Function and Distribution of $\beta_3$-Adrenoceptors in Rat, Rabbit and Human Urinary Bladder and External Urethral Sphincter

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Abstract

1 Activation of $\beta$-adrenoceptors causes relaxation of the urinary bladder and contraction of the external urethral sphincter, which consists of fast-contracting skeletal muscles. A $\beta_3$-adrenoceptor agonist, clenbuterol, recently has been developed as a therapeutic drug for the treatment of urinary incontinence, however $\beta_3$-adrenoceptor agonists have undesirable effects on cardiac and striated muscle function.

2 In this study, we compared the effects of the $\beta_3$-adrenoceptor agonist, clenbuterol and of a novel $\beta_3$-adrenoceptor agonist, GS332, on urinary bladder and external urethral sphincter function in rat, rabbit and human. We also determined the distribution of $\beta_3$-adrenoceptors in human urinary bladder and external urethral sphincter, using radioligand-binding techniques.

3 Clenbuterol induced marked relaxations in rat, rabbit and human urinary bladder smooth muscles and also induced marked contractions in rat periurethral striated muscles (external urethral sphincter), while GS332 induced marked relaxations in rat and human, but not in rabbit, urinary bladder smooth muscles and induced small contractions in rat periurethral striated muscles.

4 The radioligand-binding studies showed presence of $\beta_3$- and $\beta_3$-adrenoceptors in human urinary bladder, external urethral sphincter and abdominal rectus muscles. The affinities of GS332 were the highest in urinary bladder and the lowest in the skeletal (abdominal rectus) muscles, while the affinities of clenbuterol were similar in urinary bladder, external urethral sphincter and the skeletal (abdominal rectus) muscles.

5 These results suggest that GS332 could, similarly clenbuterol, have a role in the treatment of urinary frequency and urinary incontinence.

Key words: $\beta_3$-adrenocepter; urinary bladder; external urethral sphincter; GS332; urinary incontinence

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Introduction

The function of lower urinary tract smooth muscle is controlled in part by the autonomic nervous system, i.e. parasympathetic and sympathetic innervation. The regulatory effects of the autonomic nervous system are mediated through the interaction of the neurotransmitters, norepinephrine and acetylcholine, with adrenergic and cholinergic receptors, respectively.

Activation of $\beta$-adrenoceptors has a role in relaxation of the urinary bladder during the storage (Wein, 1994). The $\beta$-adrenoceptor-induced relaxations are mediated by $\beta_1$-adrenoceptors in rat (Suzuki et al., 1989), rabbit (Morita et al., 1990) and human (Morita et al., 1993) urinary bladder smooth muscle and by both of $\beta_1$- and $\beta_2$-adrenoceptors in canine urinary bladder smooth muscle (Morita et al., 1993). $\beta$-adrenoceptor-induced relaxation in urinary bladder smooth muscle is mediated by cAMP (Morita et al., 1990; Morita et al., 1993; Rohner & Hannigan, 1980; Levin et al., 1986) not by cGMP (Morita et al., 1993). Tension of tetanic contractions in fast contracting skeletal muscles is increased by $\beta$-adrenoceptor stimulation (Bowman & Zaimis, 1958; Holmberg & Waldeck, 1977; Tashiro, 1973). Al-Jeboory and Marshall (1977) and Fellenius et al. (1980) demonstrated that the $\beta$-adrenoceptor-induced increase in the force of subtetanic contractions of guinea pig extensor digitorum longus muscle, which is fast contracting skeletal muscle is mediated by $\beta_2$-adrenoceptors and that this is associated with significant elevations in cAMP levels. The external urethral sphincter muscles mainly consist of fast contracting skeletal muscles (Okamura et al., 1987; Okamura et al., 1991) and it has been shown that $\beta_2$-adrenoceptor agonists increase the contractile force of the external urethral sphincter (perurethral striated muscles) in human (Morita, 1989) and rabbit (Kishimoto et al., 1991).

Urinary continence is maintained by both the relaxation of the urinary bladder and the contraction of the urethra. Based on this clenbuterol hydrochloride, a $\beta_2$-adrenoceptor agonist, that has been shown to relax the human urinary bladder and contract the human external urethral sphincter has been used as a treatment for stress incontinence in Japan (Kawabe, 1995). However there have been undesirable effects of clenbuterol on the cardiac function (tachycardia, pulsation) and on the striated muscle function (finger tremor, muscle rigidity).

Pharmacological effects of $\beta$-adrenoceptor stimulation which have been observed over recent years could not be explained in terms of the conventional $\beta_1$- and $\beta_2$-subtype classification, leading to the concept of a further subpopulation of 'atypical' ($\beta_3$) $\beta$-adrenoceptors (Harms et al., 1974; De Vente et al., 1980; Arch et al., 1984). $\beta_3$-adrenoceptors have been identified on adipocytes (Wilson et al., 1988), skeletal muscle (Challiss et al., 1988) and gastrointestinal tract smooth muscles (Blue et al., 1990; McLaughlin & MacDonald, 1991; Lezama et al., 1996). $\beta_3$-adrenoceptor agonists have been reported to have little effect on human cardiac functions ( Wheeldon et al., 1994). The aim of the present study is to determine whether $\beta_3$-adrenoceptors mediate relaxation of the urinary bladder during the urine storage phase. This may aid in the development of a therapeutic drug for urinary incontinence that does not have cardiac or skeletal muscle effects, i.e. the $\beta_3$-adrenoceptor agonist, GS332.
Materials and Methods

Tissues

One-year old male and female Japanese white rabbits and 12 weeks old male and female Wistar rats were bled and killed. The urinary bladders were removed, trimmed and dissected free from the prostate in the male and from the vaginal wall in the female. The bladder dome was separated from the bladder base at the level of the ureteral orifices. Ten rabbits and eight rats were used. Human urinary bladder muscle was obtained from three patients with underwent total cystectomy as treatment for bladder cancer.

Functional studies

1. Studies for spontaneous contractions in urinary bladder muscle

Muscle strips, 3 mm wide and 10 mm long, were longitudinally dissected from bladder dome, and mounted in a 3 ml chamber containing modified Krebs solution of the following composition (mM): NaCl, 133.6; KCl, 4.7; CaCl₂·2H₂O, 1.9; MgCl₂·6H₂O, 1.2; and glucose, 8.3. The strips were gassed with 95% O₂ and 5% CO₂ (pH 7.4) at 37°C. One end of the muscle strip was attached with 4-0 silk thread to a fixed hook at the bottom of the chamber and the other end was attached to a Statham UC-2 force transducer mounted on a movable slide assembly. Muscle strips were stretched to the length of optimum tension development and were equilibrated for approximately 60 min prior to drug administration. Resting tension at this length was approximately 0.3 g and was maintained throughout the experiment.

Dose-response curves to isoproterenol, a non-selective β-agonist, clenbuterol, a selective β₁-agonist and GS332, a selective β₂-agonist were constructed by cumulatively increasing drug concentrations. 10⁻⁶ M phentolamine, an α-blocker was added to the bath 30 min before the administration of the β-agonists, because they may act as an α-agonist at high concentrations. After completion of a dose-response curve to isoproterenol, the tissue was washed four times and left in the bath for 90 min prior to the administration of the next drug, clenbuterol or GS332. The viability and stability of the preparation was confirmed finally by then showing that isoproterenol produced the same maximum relaxation as initially observed. The effect of SR59230A, a selective β₃-antagonist, on the GS332 induced-relaxation in urinary bladder smooth muscles were examined by determining Kₐ and pA₂ values.

2. Studies for electrically induced contractions of periurethral striated muscles

Periurethral striated muscles were sectioned from the urethra of one-year old male Japanese white rabbits and cut in 3 mm width transverse strips. The strips were mounted in the chamber and electrically stimulated between two parallel platinum electrodes at 40 Hz using 0.5 sec trains of 0.5 msec pulses at 15 sec intervals. Contractions of 1–2 g was evoked by the transmural field stimulation. After the amplitude of the contractile responses became stable, clenbuterol or GS332 was applied in the presence of 10⁻⁶ M phentolamine.

Radioligand binding studies

Membrane particulate preprations: Rabbit, rat and human urinary bladder muscles were
trimmed of mucosa, fat and connective tissue, in ice-cold saline, and stored at −70°C until assayed. Small amounts (1.5−2.0 g) of human external urethral sphincter and abdominal rectus muscles were obtained at the time of cystourethrectomy in three male patients with urinary bladder cancer. Abdominal rectus muscles were obtained from three mongrel dogs for comparison with data obtained from human. Striated muscles were stored at −70°C until assayed. Frozen tissues were thawed, cut into small pieces, and then homogenized in 40−50 volumes of ice-cold 50 mM tris (hydroxymethyl) aminomethane (Tris) HCl (pH 8.0 at 25°C) with two 15 sec-bursts separated by a 5 sec-cooling interval, using a polytron PT-35 set at speed six with a PTA20 probe generator. The homogenate was centrifuged for 20 min (49,000 g) at 4°C. The supernatant was discarded and the pellet was homogenized as before, filtered through a 100 µm nylon mesh and centrifuged under the same conditions. The final pellet was suspended in 60 volumes of 25 mM glycylglycine buffer (pH 7.4 at 25°C) to yield 0.2 to 0.3 mg protein/ml. The protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Saturation experiments: Saturation experiments were performed as previously described (Morita et al., 1987; Latifpour et al., 1989). In brief, aliquots of membrane particulates were incubated with increasing concentrations of 125I-iiodocyanopindolol (ICP) at 23°C for 30 min with or without 10−6 M propranolol. The incubation mixture subsequently was filtered rapidly under vacuum through Whatman GF/B glass fiber filters. The filters then were washed with 8 ml of ice-cold 50 mM TrisHCl (pH 8.0 at 25°C) and tissue-bound radioactivity was extracted from the filters using 10 ml of toluene based scintillation fluid and counted at an efficiency of 40−50%. The specific binding was defined as the difference between the binding in the presence and absence of propranolol. The maximum number of binding sites (Bmax) and the equilibrium dissociation constant (Kd) were calculated by Rosenthal's graphic method (1967) using regression of bound/free vs bound.

Inhibition experiments: The steps in the inhibition (competition) studies were identical to those used in the saturation experiments, except that aliquots of membrane preparation were incubated with a fixed concentration of 125I-ICP in the presence of increasing various concentrations of an unlabeled drug. The unlabeled drugs used were isoproterenol, clenbuterol and GS332. The concentration of the unlabeled agonist required to inhibit 50% of the binding of the labeled ligand (IC50) was calculated using a non-linear least squares curve-fitting program. The inhibition constant (Ki) was calculated from the IC50 value according to Cheng and Prusoff (1973): Ki=IC50/(1+F/Kd), where F is the free concentration of the labeled ligand and Kd is the equilibrium dissociation constant obtained from saturation studies.

Drugs

125I-ICP (99.9 Ci/mmol) was obtained from Amersham Co. (Tokyo). (+) Isoproterenol hydrochloride, phentolamine hydrochloride, dl-propranolol hydrochloride and forskolin were purchased from Sigma. Clenbuterol hydrochloride was purchased from Teijin Co. (Tokyo). GS332 and SR59230A were purchased from Tokyo Tanabe Seiyaku Co. (Tokyo). Isoproterenol, clenbuterol and GS332 were dissolved in distilled water containing 1 mg/ml ascorbic acid. Phentolamine, 10−3 M and propranolol, 10−3 M were dissolved in 70% ethanol and then diluted.
to the required concentration with distilled water.

Statistics

Statistical analysis of drug effects and differences between treatment groups was obtained with a two-way nested analysis of variance (ANOVA). The Scheffe test was used to determine what concentration of drug produced a significant effect. A non-paired t-test was used to determine significance levels between responses at any given drug concentration and also used to determine significance levels between groups. \( P<0.05 \) was regarded as the level of significance. All statistical analyses were performed on absolute data values.

Results

Functional studies

Isoproterenol, clenbuterol and GS332 caused marked dose-dependent relaxations in rat urinary bladder muscle strips. The EC\(_{50}\) values for isoproterenol, clenbuterol and GS332 were 10, 31 and 16 nM, respectively (Fig. 1). Dose response curves to GS332 in the presence of SR59230A are shown in Fig. 2, and the pA\(_2\) value calculated in Schild plot analysis (Arunlakshana & Schild, 1959) is given in Fig. 3. Clenbuterol caused marked dose-dependent relaxations in rabbit urinary bladder muscle strips with an EC\(_{50}\) of 4 nM, but GS332 caused small relaxations with an large EC\(_{50}\) value of 54 \(\mu\)M (Fig. 4).

In human urinary bladder muscle strips, clenbuterol and GS332 caused marked dose-dependent relaxations (Fig. 5). The relaxant responses induced by GS332 were greater than those induced by clenbuterol. The EC\(_{50}\) of GS332 was 0.1±0.05 \(\mu\)M (\(n=3\)) and that of clen-

![Fig. 1. Relaxant responses of rat urinary bladder muscle strips to isoproterenol, clenbuterol and GS332. 100% relaxation was induced by 1 \(\mu\)M isoproterenol. Each point represents mean±SE (\(n=6\)).](image-url)
Fig. 2. Relaxant responses of rat urinary bladder muscle strips to GS332 in controls and in the presence of various concentrations of SR59230A. 100% relaxation was induced by 1 μM isoproterenol. Each point represents mean of 4 determinations.

Fig. 3. Schild plot analysis of antagonism of SR59230A on GS332-induced relaxations of rat urinary bladder muscle strips.

buterol was 1.0 ± 0.70 μM (n = 3) for human urinary bladder strips.

In rat periurethral striated muscles, clenbuterol and to a lesser extent GS332 potentiated electrical stimulation-induced contractions significantly (Fig. 6). The effects of clenbuterol were antagonized by a relatively low concentration (10⁻⁶ M) of propranolol while the effects of GS332 were antagonized only by a relatively high concentration (10⁻⁴ M) of propranolol.
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Fig. 4. Relaxant effects of clenbuterol and GS332 on rabbit urinary bladder smooth muscles. Each point represents mean ± SE (n=6). 100% relaxation was induced by 1 μM isoproterenol.

Fig. 5. Representative tracings of relaxant responses to clenbuterol and GS332 in human urinary bladder smooth muscles.

Binding studies

The results of saturation experiments with 125I-ICP in human urinary bladder, external urethral sphincter and abdominal rectus muscles and dog abdominal rectus muscles are shown in Table 1. The data indicate similar B_max and K_D values for 125I-ICP bindings in human and dog abdominal rectus muscles. In human, the B_max values for 125I-ICP bindings in urinary bladder were significantly higher than in external urethral sphincter and abdominal rectus muscles. The K_D values, however, were significantly lower in urinary bladder and external urethral sphincter than in abdominal rectus muscles.

K_is values of isoproterenol, clenbuterol and GS332 for 125I-ICP bindings to human urinary bladder, external urethral sphincter and abdominal rectus muscles are shown in Table 2. Isoproterenol has its highest affinity in the skeletal (abdominal rectus) muscle and its lowest
Fig. 6. Potentiation by clenbuterol and GS332 of electrical stimulation-induced contractions of rat periurethral striated muscles. Each point represents mean ± SE (n = 4-5).

Table 1. Characteristics of $^{125}$I-ICP binding to human urinary bladder, external urethral sphincter and abdominal rectus muscles and dog abdominal rectus muscles. Values are mean ± SE of 3-5 experiments.

<table>
<thead>
<tr>
<th></th>
<th>Bmax (f mol/mg protein)</th>
<th>Kd (nM)</th>
</tr>
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<tbody>
<tr>
<td>Human urinary bladder</td>
<td>22.5 ± 2.4 *</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>external urethral sphincter</td>
<td>16.9 ± 3.7</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>abdominal rectus muscle</td>
<td>15.4 ± 4.4</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Dog abdominal rectus muscle</td>
<td>13.2 ± 5.8</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

* p < 0.05

Table 2. Inhibition of $^{125}$I-ICP bindings by GS332, clenbuterol and isoproterenol in human urinary bladder, external urethral sphincter and abdominal rectus muscles. Each values are mean ± SE of 3-5 experiments.

<table>
<thead>
<tr>
<th></th>
<th>Urinary bladder</th>
<th>External urethral sphincter</th>
<th>Abdominal rectus muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clenbuterol</td>
<td>6.76 ± 2.36</td>
<td>6.59 ± 4.89</td>
<td>6.99 ± 2.58</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>9.56 ± 5.17</td>
<td>3.59 ± 1.66</td>
<td>2.05 ± 0.97</td>
</tr>
</tbody>
</table>

affinity in the urinary bladder. GS332 has its highest affinity in urinary bladder and its lowest affinity in the skeletal muscle, while clenbuterol has similar affinities in human urinary bladder, external urethral sphincter and skeletal muscle.
Discussion

The present studies demonstrate the presence of qual densities of \( \beta \)-adrenoceptors in human external urethral sphincter (striated muscle) and abdominal rectus muscles (striated muscle) and of significantly greater densities of \( \beta \)-adrenoceptors in human urinary bladder smooth muscles (22.50 ± 2.40 fmol/mg protein).

Isoproterenol, a non-specific \( \beta \)-adrenoceptor agonist, clenbuterol, a specific \( \beta_2 \)-adrenoceptor agonist and GS332, a specific \( \beta_3 \)-adrenoceptor agonist caused dose-dependent relaxations in rat and human urinary bladder muscle strips. The findings that the relaxations induced by GS332 in rat bladder were competitively antagonized by SR59230A, a specific \( \beta_3 \)-antagonist (De Ponti et al., 1996), with the slope of approximately 1.0, indicate that GS332-induced relaxations are mediated by \( \beta_3 \) and not by \( \beta_2 \)-adrenoceptors. These data are consistent with \( \beta_3 \)-adrenoceptors, as well as \( \beta_2 \)-adrenoceptors (Morita et al., 1995), being involved in \( \beta \)-adrenoceptor induced relaxations in urinary bladder smooth muscle. In human urinary bladder, relaxations induced by GS332 via \( \beta_3 \)-adrenoceptor were significantly greater than those induced by clenbuterol via \( \beta_2 \)-adrenoceptor, although \( \beta_2 \)-adrenoceptor mediated relaxations were not different from \( \beta_3 \)-adrenoceptor mediated relaxations in rat urinary bladder. Clenbuterol (\( \beta_3 \)) and isoproterenol (\( \beta_2 \)) but not GS332 (\( \beta_3 \)) caused marked relaxations in rabbit urinary bladder. These data are consistent with species differences in the \( \beta \)-adrenoceptor subtypes involved in relaxation of urinary bladder smooth muscles.

Species differences also exist in the response of periurethral striated muscles to \( \beta \)-agonist subtypes. In rabbit periurethral striated muscles, clenbuterol, a \( \beta_3 \)-agonist, produced a significant potentiation of electrical stimulation-induced contractions, but GS332, a \( \beta_3 \)-agonist did not affect electrical stimulation-induced contraction of these muscles. In contrast, in the rat GS332 produced a small but significant potentiation of electrical stimulation-induced contractions of periurethral striated muscles and the potentiation of electrical stimulation-induced contractions of periurethral striated muscles by clenbuterol is significantly greater than in the rabbit.

The tension of tetanic contractions in fast contracting skeletal muscles is increased by \( \beta \)-adrenoceptor stimulation (Bowman & Zaimis, 1958; Holmberg & Waldeck, 1977; Tashiro, 1973; Bowman & Nott, 1969; Waldeck, 1985). Al-Jeboory and Marshall (1977) and Fellenius et al. (1980) demonstrated that the \( \beta_3 \)-adrenoceptor agonists, terbutaline or sulbutamol, increase the force of subtetanic contractions of guinea pig extensor digitorum longus muscle and that this was associated with a significant elevation in cAMP levels. Furthermore clenbuterol has been reported to increase the force of rabbit (Kishimoto et al., 1991) and human (Morita, 1989) external urethral sphincters, which are composed of fast contracting skeretal muscle (Okamura et al., 1987; Okamura et al., 1991). Although we did not examine the effect of GS332, a \( \beta_3 \)-adrenoceptor agonist, on the contractile responses of human external striated sphincter, the existence of \( \beta_3 \)-adrenoceptor was demonstrated using radioligand-binding techniques. The existence of \( \beta_3 \)-adrenoceptors in human external urethral sphincter had not been reported previously. The radioligand-binding data examining human urinary bladder, external urethral sphincter and abdominal rectus muscles demonstrate that the affinity of GS332, a
\(\beta_2\)-adrenoceptor agonist, is greatest in urinary bladder (detrusor) and least in abdominal rectus muscle, while the affinity of clenbuterol, a \(\beta_2\)-adrenoceptor agonist, is equal in detrusor, external urethral sphincter and abdominal rectus muscles. The binding data also demonstrate that the affinity of isoproterenol, a non-selective \(\beta\)-adrenoceptor agonist, is least in detrusor and greatest in abdominal rectus muscle.

Based on its properties to relax human detrusor and contract human external urethral sphincter (Morita, 1989), clenbuterol, a \(\beta_2\)-adrenoceptor agonist, has been available as a therapeutic drug for stress incontinence in Japan (Kawabe, 1995). However, undesirable side effects consisting of tachycardia, finger tremors and muscle rigidity, have been reported (Kawabe, 1995). These side effects may be a major problem, especially in older patients. The differences in affinities of GS332 and clenbuterol for detrusor, external urethral sphincter and abdominal rectus muscle raise the possibility that GS332 may have less side effects than clenbuterol in the treatment of stress incontinence and urinary frequency.

References


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