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The subtypes of muscarinic receptors for neurogenic bladder contraction in rats

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Abstract

We evaluated in vivo functional selectivity profiles for muscarinic M_2 and M_3 subtypes of four muscarinic antagonists: Compound A (a novel muscarinic receptor antagonist with M_2-sparing antagonistic activity), darifenacin, (a muscarinic M_3 receptor antagonist); methoctramine (a muscarinic M_2 receptor antagonist) and tolterodine (a nonselective muscarinic receptor antagonist), and compared the inhibition potency on distention-induced bladder contraction in rats. In an in vivo functional study, Compound A (0.03–10 mg/kg, i.v.) showed antimuscarinic activity with high selectivity for M_3 (salivation) over M_2 (bradycardia) (>100-fold). Darifenacin (0.01–0.3 mg/kg, i.v.) showed only slight selectivity for M_3 over M_2 (3.7-fold). Methoctramine (0.003–1 mg/kg, i.v.) showed the reverse selectivity profile (0.077-fold). Tolterodine (0.003–0.3 mg/kg, i.v.) showed less selectivity (1.2-fold). Compound A at M_3 inhibitory doses (0.1 and 0.3 mg/kg, i.v.) showed inhibition in a distention-induced neurogenic bladder contraction model, and its maximal inhibitory effects were about 60% at an even higher dose (3 mg/kg). Methoctramine at M_2 inhibitory doses (0.03 and 0.1 mg/kg, i.v.) did not significantly affect distention-induced bladder contraction. When tolterodine and darifenacin caused inhibition of distention-induced bladder contraction, its maximal inhibitory effects were similar to that of Compound A. Therefore, these findings suggest that Compound A would be an excellent pharmacological tool to give a better understanding of which subtypes of muscarinic receptors act in bladder function so far, and muscarinic M_3, but not M_2, receptors mainly mediate the cholinergic component of distention-induced bladder contraction.

Keywords: Muscarinic receptor antagonist; Urinary bladder; Micturition reflex; Bradycardia; Salivation

1. Introduction

Muscarinic receptor subtypes with distinct, but homologous, gene sequences have been cloned and classified into five receptor subtypes including m1, m2, m3, m4, and m5. Structural and pharmacological criteria suggest the presence of at least four subtypes, denoted M_1, M_2, M_3, and M_4, while a physiological role for the m5 gene product remains to be identified (Bonner et al., 1988; Buckley et al., 1989). The cholinergic nervous system is the principal excitatory innervation to the detrusor smooth muscle of the urinary bladder, and pharmacological characterization of muscarinic receptors mediating contraction of detrusor muscle have been reported in several species including humans (Tobin and Sjögren, 1995; Lai et al., 1998; Newgreen and Naylor, 1996; Hegde and Eglen, 1999). The presence of m2 and m3, but not m1, m4, or m5, was confirmed by immunoprecipitation studies using human, rabbit, guinea pig, and rat urinary bladder (Wang et al., 1995). The ratio of m2:m3 in the bladder was 9:1 in the rat, but 3:1 in all other species.

Despite of the dominant presence of m2, in vitro functional studies using several muscarinic antagonists with different subtype selectivity indicated that, postsynaptically, muscarinic M_3 receptors are most responsible for detrusor contractility. However, an indirect and complex role of presynaptic and postsynaptic muscarinic M_2 receptors has been suggested in detrusor contractility. Presynaptic muscarinic M_2 receptors were shown to inhibit the release of acetylcholine from cholinergic nerve endings in rat bladder (Somogyi and de Groat, 1992; Braverman et al., 1998).

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Postsynaptic muscarinic M_2 receptors were shown to reverse adrenergic receptor-mediated relaxation under conditions in which muscarinic M_3 receptors are selectively alkylated (Caulfield, 1993; Longhurst et al., 1995; Eglen et al., 1996). Hegde et al. (1997) reported that the cholinergic contraction in the rat distention-induced bladder contraction model was inhibited by several muscarinic receptor antagonists such as 4-diphenylacetoxy-*N*-methyl piperidine methiodide (4-DAMP), atropine, methoctramine, darifenacin, pirenzepine, and *para*-fluorohexahydrosiladifenidol (*p*-F-HHSiD), and that the in vivo inhibition potency ($ID_{35\%\ inh}$) in the rat model correlated most favorably with the K_i values of these muscarinic antagonists at human recombinant muscarinic m2, but not m3, receptors. However, the modest potency of darifenacin does not exclude a role of muscarinic M_3 receptors in distention-induced bladder contraction, and it is not clear whether these muscarinic receptor antagonists have functional in vivo selectivity between muscarinic M_2- and M_3-mediated responses. Also, there is some question as to whether muscarinic M_2 receptors are most responsible for distention-induced bladder contraction in vivo.

4-DAMP and *p*-F-HHSiD were popularly used as selective muscarinic M_3 antagonists in the pharmacological characterization of muscarinic receptor subtypes although their muscarinic M_3 selectivity over M_2 receptors is only 10- to 20-fold in binding assays to cloned human muscarinic receptors (Maclagan and Barnes, 1989; Eglen et al., 1996). The in vivo selectivity window of these classical muscarinic antagonists on muscarinic M_2- or M_3-mediated functional in vivo responses, such as muscarinic receptor agonist-induced bradycardia or salivary secretion, is not clear. Because of the limited selectivity of the antagonists between muscarinic M_2 and M_3 subtypes, their pharmacological discrimination is important. Therefore, finding additional selective muscarinic M_3 receptor antagonists would be a powerful tool for the pharmacological characterization of these muscarinic subtypes. Recently, we obtained Compound A, (2*R*)-*N*-[1-(6-aminopyridin-2-ylmethyl)piperidin-4-yl]-2-[(1*R*)-3,3-difluorocyclopentyl]-2-hydroxy-2-phenylacetamide, which is identified as a novel muscarinic receptor antagonist with M_2-sparing antagonistic activity (Mitsuya et al., 2000). In particular, Compound A has extremely high selectivity for both human and rat muscarinic M_3 receptors over M_2 receptors (190-fold and 160-fold, respectively) (Hirose et al., 2001).

In the present study, we evaluated in vivo functional selectivity profiles for four muscarinic receptor antagonists: Compound A, a novel muscarinic receptor antagonist with M_2-sparing antagonistic activity; darifenacin, a muscarinic M_3 receptor antagonist; methoctramine, a muscarinic M_2 receptor antagonist; and tolterodine, a muscarinic receptor antagonist without subtype selectivity. In order to clarify the subtypes of muscarinic receptors for distention-induced bladder contraction, we compared the inhibition potency on distention-induced bladder contraction in rats.

2. Materials and methods

All experiments complied with the Guidelines for Biological and Pharmacological Experiments approved by Tsukuba Research Institute of Banyu Pharmaceutical and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

2.1. In vitro studies

2.1.1. Binding affinity for human muscarinic receptor subtypes

Specific binding of [^{3}H] *N*-methylscopolamine (New England Nuclear, USA) was determined using membranes from Chinese hamster ovary (CHO) cells expressing cloned human m1, m2, m3, m4, or m5 receptors (Receptor Biology, USA). The membrane preparations were incubated with 0.19–0.2 nM [^{3}H] *N*-methylscopolamine in 50 mM Tris–HCl–10 mM $MgCl_2$–1 mM EDTA (pH 7.4) for 2 h at room temperature. Final protein concentrations were 22 μg/ml (human m1), 70 μg/ml (human m2), 54 μg/ml (human m3), 20 μg/ml (human m4), and 116 μg/ml (human m5). Assays were performed in a total volume of 500 μl. Nonspecific binding was measured in the presence of 1 μM *N*-methylscopolamine; it accounted for less than 2% of total binding. Free and membrane-bound [^{3}H] *N*-methylscopolamine was separated by filtration over glass filters (UniFilter-GF/C; Packard Instruments, USA) using a cell harvester (Filtermate™ 196; Packard Instruments). Radioactivity was counted by a liquid scintillation counter (TopCount™; Packard Instruments).

The K_i values were calculated from the 50% inhibitory concentration (IC_{50}) values by using the equation:

$$K_i = IC_{50}/(1 + [L]/K_d)$$

where K_d was the dissociation constant of [^{3}H] *N*-methylscopolamine in each receptor subtype, and [*L*] was the concentration of [^{3}H] *N*-methylscopolamine (Chen and Prussoff, 1973). K_d values of [^{3}H] *N*-methylscopolamine in each receptor subtype were determined by Scatchard plot analysis. The following K_d and B_{max} values were used in this study. Data of human cloned receptors were extracted from Receptor Biology's Product Information Sheets (Receptor Biology).

Human m1 receptor: $K_d = 51$ pM, $B_{max} = 1.28$ pmol/mg protein. Human m2 receptor: $K_d = 290$ pM, $B_{max} = 1$ pmol/mg protein. Human m3 receptor: $K_d = 86$ pM, $B_{max} = 0.65$ pmol/mg protein. Human m4 receptor: $K_d = 56$ pM, $B_{max} = 1.44$ pmol/mg protein. Human m5 receptor: $K_d = 200$ pM, $B_{max} = 0.59$ pmol/mg protein.

2.1.2. Antagonism activity in rat bladder and right atria

Male Sprague–Dawley rats (270–500 g, Charles River Japan, Japan) were exsanguinated. The bladder and right atria were isolated. The bladder was freed of serosal con-

nective tissue and then cut into eight longitudinal unfolded sections. The bladder and right atria were placed in 5- and 20-ml organ baths containing modified Krebs-Henseleit solution maintained at 32 °C, were continuously aerated with 95% O_2 and 5% CO_2, and were connected to isometric transducers (TB-651T; Nihon Kohden, Japan) with sutures. Mechanical responses were recorded isometrically by a multichannel polygraph (RMP-6018; Nihon Kohden). Following stabilization of the bladder and right atria with an initial tension of 0.5 g, cumulative concentration–response curves to carbachol were obtained before and after addition of the test compounds. For the bladder assay, carbachol-induced contraction was measured, and the responses were expressed as a percentage of the contractile response to carbachol (100 μM). For the right atria assay, the beating rate was measured, and the responses were expressed as a percentage of basal beating rate.

The K_B value, an index of potency, was determined for each individual curve using the equation,

$$K_B = [C]/(\text{concentration ratio} - 1)$$

where concentration ratio is the ratio of 50% effective concentration (EC_{50}) values with or without the test drug, and $[C]$ is the concentration of the test drug. The EC_{50} values were calculated as the molar concentration of agonist producing 50% of maximum response.

2.2. In vivo studies

2.2.1. Acetylcholine-induced bradycardia

Male Sprague–Dawley rats (306–490 g) were used. The animals were anaesthetized with urethane (1 g/kg) and α-chloralose (25 mg/kg) injected intraperitoneally. The trachea, carotid artery, and jugular vein were cannulated after a midline neck incision. The animals were pretreated with succinylcholine (10 mg/kg s.c.) and then ventilated (6 ml/kg; 90 strokes/min; Model 683; Harvard Apparatus, USA) with room air. Heart rate was integrated from the blood pressure signal. Bradycardia was induced by the intravenous administration of acetylcholine (10 μg/kg) delivered into the jugular vein at 5-min intervals. Once three similar responses were obtained, test drugs were administered 5 min before the acetylcholine challenge.

2.2.2. Carbachol-induced salivary secretion

Male Sprague–Dawley rats (390–490 g) were anesthetized with pentbarbital (50 mg/kg) injected intraperitoneally. For the collection of saliva, the tip of a glass capillary tube (100 μl/75 mm, Drummond, USA) was inserted into the buccal part of the oral cavity in a prone position, and saliva was drained into the capillary tube. The collection of saliva was performed for 10 min after treatment with carbachol (10 μg/kg, i.v.). Salivary secretion was defined as the total amount of saliva for the 10-min collection period. Test drugs were administered 5 min before the carbachol challenge.

2.2.3. Distention-induced rhythmic bladder contraction

Male Sprague–Dawley rats (366–468 g) were anesthetized with urethane (1.2 g/kg s.c.). A cervical or jugular vein was catheterized for administration of the drugs. The abdomen was opened through a midline section, and then a small incision was made at the vesical apex area, avoiding the observable blood vessels in the surrounding areas. A small balloon (LB-7; LMS, Japan) was then inserted into the urinary bladder. The balloon was filled with 0.1 ml/kg of saline and then pressured in order to elicit rhythmic urinary bladder contractions. Bladder pressure was measured by a transducer (DX-312; Nihon Kohden) through a polyethylene tube connected to the intravesical balloon and recorded by a polygraph (RM7000; Nihon Kohden). In all experiments, bilateral ureters were cut, and the urethra was kept intact. After stable rhythmic bladder contraction was obtained for at least 30 min, test drugs were administered. The inhibitory effects of the drugs were estimated by the mean reduction in the amplitude of the bladder contraction for 30 min after treatment with test drugs.

2.3. Expression of results

Values are expressed as mean ± S.E. unless otherwise noted. Statistical analyses were performed by analysis of variance (ANOVA), and post hoc multiple comparison was performed with the modified *t*-test (Dunnett's). In in vivo studies, the percent inhibition was calculated from vehicle-treated control values. Dose–response curves were obtained by the use of least-squares linear regression analysis on the basis of the percent inhibition at each log–dose level, and 50% inhibitory doses (ID_{50}) were calculated from the dose–response curves.

2.4. Drugs and chemicals

Compound A, (2*R*)-*N*-[1-(6-aminopyridin-2-ylmethyl) piperidin-4-yl]-2-[(1*R*)-3,3-difluorocyclopentyl]-2-hydroxy-2-phenylacetamidewas synthesized at the Tsukuba Research Institute of Banyu Pharmaceutical as described in WO9805641 (Fig. 1). Darifenacin was synthesized as described in Pfizer's published patent application (EP388054). Tolterodine was synthesized as described in Pharmacia and Upjohn's published patent application (WO890664). [^{3}H] *N*-

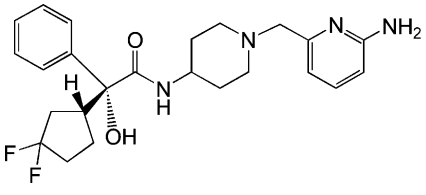

Fig. 1. Chemical structure of Compound A.

Table 1
Binding affinities of Compound A, darifenacin, methoctramine, and tolterodine to cloned human muscarinic receptor subtypes

Drug	K_i (nM) for muscarinic receptor subtypes					m2/m3 ratio
	m1	m2	m3	m4	m5	
Compound A	1.5 ± 0.1	540 ± 20.3	2.8 ± 0.03	15 ± 0.6	7.7 ± 0.3	193
Darifenacin	5.5 ± 0.4	47 ± 1.0	0.84 ± 0.03	8.6 ± 0.1	2.3 ± 0.1	56
Methoctramine	66 ± 7.6	8.2 ± 0.6	373 ± 3.3	65 ± 4.7	197 ± 14.5	0.022
Tolterodine	0.58 ± 0.06	7.2 ± 1.0	3.8 ± 0.2	2.4 ± 0.2	2.1 ± 0.2	1.9

Dissociation constants (K_i) were calculated by the method of Chen and Prusoff (1973) and are presented as the mean ± S.E. of three experiments. Hill coefficients of competition curves were not significantly different from unity.

methylscopolamine was purchased from New England Nuclear. All other reagents were purchased from Sigma (USA).

3. Results

3.1. In vitro studies

3.1.1. Binding affinity for human and rat muscarinic receptor subtypes

Compound A, darifenacin, methoctramine, and tolterodine all inhibited [^{3}H] *N*-methylscopolamine binding to cloned human muscarinic m1, m2, m3, m4, and m5 receptors expressed in CHO cells (Table 1). The Hill slopes for all the displacing ligands were not significantly different from unity. Compound A had high affinity for all muscarinic receptor subtypes, with the exception of the muscarinic m2 receptor. Its affinity for the human m3 receptors was 193-fold greater than that for human m2 receptors. Darifenacin had the highest affinity for the muscarinic m3 receptor and had 56-fold selectivity for the m3 over the m2 receptors. In contrast, methoctramine had the highest affinity for the muscarinic m2 receptor, followed by the muscarinic m1 and m4 receptors. Tolterodine displayed similar affinity for all of the subtypes of the muscarinic receptor.

3.1.2. Antagonistic activity in isolated rat bladder and right atria

Compound A showed concentration-dependent inhibition of carbachol-induced bladder contraction and carbachol-induced bradycardia (Fig. 2 and Table 2). Compound A produced parallel, rightward displacement of the concentration-effect curve to carbachol in rat right atria without significantly altering maximum responses, with a K_B value of 650 nM. However, in the bladder assay, the antagonism produced by Compound A, darifenacin, and tolterodine was characterized by nonparallel rightward displacement of the agonist curve and significant depression of the maximum response. An estimated K_B value for Compound A was calculated based on the corrected maximal response to 100%. Roughly estimated K_B values for carbachol-induced bladder contraction were 2.4 ± 0.7 nM for Compound A, 4.5 ± 2.2 nM for darifenacin, and 3.6 ± 0.6 nM for tolter-

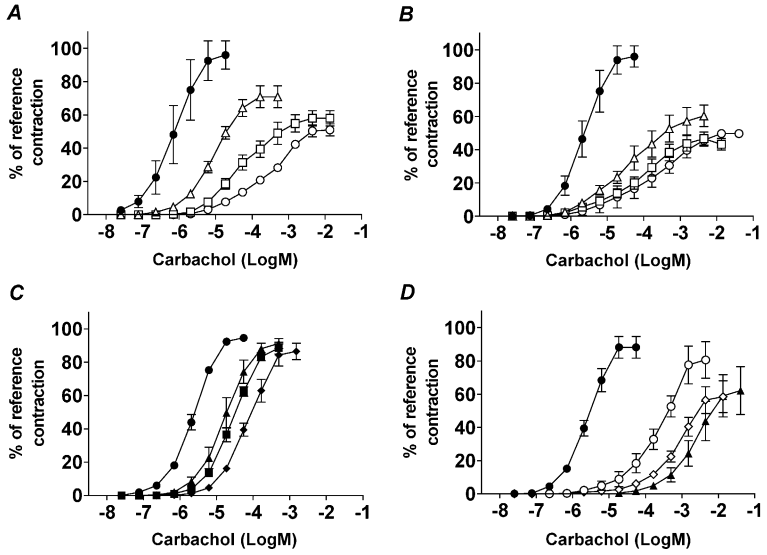

Fig. 2. Effects of Compound A, darifenacin, methoctramine, and tolterodine on the concentration–response curve to carbachol in the isolated rat bladder. (A) Compound A (●, vehicle; △, 0.03 μM; □, 0.1 μM; ○, 0.3 μM); (B) darifenacin (●, vehicle; △, 0.03 μM; □, 0.1 μM; ○, 0.3 μM); (C) methoctramine (●, vehicle; ▲, 3 μM; ■, 10 μM; ♦, 30 μM); (D) tolterodine (●, vehicle; ○, 0.3 μM; ◇, 1 μM; ▲, 3 μM). Data are presented as the mean ± S.E. of 3–5 preparations.

Table 2
Antagonism potencies (K_B values) of Compound A, darifenacin, methoctramine, and tolterodine in isolated rat bladder and right atria

Drug	K_B (nM)		Dose ratio (atria/bladder)
	Bladder	Atria	
Compound A	2.4 ± 0.7	650 ± 80	271
Darifenacin	4.5 ± 2.2	15 ± 2	3.3
Methoctramine	870 ± 110	12 ± 0.9	0.014
Tolterodine	3.6 ± 0.6	3.1 ± 0.7	0.86

For rat bladder assay, carbachol-induced contractions were measured, and the cumulative concentration–response curves to carbachol were obtained before and after addition of test drugs. The K_B values were calculated from the following equation: $K_B=[C]/(\text{Concentration Ratio}-1)$, where Concentration Ratio is the ratio of IC_{50} values with or without the antagonist and $[C]$ is the concentration of the antagonist. Dose ratio (atria/bladder) indicate the ratio of antagonist potency (K_B) for bladder and atria. The K_B values are presented as the mean ± S.E. of 3–12 preparations.

odine, respectively. Methoctramine produced a parallel, rightward shift of the concentration–response curve to carbachol without significantly altering the maximum response in the both bladder and right atria assays. The in vitro selectivity of Compound A for bladder over cardiac tissues was much greater (271-fold) than that of darifenacin (3.3-fold), methoctramine (0.014-fold), and tolterodine (0.86-fold).

3.2. In vivo studies

3.2.1. Acetylcholine-induced bradycardia

In anesthetized rats, i.v. injections of acetylcholine (10 μg/kg) caused reproducible and transient bradycardia. Four vehicle-treated groups for Compound A, darifenacin, methoctramine, and tolterodine showed almost similar acetylcholine-induced bradycardiac responses; these responses (in beats/min) were 206 ± 17 ($n=5$), 172 ± 17 ($n=5$), 186 ± 25 ($n=7$) and 218 ± 14 ($n=4$), respectively. Compound A, even at an i.v. dose of 3 mg/kg, did not affect acetylcholine-induced bradycardia (Fig. 3). However, about 50% inhibition was observed at 10 mg/kg, i.v. In contrast, darifenacin (0.03–0.3 mg/kg, i.v.), methoctramine (0.003–0.1 mg/kg, i.v.), and tolterodine (0.01–0.3 mg/kg, i.v.) potently inhibited the acetylcholine-induced bradycardia. The minimal anti-bradycardia doses were 10 mg/kg for Compound A, 0.3 mg/kg for darifenacin, 0.03 mg/kg for methoctramine, and 0.03 mg/kg for tolterodine, and their ID50 values were >10 mg/kg, 0.22 mg/kg, 0.036 mg/kg, and 0.086 mg/kg, respectively (Table 3).

3.2.2. Carbachol-induced salivary secretion

In anesthetized rats, i.v. injections of carbachol (10 μg/kg) caused salivary response. The maximal salivary flow was obtained 1–2 min after the carbachol challenge, and the salivary secretion returned to basal level (near zero) within 10 min. For a 10-min collection period, four vehicle-treated groups for Compound A, darifenacin, methoctramine, and tolterodine showed carbachol-induced salivary secretion, and the total volume (in μl) of saliva were 176 ± 14 ($n=4$), 156 ± 11 ($n=4$), 262 ± 42 ($n=6$) and 211 ± 40 ($n=4$), respectively. Intravenous injections of Compound A (0.03–0.3 mg/kg), darifenacin (0.01–0.1 mg/kg), methoctramine (0.03–1 mg/kg), and tolterodine (0.01–0.3 mg/kg) dose dependently inhibited carbachol-

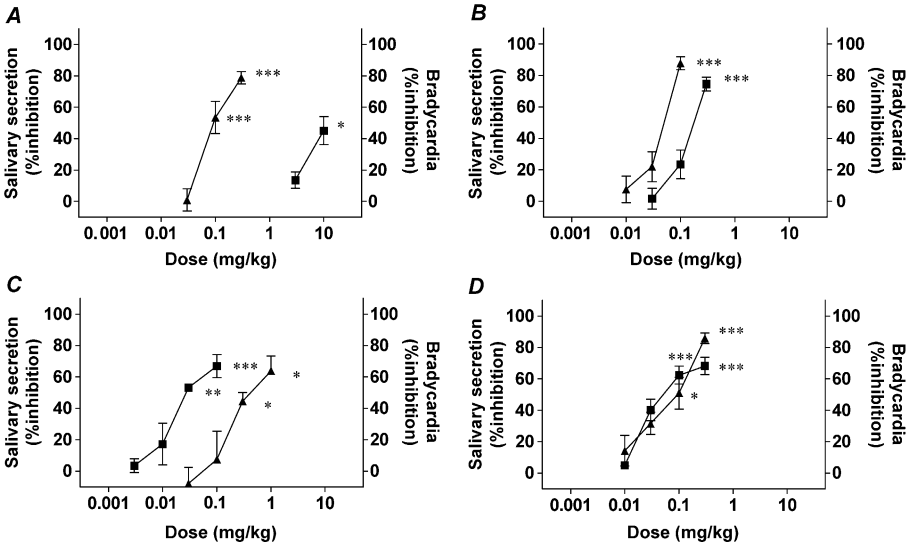

Fig. 3. Effects of Compound A, darifenacin, methoctramine, and tolterodine on carbachol-induced salivation (▲) and acetylcholine-induced bradycardia (■) in anesthetized rats. (A) Compound A; (B) darifenacin; (C) methoctramine; (D) tolterodine. The acetylcholine-induced bradycardiac response (in beats/min) in four vehicle-treated groups were 206 ± 17 for Compound A, 172 ± 17 for darifenacin, 186 ± 25 for methoctramine, and 218 ± 14 for tolterodine. The carbachol-induced salivary secretion (in μl) in four vehicle-treated groups were 176 ± 14 for Compound A, 156 ± 11 for darifenacin, 262 ± 42 for methoctramine, and 211 ± 40 for tolterodine. Data are presented as the mean ± S.E. of 4–7 animals. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$, ANOVA followed by Dunnett's test vs. vehicle. The ID_{50} values (doses required to produce 50% inhibition) of test drugs are shown in Table 3.

induced salivary secretion (Fig. 3). Their ID50 values were 0.1 mg/kg, 0.059 mg/kg, 0.47 mg/kg, and 0.071 mg/kg, respectively, and the in vivo selectivity for salivary gland over heart were >100-fold, 3.7-fold, 0.077-fold, and 1.2-fold, respectively. In addition, the minimal anti-salivation doses were 0.1 mg/kg for Compound A, 0.1 mg/kg for darifenacin, 0.3 mg/kg for methoctramine, and 0.1 mg/kg for tolterodine.

3.2.3. Distention-induced rhythmic bladder contraction

Distention of the urinary bladder by infusion of saline induced rhythmic contractions. Four vehicle-treated groups for Compound A, darifenacin, methoctramine, and tolterodine showed distention-induced rhythmic bladder contraction, and the amplitudes of the contraction (in mm Hg) were 30.4 ± 1.5 ($n=4$), 46.9 ± 7.9 ($n=4$), 35.8 ± 8.2 ($n=5$), and 31.7 ± 4.7 ($n=4$), respectively. The contractions were completely eliminated by dropping tetrodotoxin (3 μg/0.1 ml) onto the surface of bladder dome (data not shown). Intravenous injections of Compound A (0.01–0.3 mg/kg) inhibited the amplitude of rhythmic bladder contraction induced by increased intravesical volume in rats (Fig. 4). Compound A at M_3 inhibitory doses of 0.1 and 0.3 mg/kg, which significantly inhibited carbachol-induced salivary secretion, produced 60.4 ± 4.33% ($n=5$, $P<0.001$) and 52.1 ± 5.23% ($n=5$, $P<0.001$) inhibition of rhythmic bladder contraction, respectively. Similarly, darifenacin (0.01–0.1 mg/kg, i.v.) and tolterodine (0.01–0.3 mg/kg, i.v.) also inhibited rhythmic bladder contraction, and the inhibition of darifenacin (0.1 mg/kg) and tolterodine (0.3 mg/kg) occurred in 56.1 ± 7.2% ($n=5$, $P<0.001$) and 56.1 ± 4.1% ($n=5$, $P<0.001$), respectively. Methoctramine at M_2 inhibitory doses of 0.03 and 0.1 mg/kg, which significantly inhibited acetylcholine-induced bradycardia, did not show any significant inhibition of rhythmic bladder contraction; inhibition values were only 6.5 ± 9.1% ($n=4$) and 12.3 ± 9.2% ($n=5$), respectively.

Table 3
Inhibition potencies (ID_{50}) of Compound A, darifenacin, methoctramine, and tolterodine on carbachol-induced salivation and acetylcholine-induced bradycardia in anesthetized rats

Drug	ID_{50} (mg/kg, i.v.)		Dose ratio (bradycardia/salivation)
	Salivation	Bradycardia	
Compound A	0.1 (0.065–0.16)	≥ 10	≥ 100
Darifenacin	0.059 (0.041–0.083)	0.22 (0.16–0.33)	3.7
Methoctramine	0.47 (0.32–0.80)	0.036 (0.023–0.074)	0.077
Tolterodine	0.071 (0.043–0.13)	0.086 (0.057–0.14)	1.2

The ID_{50} values (doses required to produce 50% inhibition) were calculated from dose–response curves with least-squares linear regression analysis from the percent inhibition of salivation (carbachol-induced salivary secretion) or bradycardia (acetylcholine-induced decreases in heart rate) vs. the log molar doses of the drugs. The values in parentheses indicate the 95% confidence limits.

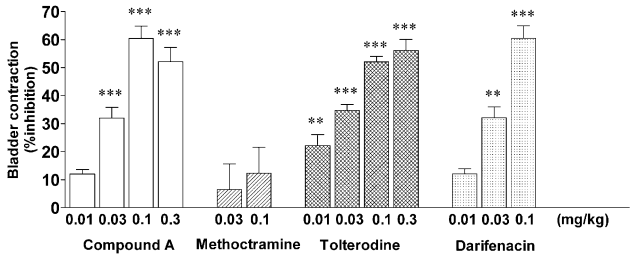

Fig. 4. Effects of Compound A, methoctramine, and tolterodine on distention-induced rhythmic bladder contraction in anesthetized rats. (A) Compound A; (B) darifenacin; (C) methoctramine; (D) tolterodine. The amplitude of distention-induced rhythmic bladder contraction (in mm Hg) in four vehicle-treated groups were 30.4 ± 1.5 for Compound A, 46.9 ± 7.9 for darifenacin, 35.8 ± 8.2 for methoctramine, and 31.7 ± 4.7 for tolterodine. Data are presented as the mean ± S.E. of 4–7 animals. ** $P<0.01$ and *** $P<0.001$, ANOVA followed by Dunnett's test vs. vehicle.

4. Discussion

The present study indicates that Compound A has a discriminative selectivity between M_2 and M_3 receptor-mediated responses in vitro and in vivo, and that the cholinergic component in rat distention-induced neurogenic urinary bladder contraction is mediated through the muscarinic M_3 receptor subtype. We have reported that Compound A binds reversibly and competitively with high affinity to muscarinic receptors in competition and saturation binding studies (Hirose et al., 2001). In the present study, Compound A inhibited carbachol-induced bladder contraction and bradycardia with K_B values of 2.4 and 650 nM, respectively. The K_i values of Compound A on cloned human muscarinic m3 and m2 were 2.8 and 540 nM, respectively. These results suggest that Compound A has >270-fold functional in vitro selectivity for bladder over cardiac muscarinic receptors, and the in vitro muscarinic antagonist potency and selectivity of Compound A for bladder and heart are nearly consistent with its affinity and selectivity for muscarinic m3 and m2 receptors obtained by binding assays. In addition, we also evaluated the functional in vitro antagonist potency and selectivity of darifenacin (high affinity for muscarinic M_3 receptors), methoctramine (high affinity for muscarinic M_2 receptors), and tolterodine (less subtype selective for distinct muscarinic receptors). Darifenacin showed slight selectivity for bladder over heart (3.3-fold). Methoctramine had the reverse selectivity profiles (0.014-fold) for bladder and heart, and tolterodine exhibited inhibition for both in vitro assays without tissue selectivity (0.86-fold). Thus, a comparison of the functional in vitro selective profiles of Compound A, darifenacin, methoctramine, and tolterodine suggests that the bladder selectivity of Compound A is much higher than that of darifenacin, methoctramine or tolterodine. It has been reported that mRNA for muscarinic m2 and m3 receptors are

present in rat urinary bladder, and that the ratio of m2 and m3 is 9:1 (Maeda et al., 1988; Wang et al., 1995). Despite of the dominant presence of m2, the inhibition potency of Compound A for bladder has a similar K_B value to that of tolterodine (2.4 vs. 3.6 nM). Therefore, in view of the M_3 selectivity of Compound A over M_2, it seems that the muscarinic agonist-induced contraction responses in bladder smooth muscle are mainly mediated through the muscarinic M_3, but not M_2, receptors. Functional in vitro data of Compound A are consistent with other investigators' results using several muscarinic antagonists, which indicated that muscarinic M_3 receptors mediated most of the detrusor contractility.

In in vivo functional studies, intravenous Compound A inhibited carbachol-induced salivation with an ID_{50} value of 0.1 mg/kg. However, Compound A, even at an i.v. dose of 3 mg/kg, did not affect acetylcholine-induced bradycardia, and 10 mg/kg of Compound A showed about 50% inhibition (ID_{50}, >10 mg/kg). These results indicate that Compound A has, at least, 100-fold in vivo selectivity for muscarinic M_3 receptors vs. M_2 receptors, and this in vivo selectivity is almost consistent with the in vitro selectivity either in binding assay (m2/m3, 193-fold) or functional tissue assay (heart M_2/bladder M_3, 271-fold). In addition, parallel in vivo studies with tolterodine showed this drug to be nonselective between salivation and bradycardia. Therefore, our in vivo assays for salivation and bradycardia correspond to the in vivo antagonism potency of functional muscarinic M_3 and M_2 receptors, respectively. It is demonstrated that Compound A has a muscarinic M_3 receptor antagonistic action without M_2 inhibition at the dose range of 0.1–3 mg/kg. Although methoctramine has in vivo muscarinic M_2 receptor antagonistic action, methoctramine has a slight selectivity for muscarinic M_2 over M_3 function in vivo (bradycardia/salivation, 0.077-fold). In this study, in vivo muscarinic M_2-selective doses of methoctramine might only be limited to the dose range of 0.03 to 0.1 mg/kg (i.v.). It has been reported that 1000 nmol/kg (0.6 mg/kg) and higher doses of methoctramine produced hypotensive responses (Hegde et al., 1997). Therefore, that the selective muscarinic M_2 inhibitory dose range of methoctamine was limited to narrow the window between 0.03 and 0.1 mg/kg (i.v.) may have some indirect and/or secondary relevance because of systemic hypotensive action. On the other hand, darifenacin has been reported as a selective muscarinic M_3 antagonist (Alabaster, 1997). In our experiments, however, darifenacin showed only a slight functional selectivity both in vitro and in vivo assays. Its in vivo selectivity of darifenacin for muscarinic M_3 over M_2 function was 3.7-fold, suggesting in vivo muscarinic M_3-selective doses of darifenacin would be limited to the narrow dose range of 0.03 to 0.1 mg/kg (i.v.). Alabaster's (1997) report suggested that darifenacin was able to differentiate between muscarinic M_3 receptors in different tissues because darifenacin showed a degree of selectivity for guinea pig ileum relative to other smooth muscle preparation such as trachea and bladder or salivary gland. The molecular mechanism for this selectivity is not still unclear because different subtypes of muscarinic M_3 receptor has not been identified from molecular sequence studies. It has been reported that there are some differences of affinity of antagonist between cloned and endogenous muscarinic receptors (Eglen et al., 1996). Therefore, it is likely that receptor configuration or antagonist binding would be affected by tissue-dependent factors such as membrane receptor glycosylation or lipid concentration, or the presence of other muscarinic receptor subtypes in the tissue membrane. Thus, Compound A is an excellent pharmacological tool to exhibit muscarinic M_3-mediated responses without affecting M_2 receptors in vivo experiments. On the other hand, methoctramine and darifenacin at higher doses should be used with caution because of its selectivity.

In order to investigate bladder contraction mediated by micturition reflex, we employed the distention-induced rhythmic bladder contraction model in rats (Maggi et al., 1986). Because the contractions were completely eliminated by local treatment with tetrodotoxin on the bladder, the distention-induced rhythmic bladder contractions were neurogenic in nature. In this study, 0.1 and 0.3 mg/kg of Compound A significantly inhibited distention-induced bladder contraction, and the maximum inhibitory effect (approximately 60% inhibition) was comparable to that observed with darifenacin and tolterodine. In addition, even higher doses of Compound A that inhibited M_2 function did not show any additional inhibition of distention-induced bladder contraction. On the contrary, 0.03 and 0.1 mg/kg of methoctramine did not significantly affect distention-induced bladder contraction. Therefore, these findings clearly suggest that muscarinic M_3 receptor activation plays a major role in the cholinergic component (~ 60%) of distention-induced bladder contraction. Hegde et al. (1997) reported that there was good correlation between in vivo potency (ID_{35}) values of the muscarinic antagonists on distention-induced bladder contraction and the corresponding binding affinity (K_i) values at the recombinant human muscarinic m2, but not m3, receptor. However, these authors used 4-DAMP, methoctramine, daifenacin, pirenzepine, and *p*-F-HHSiD as muscarinic antagonists, which have limited selectivity between M_2 and M_3 subtypes. The in vivo discrimination of these muscarinic antagonists between muscarinic M_2- and M_3-mediated responses is arguable. In fact, our results demonstrated that darifenacin and methoctramine have narrow window for selectivity between muscarinic M_3 and M_2 function in vivo. In addition, other investigators indicate that 4-DAMP and *p*-F-HHSiD show only 10- to 20-fold selectivity for muscarinic M_3 over M_2 receptors in binding assays to cloned human muscarinic receptors (MacLagan and Barnes, 1989; Eglen et al., 1996), and that darifenacin shows only 16-fold selectivity for bladder muscarinic M_3 over atria M_2 receptors in isolated tissue assays (Alabaster, 1997). The classical or available muscarinic antagonists

might have the limited selectivity between muscarinic M_2 and M_3 subtypes. Consequently, we can assert that Compound A at a selective muscarinic M_3 inhibitory dose without M_2 inhibition significantly inhibited distention-induced bladder contraction because Compound A possesses extremely high in vitro and in vivo selectivity (>100-fold) for muscarinic M_3 over M_2 receptors. However, Compound A had high affinity for not only muscarinic M_3 receptor subtype, but also M_1, M_4, and M_5. We need to take care of complemental or multiple responses of muscarinic M_1, M_4, and M_5 receptor subtypes.

We did not find any evidence for muscarinic M_2-mediated responses on distention-induced bladder contraction. However, functionally counteracting roles between pre- and postsynaptic muscarinic M_2-mediated responses have been suggested in detrusor contractility. Presynaptic muscarinic M_2 receptors inhibit the release of acetylcholine from cholinergic nerve endings, and the inhibition of muscarinic M_2 receptors enhance the neurogenic contraction induced by electrical field stimulation in rat bladder (Somogyi and de Groat, 1992; Braverman et al., 1998). In contrast, postsynaptic muscarinic M_2 receptors reduced β-adrenergic mediated relaxation, and the inhibition of muscarinic M_2 receptors would contribute indirectly to the inhibition of bladder contraction (Maggi and Meli, 1982; Caulfield, 1993; Longhurst et al., 1995; Eglen et al., 1996). Therefore, these two counteracting effects of muscarinic M_2 receptors may mask M_2 function on distention-induced bladder contraction. In general, sympathetic and parasympathetic tone is affected by sensory nerve input such as detrusor tones and intravesical pressure. The contribution of pre- and postsynaptic muscarinic M_2 receptors may be affected by experimental conditions. Further study is needed to evaluate the contribution of M_2 function under the pathological condition of hypertrophy of detrusor after partial outlet obstruction, which shows a twofold increase in muscarinic receptor binding sites (Beavington et al., 1993; Krichevsky et al., 1999).

In conclusion, Compound A would be an excellent pharmacological tool to give a better understanding of which subtypes of muscarinic receptors when compared to other antimuscarinic agents. Furthermore, Compound A shows potent antagonistic activity at bladder muscarinic receptors and significantly inhibits the in vivo distention-induced neurogenic bladder contraction at selective M_3 inhibitory doses without M_2 inhibition. Therefore, these results indicate that muscarinic M_3, not M_2, receptor activation causes distention-induced bladder contraction.

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