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b. Abbreviations: OAB, overactive bladder; RT–PCR, reverse transcription-polymerase chain reaction; L-NAME, N^G-nitro-L-arginine methylester; NO, nitric oxide; pEC_{50}, potency, negative logarithm of the concentration giving half-maximal effect.

Abstract

We investigated the presence of β-adrenoceptor subtypes in human urinary bladder urothelium and examined whether β-adrenoceptors in the urothelium modulate the relaxation responses of isolated human detrusor strips to a β-adrenoceptor agonist. Expression of β_1-, β_2- and β_3-adrenoceptor mRNA in urothelium and detrusor smooth muscle was determined by reverse transcription-polymerase chain reaction (RT-PCR), and the distribution of β_1-, β_2- and β_3-adrenoceptors in human urinary bladder urothelium was examined by immunohistochemistry. Paired human longitudinal detrusor strips with and without an intact urothelium were suspended in organ baths to construct concentration-response curves to isoproterenol. The possible involvement of urothelium-derived nitric oxide (NO) in this response was examined in additional experiments with urothelium-intact strips in the presence of N^G-nitro-L-arginine methylester (L-NAME). Results confirmed the expression of β_1-, β_2- and β_3-adrenoceptors in the human urinary bladder urothelium. Further, the presence of the urothelium caused a parallel rightward shift of the concentration-response curve to isoproterenol, with a significant reduction in potency (pEC_{50}). L-NAME failed to exert any significant effect on the relaxation response to isoproterenol in the urothelium-intact strips. These results confirm the presence of β_1-, β_2- and β_3-adrenoceptors in human urinary bladder urothelium. Further, they suggest that urothelial β-adrenoceptors induce the release of a urothelium-derived factor which inhibits the β-adrenoceptor agonist-induced relaxation of the human detrusor smooth muscle, and that this inhibitory mechanism might not involve NO.

Key Words

human urothelium, β-adrenoceptor agonist, mucosa, β_3-adrenoceptor, bladder, nitric oxide.

Introduction

Although the urothelium of the urinary bladder has been regarded as a passive barrier which protects the underlying detrusor smooth muscle from various substances in urine, recent findings suggest that the urothelium is actively involved in the regulation of bladder function (de Groat 2004). Evidence for this comes from findings that urothelial cells express various receptors such as muscarinic receptors, transient receptor potential vanilloid subtype 1 (TRPV1), adrenoceptors and purinoceptors, thereby allowing them to respond to mechanical, chemical and other stimuli (Hawthorn et al. 2000; Birder et al. 1998, 2002; Elneil et al. 2001). Further, urothelial release of neurotransmitters such as nitric oxide (NO), Ach and ATP is thought to stimulate suburothelial afferent nerves directly or *via* myofibroblasts (Birder et al. 2002; Yoshida et al. 2004; Ferguson et al. 1997; Fry et al. 2004).

Multiple lines of evidence suggest that β-adrenoceptors are abundant in the detrusor smooth muscle of the urinary bladder, and play important roles in detrusor relaxation during urinary storage in various species (Lefkowitz et al. 1996; Longhurst and Levendusky 1999; Fujimura et al. 1999; Takeda et al. 1999; Igawa et al. 1999, 2001). β-Adrenoceptors are now subclassified into β_1-, β_2- and β_3-adrenoceptor subtypes, with the predominant subtype in the urinary bladder differing among species. In humans, the β_3-adrenoceptor is predominantly responsible for detrusor relaxation (Fujimura et al. 1999; Takeda et al. 1999; Igawa et al. 1999, 2001), giving rise to the notion that β_3-adrenoceptor agonists may be highly promising agents for the treatment of overactive bladder (OAB).

Recent studies indicate that β-adrenoceptors may also be present in the urothelium of the urinary bladder. In rats, β-adrenoceptor stimulation is associated with activation of the adenylate cyclase pathway in bladder epithelial cells, leading to the initiation of an increase in intracellular Ca^{2+} concentration which in turn triggers NO production and its release (Birder et al. 1998, 2002). Using porcine isolated detrusor strips, Murakami and colleagues demonstrated that contractile responses to carbachol were inhibited by stimulating urothelial β-adrenoceptors (Murakami et al. 2007), leading them to conclude that β-adrenoceptor agonists might stimulate the urothelial release of an unidentified factor which inhibits contractions of the detrusor smooth muscle. These findings suggest that urothelial β-adrenoceptors directly or indirectly influence the function of the detrusor smooth muscle. However, the molecular biological and functional roles of β-adrenoceptors in the urothelium of the human urinary bladder have not been reported.

Here, we investigated the expression of β-adrenoceptor subtypes, particularly β_3-adrenoceptors, in human urinary bladder urothelium by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. In addition, using an *in vitro* functional technique, we also determined whether stimulation of urothelial β-adrenoceptors modulates the relaxant effect of β-adrenoceptor agonists on detrusor smooth muscle in humans, and furthermore verified whether this relaxant effect is modulated by β-adrenoceptor stimulation-induced NO release from the urothelium.

Methods

Patients and specimens

Human urinary bladder specimens were collected from 13 patients (8 men and 5 women; aged 66.2 ± 6.7, age range 57–80 years) undergoing total cystectomy for bladder carcinoma. All patients were screened by their physician as having no evidence of overactive bladder or bladder outlet obstruction. Exclusion criteria included previous pelvic radiotherapy, extensive chemotherapy or current urinary tract infection. None of the patients had diseases or used medications known to interfere with the β-adrenoceptors system. Written informed consent was obtained from all patients. The study was approved by the ethics committee of Hamamatsu University School of Medicine.

All specimens were obtained from macroscopically normal tissue in the anterior or lateral wall of the urinary bladder body *via* a longitudinal incision. They were immediately placed in pre-oxygenated Krebs' solution (composition in mM: NaCl 118.1, KCl 4.7, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11.7) at 4 °C and transported to the laboratory. After removal of the adventitia and connecting fatty tissues, the specimens were cut into paired longitudinal detrusor strips measuring approximately 15×5×5 mm for functional studies. For molecular studies, residual detrusor and urothelial tissues were separately cut into portions weighing approximately 100 mg and immediately frozen in liquid nitrogen. They were then stored at -80 °C until RNA was extracted (see below).

RT-PCR

Expression of β_1-, β_2- and β_3-adrenoceptor mRNA was independently examined by region in five detrusor and urothelium specimens (four men and one woman, age range 63–74 years). Total RNA was extracted using the TRIzol Reagent according to the manufacturer's instructions. The amount of total RNA was determined with a spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan), and only good quality samples were used for RT–PCR.

Expression of mRNA for α-smooth muscle actin (α-SMA), a smooth muscle marker, was examined in each specimen independently to confirm the separation of detrusor smooth muscle and urothelium. Expression of α-SMA gene transcripts was examined by RT-PCR in accordance with a previous report, with an expected PCR product size of 965 bp (Ueda et al. 2002). Expression of mRNA for α-SMA was high in detrusor smooth muscle RNA, but markedly low or undetectable in urothelium RNA (Fig. 1f).

Expression of β-adrenoceptor subtype transcripts in human detrusor smooth muscle and urothelium was determined by RT-PCR using the GeneAmp PCR System 9700 (Applied Biosystems, Tokyo, Japan). In the RT step, 2 μg of total RNA was mixed with the RT reagent mixtures to a final volume of 20 μl. The RT reagent mixtures consisted of 5 x RT buffer (20 mM Tris-HCl pH 8.0, 100 mM KCl, 2 mM $MgCl_2$), 10 mM dNTP, 2.5 ng random hexamer, 0.1 M DTT and 40 U RNase Out. Next, 200 U SuperScript III RT was added and the mixtures were incubated at 25 °C for 10 min, followed by additional incubation at 50 °C for 60 min. Samples were heated at 70 °C for 10 min to terminate the

reverse transcription and then stored at 4 °C until use (RT-Mixture). In the PCR step, the RT-Mixture (1 μl) was mixed with the PCR reagent mixtures to a final volume of 20 μl. The PCR reagent mixtures consisted of 10 x PCR buffer (20 mM Tris-HCl pH 8.3, 100 mM KCl, 2 mM $MgCl_2$), 0.5 μM sense primer, 0.5 μM antisense primer, 0.8 mM dNTPs, 2 mM $MgSO_4$ and 0.5 U Tac DNA polymerase.

Sequences of the sense and anti-sense primers for β_1-, β_2- and β_3-adrenoceptors were used according to a previous report (Krief et al. 1993), and a pair of primers for β-actin as internal standard was designed based on a DNA sequence. The primer sequences for β_1-, β_2-, β_3-adrenoceptors and β-actin are shown in Table 1.

The PCR reaction of β-adrenoceptor subtypes was conducted at 96 °C for 3 min and 40 cycles of 96 °C for 30 s, 57 °C for 15 s and 72 °C for 30 s (for β_1) or 20 s (for β_2 and β_3), followed by a final extension at 72 °C for 5 min. PCR products (8 μl) were visualized by electrophoresis on 3.0% agarose gels with ethidium bromide. To rule out the possibility of amplifying genomic DNA, PCR was performed with no prior RT of the RNA in all experiments. The specificity of the primers and authenticity of the corresponding PCR products were verified by digestion of PCR products with specific restriction enzymes. β_1-Adrenoceptor PCR products were digested with Rsa I (generated fragments of 67 and 198 bp); β_2-adrenoceptor with Rsa I (93 and 236 bp); and β_3-adrenoceptor with Apa I (123 and 191 bp).

Immunohistochemistry

For immunohistochemistry, paraffin sections (4 μm) of archival human surgical resection specimens of the urinary bladder obtained from six different patients (four men and two women, age range 59–79 years) were immunostained using the HistoFine SimpleStain Max-PO kit (Nichirei Bioscience, Tokyo, Japan). The tissues were composed of full-thickness sections of human urinary bladder wall taken from macroscopically and histologically normal areas of formalin-fixed specimens removed for bladder carcinoma.

Deparaffinized and rehydrated sections were pretreated to unmask epitopes (β_1- and β_2-adrenoceptor, microwave 15 min at 500 W in 10 mmol/L citrate buffer, pH 6.0; β_3-adrenoceptor, pepsin treatment for 20 min at room temperature), and to block endogenous peroxidase activity (3% H_2O_2 in methanol for 15 min at room temperature). After incubation with primary antibodies for β_1-adrenoceptor (polyclonal rabbit antibody diluted 1:50, Santa Cruz Biotechnology, California, USA; incubated overnight at 4 °C), β_2-adrenoceptor (polyclonal rabbit antibody diluted 1:50, Affinity BioReagents, Colorado, USA; incubated overnight at 4 °C), and β_3-adrenoceptor (polyclonal rabbit antibody diluted 1:50, TransGenic Inc., Hyogo, Japan; incubated for 60 min at room temperature), the slides were incubated with secondary antibodies (HistoFine SimpleStain Max-PO kit) for 30 min. The antigen-antibody reaction was visualized with 0.01% 3,3'-diaminobenzidine tetrahydrochloride (Dako Envision kit/HRP (DAB), Dako Japan, Kyoto, Japan). All sections were counterstained with Mayer's hematoxylin and mounted under coverslips. For each section, a negative control consisted of primary incubation with affinity-purified rabbit

IgG fraction (Dako Japan) at an equivalent protein concentration. Immunohistochemical staining of β_1-, β_2-, and β_3-adrenoceptors was recorded as positive or negative, with a sample considered negative when immunostaining was the same as that of the negative control.

Functional studies

Paired longitudinal strips (approximately 15×5×5 mm) of detrusor smooth muscle were isolated from the human bladder. The urothelium was carefully removed from one strip per pair. The strips were then suspended in 10-ml organ baths containing Krebs' solution (composition as above) maintained at 37 °C and gassed with 95% O_2 - 5% CO_2. One end of each strip was connected to a force-displacement transducer (TB-651T, Nihon Kohden, Tokyo, Japan) coupled to an amplifier system (EF-601G, Nihon Kohden, Tokyo, Japan), and changes in detrusor tension were recorded on a PowerLab data acquisition system using Chart software (AD Instruments Japan, Tokyo, Japan). The strips were gradually stretched until a stable resting tension of 1.0 g was obtained, and equilibrated for at least 60 min. During equilibration, the organ bath solution was changed every 10-15 minutes. After resting tension was stabilized at 1.0 g, concentration-response curves to isoproterenol, a non-selective β-adrenoceptor agonist, were constructed by cumulatively increasing drug concentration at 10-15 min intervals. One response curve was generated per preparation. All experiments were conducted in the presence of phentolamine (1 μM), a non-selective α-adrenoceptor antagonist, to eliminate the involvement of α-adrenoceptors.

In some experiments with an intact urothelium, basal concentration-response curves were drawn in the presence of 100 μM N^G-nitro-L-arginine methylester (L-NAME), a NO synthase inhibitor, for 30 min before constructing the concentration-response curve to isoproterenol. After cumulative application of isoproterenol, 10 μM forskolin was added to the organ bath.

Analysis of functional data

Results are presented as mean values ± standard error of mean (SEM). The relaxant effect of isoproterenol was expressed as a percentage of the maximal relaxation (100%) induced by 10 μM forskolin, which was used as a reference drug. Mean concentration-response curves to isoproterenol were analyzed by fitting the data to a four-parameter logistic equation using non-linear regression with GraphPad Prism software (GraphPad, California, USA). Agonist potency (pEC_{50}, negative logarithm of the concentration giving half-maximal effect) and the maximal relaxation responses were also determined using this software. Relaxation responses and pEC_{50} were compared using Student's *t*-test, with a *P* value of less than 0.05 considered statistically significant.

Drugs

All molecular reagents, including TRIzol Reagent, random hexamer, SuperScript III RT and Tac DNA polymerase, were obtained from Invitrogen Life Technologies (Carlsbad,

California, USA). Phentolamine hydrochloride, (±)-isoproterenol hydrochloride, L-NAME and forskolin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Forskolin was dissolved in dimethyl sulfoxide, and all other drugs in distilled water. Reported concentrations were the final concentrations in the organ-bath solution.

Results

Expression of β-adrenoceptor subtype mRNA in the human urinary bladder urothelium

Expression of β_1-, β_2- and β_3-adrenoceptor mRNA in human detrusor smooth muscle and urothelium was examined using RT-PCR. PCR products for β_1-, β_2- and β_3-adrenoceptors were detected in all preparations of both tissues, with the expected size of PCR products of 265, 329 and 314 bp, respectively (Fig. 1a,b). PCR product for β-actin as an internal standard was also detected in all preparations with an expected size of 353 bp. Further, PCR products without prior RT of the RNA revealed no positive bands. Using specific restriction enzymes, β_1-adrenoceptor PCR products were digested with Rsa I (generated fragments of 67 and 198 bp), β_2-adrenoceptor with Rsa I (93 and 236 bp) and β_3-adrenoceptor with Apa I (123 and 191 bp) (Fig. 1c-e).

Distribution of β-adrenoceptor subtypes in the human urinary bladder urothelium

On immunohistochemistry, urothelial cells stained positively for the presence of β_1-, β_2- and β_3-adrenoceptor (Fig. 2a-c). Positive staining for β_1-, β_2- and β_3-adrenoceptor was also identified in detrusor smooth muscle cells, but only weakly for β_1- and β_2-adrenoceptor (Fig. 2d-f). No staining of the negative control was seen in any section of either urothelial cells or detrusor smooth muscle cells (Fig. 2g,h).

Relaxation responses of detrusor strips with and without an intact urothelium to isoproterenol

Isoproterenol, a non-selective β-adrenoceptor agonist, produced significant concentration-dependent relaxation of the human detrusor strips both with and without an intact urothelium (Fig. 3). The presence of the urothelium caused a parallel rightward shift of the concentration-response curve to isoproterenol (Fig. 4), showing that the urothelium-intact detrusor strips were significantly less sensitive to isoproterenol than the urothelium-denuded strips (pEC_{50} value 5.58 ± 0.30 vs. 6.31 ± 0.23, $p < 0.01$). Nevertheless, maximal relaxation responses to isoproterenol were similar, at 80.4 ± 3.2% for the intact and 84.2 ± 3.4% for the denuded strips. L-NAME, a NO synthase inhibitor, exerted no significant effect on the relaxation responses of the intact strips to isoproterenol, with a pEC_{50} value of 5.93 ± 0.40 and maximal relaxation response of 83.2 ± 8.3% compared to the control (Fig. 5).

Discussion

Here, we used RT-PCR to provide evidence for the expression of β_1-, β_2- and β_3-adrenoceptor mRNA in human urinary bladder urothelium, and immunohistochemistry to identify the distribution of β_1-, β_2- and β_3-adrenoceptors in human urinary bladder urothelial cells. We also showed that the presence of an intact urothelium reduces the relaxant effects of isoproterenol on human detrusor strips. These findings confirm the presence of β-adrenoceptors in human urothelium, and support the notion that urothelial β-adrenoceptors play an active role in the regulation of bladder functions.

Support for a role of the urothelium in the modulation of bladder function has come from a number of molecular biological and functional studies. Detrusor contractions elicited by tachykinins in guinea pig and canine urinary bladder were strongly inhibited after removal of the mucosa (Maggi et al. 1987; Saban et al. 1992). Stimulation of urothelial α_{1D}-adrenergic receptors in rat bladder facilitated mechanosensitive bladder afferent nerve activity and the micturition reflex (Ishihama et al. 2006). Likewise, many populations of muscarinic receptors exist in the porcine and human urothelium and stimulation of the urothelium by carbachol causes the release and transfer of an unknown urothelium-derived factor, resulting in the inhibition of detrusor strip contractions (Hawthorn et al. 2000; Templeman et al. 2002; Chaiyaprasithi et al. 2003).

Although β_3-adrenoceptors are known to be abundant in the detrusor smooth muscle and play a key role in relaxation of the human urinary bladder *via* the activation of adenylate cyclase during urine storage, little is known about the expression of

β-adrenoceptor subtypes in the human urinary bladder urothelium. Harmon and colleagues identified the presence of β-adrenoceptors on immortalized human urothelial cell membranes, but not the specific subtype involved (Harmon et al. 2005). To our knowledge, the present study is the first to report the presence of β_1-, β_2- and β_3-adrenoceptors in human urinary bladder urothelium by RT-PCR and immunohistochemistry. While previous reports have demonstrated the distribution of mucosal β_3-adrenoceptor mRNA in human small intestine, stomach and colon using RT-PCR and immunohistochemical techniques (Anthony et al. 1998; Roberts SJ et al. 1997), their functional role in this mucosa remains to be elucidated.

Among other findings, we also showed that the presence of the urothelium caused a parallel rightward shift in the concentration-response curve to isoproterenol, together with a significantly greater reduction in the pEC_{50} value of urothelium-intact compared to urothelium-denuded strips. These results may suggest that stimulation of β-adrenoceptors in the human urothelium induces the release of a urothelium-derived factor which inhibits the relaxation response of the detrusor strips to isoproterenol. On the other hand, we also showed that the presence of the urothelium did not significantly inhibit the maximal relaxant response of the human detrusor strips to isoproterenol. This apparent discrepancy might be accounted for by the assumption that the strong direct relaxant effects of isoproterenol at the high concentrations used in this study masked the effect of the inhibitory factor. In this regard, Murakami and colleagues reported that the urothelium was not involved in the relaxation responses of the porcine bladder to isoproterenol, but that the presence of isoproterenol caused a parallel rightward shift of the concentration-response

curve to carbachol (Murakami et al. 2007). This discrepancy between these past and present results may be due to the different baselines used to measure the relaxant effect to isoproterenol, namely passive (resting) tension versus active (carbachol-pre-contracted) tension. Here, we did not use a pre-contraction agent such as carbachol out of concern that these might mask the effects of a urothelium-derived factor induced by stimulation of the urothelial β-adrenoceptors. Moreover, given recent studies showing the presence and functional effects of muscarinic receptor subtypes in the human urothelium and suburothelial myofibroblasts (Hawthorn et al. 2000; Chaiyaprasithi et al. 2003; Mukerji G et al. 2006; Tyagi S et al. 2006), carbachol might influence urothelial β-adrenoceptor function *via* urothelial/suburothelial muscarinic receptor signaling. On the other hand, the present study is also limited by the possibility that a urothelium-derived factor or the urothelium itself might have influenced the relaxant effects of isoproterenol on the urothelium-intact detrusor strips. Clarification of this possibility may require further studies to identify the functional role of human urothelial β-adrenoceptors.

In rats, β-adrenoceptor stimulation activates the adenylate cyclase pathway in bladder epithelial cells and initiates an increase in intracellular Ca^{2+} concentration, thereby triggering NO production and its release (Birder et al. 1998, 2002). This finding led us to speculate that urothelial-derived NO can directly or indirectly modulate detrusor smooth muscle function. However, our results unexpectedly showed that L-NAME, a NO synthase inhibitor, had no significant attenuating effect on the responses to isoproterenol in human urothelium-intact detrusor smooth muscle. Murakami and colleagues also reported that N^G-nitro-L-arginine (L-NNA), a NO synthase inhibitor, did not prevent the enhanced

inhibitory effects of isoproterenol in urothelium-intact porcine bladder (Murakami et al. 2007). While soluble guanylate cyclase is the target enzyme of NO and activation of this enzyme produces an increase in tissue levels of cyclic guanosine monophosphate (cGMP) (Mayer et al. 1994), some reports state that soluble guanylate cyclase does not exist in detrusor smooth muscle cells (Smet et al. 1996; Fathian-Sabot et al. 2001). These findings suggest that urothelial β-adrenoceptor-induced release of NO might not be involved in the relaxant effects on human detrusor smooth muscle.

In summary, we identified the presence of β_1-, β_2-, and β_3-adrenoceptors in human urinary bladder urothelium, as has been also observed in detrusor smooth muscle. In addition, we also found that the relaxant effect of isoproterenol on detrusor smooth muscle was reduced in the presence of the urothelium in humans. It is possible that β-adrenoceptor agonists stimulate the release of an unidentified inhibitory factor from the urothelium that reduces detrusor relaxation induced by β-adrenoceptor stimulation; however, further work is needed to elucidate the exact mechanisms involved.

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References

Anthony A, Schepelmann S, Guillaume JL, Strosberg AD, Dhillon AP, Pounder RE, Wakefield AJ (1998) Localization of the β(beta)$_3$-adrenoceptor in the human gastrointestinal tract: an immunohistochemical study. Aliment Pharmacol Ther 12:519-25

Birder LA, Apodaca G, De Groat WC, Kanai AJ (1998) Adrenergic- and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder. Am J Physiol 275:226-9

Birder LA, Nealen ML, Kiss S, de Groat WC, Caterina MJ, Wang E, Apodaca G, Kanai AJ (2002) β-adrenoceptor agonists stimulate endothelial nitric oxide synthase in rat urinary bladder urothelial cells. J Neurosci 22:8063-70

Chaiyaprasithi B, Mang CF, Kilbinger H, Hohenfellner M (2003) Inhibition of human detrusor contraction by a urothelium derived factor. J Urol 170:1897-900

De Groat WC (2004) The urothelium in overactive bladder: passive bystander or active participant? Urology 64:7-11

Elneil S, Skepper JN, Kidd EJ, Williamson JG, Ferguson DR (2001) Distribution of P2X(1) and P2X(3) receptors in the rat and human urinary bladder. Pharmacology 63:120-8

Fathian-Sabet B, Bloch W, Klotz T, Niggemann S, Jacobs G, Addicks K, Engelmann U (2001) Localization of constitutive nitric oxide synthase isoforms and the nitric oxide target enzyme soluble guanylyl cyclase in the human bladder. J Urol 165:1724–9

Ferguson DR, Kennedy I, Burton TJ (1997) ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes - a possible sensory mechanism? J Physiol 505:503-11

Fry CH, Ikeda Y, Harvey R, Wu C, Sui GP (2004) Control of bladder function by peripheral nerves: avenues for novel drug targets. Urology 63:24-31

Fujimura T, Tamura K, Tsutsumi T, Yamamoto T, Nakamura K, Koibuchi Y, Kobayashi M, Yamaguchi O (1999) Expression and possible functional role of the β_3-adrenoceptor in human and rat detrusor muscle. J Urol 161:680-5

Harmon EB, Porter JM, Porter JE (2005) β-adrenergic receptor activation in immortalized human urothelial cells stimulates inflammatory responses by PKA-independent mechanisms. Cell Commun Signal 3:10-21

Hawthorn MH, Chapple CR, Cock M, Chess-Williams R (2000) Urothelium-derived inhibitory factor(s) influences on detrusor muscle contractility in vitro. Br J Pharmacol 129:416-9

Igawa Y, Yamazaki Y, Takeda H, Hayakawa K, Akahane M, Ajisawa Y, Yoneyama T, Nishizawa O, Andersson KE (1999) Functional and molecular biological evidence for a possible β_3-adrenoceptor in the human detrusor muscle. Br J Pharmacol 126:819-25

Igawa Y, Yamazaki Y, Takeda H, Kaidoh K, Akahane M, Ajisawa Y, Yoneyama T, Nishizawa O, Andersson KE (2001) Relaxant effects of isoproterenol and selective β_3-adrenoceptor agonists on normal, low compliant and hyperreflexic human bladders. J Urol 165:240-4

Ishihama H, Momota Y, Yanase H, Wang X, de Groat WC, Kawatani M (2006) Activation of α_{1D} adrenergic receptors in the rat urothelium facilitates the micturition reflex. J Urol 175:358-64

Krief S, Lonnqvist F, Raimbault S, Baude B, Van Spronsen A, Arner P, Strosberg AD, Ricquier D, Emorine LJ (1993) Tissue distribution of β_3-adrenergic receptor mRNA in man. J Clin Invest 91:344-9

Lefkowitz RJ (1996) G protein-coupled receptors and receptor kinases: from molecular biology to potential therapeutic applications. Nat Biotechnol 14:283-6

Longhurst PA, Levendusky M (1999) Pharmacological characterization of β-adrenoceptors mediating relaxation of the rat urinary bladder in vitro. Br J Pharmacol 127:1744-50

Maggi CA, Santicioli P, Parlani M, Astolfi M, Patacchini R, Meli A (1987) The presence of mucosa reduces the contractile response of the guinea-pig urinary bladder to substance P. J Pharm Pharmacol 39:653-5

Mayer B (1994) Regulation of nitric oxide synthase and soluble guanylyl cyclase. Cell Biochem Funct 12:167-77

Mukerji G, Yiangou Y, Grogono J, Underwood J, Agarwal SK, Khullar V, Anand P (2006) Localization of M2 and M3 muscarinic receptors in human bladder disorders and their clinical correlations. J Urol 176:367-73

Murakami S, Chapple CR, Akino H, Sellers DJ, Chess-Williams R (2007) The role of the urothelium in mediating bladder responses to isoprenaline. BJU Int 99:669-73

Roberts SJ, Papaioannou M, Evans BA, Summers RJ (1997) Functional and molecular evidence for β_1-, β_2- and β_3- adrenoceptors in human colon. Br J Pharmacol 120:1527-35

Saban R, Keith IM, Nielsen KT, Christensen MM, Rhodes PR, Bruskewitz RC (1992) In vitro effects of bladder mucosa and an enkephalinase inhibitor on tachykinin induced contractility of the dog bladder. J Urol 147:750-5

Smet PJ, Jonavicius J, Marshall JR, de Vente J (1996) Distribution of nitric oxidesynthase-immunoreactive nerves and identification of the cellular targets of nitric oxide in guinea-pig and human urinary bladder by cGMP immunohistochemistry. Neuroscience 71:337–48

Takeda M, Obara K, Mizusawa T, Tomita Y, Arai K, Tsutsui T, Hatano A, Takahashi K, Nomura S (1999) Evidence for β_3-adrenoceptor subtypes in relaxation of the human urinary bladder detrusor: analysis by molecular biological and pharmacological methods. J Pharmacol Exp Ther 288:1367-73

Templeman L, Chapple CR, Chess-Williams R (2002) Urothelium derived inhibitory factor and cross-talk among receptors in the trigone of the bladder of the pig. J Urol 167:742-5

Tyagi S, Tyagi P, Van-le S, Yoshimura N, Chancellor MB, de Miguel F (2006) Qualitative and quantitative expression profile of muscarinic receptors in human urothelium and detrusor. J Urol 176:1673-8

Ueda T, Araki N, Mano M, Myoui A, Joyama S, Ishiguro S, Yamamura H, Takahashi K, Kudawara I, Yoshikawa H (2002) Frequent expression of smooth muscle markers in malignant fibrous histiocytoma of bone. J Clin Pathol 55:853-858

Yoshida M, Miyamae K, Iwashita H, Otani M, Inadome A (2004) Management of detrusor

dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging. Urology 63:17-23

Table 1 Primer sequences for RT-PCR of β-adrenoceptor subtypes and β-actin

Code		Primer sequence (5' → 3')	GenBank accession No.
β_1-AR	Sense:	TCGTGTGCACCGTGTGGGCC	NM_000684
	Anti-sense:	AGGAAACGGCGCTCGCAGCTGTCG	
β_2-AR	Sense:	GCCTGCTGACCAAGAATAAGGCC	NM_000024
	Anti-sense:	CCCATCCTGCTCCACCT	
β_3-AR	Sense:	GCTCCGTGGCCTCACGAGAA	NM_000025
	Anti-sense:	CCCAACGGCCAGTGGCCAGTCAGCG	
β-actin	Sense:	GCTCG CGTCGACAACGGCTC	NM_001101
	Anti-sense:	CAAACATGATCTGGGTCATCTTCTC	

Figure legends

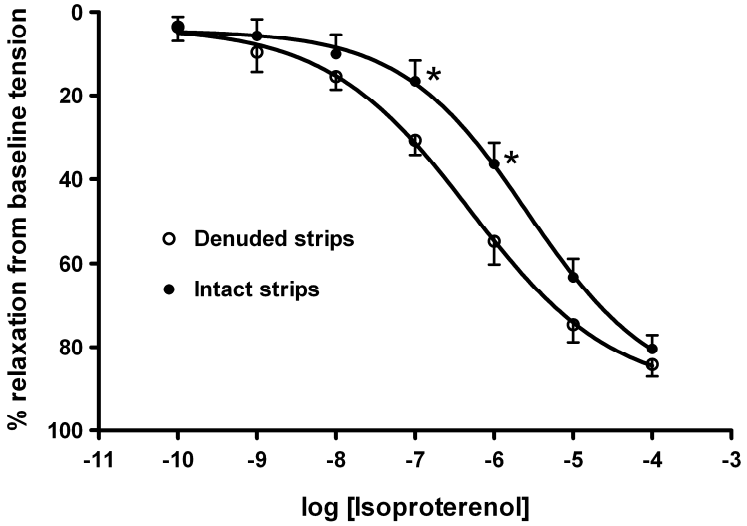

Fig. 1 Agarose gel electrophoresis shows expression of β_1-, β_2- and β_3-adrenoceptor mRNA in the human urinary bladder urothelium (**a**) and detrusor smooth muscle (**b**) obtained from four different patients (#1 - #4). Expected size of PCR products for β_1-, β_2- and β_3-adrenoceptor was 265, 329 and 314 bp, respectively. PCR products for the urothelial β-adrenoceptors were digested with specific restriction enzymes, namely β_1-adrenoceptor with Rsa I (**c**: generated fragments of 67 and 198 bp), β_2-adrenoceptor with Rsa I (**d**: 93 and 236 bp) and β_3-adrenoceptor with Apa I (**e**: 123 and 191 bp in lane 2, 4, 6 and 8; β_3-adrenoceptor PCR products in lane 1, 3, 5 and 7). Expression of mRNA for α-SMA, a smooth muscle marker, was high in human detrusor smooth muscle RNA (Musc), but undetectable in human urothelium RNA (Uroth) (**f**); 100 bp marker (MK).

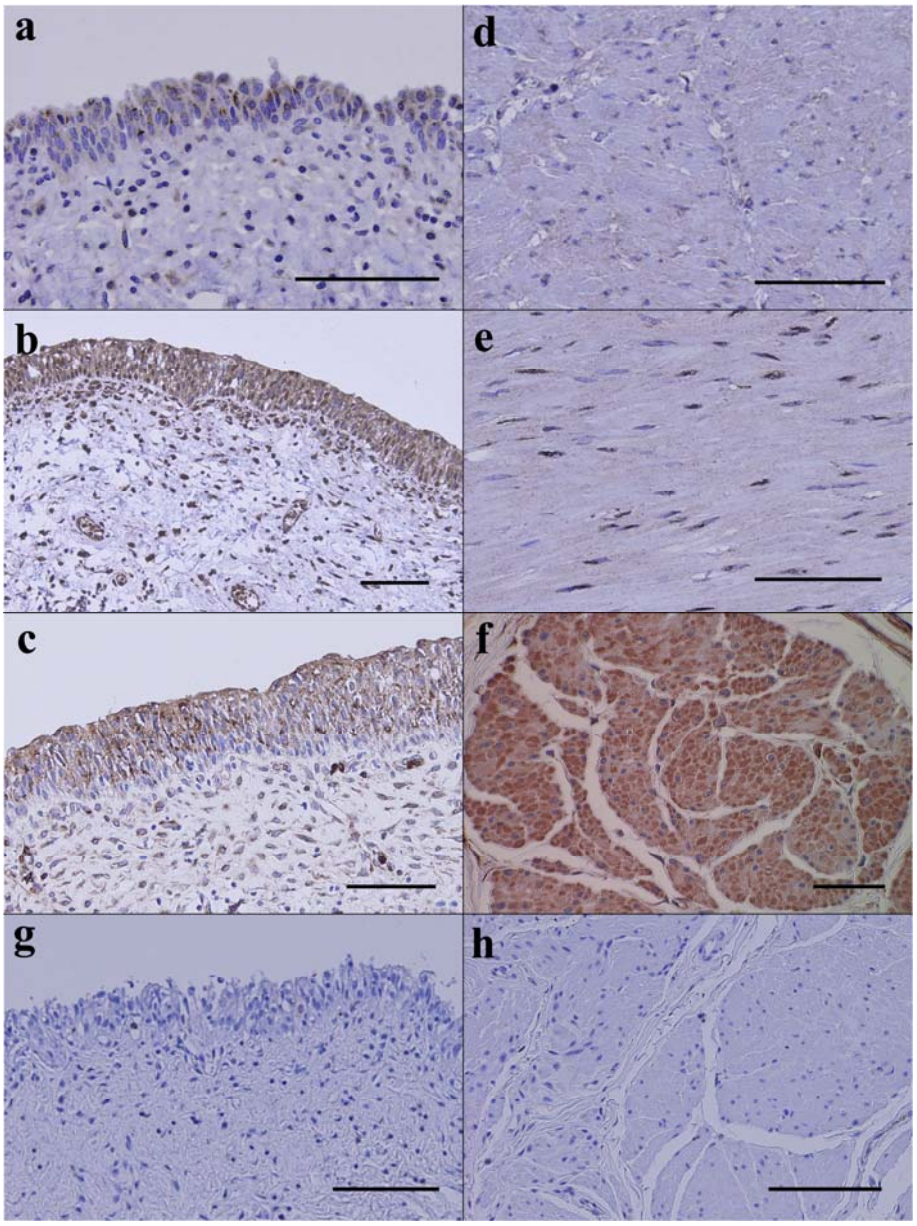

Fig. 2 Immunohistochemistry of human urinary bladder with rabbit polyclonal antibody showing positive (brown) staining of (**a)** β_1-, (**b**) β_2- and (**c**) β_3-adrenoceptor in human urinary bladder urothelial cells, and positive staining of (**d)** β_1-, (**e**) β_2- and (**f**) β_3-adrenoceptor in detrusor smooth muscle cells. Negative control of the human urinary bladder urothelium (**g**) and detrusor smooth muscle (**h**) showed no positive staining. Bar: (a)-(h) = 100 μm.

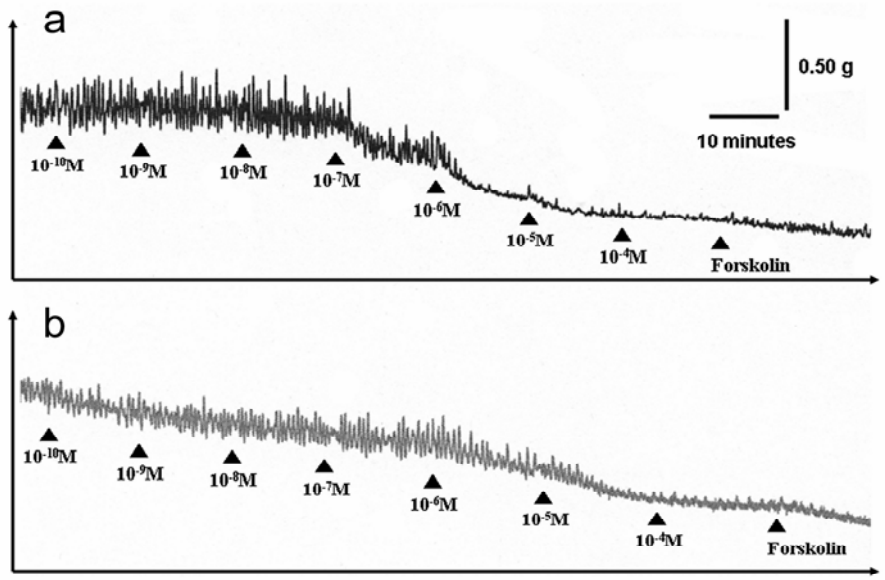

Fig. 3 Representative traces showing the effect of isoproterenol on the resting tension of human detrusor strips without (**a**) or with (**b**) an intact urothelium. Phentolamine (1 μM) was present in all experiments to inhibit α-adrenoceptors.

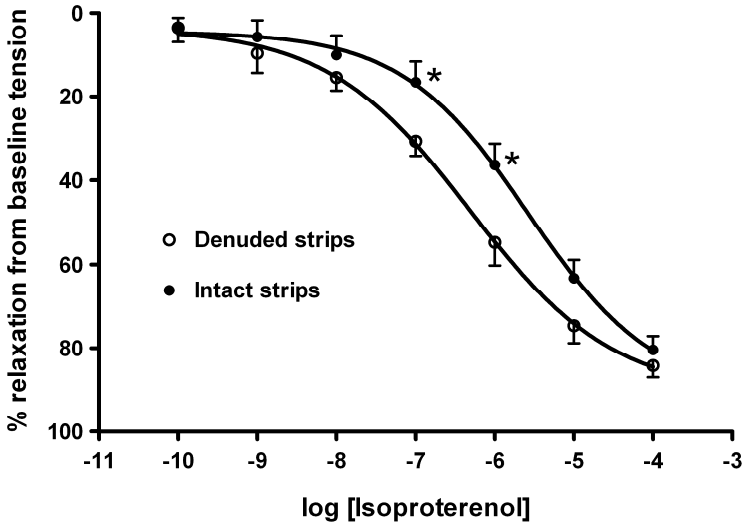

Fig. 4 Concentration-response curves of human detrusor strips to isoproterenol with (n=9) and without an intact urothelium (n=7). All experiments were performed in the presence of 1 μM phentolamine. Data are expressed as a percentage of maximal relaxation induced with 10 μM forskolin. An asterisk indicates $P < 0.05$ vs. urothelium-denuded strip responses.

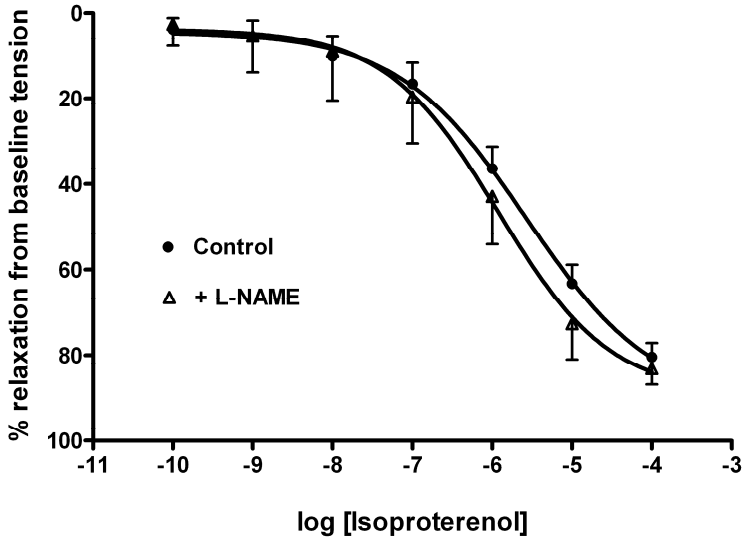

Fig. 5 Concentration-response curves of urothelium-intact human detrusor strips to isoproterenol in the presence (n=5) and absence (n=9) of 100 μM L-NAME. All experiments were performed in the presence of 1 μM phentolamine. Data are expressed as a percentage of the maximal relaxation induced by 10 μM forskolin.