ligands, an observation which is consistent with the presence of multiple muscarinic receptors in the bladder. Indeed, most smooth muscle tissues appear to express a heterogeneous population of muscarinic receptors subtypes (see Eglen *et al*., 1996a for review). Northern blot hybridization analysis in the rat, pig (Maeda *et al*, 1988) and human (Yamaguchi *et al*., 1996) bladder have shown the presence of mRNA encoding the m_2 and m_3 subtypes but not the m_1, m_5 or m_4 subtypes. This finding has been corroborated by a recent study which showed that only the m_2 and m_3 subtypes could be immunoprecipitated from human, rat, rabbit and guinea-pig bladder membranes (Wang *et al*., 1995). Furthermore, it was shown that the m_2:m_3 ratio in the bladder was 9:1 in the rat and 3:1 in other species indicating the predominance of m_2 receptors.

Pharmacological characterization of muscarinic receptors mediating contraction of detrusor muscle in rat (Longhurst *et al*., 1995), rabbit (Tobin & Sjogren, 1995), mouse (Durant *et al*., 1991), guinea-pig (Noronha-Blob *et al*., 1989) and human (Newgreen & Naylor, 1996) bladder suggest the singular involvement of M_3 receptors. Thus, the role of the dominant M_2 receptor population is unclear. Muscarinic M_3 receptor stimulation has been shown to stimulate phosphoinositide hydrolysis in guinea-pig (Noronha-Blob *et al*., 1989) and human (Andersson *et al*., 1991; Hariss *et al*., 1995) urinary bladder and this is most likely the signalling mechanism responsible for the direct contractile responses to muscarinic agonists in this tissue. Additionally, muscarinic agonists inhibit adenylyl cyclase activity in the rabbit (Ruggieri *et al*., 1987) and guinea-pig (Noronha-Blob *et al*., 1989) bladder via M_2 receptors. In other smooth muscles, such as those of the ileum (Thomas *et al*., 1993; Reddy *et al*., 1995), trachea (Thomas & Ehlert, 1996), oesophagus (Thomas & Ehlert, 1996), fundus (Thomas & Ehlert, 1996) of guinea-pig and oesophagus of rat (Eglen *et al*., 1996b), M_2 receptors have been shown to mediate reversal of tissue relaxation induced by compounds, such as β-adrenoceptor agonists, 5-HT_4 agonists or forskolin, all of which stimulate adenylyl cyclase. The mechanism for this indirect contraction involves, presumably, an M_2 receptor-mediated inhibition of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation (see Eglen *et al*., 1994; Ehlert & Thomas, 1996 for reviews) although other explanations have been proposed (Roffel *et al*., 1994). A similar mechanism may also be operative for M_2 receptors in the urinary bladder but has never been studied. Furthermore, the contribution of M_2 and M_3 receptors to the generation of reflex bladder contractions *in vivo* has seldom been investigated.

In the present study, we sought to investigate, by use of available pharmacological tools, the functional role of the dominant M_2 population and the minor M_3 population of muscarinic receptors in the rat isolated bladder. Furthermore, experiments were conducted to characterize pharmacologically the muscarinic receptor mediating volume-induced bladder contractions in the anaesthetized rat. A preliminary account of the findings has been presented to the British Pharmacological Society (Hegde *et al*., 1996).

Methods

Radioligand binding studies

Cell membranes from Chinese hamster ovary (CHO) cells expressing the recombinant human muscarinic receptors (m_1–m_5) were obtained from NEN Life Science Products (Boston, MA, U.S.A.). Identical conditions were used for binding at each receptor. The assays were conducted with 0.4 nM [^{3}H]-N-methyl scopolamine([^{3}H]-NMS; specific activity 84 Ci $mmol^{-1}$) in a final volume of 0.25 ml Tris-Krebs buffer which had the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, dextrose 11 and Tris-HCl 25; pH 7.4, 25°C. Nonspecific binding was defined with 1 μM atropine.

Assays were performed in Dynatech Microlite type 1 microplates by scintillation proximity assay (SPA) technology (Amersham) with 2.0 mg PVT-WGA SPA beads (wheat germ agglutinin covalently linked to polyvinyltoluene beads) in each well. The plates were sealed with Packard Instrument Top-Seal S, placed on an orbital shaker for 2 h at 250 r.p.m. and counted in the Topcount scintillation counter. Displacement curves were generated with 10 concentrations of test compounds.

In vitro *functional studies*

Female Sprague-Dawley rats (Charles River, Wilmington, MA), weighing between 200–300 g, were used in all experiments. The animals were killed by CO_2 asphyxiation and the urinary bladder was isolated and placed in oxygenated Krebs solution which had the following composition (in mM): NaCl 118.2, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.8 and dextrose 10. Indomethacin (10 μM) was always included in the Krebs solution in order to reduce prostaglandin-induced spontaneous activity of the tissue. In certain experiments (experimental protocols II, III and IV), a modified Krebs solution was employed in which the calcium concentration was increased to 4 mM (to obtain sustained contractile responses to KCl), cocaine (30 μM) and corticosteron (30 μM) were included (to block neuronal and extraneuronal uptake of isoprenaline) and tetrodotoxin (0.1 μM) was added (to block neuronal activity in tissues). Strips of tissue (1 cm long, 1.5 mm wide, mucosa intact) were cut from the bladder body, parallel to the longitudinal axis. The tissues were mounted in 10 ml organ baths containing Krebs solution which was maintained at 35°C and constantly aerated with 95% O_2/5% CO_2 (pH = 7.4). Isometric tension generated by the tissue was measured by a Grass FT03 transducer and recorded on a Grass 7E polygraph. The tissues were subjected to a resting tension of 1 g and allowed to equilibrate for 60 min during which time they were washed every 10 min. The viability of each tissue was evaluated by examining the contractile responses to KCl (30 mM).

Four different experimental protocols were adopted (Figure 1).

Experimental protocol I The purpose of these experiments was to define pharmacologically the receptor mediating the direct contractile effects of (+)-*cis*-dioxolane, a non-selective muscarinic antagonist, by obtaining affinity estimates (pA_2s) for a range of muscarinic antagonists. A cumulative concentration-effect curve to (+)-*cis*-dioxolane was constructed in each tissue. The tissues were then equilibrated for 90 min with Krebs solution containing the appropriate concentration of the antagonist or vehicle (time control). During this period, the tissues were periodically washed every 10 min. A second concentration-effect curve to (+)-*cis*-dioxolane was then constructed in the continued presence of antagonist or vehicle. All antagonists were evaluated at a minimum of three concentrations, each of which was tested in separate tissues.

Experimental protocol II The intent of these experiments was to determine whether the potency of (+)-*cis*-dioxolane in the rat bladder is increased under conditions of stimulated adenylyl cyclase activity (i.e., isoprenaline-induced relaxation of pre-contracted tissue) (Thomas *et al*., 1993; Reddy *et al*., 1995). After construction of the first concentration-effect curve to (+)-*cis*-dioxolane, tissues were washed with Krebs solution for 90 min. The tissues were then contracted with KCl (90 mM). Once a stable contractile tone had been attained, the

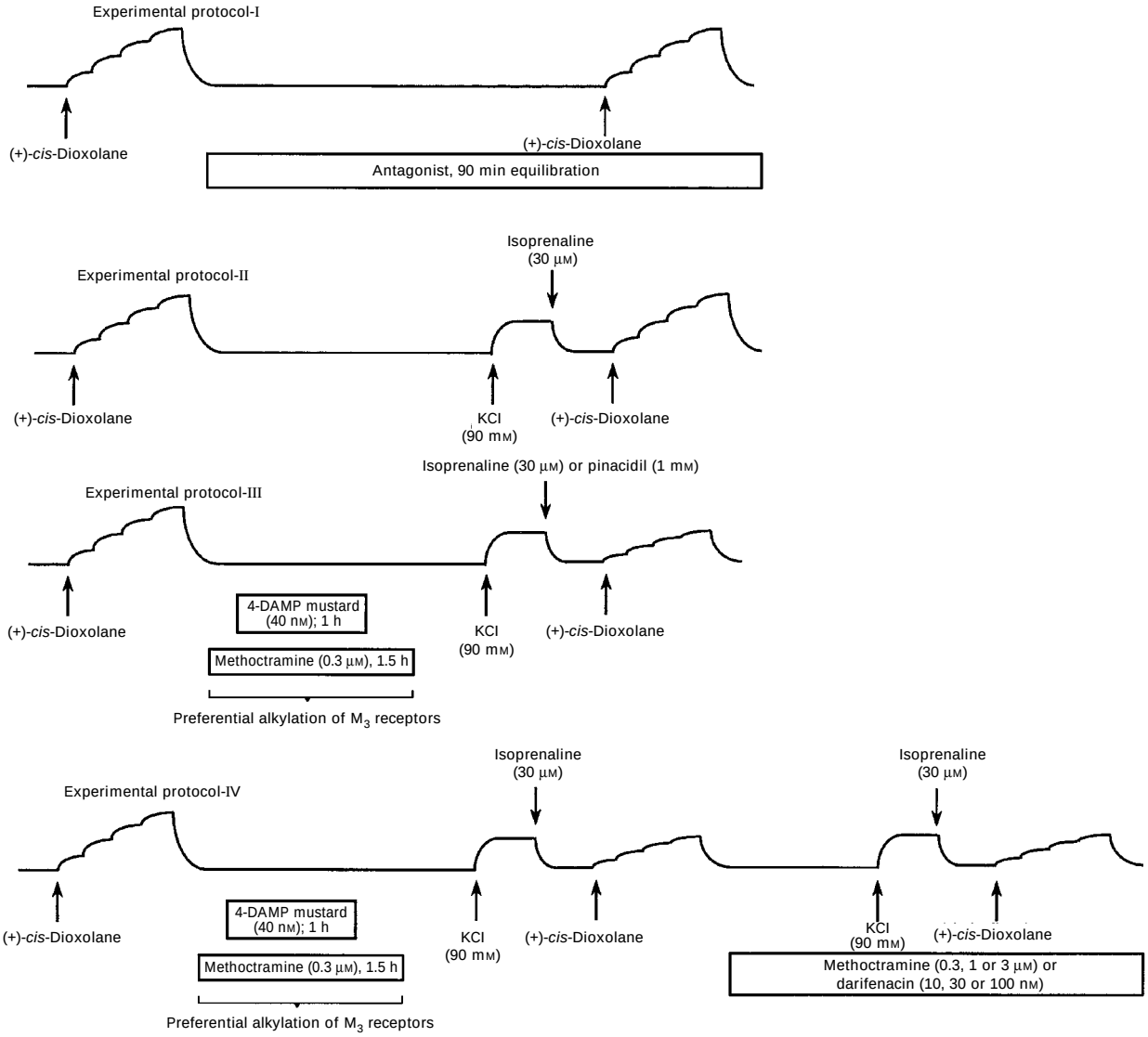

Figure 1 Illustration of the four separate experimental protocols employed for *in vitro* functional studies in the isolated bladder of rat. Refer to the Methods section for a detailed description of the experimental protocols.

tissues were relaxed to baseline tension with isoprenaline (30 μM). Ten minutes later, a second concentration-effect curve to (+)-*cis*-dioxolane was constructed.

Experimental protocol III The objective of these experiments was to determine whether (+)-*cis*-dioxolane could reverse isoprenaline-induced relaxation (i.e. cause a re-contraction) under conditions in which M_3 receptors were preferentially alkylated by use of a method described previously (Reddy *et al*., 1995; exposure to 4-DAMP mustard in the presence of methoctramine to protect M_2 receptors). After construction of the first concentration-effect curve to (+)-*cis*-dioxolane, the tissues were equilibrated with methoctramine (0.3 μM) for 60 min. The tissues were then exposed to 4-DAMP mustard (40 nM) for 60 min in the continued presence of methoctramine followed by washing for 60 min with methoctramine-containing Krebs (to remove the 4-DAMP mustard) and subsequently for 90 min with methoctramine-free Krebs (to remove the methoctramine). The tissues were then contracted with KCl (90 mM) and relaxed to baseline, via a cyclic AMP-dependent mechanism, with isoprenaline (30 μM) or, via a cyclic AMP-independent mechanism, with the potassium channel opener pinacidil (1 mM). Ten minutes later, a second concentration-effect curve to (+)-*cis*-dioxolane was constructed. Preliminary dose-response experiments showed that isoprenaline (30 μM) and pinacidil (1 mM) produced equivalent relaxation of KCl-contracted tissues.

Experimental protocol IV The aim of these experiments was to characterize pharmacologically the receptor mediating the re-contractile responses to (+)-*cis*-dioxolane obtained under experimental conditions in which M_3 receptors were preferentially alkylated and activity of adenylate cyclase was stimulated (experimental protocol III). After two consecutive concentration-effect curves to (+)-*cis*-dioxolane had been obtained under conditions identical to that described in experimental protocol III, the tissues were incubated for 90 min with Krebs solution containing the appropriate concentration of methoctramine (0.3, 1 or 3 μM), darifenacin (0.01, 0.03 or 0.1 μM) or vehicle (time control). A third concentration-effect curve to (+)-*cis*-dioxolane was then constructed.

In vivo *studies*

Female Sprague-Dawley rats (200–300 g) were anaesthetized with urethane (1.5 g kg^{-1}, s.c.) and tracheotomized to facilitate respiration. Catheters were inserted into the left femoral vein and right carotid artery for intravenous administration of drugs and measurement of cardiovascular parameters (heart rate and

arterial pressure), respectively. The pelvic viscera were exposed via a midline abdominal incision. The two ureters were ligated and cut proximal to the ligation in order to allow urine to drain into cotton wads. The urinary bladder was cannulated (PE-50) via the urethra and the cannula was tied in place around the external urethral orifice. The cannula was connected to a three-way connector to allow measurement of intravesical pressure (Gould P23XL transducer) and infusion of saline into the bladder. Continuous infusion of warm saline into the bladder (200 μl min^{-1} for $\sim$5 min followed by a maintenance infusion of 5 μl min^{-1}) evoked rhythmic volume-induced bladder contractions (VIBC; amplitude $\sim$25 mmHg) which are completely abolished by hexamethonium (data not shown) and partly inhibited ($\sim$50–60%) by muscarinic antagonists. Arterial pressure and intravesical pressure were recorded on a Gould 3800 recorder and analysed by use of Experimenter's Workbench 5.0 (Data Wave Technologies, Longmount, CO, U.S.A.) to obtain average mean arterial pressure (MAP), heart rate (HR) and amplitude of VIBC ($VIBC_{AMP}$) during 10 min intervals. After two baseline recordings had been obtained, increasing cumulative intravenous doses of the antagonist were administered at ten minute intervals. In some experiments, the effects of methoctramine and darifenacin were studied in the absence and presence of β-adrenoceptor antagonist propranolol (1 mg kg^{-1}, i.v.).

Data analysis

All data are expressed as mean $\pm$ s.e.mean or with 95% confidence intervals in parentheses.

In vitro *studies* In radioligand binding assays, data were analysed by iterative curve fitting to a four parameter logistic equation. pIC_{50} values were converted to pK_i values with the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

In functional studies, agonist concentration-effect curves were fitted by using a nonlinear iterative fitting programme (Kaleidagraph, Synergy Software, PCS Inc, Reading, PA, U.S.A.) according to the relationship described by Parker and Waud (1971). Estimates of agonist potency and maximum response are expressed as pEC_{50}s ($-$logarithm of the molar concentration of agonist producing 50% of the maximum response) and E_{max}, respectively. Concentration-ratio (CR) was determined from EC_{50} values in the presence and absence of antagonist. Antagonist affinity estimates (pA_2s) were determined by the method of Arunlakshana and Schild (1959) with at least three concentrations of the antagonist. As certain compounds produced unsurmountable antagonism conventional Schild analysis could not be used to estimate antagonist affinity. Instead, an apparent pK_B affinity estimate was obtained by use of the equation described by Furchgott (1972): $pK_B = -\log([\text{antagonist}]/(CR-1))$.

In vivo *studies* % inhibition of $VIBC_{AMP}$ from baseline was calculated for each concentration of the antagonist and the data were fitted by use of Seemingly Unrelated Non-linear regression analysis (SUNR) (Leung *et al*., 1992) to estimate inhibitory potency ($ID_{35\%inh}$, dose required to produce 35% inhibition from baseline) and maximal inhibition (I_{max}). $ID_{35\%inh}$ was estimated instead of ID_{50} (dose required to produce 50% of the maximal inhibition) because the maximal inhibitory response was greater for certain antagonists.

Drugs

(+)-*cis*-Dioxolane, atropine, propranolol, indomethacin, cocaine, corticosterone and isoprenaline were obtained from Sigma Chemical Co (MO, U.S.A.). Pirenzepine, methoctramine, p-F-HHSiD, 4-DAMP and pinacidil were obtained from Research Biochemicals Inc (MA, U.S.A.). Darifenacin was generously provided by Pfizer Central Research (Sandwich, Kent, U.K.).

Results

Radioligand binding studies

Table 1 shows the affinity estimates (pK_is) for inhibition of specific [^{3}H]-NMS binding to human recombinant muscarinic receptor subtypes expressed in CHO cells. The Hill slopes for all the displacing ligands were not significantly different from unity. Pirenzepine, methoctramine and darifenacin displayed selectivity for m_1, m_2 and m_3 receptors, respectively. 4-DAMP and p-F-HHSiD discriminated between m_2 and m_3 receptors by approximately 10 fold.

In vitro *functional studies*

Antagonist characterization of the muscarinic receptors mediating direct contractile responses to (+)-cis-*dioxolane (Experimental protocol I)* (+)-*cis*-Dioxolane produced concentration-dependent contraction of the isolated bladder of rat. The estimated pEC_{50} and E_{max} were 6.3 (6.2–6.5) and 6.0 (5.5–6.6)g, respectively. The responses were resistant to tetrodotoxin (0.1 μM) (data not shown).

Time-control experiments showed that two consecutive concentration-effect curves to (+)-*cis*-dioxolane could be constructed in the same tissue without any significant temporal change in the pEC_{50} and E_{max} estimates (data not shown), thus allowing the use of null methods for antagonist affinity estimation. Atropine, pirenzepine, methoctramine, p-F-HHSiD and 4-DAMP produced parallel, rightward displacement of the concentration-effect curve to (+)-*cis*-dioxolane without significantly altering the maximum response (Figure 2). The slopes of the Schild plots were not significantly different from unity except in the case of atropine for which the slope was significantly greater than unity (Table 2). Increasing the antagonist equilibration time (from 90 to 150 min) or incubation of the tissues with an excess (100 μM) of 4-methyl butyrate (an alternative substrate of atropine esterase) had no significant effect on the slope of the Schild plot for atropine (Table 2). The antagonism produced by darifenacin was characterized by

Table 1 Affinity estimates (pK_is) of antagonists in radioligand binding assays at recombinant human muscarinic receptor subtypes expressed in CHO cells

Antagonist	m_1	m_2	m_3	m_4	m_5
Atropine	9.11 $\pm$ 0.03	8.9 $\pm$ 0.02	9.54 $\pm$ 0.03	9.21 $\pm$ 0.02	9.11 $\pm$ 0.02
Pirenzepine	8.04 $\pm$ 0.03	6.28 $\pm$ 0.12	6.8 $\pm$ 0.02	6.98 $\pm$ 0.05	6.90 $\pm$ 0.09
Methoctramine	6.55 $\pm$ 0.09	7.56 $\pm$ 0.09	6.11 $\pm$ 0.03	6.85 $\pm$ 0.08	6.43 $\pm$ 0.02
4-DAMP	9.24 $\pm$ 0.03	8.12 $\pm$ 0.03	9.28 $\pm$ 0.03	8.43 $\pm$ 0.05	8.91 $\pm$ 0.09
p-F-HHSid	7.33 $\pm$ 0.01	6.56 $\pm$ 0.01	7.51 $\pm$ 0.01	7.24 $\pm$ 0.06	6.73 $\pm$ 0.03
Darifenacin	7.78 $\pm$ 0.15	7.00 $\pm$ 0.09	8.86 $\pm$ 0.12	7.66 $\pm$ 0.13	8.07 $\pm$ 0.03

Radioligand used was [^{3}H]-N-methyl scopolamine. Assays were conducted in Tris-Krebs buffer. pK_i estimates expressed as mean $\pm$ s.e.mean (n = 3). Hill slopes of competition curves were not significantly different from unity.

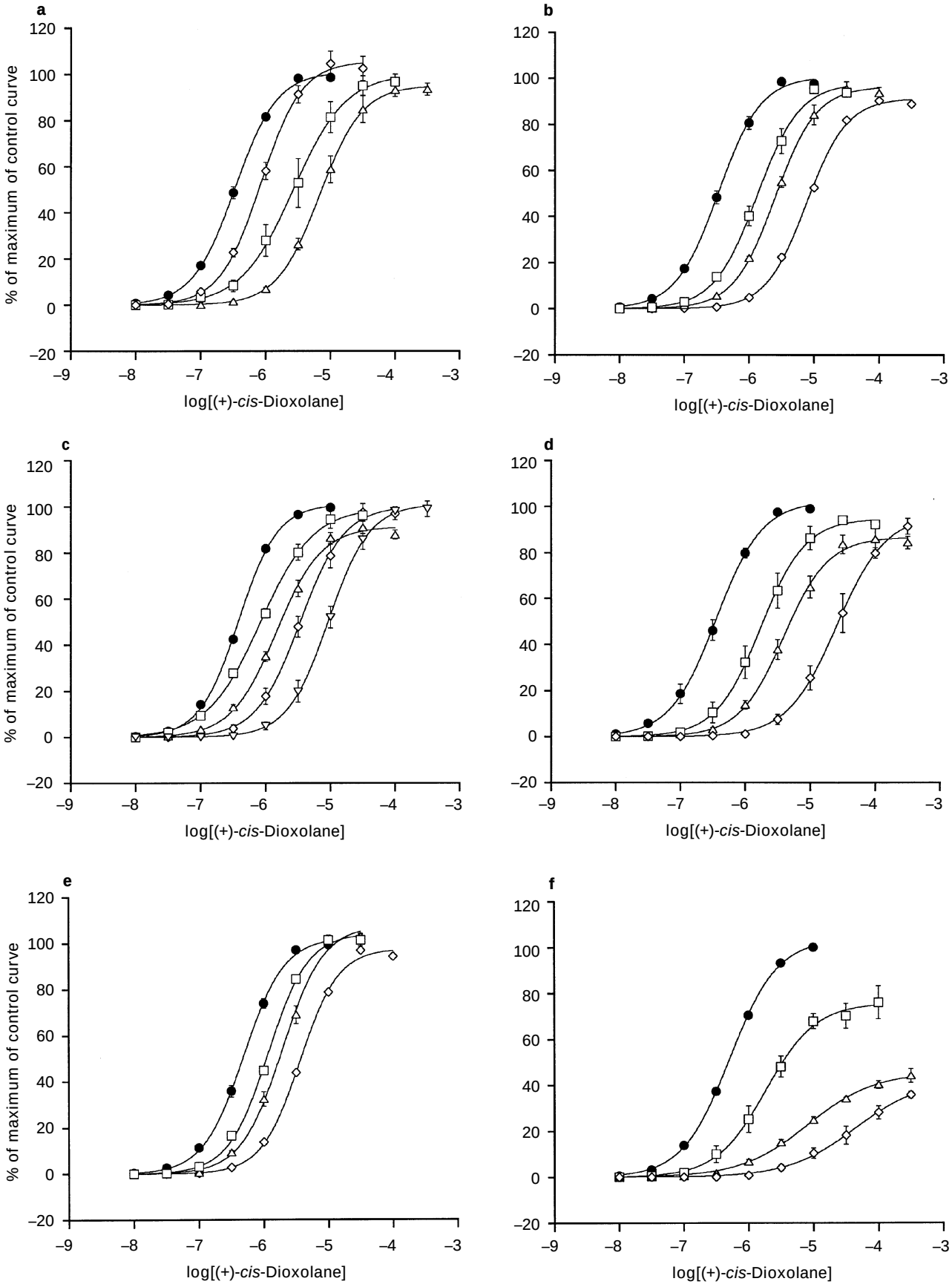

Figure 2 Effects of muscarinic receptor antagonists on the concentration-effect curve to (+)-*cis*-dioxolane (direct contractile responses) in the isolated bladder of rat. (a) Atropine, control (●), 0.001 μM (◇), 0.003 μM (□), 0.01 μM (△); (b) pirenzepine, control (●), 0.3 μM (□), 1 μM (△), 3 μM (◇); (c) methoctramine, control (●), 1 μM (□), 3 μM (△), 10 μM (◇), 30 μM (▽); (d) 4-DAMP, control (●), 0.003 μM (□), 0.01 μM (△), 0.03 μM (◇); (e) p-F-HHSiD, control (●), 0.03 μM (□), 0.1 μM (△), 0.3 μM (◇); (f) darifenacin, control (●), 0.01 μM (□), 0.03 μM (△), 0.1 μM (◇). Data are expressed as mean and vertical lines show s.e.mean, $n=4$ per group.

non-parallel rightward displacement of the agonist curve and also significant depression of the maximum response (Figure 2).

The functional affinity estimates (pA_2s) of antagonists in the rat bladder are shown in Table 2. The rank order of pA_2s of antagonists was: atropine > 4-DAMP > darifenacin > p-F-HHSiD > pirenzepine > methoctramine. Correlation analysis between the pA_2s of antagonists (excluding atropine) in the rat bladder and corresponding pK_is at human recombinant muscarinic receptors yielded correlation coefficients (r) of 0.8 ($P=0.1$), 0.31 ($P=0.61$), 0.99 ($P<0.001$), 0.92 ($P=0.03$) and 0.93 ($P=0.02$) at m_1, m_2, m_3, m_4 and m_5 receptors, respectively.

*Contractile effects of (+)-*cis*-dioxolane under conditions of stimulated adenylyl cyclase activity (Experimental protocol II)* The pEC_{50} of (+)-*cis*-dioxolane in the absence (6.0 (5.8–6.2)) and presence (6.2 (6.1–6.3)) of isoprenaline plus KCl were not significantly different from each other.

Table 2 Functional affinity (pA_2) estimates of compounds for antagonism of (+)-*cis*-dioxolane-induced direct contraction of the rat isolated bladder

Antagonist	*pA_2*	*Schild slope*
Atropine (90 min equilibration)	9.1 (9.0–9.2)	1.4 (1.3–1.5)*
Atropine (150 min equilibration)	9.2 (9.1–9.3)	1.3 (1.2–1.4)*
Atropine (+4-methyl butyrate, 100 μM)	9.2 (9.0–9.3)	1.4 (1.2–1.6)*
Pirenzepine	6.8 (6.7–6.9)	1.1 (0.9–1.2)
Methoctramine	5.9 (5.7–6.1)	1.1 (0.9–1.2)
4-DAMP	8.9 (8.7–9.2)	1.0 (0.8–1.2)
p-F-HHSiD	7.4 (7.3–7.5)	0.9 (0.8–1.1)
Darifenacin	8.5 (8.3–8.8)#	Unsurmountable

All data are expressed as mean with 95% confidence intervals in parentheses. *Significantly different from unity. #Denotes an apparent pK_B estimate.

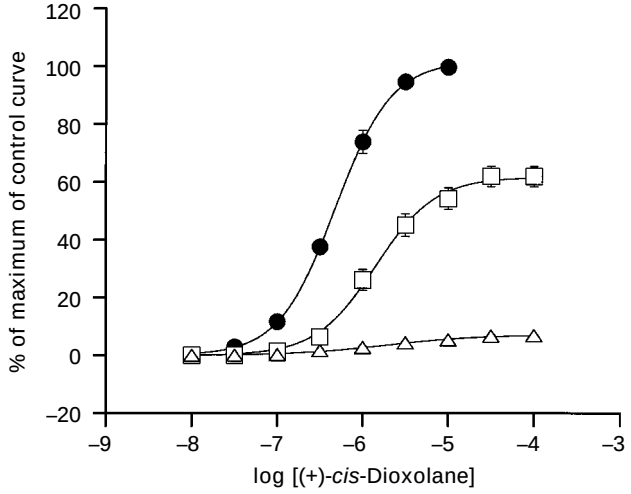

Figure 3 Effect of elevated adenylyl cyclase activity on the concentration-effect curve to (+)-*cis*-dioxolane in isolated bladder of rat after preferential alkylation of M_3 muscarinic receptors. (●) Control curve; (□) re-contractile curve after preferential alkylation of M_3 receptors (exposure of tissues to 4-DAMP mustard (40 nM) in the presence of methoctramine (0.3 μM)) and elevation of adenylyl cyclase activity (tissues were pre-contracted with KCl (90 mM) and relaxed with isoprenaline (30 μM)); (△) curve after preferential alkylation of M_3 receptors (see above) and pre-contraction of tissues with KCl (90 mM) followed by relaxation with pinacidil (1 mM). Data are expressed as mean and vertical lines show s.e.mean, $n=4$.

*Contractile responses to (+)-*cis*-dioxolane under conditions in which M_3 receptors are preferentially alkylated and adenylate cyclase activity is stimulated (Experimental protocol III)* - Under control conditions, (+)-*cis*-dioxolane produced potent contractions of the rat bladder ($pEC_{50}=6.2$ (6.1–6.4)). After preferential alkylation of M_3 receptors (exposure to 4-DAMP in presence of methoctramine), (+)-*cis*-dioxolane produced re-contractile (reversal of contraction) responses in KCl pre-contracted tissues which were relaxed with isoprenaline ($pEC_{50}=5.8$ (5.7–5.9)) (Figure 3). The maximum re-contractile response (expressed as % of the control curve) was 59.0 (52.7–65.3)%. (+)-*cis*-Dioxolane had only marginal effects in tissues which were contracted with KCl and relaxed with pinacidil (Figure 3).

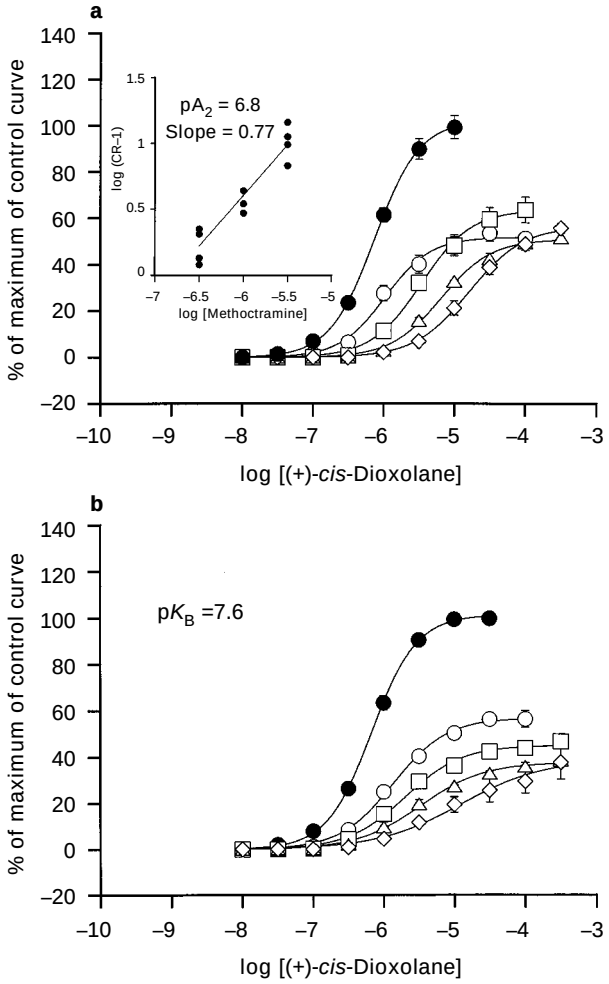

Figure 4 Effects of (a) methoctramine and (b) darifenacin on the re-contractile concentration-effect curve to (+)-*cis*-dioxolane obtained after elevation of adenylyl cyclase activity following preferential alkylation of M_3 muscarinic receptors. (a) (●) Control curve; (○) re-contractile curve obtained after preferential alkylation of M_3 receptors (exposure of tissues to 4-DAMP mustard (40 nM) in the presence of methoctramine (0.3 μM)) and elevation of adenylyl cyclase activity (tissues were pre-contracted with KCl (90 mM) and relaxed with isoprenaline (30 μM)); (□) re-contractile curve in the presence of methoctramine, 0.3 μM; (△) re-contractile curve in the presence of methoctramine, 1 μM; (◇) re-contractile curve in the presence of methoctramine, 3 μM. Inset shows Schild plot for methoctramine. (b) (●) Control curve; (○) re-contractile curve obtained after preferential alkylation of M_3 receptors and elevation of adenylyl cyclase activity; (□) re-contractile curve in the presence of darifenacin, 0.01 μM; (△) re-contractile curve in the presence of darifenacin, 0.03 μM; (◇) re-contractile curve in the presence of darifenacin, 0.1 μM. Data are expressed as mean and vertical lines show s.e.mean, $n=4$ per group.

*Antagonist characterization of receptor mediating indirect re-contractile responses to (+)-*cis*-dioxolane (Experimental protocol IV)* Time-control experiments showed that two consecutive re-contractile concentration-effect curves could be obtained in the same tissue without any temporal change in the pEC_{50} and E_{max} estimates. As shown in Figure 4a, methoctramine produced surmountable antagonism of the re-contractile response to (+)-*cis*-dioxolane. The slope of the Schild plot (0.77) was significantly less than unity. Darifenacin also antagonized the re-contractile responses to (+)-*cis*-dioxolane, although the antagonism in this case was unsurmountable (Figure 4b). The affinity estimates for methoctramine and darifenacin in the re-contractile effects were 6.8 (6.7–6.9) and 7.6 (7.4–7.9), respectively, and these were significantly different from those obtained on the direct contractile effects (experimental protocol I) of (+)-*cis*-dioxolane (5.9 (5.7–6.1) and 8.5 (8.3–8.8), respectively).

In vivo *studies*

All antagonists studied produced dose-dependent inhibition of $VIBC_{AMP}$ (Figure 5a); their $ID_{35\%inh}$ and E_{max} estimates are shown in Table 3. The rank order of potency of the antagonists was: 4-DAMP > atropine > methoctramine > darifenacin > pirenzepine > p-F-HHSiD. Correlation analysis between the potency ($-\log$ ($ID_{35\%inh}$)) of antagonists (excluding atropine) and the corresponding pK_is at human recombinant muscarinic receptors yielded correlation coefficients (r) of 0.65 ($P=0.23$), 0.89 ($P=0.04$), 0.48 ($P=0.42$), 0.74 ($P=0.16$) and 0.71 ($P=0.18$) at m_1, m_2, m_3, m_4 and m_5 receptors, respectively.

Atropine, 4-DAMP, darifenacin and pirenzepine produced maximal inhibition of $VIBC_{AMP}$ of approximately 50–55% and had no effect on MAP. In contrast, both methoctramine and p-F-HHSiD produced much greater inhibition of $VIBC_{AMP}$ and also produced significant decreases in MAP (Figure 5b). Atropine, 4-DAMP and methoctramine produced dose-dependent tachycardia (ED_{50} (nmol kg^{-1}) = 16.9 (10.6–27.3), 789.3 (133.7–4659.1) and 68.6 (51.4–102.8), respectively) whereas darifenacin, pirenzepine and p-F-HHSiD had no significant effect on heart rate (data not shown).

Pretreatment with propranolol (1 mg kg^{-1}, i.v.) significantly decreased the inhibitory potency of methoctramine on VIBC ($ID_{35\%inh}$ = 95.9 (70.3–126.9) and 404.5 (269.1–610.2) nmol kg^{-1}, i.v., in vehicle- and propranolol-treated animals, respectively) (Figure 6a). In contrast, pretreatment with propranolol had no significant effect on the inhibitory potency of darifenacin on VIBC ($ID_{35\%inh}$ = 100.6 (51.5–194.2) and 74.8 (46.8–124.0) nmol kg^{-1}, i.v., in vehicle- and propranolol-treated animals, respectively) (Figure 6b).

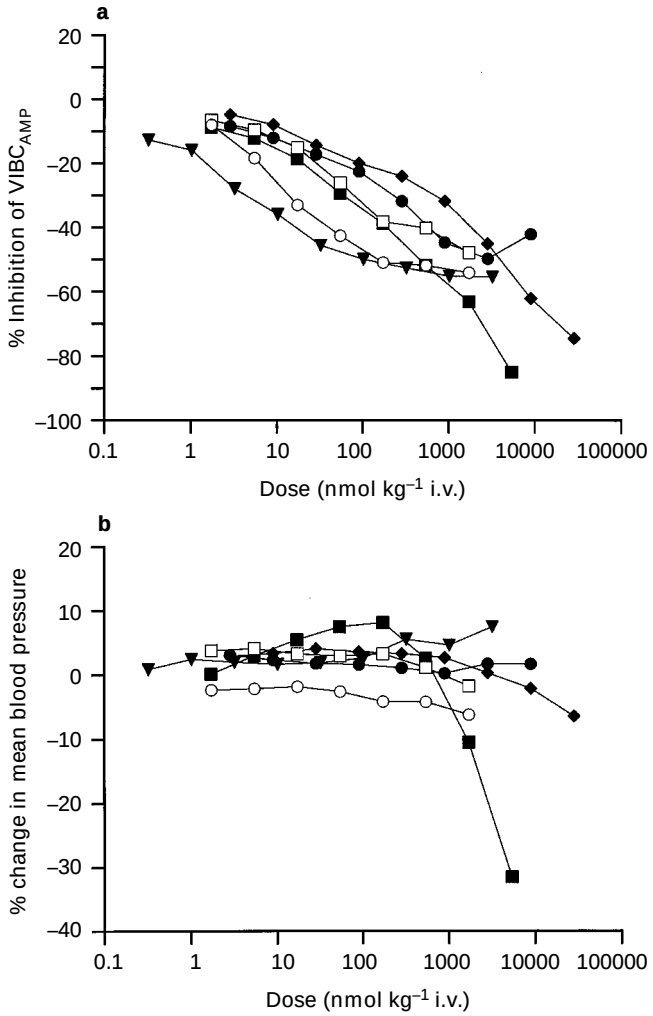

Figure 5 Effects of muscarinic receptor antagonists on (a) amplitude of volume-induced bladder contractions ($VIBC_{AMP}$) and (b) mean arterial pressure in urethane anaesthetized rats. Antagonists studied were: atropine (○); 4-DAMP (▼); methoctramine (■); darifenacin (□); pirenzepine (●); p-F-HHSiD (◆); $n=10$ per group. S.e.means were <15% of mean and have been omitted for sake of clarity. The $ID_{35\%inh}$ (dose required to produce 35% inhibition of $VIBC_{AMP}$) and I_{max} (maximal % inhibition of $VIBC_{AMP}$) estimates of antagonists are shown in Table 3.

Discussion

The findings of the present study suggest that, under the appropriate experimental conditions, both M_2 and M_3 receptors can cause contraction of the rat bladder *in vitro* via different mechanisms. Furthermore, both receptor subtypes may contribute to the generation of reflex bladder contractions *in vivo*.

Characterization of ligands in radioligand binding assays

It has been shown previously that the ionic strength of buffers can impact the binding affinity estimates of ligands for muscarinic receptor subtypes (Pedder *et al*., 1991). The radioligand binding assays were therefore conducted in Tris-Krebs buffer to enable direct comparison of binding affinity estimates with functional affinity estimates. The binding affinity estimates of all the ligands tested were comparable to those presented in the literature (Caulfield, 1993; Nunn *et al*., 1996). In particular, the striking selectivity of methoctramine and darifenacin for m_2 and m_3 receptors, respectively, lends support to their value in the operational characterization of M_2 and M_3 receptors.

Table 3 Potency estimates ($ID_{35\%inh}$) and maximal effect (I_{max}) of antagonists for inhibition of amplitude of volume-induced bladder contractions ($VIBC_{AMP}$) in anaesthetized rats

Antagonist	$ID_{35\%inh}$ (nmol kg^{-1}, i.v.)	I_{max} (% inhibition)
Atropine	20.7 (12.8–36.2)	54.8 (48.8–60.0)
Pirenzepine	369.1 (56.8–2242.8)	49.2 (34.7–63.7)
Methoctramine	119.9 (34.3–205.2)	>60
4-DAMP	8.1 (2.6–24.9)	55.9 (47.8–64.2)
p-F-HHSiD	1053.8 (398.7–2705.6)	>60
Darifenacin	283.3 (127.9–618.9)	50.1 (36.9–63.3)

Data are expressed as mean with 95% confidence intervals in parentheses. $ID_{35\%inh}$ = dose required to produce 35% inhibition of baseline $VIBC_{AMP}$. I_{max} = maximal inhibition of $VIBC_{AMP}$.

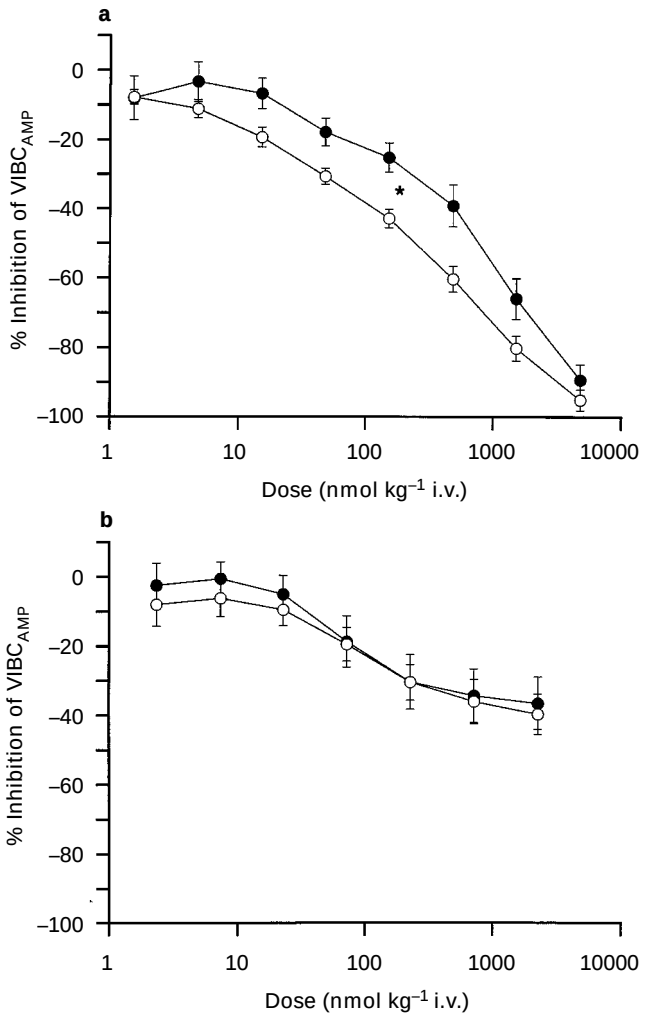

Figure 6 Effect of propranolol (1 mg kg^{-1}, i.v.) on the inhibitory effects of (a) methoctramine and (b) darifenacin on amplitude of volume-induced bladder contractions ($VIBC_{AMP}$) in urethane anaesthetized rats. (○) Vehicle-treated group; (●) propranolol (1 mg kg^{-1}, i.v.)-treated group. Data are expressed as mean and vertical lines show s.e.mean, $n=10$ per group.

Pharmacological characterization of the direct contractile and indirect re-contractile responses to (+)-cis-*dioxolane* in vitro

Under standard assay conditions, (+)-*cis*-dioxolane produced potent contractions of the rat isolated bladder which were tetrodotoxin-resistant and most likely caused by a direct increase in smooth muscle tone. Although the contractions were atropine-sensitive, the antagonism was not strictly competitive as the slope of the Schild plot was significantly greater than unity. Steep Schild plots can arise from several factors including inadequate antagonist equilibration periods and the presence of a saturable antagonist removal process in the tissue (Kenakin, 1984). The former mechanism is unlikely since the slope of the Schild plot was unaffected by increasing the antagonist equilibration period (from 90 to 150 min). With respect to the latter possibility, it is interesting to note that studies in the mouse bladder have implicated a role of atropine esterase, an enzyme which hydrolyzes atropine, in the non-competitive behaviour of the antagonist (Durant *et al*., 1991). However, this mechanism can be discounted in the rat bladder, as incubation of tissues with an excess of 4-methyl butyrate (an alternative substrate of atropine esterase) failed to modify the slope of the Schild plot. However, it should be noted that the presence of other uptake systems for muscarinic antagonists have been shown (Durant *et al*., 1991). Nevertheless, regardless of the underlying mechanism, the apparent affinity estimate of atropine (9.1) is consistent with the involvement of muscarinic receptors.

Operational characterization of muscarinic receptors is commonly done by obtaining an affinity profile of key discriminatory antagonists (see Caulfield, 1993; Eglen *et al*., 1994; 1996a for reviews). The receptor mediating the direct contractile responses to (+)-*cis*-dioxolane displayed a low affinity for methoctramine, intermediate affinity for pirenzepine and p-F-HHSiD and high affinity for 4-DAMP and darifenacin. This antagonist profile closely resembles the profile obtained at a prototypical M_3 functional assay such as the guinea-pig ileum (Ford *et al*., 1991; Eglen *et al*., 1996c). Furthermore, among the five recombinant muscarinic receptors, the m_3 was the one for which the binding affinities of antagonists correlated most strikingly with the pA_2 estimates obtained in the rat bladder. Overall, the data are consistent with the notion that the receptor mediating the direct contractile response to (+)-*cis*-dioxolane equates with the pharmacologically defined M_3 receptor.

It is intriguing that M_3 receptors mediate contractile responses in the rat bladder despite the overwhelming predominance (90%) of M_2 receptors in this tissue (Wang *et al*., 1995). This raises questions regarding the functional significance of M_2 receptors in the rat bladder. It is well documented that M_2 receptors are negatively coupled to adenylyl cyclase in most smooth muscle tissues including that of the urinary bladder (Caulfield, 1993; Eglen *et al*., 1996a). In tissues such as the ileum of guinea-pig and oesophagus of rat, M_2 receptors have been shown to reverse β-adrenoceptor-mediated relaxation (i.e. cause a re-contraction) particularly after M_3 receptors in the tissues have been selectively alkylated (Thomas *et al*., 1993; Reddy *et al*., 1995; Eglen *et al*., 1996b). Consequently, we performed experiments to investigate whether a similar mechanism operates in the rat bladder particularly since β-adrenoceptor-mediated relaxation has been previously demonstrated in this tissue (Maggi & Meli, 1982).

Initially we sought to determine whether an M_2 mediated re-contraction could be unmasked in the presence of an intact M_3 receptor population. We reasoned that if tissues were pre-contracted with KCl and relaxed with isoprenaline, any M_2 mediated re-contraction would be manifested as a leftward shift of the (+)-*cis*-dioxolane concentration-effect curve. However, the contractile potency of (+)-*cis*-dioxolane was unchanged in the presence of KCl and isoprenaline, arguing against the disclosure of any muscarinic receptor-mediated re-contraction. However, this interpretation assumes that muscarinic M_3 receptor-mediated direct contractions are unaffected by the presence of KCl and isoprenaline. It is plausible that relaxation induced by isoprenaline may have functionally antagonized the M_3 receptor-mediated direct contraction, thereby concealing the appearance of a re-contractile response.

Consequently, to avoid the confounding influence of M_3 receptors, we re-examined the involvement of M_2 receptors under experimental conditions in which M_3 receptors were preferentially depleted. This was accomplished by selectively alkylating M_3 receptors with 4-DAMP mustard in the presence of methoctramine to protect M_2 receptors. Under these conditions, (+)-*cis*-dioxolane produced concentration-dependent re-contractile responses in those pre-contracted tissues which were relaxed with isoprenaline, but had little effect in tissues which were relaxed with the potassium channel activator pinacidil. These observations are consistent with the appearance of a cyclic AMP-dependent re-contractile response resulting from reversal of isoprenaline-induced relaxation. The identity of the receptor mediating the re-contractile responses was investigated, pharmacologically, by use of methoctramine and darifenacin. Both these antagonists produced concentration-dependent antagonism of the re-contractile responses. The affinity estimates for methoctramine (6.8) and darifenacin (7.6) were intermediate between those expected for these compounds at M_2 and M_3 receptors (see Table 1). These data can

be interpreted in the following way. It is possible that alkylation of M_3 receptors was incomplete, as evidenced by the marginal contractile effects in pinacidil-relaxed tissues, thereby allowing a direct M_3 mediated contraction to contribute to the overall response. Indeed, the shallow Schild slope (0.77) of methoctramine is consistent with the involvement of M_2 and M_3 receptors. Overall, it becomes difficult to explain the atypical affinities of methoctramine and darifenacin without invoking a role of M_2 receptors. However, in order for a role of M_2 receptors to be unambiguously demonstrated, one must await the discovery of agonists which can selectively agonize the M_2 receptor.

Pharmacological characterization of reflex volume-induced bladder contractions in vivo

Reflex volume-induced bladder contractions (VIBC) can be evoked by filling the bladder rapidly with saline. VIBC are neurogenically mediated by autonomic nerves since they are abolished by the ganglion-blocker hexamethonium (data not shown). Previous studies have shown that VIBC are mediated by a spinal-bulbo-spinal reflex and thus represent micturition contractions (Maggi *et al*., 1986). In the present study, all the antagonists tested, with the exception of methoctramine and p-F-HHSiD, produced approximately 55% maximal inhibition of $VIBC_{AMP}$ suggesting the involvement of other non-cholinergic transmitter(s) in the remaining response. The greater inhibition (>60%) produced by methoctramine and p-F-HHSiD is probably a reflection of the hypotensive responses, produced by these compounds at high doses, which may have reduced perfusion to the bladder.

The potency estimates of the antagonists tested correlated most favourably with the corresponding binding affinity estimates at the recombinant human muscarinic m_2 receptor implying a major contribution of this receptor in the generation of VIBC. The potency estimate of methoctramine for inhibition of VIBC was not significantly different from that required to cause tachycardia (presumably due to antagonism of myocardial M_2 receptors), thereby supporting the involvement of M_2 receptors in the inhibitory effects of methoctramine. Despite the weak correlation at recombinant m_3 receptors, the additional involvement of this receptor cannot be excluded since darifenacin was also modestly potent in inhibiting VIBC at doses which did not affect heart rate. Pretreatment with propranolol significantly decreased the potency of methoctramine but not darifenacin implying an obligatory role of β-adrenoceptors in M_2-mediated effects. These results collectively suggest that M_3 receptors contribute to the generation of VIBC primarily by causing direct detrusor contractions whereas M_2 receptors do so by reversing β-adrenoceptor-mediated relaxation. It should be noted that an additional non-adrenergic non-cholinergic (NANC) inhibitory drive (mediated via vasoactive intestinal peptide, VIP) to the bladder has been postulated (Andersson *et al*., 1990). One, therefore, cannot dismiss the possibility that M_2 receptors may also inhibit vasoactive intestinal peptide (VIP)-induced, cyclic AMP-mediated, relaxation and this may contribute to the overall effects *in vivo*. Additional studies are required to test this possibility.

Conclusions

The data obtained in the present study may allow us to gain a better understanding of the interaction between the sympathetic and parasympathetic nervous systems during the process of urine storage and voiding. There is controversy regarding the role of the sympathetic nervous system during bladder filling (see Vaughan & Satchell, 1995 for review). Animal studies have shown that the sympathetic input to the bladder is tonically active during filling (Edwardsen, 1968; de Groat & Lalley, 1972; Satchell & Vaughan, 1988). Furthermore, hypogastric nerve section, ganglionic blockade and β-adrenoceptor blockade produce decreases in bladder compliance, bladder capacity and volume threshold for micturition, consistent with a sympathoinhibitory tone to the bladder during filling (Maggi *et al*., 1987; Flood *et al*., 1988; Vaughan & Satchell, 1992). It has recently been proposed that the sympathoinhibitory drive to the bladder partially reduces the level of bladder wall tension transduced by the bladder wall mechanoreceptors, and thus delays the time at which micturition threshold is attained (Vaughan & Satchell, 1995). In man, the failure of propranolol to decrease bladder capacity has been taken as evidence against a major contribution of β-adrenoceptors to the filling process (Andersson, 1986). However, a recent study has indicated that β-adrenoceptors in the human detrusor are of the β_3 subtype (Takeda *et al*., 1996). This subtype possesses a low affinity for propranolol (Bylund *et al*., 1994) and thus offers an explanation for the negative clinical findings with this drug.

The findings of the present study offer an explanation on how the sympathoinhibitory drive to the bladder is suppressed during voiding. It is proposed that acetylcholine, released from postganglionic parasympathetic nerves, activates M_2 receptors to reverse the sympathoinhibitory tone (and perhaps the NANC inhibitory tone as well) and this, in concert with direct M_3 receptor detrusor contraction, allows more efficient and complete voiding of urine.

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