Regular Article

Noradrenaline-Induced Relaxation of Urinary Bladder Smooth Muscle Is Primarily Triggered through the β_3 -Adrenoceptor in Rats

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β -Adrenoceptors are subclassified into 3 subtypes (β_1 – β_3). Among these, β_3 -adrenoceptors are present in various types of smooth muscle and are believed to play a role in relaxation responses of these muscles. β_3 -Adrenoceptors are also present in urinary bladder smooth muscle (UBSM), although their expression varies depending on the animal species. To date, there has been little information available about the endogenous ligand that stimulates β_3 -adrenoceptors to produce relaxation responses in UBSM. In this study, to determine whether noradrenaline is a ligand of UBSM β_3 -adrenoceptors, noradrenaline-induced relaxation was analyzed pharmacologically using rat UBSM. We also assessed whether noradrenaline metabolites were ligands in UBSM. In isolated rat urinary bladder tissues, mRNAs for β_1 -, β_2 -, and β_3 -adrenoceptors were detected using RT-PCR. In UBSM preparations contracted with methacholine (3×10^{-5} M), noradrenaline-induced relaxation was not inhibited by the following antagonists: atenolol (10^{-6} M; selective β_1 -adrenoceptor antagonist), ICI-118,551 (3×10^{-8} M; selective β_2 -adrenoceptor antagonist), propranolol (10^{-7} M; non-selective β -adrenoceptor antagonist), and bupranolol (10^{-7} M; non-selective β -adrenoceptor antagonist). In the presence of propranolol (10^{-6} M), noradrenaline-induced relaxation was competitively inhibited by bupranolol (3×10^{-7} – 3×10^{-6} M) or SR59230A (10^{-7} – 10^{-6} M; selective β_3 -adrenoceptor antagonist), with their pA_2 values calculated to be 6.64 and 7.27, respectively. None of the six noradrenaline metabolites produced significant relaxation of methacholine-contracted UBSM. These findings suggest that noradrenaline, but not its metabolites, is a ligand for β_3 -adrenoceptors to produce relaxation responses of UBSM in rats.

Key words rat urinary bladder smooth muscle; noradrenaline; β -adrenoceptor

INTRODUCTION

The urinary bladder (UB) is an organ that stores urine and discharges it outside the body. These physiological functions are controlled by the relaxation and contraction responses of UB smooth muscle (UBSM), and both responses are affected strongly by autonomic nerves. In particular, urine discharge is associated with UBSM contraction. This contraction is principally triggered by acetylcholine that is released from parasympathetic nerve endings, the activity of which predominates in the micturition phase. In contrast, urine storage (retention) is associated with UBSM relaxation. This relaxation response is generally considered to be triggered by noradrenaline that is released from sympathetic nerve endings, the activity of which predominates in the UB filling phase.^{1–3)} However, how sympathetic nerves contribute to the relaxation of UBSM and urine storage is not completely understood.

If noradrenaline is a key molecule to trigger UBSM relaxation, it is reasonable to postulate that it would target β -adrenoceptors in UBSM, as it does in other smooth muscles. β -Adrenoceptors are classified into 3 subtypes (β_1 – β_3),⁴⁾ and UBSM subtype expression is species-dependent.⁵⁾ In humans, the main β -adrenoceptor subtype in UBSM has been identified as β_3 at the mRNA level.^{5,6)} In addition, a selective β_3 -adrenoceptor agonist, mirabegron, has been reported to induce UBSM relaxation and improve overactive bladder (OAB) symptoms by increasing bladder capacity.^{7–9)} These findings suggest that β_3 -adrenoceptors play a significant role in the

regulation of the UBSM relaxation response and, thus, urine storage. However, whether noradrenaline acts as an endogenous ligand for the β_3 -adrenoceptor to induce UBSM relaxation has not been convincingly established. This is because almost all pharmacological studies on UBSM β -adrenoceptors were designed using synthetic β -adrenoceptor agonists (isoprenaline or selective β_3 -adrenoceptor agonists), but not endogenous catecholamines such as noradrenaline.

The purpose of this study was to investigate whether noradrenaline is a ligand for the β_3 -adrenoceptor using rat UBSM tissue. This tissue expresses all subtypes of β -adrenoceptors,^{10,11)} allowing the determination of whether noradrenaline can stimulate β_1 and β_2 subtypes in addition to β_3 . In this study, we also examined six metabolites of noradrenaline in order to verify whether they can induce UBSM relaxation *via* stimulation of β_3 -adrenoceptors.

MATERIALS AND METHODS

Drugs The followings drugs were used: ($\pm$)-atenolol, desipramine hydrochloride, 3,4-dihydroxymandelic acid (DOMA), DL-3,4-dihydroxyphenylglycol (DHPG), 3,5-dinitrocatechol, DL-4-hydroxy-3-methoxymandelic acid (VMA), 4-hydroxy-3-methoxyphenyl glycol (MHPG) hemipiperazinium salt, 4-hydroxy-3-methoxyphenylglycol sulfate (MHPG-S) potassium salt, indomethacin, ICI-118,551 hydrochloride, (–)-isoproterenol (ISO) hydrochloride, DL-normetanephrine (NMN) hydrochloride, *N*-methyl-*N*-propargyl-3-(2,4-dichlo-

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rophenoxy)propylamine (clorgiline) hydrochloride, DL-propranolol hydrochloride, SR 59230A (all from Sigma-Aldrich Co., St. Louis, MO, U.S.A.); ($\pm$)-phentolamine mesylate (Novartis Pharma, Basel, Switzerland); acetyl- β -methylcholine (methacholine) chloride, (*R*)-(-)-norepinephrine (noradrenaline) hydrogen tartrate monohydrate (both from Wako Pure Chemical Industries, Ltd., Osaka, Japan); and ($\pm$)-bupranolol hydrochloride (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan). All other chemicals were commercially available and reagent grade.

Atenolol was dissolved in 0.1 N hydrochloric acid (HCl) as a stock solution at 2×10^{-2} M and diluted with distilled water. Indomethacin was dissolved in pure ethanol as a stock solution at 10^{-2} M. 3,5-Dinitrocatechol was dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 4×10^{-3} M and diluted with distilled water. SR 59230A was dissolved in DMSO as a stock solution at 2×10^{-2} M and diluted with distilled water. All other drugs were prepared as aqueous stock solutions and diluted with distilled water.

Animals Male Wistar rats (8–10 weeks old; weight 165–265 g, Sankyo Labo Service Corporation, Tokyo, Japan) were housed under controlled conditions (21–22°C, relative air humidity $50 \pm 5\%$, fixed 12 h light–dark cycle (08:00–20:00)) with food and water available *ad libitum*. This study was approved by the Toho University Animal Care and User Committee (approval number: 16-52-294, accredited on May 16, 2016; approval number: 17-53-294, accredited on May 17, 2017) and was conducted in accordance with the User's Guideline to the Laboratory Animal Center of Faculty of Pharmaceutical Sciences, Toho University.

RT-PCR Analysis of β -Adrenoceptor Subtype mRNA Expression The rats were anaesthetized with isoflurane (inhalation) and euthanized by exsanguination from a carotid artery. The UB, atrium (both right and left atria), and ileum were immediately removed and placed in normal Tyrode's solution of the following composition (mM): NaCl, 158.3; KCl, 4.0; CaCl_2 , 2.0; MgCl_2 , 1.05; NaH_2PO_4 , 0.42; NaHCO_3 , 10.0; and glucose, 5.6. All tissues were stripped of surrounding adipose and connective tissue, and the bladder trigone from the UB. After washing out debris, and inserting an acrylic rod into the lumen of the ileum, the longitudinal ileal smooth muscle was isolated using tweezers and a cotton swab. The preparations were frozen in liquid nitrogen after removing moisture with filter paper. These frozen preparations were pulverized using a frozen cell crusher apparatus (Cryo-Press™; Microtec Co., Ltd., Funabashi, Chiba, Japan).

Total RNA from the resulting powders was extracted using RNAiso Plus™ (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's protocol. First-strand cDNA was synthesized by reverse transcription with 0.5 μg total RNA per 10 μL reaction mixture using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO Co., Ltd., Osaka, Japan). PCR was performed using the GoTaq® Green Master Mix (Promega Corp., Madison, WI, U.S.A.) with 0.5 μL cDNA solution per 10 μL reaction mixture in a TaKaRa PCR Thermal Cycler Dice® Touch (TaKaRa Bio Inc.). The specific oligonucleotide primers for β_1 -adrenoceptors (forward, 5'-GATCTGGTCATGGGACGTGCT-3' and reverse, 5'-AGCACTTGGGGTCTTGTAG-3'), β_2 -adrenoceptors (forward, 5'-ACCAAGAATAAGGCCGAGT-3' and reverse, 5'-GTCTTGAGGCTTTGTGCTC-3'), β_3 -adrenoceptors (forward, 5'-TGCTCGAGTGTTCTGCTAG-3' and reverse, 5'-GAA

GGCAGATTGGCATA GC-3'), and β -actin (forward, 5'-ATG GTGGGTATGGGT CAGAA-3' and reverse, 5'-ACCCTC ATAGATGGGCACAG-3') were synthesized at Eurofins Genomics (Tokyo, Japan). The PCR samples were heated for 2 min at 95°C, and then amplified by 35 cycles at 95°C for 20 s, 60°C for 5 s, and 72°C for 30 s (β -adrenoceptors) or by 25 cycles at 95°C for 20 s, 60°C for 5 s, and 72°C for 30 s (β -actin), followed by a 5-min extension at 72°C. The PCR products were separated via 1.5% agarose gel (containing ethidium bromide) electrophoresis and visualized under UV illumination.

Preparation of UB Strips and Recording of Isotonic Tension Changes The isolated rat UB, after removing the surrounding adipose tissue, connective tissue, and bladder trigone, was opened with a longitudinal incision and UB strips (approximately 2 mm in width $\times$ 25 mm in length) were prepared in normal Tyrode's solution. The UB strips were mounted under an optimal resting tension of 0.5 g in a 20-mL organ bath containing normal Tyrode's solution, aerated with 95% O_2 and 5% CO_2 , and maintained at $32 \pm 1^\circ\text{C}$. Tension changes were recorded isotonicity via a kymograph and lever. The UB preparations were equilibrated for 60 min prior to the first methacholine-induced contraction, during which time the normal Tyrode's solution was replaced every 20 min with fresh solution. After the initial 60-min incubation, the UB preparation was contracted with methacholine (3×10^{-5} M). When the contraction reached steady-state, the preparation was relaxed by applying isoprenaline. This procedure was performed twice before starting the experiment (performed 3 times in total). All experiments were carried out in the presence of indomethacin (3×10^{-6} M) to prevent any possible effects of endogenous prostaglandins.

Evaluation of Effects of β -Adrenoceptor Antagonists on Noradrenaline-Induced Relaxation After conducting the preliminary procedures described in the previous section, the UB preparations were treated with methacholine (3×10^{-5} M). When the contraction reached a steady-state level, noradrenaline was cumulatively applied to the bath solution in order to obtain a concentration-response curve. After the UB preparation had been fully recovered by washing with fresh bath solution, the preparation was again treated with methacholine (3×10^{-5} M) in the presence of the indicated β -adrenoceptor antagonists; when the methacholine-induced contraction reached steady-state, noradrenaline was cumulatively applied in order to obtain a concentration-response curve. The tested β -adrenoceptor antagonists were atenolol (10^{-6} M), ICI-118,551 (3×10^{-8} M), propranolol (10^{-7} – 10^{-6} M), bupranolol (10^{-7} – 3×10^{-6} M), and SR 59230A (10^{-7} – 10^{-6} M). When bupranolol (3×10^{-7} – 3×10^{-6} M) or SR 59230A (10^{-7} – 10^{-6} M) was administered, the experiment was performed in the presence of propranolol (10^{-6} M) according to previous studies.^{12,13)}

This series of experiments was carried out in the presence of desipramine (3×10^{-7} M) as an uptake-1 inhibitor, NMN (10^{-6} M) as an uptake-2 inhibitor, and phentolamine (10^{-6} M) as a non-selective α -adrenoceptor antagonist to prevent possible effects of noradrenaline reuptake or α -adrenoceptors. These drugs were administered 20 min before methacholine.

Evaluation of Effects of Noradrenaline Metabolites on Methacholine-Induced Contraction After conducting the preliminary procedures described in "Preparation of UB Strips and Recording of Isotonic Tension Changes," the UB

preparations were treated with methacholine (3×10^{-5} M). When the contraction reached a steady-state level, noradrenaline or the indicated noradrenaline metabolites (10^{-4} M each) was applied to the bath solution. Ten minutes after the administration, isoprenaline (10^{-4} M) was applied to confirm that the UB preparation was sufficiently relaxed.

This series of experiments was carried out in the presence of clorgiline (10^{-5} M) as a monoamine oxidase A (MAO_A) inhibitor, 3,5-dinitrocatechol (2×10^{-6} M) as a catechol-*O*-methyltransferase (COMT) inhibitor, and phentolamine (10^{-6} M) to prevent any possible effects of metabolism of noradrenaline or α -adrenoceptors. These drugs were administered 20 min before methacholine.

Data Analysis The extent of relaxation induced by noradrenaline and the six noradrenaline metabolites was calculated relative to the tone level before the application of 3×10^{-5} M methacholine (100% relaxation), and to the steady-state tone level prior to the application of each relaxant (0% relaxation).

The potencies of noradrenaline were calculated as pD_2 (pEC_{50}) values (the negative logarithm of the effective agonist concentration producing a response that is 50% of the maximum response). The data were plotted as a function of noradrenaline concentration and fitted to the equation:

$$E = E_{\max} \times A^{n_H} / (EC_{50}^{n_H} + A^{n_H})$$

where E is the % relaxation at a given concentration, $E_{\max}$ is the maximum response, A is the noradrenaline concentration, n_H is the Hill coefficient, and EC_{50} is the agonist concentration producing a 50% response. Curve-fitting was carried out using GraphPad Prism™ (Version 6.07; GraphPad Software, Inc., San Diego, CA, U.S.A.).

The β -adrenoceptor antagonist potencies are expressed as pA_2 values, which were calculated according to the method originally reported by Arunlakshana and Schild.¹⁴⁾

Data are expressed as means $\pm$ standard error of the mean (S.E.M.) or means with 95% confidence intervals (95% CIs) and n refers to the number of experiments. The significance of the differences between mean values was evaluated by two-way ANOVA or paired t -tests using GraphPad Prism™. A p -value less than 0.05 was considered statistically significant.

RESULTS

Expression of mRNAs for β -Adrenoceptor Subtypes in Rat UB Preparations Figure 1 shows representative images of agarose gels for β_1 -, β_2 -, and β_3 -adrenoceptor PCR products in rat UB, atrium (both right and left atria) (A), and ileal longitudinal smooth muscle (I), with the expected PCR products of 337, 386, and 352 base pairs, respectively. In both the UB and ileal longitudinal smooth muscle, mRNAs for all 3 β -adrenoceptors (β_1 -, β_2 -, and β_3 -) were detected. In contrast, in the atrium, β_1 - and β_2 -adrenoceptor mRNAs were clearly detected, but that of β_3 -adrenoceptor was absent or barely detected. The PCR product for β -actin, as an internal standard, was detected in all 3 preparations; this had the expected size of 375 base pairs. No bands were observed in the absence of reverse transcription (RT(-)).

Effects of Various Antagonists for β -Adrenoceptors on Noradrenaline-Induced Relaxation Figure 2A shows the effects of repeated noradrenaline administration on its

concentration-relaxation curves in rat UBSM. The response curves of noradrenaline did not change in both the first and second applications; this was evidenced by the lack of statistically significant differences in both pD_2 (5.85 ± 0.08 for first and 5.83 ± 0.05 for second, $n = 8$ each, $p > 0.05$) and $E_{\max}$ ($52.4 \pm 4.4\%$ for first and $50.3 \pm 3.0\%$ for second, $n = 8$ each, $p > 0.05$) values between the first and second applications.

Figures 2B–F show the effects of various types of β -adrenoceptor antagonists on noradrenaline-induced relaxation. The tested β -adrenoceptor antagonists were: atenolol (a selective β_1 -adrenoceptor antagonist, 10^{-6} M) (Fig. 2B), ICI-118,551 (a selective β_2 -adrenoceptor antagonist, 3×10^{-8} M) (Fig. 2C), propranolol (a nonselective β -adrenoceptor antagonist, 10^{-7} M, 10^{-6} M) (Figs. 2D, E, respectively), and bupranolol (a nonselective β -adrenoceptor antagonist, 10^{-7} M) (Fig. 2F). Noradrenaline-induced relaxation was not affected by atenolol, ICI-118,551, 10^{-7} M propranolol, or bupranolol (10^{-7} M). However, the noradrenaline-induced relaxation curve was shifted rightward by approximately 2-fold by 10^{-6} M propranolol (Fig. 2E).

Effects of Bupranolol and SR 59230A on Noradrenaline-Induced Relaxation Figure 3A shows the effects of repeated noradrenaline administration on its concentration-relaxation curves in rat UBSM in the presence of propranolol (10^{-6} M). The response curves of noradrenaline did not change in both the first and second applications; this was demonstrated by the absence of statistically significant differences in both pD_2 (5.36 ± 0.04 for first and 5.32 ± 0.03 for second, $n = 12$ each, $p > 0.05$) and $E_{\max}$ ($45.6 \pm 2.0\%$ for first and $43.6 \pm 1.9\%$ for second, $n = 12$ each, $p > 0.05$) values between the first and second applications.

Figures 3B–D show the effects of bupranolol on noradrenaline-induced relaxation in the presence of propranolol (10^{-6} M). The noradrenaline-induced relaxation was inhibited by bupranolol (3×10^{-7} – 3×10^{-6} M) in a concentration-dependent manner, shifting the corresponding concentration-response curve rightward (Figs. 3B–D). Figure 3E shows the Schild plot of bupranolol against noradrenaline based on the results of Figs. 3B–D. Schild regression analysis generated a straight line with a slope of 0.92, which was not significantly different from unity (95% CIs: 0.52–1.31, $n = 13$) (Fig. 3E). This indicates that noradrenaline-induced relaxation was antagonized competitively by bupranolol (3×10^{-7} – 3×10^{-6} M).

Fig. 1. Representative Images of Agarose Gels Showing Expression of the Indicated mRNAs in Rat Urinary Bladder (UB), Atrium (both Right and Left Atria) (A), and Ileal Longitudinal Smooth Muscle (I)

The PCR products for β_1 -, β_2 -, and β_3 -adrenoceptors, and β -actin are 337, 386, 352, and 375 bp, respectively. RT(+): reverse transcription, RT(-): no reverse transcription. These results are representative of four experiments.

Fig. 2. Effect of Various β -Adrenoceptor Antagonists on Noradrenaline-Induced Relaxation in Rat Urinary Bladder Smooth Muscle (UBSM)

A: Reproducibility of the concentration-response curves for noradrenaline-induced relaxation between successive applications. B–F: Effects of atenolol (10^{-6} M; B), ICI-118,551 (3×10^{-8} M; C), propranolol (10^{-7} M; D, 10^{-6} M; E), and bupranolol (10^{-7} M; F) on the concentration-response curves for noradrenaline-induced relaxation. Data are presented as means $\pm$ S.E.M., $n = 8$ (part A), and $n = 4$ (B–F).

in the presence of 10^{-6} M propranolol. The pA_2 value of bupranolol was calculated to be 6.64 (95% CIs: 6.40–7.17, $n = 13$).

Figure 4 shows the effects of SR 59230A on noradrenaline-induced relaxation in the presence of propranolol (10^{-6} M). The noradrenaline-induced relaxation was inhibited by SR 59230A (10^{-7} – 10^{-6} M) in a concentration-dependent manner, shifting the corresponding concentration-response curve to the right (Figs. 4A–C). Figure 4D shows the Schild plot of SR 59230A against noradrenaline based on the results of Figs. 4A–C. Schild regression analysis produced a straight line with a slope of 0.95, which was not significantly different from unity (95% CIs: 0.40–1.50, $n = 12$) (Fig. 4D). This indicates that noradrenaline-induced relaxation was antagonized competitively by SR 59230A (10^{-7} – 10^{-6} M) in the presence of 10^{-6} M propranolol. The pA_2 value of SR 59230A was calculated to be 7.27 (95% CIs: 6.92–8.38, $n = 12$).

Effects of Noradrenaline Metabolites on Methacholine (3×10^{-5} M)-Induced Contraction Noradrenaline is metabolized by MAO_A and COMT.¹⁵⁾ In this series of experiments, we investigated the effects of six metabolites of noradrenaline (*i.e.*, NMN, DOMA, DHPG, VMA, MHPG, and MHPG-S) in order to determine whether these metabolites can induce a UBSM relaxation response *via* β_3 -adrenoceptor stimulation. In the presence of clorgiline (a MAO_A inhibitor, 10^{-5} M) and 3,5-dinitrocatechol (a COMT inhibitor, 2×10^{-6} M), noradrenaline (10^{-4} M) elicited a relaxation response as shown in Fig. 5A (white column). Noradrenaline (10^{-4} M)-induced relaxation was not further augmented by isoprenaline (10^{-4} M) (Fig. 5A, black column).

In contrast, none of the 6 metabolites (NMN, DOMA, DHPG, VMA, MHPG, or MHPG-S, 10^{-4} M each) induced a

relaxation response (Figs. 5B–G). However, NMN (10^{-4} M) augmented the methacholine-induced (3×10^{-5} M) contraction of the UBSM preparation by approximately 10% (Fig. 5B).

DISCUSSION

In this study, we investigated whether noradrenaline could be a ligand for the β_3 -adrenoceptor by pharmacological identification of the β -adrenoceptor subtypes that trigger relaxation responses to noradrenaline in rat UBSM. We also examined 6 metabolites of noradrenaline (*i.e.*, NMN, DOMA, DHPG, VMA, MHPG, and MHPG-S) in order to determine whether they are able to induce UBSM relaxation responses *via* β_3 -adrenoceptor stimulation. Our pharmacological studies indicated that the predominant β -adrenoceptor subtype to mediate noradrenaline-induced relaxation is β_3 , and thus, noradrenaline was suggested to be a ligand for the β_3 -adrenoceptor in rat UBSM. In contrast, since none of the noradrenaline metabolites showed a relaxation response, these metabolites are not ligands for the β_3 -adrenoceptor.

First, we will discuss the possible β -adrenoceptor subtypes in rat UBSM. In the RT-PCR experiment, we detected mRNA expression of all 3 β -adrenoceptor subtypes (β_1 , β_2 , and β_3) (Fig. 1). This result supports the findings in previous reports.^{10,11)} In those reports, in rat UBSM, all three β -adrenoceptor subtypes were suggested to have functional significance, as supported by the following pharmacological findings: 1) rat UBSM was relaxed substantially by selective agonists for each subtype (*i.e.*, T-0509 for β_1 , terbutaline for β_2 , and BRL 37344A for β_3)¹⁶⁾; and 2) a subtype non-selective agonist for β -adrenoceptors (isoprenaline) was significantly

Fig. 3. Effect of Bupranolol on Noradrenaline-Induced Relaxation in Rat Urinary Bladder Smooth Muscle (UBSM)

A: Reproducibility between successive applications of the concentration-response curves for noradrenaline-induced relaxation in the presence of propranolol (10^{-6} M). B–D: Effects of bupranolol (3×10^{-7} – 3×10^{-6} M) on the concentration-response curves for noradrenaline-induced relaxation in the presence of propranolol (10^{-6} M). E: Schild plot of the bupranolol versus noradrenaline analyses shown in B–D. Data are presented as means $\pm$ S.E.M., $n = 12$ (part A), $n = 5$ (part C), and $n = 4$ (B, D, E). Slope and pA_2 values (part E) are presented as means with 95% confidence intervals (95% CIs).

inhibited by selective antagonists for each subtype (*i.e.*, metoprolol for β_1 , butoxamine or ICI-118,551 for β_2 , and SR 59230A for β_3).^{16–18} Our biochemical results and the previous mechanical studies with chemically-synthesized β -adrenoceptor agonists suggest that all 3 β -adrenoceptor subtypes (β_1 , β_2 , and β_3) could be the potential target for noradrenaline, and thus further pharmacological studies using subtype-selective antagonists are warranted.

Next, we will discuss the participation of β_1 - and β_2 -adrenoceptors in noradrenaline-induced relaxation. Noradrenaline-induced relaxation was not significantly inhibited by the following β -adrenoceptor antagonists (Fig. 2): atenolol (10^{-6} M), a selective β_1 -adrenoceptor antagonist at this concentration; ICI-118,551 (3×10^{-8} M), a selective β_2 -adrenoceptor antagonist at this concentration; propranolol (10^{-7} M), a β_1 - and β_2 -adrenoceptor antagonist at this concentration; and bupranolol (10^{-7} M), a β_1 - and β_2 -adrenoceptor antagonist at this concentration. The pA_2 values of each antagonist were previously calculated to be 7.01 (atenolol for β_1),¹⁹ 8.47 (propranolol for β_1),²⁰ 8.94 (bupranolol for β_1),²¹ 8.83 (ICI-118,551 for β_2),²² 8.43 (propranolol for β_2), and 8.60 (bupranolol for β_2).²³ Therefore, if either β -adrenoceptor subtype significantly contributes to noradrenaline-induced relaxation, the relaxation response should have been inhibited to some extent by these antagonists. However, since noradrenaline-induced relaxation was not affected by any of the tested antagonists, the contributions of β_1 and β_2 to this relaxation have been excluded.

Next, we will discuss the possible participation of β_3 -adrenoceptors in noradrenaline-induced relaxation. First, noradrenaline-induced relaxation was shown to be competitively antagonized by bupranolol (3×10^{-7} – 3×10^{-6} M), with a pA_2 value of 6.64 (95% CI: 6.40–7.17) (Figs. 3B–E). This pA_2 value (6.64) was deemed to be nearly identical to the values in previous reports: 6.56 against BRL37344-induced relaxation in isolated ileal longitudinal smooth muscle from guinea pigs,¹² and 6.70 against CGP 12,177-induced lipolysis in white fat cells from rats.²⁴ Second, noradrenaline-induced relaxation was shown to be competitively antagonized by SR 59230A (10^{-7} – 10^{-6} M), with a pA_2 value of 7.27 (95% CI: 6.92–8.38) (Fig. 4). This pA_2 value (7.27) was similar to values that were previously reported: 7.58 against BRL37344-induced relaxation in isolated jejunal longitudinal smooth muscle from rabbits,¹³ and 6.89 against CL 316,243-induced lipolysis in white fat cells from rats.²⁴ These findings suggest a significant contribution of β_3 -adrenoceptors, which are sensitive to both bupranolol and SR 59230A, to noradrenaline-induced relaxation in rat UBSM, and thus, noradrenaline could be a ligand for β_3 -adrenoceptors in this smooth muscle.

In our study, the pharmacological detection of β_3 -adrenoceptors with bupranolol and SR 59230A in noradrenaline-induced relaxation was carried out in the presence of 10^{-6} M propranolol, which is similar to the conditions employed in previous reports.^{12,13} In the absence of propranolol, the slope of the Schild plot regression line for bupranolol

Fig. 4. Effect of SR 59230A on Noradrenaline-Induced Relaxation in Rat Urinary Bladder Smooth Muscle (UBSM)

A–C: Effects of SR 59230A (10^{-7} – 10^{-6} M) on the concentration-response curves for noradrenaline-induced relaxation in the presence of propranolol (10^{-6} M). D: Schild plot of the SR 59230A versus noradrenaline analyses shown in A–C. Data are presented as means $\pm$ S.E.M., $n = 4$. Slope and pA_2 values (part D) are presented as means with 95% confidence intervals (95% CIs).

Fig. 5. Effect of Noradrenaline (A) and 6 Noradrenaline Metabolites (B–G) on Methacholine (3×10^{-5} M)-Induced Contraction in Rat Urinary Bladder Smooth Muscle (UBSM) in the Presence of Clorgiline (Monoamine Oxidase A (MAO_A) Inhibitor, 10^{-5} M) and 3,5-Dinitrocatechol (Catechol-*O*-methyltransferase (COMT) Inhibitor, 2×10^{-6} M)

Tested noradrenaline metabolites (10^{-4} M) are normetanephrine (NMN; B), 3,4-dihydroxymandelic acid (DOMA; C), 3,4-dihydroxyphenylglycol (DHPG; D), 4-hydroxy-3-methoxymandelic acid (VMA; E), 4-hydroxy-3-methoxyphenyl glycol (MHPG; F), and 4-hydroxy-3-methoxyphenylglycol sulfate (MHPG-S; G). Data are presented as means $\pm$ S.E.M., $n = 4$. ISO: isoprenaline.

(3×10^{-7} – 3×10^{-6} M) against noradrenaline was far less than unity; thus, we could not calculate the pA_2 value for bupranolol, necessitating the inclusion of 10^{-6} M propranolol (data not shown).

In rat UBSM, all three β -adrenoceptors have been shown to be functional,^{16–18} and bupranolol was reported to competitively inhibit isoprenaline-induced relaxation with a pA_2 value of 8.98.²⁵ This corresponds to the value for β_1 or β_2 .^{21,23}

but not to that for β_3 . Therefore, since bupranolol binds non-selectively to all 3 subtypes (β_1 , β_2 , and β_3) in rat UBSM and noradrenaline binds selectively to the β_3 -adrenoceptor, competitive antagonism of bupranolol against noradrenaline would not be expected to occur in the absence of propranolol to block β_1 and β_2 . In contrast, in the presence of propranolol (10^{-6} M), both β_1 and β_2 are occupied by propranolol, which enables bupranolol to selectively bind to β_3 and competitively antagonize noradrenaline. Thus, the slope of the Schild plot regression line for bupranolol *versus* noradrenaline becomes unity, which enables the pA_2 value to be calculated.

However, propranolol at 10^{-6} M shifted the concentration-response curve for noradrenaline-induced relaxation to the right by *ca.* 2-fold (Fig. 2E). This finding is consistent with previous reports that the pA_2 value of propranolol for the β_3 -adrenoceptor is approximately 6.^{26,27)} Therefore, it is possible that this concentration (10^{-6} M) of propranolol is able to inhibit β_3 -adrenoceptors to some extent in addition to inhibiting β_1 and β_2 , although a control experiment was also performed in the presence of propranolol (10^{-6} M) and the pA_2 values for bupranolol and SR 59230A were almost identical to the previously reported values.^{26,27)}

Finally, we will discuss the results of the noradrenaline metabolites. There were several reasons why we chose to examine their effects. Noradrenaline had been suggested to be an agonist for β_3 -adrenoceptors by pharmacological functional studies, and thus, its metabolites could also be β_3 -adrenoceptor agonists. In addition, since noradrenaline metabolites can be discharged into the urine, it is possible that they relax the bladder by stimulating internal UBSM or urothelium β_3 -adrenoceptors to promote urine storage. Previously, NMN, a metabolite of noradrenaline, was shown to have positive inotropic actions in guinea pig atrial muscle,²⁸⁾ and MHPG, another metabolite of noradrenaline, was shown to inhibit lymphocyte chemotaxis similarly to the action of noradrenaline.²⁹⁾ However, none of the six noradrenaline metabolites (*i.e.*, NMN, DOMA, DHPG, VMA, MHPG, and MHPG-S) showed relaxation responses in our study (Fig. 5). Therefore, these metabolites are unlikely endogenous agonists of the β_3 -adrenoceptor in rat UBSM; the endogenous agonist is most likely noradrenaline itself. Although NMN stimulated contraction instead of relaxation, we currently do not understand this phenomenon, which should be examined in future studies.

CONCLUSION

Noradrenaline, but not its metabolites, may be a ligand for β_3 -adrenoceptors to produce relaxation responses in the UBSM of rats. However, our present study was performed with exogenously applied, and not endogenous, noradrenaline. Therefore, further studies are required to examine whether β_3 -adrenoceptors are targets for endogenous noradrenaline.

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Conflict of Interest The authors declare no conflict of interest.

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