Comparative Evaluation of Exocrine Muscarinic Receptor Binding Characteristics and Inhibition of Salivation of Solifenacin in Mice

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Received February 27, 2006; accepted April 8, 2006

Anticholinergic agents such as oxybutynin are clinically useful in the treatment of overactive bladder. However, oral administration of oxybutynin is frequently accompanied by side effects such as dry mouth, and novel bladder-selective anticholinergic agents such as solifenacin and tolterodine are now under development. The aim of the present study was to characterize the suppression of cholinergic salivation and exocrine muscarinic receptor binding of solifenacin on oral administration to mice in comparison with those of oxybutynin. Results showed that both drugs produced a significant increase in K_d values for specific [*N*-Methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) binding in the mouse submaxillary gland, compared with control values. However, this enhancement in K_d values was significantly smaller with solifenacin than with oxybutynin. Moreover, the inhibitory effect of solifenacin on pilocarpine-induced salivary secretion was significantly weaker than that of oxybutynin. Solifenacin dissociated more readily from muscarinic receptors in the mouse submaxillary gland than oxybutynin. In conclusion, the present study indicates that the weak suppression of cholinergic salivation by solifenacin compared with oxybutynin may be partially attributed to its relatively fast dissociation kinetics from exocrine muscarinic receptors.

Key words anticholinergic agent; muscarinic receptor; submaxillary gland; dissociation kinetics; salivation

Overactive bladder is defined by the International Continence Society (ICS) as symptoms of urgency with or without urge incontinence, usually with increased frequency and nocturia.[1] Since both normal voiding processes and involuntary detrusor contractions during bladder filling are mediated by muscarinic receptors, anticholinergic agents, such as oxybutynin, are currently recommended as first-line therapy for the treatment of overactive bladder.[2,3] Although the efficacy of these agents has been demonstrated,[4,5] their side effects cause problems in geriatric patients. Especially, the dry mouth caused by oral oxybutynin, a more potent inhibitor of cholinergic salivation than bladder contraction,[6,7] often leads to the discontinuation of treatment. Bladder-selective anticholinergic agents such as solifenacin and tolterodine which may reduce or even eliminate these problems are now under development.[6—12] In particular contrast to oxybutynin, the attenuation of cholinergic responses by solifenacin has been shown to be less potent in salivary gland than in bladder detrusor muscle. However, the mechanism underlying solifenacin's relatively weak inhibition of salivary secretion has not been clarified.

The therapeutic and unwanted effects of anticholinergic agents in patients with overactive bladder stem from the blockade of muscarinic receptors in the bladder and non-target tissues, respectively. Muscarinic receptors have been classified into five subtypes (M_1—M_5), based on genetic and/or pharmacological properties.[13,14] The M_3 subtype is preferentially distributed in the salivary gland,[14,15] and has been shown using M_3 receptor knockout mice to play a key role in salivary secretion.[16] Oxybutynin and solifenacin have shown high affinity for this subtype in *in vitro* binding assays with human muscarinic receptor subtypes expressed in Chinese hamster ovary cells.[17,18] The present study was therefore conducted to characterize the suppression of cholinergic salivation and exocrine muscarinic receptor binding in mice receiving oral administration of solifenacin or oxybutynin.

MATERIALS AND METHODS

Materials [*N*-Methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS, 3.03 TBq/mmol) was purchased from Perkin Elmer Life Sciences, Inc. (Boston, MA, U.S.A.). Oxybutynin hydrochloride (oxybutynin) was donated by Meiji Milk Products Co. Ltd. (Odawara, Japan) and solifenacin succinate (solifenacin) by Astellas Pharmaceutical Company (Tsukuba, Japan). All other chemicals were purchased from commercial sources.

Animals Male ddY strain mice aged 9 to 11 weeks (Japan SLC Inc., Shizuoka, Japan) were housed under a 12 h light–dark cycle and fed laboratory chow and water *ad libitum*.

Drug Administration Mice were fasted for 16 h, and then orally administered oxybutynin (76.1 μmol/kg) or solifenacin (62.4, 208 μmol/kg) dissolved in distilled water. Control animals received vehicle alone. Measurement of salivary secretion and muscarinic receptor binding assay was carried out at 0.5 h (oxybutynin) and 2 h (solifenacin) after administration, the times of maximum receptor binding activity.[11] The study was conducted in accordance with the guidelines of the Experimental Animal Ethical Committee of the University of Shizuoka.

Measurement of Salivary Secretion Mice were anesthetized by intraperitoneal administration of pentobarbital (161 μmol/kg). Residual saliva in the oral cavity was removed with a cotton ball, and then total saliva collected in the cavity for a 10-min period was measured by absorption onto three to five cotton balls followed by immediate weighing on an electric balance to prevent moisture loss. To examine the effects of oral administration of anticholinergic agents on pilocarpine-evoked salivary secretion, pilocarpine (12.3 μmol/kg, dissolved in physiological saline) was intravenously injected 0.5 or 2 h after oral administration of vehicle or drugs, and saliva was collected for 10 min. The weight

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of the evoked saliva was estimated as the difference between the weight of cotton balls collected before and after pilocarpine application.

Tissue Preparation Mice were sacrificed by bleeding from the descending aorta under temporary anesthesia with diethyl ether, and the tissues were perfused with cold saline via the aorta. The submaxillary gland was then dissected and homogenized in a Kinematica Polytron homogenizer in 19 volumes of ice-cold 30 mM Na^+/HEPES buffer (pH 7.5), and the homogenates were centrifuged at 40000×***g*** for 20 min. The resulting pellet was finally suspended in the buffer for binding assay. Protein concentrations were measured by the method of Lowry *et al*.[19]

Muscarinic Receptor Binding Assay Binding assay for muscarinic receptors was performed using [^{3}H]NMS as previously described.[20,21] Mouse submaxillary gland homogenate was incubated with [^{3}H]NMS (125 pM) in 30 mM Na^+/HEPES buffer. Incubation was carried out for 60 min at 25 °C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel Co., Gaithersburg, MD, U.S.A.) through Whatman GF/B glass fiber filters, and the filters were then rinsed twice with 3 ml of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters by overnight immersion in scintillation fluid (2 l toluene, 1 l Triton X-100, 15 g 2,5-diphenyloxazole, 0.3 g 1,4-bis[2-(5-phenyloxazolyl)]benzene), and radioactivity was determined with a liquid scintillation counter. Specific [^{3}H]NMS binding was determined experimentally from the difference between counts in the absence and presence of 1 μM atropine. All assays were conducted in duplicate.

Dissociation Analysis from Muscarinic Receptors The dissociation experiment was performed as previously described for brain nicotinic acetylcholine receptors.[22] After homogenization of mouse submaxillary gland as described above, the homogenate was incubated with oxybutynin (3 μM) and solifenacin (30 μM) for 60 min at 25 °C. A part of each homogenate was reserved as a sample without washout (no-washout sample), and the rest was then centrifuged at 40000×***g*** for 20 min at 4 °C. A part of the suspension (homogenate) was treated with cold buffer and reserved as a single-washout sample. The residual homogenate was further centrifuged under the above conditions and the resulting pellet was resuspended in the cold buffer as the double-washout sample.

Data Analysis Analysis of [^{3}H]NMS binding data was performed as described previously.[23] The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]NMS were obtained by Rosenthal analysis of the saturation data. Inhibition of specific [^{3}H]NMS (125 pM) binding was estimated from IC_{50} values, namely the molar concentration of unlabeled drugs necessary to displace 50% of specific [^{3}H]NMS binding as determined by log probit analysis. The inhibition constant, K_i was calculated from the equation $K_i = IC_{50}/(1+L/K_d)$, where L is the concentration of [^{3}H]NMS. For analysis of dissociation kinetics, the dissociation rate (%) was determined from the difference in specific [^{3}H]NMS binding between the no- and single-washout and between the single- and double-washout samples. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. A value of $p<0.05$ was considered significant.

RESULTS

Muscarinic Receptor Binding Affinities in Submaxillary Gland Oxybutynin (1—300 nM) and solifenacin (3—300 nM) inhibited specific [^{3}H]NMS binding in the mouse submaxillary gland in a concentration-dependent manner *in vitro* (Fig. 1), with K_i values (nM) of 6.91±0.86 and 13.8±1.1 (mean±S.E., $n=5$), respectively. Thus, the K_i value for solifenacin was 2.0 times larger than that for oxybutynin. This difference was statistically significant ($p<0.01$).

Effects on Salivary Secretion Intravenous injection of pilocarpine (12.3 μmol/kg) significantly increased salivary secretion in mice. As shown in Fig. 2, oral administration of oxybutynin (76.1 μmol/kg) significantly suppressed (85.5%) this pilocarpine-induced salivation. Similarly, oral administration of solifenacin (62.4, 208 μmol/kg) also significantly and dose-dependently (30.0%, 60.5%, respectively) inhibited this salivation. The pilocarpine-induced salivary secretion was significantly greater in mice after oral administration of solifenacin at 62.4 μmol/kg than after oral oxybutynin.

Effects of Oral Administration of Anticholinergic Agents on Exocrine Muscarinic Receptors Specific

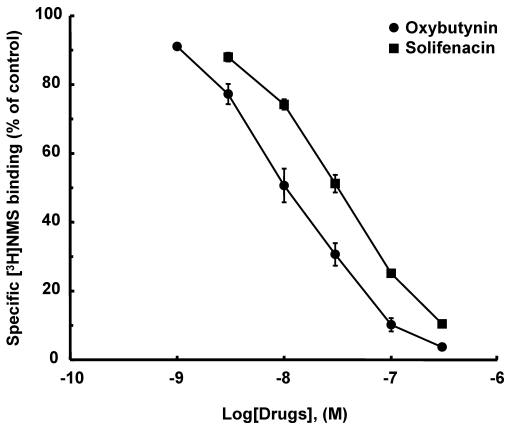

Fig. 1. Competitive Inhibition by Oxybutynin and Solifenacin of Specific [^{3}H]NMS Binding in the Mouse Submaxillary Gland

Specific [^{3}H]NMS (125 pM) binding was determined in the presence of different concentrations of these agents. Each point represents the mean±S.E. of five mice.

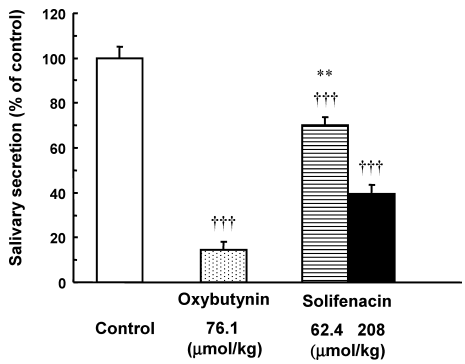

Fig. 2. Effects of Oral Administration of Oxybutynin and Solifenacin on Pilocarpine-Induced Salivary Secretion in Mice

Mice were given oxybutynin (76.1 μmol/kg) and solifenacin (62.4, 208 μmol/kg) by oral administration, followed 0.5 or 2 h later, by collection of total saliva for 10 min with absorbent cotton balls following pilocarpine stimulation (12.3 μmol/kg, i.v.). Each column represents the mean±S.E. of nine (control) and four (oxybutynin and solifenacin) mice. Daggers show a significant difference from the control value, ††† $p<0.001$. Asterisks show a significant difference from the value for oxybutynin, ** $p<0.01$.

[^{3}H]NMS binding in the submaxillary gland in mice was measured under the same anticholinergic agent protocol (dosage, time) as pilocarpine-induced salivary secretion (Fig. 2). Following oral administration of oxybutynin (76.1 μmol/kg), a significant increase (763%) was seen in the K_d value for specific [^{3}H]NMS binding in the mouse submaxillary gland compared with the control (Table 1). Oral administration of solifenacin at 62.4 and 208 μmol/kg exerted dose-dependent (291% and 499%, respectively) increases in K_d values for specific [^{3}H]NMS binding in the submaxillary gland compared with the control. The increase in K_d values by solifenacin (62.4 μmol/kg) was significantly less than that by oxybutynin (76.1 μmol/kg). Oral administration of these anticholinergic agents had little effect on B_{max} values for [^{3}H]NMS binding in the submaxillary gland.

Dissociation from Muscarinic Receptors High concentrations of oxybutynin (3 μM) and solifenacin (30 μM) inhibited specific [^{3}H]NMS binding in the mouse submaxillary gland (Fig. 1). In the ten-fold diluted assay of mouse submaxillary gland homogenates (no-washout) pretreated with these concentrations of anticholinergic agents, specific [^{3}H]NMS binding was 9.67% (oxybutynin) and 33.0% (solifenacin), respectively, of the vehicle-treated control values, with this difference being significant ($p<0.01$) (Fig. 3, open column). Moreover, specific [^{3}H]NMS binding was increased in the single- and double-washout submaxillary gland tissue homogenates pretreated with oxybutynin and solifenacin, suggesting further dissociation of anticholinergic agents from muscarinic receptor sites. Specific [^{3}H]NMS binding after single (Fig. 3, dotted column)- and double (Fig. 3, closed column)-washout was significantly greater in tissues pretreated with solifenacin (47.2%, 63.1%, respectively) than oxybutynin (16.9%, 26.2%, respectively). Further, respective increases in specific [^{3}H]NMS binding after single-washout relative to no-washout and after double-washout relative to single-washout were greater for solifenacin (14.2%, 15.9%) than oxybutynin (7.21%, 9.28%, respectively).

Table 1. K_d and B_{max} for Specific [^{3}H]NMS Binding in the Submaxillary Gland of Mice after Oral Administration of Oxybutynin and Solifenacin

Drugs	Oral doses (μmol/kg)	K_d (pM)	B_{max} (fmol/mg protein)
Control		115±5	128±5
Oxybutynin	76.1	993±133** (8.63)	138±5
Solifenacin	62.4	450±26** (3.91††)	121±8
Solifenacin	208	689±79** (5.99)	97.2±2.5

Values in parentheses represent the fold-increase in K_d values relative to controls. Asterisks show a significant difference from control values, ** $p<0.01$. Daggers show a significant difference from the value for oxybutynin (8.63), †† $p<0.01$. Values are mean±S.E. of 18 (control) and 3 or 4 (oxybutynin and solifenacin) mice.

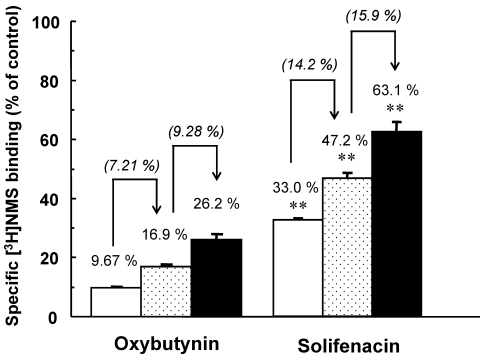

Fig. 3. Dissociation of Oxybutynin and Solifenacin from Muscarinic Receptor in the Mouse Submaxillary Gland

Homogenates of mouse submaxillary gland were pretreated with oxybutynin (3 μM) and solifenacin (30 μM), and the ten-fold diluted homogenates were used for the measurement of [^{3}H]NMS binding as the no-washout sample. Open column: specific [^{3}H]NMS binding in the tissue homogenate without washout. Dotted column: specific [^{3}H]NMS binding in the tissue homogenate after single-washout by centrifugation and suspension. Closed column: specific [^{3}H]NMS binding in the tissue homogenates after double-washout by centrifugation and suspension. Specific [^{3}H]NMS binding was expressed as a percentage of control binding in the tissue homogenate without drug treatment. Each column represents the mean±S.E. of five mice. Asterisks show a significant difference from respective values for oxybutynin, ** $p<0.01$.

DISCUSSION

The aim of this study was to compare the exocrine muscarinic receptor binding characteristics and inhibition of salivary secretion of oxybutynin and solifenacin, two agents used to treat overactive bladder.

In the *in vitro* experiments, oxybutynin and solifenacin competed with specific [^{3}H]NMS binding sites in the mouse submaxillary gland in a concentration-dependent manner. The muscarinic receptor binding affinity of solifenacin was two-fold lower than that of oxybutynin. Relative oral doses of solifenacin (62.4, 208 μmol/kg) and oxybutynin (76.1 μmol/kg) were selected based on their *in vitro* receptor binding affinities, in the subsequent experiments for the inhibition of salivation and for the *ex vivo* determination of muscarinic receptor binding activity in mice. In this regard, we recently showed that these doses of oxybutynin and solifenacin are pharmacologically relevant in terms of significant occupancy of bladder muscarinic receptors in mice.[11)]

Pilocarpine-induced salivary secretion in mice was markedly attenuated by oral administration of oxybutynin (76.1 μmol/kg) and solifenacin (62.4 and 208 μmol/kg), with that by solifenacin clearly weaker at these doses (Fig. 2). These findings have confirmed a previous observation that solifenacin attenuates cholinergic salivation in mice with significantly weaker potency than oxybutynin at doses at which they exerts approximately equivalent muscarinic receptor binding activity in the bladder.[11)]

K_d values for specific [^{3}H]NMS binding in the mouse submaxillary gland showed significant increases following oral administration of oxybutynin and solifenacin under the same protocol as used for the measurement of salivary secretion. Given that an increase in K_d for a radioligand in drug-pretreated tissues in this type of assay usually indicates competition between the agent and radioligand for the same binding sites,[21,24)] these data strongly suggest that orally administered anticholinergic agents undergo significant binding to muscarinic receptors in the mouse submaxillary gland. Based on the magnitude of increase in K_d values, the muscarinic receptor binding activity of oxybutynin in the submaxillary gland was considerably greater than that of solifenacin (Table 1). It is known that exocrine glands such as salivary gland contain predominantly the M_3 muscarinic receptor subtype[14,15)] and that oxybutynin displays high selectivity for the muscarinic M_3 subtype.[6,7,18)] Further, recent data with M_3 subtype knockout mice show that the M_3 subtype is expressed predominantly (70—80%) in the mouse submaxillary gland (Oki *et al.*, unpublished observation). It is therefore possible that the greater binding of oxybutynin to muscarinic receptors in the

mouse submaxillary gland is due to its high affinity for the M_3 subtype.

The dissociation rates of oxybutynin and solifenacin from muscarinic receptors in the mouse submaxillary gland were investigated by examining the recovery of specific [^{3}H]NMS binding due to dilution, using single- and double-washout (centrifugation and resuspension) tissue homogenates pretreated with these anticholinergic agents. This binding was stepwise increased by the ten-fold dilution (Fig. 3, open column), and by single (Fig. 3, dotted column)- and double (Fig. 3, closed column)-washout of tissue homogenates pretreated with oxybutynin and solifenacin at high concentrations which occupied completely muscarinic receptors. Notably, the degree of increase in specific [^{3}H]NMS binding in solifenacin-pretreated homogenates was consistently greater than that in oxybutynin-pretreated tissues (Fig. 3). This may indicate that the dissociation of anticholinergic agents occurs from muscarinic receptor sites in the mouse submaxillary gland by dilution and washout of the tissue homogenate, and that the dissociation rate is significantly faster in the case of solifenacin than oxybutynin. To our knowledge, therefore, these results are the first to show that the dissociation rate of anticholinergic agents from exocrine muscarinic receptors in the treatment of overactive bladder may significantly differ. Although the reason for this lower binding activity of solifenacin (Table 1) is unclear, it is likely that its readily reversible kinetics are at least in part associated with its relatively weak ability to bind muscarinic receptors in the mouse submaxillary gland and also its weak attenuation of pilocarpine-induced salivation following oral administration (Fig. 2). Similar faster dissociation kinetics have also been observed for tolterodine (Oki *et al.*, unpublished observation). Moreover, the relatively slow dissociation kinetics of oxybutynin from exocrine muscarinic receptors may be supported by our recent finding that oral oxybutynin produces long-lasting occupancy of muscarinic receptors in the rat submaxillary gland with sustained suppression of cholinergic salivation.[25)]

Solifenacin, like oxybutynin, exhibits specific selectivity for the muscarinic M_3 subtype under *in vitro* conditions,[17,18)] but its *in vivo* functional antagonism of M_3 subtype-mediated salivation in rats is considerably weaker than that of oxybutynin.[10,17)] The mechanism of this relatively weaker functional potency in the exocrine gland compared with oxybutynin has not been fully elucidated, but may be partly associated with the significant difference between these agents in their dissociation kinetics from exocrine muscarinic receptors. Alternatively, there is a possibility that the relative tissue concentration of solifenacin in the mouse submaxillary gland compared with the bladder after oral administration may be significantly lower than that of oxybutynin.

In conclusion, the present study shows that the exocrine muscarinic receptor binding activity and inhibition of salivation of solifenacin after oral administration in mice are significantly weaker than those after oral oxybutynin. This weakness of solifenacin may be at least partly attributable to its relatively fast dissociation kinetics from exocrine muscarinic receptors. These findings indicate that solifenacin may be more advantageous than oxybutynin in the treatment of patients with overactive bladder.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research (C) (2) (No. 15591703) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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