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Comparative Characterization of Lung Muscarinic Receptor Binding After Intratracheal Administration of Tiotropium, Ipratropium, and Glycopyrrolate

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Abstract. The aim of the current study was to characterize comparatively the binding of muscarinic receptor in the lung of rats intratracheally administered anticholinergic agents (tiotropium, ipratropium, glycopyrrolate) used clinically to treat chronic obstructive pulmonary disease (COPD) and asthma. Binding parameters of [*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) were determined in tissues (lung, bladder, submaxillary gland) of rats intratracheally administered tiotropium, ipratropium, and glycopyrrolate. The in vitro binding affinity of tiotropium for the receptors was 10 – 11-fold higher than those of ipratropium and glycopyrrolate. Intratracheal administration of tiotropium (0.6 – 6.4 nmol/kg) caused sustained (lasting at least 24 h) increase in the apparent dissociation constant (K_d) for [^{3}H]NMS binding in rat lung compared with the control value. Concomitantly, there was a long-lasting decrease in the maximal number of binding sites (B_{max}) for [^{3}H]NMS. Similary, ipratropium and glycopyrrolate at 7.3 and 7.5 nmol/kg, respectively, brought about a significant increase in K_d for [^{3}H]NMS binding. The effect by ipratropium was observed at 2 h but not 12 h, and that by glycopyrrolate lasted for 24 h. Both agents had little influence on the muscarinic receptors in the bladder and submaxillary gland. The present study provides the first evidence that tiotropium, ipratropium, and glycopyrrolate administered intratracheally in rats selectively bound muscarinic receptors of the lung, and tiotropium and glycopyrrolate had a much longer-lasting effect than ipratropium.

Keywords: chronic obstructive pulmonary disease, tiotropium, ipratropium, glycopyrrolate, ex vivo lung muscarinic receptor binding

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by a progressive obstruction of airflow due to persistent inflammation of the airways and lung parenchyma, caused predominantly by chronic cigarette smoking. COPD is associated with a significant level of morbidity and mortality (1, 2). In patients with COPD and asthma, bronchoconstriction and mucus secretion are increased and the airway becomes hyperresponsive to cholinergic agents. Acetylcholine released from parasympathetic nerves activates postjunctional muscarinic M_3 receptors present in airway smooth muscle to induce bronchoconstriction. Since parasympathetic activity is increased in airway inflammation and readily causes the constriction of airway smooth muscle, anticholinergic agents are important as bronchodilators in the treatment of COPD and asthma (3, 4). In clinical trials, anticholinergics were shown to improve lung function, exercise endurance, and health-related quality of life and to reduce exacerbation (5, 6). Treatment with long-acting inhaled anticholinergic agents improves the effectiveness of pulmonary rehabilitation (7, 8).

Tiotropium bromide is a synthetic quaternary anticho-

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linergic agent approved for maintenance therapy to treat stable COPD, with a once-daily dosage regime providing high patient compliance (9, 10). This agent has two important properties: it is functionally selective, targeting specific muscarinic receptors that mediate airway smooth muscle contraction, and it has a long duration of action, making it well suited for once-daily dosing (11). It is assumed that the mechanism behind its long-lasting action relates to the slow rate of dissociation from its target, the human M_3 muscarinic receptor (12). Ipratropium bromide is a short-acting antimuscarinic bronchodilator, with a maximal effect at 30 – 60 min and duration of action of 3 – 6 h (13 – 15). Glycopyrrolate bromide is also a well-known quaternary ammonium compound endowed with potent antimuscarinic activity. Clinical studies have shown that inhaled glycopyrrolate displays bronchodilator activity in COPD and asthmatic patients, an effect apparently lasting for 8 – 12 h (16 – 18). It was shown that glycopyrrolate inhibited electrical field stimulation–induced contractions of human and guinea-pig isolated airways with a longer duration of action than ipratropium and that both agents exhibited no selectivity toward M_1, M_2, and M_3 receptors (19, 20).

Because the potency and selectivity of agents for a given receptor in an in vivo setting can be dissimilar to that observed in an in vitro system, we developed an in vivo assay to simultaneously determine the absolute potency, time course, and organ selectivity of anticholinergic agents (21). In fact, our previous studies stressed the importance of characterizing the binding of ligands to receptors under the influence of various pharmacokinetic and pharmacodynamic factors (22 – 27). Although tiotropium, ipratropium, and glycopyrrolate are used clinically in inhalable forms to treat COPD and asthma, their binding of muscarinic receptors in vivo has not been measured in tissues following intratracheal administration. Therefore, the aim of the present study was to characterize comparatively muscarinic receptor binding in the lung compared with other tissues of rats treated intratracheally with tiotropium, ipratropium, and glycopyrrolate.

Materials and Methods

Materials

[*N*-Methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS, 2.59 – 2.65 TBq/mmol) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). Tiotropium bromide (tiotropium) was synthesized by Astellas Co., Ltd. (Ibaraki). Ipratropium bromide (ipratropium) and glycopyrrolate bromide (glycopyrrolate) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Kemprotec, Ltd. (Middlesbrough, UK), respectively. All other chemicals were purchased from commercial sources. Anticholinergic agents were dissolved in saline. Each solution was diluted by 25 mM Tris / 3.75 mM $MgCl_2$ buffer (pH 7.4) in the in vitro experiment.

Animals

Male Sprague-Dawley rats (250 – 300 g) at the age of 8 – 10 weeks were purchased from Shizuoka Laboratory Animal Center (Shizuoka). They were housed in the laboratory with free access to food and water and maintained on a 12-h light-dark cycle in a room with controlled temperature (24 ± 2°C). Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka.

Intratracheal administration of tiotropium, ipratropium and glycopyrrolate

Rats were fasted for 16 h and then intratracheally (by using 1 ml syringe) administered tiotropium (0.6 – 6.4 nmol/kg), ipratropium (7.3 nmol/kg), and glycopyrrolate (7.5 nmol/kg) dissolved in saline under temporary anesthesia with diethyl ether as described previously (23). The intratracheally injected volume of these agents was 1.0 ml/kg body weight. The drug diffusion after the intratracheal administration was confirmed by the complete abolishment of the airway muscle contractile response induced by the intravenous injection of acetylcholine after the tiotropium administration (data not shown) and also by significant occupancy of vasoactive intestinal peptide (VIP) receptors in the anterior and posterior lobe of lungs after the intratracheal administration of the analogues (23). The control animals received vehicle alone.

Tissue preparation

At 2 to 24 h after drug administration, rats were killed by taking the blood from the descending aorta under temporary anesthesia with diethyl ether. Then the lung, bladder, and submaxillary gland were dissected and the fat and blood vessels removed by trimming. The tissues were minced with scissors and homogenized in a Kinematica Polytron homogenizer in 19 volumes of ice-cold 25 mM Tris / 3.75 mM $MgCl_2$ buffer (pH 7.4). The homogenates were twice centrifuged at 40,000 × *g* for 20 min and the resulting pellets were finally suspended in buffer for the binding assay. In the ex vivo experiment, there was a possibility that tiotropium, ipratropium, and glycopyrrolate might partially dissociate from the receptor sites during tissue preparation (homogenization and suspension) after drug administration. Yamada et al. (28) showed that the dissociation of antagonists from receptors at 4°C was extremely slow. So, to minimize the dissociation, all steps involved in the preparation were

performed at 4°C. Protein concentrations were measured by the method of Lowry et al. (29).

Muscarinic receptor–binding assay

The binding assay for muscarinic receptors was performed using [^{3}H]NMS as described (26). The homogenates (100 – 900 μg of protein) of rat tissues were incubated with different concentrations (0.06 – 1.5 nM) of [^{3}H]NMS in 25 mM Tris / 3.75 mM $MgCl_2$ buffer (pH 7.4). Incubation was carried out for 60 min at 25°C. The reaction was terminated by rapid filtration (Cell Harvester; Brandel Co., Gaithersburg, MD, USA) through Whatman GF/B glass fiber filters, and the filters were then rinsed three times with 3 mL of ice-cold buffer. Tissue-bound radioactive material was extracted from the filters overnight by immersion in scintillation fluid [2 L toluene, 1 L Triton X-100, 15 g of 2,5-diphenyloxazole, and 0.3 g of 1,4-*bis*[2-(5-phenyloxazolyl)]benzene], and the radioactivity was measured 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.

Data analyses

The [^{3}H]NMS binding data was subjected to a nonlinear regression analysis using Graph Pad PRISM (ver. 4; Graph Pad Software, San Diego, CA, USA). The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]NMS were estimated. The ability of tiotropium, ipratropium, and glycopyrrolate to inhibit [^{3}H]NMS binding (0.5 nM) was estimated from the IC_{50}, which is the molar concentration of the anticholinergic agent necessary to displace 50% of the specific binding of [^{3}H]NMS (determined by a log probit analysis). The inhibition constant, K_i, was calculated from the equation $K_i = IC_{50} / (1 + L / K_d)$, where L represents the concentration of [^{3}H]NMS.

The statistical analysis of the receptor binding data was performed with Student's *t*-test and a one-way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons. All data are expressed as the mean ± S.E.M. Statistical significance was accepted at $P < 0.05$.

Results

In vitro effects of tiotropium, ipratropium, and glycopyrrolate on specific [^{3}H]NMS binding in rat tissues

Tiotropium (0.03 – 1.0 nM) inhibited specific [^{3}H]NMS binding in the homogenates of lung, heart, submaxillary gland, and bladder tissue in a concentration-dependent manner (Fig. 1). The respective K_i values were 0.13, 0.10, 0.42, and 0.22 nM (Table 1), reasonably consistent with previous results obstained in human airway smooth muscles (19). Thus, the inhibitory effect of tiotropium was significantly more potent in the lung, heart, and bladder than in the submaxillary gland. The

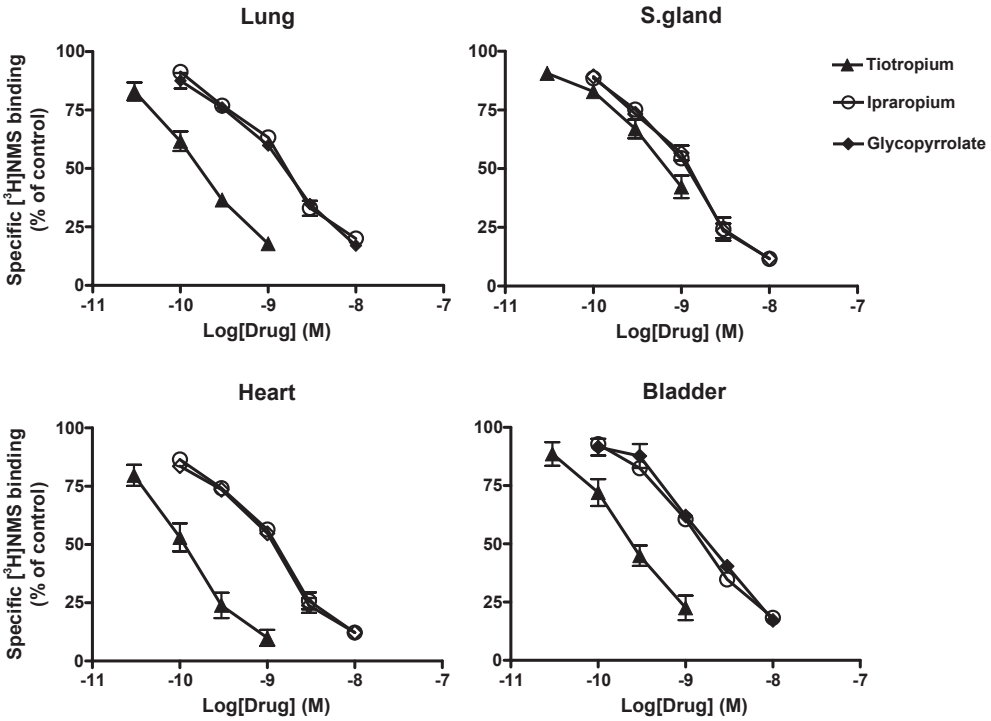

Fig. 1. In vitro inhibition by tiotropium (closed triangles), ipratropium (open circles), and glycopyrrolate (closed diamonds) of specific [*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) binding in the lung, submaxillary gland (S. gland), heart, and bladder of rats. The ordinate is [^{3}H]NMS binding (% of control) and the abscissa is logarithmic molar concentrations of tiotropium (0.03 – 1 nM), ipratropium (0.1 – 10 nM), and glycopyrrolate (0.1 – 10 nM). Each point represents the mean ± S.E.M. for 3 to 5 rats.

Hill coefficients for tiotropium in these tissues were close to one.

Similarly, ipratropium (0.1 – 10 nM), and glycopyrrolate (0.1 – 10 nM) inhibited specific [^{3}H]NMS binding in each tissue in a concentration-dependent manner (Fig. 1, Table 1). The inhibitory effects of ipratropium and glycopyrrolate were approximately 2-fold more potent in the submaxillary gland than lung, heart, or bladder. Compared with the K_i for tiotropium, the K_i values for ipratropium and glycopyrrolate were 10 – 11-fold larger in the lung and heart and about 7-fold larger in the bladder. However, there was little significant difference among the three agents in the K_i value in the submaxillary gland. The Hill coefficients for ipratropium and glycopyrrolate in these tissues were less than one.

Kinetic analysis revealed that tiotropium (0.5 nM), ipratropium (5.0 nM), and glycopyrrolate (5.0 nM) at IC_{50} concentrations (estimated from Fig. 1) increased significantly K_d values for [^{3}H]NMS binding in the lung, heart, and submaxillary gland, compared with the corresponding control values (Table 2). The extent of the increase did not differ much among the agents in the lung (9.2 – 10.7-fold) or heart (8.4 – 11.8-fold), but the increase in the submaxillary gland was approximately 3-fold greater for ipratropium and glycopyrrolate than tiotropium. Also, the increase in K_d caused by tiotropium in the submaxillary gland was about half of that in the lung or heart, while the increases caused by ipratropium and glycopyrrolate were 1.5 – 2-fold greater in the submaxillary gland than lung or heart. These agents had little

Table 1. K_i values and Hill coefficients (nH) for in vitro inhibition by tiotropium, ipratropium, and glycopyrrolate of specific [*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) binding in the lung, heart, submaxillary gland, and bladder of rats

	Tiotropium		Ipratropium		Glycopyrrolate	
	K_i (nM)	nH	K_i (nM)	nH	K_i (nM)	nH
Lung	0.13 ± 0.02	0.91 ± 0.04	1.46 ± 0.22***	0.89 ± 0.03	1.26 ± 0.14***	0.89 ± 0.06
Heart	0.10 ± 0.03	1.05 ± 0.06	1.13 ± 0.09**	0.88 ± 0.04	1.02 ± 0.08**	0.91 ± 0.06
Submaxillary gland	0.42 ± 0.12†	0.82 ± 0.08	0.64 ± 0.05††	0.85 ± 0.05	0.70 ± 0.08†	0.86 ± 0.03
Bladder	0.22 ± 0.05	0.96 ± 0.05	1.47 ± 0.10**	0.90 ± 0.05	1.57 ± 0.23**	0.94 ± 0.10

The binding of [^{3}H]NMS (0.5 nM) in rat tissues was measured in the absence or presence of different concentrations of tiotropium, ipratropium, and glycopyrrolate. The K_i and Hill coefficients were estimated. Each value represents the mean ± S.E.M. for 3 to 5 rats. **$P < 0.01$, ***$P < 0.001$ vs. tiotropium group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. lung group.

Table 2. Effect of tiotropium, ipratropium, and glycopyrrolate on K_d and B_{max} for specific [*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) binding in rat tissues

Tissues	Drug	K_d (pM)	B_{max} (fmol/mg protein)
Lung	Control	270 ± 16	35.2 ± 1.9
	Tiotropium	2882 ± 343 (10.7)***	40.2 ± 3.0
	Ipratropium	2522 ± 343 (9.34)***	39.7 ± 1.8
	Glycopyrrolate	2475 ± 93 (9.17)***	33.8 ± 4.1
Heart	Control	294 ± 11	68.4 ± 15.2
	Tiotropium	3478 ± 483 (11.8)***	71.3 ± 3.8
	Ipratropium	2456 ± 483 (8.35)***	71.6 ± 6.2
	Glycopyrrolate	2561 ± 153 (8.71)***	64.0 ± 5.5
Submaxillary gland	Control	143 ± 12	140 ± 14
	Tiotropium	702 ± 59 (4.91)***	125 ± 14
	Ipratropium	2541 ± 173 (17.8)***	123 ± 15
	Glycopyrrolate	1909 ± 210 (13.3)***	126 ± 17

The binding of [^{3}H]NMS (0.06 – 1.5 nM) in rat tissues was measured in the absence or presence of tiotropium (0.5 nM), ipratropium (5.0 nM), and glycopyrrolate (5.0 nM). Values in parentheses represent the K_d relative to controls. Each value represents the mean ± S.E.M. for 3 to 5 rats. ***$P < 0.001$ vs. control value.

effect on the B_{max} values for specific [^{3}H]NMS-binding in each tissue. Thus, an increase in the K_d for [^{3}H]NMS binding with little effect on the B_{max} (Table 2) may indicate competitive binding of muscarinic receptors by these anticholinergic agents.

Effects of intratracheal administration of tiotropium, ipratropium, and glycopyrrolate on muscarinic receptors in rat tissues

The effects of the intratracheal administration of tiotropium, ipratropium, and glycopyrrolate on specific [^{3}H]NMS binding in rat tissues were investigated. Tiotropium at doses of 0.6 – 6.4 nmol/kg significantly increased K_d values for specific [^{3}H]NMS binding in the lung (Table 3). In fact, the respective increases at 0.6, 2.1, and 6.4 nmol/kg were 61%, 66%, and 122% at 2 h and 32%, 57%, and 59% at 24 h, compared with the control values. In addition, the B_{max} values in the lungs of tiotropium (0.6 – 6.4 nmol/kg)-treated rats were markedly (43% – 79%) reduced at 2 and 24 h.

The intratracheal administration of tiotropium had little effect on the K_d and B_{max} values for specific [^{3}H]NMS binding in the bladder (Table 3). In the submaxillary gland of tiotropium-treated rats, there were slight but significant changes in the K_d and B_{max} for [^{3}H]NMS at 0.6 and 2.1 nmol/kg. On the other hand, the intratracheal administration of a high dose (6.4 nmol/kg) of this agent increased significantly (2 h: 60%, 24 h: 50%) the K_d values and decreased significantly (about 30%) the B_{max} values. The decrease in B_{max} for specific [^{3}H]NMS binding was significantly smaller in the submaxillary gland than lung.

At 2 h but not 12 h after the intratracheal administration of ipratropium (7.3 nmol/kg), there was a significant (42%) increase in the K_d value for specific [^{3}H]NMS binding in the lung compared with the control values, with no change of binding parameters in the bladder and submaxillary gland (Table 4). In these rats, there was

Table 3. K_d and B_{max} for specific [*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) binding in the lung, bladder, and submaxillary gland of rats 2 and 24 h after the intratracheal administration of tiotropium

Tissues	Drug	Dose (nmol/kg)	Time (h)	K_d (pM)	B_{max} (fmol/mg protein)
Lung	Control			254 ± 7	34.3 ± 2.0
	Tiotropium	0.6	2	409 ± 21 (1.61)**	7.17 ± 0.62 (0.21)**
			24	336 ± 33 (1.32)**	19.5 ± 3.3 (0.57)**
		2.1	2	421 ± 23 (1.66)**	7.11 ± 0.20 (0.21)**
			24	400 ± 33 (1.57)**	9.99 ± 0.65 (0.29)**
		6.4	2	565 ± 31 (2.22)**	7.13 ± 0.35 (0.21)**
			24	403 ± 26 (1.59)**	8.31 ± 0.43 (0.24)**
Bladder	Control			269 ± 8	127 ± 3
	Tiotropium	0.6	2	280 ± 5	124 ± 8
			24	272 ± 9	131 ± 4
		2.1	2	265 ± 29	128 ± 6
			24	244 ± 19	123 ± 9
		6.4	2	299 ± 12	126 ± 8
			24	264 ± 6	127 ± 9
Submaxillary gland	Control			151 ± 5	114 ± 3
	Tiotropium	0.6	2	143 ± 10	111 ± 4
			24	143 ± 9	120 ± 3
		2.1	2	143 ± 9	99.7 ± 3.8
			24	138 ± 7	104 ± 3
		6.4	2	241 ± 5 (1.60)**	80.9 ± 2.7 (0.71)**
			24	226 ± 14 (1.50)**	82.5 ± 2.9 (0.72)**

Rats received 0.6 – 6.4 nmol/kg of tiotropium intratracheally and then were sacrificed 2 and 24 h later. The binding of [^{3}H]NMS (0.06 – 1.5 nM) in rat tissues was measured. Values in parentheses represent K_d and B_{max} values relative to controls. Each value represents the mean ± S.E.M. for 4 – 16 rats. **$P < 0.01$ vs. control group.

Table 4. K_d and B_{max} for specific [*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) binding in the lung, bladder, and submaxillary gland of rats 2, 12, and 24 h after the intratracheal administration of ipratropium or glycopyrrolate

Tissues	Drug	Dose (nmol/kg)	Time (h)	K_d (pM)	B_{max} (fmol/mg protein)
Lung	Control			254 ± 7	34.3 ± 2.0
	Ipratropium	7.3	2	360 ± 8 (1.42)**	30.4 ± 3.1
			12	275 ± 19	35.7 ± 4.4
	Glycopyrrolate	7.5	2	414 ± 34 (1.63)**	30.0 ± 2.2
			24	373 ± 16 (1.47)**	34.5 ± 2.9
Bladder	Control			269 ± 8	127 ± 3
	Ipratropium	7.3	2	275 ± 17	127 ± 5
			12	247 ± 26	115 ± 14
	Glycopyrrolate	7.5	2	275 ± 17	128 ± 3
			24	272 ± 20	114 ± 12
Submaxillary gland	Control			151 ± 5	114 ± 3
	Ipratropium	7.3	2	161 ± 8	111 ± 8
			12	254 ± 7	116 ± 10
	Glycopyrrolate	7.5	2	157 ± 10	112 ± 11
			24	158 ± 11	115 ± 10

Rats received 7.3 nmol/kg of ipratropium or 7.5 nmol/kg of glycopyrrolate intratracheally and were sacrificed 2, 12, or 24 h later. The binding of [^{3}H]NMS (0.06 – 1.5 nM) in rat tissues was measured. Values in parentheses represent the K_d relative to controls. Each value represents the mean ± S.E.M. for 4 – 16 rats. $^{**}P < 0.01$ vs. control group.

little change in B_{max} values for the lung. Similarly, the intratracheal administration of glycopyrrolate (7.5 nmol/kg) increased significantly (2h: 63%, 24 h: 47%) the K_d values for specific [^{3}H]NMS binding in the lung with no change in the B_{max} (Table 4). In the bladder and submaxillary gland of glycopyrrolate-treated rats, there was little change in the binding parameters of [^{3}H]NMS.

Discussion

The major findings of this study are that the intratracheal administration of tiotropium, ipratropium, and glycopyrrolate in rats resulted in the selective binding of lung muscarinic receptors and that the effects of tiotropium and glycopyrrolate lasted much longer than the effect of ipratropium.

The heart and salivary gland exclusively contain the M_2 and M_3 muscarinic subtypes, respectively (30 – 32), whereas the lung contains both, with the M_2 subtype dominating. The M_3 subtype is considered responsible for the constriction of airway smooth muscle (33, 34). Consistent with these results, our recent study with M_1 to M_5 subtype–knockout mice showed that the M_3 subtype is expressed predominantly in the submaxillary gland and moderately in the lung and bladder, whereas M_2 is the major subtype in the lung, heart, and bladder (35). In the in vitro experiment, tiotropium, ipratropium, and glycopyrrolate competed concentration-dependently with [^{3}H]NMS for the binding sites in the lung, heart, submaxillary gland, and bladder of rats; and the potency of tiotropium compared with ipratropium and glycopyrrolate in tissues except the submaxillary gland was 10 – 11-fold greater. Although the affinity of tiotropium for muscarinic receptors was slightly higher in the lung, heart, and bladder than in the submaxillary gland, there seems to be little difference among these organs in the affinity of tiotropium, ipratropium, and glycopyrrolate in vitro (Table 1). These results are reasonably consistent with the previous finding of no selectivity among muscarinic receptor subtypes (19, 20).

The ex vivo binding in the lungs of rats after the intratracheal administration of tiotropium, ipratropium, and glycopyrrolate was characterized comparatively. The dose of each agent chosen was expected to produce significant pharmacological effects in the lung (36). The intratracheal administration of tiotropium (0.6 – 6.4 nmol/kg) brought about a significant increase in the K_d for [^{3}H]NMS-binding in the lung at 2 h, compared with control values. This effect lasted at least 24 h. Also, a significant increase in K_d values for the lung was observed at 2 h but not 12 h after the ipratropium administration, and after both 2 and 24 h after the glycopyrrolate

administration (Table 4). Given that an increase in the K_d for a radioligand in drug-pretreated tissues in this type of assay usually indicates competition between the agent and radioligand at binding sites (22, 24 – 26), these results indicate that the intratracheal administration of tiotropium, ipratropium, and glycopyrrolate resulted in significant binding to the lung muscarinic receptors with the distinct duration of action. Namely, the lung selectivity of these anticholinergic agents is reasonably considered to result from the intratracheal administration.

Notably, tiotropium caused a significant decrease in the B_{max} for [^{3}H]NMS binding in the rat lung at 2 h, and the effect remained at 24 h (Table 3). Such noncompetitive antagonism might indicate a long-lasting occupation of lung muscarinic receptors by tiotropium. Our previous study showed the sustained reduction in B_{max} values for [^{3}H]NMS binding in rat tissues after the oral administration of oxybutynin, an anticholinergic agent, to be due to the slow dissociation of this agent from receptors (26). Taken together, the sustained occupancy of lung muscarinic receptors after the intratracheal administration of tiotropium may result from binding kinetics, that is, the extremely slow rate of dissociation from receptors. The high potency and slow dissociation of tiotropium after the intratracheal administration may be closely associated with a long-lasting bronchodilation and bronchoprotection in patients with COPD and asthma (9, 10). The intratracheal administration of ipratropium and glycopyrrolate, unlike tiotropium, produced little reduction in the B_{max} for [^{3}H]NMS binding in the lung. The current results provide the first evidence that the intratracheally administered tiotropium occupies lung muscarinic receptors in a more sustained manner than glycopyrrolate and ipratropium. The observed difference in the changes (extent, duration) in binding parameters (K_d, B_{max}) after the intratracheal administration of tiotropium, ipratropium, and glycopyrrolate (Tables 3, 4) seems to coincide with the functional and in vitro receptor binding characteristics. In fact, Casarosa et al. (37) showed that intratracheal tiotropium induced considerable bronchoprotection in anesthetized dogs lasting more than 24 h, significantly longer than glycopyrrolate. Both in the recovery of carbachol-induced contraction of the isolated guinea-pig tracheum and in the offset of the bronchodilator effects of anticholinergic agents in the isolated human bronchus, the glycopyrrolate-induced bronchodilation lasted longer than the ipratropium, but not tiotropium, -induced bronchodilation (36). Furthermore, Haddad et al. (19) showed that the inhibition by glycopyrrolate of electrical field stimulation–induced contraction of guinea-pig trachea and human airways was significantly more prolonged than that by ipratropium. In relation to the in vitro receptor binding kinetics, Casarosa et al. (37) showed that the dissociation half-life ($t_{1/2}$) from the human M_3 receptor of tiotropium (27 h) was much longer than that of glycopyrrolate (6.1 h). Also, it was reported that glycopyrrolate compared with ipratropium dissociated more slowly from muscarinic receptors in human airway smooth muscle (19).

The largest dose (6.35 nmol/kg) of tiotropium produced a significant and long-lasting binding of muscarinic receptors in the submaxillary gland as shown by a significant increase of the K_d for [^{3}H]NMS-binding with a moderate decrease in the B_{max}, but it had little effect on the bladder muscarinic receptor binding parameters (Table 3). Thus, the use of supramaximally effective doses of tiotropium might cause dry mouth as a side effect. On the other hand, such significant receptor binding in the submaxillary gland was little observed by glycopyrrolate (7.5 nmol/kg). Therefore, it is suggested that tiotropium displays higher affinity to muscarinic receptors in the submaxillary gland than bladder in vivo and that glycopyrrolate has less inhibitory effect on the submaxillary gland than tiotropium. It was shown that, in CHO-K1 cells expressing human muscarinic receptor subtypes, tiotropium dissociated more slowly from M_3 receptors, which are located in bronchial smooth muscles, than from M_2 receptors (12, 38). Such kinetic distinction of muscarinic receptor binding may be responsible for the receptor-subtype selectivity in the submaxillary gland over the bladder observed in the current ex vivo binding study. The M_3-subtype selectivity of this agent was previously demonstrated (39). In addition, the inhibitory effect of tiotropium on the M_3 subtype might be evaluated by a functional study such as determining the effect on salivary secretion. In fact, Oki et al. (25) showed that pilocarpine-induced salivary secretion in mice was markedly suppressed by oral administration of M_3-selective agents such as solifenacin and oxybutynin. Thus, the effect on M_3 receptors in the submaxillary gland after intratracheal administration of tiotropium and glycopyrrolate could be clarified by the measurement of salivary secretion.

In conclusion, the current study indicates that the difference in the rate at which anticholinergic agents dissociate from the airway muscarinic receptors may underlie the difference in the duration of pharmacological action. Namely, the binding characteristics of lung muscarinic receptors after the intratracheal administration of anticholinergics in rats reflecting the clinical route may confirm and further expand previous pharmacological observations including clinical findings (9 – 20).

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