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Pharmacological Modulation of Mucosa-Related Impairment of β-Adrenoceptor-Mediated Relaxation in Human Detrusor

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Key Words
Detrusor smooth muscle · Relaxation · Urothelium · Mucosa · β-Adrenoceptors · Signal transduction

Abstract
Objectives: The mucosa of human detrusor strips impairs catecholamine-induced relaxation. In order to elucidate which signal transduction pathways are involved in this cross talk between the mucosa and detrusor, we have studied the effects of several pharmacological agonists and antagonists on noradrenaline-mediated relaxation in intact and mucosa-denuded detrusor strips. ***Patients and Methods:*** Strips of detrusor tissue were obtained from patients who had undergone cystectomy for bladder cancer and were set up for force measurement. KCl- or carbachol-precontracted strips were relaxed with increasing concentrations of noradrenaline in the absence and in the presence of nitric oxide synthase inhibitor, L-NAME; P2X-receptor antagonist, PPADS; ET_A-receptor antagonist, BQ-123; ET_B-receptor antagonist, BQ-788; cyclooxygenase inhibitor, diclofenac; AT_1-receptor antagonist, candesartan; and NK_1-receptor antagonist, L-703,606. ***Results:*** In intact strips, KCl-stimulated force was enhanced by all blockers; carbachol-stimulated force increased with L-703,606. In denuded strips, only L-NAME augmented the KCl-stimulated contraction. Noradrenaline relaxed the precontracted detrusor strips to a significantly larger extent and at lower concentrations in denuded than in intact strips. L-NAME, PPADS and BQ-123/BQ-788 had little effect on noradrenaline-induced relaxation, whereas diclofenac, candesartan and L-703,606 sensitized intact carbachol-stimulated detrusor strips to noradrenaline-induced relaxation. ***Conclusion:*** Inhibition of the noradrenaline-induced relaxation of precontracted human detrusor strips by the mucosa is attenuated by diclofenac, candesartan and L-703,606 suggesting the involvement of prostanoids, angiotensin and neurokinin pathways. Further experiments are required to unravel the exact mechanisms.

Introduction

Antimuscarinic drugs are the mainstay for the treatment of overactive bladder symptoms, and tolerability of side effects may be improved by combining selective and unselective agents [1]. Whether the recently introduced β_3-adrenoceptor (β_3-AR) agonist, mirabegron [2], will provide better long-term results has to await further experience. Future drugs may even target the modulatory role of the urothelium on urinary bladder contractility.

In ex vivo experiments, the mucosa of the human urinary bladder provides significant inhibitory effects on de-

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trusor contractions in response to various modes of stimulation, for example, muscarinic agonists [3–7], tachykinins [8] or electric field stimulation [9, 10]. Conflicting results were reported for contractions elicited by KCl depolarization, the urothelium having been associated with either a robust effect [7] or no effect at all [9]. In particular, the differences between denuded and intact detrusor strips faded during steady-state force development [6]. Upon stimulation, the mucosa apparently releases a diffusible factor, the 'urothelium-derived relaxing factor' [4, 6].

Precontracted human detrusor relaxes in response to catecholamines, and this effect is mediated via β-ARs of the β_3-AR subtype [11, 12]. As reported previously [6], noradrenaline relaxed the precontracted detrusor strips in a concentration-dependent manner, and relaxation was significantly larger and required lower concentrations in denuded than in intact strips. Therefore, the mucosa also affects catecholamine-induced relaxation of human detrusor, but in the opposite direction as expected from the release of an 'urothelium-derived relaxing factor'. In other words, the mucosa impairs relaxation. This study and those by others have shown recently that isoprenaline and noradrenaline are less effective in relaxing carbachol-precontracted intact detrusor strips than mucosa-denuded muscles, suggesting that the mucosa could also be associated with a 'contracting factor' in addition to the 'relaxing factor' described previously [6, 13, 14]. The reduced sensitivity and efficacy of catecholamines for relaxing human detrusor muscles in the presence of mucosa was mediated via β_2-ARs [6, 15]. These effects are highly species dependent, because mouse detrusor muscle relaxes mainly via β_2-AR [15, 16].

Previous efforts to unravel the nature of the 'urothelium-derived relaxing factors' have excluded the involvement of nitric oxide (NO), cyclooxygenase products, purinergic P2Y receptors, TEA-sensitive K^+ channels and small Ca^{2+}-activated K^+ channels [4, 9]. However, which signal transduction pathways contribute to the mucosa-induced impairment of relaxation remains unknown.

Stimulation of the mucosa with KCl, carbachol and catecholamines could release signaling molecules which would in turn modulate contraction. The aim of our present study was to identify some of these signaling molecules by using selective pharmacological receptor agonists and/or antagonists. Because of the analogy between 'urothelium-derived relaxing factor' and 'endothelium-derived relaxing factor', drugs that are expected to affect vascular smooth muscles were chosen, including NO synthase inhibitor, L-NAME; P2X-receptor antagonist, PPADS; ET_A-receptor antagonist, BQ-123; ET_B-receptor antagonist, BQ-788; cyclooxygenase inhibitor, diclofenac; AT_1-receptor antagonist, candesartan; and NK_1-receptor antagonist, L-703,606.

Patients and Methods

All patients donating tissue gave fully informed consent in accordance with the Declaration of Helsinki and the regulation of the local ethics committee (approval No. EK 194092004). Specimens of human detrusor muscle were obtained from the bladder dome of patients undergoing primary radical cystectomy for the treatment of carcinoma invading bladder muscle. Patients who had received chemotherapy or radiotherapy were excluded. The mean age of the 52 patients (32 male and 20 female) was 71 ± 1 years.

Preparation of Human Detrusor Strips

Care was taken, that the muscle strips were obtained from tissue free of macroscopic tumor or inflammation. The tissue was transported in buffer solution to the laboratory within 15–30 min after excision. After careful removal of the serosa, 4–8 longitudinal muscle strips (10–15 mm length and 4–6 mm width) were cut from the tissue. The mucosa was removed from one-half of the preparations, but remained intact in the other half.

Histological Studies

Tissue samples were fixed in 4% buffered formalin. Samples were embedded in paraffin and cut in 4-μm-thick sections. The tissue was dewaxed in xylene and rehydrated in water through descending graded alcohols. The slides were incubated with hematoxylin for 3 min and then washed in tap water for 5 min. The eosin staining was performed for 1 min; the slides were then washed short in water. After that, the slides were dehydrated in ascending graded alcohols, washed 3 times in xylene and covered.

Recording of Contractions

All preparations were mounted in 5-ml organ baths containing carbogen-gassed Tyrode's solution at 37°C. Isometric tension was measured with an isometric force transducer (GM 2, Föhr Medical Instruments, Seeheim/Ober-Beerbach, Germany), amplified and recorded with Chart 4.0™ (ADInstruments, Sydney, Australia). Resting load was 10 mN. All experiments were carried out in the presence of the A-AR antagonists, phentolamine (3 μM) and prazosin (1 μM), to inhibit any AR-mediated processes.

During an equilibration period of 60 min, the bath solution was changed 3 times. The detrusor strips were exposed to 40 mM KCl for 10 min followed by washout. Muscle strips were precontracted with 1 μM carbachol or 40 mM KCl, and responses became stable within 45 min. Detrusor contraction was measured as force and expressed as mN/mg wet weight of each muscle strip. Relaxation was then induced by stepwise increase of the concentration of (–)-noradrenaline, until a maximum was reached. All relaxations were expressed as percentage of maximum relaxation achieved with 10 μM forskolin at the end of each experiment. Catecholamine-induced relaxation was either measured without any extra

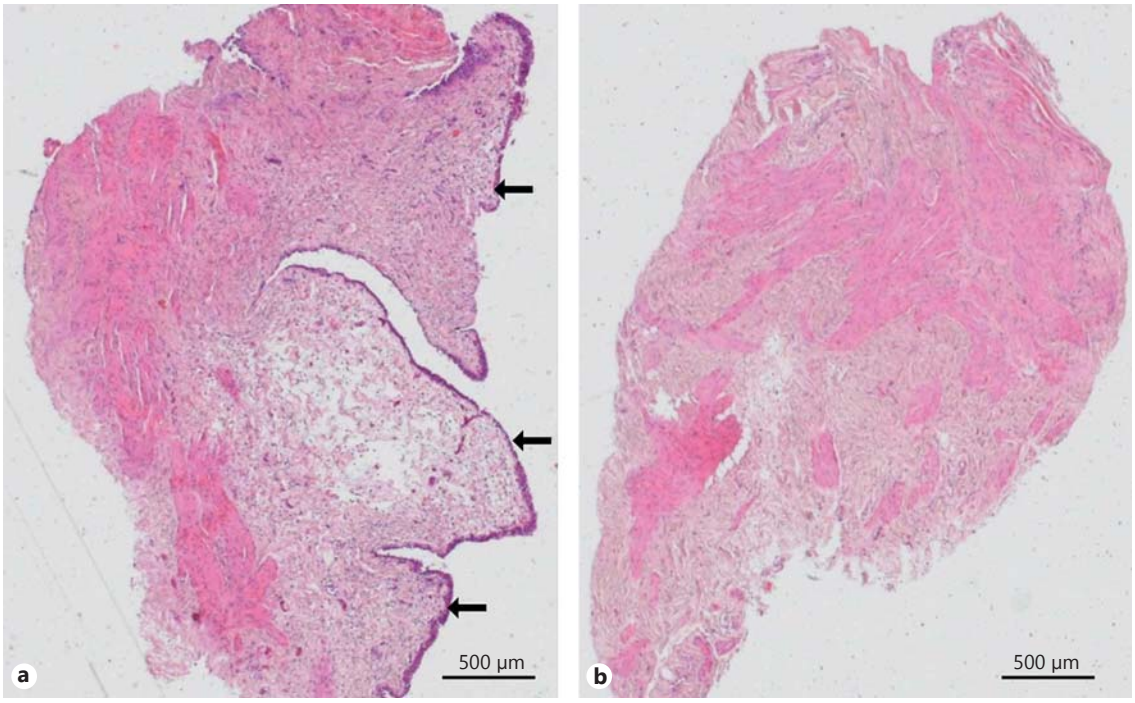

Fig. 1. Histological section of human detrusor strips with intact (**a**) and denuded urothelium (**b**). Arrows point at urothelium. Hematoxylin-eosin staining; scale bar 200 µm.

substance in the bath (time-matched controls) or in the presence of either one of the following modulators of signaling pathways – NO synthase inhibitor, L-NAME; P2X-receptor antagonist, PPADS; ET_A-receptor antagonist, BQ-123; ET_B-receptor antagonist, BQ-788; cyclooxygenase inhibitor, diclofenac; AT_1-receptor antagonist, candesartan; and NK_1-receptor antagonist, L-703,606 – added 45 min before catecholamine-induced relaxation.

Drugs and Solutions

The modified Ringer's solution contained (in mM) 149 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 0.1 NaH_2HPO_4 and 5.6 glucose. The Tyrode's solution contained (in mM) 127 NaCl, 5.4 KCl, 1.05 $MgCl_2$, 1.8 $CaCl_2$, 0.4 NaH_2HPO_4, 22 $NaCO_3$, 0.04 EDTA, 0.2 ascorbic acid and 5.6 glucose, pH 7.4, when equilibrated with 95% O_2 and 5% CO_2. Drugs and chemicals were obtained from Sigma (St. Louis, USA) and Tocris Bioscience (Bristol, UK). All drugs were dissolved in Milli-Q water (Millipore, Billerica, Mass., USA), with the exception of (–)-noradrenaline, which were dissolved in water containing 200 mM ascorbic acid and 40 mM EDTA. Forskolin was dissolved in dimethyl sulfoxide, and stock solutions were further diluted with Milli-Q water.

Data Analysis and Statistics

Concentration-response curves for catecholamines were analyzed by nonlinear regression of each individual experiment using GraphPad 4.0 (GraphPad Prism Software Inc., San Diego, USA). The negative logarithm of the molar concentration producing 50% of the maximum relaxing effect ($-\log EC_{50}$) was calculated. Results are presented as mean $-\log EC_{50}$ values ± SEM for 'n' muscle strips. Statistical differences were evaluated by using the Student's t test (paired or unpaired) and the analysis of variance. A value of $p < 0.05$ was considered statistically significant. Control experiments without addition of any drug were carried out for 2 projects that were done in parallel, that is, for our report identifying the β-AR subtype that was involved [6] and for the present one.

Results

Human intact and mucosa-denuded detrusor strips were histologically controlled for complete removal of the urothelium. Denuded strips were devoid of any urothelium which stains with hematoxylin-eosin as a continuous dark pink cell layer in intact strips (arrows in fig. 1).

Activation of intact and mucosa-denuded human detrusor strips with 40 mM KCl or 1 µM carbachol yielded the typical transient increase in force of contraction that declined to a constant level within 45 min (inset in fig. 2), with significantly less peak force of contraction upon either mode of stimulation in intact strips than denuded strips [6]. Preincubation of the preparations with the selective test drugs to block various receptors and enzymes resulted in the following responses (fig. 2). (1) Peak and

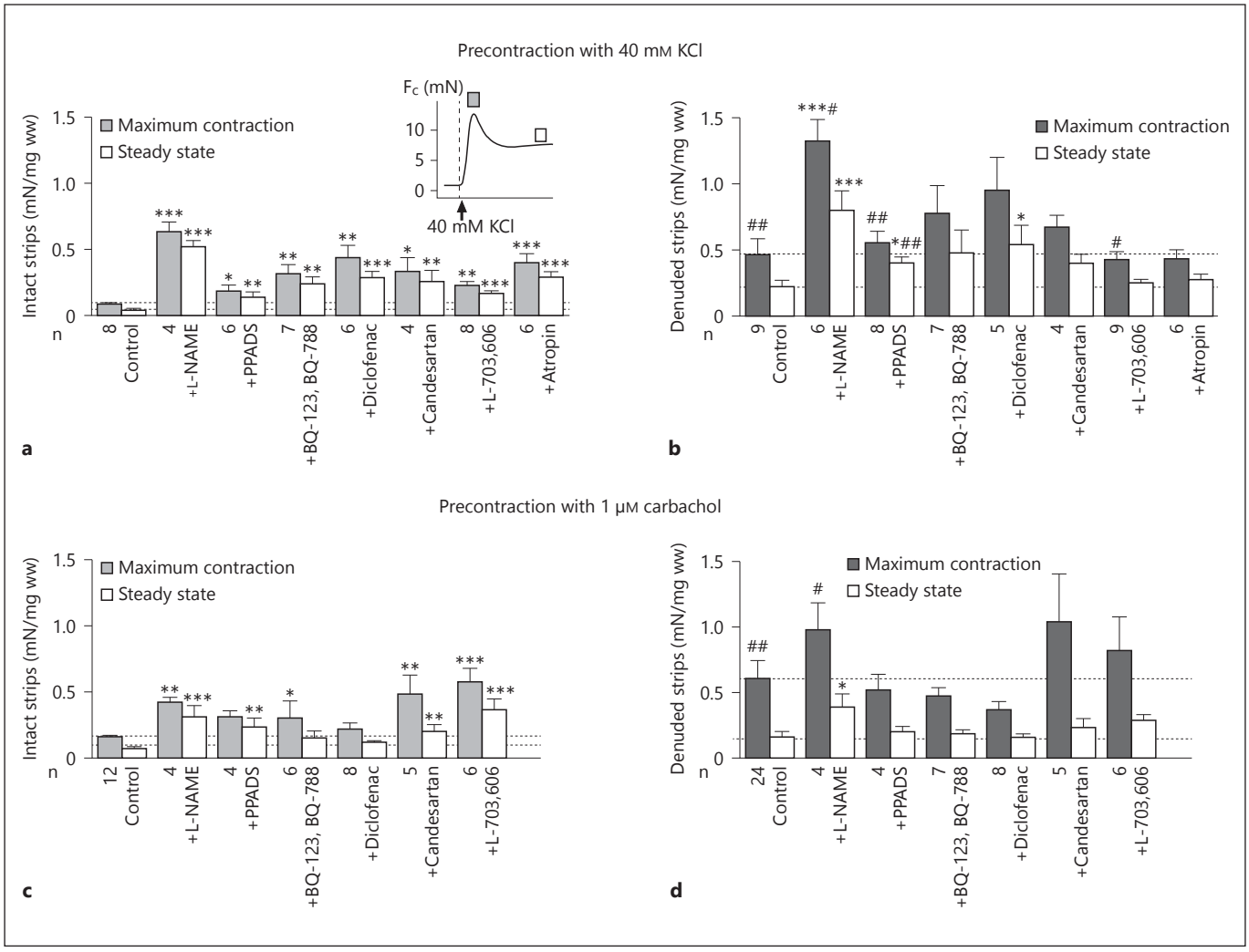

Fig. 2. Peak and steady-state force of contraction of human detrusor strips after stimulation (inset). Stimulation with KCl (40 mM) in **a** and **b**, with carbachol (1 μM) in **c** and **d**, intact strips in **a** and **c** and mucosa-denuded strips in **b** and **d**. Preparations were studied under control conditions and in the presence of various drugs. Force of contraction is given in mN/mg wet weight. Means ± SEM from n = number of investigated patients. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control force without any drug; # $p < 0.05$, ## $p < 0.01$ denuded versus intact strips.

steady-state force with KCl were enhanced by all blockers in intact strips and by L-NAME only in denuded strips. In addition, PPADS and diclofenac also increased steady-state force in the latter. (2) Like in controls, KCl-stimulated force was significantly lower in intact than in denuded preparation after preincubation with L-NAME, PPADS and L-703,606. (3) Carbachol-induced force in intact strips increased in the presence of the blockers with the exception of diclofenac, but none of the test compounds affected the peak force of denuded strips.

Control noradrenaline-induced relaxation of precontracted detrusor strips was described previously as being significantly larger and requiring lower concentrations in denuded than in intact strips [6]. These control data are used also in the present study (fig. 3; table 1 [6], with permission of the publisher). Pretreatment with L-NAME neither affected the sensitivity of the relaxation response to noradrenaline ($-\log EC_{50}$ values in table 1), nor the difference between denuded and intact detrusor, although the maximum effect expressed in percentage of relaxation elicited by 10 μM forskolin (E_{max} in table 1) was slightly, but significantly, larger after L-NAME pretreatment (intact strips stimulated with carbachol only; fig. 3). The P2X-receptor blocker, PPADS, decreased sensitivity for noradren-

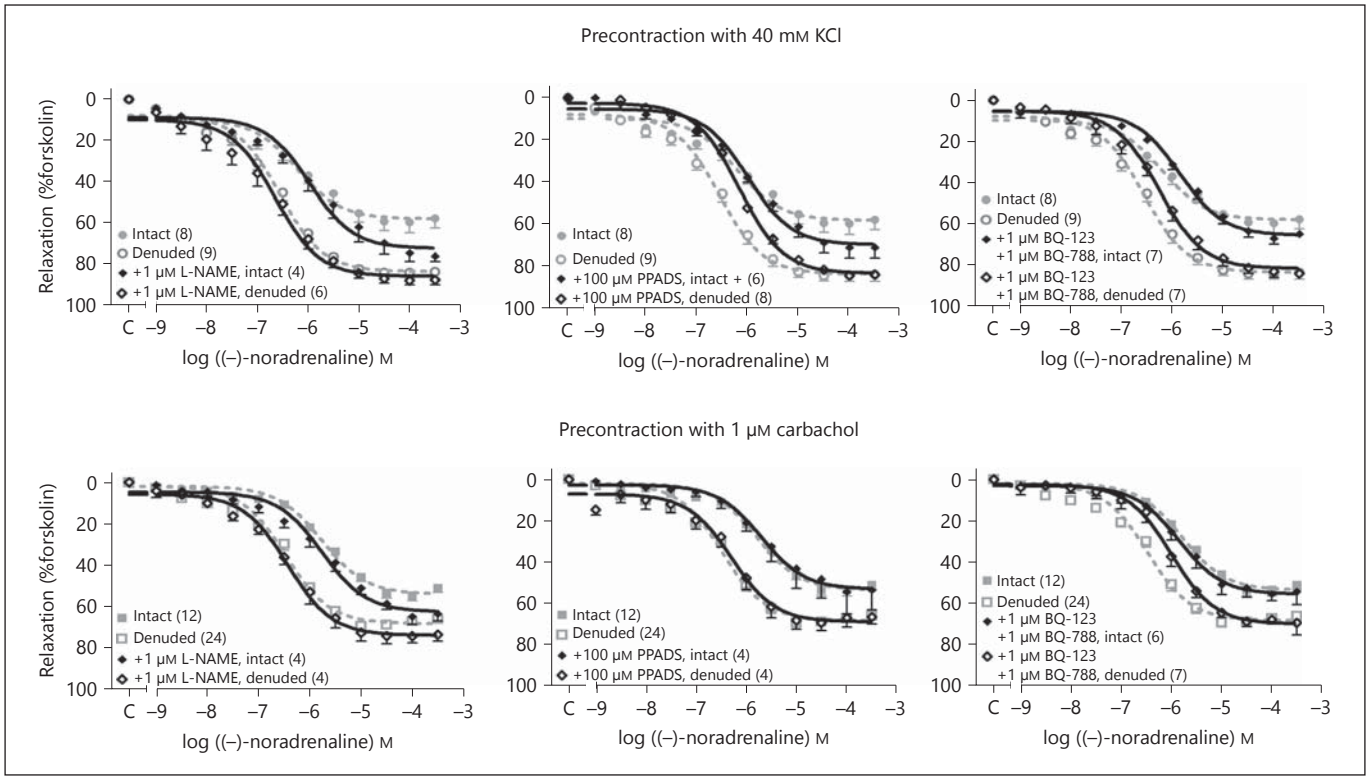

Fig. 3. Concentration-response curves for the relaxing effects of (–)-noradrenaline on KCl- and carbachol-induced contractions (upper and lower row, respectively) in human intact or mucosa-denuded detrusor strips. Control conditions (gray symbols, dashed lines) and in the presence of drug (black symbols, continuous lines), i.e. L-NAME, 1 mM; PPADS, 100 μM; and BQ-123/BQ-788, 1 μM. Data are expressed in % of maximum relaxation induced by 10 μM forskolin (= 100%). Mean ± SEM from 'n' investigated strips (numbers in parenthesis).

Table 1. Relaxing effects of noradrenaline in human detrusor strips with intact and denuded urothelium and their modulation by various drugs

Precontraction	Additional drug	Intact urothelium			Denuded urothelium		
		$-\log EC_{50}$ [M]	E_{max} [%]	n	$-\log EC_{50}$ [M]	E_{max} [%]	n
KCl, 40 mM	None (control)[1]	6.20±0.07	58±3	8	6.57±0.08$^{\#\#}$	84±3$^{\#\#\#}$	9
	L-NAME, 1 μM	5.90±0.20	74±3*	4	6.72±0.17	86±2	6
	PPADS, 100 μM	6.04±0.04	66±5	6	6.17±0.04***	83±1	8
	BQ-123, 1 mM; BQ-788, 1 mM	5.83±0.08**	66±3	7	6.27±0.10*	82±3	7
	Diclofenac, 100 μM	6.65±0.21*	85±2***	6	7.11±0.12**	85±3	5
	Candesartan, 100 μM	6.23±0.09	78±6*	4	6.77±0.15	85±3	4
	L-703,606, 1 μM	6.02±0.22	61±4	8	6.63±0.29	72±4*	9
	Atropine, 1 μM	6.20±0.12	64±4	6	6.68±0.06	86±2	6
Carbachol, 1 μM	None (control)[1]	5.76±0.06	55±3	12	6.41±0.09$^{\#\#\#}$	70±3$^{\#\#\#}$	24
	L-NAME, 1 μM	5.78±0.16	74±4**	4	6.43±0.08	63±3	4
	PPADS, 100 μM	5.68±0.11	54±11	4	6.31±0.10	69±4	4
	BQ-123, 1 mM; BQ-788, 1 mM	5.81±0.10	56±4	6	6.05±0.13*	71±2	7
	Diclofenac, 100 μM	6.20±0.15**	71±4**	8	6.66±0.0*	77±3*	8
	Candesartan, 100 μM	6.56±0.13***	76±5**	5	7.02±0.08***	85±2**	5
	L-703,606, 1 μM	6.11±0.11*	75±4***	6	6.40±0.09	83±2**	6

Precontraction by KCl or carbachol. E_{max} = Maximum response in % of response to 10 μM forskolin (= 100%); n = number of detrusor strips.
Comparison between intact and denuded urothelium: $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$. Comparison with respective control (no extra drug added): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. [1] Values taken from [4].

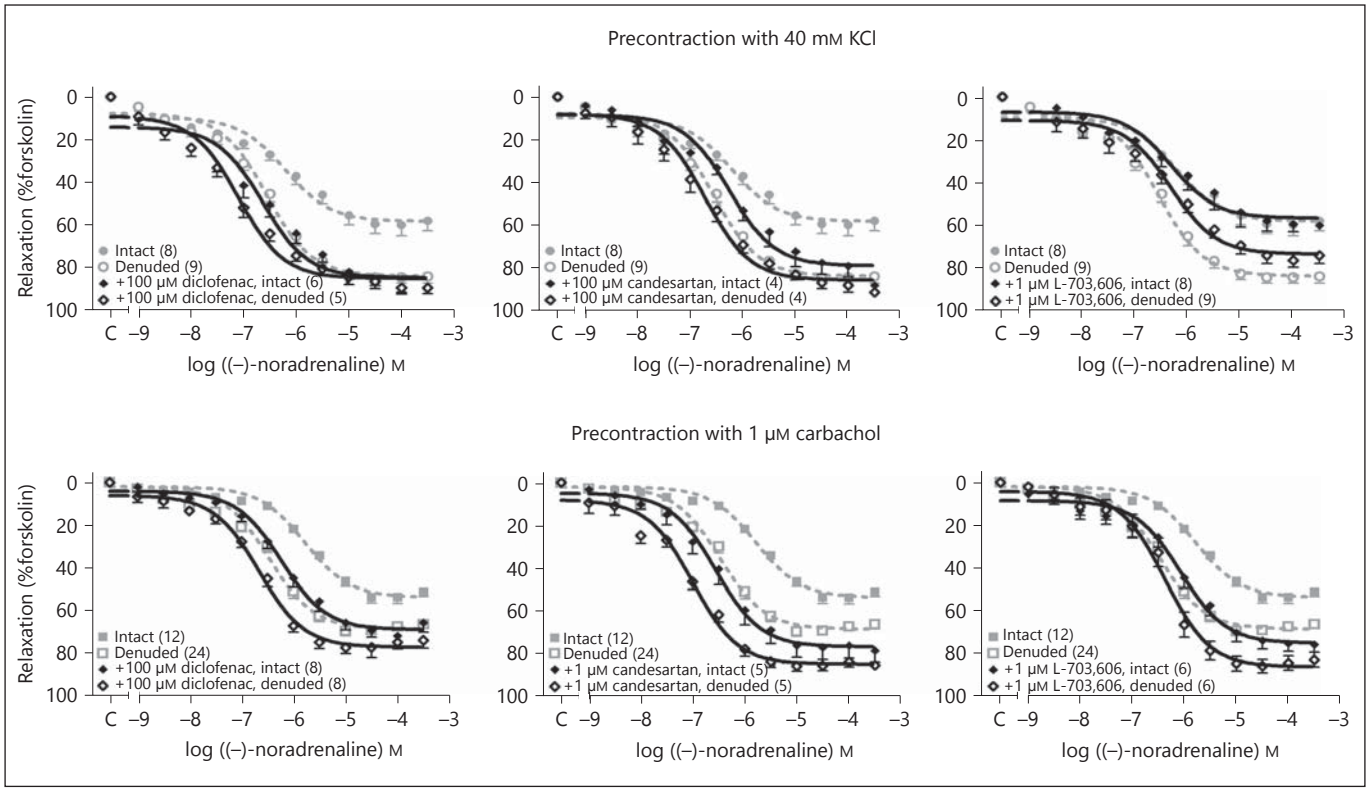

Fig. 4. Concentration-response curves for the relaxing effects of (–)-noradrenaline on KCl- and carbachol-induced contractions (upper and lower row, respectively) in human intact or mucosa-denuded detrusor. Control conditions (gray symbols, dashed lines) and in the presence of drug (black symbols, continuous lines), i.e. diclofenac, 100 µM; candesartan, 100 µM; and L-703,606, 1 µM. Data are expressed in % of maximum relaxation induced by 10 µM forskolin (= 100%). Mean ± SEM from 'n' investigated strips (numbers in parenthesis).

aline-relaxation only in denuded, KCl-depolarized muscle strips, thereby reducing the difference between intact and denuded preparations, but not in carbachol-stimulated strips. Block of ET_A- and ET_B-receptors with BQ-123/BQ-788 shifted the concentration-response curves to higher concentrations of noradrenaline without affecting the extent of relaxation. The difference between intact and denuded strips was maintained in KCl-stimulated but reduced in carbachol-stimulated muscles. In the presence of diclofenac (fig. 4), noradrenaline relaxed intact detrusor muscles to the same extent as denuded preparations and with similar potency as in control-denuded strips. In addition diclofenac-pretreated, denuded strips also became more sensitive to noradrenaline. Modulation of relaxation by diclofenac was similar in KCl- and carbachol-stimulated muscles. Candesartan, however, produced similar effects as that of diclofenac on sensitivity to, and efficacy of, noradrenaline in carbachol-stimulated strips; with KCl precontraction, the only diclofenac effect was enhancement of E_{max} in intact strips. The NK_1-receptor blocker, L-703,606, however, reduced sensitivity to noradrenaline in KCl-precontracted denuded muscles, but increased sensitivity in both intact and denuded carbachol-stimulated preparations.

Discussion

In the present study, we have investigated the influence of various pharmacological agonists and antagonists on mucosa relaxation induced with (–)-noradrenaline in human KCl- or carbachol-precontracted detrusor smooth muscles. Our major findings were the following: (1) Although NO signaling counteracts KCl contractions, it does not appear to be involved in mediating the specific mucosa effects on relaxation. (2) Inhibition of cyclooxygenase abolishes the mucosa effect on (–)-noradrenaline-induced relaxation in KCl- and carbachol-precontracted muscles. (3) AT1 receptor blocker, candesartan, and NK_1-receptor blocker, L-703,606, have similar effects, but only in carbachol-stimulated strips.

Urothelium versus Mucosa

Upon stretch, the urothelium releases active compounds, such as non-neuronal acetylcholine [17], ATP [18], tachikinins [19] and prostaglandins [20], that can either directly activate detrusor contraction or indirectly modulate contractility via stimulation of suburothelial nerves.

The histological sections in figure 1 clearly show complete removal of the urothelium; however, we cannot distinguish whether the urothelium alone or the whole mucosa is responsible for the observed differences between intact and denuded muscle strips. Human urothelial cell lines express various functional G-protein coupled receptors that are involved in the release of further signaling molecules [21, 22]. These findings suggest that urothelial cells themselves could be the source of 'urothelium-derived factors' causing either relaxation or blunting of relaxant effects of (–)-noradrenaline. On the other hand, electric field stimulation of the complete mucosa containing urothelium together with lamina propria causes neurogenic contractions that do not involve muscarinic, adrenergic, purinergic, or neurokinin neurotransmission [23].

Effects of Drugs on KCl- and Carbachol-Induced Contraction

Human detrusor preparations were activated either by depolarization with KCl and subsequent Ca^{2+} influx via activated voltage-dependent calcium channels or by stimulation of muscarinic receptors with the parasympathomimetic agent carbachol [24]. Although the major goal of our present study was to identify signal transduction pathways that may contribute to the mucosa effect of catecholamine-induced detrusor relaxation, the experiments also provided confirmation about the drug effects on the 2 modes of precontractions. Such comparison with published data seemed useful in view of some conflicting results, as for instance, whether the urothelium affects KCl-activated contractions [7] vs. [9], see Introduction.

Role of NO

Reduction of endogenous NO production by unselective inhibition of NO synthases with L-NAME strongly enhanced KCl-induced contraction, independent of the presence or the absence of urothelium. There was also a tendency to higher force development in response to carbachol, but this difference did not reach statistical significance. These results suggest that endogenous NO may attenuate the level of stimulated tension development [3] and confirm that NO is probably not the urothelium-derived relaxing factor [4, 25, 26]. Moreover, NO is unlikely to be involved in noradrenaline-induced relaxation because L-NAME had no effect on the concentration-relaxation curves with or without urothelium. This result is in good agreement with lack of NO effects on urothelium-dependent relaxation in isoprenaline-induced relaxation [14].

Role of ATP and Endothelin

Block of the P2X purinoreceptor with PPADS or of the endothelin receptors ET_A and ET_B with BQ-123 and BQ-788 had no effect on KCl- or carbachol-stimulated contraction and did not modulate the difference in contractions with and without urothelium. We observed some desensitization to noradrenaline-induced relaxation in denuded KCl- but not in carbachol-precontracted muscles with PPADS and in denuded muscles (either of the precontraction mode) with BQ-123 plus BQ-788. Therefore, we conclude that the role of purinergic compounds or endothelin is probably minor.

Role of Prostaglandins

Prostanoids are released in the human urinary bladder [27], many of which contract human detrusor muscles [28, 29]. Suppression of prostaglandin synthesis by diclofenac influenced neither KCl- nor carbachol-contractions in denuded muscles suggesting that prostaglandin release does not contribute to these 2 modes of stimulation. The finding, that diclofenac enhanced, rather than reduced, force induced in intact muscle by KCl but not by carbachol is difficult to interpret. We speculate that KCl may release another counteracting factor from the urothelium. Alternatively, a relaxing prostaglandin, such as PGD_2, is no longer produced in active concentrations [26]. Clearly, prostaglandins are involved in reducing the sensitivity of the intact and denuded detrusor to noradrenaline-induced relaxation, because diclofenac shifted the concentration-response curve to lower concentrations and enhanced efficacy in intact strips. Further experiments are required to elucidate the prostaglandins and the receptor subtypes that may be involved.

Role of Angiotensin

Angiotensin-II is known to contract detrusor muscle [3]. The lower urinary tract harbors a local renin-angiotensin system [30] that contributes to stress incontinence in animal models [31]. A post hoc analysis of antihypertensive patients treated with AT_1 receptor blockers revealed lesser lower urinary tract symptoms than the untreated subjects [32]. Here, we have demonstrated

that blocking AT_1 receptors sensitizes denuded and intact human detrusor to noradrenaline relaxation suggesting that local angiotensin II production counteracts smooth muscle relaxation by noradrenalin and is also involved in blunting the noradrenaline relaxation in the presence of urothelium. The effects were more pronounced in carbachol- than in KCl-precontracted preparations. We speculate that by promoting catecholamine-induced relaxation, AT_1 receptor blockers may be of therapeutic benefit in the overactivity of the lower urinary tract.

Role of Neurokinin

Tachykinins activated NK receptors that mediate detrusor contraction [3]. Block of NK_1 receptors significantly enhanced carbachol responses in intact but not in denuded detrusor. Similar to our arguments for diclofenac, this could hint at tachykinin-induced release of a contraction counteracting factor. In the presence of the NK_1 blocker, L-703,606, modulation of noradrenaline relaxation was only observed in the presence of urothelium including tachykinins as a putative compound involved in 'urothelium-derived contracting factor'.

Study Limitations

The different receptors involved in the relaxing effect of catecholamines in human detrusor could also be altered by pathology. For instance, β_3-ARs might be overexpressed in obstructed or diseased human bladder [33]. However, this issue cannot be resolved because detrusor tissue from healthy human probands is not available.

Conclusion

The mucosa of human detrusor strips impairs noradrenaline-induced relaxation, and this effect is attenuated by diclofenac, candesartan and L-703,606 suggesting the involvement of prostanoids, as well as AT_1 and NK_1 receptors.

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