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Full paper

# Characterization of muscarinic and P2X receptors in the urothelium and detrusor muscle of the rat bladder

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ABSTRACT

Muscarinic and purinergic (P2X) receptors play critical roles in bladder urothelium under physiological and pathological conditions. Aim of present study was to characterize these receptors in rat bladder urothelium and detrusor muscle using selective radioligands of [*N*-methyl-$^3$H]scopolamine methyl chloride ([$^3$H]NMS) and αβ-methylene ATP [2,8-$^3$H]tetrasodium salt ([$^3$H]αβ-MeATP). Similar binding parameters for each radioligand were observed in urothelium and detrusor muscle. Pretreatment with *N*-(2-chloroethyl)-4-piperidinyl diphenylacetate (4-DAMP mustard) revealed co-existence of $M_2$ and $M_3$ receptors, with the number of $M_2$ receptors being larger in the urothelium and detrusor muscle. Intravesical administration of imidafenacin and Dpr-P-4 (N → O) (active metabolite of propiverine) displayed significant binding of muscarinic receptors in the urothelium and detrusor muscle. The treatment with cyclophosphamide (CYP) or resiniferatoxin (RTX) resulted in a significant decrease in maximal number of binding sites ($B_{max}$) for [$^3$H]NMS and/or [$^3$H]αβ-MeATP in the urothelium and detrusor muscle. These results demonstrated that 1) pharmacological characteristics of muscarinic and P2X receptors in rat bladder urothelium were similar to those in the detrusor muscle, 2) that densities of these receptors were significantly altered by pretreatments with CYP and RTX, and 3) that these receptors may be pharmacologically affected by imidafenacin and Dpr-P-4 (N → O) which are excreted in the urine.



## 1. Introduction

Overactive bladder (OAB) is a chronic syndrome that is characterized by increases in frequency of micturition, urgency, and urge incontinence, having negative impacts on health-related quality of life (QoL) (1,2). Antimuscarinic agents are the first-line treatment for OAB, and their effectiveness has been documented in clinical trials (2). Physiological functions of acetylcholine in the bladder are dependent on the site of muscarinic receptors, such as detrusor muscle, pre-junctional terminals and sensory nerves, and urothelium, in which they induce bladder contractions, modulate release of chemical mediators, and influence afferent nerve activity, respectively (3). Antimuscarinics have been suggested to exert inhibitory effect on afferent nerve activity (2,4,5).

Bladder urothelium is a multifunctional tissue that acts not only as barrier between vesical contents of lower urinary tract and underlying tissues, but also as sensory organ by transducing physical and chemical stresses to the attendant afferent nervous system and underlying smooth muscle. Muscarinic receptor subtype was detected in the human bladder urothelium (6). The urothelium was shown to respond to stretch and muscarinic agonist stimuli by releasing adenosine triphosphate (ATP), nitric oxide, and acetylcholine itself, which may, in turn, modulate afferent activity through nerves and/or myofibroblasts (7,8). Therefore, urothelial

*Abbreviations:* [$^3$H]NMS, [*N*-methyl-$^3$H]scopolamine methyl chloride; [$^3$H]αβ-MeATP, αβ-methylene ATP [2,8-$^3$H]tetrasodium salt; 4-DAMP mustard, *N*-(2-chloroethyl)-4-piperidinyl diphenylacetate; CYP, cyclophosphamide; RTX, resiniferatoxin; $K_d$, apparent dissociation constant; $B_{max}$, maximal number of binding sites ($B_{max}$); $K_i$, inhibition constant; OAB, overactive bladder; ATP, adenosine triphosphate; IC, interstitial cystitis.

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muscarinic receptors may be the site of action for antimuscarinic agents. Previous studies reported that imidafenacin and an active metabolite of propiverine were excreted in human urine at sufficient concentrations to exert pharmacological effects on bladder (9,10).

Purinergic (P2X) receptors are considered to play a role in lower urinary tract function, regulating both afferent and efferent signaling pathways to control urine storage and elimination (11). Subtypes of $P2X_2$ and $P2X_3$ in the urothelium are involved in sensory functions, whereas $P2X_1$ receptor is expressed predominantly in detrusor muscle and play a role in efferent control of bladder function (12). When bladder is stretched during the filling phase, it releases ATP, which may act on receptors present on sensory nerves in the urothelium, thereby conveying information to central nervous system and initiating voiding (13). Therefore, urothelial P2X receptors are also important for micturition.

Interstitial cystitis (IC) is a chronic inflammatory disease of bladder characterized by urinary frequency, urgency, and suprapubic pain and relieved by voiding. Evidence to suggest that abnormalities in muscarinic and purinergic signaling transduction in the bladder are involved in the development of IC is increasing (11,14–16). Bladder biopsy specimens from patients with bladder obstruction or detrusor instability revealed that distribution of purinergic receptors was altered under pathological conditions (17). RTX, an extremely potent vanilloid, having desensitizing effects at nanomolar concentrations (18), was previously shown to effectively improve urinary frequency, urgency, and urge incontinence in patients (19). Antimuscarinic agents have been suggested to decrease bladder afferent activity by blocking urothelial muscarinic receptors, thereby improving OAB symptoms (20). However, muscarinic and purinergic receptors in the urothelium have not been directly identified. The present study aimed to characterize these receptors simultaneously in the urothelium and detrusor muscle of rat bladders, using selective radioligands.

## 2. Methods

### 2.1. Materials

[*N*-methyl-$^3$H]scopolamine methyl chloride ([$^3$H]NMS, 3.03 TBq/mmol) and αβ-methylene ATP [2,8-$^3$H]tetrasodium salt ([$^3$H] αβ-MeATP, 567 GBq/mmol) were purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). Imidafenacin was donated by Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan). Propiverine hydrochloride (propiverine), 1-methyl-4-piperidyl benzilate N-oxide (DPr-P-4 (N → O)), and PPADS (pyridoxal-phosphate-6-azophenyl-2′, 4′-disulphonic acid tetrasodium) were donated by Taiho Pharmaceutical Co., Ltd. (Saitama, Japan). 5-((3-phenoxybenzyl)[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]aminocarbonyl)-1,2,4-benzenetricarboxylic acid (A-317491) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-DAMP mustard was donated by Dr. Frederick J. Ehlert. All other chemicals were obtained from commercial sources.

### 2.2. Animals

Male Sprague-Dawley rats (250–300 g) at 8–10 weeks of age were purchased from Japan SLC (Shizuoka, Japan). 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 ± 24 °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 (registration number: 136023).

### 2.3. Drug administration

In experiments in which 4-DAMP mustard was used (21), the tissue homogenate was incubated with 1 μM AF-DX116 at 37 °C for 10 min to reversibly block $M_2$ receptors, followed by the addition of 10 nM cyclized 4-DAMP mustard, and the homogenate was incubated at 37 °C for 60 min to irreversibly block $M_3$ receptors and centrifuged at 40,000 × g for 10 min. The resulting pellet was finally resuspended in KRB buffer for the binding study.

In experiments in which chemical cystitis was induced by CYP, rats received a single injection of CYP (150 mg/kg, i.p.) and survived for 3 days after the injection (22). In order to intravesically instill RTX, fasted rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and their bladders were exposed. A 27-gauge needle connected to a syringe was inserted into the bladder through the bladder dome, and RTX was directly instilled in to the bladder for 30 min. In order to intravesically instill imidafenacin and DPr-P-4 (N → O), these agents were directly instilled in to the bladder for 30 min.

### 2.4. Tissue preparation and binding assay for muscarinic and P2X receptors

Rats were sacrificed by taking blood from the descending aorta under anesthesia with pentobarbital sodium (50 mg/kg, i.p.). The urothelium and detrusor muscle in the bladder were dissected, minced with scissors, and the tissue homogenates were prepared (22). Protein concentrations were measured using the BCA Protein Assay Kit (Thermo scientific, Rockford, IL).

The binding assay for muscarinic and P2X receptors was performed using [$^3$H]NMS and [$^3$H]αβ-MeATP (22), respectively. In the [$^3$H]NMS binding assay, the homogenates of rat tissues were incubated with different concentrations (0.06–1.5 nM) of [$^3$H]NMS in 30 mM $Na^+$/HEPES buffer (pH 7.5) for 60 min at 25 °C. In the [$^3$H] αβ-Me-ATP binding assay, the homogenates of rat tissues were incubated with different concentrations (0.3–10 nM) of [$^3$H]αβ-Me-ATP in 50 mM Tris–HCl buffer (pH 7.4) for 60 min at 4 °C. In the competition experiment using muscarinic and P2X antagonists, tissue homogenates were incubated with [$^3$H]NMS (0.5 nM) and [$^3$H]αβ-Me-ATP (3 nM). The reaction was terminated by rapid filtration (Cell Harvester; Brandel Co., Gaithersburg, MD) through Whatman GF/B glass fiber filters, and the filters were then rinsed three times with 3 mL of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight by immersion in scintillation fluid, and radioactivity was determined by a liquid scintillation counter. Specific binding of both radioligands was determined experimentally from differences between counts in the absence and presence of 1 μM atropine and 3 μM αβ-MeATP, respectively.

### 2.5. Data analysis

[$^3$H]NMS binding data were subjected to a non-linear regression analysis using Graph Pad PRISM (ver. 5, Graph Pad Software, San Diego, CA). The apparent dissociation constant ($K_d$) and maximal number of binding sites ($B_{max}$) were estimated for [$^3$H]NMS and [$^3$H]αβ-Me-ATP. The ability of antagonists to inhibit the specific binding of [$^3$H]NMS and [$^3$H]αβ-Me-ATP was estimated from the $IC_{50}$, which is the molar concentration of each agent needed to displace 50% of specific binding. 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 or [$^3$H]αβ-Me-ATP.

Statistical analyses were performed with the 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. Significance was accepted at $P < 0.05$.

## 3. Results

### 3.1. Identification of muscarinic and P2X receptors in urothelium and detrusor muscle

The specific binding of [$^3$H]NMS and [$^3$H]αβ-MeATP in homogenates of the urothelium and detrusor muscle increased in a concentration-dependent manner and appeared to be saturated at approximately 0.5 ([$^3$H]NMS) and 3.0 ([$^3$H]αβ-MeATP) nM. The calculated values of $K_d$ and $B_{max}$ for specific [$^3$H]NMS binding in the urothelium (259 ± 24 pM and 105 ± 7 fmol/mg protein, respectively) were similar to those in the detrusor muscle (289 ± 21 pM and 125 ± 13 fmol/mg protein, respectively). No significant differences were observed in the calculated $K_d$ or $B_{max}$ values for specific [$^3$H]αβ-MeATP binding between the urothelium (1219 ± 207 pM and 9.00 ± 0.62 fmol/mg protein, respectively) and detrusor muscle (1311 ± 97 pM and 11.1 ± 0.9 fmol/mg protein, respectively).

### 3.2. Inhibition of specific binding of [$^3$H]NMS and [$^3$H]αβ-MeATP by antimuscarinic agents and P2X receptor antagonists

Antimuscarinic agents such competed with [$^3$H]NMS for binding sites in homogenates of the urothelium and detrusor muscle in a concentration-dependent manner (Fig.1). Based on their $K_i$ values, the rank order of binding affinities of these antimuscarinic agents in the urothelium was imidafenacin > darifenacin > Dpr-P-4 (N → O) > propiverine (Table 1). No significant difference was observed in p$K_i$ values for antimuscarinic agents between the urothelium and detrusor muscle. The Hill coefficients were close to unity for antimuscarinic agents, except for darifenacin.

**Table 1**
$K_i$ values and Hill coefficients (nH) for *in vitro* inhibition by imidafenacin, darifenacin, propiverine, and DPr-P-4 (N → O) of specific [$^3$H]NMS binding in the urothelium and detrusor muscle in the rat bladder.

| Drug | Urothelium | | Detrusor muscle | |
|---|---|---|---|---|
| | $K_i$ (nM) | nH | $K_i$ (nM) | nH |
| Imidafenacin | 3.91 ± 0.84 | 1.10 ± 0.11 | 3.66 ± 0.46 | 1.04 ± 0.07 |
| Darifenacin | 17.4 ± 2.4 | 0.76 ± 0.09 | 18.0 ± 5.9 | 0.73 ± 0.06 |
| Propiverine | 328 ± 33 | 0.92 ± 0.05 | 348 ± 21 | 1.09 ± 0.17 |
| DPr-P-4 (N → O) | 186 ± 11 | 0.93 ± 0.07 | 231 ± 21 | 0.91 ± 0.04 |

The specific binding of [$^3$H]NMS (0.5 nM) in rat tissues was measured in the absence and presence of different concentrations of imidafenacin, darifenacin, propiverine, and DPr-P-4 (N → O). $K_i$ and Hill coefficients were estimated. Each value represents the mean ± SEM of three to eight rats.

αβ-MeATP, A317491, and PPADS inhibited specific [$^3$H]αβ-MeATP binding in homogenates of the urothelium and detrusor muscle in a concentration-dependent manner (Fig. 1). The $K_i$ values of each agent in the urothelium were similar to those in the detrusor muscle (Table 2). Based on their $K_i$ values, the rank order of binding affinities of these P2X receptor antagonists in the bladder was αβ-MeATP > A317491 ≈ PPADS. The Hill coefficients for αβ-MeATP, A317491, and PPADS in these tissues were close to unity.

### 3.3. Effects of 4-DAMP on muscarinic receptors

In order to estimate the relative distribution of $M_2$ and $M_3$ muscarinic receptor subtypes in the urothelium and detrusor muscle, specific [$^3$H]NMS binding was examined by a pretreatment

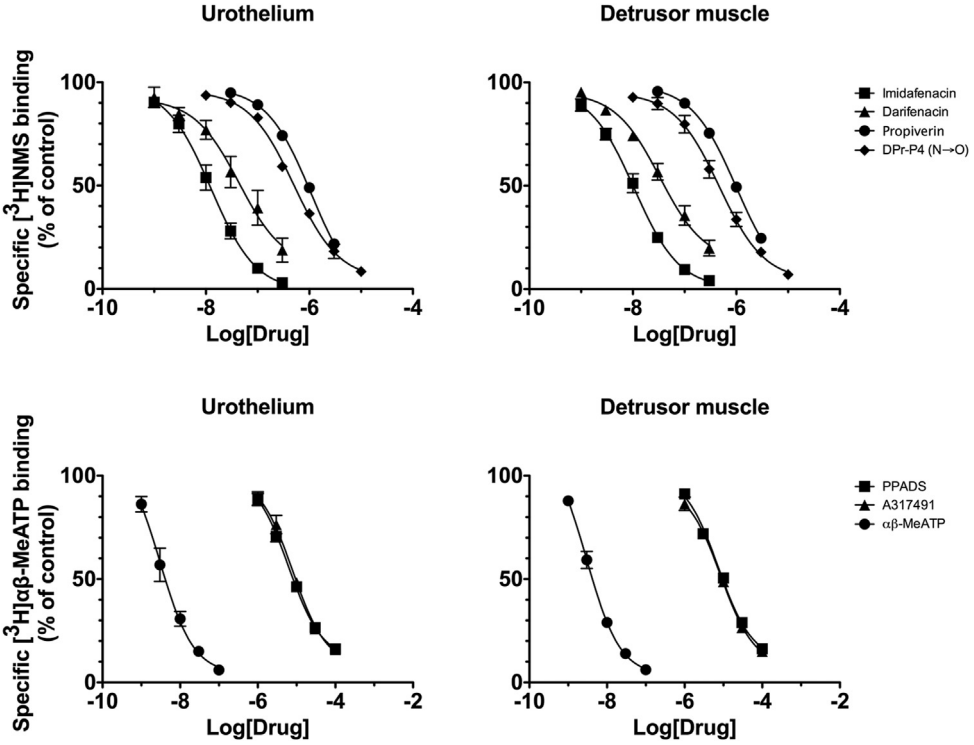


**Fig. 1.** *In vitro* inhibition by muscarinic (upper panel) and purinergic (lower panel) agents of the specific binding of [$^3$H]NMS and [$^3$H]αβ-Me-ATP in the urothelium and detrusor muscle in the rat bladder. Upper panel, muscarinic agents: imidafenacin (■), darifenacin (▲), DPr-P-4 (N → O) (◆), and propiverine (●). Lower panel, purinergic agents: αβ-MeATP (●), A317491 (▲), and PPADS (■). The ordinate is the specific binding of [$^3$H]NMS (0.5 nM) or [$^3$H]αβ-Me-ATP (3.0 nM) (% of control). The abscissa is the logarithmic molar concentrations of imidafenacin (1–300 nM), darifenacin (1–300 nM), DPr-P-4 (N → O) (0.01–10 μM), and propiverine (30–3000 nM) (upper panel), as well as those of αβ-Me-ATP (1–100 nM), A317491 (1–100 μM) and PPADS (1–100 μM) (lower panel). Each point represents the mean ± SEM of three to eight rats.

**Table 2**
$K_i$ values and Hill coefficients (nH) for *in vitro* inhibition by αβ-MeATP, A317491 and PPADS of specific [$^3$H]αβ-MeATP binding in the urothelium and detrusor muscle in the rat bladder.

| Drug | Urothelium | | Detrusor muscle | |
|---|---|---|---|---|
| | $K_i$ (nM) | nH | $K_i$ (nM) | nH |
| αβ-MeATP | 1.58 ± 0.37 | 0.94 ± 0.04 | 1.78 ± 0.30 | 0.96 ± 0.02 |
| A317491 | 2741 ± 273 | 0.85 ± 0.05 | 2992 ± 342 | 0.77 ± 0.05 |
| PPADS | 3081 ± 437 | 0.84 ± 0.08 | 3642 ± 304 | 0.93 ± 0.11 |

The specific binding of [$^3$H]αβ-MeATP (3.0 nM) in rat tissues was measured in the absence and presence of different concentrations of A317491 and PPADS. The $K_i$ and Hill coefficients were estimated. Each value represents the mean ± SEM of four to eight rats.

with 4-DAMP mustard. The treatment with 4-DAMP mustard caused significant decreases (36%, 22% and 30%, respectively) in $B_{max}$ values for specific [$^3$H]NMS binding in the urothelium and detrusor muscle compared with control values (Table 3). $B_{max}$ values of $M_3$ receptors in the urothelium and detrusor muscle (37.3 ± 10.0 and 27.2 ± 4.0 fmol/mg protein, respectively) were estimated from decreases in the $B_{max}$ values. Thus, the $B_{max}$ value was significantly higher in the urothelium than in the detrusor muscle.

### 3.4. Effects of CYP treatment on muscarinic and P2X receptors

The $B_{max}$ values for specific [$^3$H]NMS binding were significantly lower in the urothelium (39%) and detrusor muscle (19%) of CYP-treated rats than in those of control rats (Table 4). A significant decrease was also observed in $B_{max}$ values for specific [$^3$H]αβ-MeATP binding in CYP-treated rats, with decreases of 55% and 32% in the urothelium and detrusor muscle, respectively (Table 4). On the other hand, $K_d$ values for the specific binding of [$^3$H]NMS and [$^3$H]αβ-MeATP in both tissues were not significantly altered by the CYP treatment.

### 3.5. Effects of RTX treatment on muscarinic and P2X receptors

After the intravesical instillation of RTX (10, 100 nM/0.2 mL per rat) for 30 min, no significant alterations were observed from control values in the $K_d$ or $B_{max}$ values for specific [$^3$H]NMS binding in the urothelium and detrusor muscle (Table 5). On the other hand, the $B_{max}$ values for [$^3$H]αβ-MeATP binding in these tissues were significantly lower (30% and 26%, respectively) than control values after the intravesical instillation of RTX (100 nM). The instillation of a lower concentration (10 nM) of RTX did not markedly affect $B_{max}$ values for [$^3$H]αβ-MeATP binding. The $K_d$ values for specific [$^3$H]αβ-MeATP binding in each tissue were not significantly different from control values.

### 3.6. Effects of intravesical instillation of imidafenacin and DPr-P-4 (N → O) on muscarinic receptors

The $K_d$ values for specific [$^3$H]NMS binding in the urothelium and/or detrusor muscle were significantly higher than the control values following the intravesical instillation of imidafenacin (300 nM/0.2 mL/rat) for 30 min (Table 6). Increases of 27% (urothelium) and 12% (detrusor muscle) were noted. The intravesical instillation of a lower concentration of imidafenacin (30 nM/0.2 mL/rat) for 30 min significantly enhanced the $K_d$ value for specific [$^3$H]NMS binding in the urothelium. On the other hand, no significant change was observed in the $B_{max}$ values for specific [$^3$H]NMS binding in these tissues following the intravesical instillation of imidafenacin. The intravesical instillation of DPr-P-4 (N → O) at concentrations of 3 and 30 μM/0.2 mL/rat for 30 min led to significantly higher $K_d$ values for specific [$^3$H]NMS binding in homogenates of the urothelium than the control values without affecting $B_{max}$ values. Increases of 16% (3 μM) and 25% (30 μM) were noted (Table 6).

## 4. Discussion

Muscarinic and P2X receptors were identified simultaneously in the urothelium and the detrusor muscle of rats using the selective radioligands. The estimated binding parameters revealed similar affinities and densities of these receptors in the urothelium and detrusor muscle. Antimuscarinic agents displayed high affinity to specific [$^3$H]NMS binding sites. Their affinities were similar in the urothelium and detrusor muscle, suggesting that antimuscarinics exhibited their therapeutic effects by blocking muscarinic receptors in the urothelium and detrusor muscle. Similar binding properties of antimuscarinics in the urothelium (mucosa) and detrusor muscle have also been reported in humans (23).

Muscarinic subtype in the urothelium is considered to activate the afferent nerve, whereas that in the detrusor muscle is responsible for muscle contraction (3). Thomas et al. (21) reported that 4-DAMP mustard, an irreversible alkylating agent for muscarinic receptors, selectively inactivated $M_3$ receptors over $M_2$ receptors. The pretreatment with 4-DAMP mustard led to significant decreases in the $B_{max}$ of specific [$^3$H]NMS binding in the urothelium (36%) and detrusor muscle (22%) of rats (Table 3), suggesting the coexistence of $M_2$ and $M_3$ subtypes in these tissues. The $M_2$ subtype is predominant in the urothelium and detrusor muscle, whereas the $M_3$ subtype may be more prominent in the urothelium than in the detrusor muscle.

Pharmacological concentrations of imidafenacin and Dpr-P-4 (N → O) were shown to be excreted in the urine of patients with OAB following the oral administration of imidafenacin and propiverine, respectively, at clinical dosages (9,10). The present study revealed that the intravesical administration of imidafenacin and Dpr-P-4 (N → O) bound muscarinic receptors in the urothelium and detrusor muscle of rats, suggesting that these agents, which are excreted in the urine, have direct local effects on bladder overactivity. These results support the intravesical instillation of antimuscarinics reducing the distension-evoked activity of C-fiber afferents in the rat bladder (24) and intravesically infused antimuscarinic agents suppressing the carbachol-induced bladder overactivity (25).

**Table 3**
Effects of 4-DAMP mustard on $K_d$ and $B_{max}$ for specific [$^3$H]NMS binding in rat tissues.

| Tissue | Control | | 4-DAMP mustard treated | |
|---|---|---|---|---|
| | $K_d$ (pM) | $B_{max}$ (fmol/mg protein) | $K_d$ (pM) | $B_{max}$ (fmol/mg protein) |
| Urothelium | 259 ± 24 | 105 ± 7 | 243 ± 19 | 67.7 ± 7.6 (0.64)* |
| Detrusor muscle | 289 ± 11 | 125 ± 3 | 277 ± 16 | 97.8 ± 4.1 (0.78)** |

The specific binding of [$^3$H]NMS (0.06–1.5 nM) in rat tissues was measured in the absence and presence of 4-DAMP mustard (10 nM). Values in parentheses represent the $B_{max}$ relative to controls. Asterisks show a significant difference from the control values, *$P < 0.05$, **$P < 0.001$. Each value represents the mean ± SEM of three to eight rats.

**Table 4**
$K_d$ and $B_{max}$ for specific binding of [$^3$H]NMS and [$^3$H]αβ-MeATP in the urothelium and detrusor muscle of control and CYP-treated rat bladders.

| Ligand | Tissue | Control | | CYP-treated | |
|---|---|---|---|---|---|
| | | $K_d$ (pM) | $B_{max}$ (fmol/mg protein) | $K_d$ (pM) | $B_{max}$ (fmol/mg protein) |
| [$^3$H]NMS | Urothelium | 259 ± 24 | 105 ± 7 | 268 ± 21 | 64.4 ± 4.9 (0.61)* |
| | Detrusor muscle | 289 ± 11 | 125 ± 3 | 289 ± 9 | 101 ± 5 (0.81)* |
| [$^3$H]αβ-MeATP | Urothelium | 1219 ± 207 | 9.00 ± 0.62 | 1214 ± 60 | 4.01 ± 0.85 (0.45)** |
| | Detrusor muscle | 1311 ± 97 | 11.1 ± 0.9 | 1264 ± 62 | 7.56 ± 1.05 (0.68)* |

The specific binding of [$^3$H]NMS (0.06–1.5 nM) and [$^3$H]αβ-MeATP (0.3–10 nM) in rat tissues was measured. Values in parentheses represent the $B_{max}$ relative to controls. Asterisks show a significant difference from the control values, *$P < 0.05$, **$P < 0.01$. Each value represents the mean ± SEM of four to eight rats.

**Table 5**
$K_d$ and $B_{max}$ for specific binding of [$^3$H]NMS and [$^3$H]αβ-MeATP in the urothelium and detrusor muscle of control and RTX-treated rat bladders.

| | Tissue | | $K_d$ (pM) | $B_{max}$ (fmol/mg protein) |
|---|---|---|---|---|
| [$^3$H]NMS | Urothelium | Control | 246 ± 6 | 126 ± 10 |
| | | 10 nM | 269 ± 12 | 112 ± 8 |
| | | 100 nM | 254 ± 6 | 108 ± 12 |
| | Detrusor muscle | Control | 272 ± 8 | 128 ± 6 |
| | | 10 nM | 283 ± 11 | 120 ± 10 |
| | | 100 nM | 286 ± 14 | 118 ± 10 |
| [$^3$H]αβ-MeATP | Urothelium | Control | 0.94 ± 0.06 | 12.0 ± 1.3 |
| | | 10 nM | 0.99 ± 0.09 | 9.29 ± 0.93 |
| | | 100 nM | 0.96 ± 0.07 | 8.36 ± 0.70 (0.70)* |
| | Detrusor muscle | Control | 0.98 ± 0.09 | 14.0 ± 1.1 |
| | | 10 nM | 0.99 ± 0.10 | 12.3 ± 0.5 |
| | | 100 nM | 1.03 ± 0.05 | 10.4 ± 1.2 (0.74)* |

The specific binding of [$^3$H]NMS (0.1–1.5 nM) in rat tissues was measured. Each value represents the mean ± SEM of five rats. The binding of [$^3$H]αβ-MeATP (0.3–10 nM) in rat tissues was measured. Values in parentheses represent the $B_{max}$ relative to controls. Asterisks show a significant difference from the control values, *$P < 0.05$. Each value represents the mean ± SEM of five rats.

P2X and muscarinic receptors in the bladder have been proposed as promising therapeutic targets for OAB. Ford et al. (12) indicated that $P2X_1$ receptors played a significant role in the efferent regulation of detrusor muscle excitability and contraction, while $P2X_3$ and $P2X_{2/3}$ receptors mediated sensory functions, including the afferent modulation of urinary storage and elimination. The present study revealed that P2X receptors were present in the urothelium and detrusor muscle in the rat bladder. The binding parameters for specific [$^3$H]αβ-MeATP binding in the urothelium were similar to those in the detrusor muscle. Furthermore, the P2X receptor binding affinities of αβ-MeATP, PPADS (a nonselective antagonist), and A-317491 ($P2X_3$ or $P2X_{2/3}$ selective antagonist) in the urothelium were similar to those in the detrusor. Thus, these results suggested similarities in the pharmacological characteristics of P2X receptors in the urothelium and detrusor muscle.

The expression of bladder muscarinic and purinergic receptors in IC models was investigated previously by immunohistochemical, and Western and Northern blotting techniques (13,14,17). Kageyama et al. (22) showed that a treatment with CYP down-regulated the expression of muscarinic and P2X receptors in the bladders of rats. This finding was further confirmed by a significant decrease in $B_{max}$ for [$^3$H]NMS and [$^3$H]αβ-MeATP in the rat urothelium and detrusor muscle (Table 4). Muscarinic receptors are known to undergo compensatory regulation following significant changes in cholinergic neuronal activity (26). Mok et al. (27) suggested a pre-junctional effect in CYP-treated bladders that increased transmitter release in order to compensate for the down-regulation of cholinergic and purinergic receptors. Thus, a CYP treatment may significantly increase neuronal activity in the bladder, resulting in the compensatory down-regulation of pharmacological receptors.

Birder et al. (11) also showed a marked reduction in the expression of $P2X_1$ in the IC bladder. Previous studies reported that the release of ATP was significantly increased in IC rats by a CYP treatment (11,15). Another study clarified that ATP was significantly elevated in the urine of individuals with IC, and the stretch-activated release of ATP was augmented in the IC urothelium (16). Therefore, ATP release from the urothelium may be increased by a treatment with CYP, resulting in the compensatory down-regulation of P2X receptors. The results of the present study indicate that the densities of muscarinic and P2X receptors were significantly decreased not only in the detrusor muscle but also in

**Table 6**
$K_d$ and $B_{max}$ for specific [$^3$H]NMS binding in the urothelium and detrusor muscle in the rat bladder after the intravesical instillation of antimuscarinic agents for 30 min.

| Drug | | Urothelium | | Detrusor | |
|---|---|---|---|---|---|
| | | $K_d$ (pM) | $B_{max}$ (fmol/mg protein) | $K_d$ (pM) | $B_{max}$ (fmol/mg protein) |
| Control | | 273 ± 5 | 107 ± 4 | 268 ± 6 | 118 ± 4 |
| Imidafenacin | 30 nM | 307 ± 9 (1.12)* | 113 ± 9 | 283 ± 12 | 124 ± 11 |
| | 300 nM | 348 ± 15 (1.27)** | 120 ± 10 | 301 ± 12 (1.12)* | 126 ± 9 |
| DPr-P-4 (N → O) | 3 μM | 316 ± 12 (1.16)** | 118 ± 9 | 289 ± 11 | 120 ± 11 |
| | 30 μM | 341 ± 20 (1.25)** | 115 ± 11 | 253 ± 16 | 116 ± 8 |

Rats received 0.2 mL/body of an antimuscarinic agent intravesically, and were sacrificed at 30 min after the administration. Specific binding of [$^3$H]NMS (0.06–1.5 nM) in the rat bladder was measured. Values in parentheses represent fold-increases in $K_d$ values relative to controls. Asterisks show a significant difference from the control values, *$P < 0.05$, **$P < 0.01$. Each value represents the mean ± SEM of six rats.

the urothelium of rats by the CYP treatment. Furthermore, Tempest et al. (17) showed that the expression of $P2X_2$ and $P2X_3$ receptors was significantly altered in the urothelium in the bladder of patients with IC.

RTX, a vanilloid compound and agonist of transient receptor potential channel1 (TRPV1), is known for its beneficial effects on IC or detrusor overactivity (28). The ATP-induced facilitation of the micturition reflex in rats was previously shown to be effectively antagonized by a treatment with RTX (29). The intravesical instillation of RTX in patients with neurogenic detrusor overactivity led to a significant decrease in $P2X_3$ immunoreactivity in bladder specimens (30). The present study revealed that the intravesical instillation of RTX decreased the $B_{max}$ values for specific [$^3$H]αβ-MeATP binding in the urothelium and detrusor muscle in the bladder (Table 5). Lin et al. (31) previously reported that the administration of RTX markedly increased endogenous ATP levels in dorsal root ganglia and skin tissues in mice. Thus, an increase in the release of ATP from the urothelium may cause the compensatory down-regulation of purinergic receptors. Furthermore, Kuo (32) showed that the intravesical instillation of RTX was an effective treatment for patients with detrusor overactivity refractory to antimuscarinics. Therefore, our present results appear to be consistent with these functional observations.

## 5. Conclusion

The present study revealed that 1) pharmacological characteristics of muscarinic and P2X receptors in the urothelium of rat bladder were similar to those in the detrusor muscle, 2) the densities of these receptors were significantly altered by pretreatments with CYP and RTX, and 3) these receptors may be pharmacologically affected by imidafenacin and Dpr-P-4 (N → O) excreted in the urine. This is the first study to characterize pharmacologically-relevant receptors in the rat urothelium using a radioligand binding assay.

## Conflict of interest

There is no conflict of interest in this study.

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