β -Adrenoceptor-Mediated Relaxation of Carbachol-Pre-Contracted Mouse Detrusor

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Key Words

Detrusor smooth muscle · Carbachol-induced contractions · Relaxation · Mucosa · β -Adrenoceptors

Abstract

Aims: To study the β -adrenoceptor subtypes involved in the relaxation responses to (-)-isoprenaline in carbachol-pre-contracted (CCh) mouse detrusor muscle with intact and denuded mucosa. **Methods:** Isolated muscle strips from the urinary bladder of male C57BL6 mice or β_2 -adrenoceptor knockout mice were pre-contracted with CCh, 1 μ M and relaxed with increasing concentrations of the β -adrenoceptor (β -AR) agonist (-)-isoprenaline and forskolin. For estimating the β -AR subtypes involved, subtype-selective receptor blockers were used, that is, CGP 20712A (β_1 -ARs), ICI 118,551 (β_2 -ARs), and L748,337 (β_3 -ARs). **Results:** Unlike in KCl-pre-contracted muscle, the mucosa did not affect the sensitivity of the relaxation response to (-)-isoprenaline in CCh-pre-contracted murine detrusor strips. Increasing concentrations of (-)-isoprenaline produced a biphasic concentration-relaxation response without any difference both during the presence and absence of mucosa. The relaxation fraction produced by low (-)-isoprenaline concentrations was mediated by β_2 -AR as evidenced by a shift of the concentration-response curve to higher concentrations with ICI 118,551, but not with CGP 20712A and L748,337, and by the absence

of this fraction in β_2 -AR-KO mice. The relaxation response with low sensitivity to (-)-isoprenaline was not affected by any of the β -AR subtype-selective blockers and was the only response detected in detrusor strips from β_2 -AR-KO mice.

Conclusions: In CCh-pre-contracted mouse detrusor, β_2 -ARs are responsible for the relaxation component with high sensitivity to (-)-isoprenaline as indicated by the conversion of a biphasic into a monophasic CRC with ICI 118,551 or by its absence in β_2 -AR KO mice. The mucosa does not impair relaxation under these conditions.

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Introduction

The urothelium is part of the mucosa of the lower urinary tract and lines the inner surface of the urinary bladder. Besides protecting the bladder wall against aggressive constituents of the urine, the urothelium is also involved in sensing mechanical stress and regulating contractile function of the detrusor [1, 2].

The precise mechanisms by which the urothelium modulates contraction are not fully understood. In-vitro experiments with intact and mucosa-denuded detrusor strips provided conflicting results in various species. Maximum KCl-stimulated contractions of human and murine muscle strips are lower in intact strips than when

the urothelium has been removed [3, 4], although some groups did not find any difference [5]. In several species, contractions elicited via muscarinic receptor stimulation are also smaller in intact than denuded detrusor strips [4–11], suggesting that the urothelium exerts a relaxing effect on bladder muscle, which is assumed to be mediated by an unknown diffusible factor. The specific characteristic of this ‘urothelium-derived relaxing factor’ has not been determined [5, 7]. On the other hand, there is also evidence for indirect backup of contraction by the urothelium. For instance, higher concentrations of (-)-noradrenaline and (-)-isoprenaline are needed for the relaxation of carbachol (CCh)-pre-contracted intact than mucosa-denuded detrusor from pig and human bladder [4, 11, 12]. We reported similar findings in KCl-pre-contracted murine muscle strips [3], suggesting that the urothelium antagonizes relaxation mediated via β -adrenoceptors (β -AR).

Relaxation of CCh-stimulated detrusor strips with (-)-isoproterenol is mediated via different β -AR subtypes in mouse (β_2 -AR) and man (β_3 -AR) [13]. We also noted different sensitivities to β -AR stimulation between intact and denuded strips from human and mouse muscle. Interestingly, different β -AR subtypes are involved in the mucosa’s modulating properties. In man, relaxation of KCl-pre-contracted detrusor strips was mediated by β_3 -ARs but the effect of the mucosa on the relaxation response to (-)-isoprenaline mediated via mucosal β_2 -ARs, whereas in mouse, both responses are mediated by β_2 -ARs, suggesting that the activation of mucosal β_2 -ARs reduces β -AR-mediated detrusor relaxation irrespective of the β -AR sub-type involved [3, 4].

In the present study, we investigated whether the mucosa can also modulate (-)-isoprenaline-induced relaxation of CCh-pre-contracted mouse detrusor muscle and characterized the β -AR subtypes are involved. We found similar biphasic concentration-response curves for (-)-isoprenaline in intact and denuded strips and the high sensitivity fraction of the response was mediated via β_2 -ARs.

Materials and Methods

The study was conducted according to European Commission Directive 86/609/EEC regarding the protection and welfare of animals used for experimental as well as scientific purposes. All animal experiments were performed in accordance with the regulations of the local council committee (permission number 24D-9168.24–1/2007–17 of the Regierungspräsidium, Dresden).

Animals

Male C57BL6 [Charles River, Margate, Kent, UK] wild type and homozygote β_2 -AR knockout mice in mixed C57BL/6J/FVB/N background, bred in the laboratory in Würzburg, were used [14]. The age of the mice ranged from 9 to 15 months and their body weight ranged from 20–30 g. All mice were group-housed in cages with free access to food and water at least 4 days before the experiments were conducted.

Preparation of Detrusor Muscle Strips

Mice were sacrificed by cervical dislocation. The whole urinary bladder was resected at the bladder neck. After removal of the dome and the neck of the bladder, the remaining muscle ring was opened longitudinally. The mucosa was removed from half of the preparations, but remained intact in the other half. Four strips of smooth muscle around 3–4 mm long and 1–2 mm wide were prepared.

Drugs and Solutions

The modified Tyrode’s solution contained (in mM/l): NaCl 126.9, KCl 5.4, $MgCl_2$ 1.05, $CaCl_2$ 1.8, NaH_2PO_4 0.45, $NaHCO_3$ 22, EDTA 0.04, ascorbic acid 0.2, and glucose 5.6, pH 7.4, when equilibrated with 95% O_2 and 5% CO_2 . Drugs and chemicals were obtained from Sigma (St. Louis, Mo., USA) and Tocris Bioscience (Bristol, UK). All drugs were dissolved in Milli-Q water (Millipore, Billerica, Mass., USA), with the exception of (-)-isoprenaline, which was dissolved in water containing 200 mM ascorbic acid and 40 mM EDTA. L748,337 and forskolin were dissolved in dimethyl sulfoxide and stock solutions were further diluted with Milli-Q water. The maximum concentration of dimethyl sulfoxide in the organ bath did not exceed 0.1%.

Pre-Contraction and Relaxation of Urinary Smooth Muscle

All preparations were mounted in 5-ml organ baths containing carbogen-gassed, modified Tyrode’s solution at 37°C. Tension was measured with an isometric force transducer (GM 2, Föhr Medical Instruments, Seeheim/Ober-Berbach, Germany), amplified and recorded with Chart 4.0™ (ADInstruments, Sydney, Australia). Resting load was 5 mN. The force of detrusor contraction was expressed as mN/mg wet weight (w.w.) of each muscle strip.

During a 60-min episode of equilibration the Tyrode’s solution was replaced every 20 min conditioning muscle contractions were elicited to times by increasing the KCl concentration in the bath solution to 40 mM for 5 min followed by washout without compensation for increase in osmolality. To exclude any T-AR-mediated process, phentolamine (3 μ M) and prazosin (1 μ M) were added. After a resting time of 45 min, muscle strips were pre-contracted with 1 μ M CCh and responses became stable within 45 min. Relaxation was then induced by cumulatively increasing concentrations of (-)-isoprenaline (10 pM–300 μ M) and maximum relaxation was tested with 10 μ M forskolin. Involvement of β -AR subtypes in the responses was tested by incubating the strips 30 min prior to CCh stimulation with a single or a combination of β -AR subtype-selective antagonists, that is, CGP 20712A (300 nM) for β_1 -ARs, ICI 118,551 (50 nM) for β_2 -ARs, and L748,337 (100 nM, 300 nM or 1 μ M) for β_3 -ARs. Time-matched control experiments of CCh-induced detrusor contractions in the presence of phentolamine (3 μ M) and prazosin (1 μ M) were run in the absence of a β -AR agonist or antagonist.

Table 1. Relaxing effects of cumulative added (-)-isoprenaline (10 pM–300 μ M) in murine detrusor muscle strips with intact and denuded mucosa pre-contracted with 1 μ M carbachol (CCh) and the influence of selective β -AR antagonists

Subtype-selective antagonist	Intact mucosa					Denuded mucosa				
	high sensitivity component		low sensitivity component		n	high sensitivity component		low sensitivity component		n
	$-\log EC_{50}$ [M]	E_{max} [%]	$-\log EC_{50}$ [M]	E_{max} [%]		$-\log EC_{50}$ [M]	E_{max} [%]	$-\log EC_{50}$ [M]	E_{max} [%]	
none (= control)	9.21 $\pm$ 0.38	33 $\pm$ 3	5.86 $\pm$ 0.41	36 $\pm$ 3	13/13	9.21 $\pm$ 0.46	41 $\pm$ 4	5.86 $\pm$ 0.45	32 $\pm$ 2	11/11
+300 nM CGP 20712A	8.02 $\pm$ 0.16	36 $\pm$ 6	5.06 $\pm$ 0.24	42 $\pm$ 4	5/5	7.60 $\pm$ 0.83	32 $\pm$ 7	5.95 $\pm$ 0.88	48 $\pm$ 6 ^{##}	5/5
+50 nM ICI 118,551	–	–	5.93 $\pm$ 0.11	72 $\pm$ 4 ^{###}	7/7	–	–	5.93 $\pm$ 0.16	73 $\pm$ 7 ^{###}	7/7
+100 nM L 748,337	7.99 $\pm$ 0.01	49 $\pm$ 5	4.89 $\pm$ 0.16	33 $\pm$ 1	4/4	8.50 $\pm$ 0.46	46 $\pm$ 9	5.52 $\pm$ 0.65	36 $\pm$ 9	4/4
+300 nM L748,337	9.02 $\pm$ 0.76	32 $\pm$ 6	4.97 $\pm$ 1.01	36 $\pm$ 2	4/4	8.29 $\pm$ 0.12	67 $\pm$ 3 [#]	4.88 $\pm$ 0.21	56 $\pm$ 2	4/4
+1 μ M L748,337	9.73 $\pm$ 1.01	44 $\pm$ 15	7.49 $\pm$ 1.36	32 $\pm$ 8	4/4	9.64 $\pm$ 0.67	22 $\pm$ 5 [#]	6.06 $\pm$ 1.20	44 $\pm$ 2	4/4
+300 nM CGP 20712A, +100 nM L748,337	8.06 $\pm$ 0.18 [#]	30 $\pm$ 5	4.87 $\pm$ 0.16	48 $\pm$ 7	8/6	7.93 $\pm$ 0.19 [#]	30 $\pm$ 2	4.69 $\pm$ 0.08	46 $\pm$ 3 ^{##}	7/6
+300 nM CGP 20712A, +50 nM ICI 118,551	–	–	6.03 $\pm$ 0.14	72 $\pm$ 7 ^{###}	5/5	–	–	6.03 $\pm$ 0.11	80 $\pm$ 3 ^{###}	4/4
+50 nM ICI 118,551, +100 nM L748,337	–	–	5.90 $\pm$ 0.06	65 $\pm$ 4 ^{###}	5/5	–	–	5.93 $\pm$ 0.07	72 $\pm$ 4 ^{###}	5/5
+300 nM CGP 20712A, +50 nM ICI 118,551 +100 nM L748,337	–	–	5.72 $\pm$ 0.13	73 $\pm$ 5 ^{###}	8/8	–	–	5.62 $\pm$ 0.28	76 $\pm$ 2 ^{###}	7/7
β_2 -AR KO	–	–	5.77 $\pm$ 0.17	50 $\pm$ 3 ^{##}	15/8	–	–	5.57 $\pm$ 0.17	52 $\pm$ 4 ^{###}	10/8

$-\log EC_{50}$ = Negative logarithm of the concentration of (-)-isoprenaline required for half-maximum relaxation in the absence or presence of extra drug; E_{max} = maximum response in % of response to 10 μ M forskolin (100%); values are mean values obtained from curve fitting to data of individual experiments; n = number of mice/number of detrusor strips.

Values compared with respective values in control strips. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$.

Data Analysis and Statistics

Concentration-response curves (CRCs) for (-)-isoprenaline were analyzed by nonlinear regression of each individual experiment using GraphPad 4.0 (GraphPad Prism Software Inc., San Diego, Calif., USA). The negative common logarithm of the molar concentration producing 50% of the maximum relaxing effect ($-\log EC_{50}$ [M]) was calculated. The values of table 1 are mean values $\pm$ SEM from n muscle strips. Strips from the same mouse were used in different experimental groups, so that the number of strips in each group is equal to the number of mice.

The figures of nonlinear regression curves were obtained by fitting sigmoidal functions to the mean data points, whereas the $-\log EC_{50}$ values of table 1 are the mean values obtained from curve fitting to data of individual experiments. Statistical differences were evaluated by using paired or unpaired Student's t-test and ANOVA with an additional Bonferroni comparison test. A value of $p < 0.05$ was considered statistically significant.

Results

The force of contraction rapidly increased after the addition of CCh (1 μ M) to a peak value (F_{max}) and then declined to a steady state value within 30 min (F_{ss} ; fig. 1a, b). Since the preparations developed strong spontaneous ac-

tivity, tension was measured as the difference between baseline and the lowest force development during a spontaneous contraction cycle. The addition of cumulatively increasing concentrations of (-)-isoprenaline relaxed the muscle strips and maximum relaxation (R_{max}) was achieved with forskolin (10 μ M). F_{max} and F_{ss} with CCh, but also force at R_{max} in the presence of forskolin were significantly larger in urothelium-denuded than intact detrusor strips (fig. 1c). These results are in line with the existence of an 'urothelium-derived relaxing factor' [3–5, 7]. Tension decline of CCh-induced contraction in a similar time window as required for the (-)-isoprenaline application (time-matched controls, TMC) was $13 \pm 2\%$ in intact and $14 \pm 4\%$ in denuded strips.

Figure 2 depicts CRC for (-)-isoprenaline in intact and denuded strips under control conditions and in the presence of β -AR subtype selective blockers. The relaxation curves showed a highly significant better fit by a biphasic than a monophasic sigmoidal function (table 1). Approximately, half of the CCh-induced force relaxed with an EC_{50} value in the nanomolar and the other half in the micromolar concentration range ($-\log EC_{50}$ [M] of 9.21 and 5.86, respectively). There were no significant differences

Fig. 1. Relaxation of CCh-induced contraction of mouse detrusor by (-)-isoprenaline. **a** Original tracing of force contraction of intact or mucosa-denuded detrusor strips after addition of CCh, 1 μ M and cumulatively increasing concentrations of (-)-isoprenaline (increments in half log units). The force in the presence of 10 μ M forskolin defined the level of maximum relaxation (100%). **b** Sche-

matic presentation of peak ($F_{\max}$) and steady-state (F_{ss}) force of contraction induced by CCh and maximum relaxation ($R_{\max}$). **c** Mean values for CCh-induced $F_{\max}$, F_{ss} and $R_{\max}$ in intact and denuded detrusor strips. Means $\pm$ SEM, numbers indicate number of strips. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

in sensitivity to (-)-isoprenaline between intact and denuded muscles both with respect to potency and efficacy (see table 1).

Pre-incubation with the subtype selective β -AR blockers had no effect on $F_{\max}$, F_{ss} and $R_{\max}$ (data not shown). None of the β -AR blockers affected the portion of relaxation with low sensitivity to (-)-isoprenaline, neither alone nor in any combination (fig. 2). The β_1 -AR blocker CGP 20712A (300 nM) had no effect on the (-)-isoprenaline CRC in denuded detrusor, whereas the highly (-)-isoprenaline sensitive fraction of the response was shifted to higher concentrations and became slightly smaller, but this effect did not reach the level of significance (fig. 2a). The β_2 -AR blocker ICI 118,551 (50 nM) strongly shifted the high sensitivity portion of the CRC by about 2 log units to the right (fig. 2b), so that the CRCs became monophasic. They remained similar in intact and denuded strips. The β_3 -AR blocker L748,337 (100 nM) was without any effect on the CRCs (fig. 2c). We then tested whether

the combination of β -AR blockers could produce any additional effects. The combination of CGP 20712A and L748,337 significantly shifted the CRC for the high sensitivity response to the right by almost one log unit. The relative size of this portion tended to be smaller, that is, 30 versus 36% of $R_{\max}$ (see table 1), but the difference did not reach the level of significance (fig. 2d). Any combination of the other 2 blockers with ICI 118,551 produced the same effect as the β_2 -AR blocker alone (fig. 2e, f; see online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000369075) for ICI 118,551 plus L748,337. All CRCs were similar in intact and denuded muscle strips.

The above findings suggest the prominent role of β_2 -ARs in mediating (-)-Isoprenaline-induced relaxation in CCh-pre-contracted mouse detrusor. This was further confirmed in detrusor strips from β_2 -AR-KO mice (fig. 3). In these preparations, the high sensitivity component of the CRC to (-)-isoprenaline was absent, and only a low

Fig. 2. Concentration-response curves for (-)-isoprenaline-induced relaxation of intact and denuded, CCh-pre-contracted detrusor strips with and without β -AR subtype selective blockers. Monophasic or biphasic sigmoidal functions were fitted to the mean data points. **a** CGP 20712A, 300 nM (β_1 -AR). **b** ICI 118,551, 50 nM (β_2 -AR). **c** L748,337, 100 nM (β_3 -AR). **d** CGP 20712A plus

L748,337. **e** CGP 20712A plus ICI 118,551. **f** CGP 20712A plus ICI 118,551 plus L748,337. For comparative purposes, all graphs contain the same control data set. Data are expressed in % of maximum relaxation induced by 10 μ M forskolin (100%). Mean $\pm$ SEM, number of mice/number of detrusor strips.

Fig. 3. Concentration-response curves for (-)-isoprenaline-induced relaxation of intact and denuded, CCh-pre-contracted detrusor strips from WT and β_2 -AR KO mice. Same layout as in figure 2.

sensitivity response of about 50% of the maximum forskolin relaxation was observed. Both denuded and intact CCh-stimulated muscles without β_2 -AR relaxed with similar sensitivity to (-)-isoprenaline.

Discussion

The objective of the present study was to characterize β -AR-mediated relaxation in the CCh-pre-contracted detrusor muscle strips of mice. Our major findings were that (i) the mucosa does not have any influence on the relaxation response under these conditions and (ii) (-)-isoprenaline-induced relaxation occurs in two concentration ranges with the response at low concentrations being mediated via β_2 -ARs.

We have shown previously, that the presence of the mucosa attenuates the contractile response to KCl supporting the existence of an urothelium-derived relaxing factor [3, 4]; however, others could not demonstrate this effect [5]. Our study also provided evidence that the mucosa may impair relaxation in response to (-)-isoprenaline. Here we confirm that the inhibitory role of murine urothelium is also observed in CCh-stimulated muscles, but impairment of (-)-isoprenaline-induced relaxation could not be detected. It is not clear, why β -AR-stimulated relaxation is not blunted by the mucosa in CCh-pre-contracted muscles (present study), although there is a clear effect in KCl-depolarized detrusor [Propping et al., 2014]. One reason could be that the small difference in sensitiv-

ity to β -AR agonists is difficult to detect, because both intact and denuded detrusor strips develop strong spontaneous contractile activity after stimulation with CCh (see fig. 1). These spontaneous phasic contractions of detrusor muscle of many species seem to be mainly mediated via M-receptors [15, 16]. Another explanation may be sought in the well-accepted antagonism between M-receptor and β -AR stimulation on cAMP production, in which M_2 receptors and β -ARs mediate reduction of adenylyl cyclase activity via inhibitory G_i -proteins and enhancement of enzyme activity via stimulating G_s -proteins, respectively [17]. Such antagonism could also occur in the murine mucosa: the difference in the relaxation response to (-)-isoprenaline between intact and denuded murine muscles may be absent when mucosal cAMP levels are kept low by block of β_2 -ARs [3] or by the activation of M receptors in the CCh-pre-contracted muscles (present study).

We and others reported previously that CCh-pre-contracted detrusor from many species relaxed less completely with (-)-isoprenaline than KCl-pre-contracted strips [18, 19]. In the present study, the relaxation response to (-)-isoprenaline after CCh stimulation was distinctly biphasic, with about equal portions of high and low sensitivity to (-)-isoprenaline (compare table 1). Others have found that (-)-isoprenaline is a weaker relaxant against CCh- than KCl-stimulated detrusor ($-\log EC_{50}$ [M] 7.24 vs. 5.32, respectively for rat detrusor) [18]; however, a biphasic response may have escaped detection because CRCs were stopped at $10 \mu\text{M}$ (-)-isoprenaline.

In any case, less complete relaxation responses for CCh- than for KCl-pre-contracted muscles in the low (-)-isoprenaline concentration range may be explained by stimulatory effects of M receptor activation on detrusor contractions. While activation of M_3 receptors may counteract β_2 -AR-mediated relaxation by directly stimulating detrusor contractions via enhanced intracellular Ca^{2+} release [17], activation of M_2 receptors may oppose relaxation by impairment of cAMP production [20, 21]. However, some authors question a dominant contribution of cAMP increase to (-)-isoprenaline-induced relaxation, at least in rat urinary bladder [19]. The nature of the relaxation response with low sensitivity to (-)-isoprenaline is not clear, but non-cAMP-mediated signals could be involved, including the activation of Ca^{2+} -dependent BK channels (Ca^{2+} -activated K channels of big conduction) [22]. Because of the very high (-)-isoprenaline concentrations required, the low sensitivity response may not be of primary physiological importance.

The receptors mediating (-)-isoprenaline-induced relaxation of CCh-pre-contracted mouse detrusor are of the β_2 -AR subtype and thus the same as for KCl-stimulated muscles [3, 13]. This interpretation is supported by the shift in CRC with the β_2 -AR subtype-selective antagonist ICI 118,551. The CRCs were not affected by tissue exposure to either β_1 -AR- or β_3 -AR subtype selective antagonists, suggesting lack of involvement of β_1 -ARs or β_3 -ARs in relaxation of mouse detrusor. Nevertheless, β_3 -AR mediation was claimed using the β_3 -AR-selective agonist CL 316,243, but the relaxant potency of the selective β_3 -AR-agonist is low in mouse [23]. We found a statistically significant shift in the CRCs with the combination of β_1 -AR and β_3 -AR antagonists (see fig. 2d), also suggesting minor involvement of other β -AR subtype. Since pharmacological identification of β -AR subtypes heavily depends on the properties of putatively selective antagonists, which is not necessarily always the case [24].

We have also used β_2 -AR-KO mice to confirm the dominant role of this β_2 -AR subtype in CCh-precontracted muscles.

Conclusions

In murine CCh-pre-contracted detrusor muscles, (-)-isoprenaline-induced relaxation in the low concentration range is mediated via β_2 -ARs. The presence of mucosa in murine detrusor did not impair relaxation in response to (-)-isoprenaline.

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