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ORIGINAL ARTICLE

Muscarinic receptor subtypes and signalling involved in the attenuation of isoprenaline-induced rat urinary bladder relaxation

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Abstract β-Adrenoceptors are important mediators of smooth muscle relaxation in the urinary bladder, but the concomitant presence of a muscarinic agonist, e.g., carbachol, can attenuate relaxation responses by reducing potency and/or efficacy of β-adrenoceptor agonists such as isoprenaline. Therefore, the present study was designed to explore the subtypes and signalling pathways of muscarinic receptors involved in the attenuation of isoprenaline-induced isolated rat detrusor preparations using novel subtype-selective receptor ligands. In radioligand binding studies, we characterized BZI to be a M_3-sparing muscarinic agonist, providing selective M_2 stimulation in rat bladder, and THRX-182087 as a highly M_2-selective antagonist. The use of BZI and of THRX-182087 in the presence of carbachol enabled experimental conditions with a selective stimulation of only M_2 or M_3 receptors, respectively. Confirming previous findings, carbachol attenuated isoprenaline-induced detrusor relaxation. M_2-selective stimulation partly mimicked this attenuation, indicating that both M_2 and M_3 receptors are involved. During M_3-selective stimulation, the attenuation of isoprenaline responses was reduced by the phospholipase C inhibitor U 73,122 but not by the protein kinase C inhibitor chelerythrine. We conclude that both M_2 and M_3 receptors contribute to attenuation of β-adrenoceptor-mediated relaxation of rat urinary bladder; the signal transduction pathway involved in the M_3 component of this attenuation differs from that mediating direct contractile effects of M_3 receptors.

Keywords M_2 muscarinic receptor · M_3 muscarinic receptor · THRX-182087 · β-Adrenoceptor · Relaxation · Urinary bladder

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Introduction

Muscarinic receptors are the main mediator of physiological contraction of the urinary bladder. While M_2 and M_3 subtypes exist in an about 4:1 ratio in the bladder of humans and many other mammals, direct contraction responses to exogenous agonists or endogenous agonist as released by field stimulation are mediated predominantly if not exclusively by the minor fraction of M_3 receptors (Hegde 2006). While it has been questioned on theoretical grounds that subtype-selective antagonists are sufficient to support this conclusion (Ehlert 2003), it should be noted that it is consistent with findings from M_2 and M_3 receptor knockout mice (Matsui et al. 2002). M_3 receptors, including those in the urinary bladder, couple to phospholipase C (PLC) stimulation, but the direct contractile effect via M_3 receptors occurs largely independent of PLC in the bladder (Frazier et al. 2008). On the other hand, bladder smooth muscle relaxation is mediated by β-adrenoceptors, in humans and many species mostly their β_3 subtype (Michel

and Vrydag 2006). While β-adrenoceptors, including those in the bladder, couple to stimulation of an adenylyl cyclase, bladder relaxation by β-adrenergic agonists occurs largely independent of adenylyl cyclase stimulation (Frazier et al. 2008). Thus, bladder smooth muscle tone is under a dual control of M_3 muscarinic receptors and β-adrenoceptors. This is similar to smooth muscle tone regulation in many other tissues, e.g., the airways, except relaxation responses in most cases outside the bladder are mediated by the β_2 subtype (Michel and Parra 2008).

The physiological control of bladder function involves activation of β-adrenoceptors by neuronally released noradrenaline during the storage phase of the micturition cycle, whereas neuronally released acetylcholine acting on M_3 receptors mediates detrusor contraction during the voiding phase (Andersson et al. 2009). However, under pathophysiological conditions nonneuronal acetylcholine release from the urothelium may also play a role and can be present not only during the voiding but also the storage phase (Andersson 2011). The concomitant exposure of the detrusor to noradrenaline and acetylcholine during the storage phase may have important implications for bladder smooth muscle function. Thus, it has been shown that the potency and efficacy of a muscarinic agonist to elicit detrusor contraction is attenuated in the presence of a β-adrenoceptor agonist (Yamanishi et al. 2002). Perhaps even more important, muscarinic agonists can also attenuate the relaxing effects of β-adrenoceptor stimulation in the bladder (Ehlert et al. 2007; Longhurst and Levendusky 1999; Michel and Sand 2009), airways (Matsui et al. 2003; Naline et al. 2007; Sarria et al. 2002), and ileum (Matsui et al. 2003). Such attenuation has been shown across multiple species, i.e., rat (Longhurst and Levendusky 1999; Michel and Sand 2009), mouse (Ehlert et al. 2007; Matsui et al. 2003), and humans (Naline et al. 2007; Sarria et al. 2002), indicating that it is a general principle in the regulation of smooth muscle tone upon concomitant exposure to muscarinic and β-adrenoceptor agonists. While all studies agree on the existence of attenuation of β-adrenoceptor-mediated relaxation by muscarinic agonists, reduced potency and/or efficacy have been reported to underlie such attenuation. Of note, the degree of attenuation of β-adrenoceptor-mediated relaxation by muscarinic agonists exceeds that by other contractile stimuli in all cases where this has been studied, e.g., that by KCl, bradykinin, or serotonin in the bladder (Michel and Sand 2009) or that of histamine in the airways (Naline et al. 2007; Roffel et al. 1995). At least in rat bladder, this differential degree of attenuation cannot be explained by a comparison between weak and strong contractile agonists, indicating that it may involve a specific property of muscarinic receptors rather than purely reflecting functional antagonism.

The interaction between muscarinic receptors and β-adrenoceptors may also shed light on the physiological role of the numerous M_2 receptors in bladder and airways. Thus, M_2 receptor knockout mice exhibited a mitigated attenuation of relaxation responses to isoprenaline (Ehlert et al. 2007; Matsui et al. 2003) and receptor-independent elevation of cellular cAMP content such by the adenylyl cyclase activator forskolin (Pak et al. 2010). As M_2 receptors largely signal via pertussis toxin-sensitive G proteins (Caulfield and Birdsall 1998), further support for a role of M_2 receptors in the attenuation response comes from studies in which pertussis toxin treatment enhanced the relaxant effects of forskolin on oxotremorine-M-mediated contractions in the ileum and trachea (Ostrom and Ehlert 1998, 1999). In a similar vein, it has been reported that under conditions of preferential alkylation of M_3 receptors, the ability of a muscarinic agonist to reverse isoprenaline-induced relaxation is largely maintained (Ehlert et al. 2005; Hegde et al. 1997). Facing a lack of highly subtype-selective muscarinic antagonists, such alkylation protocols have been employed by several investigators to achieve reasonable subtype selectivity. While this has been successful in some cases under very specific conditions (Griffin et al. 2009), other alkylation protocols have proven to be poorly selective (Braverman and Ruggieri 1999). Moreover, in some cases, M_3 responses have also been pertussis toxin sensitive (Offermanns et al. 1994), making pertussis toxin an unreliable witness of M_2 involvement. While the knockout mice circumvent selectivity problems, they are prone to other complications such as compensatory regulation of other receptors or signal transduction pathways.

Recently, an agonist with considerable selectivity for M_2 over M_3 receptors became available, but this compound exhibits a lower degree of selectivity, if any, over other subtypes; in the bladder, where muscarinic receptor subtypes other than M_2 and M_3 are largely absent, this compound produces selective M_2 stimulation (Jasper et al. 2002; Schneider et al. 2005; Yamakawa et al. 2001a). Lacking a better term, we refer to this compound as "M_3 sparing" in the present manuscript. Moreover, we have recently discovered an M_2 receptor antagonist with unprecedented selectivity for this subtype, which we report here for the first time (Fig. 1). These two tools have enabled us to explore the role of M_2 and M_3 receptors in the attenuation of isoprenaline-induced bladder relaxation. Moreover, we have used this approach to characterize the role of PLC and protein kinase C (PKC) activation in the attenuation response.

Methods

Radioligand binding experiments

Radioligand binding assays were conducted with 1 nM [^{3}H] *N*-methyl scopolamine (GE Healthcare, Piscataway, NJ,

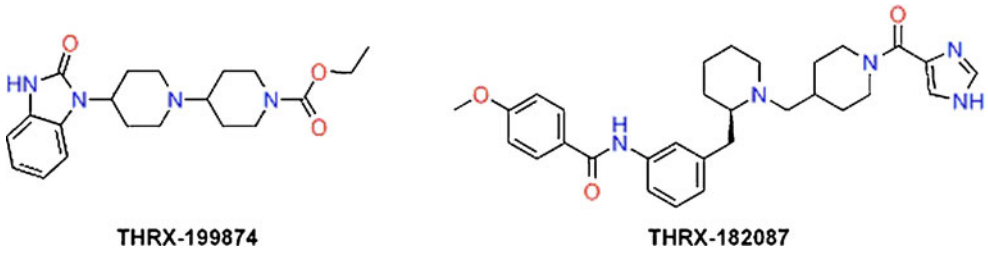

Fig. 1 Structures of THRX-199874 (BZI) and THRX-182087

USA) in a buffer consisting of 10 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, and 0.025% bovine serum albumin, pH 7.4 at 37°C. Nonspecific binding was defined in the presence of 10 μM atropine. Membrane fractions from CHO-K1 cells expressing human recombinant M_1, M_2, M_3, M_4, or M_5 muscarinic receptors were incubated with radioligand and unlabelled drugs for 1 h at 37°C in a volume of 100 μl. Receptor expression levels (B_{max}) measured by saturation binding were determined to be 2.7, 2.5, 2.4, 2.0, and 3.2 pmol/mg protein for human recombinant M_1, M_2, M_3, M_4, or M_5 muscarinic receptors, respectively. After separation by vacuum filtration onto GF/B filter plates presoaked with 0.3% polyethyleneimine, the quantity of membrane-bound radioligand was measured by scintillation counting.

Organ bath experiments

Tissue preparation Adult male Wistar rats weighing 300± 22 g were purchased from Charles River (Maastricht, The Netherlands). Animals were anesthetized using pentobarbital (75 mg/kg, i.p.) and sacrificed by decapitation. The bladders were harvested, and adipose and soft connective tissues were removed. After removal of the dome and the trigonum, the middle parts of the cleaned bladders (weight of 96±15 mg) were cut transversally in four equal strips. The strips had a length of 19.9±3.6 mm and a weight of 9.5± 2.7 mg (n=76). All experimental procedures were in line with European Union guidelines for the use of laboratory animals and approved by the Animal Care Committee of Academisch Medisch Centrum.

Relaxation experiments Experiments were performed as previously described (Frazier et al. 2011) with minor modifications. Briefly, the bladder strips were mounted under a resting tension of 10 mN in organ baths containing 7 ml of Krebs–Henseleit buffer of the following composition: 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 0.025 mM Na_4EDTA, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 5.6 mM glucose at a temperature of 37°C, yielding a total potassium concentration of 5.9 mM. The organ baths were continually gassed 95% O_2/5% CO_2 to maintain a pH of 7.4. The bladder strips were equilibrated for 120 min, during which the buffer solution was refreshed every 15 min until a steady contractile state had been reached. Following the equilibration, the tissues were challenged with 50 mM KCl for 6 min (maintaining isoosmolarity by reducing NaCl concentration from 116.8 to 68.5 mM). After the first challenge, we equilibrated the strips again at passive tension of 10 mN for 90 min and challenged the strips again with 50 mM KCl for 6 min. After 60 min equilibration at 10 mN after the second KCl challenge, we added vehicle or substance of interest. Strips with a KCl response of <20 mN were excluded. Carbachol was used at a concentration of 1 μM, BZI and THRX at 100 nM. The enzyme inhibitors U 73,122 (10 μM) and chelerythrine (1 μM) were added 5 min prior to the administration of THRX. Ten minutes later carbachol (1 μM) was added, which was given an incubation time of 15 min to reach steady contractile stadium. Thereafter, cumulative concentration–response curves were generated for the β-adrenoceptor agonist isoprenaline. As isoprenaline-induced rat bladder relaxation can exhibit desensitization (Vrydag and Michel 2009), only one isoprenaline concentration–response curve was constructed per bladder strip; however, conditions being compared were always tested in parallel using strips from the same animal, and those paired comparisons were the basis of our statistical analysis (see below).

Chemicals

The M_3-sparing agonist BZI [also known as THRX-199874, 4-(2-oxo-2,3-dihydro-benzimidazol-1-yl)-1,4′-bipiperidinyl-1′-carboxylic acid ethyl ester] was synthesized at Theravance as described (Yamakawa et al. 2001b). The M_2-selective antagonist THRX-182087 [*N*-(3-{(R)-1-[1-(1*H*-imidazole-4-carbonyl)-piperidin-4-ylmethyl]-piperidin-2-ylmethyl}-phenyl)-4-methoxy-benzamide] was synthesized in house as follows: Preparation of THRX-182087 was initiated from the condensation of 4-methoxyl benzoyl chloride and 3-bromo aniline. Metal halogen exchange with *n*-butyllithium in the presence of triethylchlorosilane and addition to (R)-2-formyl-1-Cbz-piperidine, followed by decarboxylation with palladium on carbon and potassium formate. Reductive alkylation with 4-formyl-1-Cbz-piperidine and Cbz removal with palladium on carbon, followed

Table 1 Affinity estimates of BZI and THRX-182087 at human muscarinic receptor subtypes as determined in competition radioligand binding studies

	BZI	THRX-182087
M_1	7.27±0.04	6.82±0.02
M_2	8.59±0.05	9.06±0.02
M_3	<5	6.61±0.02
M_4	8.01±0.05	7.34±0.02
M_5	6.11±0.05	5.46±0.02

Data are means±SEM of 19–27 experiments and shown as pK_I values

by acylation with 1*H*-imidazole-4-carbonyl chloride provided THRX-182087 (for detailed synthetic route see Stangeland et al., manuscript in preparation). U 73,122 [1-(6-[([17ß]-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino] hexyl)-1*H*-pyrrole-2,5-dione] was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and chelerythrine HCl from Calbiochem (via VWR, Amsterdam, The Netherlands).

Data analysis

Bound radioactivity in counts-per-minute data was normalized to percent specific binding and analyzed using a four-parameter logistic equation in Prism 3.0 (GraphPad Software, San Diego, CA, USA). Because Hill coefficients did not significantly differ from unity, IC_{50}s were determined with slopes fixed to 1. Inhibition binding constants (K_I) for test compounds were calculated from the IC_{50} values using the Cheng and Prusoff correction (Cheng and Prusoff 1973) and reported as the mean negative logarithm of the inhibition binding constants (pK_I) ± SEM.

Nonlinear regression was used to fit sigmoidal curves to the isoprenaline concentration–response curves to determine agonist potency (pEC_{50}) and maximum effects (E_{max}) using Prism 5.0. The force of contraction immediately prior to addition of the first isoprenaline concentration within a given experiment was defined as 0% relaxation, and a force of contraction of 0 mN was defined as 100% relaxation.

All data are expressed as mean±SEM of *n* experiments. Statistical significance of inhibitor effects on the E_{max} or pEC_{50} of isoprenaline was assessed by paired two-tailed *t* tests as compared to the indicated control condition, with treated and control conditions being measured in paired strips prepared from the same bladder. All statistical analyses were calculated using the Prism program, and a $p<0.05$ was considered statistically significant.

Results

The affinities of BZI and THRX-182087 for human recombinant M_1, M_2, M_3, M_4, and M_5 muscarinic receptors as determined in competition radioligand binding experiments are shown in Table 1, most notably demonstrating a >100-fold selectivity for M_2 over M_3 receptors for both compounds.

Five series of functional experiments were performed. In the first series, we reinvestigated the role of muscarinic receptor stimulation vs. receptor-independent bladder contraction, induced by KCl, for isoprenaline-induced relaxation. Carbachol and KCl caused a comparable starting tension (Table 2). The maximum relaxation by isoprenaline was significantly smaller in carbachol- than KCl-precontracted strips, whereas the potency of isoprenaline

Table 2 Starting tension of relaxation experiments in the absence and presence of muscarinic agonists and antagonists and/or signal transduction inhibitors

Series 1: KCl vs. mixed muscarinic stimulation		
KCl, 50 mM	2.86±0.22	*n*=8
Carbachol, 1 μM	2.46±0.12	*n*=8
Series 2: passive tension vs. M_2 stimulation		
Passive	0.95±0.15	*n*=6
BZI, 100 nM	1.08±0.10	*n*=6
Series 3: mixed vs. M_3 stimulation		
Carbachol, 1 μM	2.60±0.13	*n*=6
Carbachol, 1 μM + THRX-182087, 100 nM	2.14±0.22	*n*=6
Series 4: M_3 stimulation in absence and presence of phospholipase C inhibitor		
Carbachol, 1 μM + THRX-182087, 100 nM	2.90±0.21	*n*=6
Carbachol, 1 μM + THRX-182087, 100 nM + U 73,122, 10 μM	2.33±0.12*	*n*=6
Series 5: M_3 stimulation in absence and presence of protein kinase C inhibitor		
Carbachol, 1 μM + THRX-182087, 100 nM	2.89±0.51	*n*=6
Carbachol, 1 μM + THRX-182087, 100 nM + chelerythrine, 1 μM	2.90±0.22	*n*=6

Data are means±SEM of the indicated number of experiments and shown in millinewtons per milligram strip weight

*$p<0.05$ vs. test condition (KCl, carbachol, or passive tension) in a paired, two-tailed *t* test

did not differ significantly between the two conditions (Fig. 2).

The second series of experiments explored whether selective M_2 receptor stimulation mimics the effect of carbachol. Based upon previous data that BZI alone causes little bladder contraction (Schneider et al. 2005), we compared BZI with passive tension and confirmed the lack of effect of BZI on detrusor tone (Table 2). While BZI did not affect maximum isoprenaline-induced relaxation, it significantly reduced its potency (Fig. 3).

The third series addressed the reverse question, i.e., whether selective M_3 receptor stimulation (carbachol in presence of THRX-182087) mimics the carbachol effect. Both conditions caused comparable starting tension (Table 2).

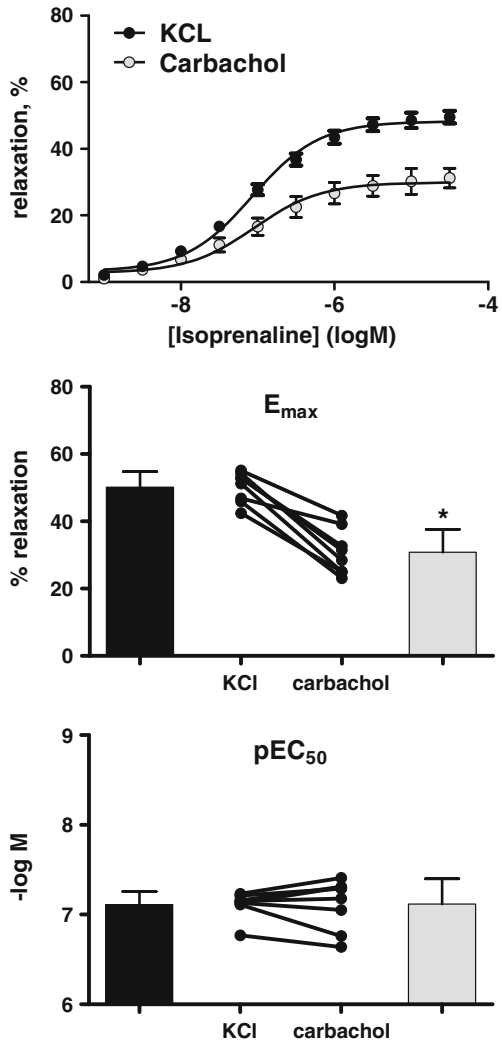

Fig. 2 Comparison of isoprenaline-induced relaxation against tension induced by 50 mM KCl and 1 μM carbachol. *Upper panel* data are means±SEM (n=6). *Middle and lower panel* bars showing E_{max} and pEC_{50} as derived from the curves in the *upper panel* indicate means±SEM, whereas *filled circles* represent individual experiments. *p<0.05 vs. KCl in a paired t test

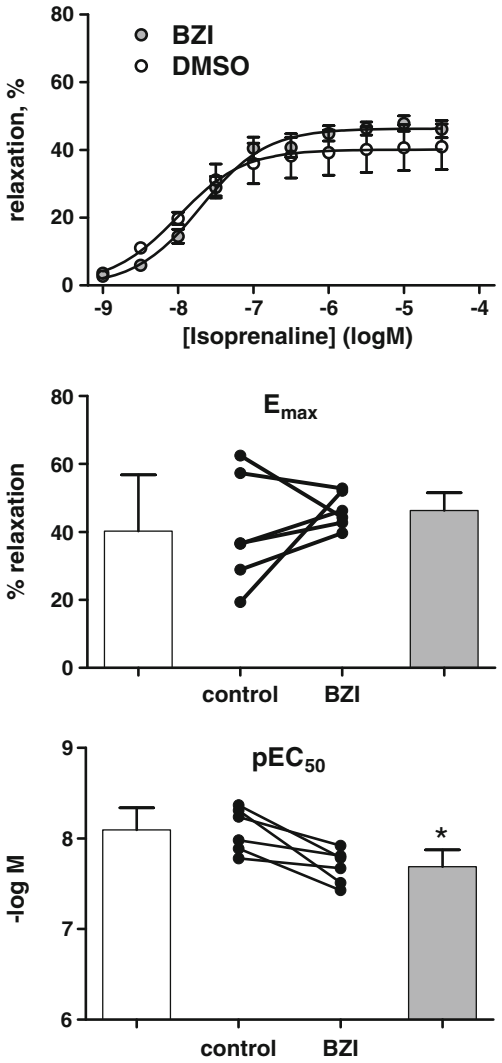

Fig. 3 Comparison of passive tension and M_2-selective muscarinic stimulation by 100 nM BZI on isoprenaline-induced relaxation. *Upper panel* data are means±SEM (n=6). *Middle and lower panel* bars showing E_{max} and pEC_{50} as derived from the curves in the *upper panel* indicate means±SEM, whereas *filled circles* represent individual experiments. *p<0.05 vs. passive tension in a paired t test

The potency and efficacy of isoprenaline were significantly greater upon M_3 selective as compared to general muscarinic receptor stimulation, confirming a contribution of M_2 receptors to the attenuation of the isoprenaline response (Fig. 4). However, the enhancement of isoprenaline responses by M_2 blockade (Fig. 4) was quantitatively less than the attenuation by carbachol (Fig. 2), indicating that both subtypes contribute to the attenuation of isoprenaline responses by carbachol.

The fourth and fifth series of experiments explored whether PLC and/or PKC contribute to the attenuation of isoprenaline responses by M_3-selective stimulation. In the presence of the PLC inhibitor U 73,122 starting tension was significantly smaller than with M_3-selective stimulation

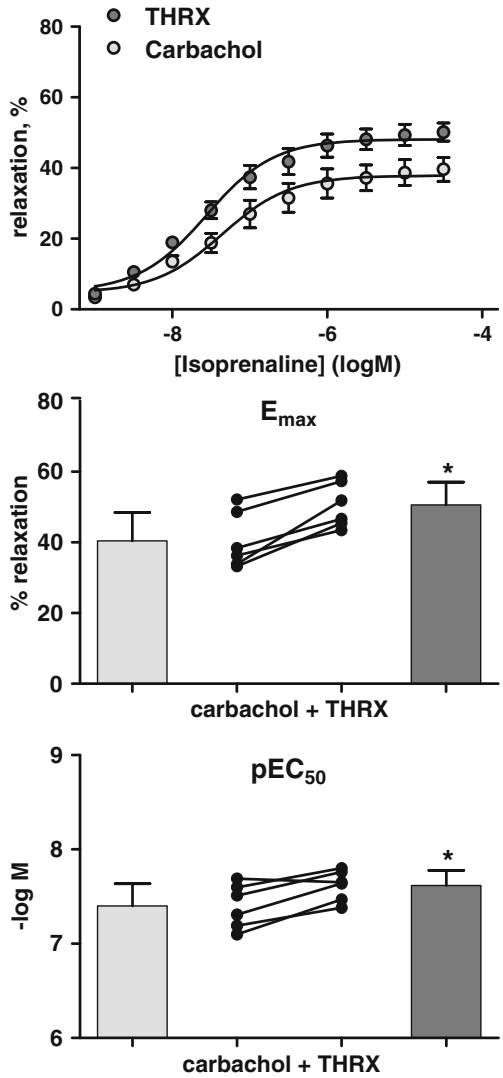

Fig. 4 Comparison of mixed (1 μM carbachol) vs. M_3-selective muscarinic stimulation (1 μM carbachol + 100 nM THRX-182087) on isoprenaline-induced relaxation. *Upper panel* data are means±SEM (n=6). *Middle and lower panel* bars showing E_{max} and pEC_{50} as derived from the curves in the *upper panel* indicate means±SEM, whereas *filled circles* represent individual experiments. *p<0.05 vs. carbachol alone in a paired t test

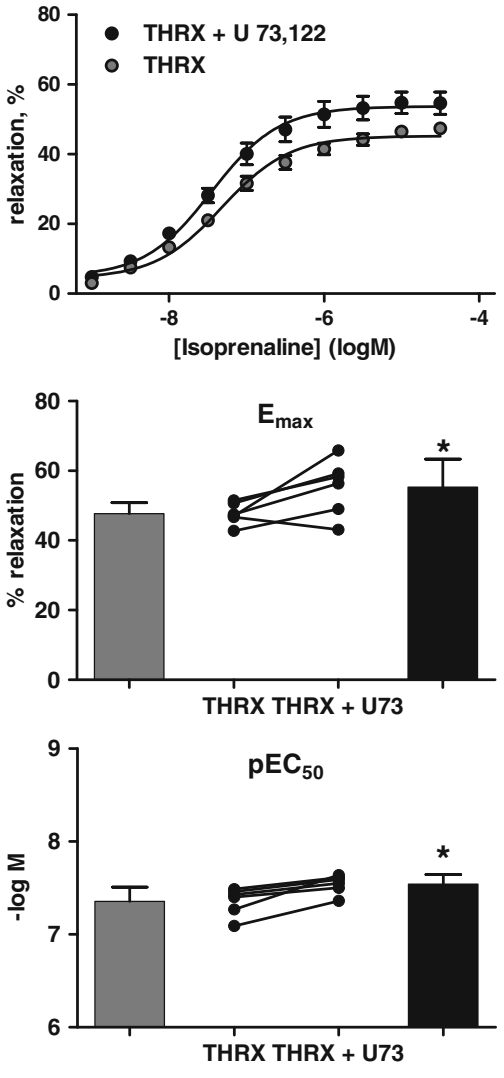

Fig. 5 Effect of the phospholipase C inhibitor U 73,122 (10 μM) on isoprenaline-induced relaxation during M_3-selective stimulation (1 μM carbachol + 100 nM THRX). *Upper panel* data are means±SEM (n=6). *Middle and lower panel* bars showing E_{max} and pEC_{50} as derived from the curves in the *upper panel* indicate means±SEM, whereas *filled circles* represent individual experiments. *p<0.05 vs. data in absence of signalling inhibitor in a paired t test

alone (Table 2). Potency and efficacy of isoprenaline were significantly greater in the presence of U 73,122 (Fig. 5). In contrast, the PKC inhibitor chelerythrine affected neither starting tension (Table 2) nor potency or efficacy of isoprenaline-induced relaxation (Fig. 6).

Discussion

Our study introduces a novel highly M_2-selective antagonist (THRX-182087) and uses it together with the M_3-sparing agonist BZI to explore the muscarinic receptor subtypes involved in the attenuation of isoprenaline effects in rat urinary bladder as well as the signalling pathways mediating such attenuation. According to our competition binding data, THRX-182087 is 281-fold selective for M_2 over M_3 receptors. Selectivity over other muscarinic receptors is 50–4,000-fold. This compares favorably to the limited M_2 selectivity of other compounds which have been used in this field such as methoctramine which is only 30-fold selective (Hegde et al. 1997). While the selectivity of THRX-182087 over M_4 receptors is less pronounced, these receptors are of little importance in the regulation of bladder smooth muscle tone (Hegde 2006). Hence, for practical purposes, THRX-182087 is a highly selective M_2 antagonist in our experimental setting. The THRX-182087

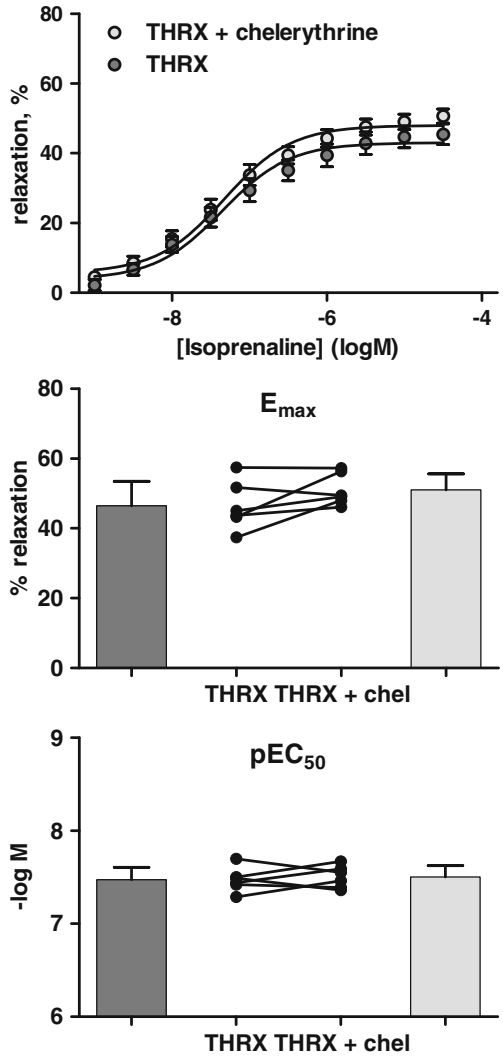

Fig. 6 Effect of the the protein kinase C inhibitor chelerythrine (1 μM) on isoprenaline-induced relaxation during M_3-selective stimulation (1 μM carbachol + 100 nM THRX-182087). *Upper panel* data are means±SEM (n=6). *Middle and lower panel* bars showing E_{max} and pEC_{50} as derived from the curves in the *upper panel* indicate means±SEM, whereas *filled circles* represent individual experiments. Data in the absence and presence of the signalling inhibitor were not significantly different in a paired t test (p>0.05)

concentration of 100 nM used in our studies produces an almost complete occupancy of M_2 receptors, and accordingly the combination of carbachol with THRX-182087 provides selective M_3 agonism.

BZI has been introduced as an M_3-sparing agonist (Jasper et al. 2002; Yamakawa et al. 2001a), and our binding data show a more than 3,000-fold selectivity for M_2 over M_3 receptors. Selectivity over other muscarinic receptors is 3–390-fold. Accordingly, BZI provides selective stimulation of M_2 receptors in bladder smooth muscle, where only M_2 and M_3 receptors are functionally relevant (Hegde 2006). The combined use of THRX-182087 and BZI has enabled us to explore the relative roles of M_2 and M_3 receptors in the attenuation of isoprenaline-induced relaxation.

Our data on starting tension demonstrate that inhibition of the M_2 receptors did not attenuate carbachol responses, whereas selective activation of M_2 receptors did not induce contraction as also observed in previous studies (Schneider et al. 2005). These data confirm a large body of evidence that despite the much larger presence of M_2 receptors in the urinary bladder, direct contractile responses are mediated predominantly if not exclusively by the M_3 receptor (Hegde 2006).

Studies in multiple tissues and species had demonstrated that muscarinic receptors can attenuate relaxation responses to the β-adrenoceptor agonist isoprenaline (see "Introduction" section), and this is confirmed in the present data. While such attenuation was found very consistently, those previous studies had been inconsistent with regard to the question whether such attenuation affects the potency and/or efficacy of isoprenaline, and our data also are not fully consistent in this regard.

Studies based on knockout mice had indicated that the M_2 subtype, which contributes little to direct detrusor contraction, plays a role in the attenuation of the relaxation response (Ehlert et al. 2005; Matsui et al. 2003; Pak et al. 2010). Using the complementary approach of selective pharmacological stimulation by BZI, we confirm a role of M_2 receptors in the attenuation of relaxation. Our finding that selective inhibition of M_2 receptors by THRX-182087 in the presence of carbachol enhances relaxation by isoprenaline further corroborates this idea. However, it should be noted that neither the effect of BZI nor that of THRX-182087 can fully explain the attenuation obtained by mixed muscarinic stimulation using carbachol alone, suggesting that the attenuation response may also contain an M_3 component.

Stimulation of the PLC/PKC pathway is a prototypical signalling response of M_3 receptors (Caulfield and Birdsall 1998; Ehlert et al. 1997) which also was detected in the bladder as being mediated predominantly if not exclusively via M_3 receptors (Kories et al. 2003; Nelson et al. 2004). Nevertheless, M_3 receptor-mediated bladder contraction has been shown to be insensitive to inhibition of PLC or PKC in rats, mice, and humans (Frazier et al. 2008). In the present study, we have used the PLC inhibitor U 73,122 in a concentration where it fully suppresses inositol phosphate formation in the bladder but does not affect rat or human bladder contraction (Schneider et al. 2004a, b). Interestingly, U 73,122 significantly enhanced isoprenaline-induced relaxation in the presence of M_3-selective stimulation. Thus, PLC may be involved in the M_3 component of attenuation of relaxation but not in direct bladder contraction mediated by the same receptor subtype. According to our data, PKC is

not involved in either response, indicating that the involvement of PLC in the attenuation of relaxation occurs via a PKC-independent pathway.

In conclusion, we have introduced a novel antagonist with very high selectivity for M_2 over M_3 receptors, THRX-182087. Using this compound as well as the M_3-sparing agonist BZI, we confirm a role for M_2 receptors in the attenuation of isoprenaline-induced bladder relaxation, which previously was mainly supported by genetic evidence, by a pharmacological approach. Our data also suggest involvement of M_3 receptors in this attenuation. Thus, muscarinic receptors cause direct contraction and inhibition of relaxation in the bladder, but the two responses involve different subtypes and, at least for M_3 receptors, different signalling pathways. This interaction may become clinically relevant under pathophysiological conditions when acetylcholine is being released in the bladder during the storage phase of the micturition cycle.

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