*Full Paper***Assessment of Muscarinic Receptor Subtypes in Human and Rat Lower Urinary Tract by Tissue Segment Binding Assay**

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Abstract. Muscarinic receptors in the human and rat lower urinary tract (urinary bladder detrusor muscle and mucosa, and prostate) were identified by intact tissue segment binding assays with two radioligands, and the effects of prolonged receptor activation in vitro on muscarinic receptors were examined. Hydrophilic [³H]-NMS and hydrophobic [³H]-QNB bound to the detrusor muscle segments with the same density, suggesting that the muscarinic receptors were localized at the plasma membrane. While the density of muscarinic receptor was higher in detrusor muscle than in the bladder mucosa and prostate, there was no species-specific difference either in density or in subtype distribution (M₁, M₂, and M₃ subtypes in detrusor; M₂ and M₃ subtypes in bladder mucosa; and M₁ and M₂ subtypes in prostate). Incubation of detrusor strips with carbachol decreased [³H]-NMS binding sites within 20 min, followed by a reduction of [³H]-QNB binding sites after a 60-min lag phase. The loss of the binding sites over 3 h after carbachol treatment was the same (approximately 40%) for both radioligands. The present intact tissue segment binding assay reveals tissue-specific and plasma membrane distribution of distinct muscarinic receptor subtypes and their dynamic changes (internalization and down-regulation) in lower urinary tract of humans and rats.

Keywords: urinary bladder (detrusor, mucosa), prostate, muscarinic receptor, tissue segment binding assay, down-regulation

Introduction

Five different muscarinic receptors (M₁ – M₅ subtypes) have been cloned, among which three subtypes (M₁, M₂, and M₃) have been pharmacologically well characterized (1, 2). Muscarinic receptors are the main receptors regulating the muscle tension of the urinary bladder, and their dysfunction may lead to micturition disorders (3, 4). A number of radioligand binding studies, as well as functional studies, have revealed the subtype distribution in the detrusor muscle, and the expression of M₃ and M₂ subtypes as well as a minor proportion of M₁ subtype has been recently demonstrated in radioligand-binding studies (5 – 8). Although selective antagonists of M₃ muscarinic receptor subtype,

which is now known to be primarily responsible for bladder contraction (9, 10), are widely used for over-active bladder (OAB) and have been proved to be effective, the problem of drug tolerance sometimes hinders the long-term treatment with these drugs. Thus, it is necessary to examine not only the subtype distribution of the muscarinic receptors, but also the dynamic change of the distribution such as after long-term drug treatment. In addition, significant amounts of muscarinic receptors have recently been detected in the bladder urothelium, and their physiological roles have been explored (11 – 13).

To quantify the receptor density and to assay the subtype population, radioligand binding assays with homogenates or microsomal fractions of tissues have conventionally been used (14). Tissue homogenization and fractionation can yield membrane-rich preparations but may cause receptor disintegration and/or deformation, resulting in a significant loss of yield and

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underestimation of receptors (15, 16). In order to overcome these problems, an intact tissue segment binding method without homogenization was recently developed (17). Furthermore, it was demonstrated that the intact segment binding assay with hydrophilic radioligand can selectively identify plasma membrane receptors of intact tissues without contamination by intracellular receptors (16, 18).

The purpose of the present study was to evaluate the muscarinic receptors at the plasma membrane, separately from those in the intracellular compartment, in the lower urinary tract (detrusor muscle and mucosa of the urinary bladder, and prostate) of both humans and rats by intact tissue segment binding assays. To separately identify surface and intracellular muscarinic receptors, a membrane-impermeable hydrophilic radioligand, [^3H]-NMS (1-[*N*-methyl- ^3H]-scopolamine methyl chloride), and a membrane-permeable hydrophobic radioligand [^3H]-QNB (1-quinuclidinyl-[phenyl-4- ^3H]-benzilate), were used (19). The results obtained clearly show that the muscarinic receptors in the human and rat lower urinary tract are exclusively localized on the plasma membrane at different densities and with different subtype populations. Furthermore, it was shown that global activation of three coexisting plasma membrane muscarinic receptor subtypes in detrusor muscle caused down-regulation that was closely associated with the reduction in muscarinic contraction.

Materials and Methods

Sample preparation

Human urinary bladder and prostate were obtained during the course of prescribed surgery. University of Fukui Ethics Committee approval and patient consent were obtained for each case. The donors were 13 males and 4 females with ages ranging from 50- to 77-year-old; all of them underwent surgery for tumors of the urinary bladder or prostate. No patient had received radiotherapy or chemotherapy before intervention. All specimens collected were macroscopically normal without any signs of tumor or inflammation. The urinary bladder and prostate of male Wistar rats (approximately 300 g) were also isolated after pentobarbital anesthesia. Human and rat tissues were immediately placed in ice-cold modified Krebs-Henseleit solution composed of 112 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl_2 , 2 mM CaCl_2 , 1.2 mM NaHPO_4 , 25 mM NaHCO_3 , and 11.5 mM glucose (pH 7.4) gassed with 95% O_2 and 5% CO_2 . The detrusor muscle and the mucosa (consisting of the urothelium and lamina propria) of the urinary bladder and the prostate were carefully dissected out under a dissection microscope.

Tissue segment binding experiments with urinary bladder and prostate

Tissue segment binding experiments were performed according to the method described by Muramatsu et al. (17). The sizes of tissue segments were approximately $4 \times 3 \times 3$ mm for human detrusor muscle and prostate, $3 \times 3 \times 1$ mm for human urinary bladder mucosa and rat prostate, and $1.5 \times 1.5 \times 1$ mm for rat detrusor muscle and mucosa. Incubation was carried out for 16 h at 4°C in 1 ml of the incubation buffer, which was similar to modified Krebs-Henseleit solution except that NaHCO_3 was reduced to 10.5 mM to adjust the pH to 7.4 in air, and the decreased osmolarity was compensated by adding NaCl to 135.7 mM. In saturation binding experiments, [^3H]-NMS and [^3H]-QNB were used at concentrations ranging from 50 – 5000 pM and used at 300 pM for competition experiments. After incubation, the tissue segment was quickly moved into a plastic tube containing 1.5 ml of incubation buffer and vortexed at 4°C for 1 min. By this procedure, most of the unbound radioligand was released from the segment into the washing buffer (17). The segments were then solubilized in 0.3 M NaOH solution and subjected to counting of the radioactivity and measurement of the protein content. Nonspecific binding was determined in the presence of $1 \mu\text{M}$ atropine. The bound radioactivity was measured using a liquid scintillation counter (Aloka, Tokyo). The protein content was determined by the method of Bradford (20).

In order to examine the effects of prolonged incubation with carbachol on muscarinic receptors, after 1 h of equilibration in modified Krebs-Henseleit solution at 37°C , the detrusor strips were treated with $100 \mu\text{M}$ carbachol for 3 – 180 min. Some strips of rat detrusor were also incubated in the presence of $10 \mu\text{M}$ cycloheximide to inhibit new synthesis of muscarinic receptors during incubation at 37°C . Then, the strips were washed with ice-cold Krebs-Henseleit solution without carbachol and cut into segments and then assayed by the tissue segment binding method with both radioligands at the concentration of 1500 pM for examination of the time course. Saturation and competition binding of [^3H]-NMS were estimated using human and rat detrusor muscle after 3 h of treatment with carbachol.

Functional experiments

Human and rat detrusor muscles were excised into small strips (1.0 – 1.5 cm in length, 3 mm in width, 3 mm in thickness for human muscle strips and 1.0 cm in length, 3 mm in width and 1 mm in thickness for rat muscle strips). Strips were mounted under a tension of 1 g in a 10-ml organ bath containing modified Krebs-Henseleit solution, which was aerated with 95% O_2

and 5% CO₂ at 37°C. Contractile responses were recorded as changes in isometric force, as measured using a T7-30 isometric force transducer (Orientec, Tokyo). After 60 min of equilibration, carbachol was added to the bath in a cumulative manner to obtain concentration-response curves. The concentration-response curves were obtained twice with a 1-h interval, and the maximum contractile amplitude in the second concentration-response curve for carbachol was taken as 100% in each preparation. After recording the second response curve, various muscarinic receptor antagonists were added and the third concentration-response curve was obtained. In parallel with these experiments, time control responses were obtained from different strips in the absence of antagonist. For continuous treatment with carbachol, the strips were exposed to 100 μ M carbachol without changing the bath solution after recording the second concentration-response curves for carbachol. Thereafter, the strips were washed with carbachol-free solution for 1 h and then the third concentration-response curves were recorded.

Data analyses

Binding data were analyzed using commercially available software (Graph Pad PRISM®, Ver. 3.00; Graph Pad Software, San Diego, CA, USA). Briefly, the data were first fitted to a one- and then a two-site model, and if the residual sums of squares were significantly lower for a two-site fit of the data than for a one-site, as determined by an F-test comparison, then a two-site model was accepted. *P* values less than 0.05 were considered significant. The density of muscarinic receptors in lower urinary tract tissues was represented as binding capacity per mg of total tissue protein ($B_{\max}$ in fmol/mg of total tissue protein).

In functional studies, antagonist affinity estimates

(pK_B values) were determined for a single concentration of antagonist by the concentration ratio method, as reported previously (21). We used a one-way analysis of variance (ANOVA), followed by Scheffé's post-hoc test, or Student's paired or unpaired *t*-test where appropriate. The statistical method applied in each analysis was described in each figure legend. Results were considered to be significant when *P* values were less than 0.05.

Drugs

The following drugs were used in the present study: 1-[*N*-Methyl-³H]-scopolamine methyl chloride ([³H]-NMS, specific activity 3.00 TBq/mmol) and 1-quinuclidinyl-[phenyl-4-³H]-benzilate ([³H]-QNB, specific activity 1.81 TBq/mmol) (Amersham Biosciences, Buckinghamshire, UK); atropine sulfate (Nacalai Tesque, Kyoto); darifenacin (Ono Pharm Co, Osaka), carbachol chloride, and pirenzepine (Sigma-Aldrich Co., St. Louis, MO, USA); AF-DX 116 (Tocris, Ellisville, MO, USA); and cycloheximide (Nacalai Tesque, Kyoto).

Results

Intact tissue segment binding of [³H]-NMS and [³H]-QNB to urinary bladder and prostate

Figure 1 shows representative saturation curves for [³H]-NMS and [³H]-QNB in intact segments of human detrusor muscle. The nonspecific binding of [³H]-NMS in the presence of 1 μ M atropine was less than 5% of total binding at 1000 pM, but that of [³H]-QNB was approximately 30% at the same concentration as [³H]-NMS. These results together with the results obtained from competition experiments with other antagonists (see below) suggested that nonspecific contamination or accumulation in tissue segments was extremely low and

Fig. 1. Representative saturation curves for [³H]-NMS (A) and [³H]-QNB (B) binding in intact tissue segments of human urinary bladder detrusor muscle. Each point represents the mean of duplicate determinations. The experiments were replicated four or five times with similar results. The nonspecific binding was determined in the presence of 1 μ M atropine.

did not influence the estimation of specific binding. This was further confirmed in dissociation experiments, in which [3 H]-NMS binding after 16-h incubation was completely reduced to the nonspecific binding level by addition of 1 μ M atropine (data not shown). Binding saturation isotherms revealed that the specific binding of both radioligands met the criteria for binding to a single set of sites. Similar saturation curves for [3 H]-NMS and [3 H]-QNB were obtained in rat detrusor muscle and in the human urinary bladder mucosa, and these data are summarized in Table 1. The $B_{\max}$ values in each tissue of urinary bladder were almost the same between human and rat, but the densities in the detrusor muscle were around 7 times higher than those in the mucosa. Similar estimations of hydrophilic [3 H]-NMS and hydrophobic [3 H]-QNB binding sites suggested that the muscarinic receptors in these tissues were exclusively distributed on the plasma membrane.

[3 H]-NMS also bound to the human and rat prostate segments in a concentration-dependent manner. The $B_{\max}$ values in the prostate were significantly lower than those in the detrusor muscle but were the same level as those in the mucosa (Table 1). [3 H]-QNB binding experiments were not performed in the human and rat prostate.

Since the densities of the [3 H]-NMS and [3 H]-QNB binding sites were found to be the same level, the

pharmacological profiles of [3 H]-NMS binding sites were examined in competition binding experiments with intact tissue segments. Figure 2 shows representative competition curves in the human detrusor muscle, mucosa, and prostate for three drugs that have been widely used for the determination of muscarinic receptor subtypes (pirenzepine, AF-DX 116, and darifenacin for the M_1 , M_2 , and M_3 subtype, respectively) (1, 8). The binding of [3 H]-NMS was biphasically competed by pirenzepine in the detrusor muscle and prostate, but its binding in the mucosa was monophasically competed against by pirenzepine with low affinity (Fig. 2A). The slope of the competition curves for AF-DX 116 were shallow in the three tissues (Fig. 2B), resulting in two different affinity estimates by computer analysis (Table 2). Darifenacin showed biphasic competition in the detrusor muscle and mucosa, but high affinity binding of darifenacin could not be detected in the prostate (Fig. 2C). The results obtained from competition experiments are summarized in Table 2. The proportions of high affinity sites for pirenzepine, AF-DX 116, and darifenacin, indicating the M_1 , M_2 , and M_3 subtype, respectively, were approximately 30%, 40%, and 30%, respectively, in human and rat detrusor muscles. In the mucosa of human and rat, M_1 was not detected, whereas in the prostate of human and rat, M_1 and M_2 , but not M_3 , were detected. These results

Table 1. Characteristics of [3 H]-NMS and [3 H]-QNB binding sites in human and rat lower urinary tract

		Detrusor		Mucosa		Prostate	
		$B_{\max}$	K_d (pM)	$B_{\max}$	K_d (pM)	$B_{\max}$	K_d (pM)
[3 H]-NMS	Human	706 $\pm$ 52	568 $\pm$ 95	100 $\pm$ 7	220 $\pm$ 22	114 $\pm$ 6	190 $\pm$ 12
	Rat	621 $\pm$ 36	139 $\pm$ 27	146 $\pm$ 13	288 $\pm$ 55	151 $\pm$ 17	398 $\pm$ 78
[3 H]-QNB	Human	704 $\pm$ 39	698 $\pm$ 97	102 $\pm$ 6	102 $\pm$ 15	—	—
	Rat	642 $\pm$ 43	277 $\pm$ 39	—	—	—	—

Mean $\pm$ S.E.M. of 4 to 5 experiments. $B_{\max}$ values are expressed as fmol/mg of total tissue protein. —: not determined.

Fig. 2. Competition binding experiment of pirenzepine (A), AF-DX 116 (B), and darifenacin (C) at 300 pM of [3 H]-NMS binding sites in the intact segments of detrusor muscle and mucosa of human urinary bladder, and prostate. Data are each the mean $\pm$ S.E.M. of 4–5 experiments.

Table 2. Binding profiles of [³H]-NMS binding sites in human and rat lower urinary tract

Drug	Species	Detrusor		Mucosa		Prostate	
		pK _i _{high} (%)	pK _i _{low}	pK _i _{high} (%)	pK _i _{low}	pK _i _{high} (%)	pK _i _{low}
Pirenzepine	Human	8.4 ± 0.3 (30)	6.1 ± 0.2		6.7 ± 0.3	8.5 ± 0.2 (73)	6.2 ± 0.2
	Rat	8.4 ± 0.3 (23)	6.4 ± 0.3		6.5 ± 0.2	—	—
AF-DX 116	Human	7.1 ± 0.2 (41)	5.2 ± 0.1	6.9 ± 0.3 (56)	5.3 ± 0.2	7.0 ± 0.3 (27)	5.3 ± 0.3
	Rat	7.0 ± 0.2 (47)	5.1 ± 0.3	7.2 ± 0.3 (67)	5.1 ± 0.1	—	—
Darifenacin	Human	8.2 ± 0.2 (34)	6.1 ± 0.2	8.4 ± 0.1 (41)	5.9 ± 0.1		7.2 ± 0.3
	Rat	8.7 ± 0.2 (31)	6.2 ± 0.1	8.8 ± 0.2 (35)	5.7 ± 0.2	—	—

Values are each the mean ± S.E.M. obtained from 4–5 experiments. pK_i_{high} and pK_i_{low}: negative logarithm of equilibrium constants at high and low affinity sites, respectively, for tested drugs. (): Proportion of high affinity sites. —: Not determined.

Fig. 3. Effects of prolonged treatment with carbachol on [³H]-NMS and [³H]-QNB binding sites in rat and human detrusor muscles. The muscle strips were incubated with 100 μM carbachol at 37°C, and subsequently [³H]-NMS and [³H]-QNB binding sites were detected by the intact segment binding assay. A: Specific binding sites of [³H]-NMS (1500 pM) and [³H]-QNB (1500 pM) in rat detrusor muscle. Each data value is the mean ± S.E.M. of 4 experiments. **P* < 0.05: significantly different from 0 min (control) by the paired *t*-test. B: The B_{max} values of [³H]-NMS binding sites and the subtype proportions in human and rat detrusor muscles after incubation with or without 100 μM carbachol for 3 h. The same experiments were conducted in the rat detrusor muscle in the presence of 10 μM cycloheximide. The values represent mean values estimated from saturation and competition experiments. Each experiment was repeated 4 times. * and †: significantly different (*P* < 0.05) from the time control without carbachol treatment, and ‡: significantly different (*P* < 0.05) from the time control in the presence of cycloheximide. Data were analyzed by ANOVA followed by Scheffe's post-hoc tests.

suggested that the distribution of muscarinic receptor subtypes varies significantly among the lower urinary tract tissues.

Effects of incubation with carbachol on [³H]-NMS and [³H]-QNB binding sites in human and rat detrusor muscle

Since three different muscarinic receptor subtypes were co-localized on the plasma membrane of detrusor muscles of humans and rats, the effects of persistent activation of these subtypes were examined. Detrusor

muscle strips were incubated with 100 μM carbachol for various durations (up to 3 h), and then they were cut into tissue segments. Figure 3A shows the specific binding of 1500 pM of [³H]-NMS or [³H]-QNB to these segments versus the time of incubation with carbachol. The concentration (1500 pM) of both radioligands was 10 times as high as the K_d values of these ligands (Table 1) and the resulting specific bindings were very close to the B_{max} values. [³H]-NMS binding sites were reduced within 20 min after treatment with carbachol and subsequently reached 60% of the original density. On

Table 3. Pharmacological characteristics of carbachol in detrusor muscle of human and rat urinary bladder

	Human		Rat	
	Time control	CCh-treated	Time control	CCh-treated
Binding affinity (pK_i)	—	—	6.5 ± 0.1 4.5 ± 0.1	5.9 ± 0.1 4.2 ± 0.1
Function (pEC_{50})	7.3 ± 0.1	5.4 ± 0.2	6.4 ± 0.1	5.8 ± 0.2
Contractility (%)	101 ± 3	63 ± 4	98 ± 3	68 ± 3

Detrusor muscles were incubated for 3 h in the absence (time control) or presence of $100 \mu\text{M}$ carbachol (CCh-treated). Carbachol competed biphasically with the 300 pM [^3H]-NMS binding, resulting in high and low affinities. —: not determined. Contractility (%): The maximum contraction induced by $100 \mu\text{M}$ carbachol after 3-h incubation compared with that (100%) before the incubation in the same preparation. Values are each the mean $\pm$ S.E.M. of 3–6 experiments.

Table 4. Functional affinities for muscarinic antagonists in detrusor muscle of human and rat urinary bladder before and after carbachol pretreatment

Antagonists	Human (pK_B)		Rat (pK_B)	
	before	after	before	after
Pirenzepine	6.9 ± 0.2	7.0 ± 0.2	6.8 ± 0.2	7.0 ± 0.2
AF-DX 116	6.2 ± 0.2	6.2 ± 0.2	6.0 ± 0.2	6.3 ± 0.1
Darifenacin	8.0 ± 0.2	7.9 ± 0.2	8.9 ± 0.2	9.0 ± 0.2

Each reported value is the mean $\pm$ S.E.M. of 4–6 experiments.

the other hand, the density of [^3H]-QNB binding sites was constant for the first 60 min of treatment, but the density was reduced to the same level (60%) as that of [^3H]-NMS binding sites at 3 h of incubation (Fig. 3A). Figure 3B shows the density and subtype populations of [^3H]-NMS binding sites in the segments of human and rat detrusor muscles after 3 h of incubation. Incubation for 3 h without carbachol (that is, the time control) did not change the binding density or subtype population of [^3H]-NMS binding sites. However, incubation with $100 \mu\text{M}$ carbachol significantly reduced the binding density by 50% or 40% in human and rat detrusor muscles, respectively (Fig. 3B). The proportions of muscarinic receptor subtypes (M_1 , M_2 , and M_3 , which were identified as high affinity sites for pirenzepine, AF-DX 116, and darifenacin, respectively) were not different between the time control and carbachol-treated strips.

The rat detrusor muscle was also incubated in the presence of $10 \mu\text{M}$ cycloheximide to rule out a possible involvement of new synthesis of muscarinic receptors during 3-h incubation. The treatment with cycloheximide slightly reduced the total density of muscarinic receptors without affecting either the subtype proportion or the carbachol-induced down-regulation (Fig. 3B).

Next, the binding affinities for carbachol were also examined in the rat detrusor muscle incubated for 3 h in the absence and presence of carbachol. Carbachol competed with the [^3H]-NMS binding sites biphasically, with pK_i estimates being 6.5 and 4.5 in time control

segments and 5.9 and 4.2 in carbachol-treated segments (Table 3).

Contractile responses to carbachol in human and rat detrusor muscles

Carbachol (0.01 – $100 \mu\text{M}$) produced concentration-dependent contractions in human and rat detrusor muscle strips ($EC_{50} = 0.07 \pm 0.01$ and $0.5 \pm 0.2 \mu\text{M}$, respectively; $n = 8$ – 12). These contractile responses were competitively antagonized by low concentrations of darifenacin (10 – 1000 nM) and by higher concentrations of AF-DX 116 (1 – $30 \mu\text{M}$) or pirenzepine (0.1 – $10 \mu\text{M}$). The estimated pK_B values were more than 8 for darifenacin, approximately 6.2 for AF-DX 116, and 7.0 for pirenzepine in both human and rat detrusor muscles (Table 4). After recording the control responses to carbachol, some strips of human and rat detrusor muscles were incubated with $100 \mu\text{M}$ carbachol for 3 h and then washed for 1 h with carbachol-free Krebs solution. Thereafter, the contractility of these strips in response to carbachol was again examined. As shown in Fig. 4 and Table 3, both the potency of carbachol and the maximum contraction were markedly reduced, resulting in a right and downward shift of the concentration–response curves for carbachol. The residual contractile response to carbachol after 3 h incubation with $100 \mu\text{M}$ carbachol was also highly sensitive to darifenacin but not to pirenzepine and AF-DX 116 (Table 4).

Fig. 4. Effects of prolonged incubation with carbachol on the contractile responses to carbachol in human and rat detrusor muscles. Contraction in each strip was normalized by the maximum contraction induced by carbachol before the prolonged incubation. Concentration–response curves for carbachol in human (A) and rat (B) detrusor muscles after incubation with (open circles) or without (closed squares, time control) 100 μ M carbachol for 3 h are shown. Data each represent the mean $\pm$ S.E.M. of 4–6 experiments. Maximum responses after the treatment of carbachol for 3 h significantly decreased (tested by unpaired *t*-test, $P < 0.05$).

Discussion

Two different muscarinic radioligands ($[^3\text{H}]\text{-NMS}$ and $[^3\text{H}]\text{-QNB}$) have been widely used to detect native and recombinant muscarinic receptors in binding assays. If the cells and tissues are intact, it is likely that hydrophilic $[^3\text{H}]\text{-NMS}$ can selectively detect the muscarinic receptors located on plasma membrane, while hydrophobic $[^3\text{H}]\text{-QNB}$ can bind to the receptors at both the cell surface and intracellular regions (19, 22). In the present study, both radioligands were applied to intact segments of lower urinary tract tissues and the same densities were estimated from the specific binding of $[^3\text{H}]\text{-NMS}$ and $[^3\text{H}]\text{-QNB}$. This result indicates that the muscarinic receptors in the human and rat lower urinary tract are exclusively localized on the plasma membrane and that the intracellular receptors are negligible under normal conditions. This is in contrast to the recombinant muscarinic receptors artificially expressed in cultured cells, where significant proportion of the muscarinic receptors have been found to distribute not only at the cell surface but also in intracellular sites, even though the same binding protocol as the present study was used (19, 23, 24). We have also observed intracellular distribution of the muscarinic receptors in CHO cells where M_1 and M_3 subtypes were independently expressed (unpublished observations).

Although significant amounts of muscarinic receptors were present in the urinary tract, the densities varied among the tissues. The following order of density was seen in the human and rat urinary tract: detrusor muscle > bladder mucosa $\cong$ prostate. The density in detrusor muscles of both species was approximately 700 fmol/mg total tissue protein (Table 1), which was

several times higher than those reported in conventional membrane binding assays: 172 ± 11 fmol/mg membrane protein in human detrusor muscles (8), 169 ± 22 fmol/mg membrane protein in mouse detrusor muscle (25), and 81.3 ± 15.3 fmol/mg membrane protein in pig detrusor muscle (11). In our preliminary experiments, the density of $[^3\text{H}]\text{-NMS}$ binding sites estimated in the crude membrane fraction of human detrusor muscle was also as low as 82 ± 20 fmol/mg membrane protein ($n = 5$, unpublished observations). Since $[^3\text{H}]\text{-NMS}$ is hydrophilic and the nonspecific binding was extremely low (Fig. 1A), it is not likely that the high density estimated in the present study is an overestimation due to nonspecific accumulation of the radioligand into intact tissues and cells. Rather, underestimation may be caused by the conventional binding method with membrane preparations, as reported previously in the rat heart (16) and rabbit ear artery (21). We are now examining this discrepancy between different binding assay methods.

Since their density in intact segments of urinary bladder mucosa was approximately one seventh of that in the detrusor muscle (Table 1), the physiological significance of urothelial muscarinic receptors may be limited. However, specific localization at the afferent nerve endings and specific functions such as regulation of detrusor muscle contractility has been suggested for urothelial muscarinic receptors (9, 13, 26, 27). Muscarinic receptors were also present in the human prostate, and their density was slightly higher than the density of α -1 adrenergic receptors (76 ± 24 fmol/mg total tissue protein, estimated in intact prostate segments) (28). The finding here of a substantial population of muscarinic receptors may also suggest unknown roles of muscarinic receptors in the prostate.

The muscarinic receptors of lower urinary tract were pharmacologically subclassified by competition experiments into the M₁ subtype (pirenzepine: high-affinity sites), M₂ subtype (AF-DX 116: high-affinity sites), and M₃ subtype (darifenacin: high-affinity sites) (8). The results clearly showed that the majority of muscarinic receptors are M₂ in the human and rat urinary bladder. This is consistent with the previous results obtained by conventional membrane binding assays (29–31). In addition, the present results and the previous reports showed the tissue-specific distribution of different subtypes in lower urinary tract. In the detrusor muscle, M₁, M₂, and M₃ subtypes co-existed. In the mucosa of urinary bladder, M₁ was absent, but M₂ was still predominant. In the prostate, M₁ and M₂ co-existed, but M₃ was absent. Our functional experiments showed that the M₃ subtype plays a major role in detrusor muscle contraction. In spite of its major occurrence, the M₂ receptor may have an indirect effect on the muscarinic contraction in urinary bladder and the adrenergic contraction in the prostate (32). Braverman et al. found that the contraction in the denervated urinary bladder is mainly mediated through M₂ (33).

Another interesting finding in this study was the time-dependent dynamic changes in the muscarinic receptor after *in vitro* incubation with carbachol, which were clearly detected by the intact tissue segment binding assay. As shown in Fig. 3A, carbachol treatment rapidly reduced [³H]-NMS binding sites before decreasing [³H]-QNB binding sites in detrusor muscle. Since hydrophobic [³H]-QNB can bind to cell surface and intracellular muscarinic receptors indiscriminately (22), the difference in binding amounts of hydrophilic [³H]-NMS and hydrophobic [³H]-QNB at the early phase of incubation seemed to reflect the receptors leaving the plasma membrane, as a result of internalization. Subsequently, at 3-h incubation, both [³H]-NMS and [³H]-QNB binding sites were equally reduced. This indicates that the internalized receptors became unable to bind [³H]-QNB gradually, suggesting that the down-regulation of muscarinic receptors may occur slowly after the internalization (34). The experiments with cycloheximide further suggest that such dynamic changes in muscarinic receptors are in part covered by newly synthesized receptors, even though the tissues were incubated *in vitro*. Since the subtype proportions of the residual muscarinic receptors on the plasma membrane were not changed after prolonged incubation (Fig. 3), it is indicated that internalization/down-regulation may have occurred in all the subtypes of muscarinic receptors when global activation of the muscarinic receptors occurred in the native tissues after introduction of muscarinic agonist to the organ bath.

Comparison with contractile responses to carbachol revealed that human detrusor muscle is approximately 10 times sensitive to carbachol than rat muscle (EC₅₀: 0.07 μ M in human vs 0.5 μ M in rat and results shown in Fig. 4). Since no difference in binding and functional affinities for carbachol was seen in rat detrusors (Table 3), it is likely that there is not a significant spare receptor in rat detrusor. A marked downward and rightward shift of concentration–response curves for carbachol after prolonged treatment with carbachol may be accounted for by not only down-regulation but also desensitization of muscarinic receptors (Fig. 4, Table 3), although the detailed mechanisms should be analyzed in further studies.

The density and/or subtype distribution of muscarinic receptors may also change according to age or in various pathophysiological conditions such as overactive bladder (OAB). However, very little information is available in the literature concerning the effects of age and OAB on the density and distribution of total or individual muscarinic receptor subtypes in the human urinary bladder and the results reported are conflicting (8, 26, 35). Our current binding assay with intact tissue segments and with different physicochemical radioligands will be a powerful tool for the analysis of dynamic changes of receptor distribution and for the understanding of receptor-related pathophysiological conditions (17).

In conclusion, the present study shows that the current binding assay with intact tissue segments can detect distinct densities and different subtype populations of plasma membrane muscarinic receptors in the lower urinary tract (urinary bladder detrusor muscle and mucosa, and the prostate) in humans and rats in higher quality and quantity than the conventional membrane binding assay. The intact tissue binding assay also clearly revealed that global activation of muscarinic receptors produced subtype-independent internalization followed by down-regulation in native tissues.

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