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Characterization of the functional muscarinic receptors in the rat urinary bladder

[1]Penelope A. Longhurst, Robert E. Leggett & Janice A.K. Briscoe

Division of Urology, Hospital of the University of Pennsylvania, 3400 Spruce Street, Philadelphia, Pennsylvania 19104, U.S.A.

1 Muscarinic receptors mediating contraction of the rat urinary bladder were characterized functionally *in vitro* by use of atropine, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP methiodide), 4-diphenylacetoxy-N-(2-chloroethyl)-piperidine hydrochloride (4-DAMP mustard), hexahydro-sila-diphenidol hydrochloride (HHSiD), the *p*-fluoro analogue of hexahydro-sila-diphenidol hydrochloride (*p*-F-HHSiD), methoctramine, and pirenzepine.

2 (+)-*cis*-Dioxolane contracted bladder strips in a concentration-dependent manner with an EC_{50} of 0.169 ± 0.018 μM and an E_{max} of 7.84 ± 0.67 g.

3 Concentration-effect curves to (+)-*cis*-dioxolane were shifted to the right in the presence of the antagonists in a concentration-dependent manner. The rank order of antagonist affinities against the (+)-*cis*-dioxolane response was (pA_2 values in the parentheses) atropine (9.28) $\geq$ 4-DAMP methiodide (9.04) > HHSiD (8.01) > *p*-F-HHSiD (7.28) = pirenzepine (7.12) $\geq$ methoctramine (6.77, 7.25). The profile resembles that associated with the M_3 receptor subtype.

4 Atropine, 4-DAMP methiodide, pirenzepine, and methoctramine had no effects on the contractile response to 120 mM KCl. However, HHSiD and *p*-F-HHSiD decreased the response to KCl, and 4-DAMP mustard increased it.

5 Contractile responses to electrical field stimulation (1–32 Hz, 0.05 ms pulse duration) were biphasic in nature. The tonic response was suppressed more than the phasic response by all antagonists except methoctramine. The suppression was not always concentration-dependent, and did not seem to be related to antagonism of any one receptor subtype.

6 Our findings are consistent with the minority M_3 receptors mediating the contractile response to muscarinic stimulation by (+)-*cis*-dioxolane in the rat bladder.

Keywords: Rat urinary bladder; M_3 muscarinic receptors

Introduction

Although elucidation of the muscarinic receptor subtypes responsible for responsiveness to cholinoceptor agonists has been hampered by the lack of subtype-selective agonists or antagonists, at least 4 different subtypes of muscarinic receptor have been identified pharmacologicallly on the basis of differing antagonist affinities (Hulme *et al.*, 1990; Caulfield, 1993; Eglen *et al.*, 1994). These have been designated M_1, M_2, M_3, and M_4. Gene products corresponding to the pharmacologically identified muscarinic receptor subtypes have been identified and are designated m_1, m_2, m_3, and m_4 (Hulme *et al.*, 1990; Hosey, 1992).

Like intestinal and respiratory smooth muscles (Thomas & Ehlert, 1994; Watson & Eglen, 1994a; Reddy *et al.*, 1995), the urinary bladder contains heterogeneous populations of muscarinic receptors. The number of muscarinic M_2 receptors in the rat urinary bladder is reported to be greater than the number of M_3 receptors in radioligand binding studies (Monferini *et al.*, 1988; Kamai *et al.*, 1994). Using receptor-specific antibodies, Wall and co-workers found that m_2 receptors accounted for 86% of the expressed receptors in rat bladder, m_3 receptors accounted for the remainder and m_1 receptors were undetectable (Wall *et al.*, 1991). Northern blot studies showed that porcine bladder expresses m_2 and m_3 mRNA (Maeda *et al.*, 1988).

Despite these studies examining the numbers of muscarinic receptors in the bladder, there is a paucity of information concerning the functional muscarinic receptor subtype(s) responsible for bladder contraction. Studies on guinea-pig bladders have shown that *in vitro* responses to carbachol and *in vivo* micturition contractions result from stimulation of M_3 receptors (Noronha-Blob *et al.*, 1989a,b). The aim of this paper was to identify the muscarinic receptors responsible for contraction of the rat bladder after cholinergic stimulation.

Methods

Animals

Adult male Sprague-Dawley rats (500–600 g) obtained from Ace Animals Inc. (Boyertown, PA, U.S.A.) were used throughout the study. All animals received food and water *ad libitum*.

Tissue preparation

Rats were anaesthetized with a mixture of ketamine (90 mg kg^{-1}) and xylazine (11 mg kg^{-1}). The urinary bladder was removed and placed in ice-cold Krebs-Henseleit buffer of the following composition (mM): NaCl 113, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and dextrose 5.6. Four equally sized longitudinal strips of approximately 2 mm × 10 mm were cut from the bladder body, suspended on 000 sutures between a pair of platinum ring electrodes, 8 mm apart, and placed in 10 ml organ baths containing Krebs-Henseleit solution equilibrated with 95% O_2 5% CO_2 at 37°C. The sutures were connected to Grass force displacement transducers (FT03) and the resting tension was adjusted to 2 g. Responses were recorded on a Grass Model 7E polygraph. All tissues were then given a 30 min equilibration period during which they were washed and the resting tension was adjusted every 10 min.

[1] Author for correspondence.

Contractile studies

Frequency-response curves were elicited by stimulating the strips for 15 s with pulses of 0.05 ms width at 100 V every 3 min with a Grass S88 stimulator. These responses have previously been shown to be sensitive to tetrodotoxin (Tammela *et al.*, 1994). Then non-cumulative concentration-effect curves to (+)-*cis*-dioxolane were generated. Tissues were washed at least twice between each incremental concentration. The response to 120 mM KCl was then measured. After washing out KCl, one strip from each rat was incubated with one of the following concentrations of antagonist for 60 min, with washing every 15 min: atropine, 1, 3, 10, 30 nM; 4-DAMP methiodide, 1, 3, 10, 30 nM, HHSiD, 0.03, 0.1, 0.3, 1 μM; *p*-F-HHSiD, 0.1, 0.3, 1, 3 μM; pirenzepine, 0.1, 1, 3, 10 μM; methoctramine, 0.1, 0.3, 1, 3 μM. In some studies, strips were incubated with 4-DAMP mustard, 0.1, 0.3, 1, 3, 10, 30 nM before repeating stimuli. Each strip was exposed to only one concentration of antagonist. Stimulation with field stimulation, (+)-*cis*-dioxolane, and KCl was then repeated. In separate experiments, the effect of time on contractile responses was measured by repeating the experiment in the absence of antagonists.

Drugs

Atropine was obtained from Sigma Chemical Company (St. Louis, MO. U.S.A.). 4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP methiodide), 4-diphenylacetoxy-N-(2-chloroethyl)-piperidine hydrochloride (4-DAMP mustard), (+)-*cis*-dioxolone (a 60:40 mixture of *cis*:*trans* diastereomers), hexahydro-sila-diphenidol hydrochloride (HHSiD), the *p*-fluoro analogue of hexahydro-sila-diphenidol hydrochloride (*p*-F-HHSiD), methoctramine tetrahydrochloride, and pirenzepine dihydrochloride were obtained from Research Biochemicals International (Natick, MA, U.S.A.).

Statistical analysis

Data are normalized to the maximal response generated during the first (no antagonist) control curve, and are expressed as means ± s.e.mean. Geometric mean EC_{50} values were obtained by probit analysis (Fleming *et al.*, 1972). pA_2 values for the muscarinic receptor antagonists were determined by Schild regression (Arunlakshana & Schild, 1959). Confidence intervals for pA_2 values and the slope were calculated as described by Tallarida & Murray (1987). Phasic (maximum within 10 s) and tonic (response at 15 s) contractile responses to field stimulation were measured. Comparisons between responses before and after incubation with antagonists were made by the paired *t* test with a P value <0.05 considered significant.

Results

Responses to (+)-cis-dioxolane

(+)-*Cis*-dioxolane caused concentration-dependent contractions of the rat bladder with an E_{max} of 7.84 ± 0.67 g and EC_{50} values of 0.169 ± 0.018 μM. Neither tension nor the EC_{50} was affected by time. The E_{max} for the second curve was 7.31 ± 0.59 g and the EC_{50} was 0.201 ± 0.020 μM.

All competitive antagonists caused parallel shifts of the concentration-effect curves to (+)-*cis*-dioxolane, generally without suppression of the maximal response (Figure 1). Schild regression analysis was linear for all antagonists except methoctramine, with a slope of unity (Table 1). Schild plots are illustrated in Figure 2. The rank order of antagonist affinities was: atropine ≥ 4-DAMP > HHSiD > *p*-F-HHSiD = pirenzepine ≥ methoctramine. Comparison of the values obtained in the present study with those reported for muscarinic receptors in the literature indicates that the profile resembles that associated with the M_3 receptor subtype.

Incubation with the irreversible M_3-antagonist, 4-DAMP mustard, produced significant suppression of the contractile response to (+)-*cis*-dioxolane with high potency (Figure 3), further supporting the role of M_3 receptors mediating contraction in the rat urinary bladder.

Responses to KCl

Incubation with atropine, 4-DAMP methiodide, pirenzepine, and methoctramine had little effect on the contractile response to 120 mM KCl. Compared to control responses in the absence of antagonist, responses to KCl were 97.5 ± 5.3% after 30 nM atropine, 121.4 ± 14.4% after 30 nM 4-DAMP methiodide, 97.7 ± 5.7% after 3 μM methoctramine, and 104.3 ± 11.8% after 10 μM pirenzepine. All concentrations of HHSiD significantly reduced the response to KCl (83.2 ± 3.7% after 1 μM), but *p*-F-HHSiD significantly reduced the response to KCl at only 0.3 (71.3 ± 5.05%) and 3 μM (83.7 ± 4.1%). Incubation with increasing concentrations of 4-DAMP mustard caused a progressive increase in the response to KCl, which was significant at 1, 3, and 30 nM (151.2 ± 10.7% after 30 nM).

Responses to field stimulation

There was a small but significant decrease in magnitude of both the phasic and tonic components of the contractile response to field stimulation during the second frequency-response curve. The E_{max} for the phasic component was 7.17 ± 0.72 g during the first curve and 6.02 ± 0.73 g during the second curve. The tonic component had an E_{max} of 5.72 ± 0.67 g during the first curve and 5.04 ± 0.62 g during the second curve.

The effects of the antagonists on the responses to field stimulation were qualitatively very similar. Therefore only representative examples are shown (Figure 4). Wherever possible in the descriptions of the effects of the antagonists, we compare concentrations which were roughly equieffective at suppressing the response to (+)-*cis*-dioxolane (see Table 2). All concentrations of 4-DAMP methiodide reduced the phasic response to field stimulation (Figure 4a). Although 1 nM 4-DAMP methiodide had no effects on the tonic response, 3–30 nM did reduce the response. There was a 35% suppression of the phasic response and 49% suppression of the tonic response to 32 Hz stimulation at 10 nM 4-DAMP methiodide, a concentration which produced a 20 fold shift in the EC_{50} value for (+)-*cis*-dioxolane (Figure 1b, table 2).

The effects of *p*-F-HHSiD on the response to field stimulated were similar to those of 4-DAMP methiodide (Figure 4b). The tonic response (55% suppression of the response to 32 Hz after 1 μM) was more susceptible to *p*-F-HHSiD than was the phasic response (35% suppression). This concentration produced a 16 fold shift in the EC_{50} value for (+)-*cis*-dioxolane (Figure 1d, Table 2).

The effects of pirenzepine on the contractile response to field stimulation were more variable than those of the other antagonists, particularly at the lowest concentrations (Figure 4c). Like 4-DAMP methiodide and and *p*-F-HHSiD, all concentrations of pirenzepine reduced the phasic response, but the effects of the lowest concentrations on the tonic response were much more variable. There was a 34% suppression of the phasic response and 49% suppression of the tonic response to 32 Hz stimulation with 1 μM pirenzepine, a concentration which caused a 12 fold shift in the EC_{50} value for (+)-*cis*-dioxolane (Figure 1e, Table 2).

Methoctramine suppressed the response to field stimulation less than the other antagonists (Figure 4d). Methoctramine 3 μM, a concentration which like pirenzepine and atropine, caused a 12 fold shift in the EC_{50} value for (+)-*cis*-dioxolane (Figure 1f), inhibited both phasic and tonic components by 20–25%. In separate experiments with 10 μM methoctramine, we could find no additional suppression of the response to field stimulation, and only a small additional decrease in the EC_{50} value for (+)-*cis*-dioxolane.

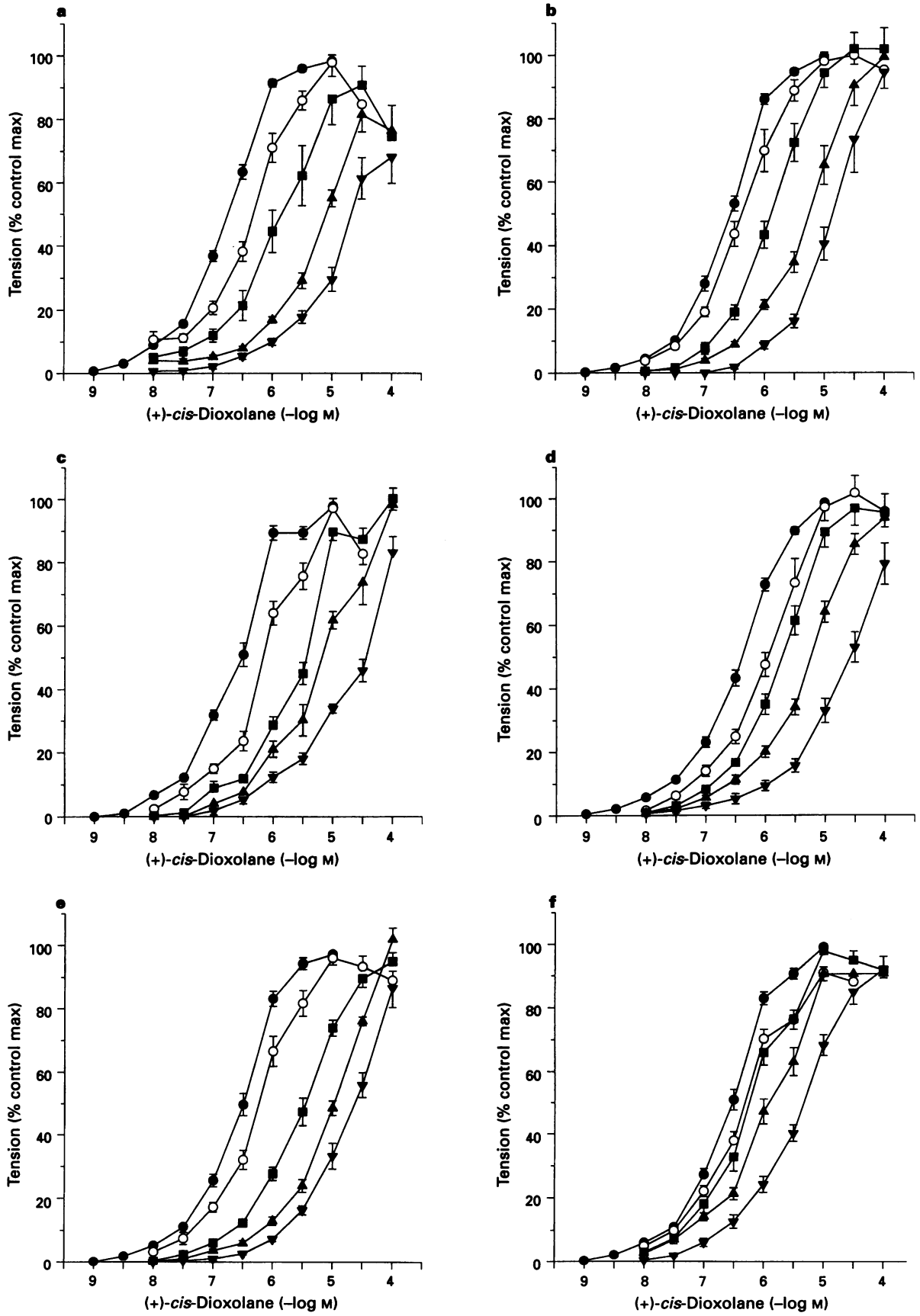

Figure 1 Contractile response of rat bladder body strips to (+)-*cis*-dioxolane in the absence (●) and presence of (a) atropine (○) 1 nM, (■) 3 nM, (▲) 10 nM, (▼) 30 nM; (b) 4-DAMP methiodide (○) 1 nM, (■) 3 nM, (▲) 10 nM, (▼) 30 nM; (c) HHSiD (○) 0.03 μM, (■) 0.1 μM, (▲) 0.3 μM, (▼) 1 μM; (d) *p*-F-HHSiD (○) 0.1 μM, (■) 0.3 μM, (▲) 1 μm, (▼) 3 μM; (e) pirenzepine (○) 0.1 μM, (■) 1 μM, (▲) 3 μM, (▼) 10 μM, and (f) methoctramine (○) 0.1 μM, (■) 0.3 μM, (▲) 1 μM, (▼) 3 μM. Responses are normalized to the maximal response generated by each strip during the first curve in the absence of antagonist. Each point represents the mean ± s.e.mean of 4 to 12 individual strips.

Table 1 Comparison of pA_2 values for antagonists at receptors mediating contraction of rat urinary bladder in response to (+)-*cis*-dioxolane

Antagonist	*pA_2*	*95% CL*	*Slope*	*95% CL*
Atropine	9.28	9.10–9.46	1.1558	0.98–1.33
4-DAMP methiodide	9.04	8.97–9.11	1.1002	0.96–1.24
HHSiD	8.01	7.65–8.36	0.9230	0.68–1.16
p-F-HHSiD	7.28	7.03–7.53	0.9688	0.76–1.18
Pirenzepine	7.12	6.90–7.34	0.9448	0.80–1.09
Methoctramine	7.25	6.85–7.65	0.5322	0.35–0.72
Methoctramine*	6.77	6.62–6.92	1.0000	

Values were determined by Schild regression, as described in the text. 95% CL: 95% confidence limits. For abbreviations, see text. *After constraining slope to unity.

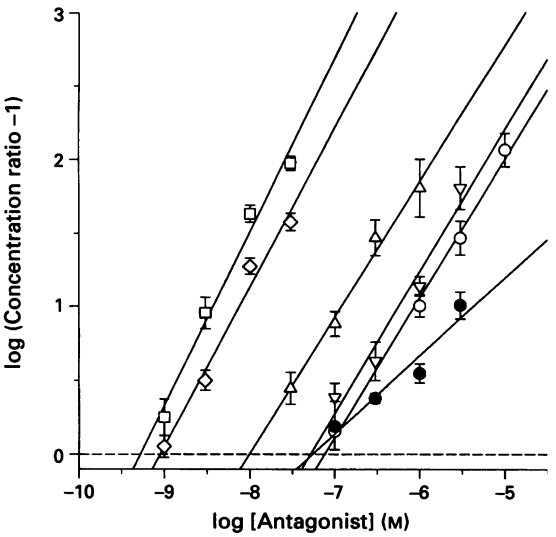

Figure 2 Schild analysis for atropine (□), 4-DAMP methiodide (◇), HHSiD (△), *p*-F-HHSiD (▽), pirenzepine (○), and methoctramine (●). Each point represents the mean ± s.e.mean of 4 to 12 individual observations. The pA_2 values and slopes for the regression lines are given in Table 1.

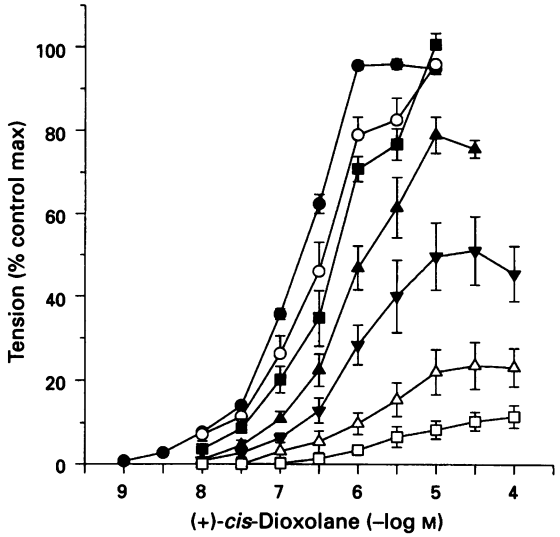

Figure 3 Contractile response of rat bladder body strips to (+)-*cis*-dioxolane in the absence (●) and presence of 4-DAMP mustard (○) 0.1 nM, (■) 0.3 nM, (▲) 1 nM, (▼) 3 nM, (△) 10 nM, and (○) 30 nM. Responses are normalized to the maximal response generated by each strip in the absence of antagonist. Each point represents the mean ± s.e.mean of 4 to 8 individual strips.

Responses in the presence of atropine (1–30 nM) and HHSiD (0.03–1 μM) (data not shown) were similar to those observed after 4-DAMP methiodide. In all instances there was a large antagonist-resistant component to the phasic response to 32 Hz (36% suppression after 3 nM atropine and 26% suppression after 0.1 μM HHSiD). The suppression of the phasic component was not particularly concentration-dependent, and similar degrees of inhibition were seen with lower concentrations of antagonists. The tonic response was suppressed by 40% after 3 nM atropine and 42% after 0. 1 μM HHSiD. These same concentrations of antagonists caused 12 and 9 fold shifts in the EC_{50} values for (+)-*cis*-dioxolane (Figure 1a and c, Table 2).

Incubation with 4-DAMP mustard caused concentration-dependent decreases in both phasic and tonic components (Figure 4e). Maximal suppression of the phasic response to 32 Hz stimulation (50%) was achieved with 3–30 nM, while 10 and 30 nM caused 70% suppression of the tonic response.

Discussion

Previous biochemical studies have shown that the predominant muscarinic receptor in the rat urinary bladder is the M_2 subtype (Monferini *et al.*, 1988; Wall *et al.*, 1991; Kamai *et al.*,

Table 2 Comparison of inhibitory effects of antagonists in response to (+)-*cis*-dioxolane with suppression of phasic and tonic response to 32 Hz field stimulation

Antagonist	[concentration]	*A'/A*	*Phasic*	*Tonic*
HHSiD	[0.1 μM]	9.29 ± 1.57	73.5 ± 7.8	58.8 ± 6.9
Atropine	[3 nM]	11.71 ± 3.26	64.2 ± 8.5	59.3 ± 9.2
Pirenzepine	[1 μM]	12.06 ± 1.74	66.0 ± 3.0	51.3 ± 5.5
Methoctramine	[3 μM]	12.32 ± 2.45	75.4 ± 8.7	79.7 ± 9.8
p-F-HHSiD	[1 μM]	16.43 ± 2.44	64.6 ± 3.0	45.5 ± 3.9
4-DAMP methiodide	[10 nM]	20.52 ± 2.05	65.1 ± 3.8	51.4 ± 4.8

Values shown represent response remaining after incubation with equieffective concentrations of antagonists. Data are presented as the % of original phasic or tonic response remaining after incubation with each antagonist. A'/A is the agonist dose-ratio determined at 50% of maximal response to (+)-*cis*-dioxolane in the absence (A) and presence (A') of the concentration of antagonist described in the table.

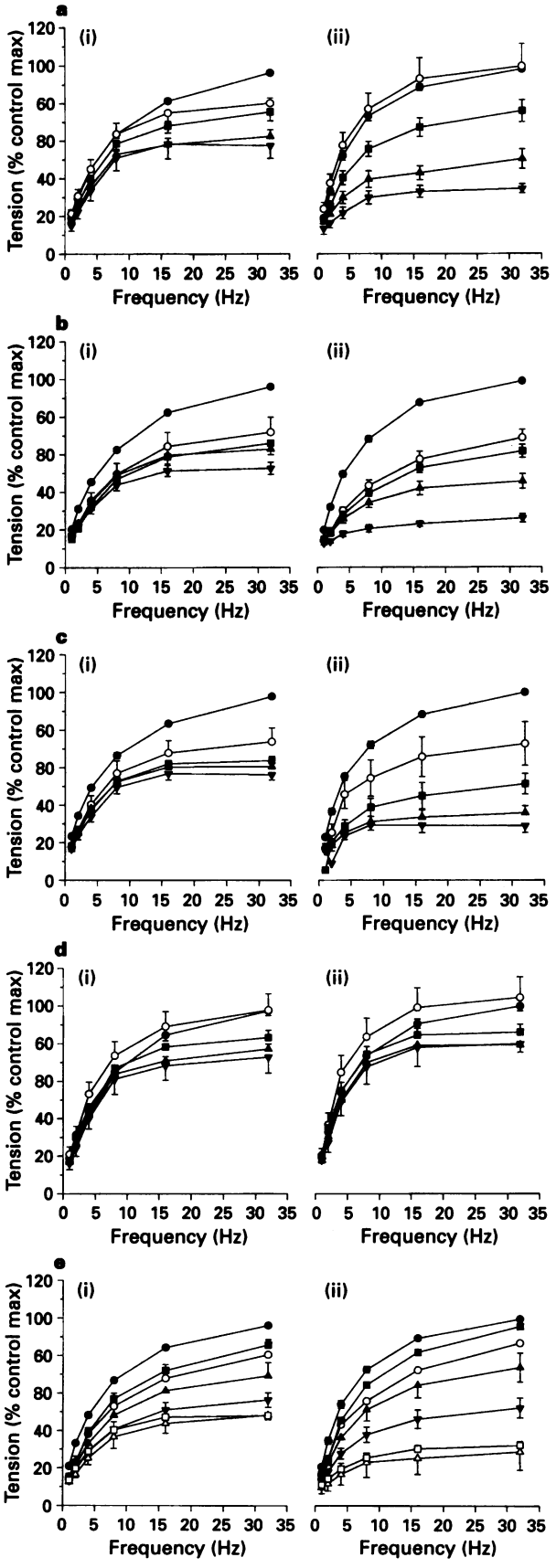

Figure 4 Contractile response of rat bladder body strips to field stimulation in the absence (●) and presence of (a) 4-DAMP methiodide (○) 1 nM, (■) 3 nM, (▲) 10 nM, (▼) 30 nM; (b) *p*-F-HHSiD (○) 0.1 μM, (■) 0.3 μM, (▲) 1 μM, (▼) 3 μM; (c) pirenzepine (○) 0.1 μM, (■) 1 μM, (▲) 3 μM, (▼) 10 μM, (d) methoctramine (○) 0.1 μM, (■) 0.3 μM, (▲) 1 μM, (▼) 3 μM; and (e) 4-DAMP mustard (○) 0.1 nM, (■) 0.3 nM, (▲) 1 nM, (▼) 3 nM, (△) 10 nM, and (□) 30 nM. Responses are normalized to (i) the maximal phasic (within 10 s) or (ii) tonic (after 15 s) response generated by each strip in the absence of antagonist. Each point represents the mean ± s.e.mean of 4 to 12 individual strips.

1994). The role of M_2-receptors in the bladder is unknown. In some smooth muscle tissues, such as the uterus, stimulation of M_2-receptors results in contraction (Eglen *et al.*, 1989; Doods *et al.*, 1993). However, activation of M_2 receptors is associated with inhibition of adenylyl cyclase, which in most smooth muscles would be expected to result in relaxation. Until recently it has been difficult to characterize the muscarinic receptor subtypes mediating functional responses of muscle preparations. However, the development of a number of relatively subtype-selective muscarinic antagonists has meant that it is now much easier to characterize pharmacologically the functional responses to muscarinic stimulation.

The muscarinic agonist (+)-*cis*-dioxolane caused reproducible contractions of the rat bladder. This agonist is thought to be non-specific in its selectivity for M_1, M_2, and M_3 receptors. The EC_{50} value obtained in this study was similar to that found for the guinea-pig bladder by Dorofeeva and co-workers (0.145 μM) (Dorofeeva *et al.*, 1992). This was considerably lower than for the endothelium-denuded rabbit aorta (1 μM) (Watson & Eglen, 1994b), but higher than in guinea-pig ileum (15 nM), heart (2–7 nM) (Dorofeeva *et al.*,1992; Watson & Eglen, 1994b), or trachea (2 nM) (Dorofeeva *et al.*,1992). Dorofeeva and co-workers postulated that the relatively low potency of muscarinic agonists on the guinea-pig bladder might be due to the presence of a heterogeneous population of M_3-receptors. However, in our study the potency of (+)-*cis*-dioxolane was similar to that previously reported for acetylcholine (0.21 μM), and lower than that of carbachol (1.08 μM), and bethanechol (12.15 μM) in the rat bladder (Latifpour *et al.*, 1989), and may simply reflect tissue-specific differences in receptor-effector coupling efficiency. In comparison to other smooth muscles, the urinary bladder is known to be relatively insensitive to contractile agents (Longhurst *et al.*, 1984).

The rank order of antagonist affinities, atropine ≥ 4-DAMP methiodide > HHSiD > *p*-F-HHSiD = pirenzepine ≥ methoctramine, is consistent with stimulation of M_3 receptors causing contraction with no involvement of M_2 receptors. 4-DAMP methiodide has high affinity for both M_1 and M_3 receptors. However, the low affinity for pirenzepine suggests that M_1 receptors are not involved in the contractile response to muscarinic agonists. The pA_2 value for *p*-F-HHSiD obtained in this study (7.3) was somewhat lower than that originally reported for interactions with M_3 receptors (Lambrecht *et al.*, 1989), but similar to values for M_3 interactions in rabbit aorta (7.4) (Watson & Eglen, 1994b), and bovine (7.36) or rabbit (7.09) trachea (Eltze *et al.*, 1992; Roffel *et al.*, 1994). Both HHSiD and *p*-F-HHSiD caused significant decreases in the contractile response to KCl, an effect which was not seen with the other antagonists. We are not aware of any previous reports of this non-specific effect, but it might contribute to the reduced pA_2 value observed in this study. The reasons for the decreased response to KCl are not known. The low pA_2 value of methoctramine (7.25) suggests that the predominant M_2 receptors are not normally involved in contraction of the rat bladder.

In trachea and intestinal smooth muscle, similar to the urinary bladder, M_2 receptors predominate over the M_3 receptors which mediate contraction. In both cases, stimulation of M_2 receptors is thought to impart a modulatory action on β-adrenoceptor relaxant responses, by coupling to the pertussis toxin-sensitive guanine nucleotide regulatory protein G_i and inhibiting adenylyl cyclase activation. Inhibition of tracheal M_2 receptors by methoctramine increases the relaxant potency of isoprenaline (Watson & Eglen, 1994a). Protection of M_2 receptors on guinea-pig ileum by AF-DX 116 during M_3 re-

ceptor alkylation with 4-DAMP mustard revealed a M_2-mediated contractile response to oxotremorine, which was pertussis toxin and AF-DX 116-sensitive (Thomas & Ehlert, 1994). Similar results have been reported for the guinea-pig ileum by Reddy and co-workers (1995). Protection of M_2 receptors by methoctramine during alkylation with 4-DAMP mustard revealed an oxotremorine-induced contraction which was inhibited by the M_2-antagonist, methoctramine. β-Adrenoceptors predominate over α-adrenoceptors in the urinary bladder body, where their tonic stimulation is thought to facilitate the storage phase of micturition by relaxing the detrusor smooth muscle (Bissada & Finkbeiner, 1982; Wein, 1992). During bladder emptying, acetylcholine release from efferent parasympathetic pelvic nerve endings results in a sustained contraction of the bladder. If similar mechanisms to those present in trachea and ileum exist in the bladder, activation of M_2 receptors during micturition may oppose inhibitory sympathetic activation of β-adrenoceptors, resulting in more efficient bladder emptying. Alterations in this modulatory action could result in bladder emptying dysfunction.

Radioligand binding and molecular studies have been unable to identify M_1 receptors in the urinary bladder. However, studies by Somogyi have shown that continuous stimulation of postganglionic cholinergic nerves in the rat urinary bladder leads to the activation of presynaptic facilitatory M_1 receptors which enhance acetylcholine release (Somogyi *et al.*, 1994). Under low stimulation conditions, M_2-inhibitory receptors have the major influence on transmitter release (Somogyi & de Groat, 1992). The contraction of the urinary bladder in response to electrical field stimulation is biphasic in nature, characterized by a rapid phasic component and a sustained tonic component. Although there are some species differences in the relative proportions of these phasic and tonic components, and they also are dependent to some extent on the frequency of stimulation, there is general agreement that the phasic portion, which is lost after desensitization of P_{2X}-purinoceptors, results primarily from ATP release (Burnstock *et al.*, 1978; Brading & Williams, 1990; Luheshi & Zar, 1990; Tammela *et al.*, 1994). The tonic portion is reduced by atropine treatment, indicating that it results from acetylcholine release (Longhurst *et al.*, 1984; Maggi *et al.*, 1985; Levin *et al.*, 1986).

Although there are many studies of the influence of atropine on the response of urinary bladder strips to field stimulation, we are not aware of any previous work using subtype-selective antagonists. The figures presented in this paper illustrates the effects of 4-DAMP methiodide (M_1/M_3-selective), *p*-F-HHSiD (M_3-selective), 4-DAMP mustard (M_3 irreversible), methoctramine (M_2-selective), and pirenzepine (M_1-selective) on both the phasic and tonic components of contraction. The responses after atropine (non-selective), HHSiD (M_3-selective), *P*-F-HHSiD (M_3-selective), 4-DAMP methiodide, and 4-DAMP mustard were very similar. The phasic response was less sensitive to the antagonists than the tonic component, and similar degrees of antagonist resistance were noted. The M_1 antagonist, pirenzepine, produced a quantitatively similar suppression of the response to field stimulation compared to the M_3 antagonists, probably due to blockade of M_1 receptor-mediated facilitation of acetylcholine release (Somogyi *et al.*, 1994). However, the M_2 antagonists, methoctramine, was relatively ineffective compared to the M_1 or M_3 antagonists at suppressing the response to field stimulation, even at concentrations which produced similar shifts in the EC_{50} values for (+)-*cis*-dioxolane. This lack of effect may be because of blockade of M_2 receptors which normally inhibit acetylcholine release (Somogyi & de Groat, 1992).

Our study demonstrates that the M_3 receptors, which constitute the minority of muscarinic receptors in the rat urinary bladder, mediate contraction in response to muscarinic stimulation. This heterogeneity of receptors with the minority M_3 receptor mediating contraction has been described in the guinea-pig bladder and other smooth muscles, but has not previously been demonstrated in the rat urinary bladder.

Note added in proof

A recent report by Wang *et al.*, (*J. Pharmacol. Exp. Ther.*, **273**, 959–966, 1995) similarly demonstrates that the functional muscarinic receptor in the rat bladder is the M_3 subtype.

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