Effect of an Atypical Adrenergic $\beta_3$-Agonist, GS-332: Sodium (2R)-[3-[3-[2-(3-Chlorophenyl)-2-
Hydroxyethylamino]Cyclohexyl]Phenoxy]Acetate, on Urinary Bladder Function in Rats

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Abstract

We have developed an atypical adrenergic $\beta_3$-agonist, GS-332: Sodium (2R)-[3-[3-[2-(3-Chlorophenyl)-2-hydroxyethylamino]cyclohexyl]phenoxy]acetate, which has a unique structure compared to other $\beta_3$-agonists. In vitro study, we compared effects of GS-332 on rat urinary bladder muscle strip contractility with those of clenbuterol hydrochloride (clenbuterol), an adrenergic $\beta_2$-agonist. GS-332 relaxed isolated rat urinary bladder strips in a concentration dependent manner with 50% effective concentration (EC₅₀) of 15.7 nM, and the relaxant activity of GS-332 was as potent as that of clenbuterol (EC₅₀; 30.8 nM). The concentration-response curve of GS-332 on isolated rat urinary bladder was competed by a specific $\beta_3$-antagonist, SR59230A in a concentration-dependent manner, in which Schild slope was 1.1 and pA₂ value of SR59230A was 7.1. In vivo study, cystometory investigated in anesthetized rats demonstrated that GS-332 was more potent in increasing the urinary storage volume than clenbuterol and less potent in inhibiting the contractile force of urinary bladder at micturition reflex than clenbuterol. These data demonstrate that GS-332, a new adrenergic $\beta_3$-agonist, may be more useful to maintain continence than clenbuterol, an adrenergic $\beta_2$-adrenergic agonist.

Key Words: urinary bladder, $\beta_3$-adrenoceptor, cystometrogram, GS-332.

Introduction

Rich adrenergic $\beta$-receptors locate on urinary bladder smooth muscles (Todd et al., 1969; Negard and Boreus, 1972; Awad et al., 1974; Downie et al., 1975; Levin et al., 1979, 1980, 1983, 1988; Rohner and Hangan, 1980; Morita et al., 1986), and subtypes of the receptor which involve relaxation of urinary bladder smooth muscles are thought to be $\beta_2$ (Morita, 1989a, 1989b). Accordingly, clenbuterol hydrochloride ( clenbuterol), an adrenergic $\beta_2$-agonist, has

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been used clinically for the treatments of urinary incontinence (Shimazaki et al., 1989) and of urinary frequency (Kondo et al., 1995) in Japan.

Recently, it is reported that there exits an atypical adrenergic β receptor in adipose and gastrointestinal tract tissues, which is thought to be neither β1 nor β2 but third receptor (β3). It mediates lipolysis in adipose tissue and affects on gastrointestinal smooth muscles contractility (Farness and Costa, 1974; Arch et al., 1984; Wilson et al., 1984; Cori et al., 1987; Bloom et al., 1992). However, the roles and the functions of β3 receptor in urinary bladder have not been understood.

GS-332: sodium (2R)-[3-3-[2-(3-chlorophenyl)-2-hydroxyethylamino] cyclohexyl] phenoxyacetate, was synthesized and developed in Tokyo Tanabe Co., Ltd. as a new β3-agonist with an unique structure compared to other typical β3-agonists, BRL35135 (Wilson et al., 1984), SR58611A(Cori et al., 1987) and CL316,243 (Bloom et al., 1992).

In this study, we investigated in vitro and in vivo effects of GS-332 on urinary bladder function by comparing with those of clenbuterol, a β2-agonist and discussed the possibility of usefulness of GS-332, a β3-agonist, for the treatment of incontinence and urinary frequency.

Methods

Animals

Male Wistar rats aged 12-14 weeks were obtained from Charles River Japan (Tokyo, Japan).

In vitro study of isolated bladder muscle strips relaxation

After rats were sacrificed by bled, abdominal midline was incised to explore bladder, and picking the top of bladder up to cut trigonal region of bladder off was followed. Two muscle strip preparations (3×8 mm) were made from a bladder segment. Strips were mounted in organ bath filled with modified Krebs solution of following composition (mM): NaCl 118.6, KCl 4.7, CaCl2 • 2H2O 1.9, MgCl2 • 6H2O 1.2, NaHCO3 25.0, and glucose 8.3, pH 7.4; gassed by 95% O2+5%O2, equilibrated at 37°C. One end of the strip was connected to the isometric transducer (TB-611T, Nihon Koden, Japan) via silk thread and the other end was fixed at the bottom of organ bath. An initial tension of 1 g was applied and the strips were allowed to equilibrate for at least 1 hour. The change of tension was monitored via the isometric transducer, amplified with an amplifier (AP-621G, Nihon Koden, Japan) and recorded on a pen recorder(WT-645G, Nihon Koden, Japan).

GS-332 and clenbuterol were dissolved in distilled water and adjusted to correct concentrations. Drugs were added cumulatively to organ bath at an interval of 10 min. Efficacy of drugs were represented as 50% of effective concentration (EC50; nM), in which the relaxation induced by 10-6M (+)-isoproterenol was regarded as 100% relaxation.
Effect of GS-332 on urinary bladder function

In vitro study of effect of a $\beta_3$-antagonist, SR59230A, on relaxant effects induced by GS-332 in isolated rat bladder

SR59230A is a specific $\beta_3$-antagonist developed by Sanofi, Co., Ltd. Experimental procedure was performed briefly according to previous report (Manara et al., 1996). SR59230A was dissolved in dimethyl sulfoxide (DMSO) and adjusted to correct concentrations with distilled water. One hour after the addition of each concentration of SR59230A, GS-332 was added cumulatively. The change of tension was monitored via the isometric transducer (TP-400, Nihon Koden, Japan) and its output was loaded to magnus assist system (MAGMATE®, Medical Research Equipment Co Ltd., Japan), in which, EC$_{50}$ value was calculated from its area under the curve (AUC) of each concentration-response curve.

In vivo study of effects of GS-332 on bladder pressure, which was maintained in low or high

Experiment was performed according to the method of previous report (Sato et al., 1975). Briefly, rats were anesthetized with urethane (1.15 g/kg) and placed on electrically heated pad. After bladder was explored with midline incision, 1.0 ml volume of balloon with SP-45 catheter made by latex rubber was inserted through the incision of top of bladder. The balloon catheter was connected to force transducer (AP-621G, Nihon Koden, Japan), coordinating insertion of SP-45 catheter for drainage of urine. Intravesical pressure was monitored via the balloon catheter, and was recorded on pen recorder (WT-645G, Nihon Koden, Japan). In the experiment of effect of GS-332 on bladder of which intravesical volume maintained relatively small, about 0.4ml of saline was infused to balloon catheter to keep intravesical pressure about 100 mmH$_2$O, in which little autonomic nervous control is regulating intravesical pressure. In the experiment of effect of GS-332 on high intravesical pressure, about 0.8 ml of saline was infused to balloon to cause increases of intravesical pressure over 150-200 mmH$_2$O. At this condition, rat urinary bladder exhibited rhythmic micturition contraction regulated by autonomic nervous systems as already reported (Sato et al., 1975).

Effects of GS-332 and clenbuterol were evaluated in every two experiments by the changes of intravesical pressure.

Effects of GS-332 on micturition intervals and micturition reflex in anesthetized rat cystometory

Experiment was performed according to the method of Maggi et al., 1987. Briefly, rats were anesthetized with urethane, and bladder was catheterized through incision of top of bladder by SP-45 polyethylene tube. The tube was connected to force transducer (AP-621G, Nihon Koden, Japan) and infusion pump (55-1111, Harvard apparatus, USA) via Y shaped connector. Saline was infused into bladder at a rate of 3.4 ml/hour by the pump to obtain a cystometrogram recorded on the pen recorder. Effects of GS-332 and clenbuterol were evaluated by increases in the micturition intervals and by decreases in the magnitude of micturition reflex.

Drugs

GS-332, SR59230A were synthesized in Research Laboratory of Tokyo Tanabe Co, Ltd. (Japan). (+)-Isoproterenol hydrochloride, clenbuterol hydrochloride were purchased from
Sigma. Urethane, DMSO and other reagents were purchased from Wako chemical Co. Ltd. (Japan).

GS-332 and clenbuterol were dissolved in distilled water, and SR59230A was dissolved in DMSO. \textit{In vivo} experimental study, each subject was dissolved at a volume of 0.5 ml/kg body weight, and intravenously injected additively.

\textit{Statistical analysis}

The experimental data were expressed as the mean±S.E. Statistical differences were analyzed by using paired t-test for comparing to predosing value, in which \( p < 0.05 \) were taken as significant.

\textbf{Results}

\textit{In vitro study}

\((\pm)-\text{Isoproterenol}\), a non specific \( \beta \)-adrenoceptor agonist, relaxed isolated rat bladder strips in a concentration-dependent manner with the \( EC_{50} \) value of 10\( \pm \)3.1 nM \((n=3)\). GS-332, \( \beta_3 \)-adrenoceptor agonist, relaxed isolated rat bladder strips in a concentration-dependent manner with the \( EC_{50} \) value of 16\( \pm \)1.6 nM \((n=4)\). Clenbuterol, \( \beta_2 \)-adrenoceptor agonist, relaxed isolated rat bladder strips in same fashion as well as above two compounds with the \( EC_{50} \) value of 31\( \pm \)4.7 nM \((n=3)\) (Fig. 1).

The concentration–response curve of GS–332 was shifted to the right in the presence of SR59230A, a specific \( \beta_3 \)-antagonist, in concentration-dependent fashion (Fig. 2). The slope was 1.1\( \pm \)0.2 and \( pA_2 \) value of SR59230A was 7.1\( \pm \)0.2 \((n=5)\).

![Fig. 1. Effects of GS-332 and clenbuterol on isolated rat bladder. Each value represents the mean ± S.E. of 4 determination. 100% relaxation is induced by 1 \( \mu \)M isoproterenol. GS-332 (△), clenbuterol (●) and isoproterenol (○) were added cumulatively at an interval of 10 min. \( EC_{50} \) values (nM) were GS-332, 16\( \pm \)1.6; clenbuterol, 31\( \pm \)4.7; isoproterenol, 10\( \pm \)3.1, respectively.](image-url)
Effect of GS-332 on urinary bladder function

In the study of effect on the bladder with relatively low intravesical pressure, GS-332 lowered intravesical pressure dose-dependently (0.01 mg/kg – 1 mg/kg) and the decreases induced by GS-332 were statistically significant from a dose of 0.01 mg/kg. On the other hand, clenbuterol showed a little tendency to lower the intravesical pressure, but there was not significance at doses we studied (Fig. 3). Accordingly, it was suggested that the lowering effect of GS-332 was greater than those of clenbuterol.

**In vivo study**

Fig. 2. Effect of SR59230A on relaxing effect of GS-332 on isolated rat bladder. Each value represents the mean of 4 determination. 100% relaxation is induced by 1 μM isoproterenol. The concentration-response curve elicited by increasing concentration of GS-332 was recorded in the absence (▲) and in the presence of SR59230A at concentrations of 10^-8 (○), 10^-7 (△), 10^-6 (□) and 10^-5 M (◆). The pA2 value of SR59230A was 7.1 and slope of GS-332 calculated by Schild plot analysis was 1.1.

Fig. 3. Effects of GS-332 and clenbuterol on intravesical pressure maintained at relatively low in anesthetized rat. Each value represents the mean ± S.E. of 4 determination. *, ** means significant difference vs pretreatment (C) of drugs at p < 0.05, 0.01, respectively.
In the study of effect on the bladder with relatively high intravesical pressure, GS-332 dose-dependently reduced the magnitude of micturition reflex. Clenbuterol also showed reducing effects in the same fashion as GS-332. The reducing effects of these two compounds were significant at a dose of 1 mg/kg, respectively. The reducing effects of the two components were not different at 1 mg/kg. However, GS-332 was significantly less potent than clenbuterol at high concentration, 10 mg/kg (Fig. 4).

In the experiment of cystometory, as shown in Fig. 5, effect of clenbuterol was examined.
Effect of GS-332 on urinary bladder function

Fig. 6. Effects of GS-332 and clenbuterol on micturition intervals in cystometrogram of anesthetized rat. Cystometrogram was obtained by continuously infused saline at a rate of 3.4 ml/hr. Effect of clenbuterol was evaluated by the two time interval; until beginning over flow and until complete micturition. Each value represents the mean±S.E. of 4 determination. * means significant difference vs pretreatment (C) of each drugs at p<0.05.

at doses of 1, 3, and 10 mg/kg. At 10 mg/kg, irregular bladder contractions were observed and were accompanied by overflow incontinence in the filling phase before complete micturition. Thus, effect of 10 mg/kg, clenbuterol in cystometrogram was evaluated by measuring the two time intervals; one is the time until overflow begins, and the other is the time until complete micturition occurs.

Fig. 7. Effects of GS-332 and clenbuterol on magnitude of micturition reflex in cystometrogram of anesthetized rat. Cystometrogram was obtained by continuously infused saline at a rate of 3.4 ml/hr. Effect was evaluated by measuring the magnitude of micturition reflux in which complete micturition occurred. Each value represents the mean±S.E. of 4 determination. *, ** means significant difference vs pretreatment (C) of each drugs at p<0.05, 0.01 respectively.
Clenbuterol did not increase the micturition intervals until beginning of over flow. Furthermore clenbuterol did not significantly increase the micturition intervals until complete micturition. On the other hand, GS-332 never showed the cystometrogram like clenbuterol, in which over flow occurs before micturition. GS-332, in the doses from 1 to 10 mg/kg, significantly increased micturition intervals, however, the effect of GS-332 became maximum at a dose of 3 mg/kg. There could not be found dose-dependency in the effects of GS-332 (Fig. 6).

As for the effect on magnitude of micturition reflex in the cystometrogram, both of clenbuterol and GS-332 significantly reduced the magnitude of micturition reflex, but their effects were not found to be dose-dependent. There was no significant difference between inhibitory effects of GS-332 and clenbuterol on magnitude of micturition reflex (Fig. 7).

**Discussion**

It is reported that the mechanisms of effectiveness of clenbuterol, $\beta_2$-agonist on urge incontinence ascribed to 1) the contractile effect on external urethral sphincter, to 2) the relaxant effects on smooth muscles of urinary bladder which cause the increases in bladder volume collecting urine, and to 3) the inhibitory effects on magnitude of micturition reflex (Grunberger, 1984; Morita, 1989; Suzuki et al., 1989; Shimazaki et al., 1989; Morita et al., 1995). In present in vitro study, any of isoproterenol, a non-specific $\beta$-adrenoceptor agonist, clenbuterol, a specific $\beta_2$-adrenoceptor agonist and GS-332, a specific $\beta_3$-adrenoceptor agonist, relaxed isolated rat bladder muscle strips. The relaxant effects of GS-332, a $\beta_3$-agonist on rat bladder strips were as potent as those of clenbuterol, a $\beta_2$-agonist. The relaxant effects of GS-332 were antagonized by SR59230A, a specific $\beta_3$-antagonist in concentration-dependent fashion and the GS-332 induced-maximum magnitude of relaxations were not affected by SR59230A. The slope was not different from 1.0, the $pA_2$ value of SR59230A was 7.1, which was a little weak activity than that in the study isolated rat proximal colon (Manara et al., 1996). Thus the relaxant effects of GS-332 on isolated rat bladder contribute to its effect through the $\beta_3$-adrenoceptors.

In present in vivo study, we evaluated the effects of GS-332 on bladder pressure, which was maintained low or high, and on cystometory. GS-332 as well as clenbuterol decreased the intravesical pressure when the pressure was kept relatively low. This potency of GS-332 was significantly greater than that of clenbuterol. GS-332 as well as clenbuterol significantly suppressed the magnitude of micturition reflex when the pressure was kept relatively low but GS-332 was less potent than clenbuterol at high dosage. These data clearly demonstrate that $\beta_2$- and $\beta_3$-adrenoceptors mediate relaxant responses in rat urinary bladder as well as $\beta_2$-adrenoceptor.

In vitro experiment, GS-332 and clenbuterol showed almost same potency in relaxing the isolated rat bladder muscle strips. The $EC_{50}$ values (nM) were 15.7 in GS-332, and 30.8 in clenbuterol, respectively. However, in the in vivo study of lowering intravesical pressure at relatively small bladder volume, potency of GS-332 was significantly greater than that of clenbuterol. These in vivo and in vitro results contain a little inconsistency. One of the
explanations for the inconsistency is that lowering function of intravesical pressure does not reflect only the relaxant effect on in vitro smooth muscles. The reports that there exists possibility of localization of β₁-receptor on post synaptic nerve endings in sympathetic nerves (Fujimoto, 1992) and that clenbuterol does not affect to the bladder function via upper nervous systems (Fukuda et al., 1984) may support our explanation. Accordingly, the facts that GS-332 showed more potent effect in lowering intravesical pressure at low volume bladder than clenbuterol might attribute to the effects of GS-332 via upper nervous systems.

β₂-adrenoceptors locate on the external urethral sphincter, and stimulation of β₂-adrenoceptors cause increases in contractile force of external urethral sphincter (Kishimoto et al., 1991). They are the reason why clenbuterol has a therapeutic effect on stress incontinence in Japan (Morita, 1989; Shimazaki et al., 1989; Morita et al., 1995). Clenbuterol induced irregular over flow like dripping of urine in the filling phase of cystometrogram in the present study. It might be speculated that this phenomenon in the cystometrogram originated from the contractile effect of clenbuterol on external urethral sphincter. On the other hand, GS-332 did not show this urine-dripping phenomenon but significantly increased micturition interval with little influence on magnitude of micturition reflex.

It was found that GS-332, a β₁-adrenoceptor agonist increases urinary bladder volume without significant inhibition on micturition reflex. The effects to increase urinary bladder volume were significantly greater in GS-332, a β₁-adrenoceptor agonist, than in clenbuterol, a β₂-adrenoceptor agonist, though the effects to suppress micturition reflex were significantly smaller in GS-332 than in clenbuterol. These data suggest that GS-332 shows increasing effect on bladder volume without affecting micturition reflex, which is the important factor inducing residual urine. Furthermore, it is expected that GS-332 does not elicit finger tremor, nor increase heart rate, which are commonly observed in patients with stress incontinence treated with clenbuterol, because there exists small amount of β₁-adrenoceptors in striated and heart muscles (Grunberger, 1984; Morita, 1989; Shimazaki et al., 1989; Wheelden et al., 1994; Morita et al., 1997a, 1997b).

Furthermore, there remains to possibility that GS-332 may show contractile effects on human external urethral sphincter as well as clenbuterol because β₁-adrenoceptor locates on external urethral sphincter as well as bladder in human (Morita et al., 1997a; 1997b). Thus, there is a great possibility that GS-332 has therapeutic effects in the treatments of urge and stress incontinence and urinary frequency without significant side effects.

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