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Prejunctional muscarinic inhibitory control of acetylcholine release in the human isolated detrusor: involvement of the M_4 receptor subtype

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1 Experiments were carried out in human detrusor strips to characterize muscarinic receptor subtypes involved in the prejunctional regulation of acetylcholine (ACh) release from cholinergic nerve terminals, and in the postjunctional smooth muscle contractile response.

2 In detrusor strips preincubated with [^{3}H]-choline, electrical field stimulation (600 pulses) delivered in six trains at 10 Hz produced a tritium outflow and a contractile response. In the presence of 10 μM paraoxon (to prevent ACh degradation) the tritium outflow was characterized by HPLC analysis as [^{3}H]-ACh (76%) and [^{3}H]-choline (24%).

3 Electrically-evoked [^{3}H]-ACh release was abolished by tetrodotoxin (TTX: 300 nM) and unaffected by hexamethonium (10 μM), indicating a postganglionic event. It was reduced by physostigmine (100 nM) and the muscarinic receptor agonist, muscarone (10 nM – 1 μM), and enhanced by atropine (0.1 – 100 nM). These findings indicate the presence of a muscarinic negative feedback mechanism controlling ACh release.

4 The effects of various subtype-preferring muscarinic receptor antagonists were evaluated on [^{3}H]-ACh release and muscle contraction. The rank potency ($-\log EC_{50}$) orders at pre- and postjunctional level were: atropine $\geqslant$4-diphenyl-acetoxy-N-piperidine (4-DAMP) > mamba toxin 3 (MT-3) > tripitramine > *para*-fluorohexahydrosiladiphenidol (pF-HHSiD) $\geqslant$ methoctramine $\geqslant$ pirenzepine > tripinamide, and atropine $\geqslant$ 4-DAMP > pF-HHSiD >> pirenzepine = tripitramine > tripinamide > methoctramine >> MT-3, respectively.

5 The comparison of pre- and post-junctional potencies and the relationship analysis with the affinity constants at human cloned muscarinic receptor subtypes indicates that the muscarinic autoreceptor inhibiting ACh release in human detrusor is an M_4 receptor, while the receptor involved in muscular contraction belongs to the M_3 subtype.

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Abbreviations: ACh, acetylcholine; CHO cells, Chinese hamster ovary cells; 95% CL, confidence limits at 95% probability; 4-DAMP, 4-diphenyl-acetoxy-N-piperidine; HPLC, high pressure liquid chromatography; MT-3, mamba toxin 3; pF-HHSiD, *para*-fluorohexahydrosiladiphenidol; TTX, tetrodotoxin

Introduction

It is widely accepted that in most mammalian species, neurogenic contractions of the detrusor depend on the concomitant release of acetylcholine (ACh) and adenosine triphosphate (ATP) from postganglionic parasympathetic fibres (Andersson, 1993), where the two transmitters are thought to be co-stored (Brading, 1993). Conversely, the excitatory transmission in the human isolated detrusor is primarily due to ACh release (Sibley, 1984; Palea *et al*., 1995; Tagliani *et al*., 1997), with a minor ATP-mediated component generally observed in tissues from patients with neurogenic bladder or interstitial cystitis (Ruggieri *et al*., 1990; Palea *et al*., 1993). In support to this evidence, atropine and other antimuscarinics are known to markedly reduce bladder contractility either in healthy subjects (Cullumbine *et al*., 1955) or patients with an unstable bladder (Turner & Brading, 1997).

In rodents, the detrusor contraction in response to released or applied ACh is mediated by a postjunctional muscarinic receptor, which has been recognized as an M_3 receptor (D'Agostino *et al*., 1993; Longhurst *et al*., 1995; Alberts, 1995), whereas in the human detrusor such a receptor remains to be characterised in detail (Poli *et al*., 1992; Newgreen & Naylor, 1996).

In addition to activate postjunctional muscarinic receptors, ACh controls its own release *via* muscarinic autoreceptors located at a prejunctional site. In rodents, the latter sites have been classified as M_1 excitatory (Somogyi *et al*., 1994) and M_4 inhibitory autoreceptors (Alberts, 1995; D'Agostino *et al*., 1997a). To our knowledge, functional studies to ascertain the presence of presynaptic muscarinic autoreceptor(s) in the human detrusor are still lacking, even though biochemical and pharmacological evidence suggests the presence of an heterogeneous population of muscarinic receptor subtypes in the human bladder (Wang *et al*., 1995; Kondo *et al*., 1995; Yamaguchi *et al*., 1996).

The present study was designed to characterize with the use of various subtype-preferring muscarinic receptor antagonists the muscarinic autoreceptor(s) regulating ACh release, and postjunctional receptors mediating smooth muscle contraction

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in the human isolated detrusor. The presence of a feedback mechanism was investigated by measuring the outflow of [^{3}H]-ACh after labelling ACh stores with [^{3}H]-choline.

A preliminary report of this work has already been published (D'Agostino *et al*., 1997b).

Methods

Specimens from the anterior part of the bladder dome were obtained from 76 male patients (age: 70 ± 2.1 years; range 45–85 years) undergoing total cystectomy due to bladder base malignancy. It was previously shown that the density of muscarinic receptors and the affinity of muscarinic antagonists were not influenced by age, sex and radiotherapy (Nilvebrant *et al*., 1997). The use of human tissue was approved by the Ethics Committee of the University of Pavia.

Tissue preparation

The specimens were transported to the laboratory in oxygenated Krebs solution cooled at 5–6°C and removed of serosal and mucosal layers. Detrusor strips (20 mm long, 4 mm wide), with a good alignment of muscle bundles, were prepared and stored overnight at 4°C. Each preparation was used for distinct protocols.

Postjunctional affinities of muscarinic receptor antagonists

Detrusor strips were suspended isometrically in a 10 ml organ bath containing Krebs solution at 37°C, and bubbled with 95% O_2 + 5% CO_2. The tissues were allowed to equilibrate for 60 min under a tension of 20 mN. The preparations were then primed with a submaximal concentration of muscarone (1 μM), a potent muscarinic receptor agonist in the urinary bladder (D'Agostino *et al*., 1997a). After a washout of 30 min, three cumulative concentration-response curves to muscarone were carried out 60 min spaced by increasing the agonist concentration by 0.5 logarithmic units. The last two curves were generally superimposable. At the end of the first concentration-response curve, the preparation was washed at 12 min intervals for 60 min, during which time the tension returned to baseline level. After the second curve was allowed, the preparation was exposed for 30–45 min to a given antagonist, and a third curve to muscarone was constructed. Only one concentration of antagonist was tested in each preparation.

Labelling and [^{3}H]-outflow experiments

Neuronal ACh stores were labelled according to a procedure described previously, with minor modifications (D'Agostino *et al*., 1997a). Briefly, detrusor strips were incubated for 45 min with [^{3}H]-choline (74 Kbequerel ml^{-1}) in 2 ml organ bath and stimulated by means of 10 s pulse trains at 10 Hz (0.2 ms duration, 60 V cm^{-1}, 60 s apart). At the end of the labelling period, the preparation was washed out for 120 min by superfusion at a constant rate of 2 ml min^{-1} (Gilson Minipulse 2HP8 flow inducer). Hemicholinium-3 (10 μM) was present in the washout solution throughout the experiment. Starting at the 121st min (zero time), superfusion fluid was collected continuously in 3 min periods (6 ml samples). The strip was stimulated two times (S_1 and S_2) beginning 9 (S_1) and 69 (S_2) min after zero time. The release was evoked by 600 square wave pulses delivered in six trains at 10 Hz (1 ms duration, 15 V cm^{-1}, 30 s apart). Aliquots (1 ml) of the superfusate were added to 5 ml of Ultima Gold (Packard) and the tritium content was measured by liquid scintillation spectrometry (Packard 1900). Quench correction curves were established and external standardization was used for counting efficiency. Both resting and stimulation-evoked outflow of radioactivity was expressed as disintegration s^{-1} (bequerel: Bq) per g of tissue (Bq g^{-1}). The increase in the release caused by stimulation was obtained from the difference between the total [^{3}H]-outflow during 3 min stimulation plus the following 12 min (stimulation outflow period) and the calculated spontaneous outflow. The decline of the spontaneous outflow was calculated by fitting a linear regression line to the values (expressed in Bq g^{-1}) of three 3 min samples before and after the stimulation outflow period.

Pre- and postjunctional experimental protocols

Muscarinic ligands were added 9 min (agonist) or 30–45 min (antagonist) before the onset of S_2. The prejunctional effect of a compound was expressed as the ratio (S_2/S_1) of the stimulation-induced [^{3}H]-outflow. Electrically-evoked contractions of detrusor strips (postjunctional effects) were recorded isometrically (tension: 20 mN) and displayed on a poligraph. The sum of peak tensions developed by pulse train during S_1 was calculated and compared to that obtained during S_2. This procedure allowed to concomitantly estimate both pre- and postjunctional effects in each experiment.

Concentration-response curve for pre- and postjunctional effects were constructed by expressing the S_2/S_1 ratio in the presence of a given concentration of muscarinic receptor antagonist as a percentage of the equivalent ratio obtained in the absence of the antagonist (control experiment).

Separation of labelled choline and acetylcholine

Radioactive compounds (choline and ACh) were separated either from the incubation medium or from the tissue according to the method described by Wessler & Werhand (1990). Experiments were carried out in the presence of 10 μM paraoxon to irreversibly inhibit tissue acetylcholinesterase activity, and thus ACh degradation. In addition, a high concentration (1 μM) of 4-dyphenyl-acetoxy-N-piperidine (4-DAMP) was added to the medium to prevent inhibitory feedback mechanisms by blocking muscarinic autoreceptors. Tissues, which were frozen in liquid nitrogen after the labelling and washout period, were dissolved in 1 ml ice-cold 0.4 M perchloric acid and the resultant homogenate was centrifuged (20,000 × *g*, 10 min). Aliquots of the supernatant (20 μl) or the superfusate (200 μl) were analysed by HPLC (Shimadzu LC-9A; reverse phase C18 column Shiseido SG128, 250 × 4.6 mm). Phosphate buffer (0.1 M, pH 4.7), containing methanol (6% vol) and tetramethylammonium chloride (0.9 mM), was used. The flow was 0.4 ml min^{-1} and the effluent was collected in 30 s fractions.

For the estimation of the retention times, standards of [methyl-^{3}H]-choline and [acethyl-^{14}C]-ACh were injected. In the same individual samples, [methyl-^{14}C]-choline was also used as internal standard. Radioactivity was measured by a Packard 1900 *beta*-counter. External standardization was used to correct counting efficiency.

Solutions and drugs

The composition of modified Krebs solution was (mM): NaCl 120, KCl 4.7, $MgSO_4$ 0.6, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.0, glucose 10. The following compounds were purchased:

[methyl-^{3}H]-choline chloride (78 Ci mmol^{-1}; 2.89 TB g mmol^{-1}), [methyl-^{14}C]-choline chloride and [1-^{14}C]-acetylcholine chloride (58.3 Ci mmol^{-1}; 1.99 GB g mmol^{-1}) from Amersham (Arlington Heights, IL, U.S.A.); tetramethylammonium chloride, hemicholinium-3, hexamethonium chloride, physostigmine hemisulphate, tetrodotoxin, atropine sulphate, pirenzepine dihydrochloride monohydrate, diethyl-para-nitrophenyl phosphate (paraoxon) from Sigma Chemical Company (St. Louis, MO, U.S.A.); para-fluorohexahydrosila-diphenidol hydrochloride (pF-HHSiD), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), from Research Biochemicals International (Natick, MA, U.S.A.); the mamba toxin, MT-3, from Alomone Labs (Jerusalem, Israel). Methoctramine hydrochloride, tripitramine sesquifumarate and tripinamide dihydrochloride were synthesized by Prof C. Melchiorre. Racemic muscarone was a gift of Prof C. De Micheli (University of Milan, Italy).

Data analysis

Drug potency estimates were evaluated as $-\log EC_{50}$ values (negative log of the molar concentration producing half-maximal effect) by nonlinear curve fitting (GraphPAD Prism, Version 1.3, GraphPAD Software, San Diego, CA, U.S.A.). Values from individual experiments were averaged, and the s.e.mean or confidence limits (CL) at 95% probability were calculated. Antagonist affinity estimates (pA_2 values) were calculated following Schild regression analysis (Arunlakshana & Schild, 1959) using ($\pm$) muscarone concentration ratios (CR) determined at EC_{50} levels in control and test curves. CL at 95% probability for the slope of the regression were evaluated by using a computer programme based on a manual for pharmacological calculations (PHARM/PCS, Version 4.1; Tallarida & Murray, 1986). The classification of prejunctional and postjunctional muscarinic receptor subtypes was carried out by comparing the potency or affinity estimates for antagonists obtained in the bladder with the constants (pK_i) present in the literature for the same compounds for cloned muscarinic receptors (correlation analysis) (Kenakin, 1993). Original antagonist pK_i values (single values) are derived from the following studies (atropine: Bolden *et al*., 1992; 4-DAMP, pF-HHSiD, pirenzepine and methoctramine: Dorje *et al*., 1991; tripitramine: Maggio *et al*., 1994; MT-3: Jolkkonen *et al*., 1994; tripinamide: Melchiorre, unpublished data). Data sets were tested for significance using Student's *t*-test for unpaired data. The level of significance was set at $P<0.05$.

Results

Postjunctional affinities of muscarinic receptor antagonists vs muscarone

Muscarone caused concentration-dependent contractions with a $-\log EC_{50}$ of 7.33 ± 0.056 ($n=94$). Parallel shifts of the concentration-response curves to muscarone, without depression of the maximum response, were obtained with all the muscarinic receptor antagonists (atropine: 1–100 nM, $n=4$–5; 4-DAMP: 3–100 nM, $n=3$–6; pF-HHSiD: 10 nM–1 μM, $n=5$; tripitramine: 100 nM–1 μM, $n=3$–6; pirenzepine: 300 nM–3 μM, $n=3$–5; tripinamide: 100 nM–10 μM, $n=3$–5; methoctramine: 1–10 μM, $n=3$–5). Schild regression analysis was linear with a slope not significantly different from unity (Table 1). The rank order of antagonist affinities (pA_2 values) was: atropine $\geq$ 4-DAMP > pF-HHSiD > tripitramine > pirenzepine > tripinamide > methoctramine > (Table 1).

[^{3}H]-ACh synthesis, [^{3}H]-outflow and smooth muscle contraction

As observed in other peripheral neuromuscular preparations (Starke *et al*., 1989), in human detrusor strips incubated with tritiated choline, the electrical stimulation induced the formation and neuronal storage of newly-synthetized [^{3}H]-ACh (D'Agostino *et al*., 1997b). After a 120 min washout period tissues were homogenized and the homogenate was assayed for [^{3}H]-ACh, [^{3}H]-choline and [^{3}H]-content by HPLC. Total radioactivity accounted for $525{,}225\pm23{,}245$ Bq g^{-1} ($n=8$), of which 15.7% and 76% were recognized as [^{3}H]-ACh and [^{3}H] -choline, respectively. The sum of labelled choline and ACh accounted for 91.9% of the total [^{3}H]-activity in the tissue.

Electrical stimulation applied in six trains (600 pulses) at 10 Hz (S_1) induced contractile responses (1586 ± 109 mN g^{-1}) and increased [^{3}H]-outflow (4765 ± 808 Bq g^{-1} *vs* a basal outflow of 485 ± 94 Bq g^{-1}, $n=13$). An example of the experiment is shown in Figure 1a,b. A second stimulation (S_2) delivered 60 min after S_1 produced a contractile response, which was superimposable to that caused by S_1. By contrast, a decay in [^{3}H]-outflow was observed (S_2/S_1 ratio: 0.706 ± 0.002, $n=13$) due to a partial depletion of stored [^{3}H]-ACh. To identify the nature of the radioactivity present in the superfusate, labelled choline and ACh were determined in the presence of 10 μM paraoxon and 1 μM 4-DAMP in pooled samples collected during the resting (15 min) and the stimulation period. The resting [^{3}H]-outflow accounted mainly for [^{3}H]-choline (97 ± 3.2% of total [^{3}H]-activity), whereas the electrical stimulation caused an [^{3}H]-outflow that consisted of 75.7 ± 3.8% of [^{3}H]-ACh and 24.3 ± 1.8% [^{3}H]-choline ($n=7$).

Pre- and postjunctional effects of drug treatment

The electrically-evoked [^{3}H]-outflow and smooth muscle contraction were studied in the absence and in the presence of 300 nM tetrodoxotin (TTX). This treatment prevented the responses induced by S_2 ($n=4$, data not shown), indicating that the electrically-evoked [^{3}H]-outflow (and the resultant muscle contraction) is a nerve-mediated event. The ganglionic blocking agent hexamethonium (10 μM) did not affect either the [^{3}H]-ACh release or the contraction induced by S_2 ($n=4$, data not shown). Therefore, under our experimental conditions, the release of [^{3}H]-ACh has to be considered as postganglionic. In the presence of the acetylcholinesterase inhibitor, physostigmine (100 nM), the S_2-evoked release decreased by 30.4 ± 5.0% ($n=4$) (Figure 1d). Conversely, the resultant contractile response was increased by about four

Table 1 Postjunctional affinity values of muscarinic antagonists at receptors mediating contraction of human detrusor strips in response to muscarone

Antagonist	*pA_2*	*95% CL*	*Slope*	*95% CL*
Atropine	9.21	8.96–9.46	0.98	0.82–1.13
4-DAMP	9.02	8.68–9.36	0.99	0.74–1.24
pF-HHSiD	7.68	7.53–7.84	1.03	0.76–1.31
Tripitramine	7.24	7.07–7.40	0.91	0.73–1.08
Pirenzepine	6.90	6.68–7.11	1.00	0.85–1.14
Tripinamide	6.60	6.41–6.78	0.91	0.77–1.05
Methoctramine	6.22	6.08–6.35	0.99	0.87–1.11

Values were determined by Schild regression analysis, as described in Methods. 95% CL (confidence limits at 95%).

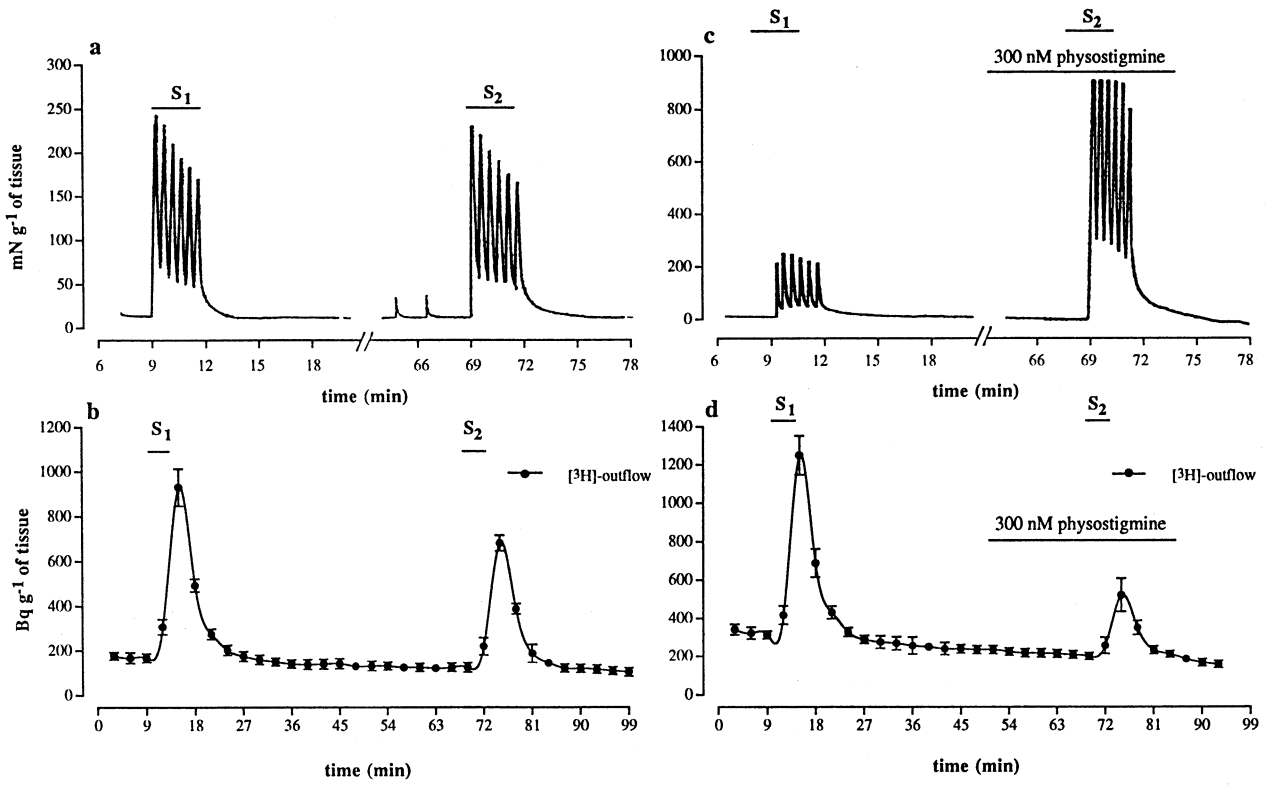

Figure 1 Electrically-evoked contractile responses and [^{3}H]-ACh outflow in control conditions (a,b) and in the presence of physostigmine (c,d). After a 120 min washout period, human detrusor strips were stimulated twice with 600 pulses in six trains at 10 Hz (1 ms, 15 V, 30 s apart). Stimulation periods (S) are indicated at the top of the figure. Horizontal bar indicates superfusion with 300 nM physostigmine (c,d). In release experiments (b,d), the radioactivity was detected in the superfusate which was collected in 3 min samples.

times (Figure 1c). TTX and hexamethonium did not affect the basal tone and the spontaneous radioactivity outflow. Physostigmine (100 nM) had no effect on spontaneous radioactive outflow but slightly enhanced the basal tone and phasic activity in one out of four preparations. This implies that prevention of ACh degradation under basal conditions does not usually allow to reach effective transmitter concentrations to produce detectable changes of basal tone. Muscarone (10 nM – 1 μM), which *per se* contracted the preparation, inhibited in a concentration-dependent manner the transmitter release evoked by electrical stimulation (maximal inhibition: 50% with a $-\log EC_{50}$ value of 7.30 ± 0.06, $n=4$) and the resultant contraction (inhibition range: 10 – 50%). Atropine (0.1 – 100 nM) enhanced in a concentration-dependent manner the electrically-evoked release and reduced the contraction (Figure 2a,b; $n=4$). The pre- and postjunctional $-\log EC_{50}$ values for atropine are given in Table 2. Maximal facilitation of release ($50.1 \pm 3.2\%$, $n=9$) and reduction of contractile response (74.4 ± 6.0, $n=9$) was observed at 100 nM. This atropine concentration totally counteracted the inhibitory effect of 1 μM muscarone on the evoked release, since the S_2/S_1 ratio (0.71 ± 0.04, $n=3$) in the presence of the two drugs was not significantly different from control. Atropine did not affect the resting tension and the spontaneous radioactivity outflow.

Pre- and postjunctional potencies of selective muscarinic receptor antagonists

A series of subtype-preferring muscarinic receptor antagonists (see Table 2 for their receptor selectivity profile), including pirenzepine (1 nM – 10 μM), 4-DAMP (0.1 nM – 100 μM), pF-HHSiD (10 nM – 10 μM), tripitramine (0.3 nM – 1 μM), methoctramine (3 nM – 10 μM), tripinamide (1 nM – 10 μM) and MT-3 mamba toxin (1 – 10 nM) increased in a concentration-dependent manner the [^{3}H]-outflow evoked by electrical stimulation (see Figure 2d for the effect of MT-3). The maximal facilitation was approximately 50% for all the antagonists. Concomitantly, all the antagonists, with the exception of MT-3 (Figure 2c, $n=5$), inhibited the resultant contractile response with a maximal inhibition of approximately 80%. Figure 3 illustrates the effects of muscarinic receptor antagonists on electrically-evoked [^{3}H]-outflow and smooth muscle contraction. Conversely, the basal [^{3}H]-outflow and the resting tension of the preparation were not affected during the exposure to all the antagonists.

The potency estimates of antagonists at pre- and postjunctional level are shown in Tables 2 and 3. In the latter table, the postjunctional affinity values (calculated in a separate set of experiments) are also listed for comparison purposes. The rank orders of potency of antagonists at pre- and postjunctional level were: atropine $\geqslant$ 4-DAMP > MT-3 > tripitramine > pF-HHSid $\geqslant$ methoctramine $\geqslant$ pirenzepine > tripinamide, and atropine $\geqslant$ 4-DAMP > pF-HHSid > > pirenzepine = tripitramine > tripinamide > methoctramine > > MT-3, respectively.

Table 4 summarizes the correlation analysis of antagonist postjunctional affinities (pA_2 values against the contractile response to muscarone), postjunctional potencies (inhibition of electrically-evoked contractions) and prejunctional potencies (facilitation of [^{3}H]-ACh release) in the human detrusor muscle with available constants (pK_i values) for human cloned muscarinic receptor subtypes. The best correlation was found for a postjunctional M_3 receptor and a prejunctional M_4 receptor, respectively.

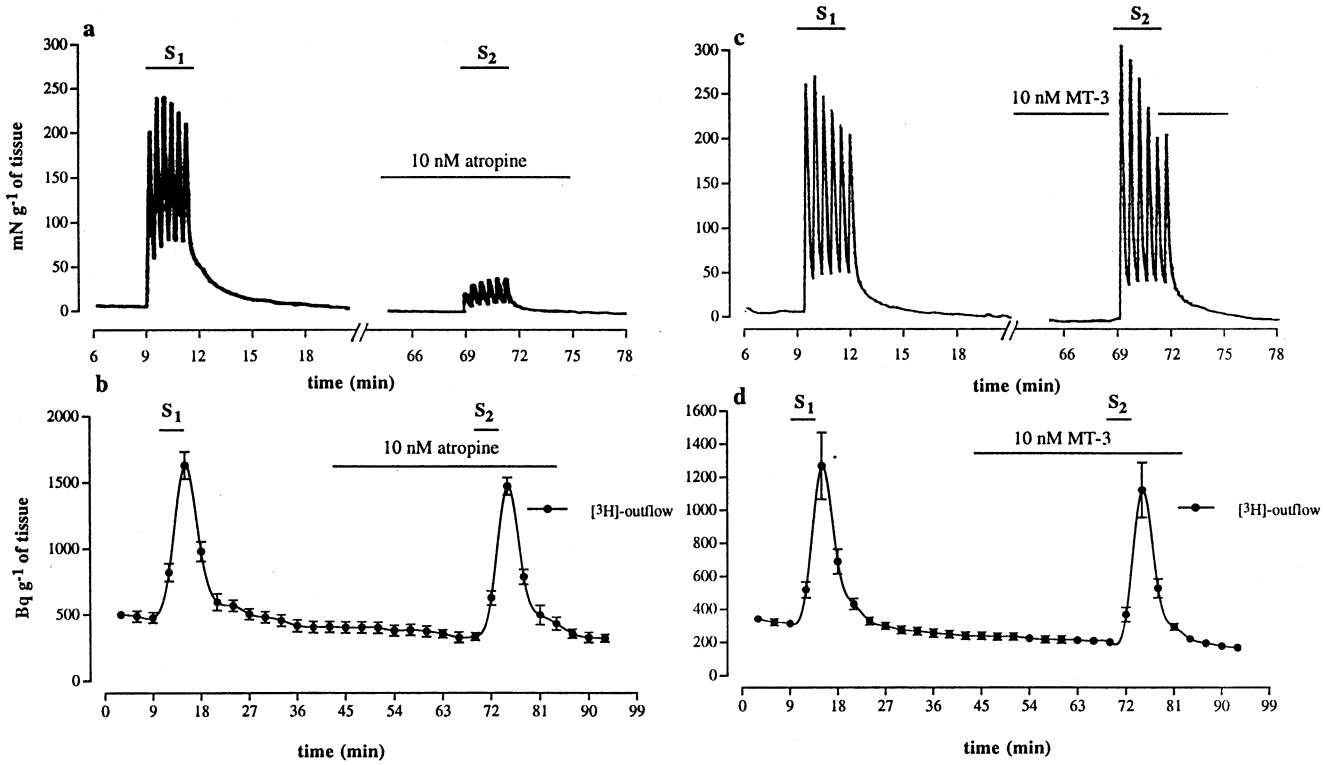

Figure 2 Electrically-evoked contractile responses and [^{3}H]-ACh outflow in the presence of atropine (a,b) and the muscarinic toxin MT-3 (c,d). After a 120 min washout period, human detrusor strips were stimulated twice with 600 pulses in six trains at 10 Hz (1 ms, 15 V, 30 s apart). Stimulation periods (S) are indicated at the top of the figure. Horizontal bar indicates superfusion with 10 nM atropine (a,b) and 10 nM MT-3 (c,d). In release experiments, the radioactivity was detected in the superfusate which was collected in 3 min samples.

Table 2 Comparison between pre- and postjunctional potencies ($-\log EC_{50}$) of muscarinic antagonists in human detrusor strips and their selectivity profile

Antagonist	*$-\log EC_{50}$ pre (95% CL)*	*$-\log EC_{50}$ post (95% CL)*	*Selectivity profile*
Atropine	9.12 (8.91–9.33)	9.09 (8.75–9.42)	$M_1=M_2=M_3=M_4=M_5$
4-DAMP	9.07 (8.87–9.27)	9.00 (8.69–9.31)	$M_3=M_4=M_1=M_5>M_2$
pF-HHSiD	7.50 (7.37–7.63)	7.57 (6.92–8.24)	$M_3=M_4=M_1=M_5>M_2$
Pirenzepine	7.33 (7.15–7.51)	6.97 (6.68–7.26)	$M_1>M_4\geqslant M_3\geqslant M_2=M_5$
Methoctramine	7.43 (7.15–7.65)**	5.85 (5.69–6.01)	$M_2\geqslant M_4\geqslant M_1>M_5>M_3$
Tripitramine	8.20 (8.06–8.34)**	6.92 (6.67–7.17)	$M_2>M_1>M_4>M_5=M_3$
Tripinamide	7.00 (6.80–7.21)*	6.54 (6.29–6.79)	$M_2>M_1>M_4>M_3$
MT-3	8.50 (8.31–8.69)**	<6	$M_4>>M_1>>M_2=M_3=M_5$

The $-\log EC_{50}$ values are mean, 95% confidence limits, of 4–12 experiments. Comparison between prejunctional and postjunctional potencies: *$P<0.05$,**$P<0.01$. For the selectivity profile of antagonists see Caulfield & Birdsall (1998).

Discussion

A modulatory role of presynaptic inhibitory muscarinic receptors on ACh release from cholinergic nerve terminals has been demonstrated in the urinary bladder of several rodents (see Andersson, 1993 for review). In this study, we describe the occurrence of a negative feedback mechanism regulating [^{3}H]-ACh release in the human isolated detrusor, and the pharmacological profile of muscarinic receptors involved in the inhibition of transmitter release and smooth muscle contraction.

Electrical field stimulation of human detrusor strips that had been preincubated with [^{3}H]-choline produced an outflow of [^{3}H]-activity, which can be regarded as a reliable indicator of [^{3}H]-ACh release from postganglionic cholinergic nerve terminals for the following reasons. Most of the evoked [^{3}H]-activity was recognized as [^{3}H]-ACh by HPLC analysis; the [^{3}H]-ACh outflow was abolished by TTX, a compound known to suppress action potential-mediated transmitter release, but was unaffected by hexamethonium, a ganglionic blocking agent. In addition to evoke transmitter release, electrical field stimulation induced a contractile response of detrusor strips. Therefore, any drug-induced change of either [^{3}H]-activity and/or contraction was considered as due to indirect or direct interaction with muscarinic receptors located at prejunctional and postjunctional level, respectively.

Physostigmine reduced the evoked outflow of [^{3}H]-ACh, but the postjunctional contractile response to electrical stimulation was enhanced despite the decrease of transmitter release. Acetylcholinesterase inhibition caused by physostigmine may result in an increased concentration of ACh in the junctional cleft and hence in a greater postjunctional effectiveness, which is compensative for the decrease of transmitter release (D'Agostino *et al.*, 1986; Starke *et al.*, 1989).

Muscarone, a potent muscarinic receptor agonist, reduced to a similar extent and in a concentration-dependent manner

both the evoked [^{3}H]-ACh outflow and contraction. These effects may mostly depend on muscarone interaction with prejunctional muscarinic receptors (D'Agostino *et al*., 1997a). In the presence of a negative feedback mechanism, blockade of muscarinic inhibitory autoreceptors should be associated with an increase in transmitter release. Indeed, atropine enhanced in a concentration-dependent manner the evoked [^{3}H]-ACh outflow with a parallel reduction of the postjunctional contractile response. Furthermore, the administration of atropine and muscarone, combined at concentration exerting opposite effects, produced a final effect on release not significantly different from controls through an apparent competitive interaction.

Taken together, the above findings fulfil the criterion (see Starke *et al*., 1989 for review) for the presence of a muscarinic feedback mechanism inhibiting the release of ACh from cholinergic terminals in the human detrusor.

Pharmacological characterization of muscarinic receptor subtypes has long been hampered by the lack of selective antagonists. Due to the narrow selectivity window of available antagonists, convincing evidence for the involvement of a particular receptor necessitates the use of a series of subtype-preferring antagonists. Recently, however, some snake toxins from the venom of green and black mambas have been reported to be important tools in discriminating muscarinic receptor subtypes (Adem & Karlsson, 1997). To determine the pharmacological profile of muscarinic receptors involved in the inhibitory control of transmitter release and detrusor contraction, we used classical muscarinic receptor antagonists (atropine, pirenzepine, 4-DAMP, pF-HHSiD), and recently developed agents such as tripitramine (Maggio *et al*., 1994) and tripinamide (Bolognesi *et al*., 1998) (see Table 2 for their selectivity profile). In addition, we employed a highly selective M_4 receptor antagonist, the MT-3 mamba toxin (Jolkkonen *et al*., 1994), for an unambiguous muscarinic receptor classification in the human detrusor.

Previous biochemical and pharmacological investigations revealed that human detrusor muscle co-expresses muscarinic m_2 and m_3 receptors (Kondo *et al*., 1995; Wang *et al*., 1995; Yamaguchi *et al*., 1996) and suggested that functional responses to agonists are mediated by the M_3 subtype (Poli *et al*., 1992; Newgreen & Naylor, 1996). The presence of postjunctional excitatory receptors belonging to the M_3 receptor subtype was further confirmed in the present investigation. In our experimental conditions, the rank order of potencies and affinities at postjunctional level of seven subtype-preferring antagonists (4-DAMP >> pF-HHSiD > tripitramine > pirenzepine > tripinamide > methoctramine > MT-3) is similar (Table 3). The comparison of postjunctional values with the affinity constants of the five human cloned

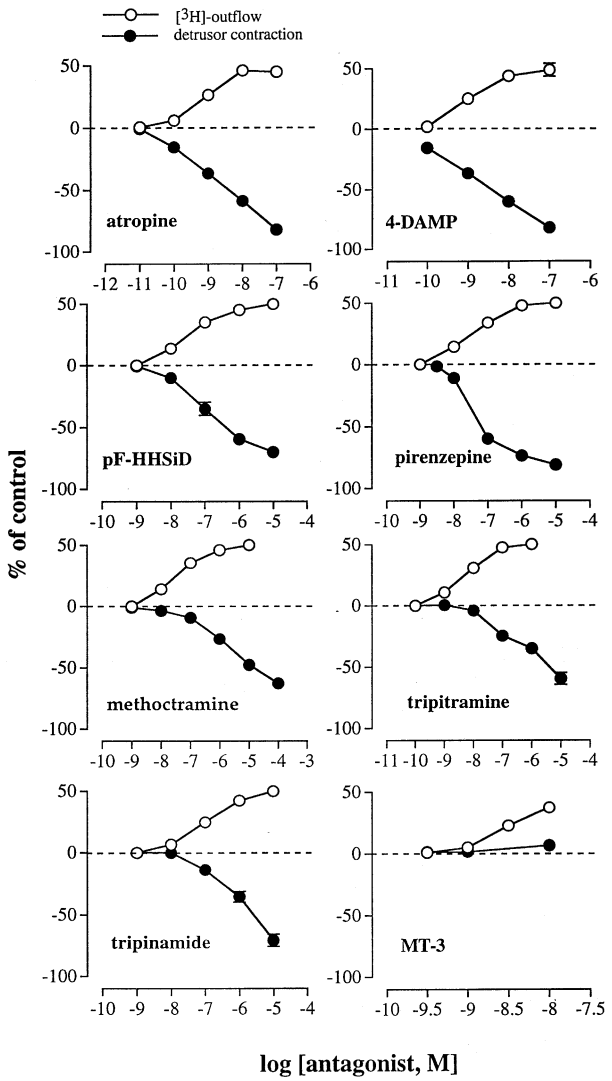

Figure 3 Effect of muscarinic receptor antagonists on [^{3}H]-ACh outflow and smooth muscle contraction evoked by electrical field stimulation (600 pulses in six trains at 10 Hz) in human detrusor strips. Control experiments were carried out in parallel, and the effects of the antagonists are given as a percentage of the corresponding control value. Given are means ± s.e.mean of 3–9 experiments.

Table 3 Comparison of pre- and postjunctional potencies ($-\log EC_{50}$) and postjunctional affinities (pA_2) of muscarinic antagonists in human detrusor strips with their affinity values (pK_i) at human cloned muscarinic receptors

	pK_i (nM)					(nM)		
Antagonist	m_1	m_2	m_3	m_4	m_5	pA_2 post	$-\log EC_{50}$ post	$-\log EC_{50}$ pre
Atropine	9.30	9.04	8.96	9.22	8.76	9.21	9.09	9.12
4-DAMP	9.24	8.42	9.28	8.93	8.98	9.02	9.00	9.07
pF-HHSiD	7.65	6.88	7.81	7.50	7.03	7.68	7.57	7.50
Pirenzepine	8.20	6.65	6.86	7.43	7.05	6.90	6.97	7.33
Methoctramine	7.30	7.90	6.70	7.50	6.87	6.22	5.67	7.43
Tripitramine	8.80	9.57	7.41	8.19	7.47	7.24	6.92	8.20
Tripinamide	7.32	8.56	6.46	6.88	n.d.	6.60	6.54	7.00
MT-3	7.10	< 6	< 6	8.70	< 6	n.d.	< 6	8.50

Affinity values (pK_i) refer to radioligand binding studies at human cloned muscarinic receptors expressed in chinese hamster ovary (CHO) cells (data from Dorje *et al*., 1991; Bolden *et al*., 1992; Jolkkonen *et al*., 1994; Maggio *et al*., 1994). Tripinamide (Melchiorre, unpublished data). Postjunctional affinity estimates (pA_2 post) were determined by Schild regression analysis in experiments against muscarone. See Methods for details.

Table 4 Correlation of postjunctional affinities, postjunctional potencies (inhibition of contractile response) and prejunctional potencies (facilitation of [^{3}H]-ACh release) in the human detrusor with available constants for human cloned muscarinic receptors

	Correlation coefficient (r)					*Slope*				
Constant	m_1	m_2	m_3	m_4	m_5	m_1	m_2	m_3	m_4	m_5
pA_2 post	0.847*	0.319	0.969***	0.408	0.928**	0.633*	0.292	0.955***	0.377	0.730***
$-\log EC_{50}$ post	0.868	0.389	0.954**	0.635	0.808	0.605	0.372	1.010**	0.419	0.723
$-\log EC_{50}$ pre	0.705	0.250	0.615	0.987***	0.564	0.778	0.414	0.976	1.033***	0.878

Postjunctional affinities (pA_2 post), postjunctional ($-\log EC_{50}$ post) and prejunctional potencies ($-\log EC_{50}$ pre) of muscarinic receptor antagonists (4-DAMP, p-HHSiD, pirenzepine, MT-3, tripinamide, methoctramine and tripitramine) were taken from Table 3. Constants related to the same antagonists at human cloned muscarinic receptors (pK_i values in Table 3) refer to binding experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

receptor subtypes (Table 3) is consistent with the pharmacological profile of the M_3 receptor subtype (Caulfield & Birdsall, 1998). This notion is further corroborated by the relationship analysis, in which the best correlation coefficient ($r=0.969$) was found for the M_3 subtype (Table 4).

From the comparison of pre- and postjunctional potencies of muscarinic receptor antagonists (methoctramine, tripitramine, tripinamide and MT-3 toxin) evaluated concomitantly in the same experiments, it is evident that muscarinic receptors located at pre- and postjunctional level are pharmacologically different (Table 2). The comparison of prejunctional potencies with the affinity constants at human cloned muscarinic receptor subtypes indicates that the muscarinic autoreceptor involved in the control of ACh release in human detrusor belongs to the M_4 subtype. In fact, the prejunctional potency values of pirenzepine (7.33), methoctramine (7.43), tripitramine (8.2) and tripinamide (7.0) (Table 3) suggest as unlikely the participation of M_1 and M_2 receptors. Furthermore, the potency values of all these antagonists do not fit with the values reported in the literature for the M_3 receptor (Caulfield & Birdsall, 1998). Conversely, the prejunctional potencies of the aforementioned antagonists do not fit with the values reported in the literature for the M_3 receptor (Caulfield & Birdsall, 1998). Conversely, the prejunctional potencies of the aforementioned antagonists are similar to their affinity values for the human M_4 muscarinic receptor (Table 3). In particular, the potency value (8.5) obtained in the human detrusor with the MT-3 toxin, a compound possessing an affinity ($pK_i=8.7$) 30–1000 fold higher for M_4 muscarinic receptor compared to other receptor subtypes (Caulfield & Birdsall, 1998), makes the classification of the prejunctional autoreceptor unambiguous. Finally, the involvement of an M_4 autoreceptor is corroborated further by the relationship analysis, in which the best correlation coefficient ($r=0.987$) was found for the M_4 subtype (Table 4).

Based on previous evidence, the muscarinic autoreceptors controlling negative feedback mechanisms in peripheral cholinergically innervated tissues belong to the M_2 or M_4 subtype (see Eglen & Watson, 1996 for review). In particular, muscarinic inhibitory autoreceptor in the urinary bladder of the guinea-pig and rat has been recently characterized as an M_4 receptor (Alberts 1995; D'Agostino *et al*., 1997a). The evidence that an M_4 receptor is also involved in the human bladder indicates that the negative feedback mechanism regulating ACh release in the mammalian bladder is under the control of this receptor subtype.

In conclusion, our results are compatible with the notion that in the human urinary bladder the prejunctional muscarinic autoreceptor, which negatively regulates ACh release from cholinergic nerve terminals belongs to the M_4 receptor subtype. This receptor seems to be different from that mediating detrusor contraction, which has been characterized as an M_3 receptor. The knowledge of diversity between receptor subtypes mediating the effects of ACh at pre- and postjunctional level is an important prerequisite for the development of new muscarinic receptor ligands for the treatment of unstable bladder due to overactive detrusor (Eglen & Watson, 1996; Turner & Brading, 1997).

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