ELSEVIER

European Journal of Pharmacology 281 (1995) 1–8

ejp

In vivo and in vitro effects of muscarinic receptor antagonists on contractions and release of [^{3}H]acetylcholine in the rabbit urinary bladder

Gunnar Tobin [a,*], Christer Sjögren [b]

[a] *Department of Pharmacology, Institute of Physiology and Pharmacology, University of Göteborg, Medicinaregatan 7, 413 90 Göteborg, Sweden*
[b] *Perstorp Pharma, Ideon, Lund, Sweden*

Received 31 October 1994; revised 31 March 1995; accepted 4 April 1995

Abstract

The functional effects of muscarinic receptor antagonists were examined in vivo and in vitro on the rabbit urinary bladder. Inhibitory effects on carbachol-evoked contractions of detrusor strips were pronounced for 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP; $-\log IC_{50}$: 8.64), *p*-fluoro-hexahydro-sila-diphenidol (pFHHSiD; 7.84) and atropine (8.27), while they were less pronounced for pirenzepine (6.62) and methoctramine (5.36). 4-DAMP and methoctramine increased ^{3}H overflow from [^{3}H]choline-labelled strips in response to electrical stimulation, contrary to pirenzepine, which decreased the overflow. Concomitant contractions were markedly reduced by 4-DAMP and by pirenzepine, but not by methoctramine. The $-\log IC_{50}$ estimations for atropine-sensitive electrically evoked contractions revealed methoctramine (4.85) to be less potent on nerve-evoked contractions than on carbachol-evoked contractions, in contrast to pirenzepine (7.15) and 4-DAMP (9.15). The effects of the antagonists in anaesthetized rabbits resembled those in vitro. Thus, muscarinic receptors in the rabbit urinary bladder are heterogeneous; prejunctional facilitatory (M_1) and inhibitory (M_2) for acetylcholine release, and postjunctional muscarinic M_3 receptors mediating contractile responses.

Keywords: Urinary bladder, rabbit; Muscarinic receptor subtype; Contractile response; Acetylcholine release; Muscarinic receptor antagonist

1. Introduction

The contractile response of the detrusor muscle during micturition is primarily mediated by acetylcholine acting on muscarinic receptors (Andersson and Sjögren, 1982). In vitro studies have shown that muscarinic receptor antagonists affect not only responses postjunctionally, but also the neuronal release of acetylcholine elicited by electrical field stimulation of detrusor muscle preparations (D'Agostino et al., 1989). The existence of muscarinic receptors located prejunctionally in the urinary bladder is in accordance with findings in other organs (Kilbinger, 1984).

On the basis of experiments studying the affinities of different antagonists, at least four different muscarinic receptor subtypes have been proposed: M_1, M_2, M_3 and M_4 receptors (Levine and Birdsall, 1993). In the urinary bladder several studies have shown the existence of binding sites for muscarinic 'M_3-selective' receptor antagonists (Batra, 1987; Nilvebrant and Sparf, 1988; Mutschler et al., 1989). Notably, it has been found that bladder tissue contains not only mRNA encoding the m3 receptor species, but also mRNA encoding the m2 species (Maeda et al., 1988). Also binding experiments have indicated that the muscarinic receptor population of this organ is not homogeneous (Monferini et al., 1988). Since the muscarinic M_3 receptor is quantitatively the most conspicuous subtype, it has been suggested to be the functional receptor responsible for the bladder contractile response to acetylcholine (Ladinsky et al., 1988). However, in vitro functional studies have revealed that cholinergic nerve

* Corresponding author. Tel. +31 773 38 35, fax +31 773 38 32, email Odopharm@odontologi.gu.se.

SSDI 0014-2999(95)00221-9

terminals in the urinary bladder may exhibit muscarinic M_1 and muscarinic M_2 receptors (Somogyi and De Groat, 1992). The functional significance of a heterogeneity of the muscarinic receptor population regarding detrusor contractile responses has not been explored. The aims of the present study were therefore to examine the effects of different 'selective' antagonists on functional responses, contractions and release of acetylcholine, and further, to compare the in vitro effects of the antagonists with their effects in vivo. For this purpose different antagonists were used; pirenzepine ('M_1-selective'; Hammer et al., 1980; Eglen and Whiting, 1988), methoctramine ('M_2-selective'; Melchiorre et al., 1987), gallamine ('M_2-selective'; Mitchelson, 1984), 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP; 'M_3-selective'; Barlow and Shepard, 1986; Doods et al., 1987) and *p*-fluoro-hexahydro-sila-diphenidol (pFHHSiD; 'M_3-selective'; Mutschler and Lambrecht, 1984; Lambrecht et al., 1989).

2. Materials and methods

2.1. Surgical and experimental procedures

In the study, 149 adult female rabbits of the Danish Land race were used (range of body weights: 2.5–3.7 kg). For the in vivo experiments, the rabbits were anaesthetized with sodium pentobarbitone injected through the posterior ear vein (40 mg/kg). During the experiments pentobarbitone was infused via the femoral vein for maintenance of anaesthesia (10 mg/kg/h), and body temperature was kept at 38° C by means of a thermostatically controlled blanket. Mean arterial blood pressure was monitored via a catheter inserted into the femoral artery. For pressure recordings in the urinary bladder, an open-ended, fluid-filled catheter was inserted in the bladder through the urethra. The urinary bladder was emptied of urine and filled with saline in such a volume that the basal pressure was about 5 mm Hg. Carbachol was given intravenously in two doses, 2.5 and 5 nmol/kg, first in the absence of antagonists and then in the presence. Muscarinic receptor antagonists were administered via the femoral vein in the doses of 0.25 and 1.25 μmol/kg at least 15 min prior to the injections of carbachol. At the end of the experiments the animals were killed with an overdose of pentobarbitone.

For the in vitro experiments, the animals were killed by cervical fracture and thereafter exsanguinated. The urinary bladder was removed from the animal, placed in Krebs bicarbonate solution and cut transversely at the level of the ureteral orifices. The upper part was centro-dorsally dissected free of vesicle mucosa, perivesicle fat and connective tissue. Then strip preparations measuring approximately $1 \times 3 \times 6$ mm (17.4 ± 1.0 mg; mean tissue weight) were cut in a coronal-caudal direction.

For examination of contractile responses, the strip preparations were mounted on two thin steel rods, one fixed and the other adjustable, in 10 ml organ baths, containing Krebs bicarbonate buffer, which was continuously gassed with 5% CO_2 in O_2 and kept at 38° C by a thermoregulated water circuit. The mechanical activity of the detrusor strip was recorded isometrically. The tension of the preparations was adjusted during an equilibration period of at least 45 min to a level of 7–8 mN. In the experiments, where carbachol was used as stimulus, a concentration of 10^{-5} M (E_{max}; concentration-response curves) or 10^{-6} M (EC_{70}; inhibitory effects on the EC_{70} of carbachol) was initially administered in order to provide a reference response. When performing concentration-response curves, carbachol was added cumulatively twice to each strip, first in the absence of a muscarinic receptor antagonist, and then in the presence of antagonist. The antagonist was added at just one concentration to each muscle strip, but at different concentrations to the muscle strips (3–4) from one bladder; these strips were then regarded as a group. In a series of control experiments ($n = 8$), a second cumulative administration of carbachol in the absence of muscarinic receptor antagonists resulted in a concentration-response curve which was not significantly different from the first. When a single concentration of carbachol (EC_{70}) was given repeatedly, with the successive addition of increasing concentrations of an antagonist, the tension of the strip was always allowed to reach baseline before the next administration of carbachol. Carbachol at EC_{70} was administered once at each concentration of the antagonist. Drugs were added to the organ baths in volumes of 100 μl.

For electrical field stimulation, the detrusor preparations were placed in the organ baths, equipped with two platinum electrodes. The stimulation was performed with supramaximal voltage (50–60 V), delivered as square wave pulses with a duration of 0.8 ms. Initially a frequency-response curve was performed in order to find EF_{70} (12 ± 1 Hz, $n = 52$). The EF_{70} was then given repeatedly every minute for 3 s in the absence and presence of different concentrations of the antagonists.

For examination of how electrical field stimulation-evoked overflow of ^3H was affected by the presence of different antagonists, the preparations were labelled with [^{3}H]choline (7 μCi/ml, 76 nM, Amersham Sweden). Before the labelling period of 45 min, the tissue preparations had been preincubated in Krebs bicarbonate buffer (1.5 ml, continuously gassed at 38° C) in 5 ml organ baths for 30 min. Following the labelling period the detrusor strips were mounted in electrode-equipped 2 ml organ baths. The baths were perifused with a Krebs solution containing 10 μM hemi-

cholinum-3 at a flow rate of 3 ml/min. The preparations were rinsed over a period of 60 min, and the muscle strips were allowed to reach a tension of 7–8 mN. Thereafter, fractions of 3 ml of the superfusate were collected over periods of 1 min. Electrical stimulation was performed at 10 Hz, supramaximal voltage and 0.8 ms. The square wave pulses were delivered for 1 min at 18 min intervals. Two initial stimulations were used to provide reference values for the amount of released substance. Antagonists were administered 15 min before the third stimulation. To each vial 10 ml of a scintillation liquid (OptiPhase II, LKB Scintillation Products, Sweden) was added and for counting a liquid scintillation spectrometer (Packard 2000 CA) was used.

2.2. Materials

The following drugs were used: atropine sulphate (Sigma, St. Louis, MO, USA), carbamylcholine chloride (carbachol; Sigma), 4-diphenylacetoxy-*N*-methylpiperidine methobromide (4-DAMP; a kind gift from Dr. Barlow, University of Bristol, UK), *p*-fluoro-hexahydro-sila-diphenidol hydrochloride (pFHHSiD; Research Biochemicals, Natick, MA, USA), gallamine triethiodide (Sigma), hemicholinium-3 (Sigma), methoctramine hydrochloride (Research Biochemicals), phentolamine methansulphate (Ciba-Geigy, Basel, Switzerland), pirenzepine dihydrochloride (Sigma), propranolol hydrochloride (ICI Pharmaceuticals, UK), tetrodotoxin (Sigma).

The Krebs bicarbonate buffer had the following composistion (mM): NaCl 118, KCl 4.6, $CaCl_2$ 1.25, KH_2PO_4 1.15, $MgSO_4$ 1.15, $NaHCO_3$ 25, glucose 5.5.

In the in vitro experiments the α-adrenoceptor blocker phentolamine and the β-adrenoceptor blocker propranolol were present at the concentration of 5×10^{-6} M, to exclude any adrenergic effects.

2.3. Calculations and statistics

Schild plots for competitive antagonism were constructed from the concentration-ratios (DR) of carbachol, obtained from at least three different antagonist concentrations [B], to estimate the pA_2 values (Arunklakshana and Schild, 1959). For each antagonist and set of conditions, log(DR − 1) was plotted against −log[B], and a least-squares linear regression analysis was performed. Statistical significance was determined by Student's *t*-test for unpaired data and for paired data. When multiple comparisons with the same variable were made a *t*-test according to the Bonferroni method was used (Wallenstein et al., 1980). *P* values of 0.05 or less were regarded as statistically significant. Values are presented in the form of means ± S.E.M.

3. Results

3.1. In vivo experiments

An intravenous injection of carbachol elicited a well-defined pressure increase in the bladder. The contractile response to carbachol 2.5 nmol/kg i.v. and 5 nmol/kg i.v. was 2.6 ± 0.6 mm Hg and 4.6 ± 0.7 mm Hg above basal pressure, respectively. The mean arterial blood pressure was before administration of carbachol 114 ± 2 mm Hg ($n = 34$). The intravenous injections of carbachol, at both doses, lowered the blood pressure markedly, and within 30 s the pressure fell to 60–80 mm Hg. However, recovery occurred rapidly, and after 60–90 s, the blood pressure had reached the original level.

The carbachol-induced bladder contractions were completely or almost completely abolished by 4-DAMP (0.25 and 1.25 μmol/kg reduced the carbachol-induced contractions by 85–100%; Fig. 1). Also the decrease in blood pressure concomitant with the carbachol injections was completely inhibited by the higher dose of 4-DAMP. Whereas pirenzepine reduced the carbachol-induced bladder contractions by only 10–35%, methoctramine had a more pronounced effect on the contractile responses (Fig. 1). In the presence of methoctramine 1.25 μmol/kg, the carbachol (5 nmol/kg)-evoked bladder contractions were reduced by 82 ± 8% ($n = 12$). Methoctramine had no effect on the fall in blood pressure following carbachol injections. In the presence of the higher dose of pirenzepine

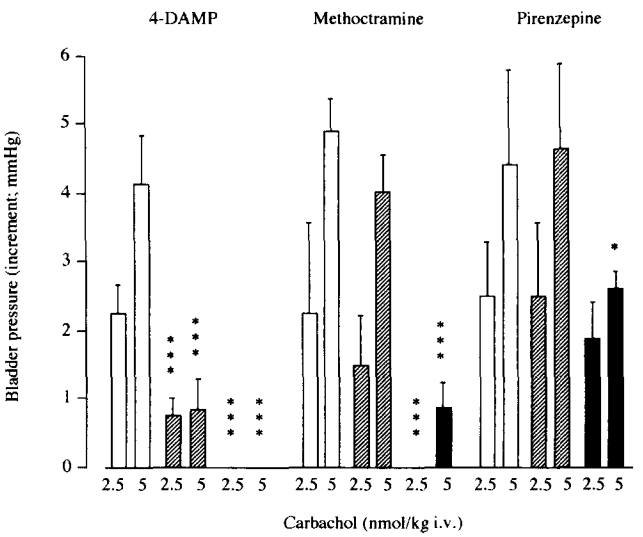

Fig. 1. Mean increases in bladder pressure in anaesthetized rabbits in response to carbachol i.v. in the absence of muscarinic antagonists (open columns) and in the presence of 4-DAMP ($n = 12$), methoctramine ($n = 12$) and pirenzepine ($n = 10$) at the doses of 0.25 μmol/kg (hatched columns) and 1.25 μmol/kg (black columns). Vertical bars represent S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

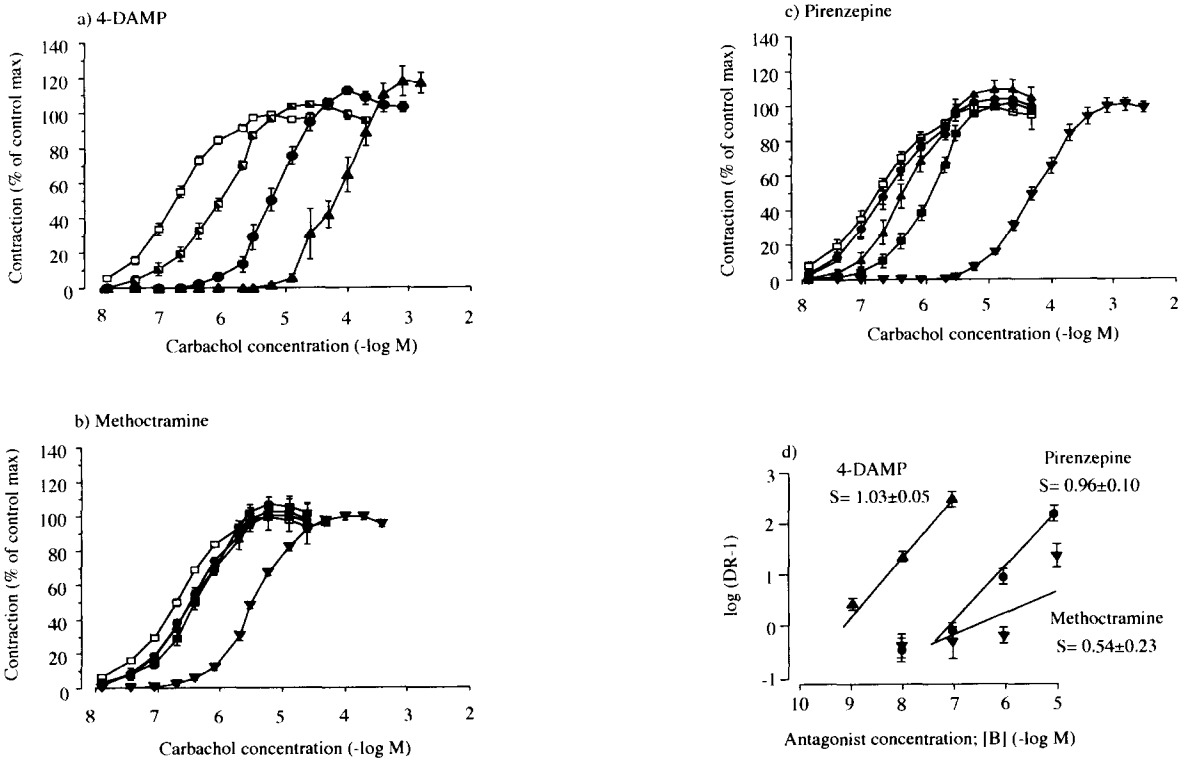

Fig. 2. (a–c) Mean contractile responses to carbachol of isolated detrusor strips in the absence of antagonists (open squares) and in the presence of (a) 4-DAMP ($n = 24$), (b) methoctramine ($n = 28$) and (c) pirenzepine ($n = 28$) at different concentrations (semi-solid squares: 10^{-9} M; solid circles: 10^{-8} M; solid triangles up: 10^{-7} M; solid squares: 10^{-6} M; solid triangles down: 10^{-5} M). Contractile responses are expressed as percentages of an initially evoked reference carbachol response (10^{-5} M). (d) Schild regressions for the antagonism in (a–c) (solid triangles up: 4-DAMP; solid triangles down: methoctramine; solid circles: pirenzepine) and slopes ± S.E.M. for the regression lines (S). Experiments performed in the presence of α- and β-adrenoceptor antagonist. Vertical bars represent S.E.M.

the decrease in blood pressure was reduced by 10–50% ($n = 10$). With respect to the mean arterial blood pressure and the basal tone of the bladder, neither of the muscarinic receptor antagonists caused any statistically significant change per se. However, methoctramine at the lower dose (0.25 μmol/kg) caused in some preparations an increase in the amplitude of the spontaneous basal activity. The increase vanished within 10 min.

3.2. *In vitro experiments; contractile responses*

The maximal tension developed by carbachol was 5.1 ± 0.4 mN/mg tissue ($n = 80$). The maximal response was elicited at a concentration of carbachol of 10^{-5} M and the EC_{50} value was 100 nM ($pD_2 = 7.0 \pm 0.04$; $-\log EC_{50}$). 4-DAMP, methoctramine and pirenzepine shifted the carbachol-induced concentration-response curves to the right (Fig. 2a–c). 4-DAMP showed the largest antimuscarinic effect on the carbachol-evoked concentration-response curves, which was reflected by the pD_2 values for carbachol. In the presence of 4-DAMP, methoctramine and pirenzepine (at the concentration of 10^{-7} M), the pD_2 values were 4.45 ± 0.01, 6.60 ± 0.17 and 6.52 ± 0.04, respectively. Schild analysis of the data was consistent with competitive antagonism for 4-DAMP and pirenzepine, since the slopes of the Schild plots were not significantly

Table 1
In vitro antimuscarinic effect on isolated detrusor strips expressed either as means of dissociation contants (pA_2) for carbachol-evoked concentration-response curves or as means of inhibitory constants of contractile responses evoked by carbachol at EC_{70} (Carb $-\log IC_{50}$) or by electrical field stimulation at EF_{70} (Nerve $-\log IC_{50}$; estimated for the atropine-sensitive part of the electrically evoked contractions

	pA_2	Carb $-\log IC_{50}$	Nerve $-\log IC_{50}$	Ratio (Carb IC_{50}/Nerve IC_{50})
4-DAMP	9.10 ± 0.09 ($n = 24$)	8.64 ± 0.12 ($n = 12$)	9.15 ± 0.10 ($n = 12$)	3.2
Methoctramine	6.74 ± 0.25 ($n = 28$)	5.36 ± 0.07 ($n = 12$)	4.85 ± 0.10 [a] ($n = 12$)	0.3
Pirenzepine	7.08 ± 0.06 ($n = 28$)	6.62 ± 0.05 ($n = 12$)	7.15 ± 0.15 ($n = 12$)	3.4
pFHHSiD		7.84 ± 0.14 ($n = 12$)		
Atropine		8.27 ± 0.08 ($n = 10$)	8.69 ± 0.12 ($n = 10$)	2.6

The ratios of Carb IC_{50}/Nerve IC_{50} were calculated from the antilog of the means. [a] Estimation from extrapolated maximum.

different from unity (Fig. 2d). However, the slope for methoctramine was different ($P < 0.05$). Notably, the pA_2 value for 4-DAMP was significantly different from those of pirenzepine and methoctramine ($P < 0.01$–0.001; pA_2, Table 1).

The inhibitory potencies of 4-DAMP and pFHHSiD were about the same as that of atropine, when the antagonists were tested on the contractions elicited by the EC_{70} concentration of carbachol, as judged by the $-\log IC_{50}$ values (carbachol $-\log IC_{50}$: 7.84 and 8.64 vs. 8.27, $P > 0.05$; Table 1). The $-\log IC_{50}$ values for pirenzepine and methoctramine showed that thay had significantly lower inhibitory potencies than atropine (carbachol $-\log IC_{50}$: 6.62 and 5.36 vs. 8.27, $P < 0.05$–0.01; Table 1). Whereas 4-DAMP and pFHHSiD reduced the carbachol (EC_{70})-evoked contractions already at concentrations of 5×10^{-10} M ($-16 \pm 3\%$; $P < 0.001$, $n = 12$) and 5×10^{-9} M ($-20 \pm 5\%$; $P < 0.01$, $n = 12$), respectively, pirenzepine had to be present at a concentration of 10^{-7} M ($-19 \pm 2\%$; $P < 0.001$, $n = 12$), and methoctramine at even higher concentrations to cause significant reductions ($-58 \pm 4\%$ at 5×10^{-6} M; $P < 0.001$, $n = 12$; Fig. 3a). Furthermore, gallamine did not cause any significant change at all; at 10^{-4} M of gallamine the carbachol-evoked response was $96 \pm 3\%$ ($n = 6$) of that in the absence of gallamine.

The maximal tension developed in response to electrical field stimulation was 4.3 ± 0.2 mN/mg tissue ($n = 52$), which was obtained at a stimulation frequency range of 20–40 Hz. The presence of the α- and β-adrenoceptor blockers reduced this response to the stimulation by $18 \pm 1\%$ ($n = 52$), and the presence of tetrodotoxin (2.25 μM) abolished the contractile response. The maximal reduction of the EF_{70} (12 ± 1 Hz)-evoked contractions, in the presence of atropine, amounted to $-50 \pm 3\%$ (10^{-7} M; $P < 0.001$, $n = 10$). Whereas the reductions in the presence of 4-DAMP were of the same magnitude, pirenzepine had to be present at a concentration of 10^{-6} M to cause a reduction of 50% (Fig. 3b). The effective threshold concentration of 4-DAMP was 5×10^{-10} M ($-14 \pm 5\%$; $P < 0.05$, $n = 12$), while that of pirenzepine was 10^{-8} M ($-8 \pm 2\%$; $P < 0.05$, $n = 12$). Notably, methoctramine caused only significant reductions at a concentration of 5×10^{-5} M ($-31 \pm 4\%$, $P < 0.05$, $n = 12$). Inhibitory constants for the antagonists were estimated with respect to the atropine-sensitive part of the EF_{70}-evoked contractions (nerve $-\log IC_{50}$; Table 1). While the ratio (carbachol IC_{50}/nerve IC_{50}) showed larger inhibitory potencies for pirenzepine, 4-DAMP and atropine on electrically evoked contractions than on carbachol-evoked contractions (ratio > 1), it showed lower potencies for methoctramine on the electrically than on the carbachol-evoked contractions (ratio < 1; Table 1). The differences between the IC_{50} values were significant with respect to pirenzepine, 4-DAMP and methoctramine ($P < 0.01$–0.001; Table 1).

3.3. *In vitro experiments; ^3H overflow and contractile responses*

Electrical field stimulation at 10 Hz induced an increase in outflow of tritium from the detrusor preparations preloaded with [^{3}H]choline, which successively decreased during repetition of the stimulations. In the control experiments ($n = 64$), the release of tritium amounted to 1350 ± 104, 1050 ± 30 and 777 ± 25 d.p.m

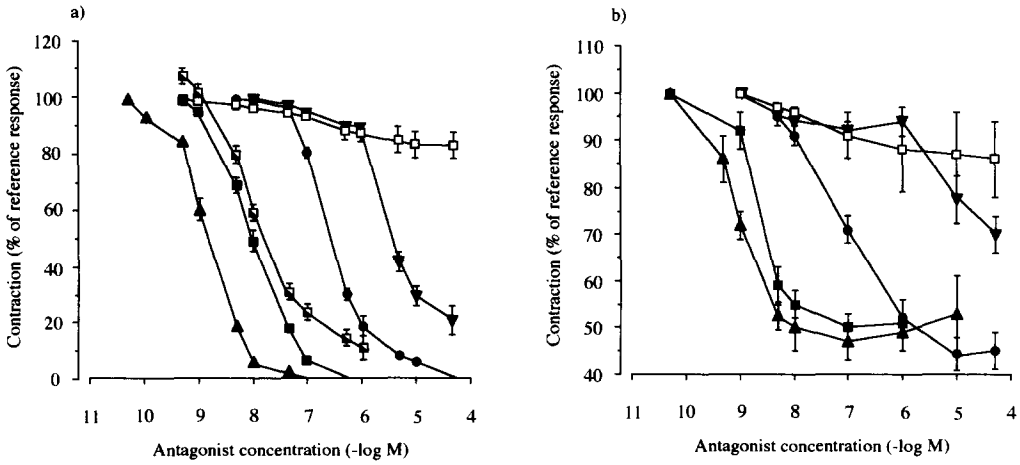

Fig. 3. Mean contractile responses (a) to carbachol at EC_{70} ($n = 10$–12) and (b) to electrical field stimulation at EF_{70} ($n = 10$–12) of isolated detrusor strips in the absence of muscarinic antagonists (open squares: addition of vehicle) and in the presence of 4-DAMP (solid triangles up), pFHHSiD (semi-solid squares in (a)), methoctramine (solid triangles down), pirenzepine (solid circles) and atropine (solid squares). Contractile responses are expressed as percentages of an initial reference contractile response (mean of two EC_{70} or five EF_{70} contractions). Vertical bars represent S.E.M. Experiments performed in the presence of α- and β-adrenoceptor antagonist.

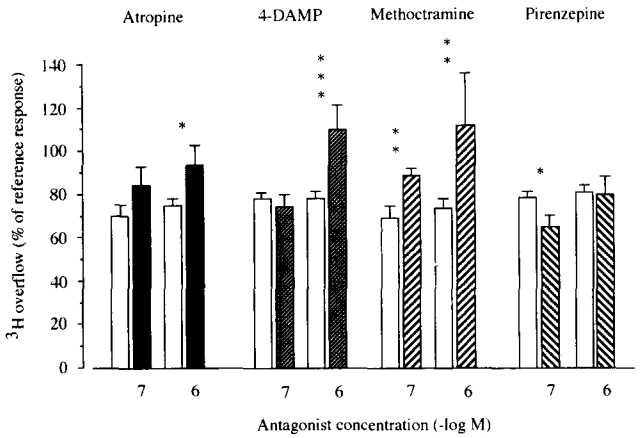

Fig. 4. Mean ^{3}H overflow from isolated detrusor strips in response to electrical field stimulation at 10 Hz. The overflow during a third stimulation period is expressed as a percentage of the overflow during the second stimulation period, in the absence of antagonists (open columns: controls) and in the presence of atropine (black columns), 4-DAMP (hatched columns), methoctramine (dark left oblique hatched columns) and pirenzepine (dark right oblique hatched columns). Each column represents the mean of eight observations and vertical bars S.E.M. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Control experiments were performed in parallel to each experiment in which antagonists were added. Experiments performed in the presence of α- and β-adrenoceptor antagonist.

during the first, second and third stimulation periods, respectively. The release during the third stimulation period was $74 \pm 2\%$ of that in response to the second stimulation. Inbetween the stimulation periods there was a spontaneous release of ^{3}H, which was in the range of 110–652 d.p.m. The addition of tetrodotoxin (2.25 μM) did not change the spontaneous release, but abolished the increase in response to electrical stimulation. No contractile response occurred in the presence of tetrodotoxin.

The presence of atropine, 4-DAMP or methoctramine at the concentration of 10^{-6} M during the third stimulation period significantly increased the ^{3}H overflow compared to that in control experiments (Fig. 4). Notably, methoctramine caused an increase also at 10^{-7} M. In the presence of pirenzepine at a concentration of 10^{-7} M, a significant decrease in ^{3}H overflow was observed. Neither of the antagonists caused any detectable change at concentrations lower than 10^{-7} M.

The concomitant contractile response to the electrical field stimulation was in controls decreased by $9 \pm 2\%$ during the third stimulation period in comparison with the second response. Since the stimulation frequency was close to the EF_{70} (10 Hz vs. 12 ± 1 Hz), the contractile responses in the presence of the antagonists (10^{-7}, 10^{-6} M) were almost similar to those previously described. So 4-DAMP and atropine decreased contractile force by 50–55% and pirenzepine by 25–45%, while methoctramine did not cause any significant change in contractile force. However, there was a tendency for an increase ($92 \pm 3\%$ and $91 \pm 2\%$ in controls vs. $97 \pm 5\%$ and $96 \pm 2\%$ in the presence of methoctramine at 10^{-7} and 10^{-6}, respectively, $P < 0.2$–0.1).

4. Discussion

The present study shows that the muscarinic receptor population of the rabbit urinary bladder is heterogeneous, located pre- and postjunctionally, and that different receptor subtypes mediate different functional responses. The activation of the muscarinic M_3 receptor subtype evoked detrusor muscle contraction, while the activation of the M_2 and M_1 subtypes modulated the release of acetylcholine. The prejunctional muscarinic M_2 and muscarinic M_1 receptor subtypes were inhibitory and facilitatory, respectively, for the release of the transmitter. Furthermore, the in vivo and the in vitro results of the study were similar.

The muscarinic M_3 receptor mediation of detrusor contractions is in accordance with previous reports describing binding sites, as well as blocking effects on contractile responses of muscarinic 'M_3-selective' receptor antagonists in the urinary bladder of several species (Batra, 1987; Nilvebrant and Sparf, 1988; Peterson et al., 1990; Monferini et al., 1988). In the binding studies, performed on urinary bladder tissue from rats, guinea pigs and rabbits, dissociation constants of 8.2–8.5 for 4-DAMP and of 6.2–6.6 for pirenzepine were reported. Generally, in tissues containing mostly muscarinic M_3 receptor subtypes, the pK_B values for 4-DAMP and pirenzepine have been estimated to 9.0 and 6.8, respectively (Eglen and Whiting, 1990). Thus, the pA_2 values for 4-DAMP (9.10) and pirenzepine (7.08) of this study, with respect to the contractile responses, correspond with muscarinic M_3, and not M_1, receptor subtypes mediating the cholinergic contraction of the urinary bladder. The Schild analysis of the data indicated the 4-DAMP and the pirenzepine antagonism to be competitive, whereas it indicated a non-competitive antagonism for methoctramine. However, competitive antagonists may behave noncompetitively at high concentrations (Melchiorre, 1988). Since the m2 receptor only weakly stimulates the formation of inositol-trisphosphate (Goyal, 1989), this subtype is unlikely to cause contractions when activated, which is in line with the low inhibitory effects of methoctramine and gallamine in the present study. For further comparisons, examinations were also made of the inhibitory effects of pFHHSiD, which shows a somewhat

larger selectivity ratio for muscarinic M_3 over muscarinic M_1 receptor subtypes than 4-DAMP (Dörje et al., 1991), and of the unselective antagonist atropine. Overall, the muscarinic 'M_3-selective' receptor antagonists and atropine showed, compared to the 'M_1-' and the 'M_2-selective' antagonists, large inhibitory effects on contractions evoked both by the cholinomimetic agent and by electrical field stimulation. Importantly, the atropine-resistant part of the parasympathetically nerve-evoked detrusor contractions is in the rabbit about 50% (Husted et al., 1980), as we also found. Since the electrical stimulation in this study was performed in the presence of adrenoceptor blockers, the inhibition caused by 4-DAMP (and atropine) and pirenzepine is likely to have been a complete blockade of the cholinergic part of the detrusor contractile response to parasympathetic stimulation.

The origin of the overflow of ^3H in response to electrical field stimulation of rat bladder tissue labelled with [^3H]choline has been discussed thoroughly by Somogyi et al. (1994), and in this tissue they could exclude a non-neuronal source for the release. The finding in the rabbit bladder that some muscarinic receptor antagonists increased, and not decreased, the efflux of ^3H (in the presence of adrenoceptor blockers) makes it likely that the overflow originated from nerve terminals. Furthermore, the electrical field stimulation seemed to have activated nerves rather than the smooth muscle cells directly, since tetrodotoxin abolished both the release and the contractile response.

Both muscarinic 'M_2-' and muscarinic 'M_3-selective' receptor antagonists, as well as atropine, significantly increased the efflux of ^3H in response to the electrical stimulation. The muscarinic M_1 receptor antagonist pirenzepine, on the other hand, caused a decrease in the efflux. These findings, and notably atropine causing a smaller increase than methoctramine, speak in favour of the existence of both facilitatory and inhibitory muscarinic autoreceptors for the release of acetylcholine in the rabbit bladder. Although the presence of 4-DAMP could cause an increase in the ^3H overflow, the effect of 4-DAMP was comparable to, or at least not larger than, that of methoctramine. Since this is in accordance with the relative inhibitory potencies of the two antagonist found on prejunctional muscarinic M_2 receptors in the guinea-pig trachea (Kilbinger et al., 1991), the 4-DAMP effect is likely to have been exerted on muscarinic M_2 receptors. Furthermore, the different IC_{50} values, based on carbachol and on nerve (electrically)-evoked contractile responses, also support the existence of facilitatory and inhibitory autoreceptors for acetylcholine release. The ratio between the two kinds of IC_{50} values indicated that pirenzepine and 4-DAMP had greater, and methoctramine lower, inhibiting potency on an endogenous cholinergic response than on a carbachol-evoked response. The explanation for this may be a blockade of prejunctional receptors simultaneously with the blockade of postjunctional receptors, with respect to the nerve-evoked response. Considering the fact that 4-DAMP has almost as high an affinity for muscarinic M_1 as for muscarinic M_3 receptors (and $M_1 > M_2$; Dörje et al., 1991), the effects of simultaneous blockade by the different antagonists are in agreement with prejunctional muscarinic M_1 and muscarinic M_2 receptor subtypes facilitating and inhibiting the release of acetylcholine, respectively. In the rat urinary bladder, it has been reported that cholinergic nerve terminals exhibit muscarinic M_1 facilitatory and muscarinic M_2 inhibitory receptors and that these receptors are activated to varying degrees depending on the frequency and pattern of the stimulation (D'Agostino et al., 1986; Somogyi and De Groat, 1992; Somogyi et al., 1994). With respect to the comparison of the IC_{50} values and the ^3H overflow, the results for 4-DAMP were ambiguous in the present study. The effect of 4-DAMP on the IC_{50} values was similar to that of pirenzepine, whereas the effect on ^3H overflow resembled that of methoctramine. However, the pirenzepine-like effect occurred at a lower concentration range of 4-DAMP than the methoctramine-like effect did, which is in line with 4-DAMP preferentially binding to muscarinic M_1 rather than muscarinic M_2 receptors. Furthermore, it has been observed that, in the rat urinary bladder, the release of acetylcholine per volley decreases during longer periods of stimulation (for 10 s and longer at 10 Hz; Somogyi et al., 1994). Therefore, muscarinic M_2 receptor blockade would under the present experimental circumstances have an advantage over muscarinic M_1 receptor blockade (i.e. the stimulations for 1-min periods at 10 Hz may have caused a low release of acetylcholine).

The muscarinic 'M_3-selective' receptor antagonist 4-DAMP was also in vivo the most potent inhibitor of bladder contractions and at the lower dose tested (0.25 μmol/kg), 4-DAMP inhibited carbachol-evoked contractions in contrast to pirenzepine and methoctramine. The inhibitory effects of pirenzepine and methoctramine at the higher dose are likely to have been exerted on receptors other than muscarinic M_1 and muscarinic M_2 receptors, respectively, since the in vitro findings showed that the postjunctional response to carbachol was exerted via muscarinic M_3 receptors. However, with respect to methoctramine and pirenzepine there were discrepancies compared to the in vitro findings. In vivo methoctramine showed a larger inhibitory effect than pirenzepine. In contrast to the in vitro experiments, no adrenoceptor blockers were present in the in vivo experiments. Since methoctramine has been reported to act, at certain dose levels, as a β-adrenoceptor agonist (Eglen et al., 1988), effects via adrenoceptors may have occurred.

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council (project no 14X-05927). We acknowledge the competent technical assistance of Mrs. Gull-Britt Ask, Mrs. Lillemor Halvarsson and Mrs. Ingrid Persson.

References

Andersson, K.-E. and C. Sjögren, 1982, Aspects on the physiology and pharmacology of the bladder and urethra, Prog. Neurobiol. 19, 71.

Arunklakshana, O. and H.O. Schild, 1959, Some quantitative uses of drug antagonists, Br. J. Pharmacol. 14, 48.

Barlow, R.B. and M.K. Shepard, 1986, A further search for selective antagonists at M_2-muscarinic receptors, Br. J. Pharmacol. 89, 837.

Batra, S., 1987, Comparison of muscarinic acetylcholine binding in the urinary bladder and submandibular gland of the rabbit, Eur. J. Pharmacol. 138, 83.

D'Agostino, G., H. Kilbinger, C.M. Chiari and E. Grana, 1986, Presynaptic inhibitory muscarinic receptors modulating ^{3}H-Ach release in the rat urinary bladder, J. Pharmacol. Exp. Ther. 239, 522.

D'Agostino, G., M.C. Chiari and E. Grana, 1989, Prejunctional effects of muscarinic agonists on ^{3}H-acetylcholine release in the rat urinary strip, Naunyn-Schmied. Arch. Pharmacol. 340, 76.

Doods, H.N., M.-J. Mathy, D. Davidesko, K.J. Van Charldorp, A. De Jonge and P.A. Van Zwieten, 1987, Selectivity of muscarinic antagonists in radioligand and in vivo experiments for the putative M_1, M_2 and M_3 receptors, J. Pharmacol. Exp. Ther. 242, 257.

Dörje, F., J. Wess, G. Lambrecht, R. Tacke, E. Mutschler and M.R. Brann, 1991, Antagonist binding profiles of five cloned human muscarinic receptor subtypes, J. Pharmacol. Exp. Ther. 256, 727.

Eglen, R.M. and R.L. Whiting, 1988, Comparison of the guinea-pig oesophageal muscularis mucosae and trachea in vitro, J. Auton. Pharmacol. 8, 181.

Eglen, R.M. and R.L. Whiting, 1990, Heterogeneity of vascular muscarinic receptors, J. Auton. Pharmacol. 19, 233.

Eglen, R.M., W.W. Montgomery, I.A. Dainty, L.K. Dubuque and R.L. Whiting, 1988, The interaction of methoctramine and himbacine at atrial, smooth muscle and endothelial muscarinic receptors in vivo, Br. J. Pharmacol. 95, 1031.

Goyal, R.K., 1989, Muscarinic receptor subtypes. Physiological and clinical implications, New Engl. J. Med. 321, 1022.

Hammer, R., C.P. Berric, N.J.M. Birdsall, A.S.V. Burgen and E.C. Hulme, 1980, Pirenzepine distinguishes between different subclasses of muscarinic receptors, Nature 283, 90.

Husted, S., C. Sjögren and K.-E. Andersson, 1980, Role of prostaglandins in the response of rabbit detrusor to non-cholinergic, non-adrenergic nerve stimulation and to ATP, Arch. Int. Pharmacodyn. Ther. 246, 84.

Kilbinger, H., 1984, Presynaptic muscarinic receptors modulating acetylcholine release, Trends Pharmacol. Sci. 5, 103.

Kilbinger, H., R. Schneider, H. Siefken, D. Wolf and G. D'Agostino, 1991, Characterization of prejunctional muscarinic autoreceptors in the guinea-pig trachea, Br. J. Pharmacol. 103, 1757.

Ladinsky, H., E. Giraldo, E. Monferini, G.B. Schiavi, M.A. Vigano, L. De Conti, R. Micheletti and R. Hammer, 1988, Muscarinic receptor heterogenity in smooth muscle muscle: binding and functional studies with AF-DX 116, Trends Pharmacol. Sci. 9 (Suppl.: Subtypes of muscarinic receptors III), 44.

Lambrecht, G., R. Feifel, U. Moser, M. Wagner-Röder, L.K. Choo, J. Camus, M. Tastenoy, M. Waelbroeck, C. Strohmann, R. Tache, J.F. Rodriges de Miranda, J. Cristophe and E. Mutschler, 1989, Pharmacology of hexahydro-difenidol, hexahydro-sila-difenidol and related selective muscarinic antagonists, Trends Pharmacol. Sci. 10 (Suppl.: Subtypes of muscarinic receptors IV), 60.

Levine, R.R. and N.J.M. Birdsall, 1993, Subtypes of muscarinic receptors V, Life Sci. 52, 405.

Maeda, A., T. Kubo, M. Mishina and S. Numa, 1988, Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes, FEBS Lett. 239, 339.

Melchiorre, C., 1988, Polymethylene tetramines: a new generation of selective muscarinic antagonists, Trends Pharmacol. Sci. 9, 216.

Melchiorre, C., A. Cassinella and N. Quaglia, 1987, Differential blockade of muscarinic receptor subtypes by polymethylene tetramines. Novel class of selective antagonists of cardiac M_2 muscarinic receptors, J. Med. Chem. 30, 201.

Mitchelson, F., 1984, Heterogeneity in muscarinic receptors: evidence from pharmacological studies with antagonists, Trends Pharmacol. Sci. 5 (Suppl.: Subtypes of muscarinic receptors II), 12.

Monferini, E., E. Giraldo and H. Ladinsky, 1988, Characterization of the muscarinic receptor subtypes in the rat urinary bladder, Eur. J. Pharmacol. 147, 453.

Mutschler, E. and G. Lambrecht, 1984, Selective muscarinic agonists and antagonists in functional tests, Trends Pharmacol. Sci. 5 (Suppl.: Subtypes of muscarinic receptors II), 39.

Mutschler, E., U. Moser, J. Wess and G. Lambrecht, 1989, Muscarinic receptors subtypes: agonists and antagonists, Prog. Pharmacol. Clin. Pharmacol. 7, 13.

Nilvebrant, L. and B. Sparf, 1988, Receptor binding profiles of some selective muscarinic antagonists, Eur. J. Pharmacol. 151, 83.

Peterson, J.S., A.J. Patton and L. Noronha-Blob, 1990, Mini-pig urinary bladder function: comparisons of in vitro anticholinergic responses and in vivo cystometry with drugs indicated for urinary incontinence, J. Auton. Pharmacol. 10, 65.

Somogyi, G.T. and W.C. De Groat, 1992, Evidence for inhibitory and facilitatory muscarinic receptors on cholinergic nerve terminals of that rat urinary bladder, J. Auton. Nerv. Syst. 37, 89.

Somogyi, G.T., M. Tanowitz and W.C. De Groat, 1994, M1 muscarinic receptor-mediated facilitation of acetylcholine release in the rat urinary bladder, J. Physiol. 480, 81.

Wallenstein, S., C.L. Zucker and J.L. Fleiss, 1980, Some statistical methods useful in circulation research, Circ. Res. 47, 1.