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Tolterodine – a new bladder-selective antimuscarinic agent

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Abstract

Tolterodine is a new muscarinic receptor antagonist intended for the treatment of urinary urge incontinence and other symptoms related to an overactive bladder. The aim of the present study was to compare the antimuscarinic properties of tolterodine with those of oxybutynin, in vitro and in vivo. Tolterodine effectively inhibited carbachol-induced contractions of isolated strips of urinary bladder from guinea pigs (K_B 3.0 nM; pA_2 8.6; Schild slope 0.97) and humans (K_B 4.0 nM; pA_2 8.4; Schild slope 1.04) in a concentration-dependent, competitive manner. The affinity of tolterodine was similar to that derived for oxybutynin (K_B 4.4 nM; pA_2 8.5; Schild slope 0.89) in the guinea-pig bladder. Tolterodine (21–2103 nmol/kg (0.01–1 mg/kg); intravenous infusion) was significantly more potent in inhibiting acetylcholine-induced urinary bladder contraction than electrically-induced salivation in the anaesthetised cat. In contrast, oxybutynin displayed the opposite tissue selectivity. Radioligand binding data showed that tolterodine bound with high affinity to muscarinic receptors in urinary bladder (K_i 2.7 nM), heart (K_i 1.6 nM), cerebral cortex (K_i 0.75 nM) and parotid gland (K_i 4.8 nM) from guinea pigs and in urinary bladder from humans (K_i 3.3 nM). Tolterodine and oxybutynin were equipotent, except in the parotid gland, where oxybutynin bound with 8-times higher affinity (K_i 0.62 nM). Binding data on human muscarinic m1–m5 receptors expressed in Chinese hamster ovary cells showed that oxybutynin, in contrast to tolterodine, exhibits selectivity (10-fold) for muscarinic m3 over m2 receptors. The K_B value determined for oxybutynin (4.4 nM) in functional studies on guinea-pig bladder correlated better with the binding affinity at muscarinic M_2/m2 receptors (K_i 2.8 and 6.7 nM) than at muscarinic M_3/m3 receptors (K_i 0.62 and 0.67 nM). The tissue selectivity demonstrated for tolterodine in vivo cannot be attributed to selectivity for a single muscarinic receptor subtype. However, the combined in vitro and in vivo data on tolterodine and oxybutynin may indicate either that muscarinic M_3/m3 receptors in glands are more sensitive to blockade than those in bladder smooth muscle, or that muscarinic M_2/m2 receptors contribute to bladder contraction.

Keywords: Urinary bladder contraction; Salivation; Muscarinic receptor subtype; Muscarinic receptor antagonist; (Guinea pig); (Cat); (Human)

1. Introduction

Tolterodine (Fig. 1) is a new muscarinic receptor antagonist intended for the treatment of urinary urge incontinence and other symptoms of an overactive bladder. The pharmacological treatment of urge incontinence has for a long time been based on muscarinic receptor antagonists, e.g., propantheline, emepronium and oxybutynin (Andersson, 1988; Wein et al., 1994), and it is generally agreed that contractions of the human urinary bladder are mediated mainly through stimulation of acetylcholine muscarinic receptors (Andersson, 1993). Oxybutynin is currently considered to be the drug of choice in the treatment of bladder overactivity (Yarker et al., 1995). The effectiveness of oxybutynin has been demonstrated in several controlled clinical studies, but the incidence of classical antimuscarinic side effects often leads to discontinuation of treatment. Dryness of the mouth is the most prevalent side effect, experienced by at least 50% of patients on oxybutynin therapy (Yarker et al., 1995).

The existence of molecularly distinct muscarinic receptor subtypes has been well established (Hulme et al., 1990; Caulfield, 1993). Thus, five genes encoding for muscarinic receptors (m1–m5) have been cloned and expressed in cell lines, while three muscarinic receptor subtypes (M_1–M_3)

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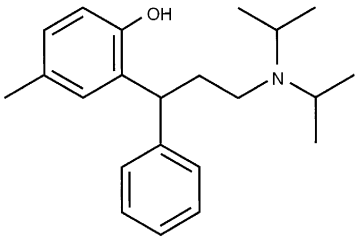

Fig. 1. Chemical structure of tolterodine.

can be distinguished by pharmacological means. A fourth pharmacological subtype (M_4) has also been defined, although muscarinic M_4-selective antagonists are not yet available. Roughly, muscarinic M_1 receptors prevail in neuronal tissues while the heart appears to contain a homogenous population of muscarinic M_2 receptors and exocrine glands are considered to contain mainly muscarinic M_3 receptors. The muscarinic M_4 receptors are found in striatum, cortex and peripheral rabbit lung. However, it is commonly found that more than one receptor subtype coexist within a tissue or cell (Hulme et al., 1990; Caulfield, 1993).

Many smooth muscles contain both muscarinic M_2 ('cardiac') and muscarinic M_3 ('glandular') receptors (for review, see Eglen et al., 1994). The presence of mRNA for muscarinic m2 and m3 receptors has been demonstrated in the urinary bladder of rat, pig (Maeda et al., 1988) and humans (Yamaguchi et al., 1994). Radioligand binding data indicate the presence of muscarinic M_2 and M_3, as well as muscarinic M_1, receptors in human bladder tissue and that muscarinic M_3 receptors may predominate (Kondo et al., 1995; Wammack et al., 1995). On the other hand, immunoprecipitation data indicate that the proportion of muscarinic m2 and m3 receptors is 3:1 in the bladders from humans, guinea pigs and rabbits, and 9:1 in the rat bladder (Wang et al., 1995). Radioligand binding data on selective antagonists in the guinea-pig bladder (Nilvebrant and Sparf, 1986, 1988) and other smooth muscles (Giraldo et al., 1987; Michel and Whiting, 1988; Eglen et al., 1994) most often reflect the pharmacology of the muscarinic M_2 receptors. This probably reflects a predominance of muscarinic M_2/m2 receptors in smooth muscles and the rather limited subtype selectivity of available antagonists. However, functional in vitro data on a number of selective antagonists indicate that the contraction of most smooth muscles, including the urinary bladder, is mediated by muscarinic M_3 receptors (Caulfield, 1993; Eglen et al., 1994; Tobin and Sjögren, 1995; Wang et al., 1995). The functional role of muscarinic M_2 receptors is still mysterious, but emerging evidence suggests that the importance of the muscarinic M_2/m2 receptors in various smooth muscles might have been underestimated (Zhang and Buxton, 1991; Griffin and Ehlert, 1992; Caulfield, 1993; Thomas et al., 1993; Eglen et al., 1994; Chen et al., 1995; Reddy et al., 1995).

The aim of the present study was to compare the antimuscarinic properties of tolterodine and oxybutynin. Oxybutynin has been characterised as a muscarinic M_1/M_3-selective antagonist (Nilvebrant and Sparf, 1982, 1983b, 1986; Lazareno et al., 1990; Noronha-Blob and Kachur, 1991). For purposes of comparison, the classical non-selective muscarinic receptor antagonist atropine was also included. The inhibitory effects of tolterodine, oxybutynin and atropine on urinary bladder contraction and salivary secretion in vivo were studied in the anaesthetised cat. The in vitro studies comprised radioligand binding and functional studies on bladders from guinea pigs and humans. Radioligand binding studies were also carried out on parotid gland, heart and cerebral cortex from guinea pigs, as well as on human muscarinic m1–m5 receptors expressed in Chinese hamster ovary (CHO) cells.

2. Materials and methods

2.1. In vitro studies

2.1.1. Guinea-pig tissues

Male guinea pigs (Dunkin Hartley, weighing 300–500 g) were killed by a blow on the neck and exsanguinated. The cerebral cortex was dissected on ice and the other tissues in Krebs-Henseleit solution, as previously described (Nilvebrant and Sparf, 1982, 1983a, 1986). Urinary bladders for functional in vitro studies were cut into strip preparations ($3 \times 3 \times 12$ mm). Tissues for radioligand binding studies were homogenised, as described below.

2.1.2. Human urinary bladder

Detrusor specimens from the dome of the bladder were obtained from 23 patients (mean age 63 ± 2 years; range 44–75 years) undergoing surgery for bladder malignancy. Some of the patients had received preoperative radiation therapy (20 Gy). However, the muscarinic receptor density in the human bladder and the affinity for muscarinic receptor antagonists does not appear to be influenced by age, sex and radiotherapy (Nilvebrant et al., 1985). All specimens were taken from a macroscopically normal part of the detrusor. No mucosal tissue was included. For functional in vitro studies, dissected bladder specimens were cut into strip preparations ($1 \times 1 \times 8$ mm; 6–12 strips were obtained from each patient). Tissue for radioligand binding studies was frozen at -70°C and stored at this temperature until used in the assays.

2.1.3. Chinese hamster ovary (CHO) cells

CHO cell lines transfected with genes encoding the human muscarinic receptor subtypes m1, m2, m3 and m4, respectively, were obtained from Dr T. Bonner, National Institutes of Health, USA, while cells expressing the m5 subtype were obtained from Dr M. Brann, University of Vermont, USA (Buckley et al., 1989). The cells were

cultured in a medium consisting of equal parts of Dulbecco's modified Eagle's medium (DMEM) and HAM's F12, respectively. The medium was supplemented with foetal bovine serum (10%), L-glutamine (4 mM) and penicillin/streptomycin (100 U/ml), at 37°C in a humidified atmosphere (7% CO_2, 93% air) in tissue culture flasks or roller bottles. Cultures were continuously harvested every second or third day and stored at −70°C until used in radioligand binding studies.

2.1.4. Functional in vitro studies

Guinea-pig and human bladder strips were transferred to jacketed tissue baths (5 ml) and mounted between two hooks. One of the hooks was connected to a force transducer (FT 03, Grass Instruments). The baths contained a temperature-controlled (37°C) Krebs-Henseleit solution, which was continuously aerated with carbogen (93.5% or 95% O_2/6.5% or 5% CO_2) to maintain pH at 7.4. Isometric tension was recorded by a Polygraph (Grass, model 79 D). A resting tension of 5 mN was initially applied to each preparation. During stabilisation (45–60 min) the strips were repeatedly washed and the resting tension was adjusted. The Krebs-Henseleit solution used for guinea-pig bladder contained (mM): NaCl (119), KCl (4.6), $CaCl_2$ (1.5), $MgCl_2$ (1.2), $NaHCO_3$ (20), NaH_2PO_4 (1.4) and glucose (11). The Krebs-Henseleit buffer used for human preparations had a slightly different composition (mM): NaCl (118), KCl (4.6), $CaCl_2$ (1.5), $MgSO_4$ (1.2), $NaHCO_3$ (24.9), KH_2PO_4 (1.4) and glucose (11). When responses of human strips to K^+ were studied, the NaCl in the Krebs solution was replaced by KCl to give a K^+ concentration of 124 mM.

Guinea-pig preparations were exposed to a standard concentration of the muscarinic receptor agonist carbachol (3 μM; EC_{80}) to establish reproducible responses (three subsequent contractions with ≤10% variation in amplitude) before a concentration–response curve to carbachol (control) was generated using cumulative addition. After exposure of the bladder strips to a fixed concentration of antagonist for 60 min (Nilvebrant, 1986), the concentration–response curve to carbachol was repeated in the presence of antagonist. Responses were expressed as a percentage of the maximal contractile response elicited by carbachol in the control curve.

The viability of human bladder preparations was controlled by exposure to K^+ (124 mM) before cumulative concentration–response curves to carbachol were established (control). Two consecutive control curves were generated before incubation with tolterodine, but all responses were expressed as a percentage of the maximal contractile response in the control curve immediately preceding incubation with tolterodine. The strips were exposed to tolterodine for 20 min before the concentration–response curve to carbachol was repeated.

When human bladder strips were stimulated electrically, two electrodes were placed on each side of the preparation and parallel to it. Stimulation was delivered by a Grass S88 electrical stimulator as single square-wave pulses with a duration of 0.8 ms. The voltage was supramaximal and the stimulation was given in 5 s trains with an interval of 2 min. The frequency interval used was 1–45 Hz. The frequency-response relations were established for each strip, and a frequency producing 70–80% of maximum response was selected. The strips were stimulated at this frequency at 2-min intervals, and tolterodine was then added cumulatively.

EC_{50} values for carbachol were determined in the absence and presence of antagonist, respectively. The affinity of the antagonist was calculated as the dissociation constant K_B (Schild, 1949). The mean ± S.E.M. of the K_B values determined using different antagonist concentrations in (n) preparations was calculated. The IC_{50} value for tolterodine in electrically stimulated preparations was calculated by linear interpolation.

2.1.5. Membrane preparations for radioligand binding studies

All preparation steps were carried out on ice. Human bladder specimens obtained from different patients were never pooled. Thawed human bladder samples, dissected guinea-pig bladders, parotid glands and hearts were homogenised in ten volumes of ice-cold sodium-potassium phosphate buffer (50 mM; pH 7.4) containing 1 mM phenylmethylsulphonyl fluoride, using a Polytron PT-3000 (guinea-pig urinary bladder and parotid gland: 26 000 rpm, 2 × 15 s; guinea-pig heart: 1300 rpm, 2 × 15 s; human urinary bladder: 26 000 rpm, 3 × 15 s; with a 30 s cooling interval between bursts). Guinea-pig cerebral cortex was homogenised using a Potter-Elvehjem Teflon/glass homogeniser. Before use in the assays, tissue homogenates were diluted with ice-cold phosphate/phenylmethylsulphonyl fluoride buffer to a final protein concentration of ≤0.3 mg/ml. Homogenates of CHO cells containing muscarinic m1–m5 receptors were prepared in 1–2 ml of ice-cold sodium phosphate buffer (25 mM, pH 7.4) containing 5 mM $MgCl_2$ (Buckley et al., 1989) and 1 mM phenylmethylsulphonyl fluoride, using a glass-glass homogeniser. The cell homogenates were diluted with ice-cold phosphate/phenylmethylsulphonyl fluoride buffer to 1000–10 000 times the original wet weight (protein concentrations of 4–190 μg/ml) before use in the assays. Protein concentration in the final tissue homogenates and in cell homogenates containing >25 μg/ml were determined by the method of Lowry et al. (1951). The BioRad Protein Assay (Bradford, 1976) was used for cell homogenates with protein concentrations below 25 μg/ml. Bovine serum albumin was used as standard for both methods.

2.1.6. Radioligand binding assays

The affinities of the unlabelled drugs were determined by competition experiments, using [^{3}H](−)-1-quinuc-

lidinyl benzilate as tracer. Non-specific binding was determined in the presence of 10 μM atropine. Total and non-specific binding of [^{3}H](−)-1-quinuclidinyl benzilate was determined in six samples each, while samples containing unlabelled antagonist were run in triplicate.

Tissue homogenates (1.0 ml) were incubated with [^{3}H](−)-1-quinuclidinyl benzilate (2 nM) and different concentrations of unlabelled antagonist under conditions of equilibrium: guinea-pig urinary bladder, 25°C for 60 min (Nilvebrant and Sparf, 1983a); parotid gland, 25°C for 210 min (Nilvebrant and Sparf, 1982); heart and cerebral cortex, 25°C for 80 min (Nilvebrant and Sparf, 1986); and human bladder, 25°C for 180 min (Nilvebrant et al., 1985). Incubations were terminated by centrifugation (Nilvebrant and Sparf, 1983a). The pellets were washed and the tubes allowed to dry before the tips containing the pellets were cut off and transferred to scintillation vials. Soluene-100 (Packard) was added (200 μl/tube) and the samples were left overnight for solubilisation of the pellet. A scintillation cocktail (Instafluor or Ultima Gold, Packard) was added and radioactivity determined by liquid scintillation spectrometry (Packard TriCarb model 2200 CA or 2500 TR). A quench correction curve was used for the conversion of cpm to dpm.

Binding studies on CHO cell lines containing human muscarinic m1–m5 receptors were run in 24-well cultures plates. Cell homogenates (1.0 ml) were incubated with [^{3}H](−)-1-quinuclidinyl benzilate (1 nM) and different concentrations of unlabelled drug under conditions of equilibrium (37°C for 300 min). The optimal protein concentrations and the time required to reach equilibrium for each cell line was determined in a separate series of experiments (data not shown). Incubations were terminated by filtration through a cell harvester (Packard) onto GF/C filters (Packard). The filter plates were dried, 200 μl of Microscint (Packard) added and the radioactivity was determined in a Packard Top Count scintillation counter (efficiency of 44–46%).

Data are presented as mean ± S.E.M. of (n) experiments. In the competition binding experiments, IC_{50} values for the antagonists were determined from the experimental concentration–inhibition curves. Dissociation constants (K_i) were calculated by correcting the IC_{50} values for the radioligand-induced parallel shift and the differences in receptor concentration, using the method described by Jacobs et al. (1975); see also Nilvebrant and Sparf (1982, 1983b) for details.

In order to verify that the density of muscarinic receptors in the human bladder and the affinity (K_d) for [^{3}H](−)-1-quinuclidinyl benzilate were within the ranges previously reported (Nilvebrant et al., 1985), saturation experiments were performed in specimens from six individuals. Samples containing different concentrations of [^{3}H](−)-1-quinuclidinyl benzilate (0.05–5 nM) were incubated under conditions of equilibrium (25°C for 180 min), using atropine for determination of non-specific binding. Receptor density and K_d were determined by Scatchard analysis of the saturation data. These parameters (receptor density 122 ± 9 fmol/mg protein (range 93–146); K_d 0.12 ± 0.02 nM) were within the range of those previously determined in a series of 14 patients (receptor density 164 ± 15 fmol/mg protein (range 77–231); K_d 0.14 ± 0.02 nM) (Nilvebrant et al., 1985).

2.2. In vivo studies

2.2.1. Animals and anaesthesia

Twenty-five female European short-haired cats (2.2–3.4 kg; 6–10 months old) were used. The animals were deprived of food overnight before the experiment. They were initially anaesthetised with an intraperitoneal injection of pentobarbital sodium (30–39 mg/kg), followed by intravenous (i.v.) administration of α-chloralose (25 mg/ml in 5% sodium tetraborate; 25 mg/kg).

The animal was placed on an electrically heated pad and the body temperature was controlled by a feedback loop from a thermometer in the anus. Additional doses of α-chloralose were given (176–312 μl/kg per h) when required to maintain anaesthesia. In order to maintain homeostasis, a Ringer solution was administered (2.2–7.8 ml/kg per h).

2.2.2. Surgery

The animal was tracheotomised and a glass respiratory cannula was inserted and connected to a volumetric low-pressure transducer (Grass PT5A) for recording of respiratory volume. Both femoral arteries were catheterised with polyethylene tubing (PE90). One of these was connected to a blood pressure transducer (Statham P23DC). The other was used for intra-arterial (i.a.) administration of acetylcholine and collection of blood samples for determination of haematocrit and blood gases. The right femoral vein was catheterised (PE90) and connected to a 3-way stopcock for i.v. infusion of test compounds and Ringer solution.

The hypogastric and pelvic nerves were identified and transected. The ureters were cut and catheterised (PE10). A catheter (PE240) inserted into the urinary bladder through an incision in the proximal urethra. At the beginning of the experiment, this catheter was connected to an open vessel filled with temperature-controlled (37°C) saline that was placed approximately 15 cm above the animal. During stabilisation (about 1 h), the bladder relaxed and gradually became filled with saline under constant hydrostatic pressure. The bladder catheter was later connected to a pressure transducer (Statham p23A) for recording of intravesical pressure. The duct of the submandibular gland was exposed in the neck and catheterised (PE10). The parasympathetic chorda-lingual nerve was exposed and cut as proximally as possible. The peripheral nerve stump was placed on a bipolar electrode. Intravesical pressure, blood pressure, heart rate (derived from the blood pressure sig-

nal) and respiration rate were recorded on a Polygraph (Grass model 7D). Blood pressure, heart rate, respiration, blood gases and haematocrit were measured in order to monitor the general condition of the animal during the course of the experiment.

2.2.3. Experimental procedure

The cats were divided into four groups receiving tolterodine ($n = 5$), oxybutynin ($n = 5$), atropine ($n = 5$), or saline ($n = 10$; control group), respectively. Tolterodine (21–2103 nmol/kg (0.01–1 mg/kg)), oxybutynin (25–2538 nmol/kg (0.01–1 mg/kg)), atropine (0.86–86 nmol/kg (0.0003–0.03 mg/kg)) or saline were administered by i.v. infusion at a rate of 1 ml/kg per min. The animals were left to recover for at least 24 min between doses. Urinary bladder contractions were evoked by i.a. injection of a standard (submaximal) dose of acetylcholine (1–4 μg/kg), which produced a short-lasting (< 1 min), large (> 9 cmH_2O) and reproducible (< 15% variation) increase in intravesical pressure. This standard dose was selected from a dose-response curve to acetylcholine (0.5–8 μg/kg) generated at the beginning of each experiment. Acetylcholine was administered before and approximately 9 and 16 min after each dose of antagonist. Salivation was induced by supramaximal electrical stimulation (6 V, 2 ms, 5 Hz) of the chorda-lingual nerve. The frequency-response relationship was established at the beginning of each experiment using frequencies of 0.5, 1, 2 and 5 Hz over 2 min. The frequency used in the experiments (5 Hz) produced a mean salivary secretion of 372 ± 24 μl during 2 min. Electrical stimulation of the chorda-lingual nerve was performed before and approximately 7 min after each dose of antagonist. Arterial blood samples for determination of haematocrit and blood gases were drawn before the administration of each dose of test compound.

2.3. Calculations and statistics

Effects of drugs on urinary bladder contractions and salivary secretion are expressed as percentage inhibition of the control responses. Data are presented as mean ± S.E.M. of (n) animals. Differences between the inhibitory effect exerted by the antagonist on urinary bladder contractions and salivary secretion were analysed for each dose level using a paired Student's t-test. Differences between the doses of antagonist causing a 50% inhibition of these responses (IC_{50}) were also analysed. P values < 0.05 were considered significant.

2.4. Drugs and chemicals

The following drugs and chemicals were used: tolterodine tartrate [(*R*)-N,*N*-diisopropyl-3-(2-hydroxy-5-methylphenyl)-3-phenylpropanamine hydrogen L-(+)-tartrate], saline and Ringer solution (Pharmacia&Upjohn, Sweden); carbamylcholine chloride (carbachol), acetylcholine chloride, phenylmethylsulphonyl fluoride, oxybutynin hydrochloride and atropine sulphate (Sigma, USA); [^{3}H](−)-1-quinuclidinyl benzilate, 32–52 Ci/mmol (equivalent to 1.18–1.94 TBq/mmol) (Amersham, UK or Du Pont NEN, USA); pentobarbital sodium (Apoteksbolaget, Sweden); α-chloralose (Merck, Germany); sodium borate (Mallinkrodt, USA); DMEM and HAM's F12 (National Veterinary Institute, Sweden); foetal bovine serum albumin (HyClone Lab, USA); L-glutamine and penicillin/streptomycin (ICN Biomedicals, USA). Other chemicals used (analytical grade) were purchased from general commercial sources.

The radioligand [^{3}H](−)-1-quinuclidinyl benzilate was diluted in absolute ethanol. Stock solutions of tolterodine, oxybutynin, atropine and carbachol for functional in vitro studies were prepared and diluted in redistilled water. Stock solutions of tolterodine, oxybutynin, atropine and acetylcholine for in vivo studies were prepared and diluted in saline. Fresh solutions were prepared for each experiment.

3. Results

3.1. In vitro studies

3.1.1. Functional studies on isolated urinary bladder

Tolterodine effectively inhibited carbachol-induced contractions of guinea-pig isolated urinary bladder strips in a concentration-dependent manner (Fig. 2). The Schild plot (not shown) was linear (slope 0.97; pA_2 8.6), indicating a simple competitive blockade of the bladder muscarinic receptors. Similar data were obtained for oxybutynin (Schild slope 0.89; pA_2 8.5). Tolterodine was thus equipotent to oxybutynin, but less potent than atropine (Table 1).

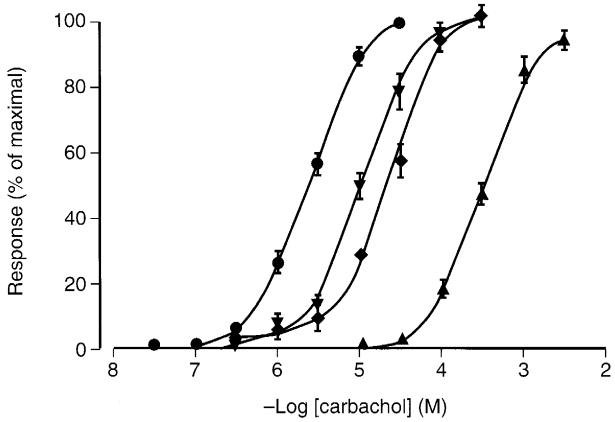

Fig. 2. Effect of tolterodine on the concentration-response curve to carbachol in guinea-pig isolated urinary bladder. The contractile responses elicited by increasing concentrations of carbachol (cumulative additions) were recorded in the absence (control) and presence, respectively, of tolterodine at concentrations of 10 nM (▼), 30 nM (♦) and 500 nM (▲). The time of incubation with tolterodine was 60 min. Control data (●) represent the mean (±S.E.M.) of 14 individual experiments. The EC_{50} value for carbachol was 2.4 ± 0.3 μM.

Table 1
Affinities, expressed as the dissociation constants (K_i), for muscarinic receptors in guinea-pig cerebral cortex, heart and parotid gland

Compound	Urinary bladder			Cerebral cortex		Heart		Parotid gland	
	K_B (nM)	K_i (nM)	n_H	K_i (nM)	n_H	K_i (nM)	n_H	K_i (nM)	n_H
Tolterodine	3.0 ± 0.2 (15)	2.7 ± 0.2 (6)	1.02 ± 0.03 (6)	0.75 ± 0.01 (5)	1.05 ± 0.03 (5)	1.6 ± 0.04 (5)	1.04 ± 0.06 (5)	4.8 ± 0.3 (5)	1.04 ± 0.03 (5)
Oxybutynin	4.4 ± 0.6 (14)	4.0 ± 0.4 (6)	0.99 ± 0.01 (6)	0.41 ± 0.05 (5)	0.97 ± 0.01 (5)	2.8 ± 0.3 (5)	1.05 ± 0.03 (5)	0.62 ± 0.05 (6)	1.01 ± 0.03 (6)
Atropine	0.7 ± 0.1 (10)	1.6 ± 0.1 (7)	1.00 ± 0.02 (7)	0.32 ± 0.02 (4)	1.00 ± 0.003 (4)	0.89 ± 0.06 (3)	1.05 ± 0.06 (3)	0.85 ± 0.05 (9)	0.95 ± 0.01 (9)

Data on urinary bladder muscarinic receptors were derived from functional in vitro studies (K_B) on carbachol-induced contractions of bladder strips and from competition binding studies (K_i) in bladder homogenates, respectively. K_i values were derived from competition binding experiments with [^{3}H](−)-1-quinuclidinyl benzilate, as described in Section 2. Abbreviation: n_H, Hill coefficient. Results are mean ± S.E.M. Numbers in parentheses indicate the number of experiments. Data for atropine from Nilvebrant and Sparf (1983a, 1986).

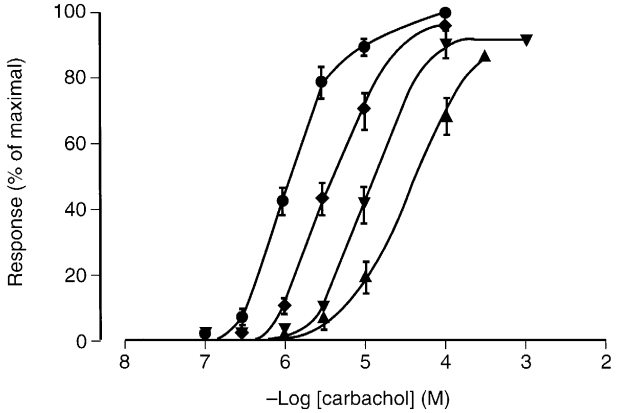

Fig. 3. Effect of tolterodine on the concentration-response curve to carbachol in human isolated urinary bladder. The contractile responses elicited by increasing concentrations of carbachol (cumulative additions) were recorded in the absence (control) and presence, respectively, of tolterodine at concentrations of 10 nM (♦), 30 nM (▼) and 100 nM (▲).The time of incubation with tolterodine was 20 min. Control data (●) represent the mean (±S.E.M.) of 21 individual experiments. The EC_{50} value for carbachol was 1.7±0.2 μM.

Tolterodine acted as a potent and competitive muscarinic receptor antagonist also in human isolated urinary bladder (Fig. 3). The Schild slope was 1.04 and pA_2 8.4. The affinity of tolterodine, expressed as the K_B, was 4.0 ± 0.5 nM ($n = 16$). In addition, tolterodine effectively reduced the contractile response of human detrusor strips induced by electrical stimulation (IC_{50} 64 nM) (Fig. 4).

3.1.2. Radioligand binding studies in tissue homogenates

The receptor-specific binding of [^{3}H](−)-1-quinuclidinyl benzilate in homogenates of human bladder, guinea-pig bladder (Fig. 5a), parotid gland (Fig. 5b), heart and cerebral cortex was effectively displaced by tolterodine. The dissociation constants (K_i) were in the nanomolar range and the Hill slopes were close to unity in all tissues (Table 1). The affinities determined for tolterodine (K_i 3.3 ± 0.4 nM; $n = 6$) and oxybutynin (K_i 4.5 ± 0.7 nM; $n = 6$) in human bladder were comparable to those in the guinea-pig bladder (Table 1). The K_i value for tolterodine (3.3 nM) in the human bladder was similar to the K_B value (4.0 nM) derived from the functional studies. This correlation between radioligand binding and functional data was also shown for both tolterodine and oxybutynin in guinea-pig bladder, whereas atropine was slightly less potent in binding than in functional studies (Table 1).

The binding affinities determined in the bladder were similar to those in the heart, which can be assumed to contain only muscarinic M_2 receptors. Tolterodine was roughly equipotent to oxybutynin and atropine in the

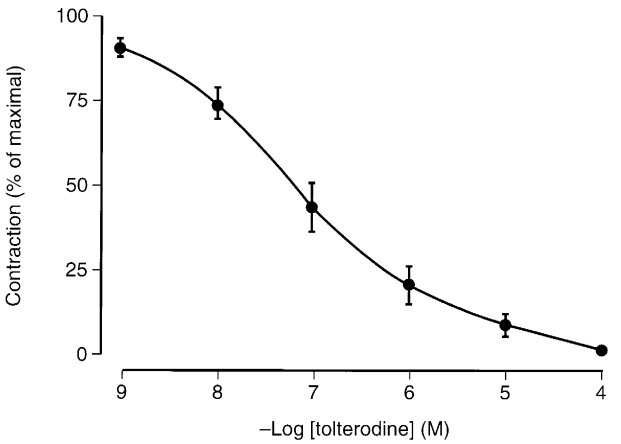

Fig. 4. Effect of tolterodine on electrically induced contraction (frequency 20–25 Hz, stimulus duration 0.8 ms, train duration 5 s, supramaximal voltage, stimulation interval of 2 min) of isolated preparations of human detrusor muscle ($n = 6$) from one individual. The IC_{50} value was determined to be 64 nM.

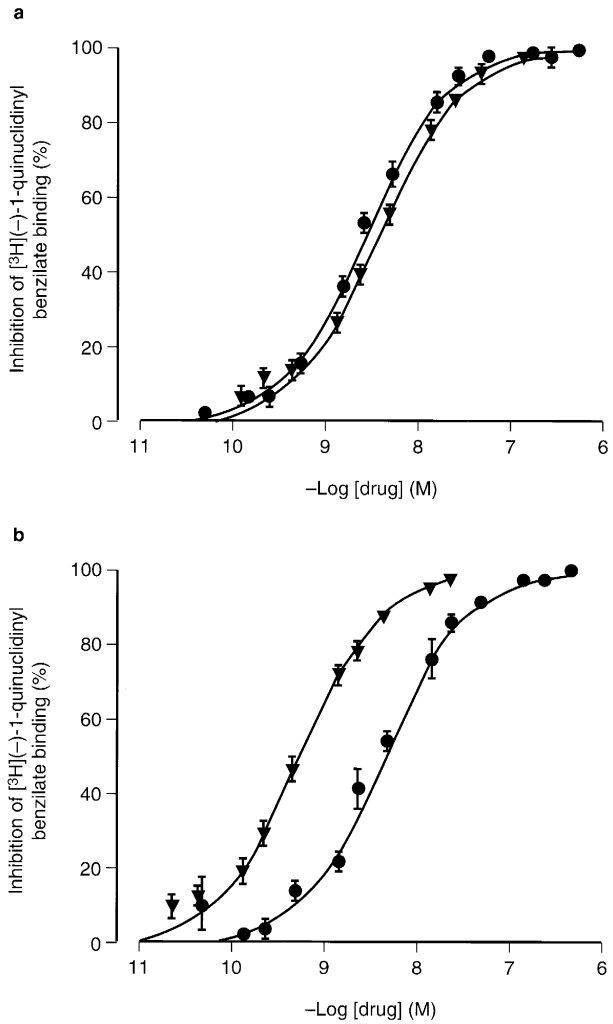

Fig. 5. Concentration-inhibition curves for tolterodine (●) and oxybutynin (▼) in homogenates of guinea-pig urinary bladder (a) and parotid gland (b). Data represent mean (±S.E.M.) of 5–6 individual experiments. The inhibition curves, corrected for the radioligand-induced parallel shift as described in Section 2, represent the theoretical curves expected for an interaction with a single homogeneous population of binding sites. Binding parameters are shown in Table 1.

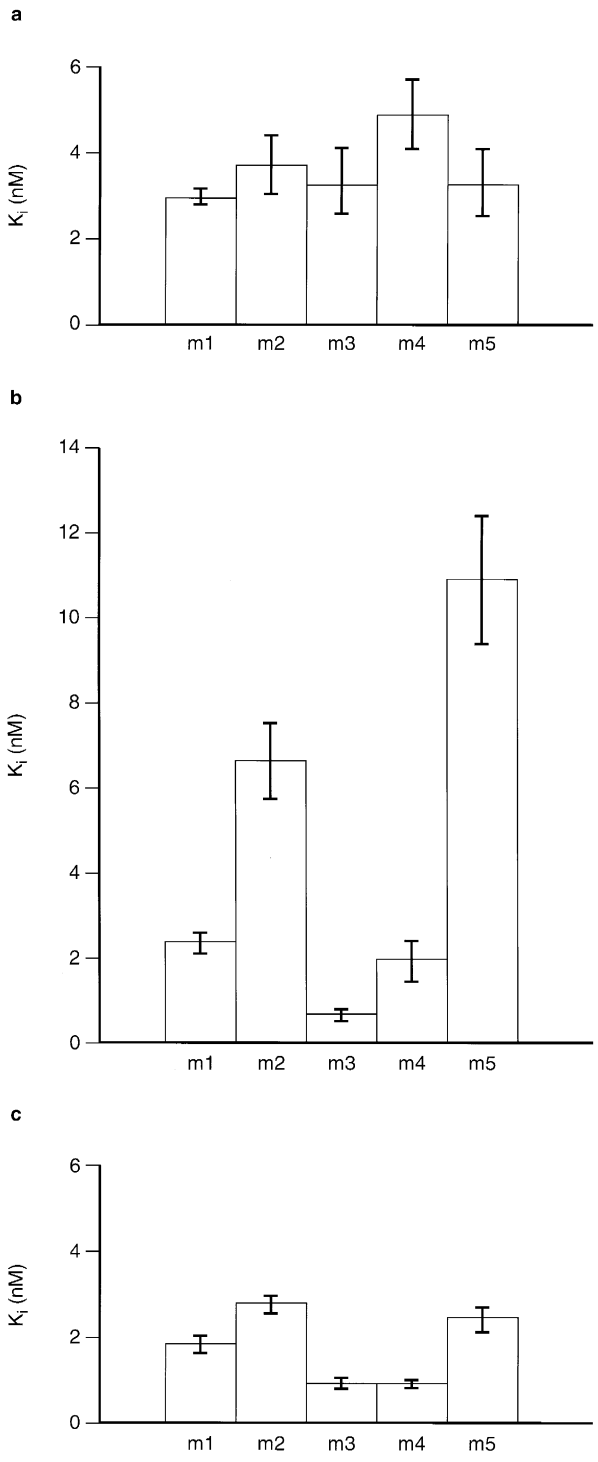

Fig. 6. Affinity profiles of tolterodine (a), oxybutynin (b) and atropine (c) at human m1–m5 muscarinic receptors expressed in Chinese hamster ovary cells. Binding studies were carried out under conditions of equilibrium, as described in Section 2. K_i values are shown in Table 2.

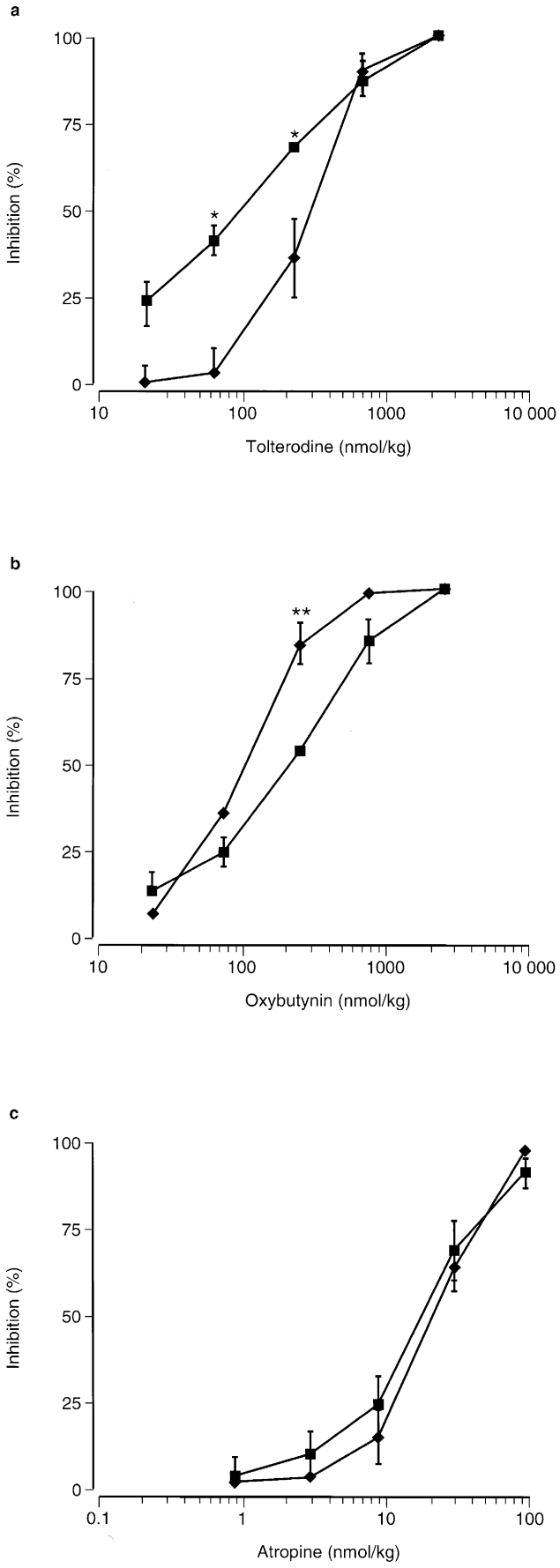

Fig. 7. Effect of tolterodine (a), oxybutynin (b) and atropine (c) on acetylcholine-induced urinary bladder contractions (■) and electrically induced salivation (◆) in the anaesthetised cat. The drugs were administered by intravenous infusion. Data are mean (±S.E.M.) of 5 animals. ID_{30} (threshold dose) and ID_{50} values are shown in Table 3.

Table 2
Affinities, expressed as the dissociation constants (K_i), for human muscarinic m1–m5 receptors expressed in Chinese hamster ovary cells

Compound	Parameter	Muscarinic receptor subtype				
		m1	m2	m3	m4	m5
Tolterodine	K_i (nM)	3.0 ± 0.2	3.8 ± 0.7	3.4 ± 0.8	5.0 ± 0.8	3.4 ± 0.8
	n_H	1.03 ± 0.04	1.00 ± 0.04	1.06 ± 0.03	1.05 ± 0.07	1.00 ± 0.05
Oxybutynin	K_i (nM)	2.4 ± 0.2	6.7 ± 0.9	0.67 ± 0.05	2.0 ± 0.5	11 ± 1.5
	n_H	1.03 ± 0.03	0.95 ± 0.03	1.06 ± 0.02	1.00 ± 0.03	1.04 ± 0.03
Atropine	K_i (nM)	1.9 ± 0.2	2.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.02	2.6 ± 0.3
	n_H	1.01 ± 0.04	0.95 ± 0.05	0.90 ± 0.03	1.01 ± 0.05	0.99 ± 0.03

K_i values were derived from competition binding experiments with [^{3}H](−)-1-quinuclidinyl benzilate, as described in Section 2. Abbreviation: n_H, Hill coefficient. Results are expressed as mean ± S.E.M. of 5–6 separate experiments.

guinea-pig bladder (Fig. 5a), heart and cerebral cortex (Table 1). However, in the parotid gland (M_3), tolterodine exhibited 8-times lower affinity than oxybutynin (Fig. 5b) and it was 6-times less potent than atropine (Table 1).

3.1.3. Radioligand binding studies in CHO cells

Tolterodine, oxybutynin and atropine were potent inhibitors of [^{3}H](−)-1-quinuclidinyl benzilate binding in homogenates of CHO cells expressing human muscarinic m1–m5 receptors (Table 2). In contrast to tolterodine, oxybutynin showed a distinct affinity profile, exhibiting the highest affinity for muscarinic m3 receptors and the lowest affinity for muscarinic m2 and m5 receptors (Fig. 6). The affinity of atropine at muscarinic m3 receptors was 2.9-times higher than at muscarinic m2 receptors. Oxybutynin, however, showed a 10-fold selectivity for muscarinic m3 over m2 (Table 2 and Fig. 6). In agreement with the data on cortical and cardiac homogenates, the affinity of tolterodine for muscarinic m1 and m2 receptors differed by less than a factor of two from those of oxybutynin and atropine. At muscarinic m3 receptors, oxybutynin was 5-times and atropine 3.4-times more potent than tolterodine.

3.2. In vivo studies

Intravenous infusion of tolterodine (21–2103 nmol/kg) in anaesthetised cats resulted in dose-dependent inhibition of acetylcholine-induced urinary bladder contractions and electrically induced salivation. However, the effect on urinary bladder contractions occurred at significantly lower doses than the effect on salivary secretion (Fig. 7), showing that tolterodine exhibited favourable tissue selectivity with respect to these two responses. Thus, the threshold dose (ID_{30}) for inhibition of bladder contractions was 40 nmol/kg, while 132 nmol/kg was required to produce a corresponding effect on salivary secretion (Table 3 and Fig. 7). This selectivity persisted at higher dose levels, i.e., when the bladder response was blocked by 75%, salivation was only inhibited by approximately 50% (Fig. 7). Tolterodine (21–631 nmol/kg) did not affect basal heart rate, while a slight decrease (< 10%) was observed after the highest dose (2103 nmol/kg).

In contrast to tolterodine, oxybutynin was significantly more potent in inhibiting salivary secretion than urinary bladder contractions (Table 3 and Fig. 7). Thus, salivation was blocked by 80% at the dose required for 50% inhibition of the urinary bladder response. At lower doses ($\leq ID_{30}$), urinary bladder contractions and salivary secretion were affected to a similar degree. Atropine showed no selectivity in this in vivo model (Table 3 and Fig. 7). Neither oxybutynin nor atropine showed any effect on heart rate. Infusion of saline to control animals ($n = 10$, five infusions per experiment) caused little or no effect per se.

Table 3
Inhibitory effects of tolterodine, oxybutynin and atropine on acetylcholine-induced urinary bladder contractions and electrically induced salivary secretion in the anaesthetised cat

Compound	ID_{30} (nmol/kg)		ID_{50} (nmol/kg)	
	Urinary bladder	Salivation	Urinary bladder	Salivation
Tolterodine	40 ± 8 [a]	132 ± 29	101 ± 8 [a]	257 ± 44
Oxybutynin	83 ± 13	50 ± 7	200 ± 23 [b]	104 ± 12
Atropine	9 ± 3	12 ± 2	18 ± 4	21 ± 4

The antagonists were administered by intravenous infusion. ID_{30} (threshold dose) and ID_{50} values were graphically determined. For purposes of comparison all data are given as nmol/kg. Results are mean ± S.E.M. of 5 separate experiments. [a] $P < 0.05$ vs. effect on salivation, [b] $P < 0.01$ vs. effect on salivation.

4. Discussion

Tolterodine is a new muscarinic receptor antagonist intended for the treatment of urinary urge incontinence and other symptoms of an overactive bladder. The aim of the present study was to compare the antimuscarinic properties of tolterodine with those of oxybutynin, the antimuscarinic drug most widely used in the treatment of urge incontinence (Wein et al., 1994; Yarker et al., 1995). The results of the in vitro studies showed that tolterodine is a potent and competitive antagonist at muscarinic receptors in urinary bladder preparations from guinea pigs and humans. Tolterodine was equipotent to oxybutynin, as shown by both functional and radioligand binding data in the guinea-pig bladder.

The potent antimuscarinic actions of tolterodine and oxybutynin on the bladder were confirmed in vivo in the anaesthetised cat. However, the most interesting finding was that tolterodine was significantly more potent in inhibiting urinary bladder contraction than salivation. Oxybutynin showed the reverse selectivity profile, while atropine was non-selective. Bladder contractions were induced by i.a. administration of acetylcholine and salivary secretion was induced by endogenous acetylcholine, released from the chorda-lingual nerve by electrical stimulation (Darke and Smaje, 1972). It may be argued that it would have been preferable to induce both responses using the same type of stimulation, i.e., either electrically or chemically with a muscarinic agonist given intravenously. However, both of these methods have practical limitations. Thus, bladder contractions induced by electrical stimulation of the pelvic nerve are resistant to blockade by atropine and other muscarinic receptor antagonists, i.e., the cholinergic component constitutes only about 30% of the response (unpublished observations), while the remainder is non-cholinergic non-adrenergic (NANC) mediated. This is well known to be the case in animal bladders, as shown in numerous in vitro and in vivo studies (Andersson, 1988, 1993; Wein et al., 1994). However, since it is generally accepted that bladder contractions in humans are mediated mainly by muscarinic receptors (Andersson, 1993; Wein et al., 1994), we found it more relevant to study only the cholinergic component of the bladder response in the cat. To avoid degradation of acetylcholine, it must be administered close to the target organ. Thus, we used i.a. injections because it is not possible to simultaneously stimulate both the bladder and the salivary glands by i.v. administration of acetylcholine without using doses high enough to kill the animals. We also tried to give stable analogues of acetylcholine (e.g., carbachol, methacholine) by i.v. injection. In general, however, this method resulted in an unstable baseline for the bladder response over time, together with a low and variable salivary secretion. The inhibition exerted by muscarinic antagonists could therefore not be reliably determined and reproduced between animals (data not shown).

In our hands, stimulation of the bladder with i.a. acetylcholine and stimulation of the salivary gland by electrical means turned out to be the methods of choice, in terms of reliable and consistent responses. Both responses were elicited by the natural transmitter, acetylcholine, but the concentration of acetylcholine at the muscarinic receptors in the bladder and salivary gland is obviously unknown, as always in in vivo experiments. Thus, it cannot be excluded that a difference in end-organ activation to some extent can explain the bladder selectivity of tolterodine. However, in this context, it should be noted that the corresponding in vivo data on the two reference compounds, atropine and oxybutynin, were clearly in line with the general clinical experience with these drugs (Andersson, 1988; Wein et al., 1994; Yarker et al., 1995).

The selectivity profiles in vivo were reflected in the radioligand binding studies. Thus, the affinity profile of tolterodine (cerebral cortex $\geq$ heart $\approx$ urinary bladder $>$ parotid gland) differed from those of oxybutynin (cerebral cortex $\approx$ parotid gland $>$ heart $\approx$ urinary bladder) and atropine (cerebral cortex $\geq$ parotid gland $\approx$ heart $\approx$ urinary bladder). In the cerebral cortex, heart and bladder, the three drugs were more or less equipotent. However, in the parotid gland, tolterodine was 6- and 8-times less potent, respectively, than atropine and oxybutynin. The guinea-pig urinary bladder contains a mixed population of muscarinic M_2/m2 and M_3/m3 receptors (Wang et al., 1995). The binding data on the bladder seemed to reflect the pharmacology of the muscarinic M_2 receptors, since oxybutynin exhibited a similar affinity in bladder (K_i 4.0 nM) and heart (M_2: K_i 2.8 nM). The K_i values and the selectivity for cortical (K_i 0.41 nM) and glandular (M_3: K_i 0.62 nM) muscarinic receptors found for oxybutynin are in agreement with previous reports (Nilvebrant and Sparf, 1982, 1983b, 1986; Lazareno et al., 1990).

The selectivity of oxybutynin for muscarinic M_3 over M_2 receptors was confirmed in radioligand binding studies on CHO cells expressing the human muscarinic receptor subtypes. Thus, while tolterodine was non-selective, oxybutynin showed a 10-fold selectivity for muscarinic m3 over m2 receptors (Table 2 and Fig. 6). The same rank order of potency was found in the cell lines containing muscarinic m1–m3 receptors as in the tissue homogenates, i.e., cerebral cortex and muscarinic m1 receptors: atropine $\approx$ oxybutynin $\approx$ tolterodine; heart and muscarinic m2 receptors: atropine $\approx$ tolterodine $\geq$ oxybutynin; parotid gland and muscarinic m3 receptors: oxybutynin $\approx$ atropine $>$ tolterodine. The only consistent difference between the drugs was found at the muscarinic m3 receptors, where tolterodine (K_i 3.4 nM) was less potent than either oxybutynin (K_i 0.67 nM) or atropine (K_i 1.0 nM). Thus, tolterodine showed the same affinity at muscarinic m2 and m3 receptors (K_i m2/K_i m3: 1.1), while both oxybutynin (K_i m2/K_i m3: 9.6) and atropine (K_i m2/K_i m3: 2.9) had higher affinity at the muscarinic m3 receptors.

The selectivity of oxybutynin for muscarinic M_3/m3 over M_2/m2 receptors may indicate that the reverse tissue selectivity demonstrated for tolterodine and oxybutynin in vivo can be explained by their different affinities for muscarinic M_3/m3 receptors (Fig. 6). This explanation is, however, not in line with the current concept that contraction of most smooth muscles, including the bladder, is mediated only by the muscarinic M_3/m3 receptors (Hulme et al., 1990; Caulfield, 1993; Eglen et al., 1994; Wang et al., 1995; Tobin and Sjögren, 1995). On the other hand, according to this concept, tolterodine would have been expected to be less potent than oxybutynin in inhibiting contraction of isolated urinary bladder, since the affinity of oxybutynin for muscarinic M_3/m3 receptors was 5–8-times higher than that exhibited by tolterodine. However, the two compounds were equipotent in functional studies

on guinea-pig bladder and there was no disparity between functional (K_B) and binding (K_i) data, neither for oxybutynin (K_B 4.4 nM; K_i 4.0 nM) nor for tolterodine (K_B 3.0 nM; K_i 2.7 nM). The K_B value for tolterodine was similar to K_i values determined for both the M_2/m2 (1.6 and 3.8 nM, respectively) and muscarinic M_3/m3 receptors (4.8 and 3.4 nM, respectively), which is consistent with the lack of selectivity for either of these subtypes. The affinity of oxybutynin derived from functional studies (K_B 4.4 nM), however, correlated better with its binding affinity at M_2/m2 (K_i 2.8 and 6.7 nM, respectively) than at muscarinic M_3/m3 receptors (K_i 0.62 and 0.67 nM, respectively). Thus, oxybutynin was 6–7-times less potent in inhibiting bladder contractions than in binding to muscarinic M_3/m3 receptors. Similar observations have been made with dicyclomine, i.e., the functional K_B value (24 nM) in the bladder is identical to the affinity for muscarinic M_2 receptors (24 nM) in the heart, but not to that determined for muscarinic M_3 receptors (2.8 nM) in the parotid gland (unpublished observations).

The tissue selectivity demonstrated for tolterodine in vivo cannot obviously be attributed to selectivity for a single muscarinic receptor subtype. However, the data on oxybutynin and atropine may indicate either that the muscarinic M_3/m3 receptors in glands are more sensitive to blockade than those in the urinary bladder, or that the muscarinic M_3/m3 receptors are heterogeneous (Eglen and Watson, 1996). An alternative explanation may be that also the muscarinic M_2/m2 receptors are involved in bladder contraction. The function of the large population of muscarinic M_2/m2 receptors present in most smooth muscles remains to be delineated, but it is clear that they mediate contraction under certain circumstances. Thus, muscarinic M_2 receptors mediate contraction of the uterus, which contains no muscarinic M_3 receptors (Eglen et al., 1989; Doods et al., 1993). In rat and guinea-pig ileum, stimulation of muscarinic M_2 receptors results in contraction when the muscarinic M_3 receptors have been selectively inactivated by alkylation (Thomas et al., 1993; Reddy et al., 1995). This has been interpreted as an indirect contraction caused by functional inhibition of β-adrenoceptor-mediated relaxation, since activation of muscarinic M_2 receptors counteracts the accumulation of cAMP induced by β-adrenoceptor stimulation (Griffin and Ehlert, 1992; Thomas et al., 1993; Eglen et al., 1994; Reddy et al., 1995). Similarly, it has been suggested that activation of muscarinic M_2 receptors in canine colon circular muscle directly enhances the effect of muscarinic M_3 receptor stimulation by preventing relaxation via inhibition of cAMP production (Zhang and Buxton, 1991). It is thus possible, as pointed out by Eglen et al. (1994), that both muscarinic M_2 and M_3 receptors modulate smooth muscle contraction and that the balance between the relaxant and contractile state is dependent on the prevailing parasympathetic or sympathetic drive. This is interesting with respect to urinary bladder function, since urine storage in some species is facilitated by sympathetic reflexes that mediate an inhibition of bladder activity during the filling phase (De Groat et al., 1993; Khadra et al., 1995). Carbachol inhibits adenylyl cyclase activity and both carbachol and acetylcholine stimulate the production of inositol phosphates in human detrusor (Andersson, 1993). Thus, in analogy with intestinal smooth muscles, activation of the muscarinic M_2 receptors in the bladder may contribute to the micturition contraction by counteracting the sympathetic relaxation.

Another candidate role for muscarinic M_2/m2 receptors in smooth muscle contraction has been indicated by Caulfield (1993), who suggested that low concentrations of agonist activate the muscarinic M_2/m2 receptors to evoke contraction via an inward current, causing depolarisation and opening of voltage-gated calcium channels, while higher concentrations of the agonist activate muscarinic M_3/m3 receptors to elicit contraction via an increase in IP_3 and intracellular calcium. Similarly, both muscarinic M_2 and M_3 receptors seem to be involved in the contractile response of the gall bladder. The muscarinic M_2 receptors appear to be linked to calcium influx, while the muscarinic M_3 receptors are preferentially associated with intracellular calcium release (Chen et al., 1995).

Muscarinic receptors involved in the regulation of transmitter release are present on both adrenergic and cholinergic nerves in the detrusor of different species (Andersson, 1993). The inhibitory muscarinic receptors on adrenergic neurones remain to be subclassified. However, postganglionic cholinergic nerves in the rat bladder (Somogyi and De Groat, 1992; Somogyi et al., 1994) and cholinergic nerves in rabbit detrusor (Tobin and Sjögren, 1995) exhibit a mixed population of inhibitory muscarinic M_2 and facilitatory muscarinic M_1 receptors. Facilitatory prejunctional muscarinic M_1 receptors have also been demonstrated in the human detrusor (Flood et al., 1994), and it has been suggested that they may serve as an amplification mechanism during micturition (Somogyi and De Groat, 1992; Somogyi et al., 1994; Flood et al., 1994). In guinea-pig bladder, the inhibitory muscarinic receptors on cholinergic nerves are reported to be of the muscarinic M_4 subtype (Alberts, 1995). Thus, the regulation of bladder function is complex and the relative importance of the different muscarinic receptor subtypes in vivo, under normal and various pathological conditions, is still unknown.

In conclusion, tolterodine was shown to be a potent and competitive antagonist at bladder muscarinic receptors. The results on tolterodine and oxybutynin showed that it is possible to separate the antimuscarinic effects on the bladder and salivary glands in vivo. The in vitro data indicated that the reverse selectivity patterns observed for tolterodine and oxybutynin may be related to the difference in relative affinity for muscarinic M_3/m3 and M_2/m2 receptors, and that the muscarinic M_2/m2 receptors may contribute to bladder contraction. The muscarinic receptor function in smooth muscles is obviously more complex than previ-

ously believed (Caulfield, 1993; Eglen et al., 1994) and, with respect to the urinary bladder, the relative functional importance of the postjunctional muscarinic M_2/m2 and M_3/m3 receptors and the prejunctional inhibitory (M_2/M_4) and facilitatory (M_1) receptors remains to be clarified, particularly in vivo.

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