

Muscarinic receptors in isolated urinary bladder smooth muscle from different mouse strains

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1 The pharmacological characteristics of muscarinic receptors in male and female mouse urinary bladder smooth muscle from different strains (C57Bl/6, 129/SvJ and hybrid backcross N1F2) were studied.

2 (+)-Cis-dioxolane, oxotremorine-M, acetylcholine, carbachol and pilocarpine induced concentration-dependent contractions of the urinary bladder smooth muscle (range for pEC_{50} = 6.4–6.6, 6.2–6.7, 6.2–6.4, 5.4–6.0 and 0.0–5.1, T_{max} = 1.9–4.7 g, 1.3–3.4 g, 1.0–3.0 g, 1.4–2.4 and 0.0–0.3 g, respectively, n = 4–6 depending on the gender and the strain). In females, these contractions were competitively antagonized by a range of muscarinic receptor antagonists (pK_B value range, depending on the strain): atropine (8.0–8.9), pirenzepine (6.1–6.4), 4-DAMP (7.6–8.4), methoctramine (5.6–6.1), p-F-HHSiD (7.5–7.7), zamifenacin (7.7–8.4) and darifenacin (8.2–8.7).

3 In recontraction studies, in which the muscarinic M_3 receptor population was decreased, and conditions optimized to study M_2 receptor activation, methoctramine exhibited an affinity estimate consistent with muscarinic M_3 receptors (pK_B = 6.26 ± 0.08 , pA_2 = 6.31 ± 0.07 ; pK_B = 6.09 ± 0.22 , pA_2 = 6.08 ± 0.01 for female inbred strain 129/SvJ and hybrid backcross N1F2, respectively) or intermediate between the one expected for this compound at M_2 and M_3 receptors, (pK_B = 6.66 ± 0.08 , pA_2 = 7.00 ± 0.27 for female inbred strain C57BL/6).

4 These data study suggest that muscarinic M_3 receptors are the predominant, if not the exclusive, subtype mediating contractile responses to muscarinic agonists in female mouse urinary bladder smooth muscle, with strain differences.

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Keywords: Muscarinic receptors; M_3 -receptor; urinary bladder smooth muscle; gender; mouse strain

Abbreviations: 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine; p-F-HHSiD, para fluoro hexahydrosiladifenidol

Introduction

Muscarinic receptors consist of five subtypes, M_1 , M_2 , M_3 , M_4 and M_5 defined pharmacologically and encoded by five different genes (Eglen *et al.*, 1996; Caufield & Birdsall, 1998; see Eglen & Nahorski, 2000 for review). Muscarinic receptors are distributed widely and play a key physiological role in peripheral organs, including the urinary bladder. In most smooth muscle preparations (Eglen *et al.*, 1996), the muscarinic M_3 receptor subtype subtype, which forms only 20–30% of the receptor population in the bladder for example, has been demonstrated to play dominant roles in eliciting contraction *in vitro* (despite the fact that the M_2 receptor subtype accounts for 70–80% of the receptor population). Therefore, it was proposed that muscarinic M_3 receptor activation primarily causes direct contraction of the smooth muscle and the muscarinic M_2 receptor contracts the tissue indirectly, by reversing sympathetically mediated relaxation (Thomas *et al.*, 1993; Thomas & Ehlert, 1994; Hegde *et al.*, 1997).

Muscarinic receptors mediating contraction of rat (Longhurst *et al.*, 1995; Hegde *et al.*, 1997), rabbit (Tobin & Sjögren, 1995; Choppin *et al.*, 1998), guinea-pig (Noronha-Blob *et al.*, 1989) and human (Newgreen & Naylor, 1996)

detrusor muscle have been pharmacologically characterized. Recently, the availability of mutant mouse strains that lack functional receptors has provided the means to examine the physiological role of muscarinic receptors in native tissue. Nevertheless, only few investigations of the muscarinic receptors mediating contractions of wild-type mouse bladder have been undertaken (Durant *et al.*, 1991; Paravicini *et al.*, 2000; Welsh *et al.*, 2000; Choppin & Eglen, 2001; Eglen & Choppin, 2001), but suggest a major role of the muscarinic M_3 receptor in the contractile response, with the role of the M_2 receptor, if any, being unresolved.

Since the gender and the genetic background of transgenic mice is rarely the same across studies, the objective of the present study was to examine, using a range of defining antagonists, the pharmacological characteristics of muscarinic receptors present in mouse urinary smooth muscle from both gender and from different strains, in isolated tissue studies.

Methods

In vitro contractile studies

Mice (both gender, inbred strains C57Bl/6, 129/SvJ or hybrid N1F2; 25–30 g; 6–8-weeks-old; Jackson laboratories) were euthanized by CO₂ asphyxiation. The urinary bladder was

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isolated, cleared of adhering adipose tissue and placed in oxygenated Krebs solution (composition in mM: NaCl 118.2, KCl 4.6, CaCl₂ 2.5, MgSO₄ · 7H₂O 1.2, KH₂PO₄ 1.2, NaHCO₃ 24.8 and dextrose 10.0). The physiological solution contained indomethacin (10 μM) in order to reduce prostaglandin-induced spontaneous activity of the tissues. Four strips of urinary bladder smooth muscle were cut from the supratrigonal portion of the bladder (longitudinal section). The tissues were mounted in 10 ml organ baths (Radnoti Glass Technology Inc, Monrovia, CA, U.S.A.) containing Krebs solution, maintained at 37°C and constantly aerated with 95% O₂ /5% CO₂ (pH=7.4). Grass FT03 transducers were used to measure changes in isometric tension of the tissues, which were displayed on a Grass 7E polygraph. The tissues were maintained at a resting tension of 1 g during an equilibration period of 60 min. Tension adjustments were made as necessary. The tissues were washed every 15 min.

The viability of each tissue was assessed by determining the contractile response to KCl (30 mM) at the start of the experimental protocol. After washing, tissues were re-equilibrated for 10 min and allowed to regain baseline tension. Cumulative concentration–effect curves to agonists ((+)-cis-dioxolane, oxotremorine-M, acetylcholine, carbachol and pilocarpine; 1 nM–0.1 mM) were then constructed in each tissue. Thereafter, tissues were equilibrated in either the absence (time control) or presence of antagonist for a 90 min period during which tissues were washed every 10 min. Subsequently, a second concentration–effect curve to the same agonist was constructed.

Recontraction experiments

After an initial concentration–response curve to (+)-cis-dioxolane was established, the tissues were washed and equilibrated with 4-DAMP mustard (40 nM) for 60 min in the presence of methoctramine (0.3 μM). This procedure enabled selective alkylation of M₃ but not M₂ receptors (Eglen *et al.*, 1994; Ehlert, 1996; Hegde *et al.*, 1997; Braverman & Ruggieri, 1999; Choppin & Eglen, 2001). 4-DAMP mustard was then removed from the tissues by overflow with Krebs solution containing methoctramine (0.3 μM) every 10 min for 60 min and subsequently with methoctramine-free Krebs solution every 10 min for 90 min. The tissues were then contracted with 90 mM of KCl and subsequently relaxed with isoproterenol (30 μM). Once the tissues had relaxed to baseline, a cumulative concentration–effect curve to (+)-cis-dioxolane (1 nM–0.1 mM) was constructed.

Effects of an M₂ antagonist (methoctramine) on the recontractile responses to (+)-cis-dioxolane

After constructing two concentration–effect curves to (+)-cis-dioxolane under conditions described above, a third cumulative concentration effect curve to (+)-cis-dioxolane (1 nM–0.3 mM) was constructed after equilibration of tissue in absence (time control) or presence of methoctramine (0.1–1.0 μM) for 90 min.

Data analysis

Contractions were recorded as changes in tension from baseline and expressed as a percentage of the maximum

response of the first agonist concentration–effect curve. Agonist concentration–response curves were fitted using a nonlinear iterative fitting program (Origin, Microcal Software, Inc., Northampton, MA, U.S.A.) using the relationship of Parker & Waud (1971). Agonist potencies and maximum response are expressed as pEC₅₀ (– logarithm of the molar concentration of agonist producing 50% of the maximum response) and T_{max}, respectively. Concentration ratios (CRs) were determined from EC₅₀ values in the presence and absence of antagonist. Antagonist affinity estimates (pK_B values) were determined with the equation described by Furchgott (1972); $pK_B = -\log ([\text{antagonist}]/\text{CR}-1)$ or using the method of Arunlakshana & Schild (1959) using at least three concentrations of the antagonist (pA₂ values). In cases where the slope of the linear regression was not significantly different from unity, the slope was constrained to unity and the data expressed as the pK_B value. All data are expressed as mean ± s.e.mean. Pearson correlation coefficients (*r*) and associated *P*-values were calculated using the method described by Dixon & Massey (1983). The sum of squares of differences in affinity estimates for each plot (Σ (y–x)², noted *ssq*) defines the proximity of the data points to the line of identity (y=x).

Compounds used

Atropine sulphate, indomethacin and isoproterenol were obtained from Sigma Chemical Co (MO, U.S.A.). (+)-Cis-dioxolane, acetylcholine, carbachol, oxotremorine-M, pilocarpine, pirenzepine dihydrochloride, methoctramine hydrochloride, 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) methiodide, 4-DAMP mustard and para fluoro hexahydro-siladifenidol (p-F-HHSiD) hydrochloride were obtained from Research Biochemicals Inc. (MA, U.S.A.). Darifenacin hydrobromide and zamifenacin fumerate were generously provided by Pfizer Central Research (Sandwich, Kent, U.K.).

All compounds were diluted in distilled water except indomethacin, which was diluted in polyethylene glycol.

Results

Characterization of muscarinic receptors mediating contractions of the mouse isolated urinary bladder smooth muscle

(+)-Cis-dioxolane, oxotremorine-M, acetylcholine, carbachol and pilocarpine induced concentration-dependent contractions of the mice urinary bladder smooth muscle (pEC₅₀ and T_{max} are summarized in Table 1, *n*=4–6). Two consecutive concentration–effect curves to these agonists could be constructed in the same tissue with no significant temporal change in the agonist potency and maximum response (time–control; Figure 1).

The muscarinic receptor(s) mediating direct contractions was identified by pharmacological determination of antagonist affinities. Atropine, pirenzepine, 4-DAMP, methoctramine, p-F-HHSiD, zamifenacin and darifenacin were tested for their ability to inhibit (+)-cis-dioxolane-induced responses. All these compounds surmountably antagonized cumulative agonist concentration–response curves, in a

Table 1 Potency and efficacy estimates for muscarinic agonists in mouse urinary bladder smooth muscle

Agonist	parameter	C57BL/6		129/SvJ		N1F2	
		male ^a	female	male	female	male	female
(+) -cis-dioxolane	pEC ₅₀	6.6±0.1	6.4±0.1	6.5±0.1	6.4±0.1	6.6±0.1	6.5±0.0
	T _{max} (in g)	3.2±0.8	1.9±0.6	4.7±0.5†	3.6±0.7	2.3±0.2	2.7±0.8
Oxotremorine-M	pEC ₅₀	6.9±0.1	6.2±0.1**	6.2±0.1**	6.2±0.1‡	6.4±0.1**	6.7±0.3
	T _{max} (in g)	2.7±0.4	1.3±0.1**	3.4±0.9	2.9±0.4¶	3.3±0.4	2.3±0.6
Acetylcholine	pEC ₅₀	6.7±0.1	6.3±0.1*	6.2±0.1**	6.2±0.1	6.4±0.1**	6.2±0.1
	T _{max} (in g)	1.0±0.1	1.0±0.3	3.0±0.4†	2.5±0.4¶	1.5±0.3	1.8±0.3
Carbachol	pEC ₅₀	5.8±0.1	6.0±0.1	ND	5.4±0.2	ND	5.5±0.1
	T _{max} (in g)	2.7±0.3	1.6±0.3	ND	2.4±0.7	ND	1.4±0.3
Pilocarpine	pEC ₅₀	5.8±0.1	5.0±0.1**	5.0±0.2**†	5.1±0.1‡	0.0±0.0**	0.0±0.0¶
	T _{max} (in g)	0.9±0.2	0.1±0.0**	0.3±0.1**†	0.2±0.1	0.0±0.0**	0.0±0.0

Values shown are mean±s.e.mean, $n=4-6$. ND: not determined; * $P<0.05$ versus male C57BL/6; ** $P<0.01$ versus male C57BL/6; † $P<0.01$ versus male N1F2; ¶ $P<0.01$ versus female C57BL/6; ‡ $P<0.01$ versus female N1F2. ^aValues taken from Choppin & Eglen (2001).

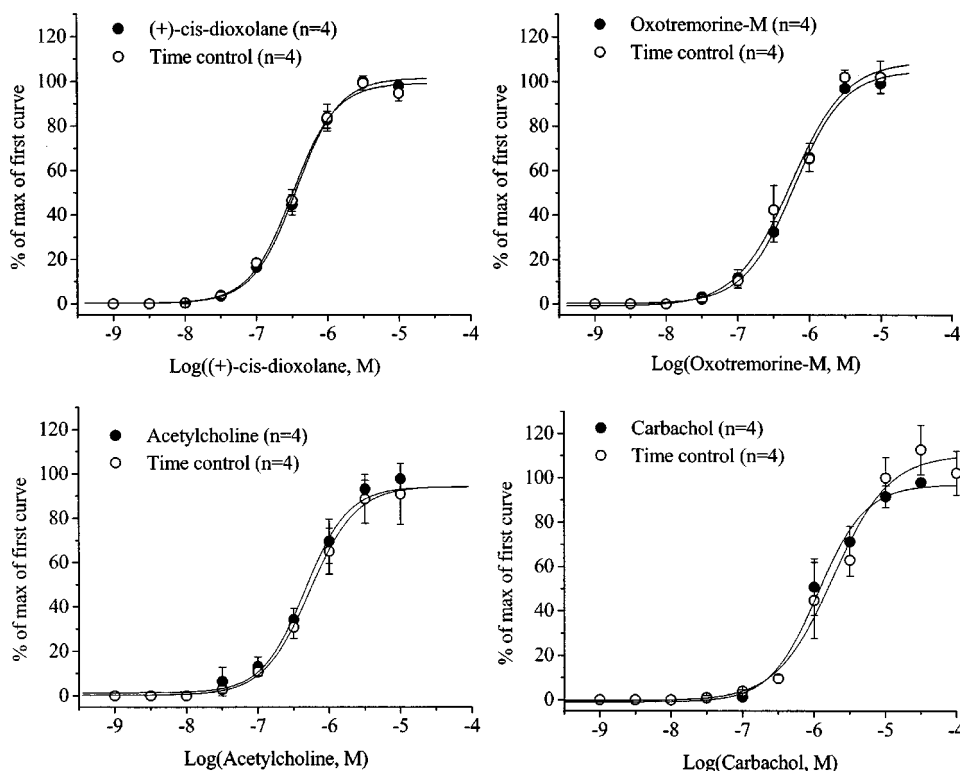


Figure 1 Effects of muscarinic agonists on female C57BL/6 mouse urinary bladder smooth muscle. Contractile effects were expressed as percentages of the maximum response of the control curve. The values shown are mean±s.e.mean, $n=4$ animals.

concentration-dependent fashion, with parallel rightward displacements. Their functional affinity estimates (pK_B) are summarized in Table 2.

Comparison of functional data for female mouse urinary bladder smooth muscle with binding data at human recombinant muscarinic receptors

The analysis between the affinities of the antagonists at muscarinic receptors in the female mouse urinary bladder smooth muscle and the affinities at human recombinant muscarinic receptors showed a significant correlation at m_3 ($r=0.93$, $P=0.002$, $ssq=7.95$; $r=0.76$, $p=0.05$, $ssq=6.10$; $r=0.92$, $P=0.004$, $ssq=6.30$; for female 129/SvJ, C57BL/6

and N1F2, respectively). In contrast, poor correlations were observed at m_1 , m_2 , m_4 and m_5 (Figure 2).

Characterization of muscarinic receptors mediating the reconstrictions in female mouse urinary bladder smooth muscle

(+)-Cis-dioxolane produced concentration-dependent contractions of female mouse urinary bladder smooth muscle under control conditions. After preferential alkylation of M_3 receptor (exposure to 4-DAMP mustard in presence of methoctramine), (+)-cis-dioxolane produced recontractile (reversal of contraction) responses of KCl-precontracted tissues, which were relaxed with isoproterenol. No time-

Table 2 Affinity estimates (pK_B) for muscarinic antagonists in mouse urinary bladder smooth muscle

Antagonist	m_1	m_2	m_3	m_4	m_5	Bladder C57BL6		129SvJ	N1F2
	pK_i	pK_i	pK_i	pK_i	pK_i	male	female	female	female
atropine (3 nM)	9.1 ^a	8.9 ^a	9.5 ^a	9.2 ^a	9.1 ^a	9.22±0.09 ^b	8.89±0.18	7.98±0.24	8.37±0.13
pirenzepine (1 μM)	8.0	6.3	6.8	7.1	6.9	6.85±0.08 ^b	6.14±0.45	6.25±0.06	6.41±0.01
4-DAMP (10 nM)	9.2 ^a	8.1 ^a	9.3 ^a	8.4 ^a	8.9 ^a	8.42±0.14 ^b	8.36±0.09	7.64±0.30	7.97±0.13
methoctramine (10 μM)	6.7	7.7	6.1	7.0	6.3	5.96±0.05 ^b	5.99±0.12	6.09±0.15	5.62±0.11
p-F-HHSiD (0.3 μM)	7.3 ^a	6.6 ^a	7.5 ^a	7.2 ^a	6.7 ^a	7.48±0.09 ^b	7.54±0.08	7.69±0.05	7.71±0.12
zamifenacin (0.1 μM)	7.6	7.2	7.9	6.9	7.3	8.30±0.17 ^b	8.30±0.13	8.37±0.31	7.73±0.13
darifenacin (10 nM)	7.8	7.0	8.8	7.7	8.0	8.70±0.09 ^b	8.31±0.23	8.73±0.12	8.23±0.08

Values shown are mean ± s.e.mean, $n=4$. pK_i values are from Loury *et al.* (1999). ^a pK_i values taken from Hegde *et al.* (1997). ^b pK_B values taken from Choppin & Eglen (2001).

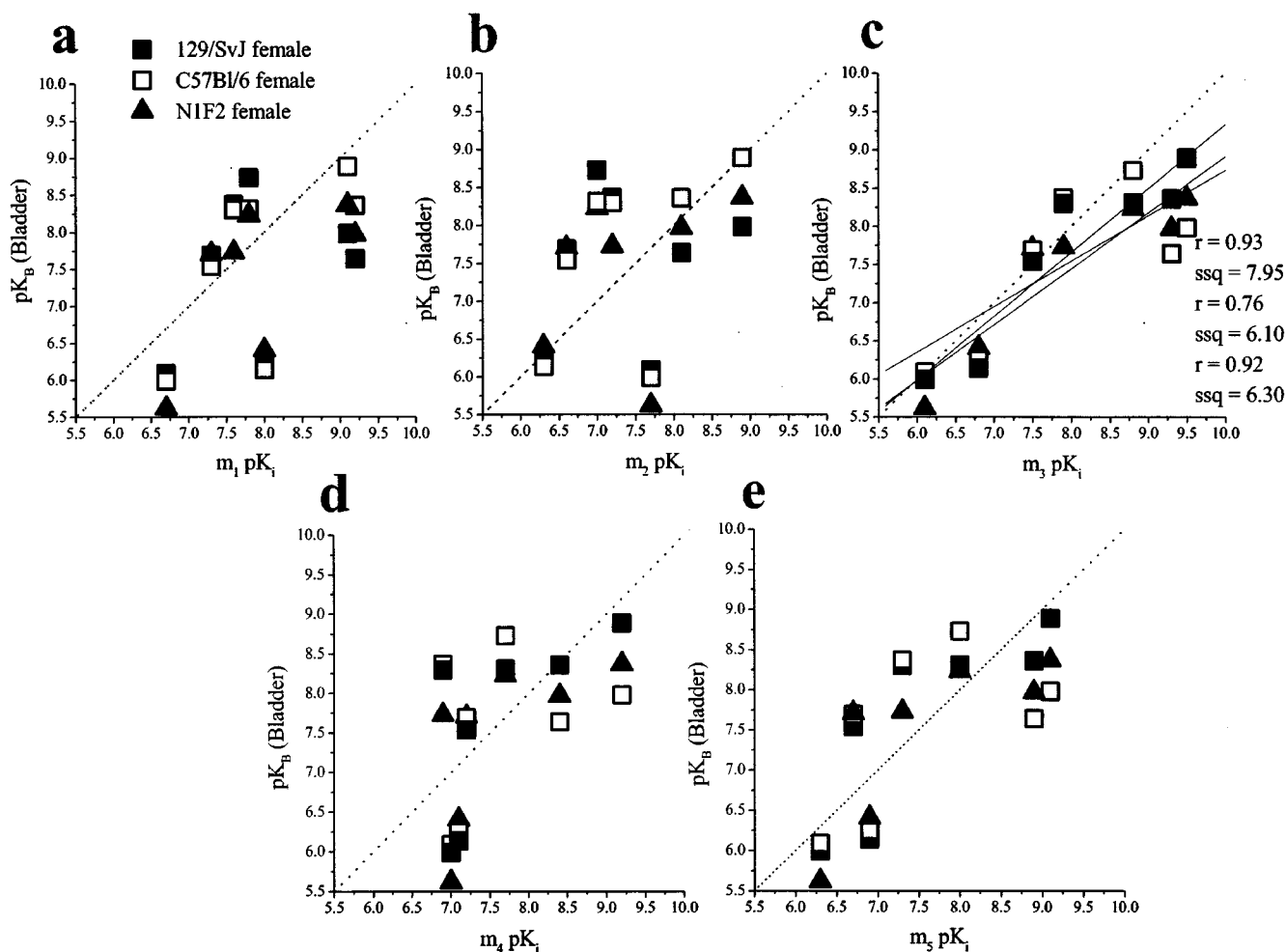


Figure 2 Correlation between the functional affinities (pK_B values) of muscarinic antagonists at muscarinic receptor in female mouse isolated urinary bladder smooth muscle and binding affinities (pK_i values) at human recombinant muscarinic receptors (m_1 – m_5 ; a–e respectively). The binding data were taken from Dörje *et al.*, 1991; Eglen *et al.*, 1997; Hegde *et al.*, 1997; Nilvebrant *et al.*, 1996. The broken line is the line of identity ($x=y$) while the solid line is the correlation plot (the inserts give the correlation factors (r) and the sum of squares values (ssq)).

dependent changes in agonist sensitivity were observed during the construction of two consecutive concentration-recontractile effect curves. As shown in Figure 3 for female N1F2 mice, methoctramine produced surmountable antagonism of the retractorile response to (+)-cis-dioxolane. The affinity estimates (pK_B/pA_2) for methoctramine and the slope of the Schild plots (not significantly different than unity) are summarized in Table 3.

Discussion

Muscarinic antagonists are still the first line of treatment for overactive bladder, which occurs predominantly in females (Wein, 1995). Although gender differences in responsiveness of the bladder to muscarinic stimulation have been reported in rat (Chun *et al.*, 1990; Longhurst *et al.*, 1992; Longhurst & Levendusky, 2000), the receptor subtypes that are responsible

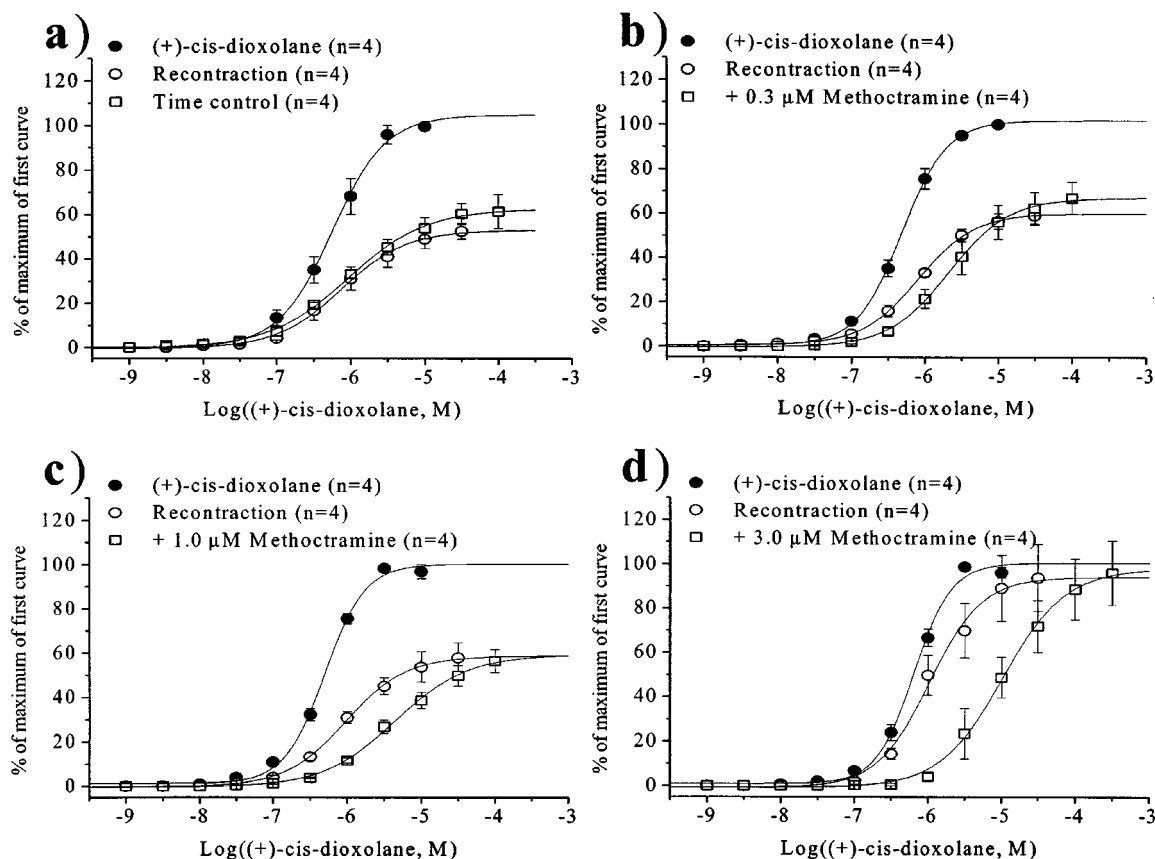


Figure 3 Recontraction experiments in female N1F2 mouse urinary bladder smooth muscle: effect of methoctramine on the recontractile concentration-effect to (+)-cis-dioxolane obtained after elevation of adenylyl cyclase activity following preferential alkylation of muscarinic M_3 receptors ($n=4$). (a) Time control; (b) $+0.3 \mu\text{M}$ methoctramine; (c) $+1.0 \mu\text{M}$ methoctramine; (d) $+3.0 \mu\text{M}$ methoctramine.

Table 3 Affinities (pK_B/pA_2) and slopes of the Schild plots for methoctramine after recontraction in mouse urinary bladder

	C57BL6		129SvJ	N1F2
	male ^a	female	female	female
pK_B	6.23 ± 0.14	6.66 ± 0.08	6.26 ± 0.08	6.09 ± 0.22
pA_2	6.16 ± 0.03	7.00 ± 0.27	6.31 ± 0.07	6.08 ± 0.01
Slope	NS	0.65 ± 0.18	0.82 ± 0.19	1.08 ± 0.22

Values are mean \pm s.e.mean. ^aData from Choppin & Eglen, 2001. NS: not significantly different than unity.

have not been identified. Previous studies using mouse urinary bladder (Durant *et al.*, 1991; Lundbeck & Sjögren, 1992) have also demonstrated a muscarinic-induced contractile response but did not characterize the receptor subtype(s) involved. Thus, the aim of this study was to examine the influence of gender as well as strains on muscarinic responsiveness of mouse bladder strips.

Using five different muscarinic agonists (including the partial agonist pilocarpine), the potencies and maximal responses obtained in mouse urinary bladder smooth muscle suggest gender and strain differences (range for pEC_{50} = 6.4–6.6, 6.2–6.9, 6.2–6.7, 5.4–6.0 and 0.0–5.8, T_{max} = 1.9–4.7 g, 1.3–3.4 g, 1.0–3.0 g, 1.4–2.7 and 0.0–0.9 g, for (+)-cis-dioxolane,

oxotremorine-M, acetylcholine, carbachol and pilocarpine, respectively, $n=4-6$, depending on the gender and the strain; see Table 1). Amongst these agonists, (+)-cis-dioxolane was the only one producing bladder contractions of similar potency and maximal response in both gender and strains. Therefore, it was used to determine the affinity estimates of a range of muscarinic antagonists (see Table 2): the agonist-induced concentration-dependent contractions were inhibited in a competitive fashion. The pK_B values of these antagonists correlated most strikingly with the binding affinities of the antagonists at m_3 recombinant muscarinic receptors (pK_i are: atropine 9.5; pirenzepine 6.8; 4-DAMP DAMP 9.3; methoctramine 6.1; p-F-HHSiD 7.5; zamifenacin 7.9 and darifenacin 8.8; Hegde *et al.*, 1997; Loury *et al.*, 1999; Since ionic strength influences antagonist binding affinity, it is important to mention that these values were measured in Tris–Krebs buffer; Pedder *et al.*, 1991). This finding suggests the exclusive involvement of M_3 muscarinic receptors in the direct contractile response to muscarinic agonists in female mouse bladder. This accords with preliminary results described by Paravicini *et al.* (2000) but also in male mice (Choppin & Eglen, 2001) and other species: rabbit (Tobin, 1995; Tobin & Sjögren, 1995), rat (Longhurst *et al.*, 1995; Hegde *et al.*, 1997) and human (Newgreen & Naylor, 1996) bladder.

If present, a functional role of M_2 receptors could be unmasked in recontraction experiments (with preferential

alkylation of M₃ receptors and elevated adenylyl cyclase). Under these conditions, methoctramine exhibited a low affinity in the mouse strains tested ($pA_2=6.31$ and 6.08 in female 129/SvJ and N1F2, respectively), which argues against the involvement of an M₂ receptor. As previously described in male mouse bladder (Choppin & Eglen, 2001), no indirect contractile role of M₂ receptors was revealed by functional studies (recontraction experiments) in female mouse bladder, and only M₃ receptor activation induced bladder contraction. The remaining response observed with (+)-cis-dioxolane after recontraction is likely due to an incomplete alkylation of muscarinic M₃ receptors. However, in female C57BL/6, methoctramine yielded a pA_2 value (7.00) intermediate between the one expected for this compound at M₂ and M₃ receptors, suggesting the involvement of both of these subtypes. Similar observations have been seen in rat urinary bladder (Hegde *et al.*, 1997) and several gastrointestinal smooth muscle tissues.

Recently, knockout male mice for M₂ (Stengel *et al.*, 2000; Zhou *et al.*, 2002) and M₃ (Matsui *et al.*, 2000) receptors were reported. Data from these studies suggest, on one hand, that muscarinic M₂ receptors play a minor role in carbachol-induced contraction of isolated bladder smooth muscle (the potency of muscarinic agonists was only modestly reduced,

and the maximal response unaffected; Stengel *et al.*, 2000). On the other hand, Matsui *et al.* (2000) demonstrated a predominant involvement of muscarinic M₃ receptor in bladder contractions, as these contractions *in vitro* were virtually abolished and urinary retention was marked in male mice *in vivo*, in transgenic mice lacking the muscarinic M₃ receptor. These data suggest that a dominant, if not exclusive, role of this subtype prevails and are consistent with the findings obtained in the present study. Similarly in rat bladder, it has been shown that gender influences the sensitivity to muscarinic stimulation (Longhurst & Levensky, 2000).

Conclusions

The present data suggest that only M₃ receptors play a role in mouse urinary bladder smooth muscle contraction, in accord with emerging data from knockout animals. The pharmacological antagonist profile of the muscarinic receptors present in the mouse bladder equates most closely with the M₃ muscarinic receptor, with however gender and strain differences. It thus appears that one should be careful when comparing results obtained from different studies using various strains.

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