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ET_{B2} Receptor Subtype Stimulation Relaxes the Iris Sphincter Muscle

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Summary

Effects of ET_B receptor stimulation and its subcellular pathways were evaluated in carbachol pre-contracted rabbit iris sphincter muscles (n=51). ET_B stimulation with sarafotoxin (SRTX-c; 10^{-10}-10^{-6} M) was tested in the absence (n=7) or presence of 10^{-5} M of: BQ-788 (ET_{B2} receptor antagonist; n=6), L-NA (NOS inhibitor; n=7) or indomethacin (cyclooxygenase inhibitor; n=10). Effects of ET_B stimulation by endothelin-1 (ET-1; 10^{-10}–10^{-7} M) in the presence of an ET_A receptor antagonist (BQ-123; 10^{-5} M; n=7) and of ET_{B1} stimulation by IRL-1620 (10^{-10}–10^{-7} M; n=7) were also tested. Finally, the effects of SRTX-c (10^{-9}–10^{-7} M) in electric field stimulation (EFS) contraction were evaluated (n=7). ET_B receptor stimulation by SRTX-c or ET-1 in presence of BQ-123 promoted a concentration-dependent relaxation of the rabbit iris sphincter muscle by 10.8±2.0 % and 9.4±1.8 %, respectively. This effect was blocked by BQ-788 (-2.3±2.0 %), L-NA (4.5±2.3 %) or indomethacin (2.3±2.9 %). Selective ET_{B1} stimulation by IRL-1620 did not relax the iris sphincter muscle (0.9±5.4 %). EFS elicited contraction was not altered by SRTX-c. In conclusion, ET_B receptor stimulation relaxes the carbachol precontracted iris sphincter muscle, an effect that is mediated by the ET_{B2} receptor subtype, through NO and the release of prostaglandins.

Key words

Peptide hormones • Iris • Muscle fibers • ET_{B2} • Sarafotoxin-s6c

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Introduction

Endothelin-1 (ET-1) is an endogenous vasoactive peptide with 21 amino acids, secreted by vascular endothelial cells (Yanagisawa *et al.* 1988, 1989). ET-1 is a member of related peptide family, which includes endothelin-2 (ET-2), endothelin-3 (ET-3), sarafotoxin-s6c (SRTX-c) and vasoactive intestinal contractors. This peptide potently promotes the contraction of both vascular and non-vascular smooth muscles (Eglen *et al.* 1989).

In humans, these three isoforms of ET mediate their biological actions *via* two different receptors, ET_A and ET_B (Inoue *et al.* 1989, Arai *et al.* 1990, Sakurai *et al.* 1990). The ET_A receptor, located on vascular smooth muscle, mediates a potent vasoconstrictor action, promotes miosis and mitogenesis, and binds preferably to ET-1 (Masaki 1991), while the ET_B receptor binds equipotently the three isoforms and mediates vasodilation and ocular hypotension (Haque *et al.* 1995), probably through stimulation of nitric oxide and prostaglandins release (Inoue *et al.* 1989, Masaki 1991). This receptor has two isotypes: ET_{B1} or endothelial, and ET_{B2} or muscular (Sudjarwo *et al.* 1994, Nishiyama *et al.* 1995). Another receptor, the ET-C receptor, cloned from *Xenopus melanophores* has greater affinity for ET-3 than for ET-1 or ET-2 (Karne *et al.* 1993, Yorio *et al.* 2002).

Endothelin-1 is widely distributed in mammalian ocular tissues including cornea, ciliary body epithelium and retina (Ripodas *et al.* 2001, Yorio *et al.* 2002). ET-1 mRNA was identified by *in situ* hybridization in human ciliary body, ciliary muscle, iris sphincter muscle, stroma and iris vessels. ET-1 was detected in aqueous humor, endothelial and non-pigmented ciliary epithelium

(Fernandez-Durango *et al.* 2003). In terms of ET receptors expression in the human iris, almost two thirds of them are of the ET_B subtype (Fernandez-Durango *et al.* 2003). The iris is the main tissue expressing ET receptors, followed by the ciliary muscle and ciliary processes (Fernandez-Durango *et al.* 2003). Immunoreactivity studies detected ET-3 in the retina (De Juan *et al.* 1995). In the pig and cat iris, ET produces a concentration-dependent contraction mediated by the ET_A receptor subtype (Geppetti *et al.* 1989). When injected in the posterior compartment of the eye, ET promotes iNOS stimulation, optic nerve ischemia and lowering of axonal transport, with destruction of optic nerve and increase of intraocular pressure (Yorio *et al.* 2002). ET administration in the eye's anterior segment reduces intraocular pressure, independently of prostaglandin´s production (Haque *et al.* 1995). The production of aqueous humor is also affected by ET-1, which inhibits the Na^+/K^+ ATPase (Prasanna *et al.* 2001).

The aim of this work was to determine the role of ET_B receptor stimulation in the modulation of iris sphincter muscle contraction, its subcellular pathways and the ET_B receptor subtype involved.

Methods

Specimens preparation

The study was performed in isolated iris sphincter (n=51) muscles from male New Zealand white rabbits (*Oryctolagus cuniculus*; 2.0-3.0 kg). All animal procedures were performed in accordance with the ARVO statement for the *Use of Animals in Ophthalmic and Vision Research.* Animals were euthanized after an injection of pentobarbital sodium salt (50 mg/kg) into the marginal ear vein. The eyes were immediately enucleated and placed in modified Krebs-Ringer (KR) solution at 35 ºC, with the following composition in mM: NaCl 98; KCl 4.7; $MgSO_4$ 2.4; KH_2PO_4 1.2; glucose 4.5; $CaCl_2$ 2.5; $NaHCO_3$ 17; $C_3H_3NaO_3$ 15 and CH_3COONa 5. After removal of the cornea, the iris sphincter muscle was quickly excised and immersed in the KR solution. After dissection, the ends of each piece were tied with silk thread for mounting in a 15 ml plexi glass organ bath containing the above-described solutions. One end of the specimen was connected to an electromagnetic length-tension transducer (University of Antwerp, Belgium), and the other was secured to a clip at the wall of the organ bath. All the surgical procedures were performed under microscope (Zeiss, Stemi 2000C, Germany). Solutions were bubbled with 95 % O_2 and 5 % CO_2 and pH was maintained between 7.38-7.42.

Iris sphincter muscles were always stabilized at the same preload (1.0 mN) and bath solutions were continuously replaced until muscle length stabilization. They were then switched to isometric conditions and the protocols initiated when muscle tension was stabilized.

Effect of ET_B stimulation on the pre-contracted iris sphincter muscle

After stabilization, the rabbit iris sphincter muscles were contracted by adding carbachol (10^{-6} M) to the organ bath. The effects of ET_B receptor stimulation on the pre-contracted iris sphincter muscle were studied by evaluating its response to: i) the ET_B agonist SRTX-c (10^{-10}–10^{-6} M; n=7), ii) endothelin-1 (ET-1;10^{-10}–10^{-7} M; n=7) in the presence of an ET_A receptor antagonist (BQ-123; 10^{-5} M) iii) the selective ET_{B1} agonist IRL-1620 (10^{-10}–10^{-7} M; n=7). Furthermore, the response to SRTX-c (10^{-10}–10^{-6} M) was also assessed in the presence of: i) ET_{B2} receptor antagonist, BQ-788 (10^{-5} M; n=6); ii) a NO synthase inhibitor, L-nitro-L-arginine (L-NA; 10^{-5} M; n=7); iii) a cyclooxygenase inhibitor, indomethacin (indo; 10^{-5} M; n=10). In each muscle, two carbachol-induced contractions were studied. One of these contractions was randomly selected to test the effects of the studied drugs, while the other one was used as control, having been studied in the presence of the vehicle solution alone. In each protocol, each concentration was added to the bath solution only after recording the maximal effect of the previous one.

Effect of ET_B stimulation on the EFS-elicited contraction

After stabilization, rabbit iris sphincter muscles (n=7) were contracted by placing them in an electric field stimulation of 10 V, 5 Hz and 1 ms duration. Developed tension was recorded in five consecutive contractions (3 min apart). After completing the acquisition in baseline conditions, SRTX-c (10^{-9} M) was added to the organ bath and a new electric field stimulation was applied 15 min later. The drug was then washed out and a new control contraction obtained. After that, the second concentration of SRTX-c (10^{-8} M) was added to the bath and a new electric field stimulation was performed 15 min later. The procedure was then repeated to test the concentration of 10^{-7} M. Finally, the drug was washed out again and another control contraction was obtained to confirm the preservation of muscle performance.

Materials

All chemicals were obtained from Sigma Chemical Co (St. Louis, MO, USA). Peptides were prepared in aliquots and stored at –20 ºC.

Statistical analysis

Data presented as means ± S.E.M. EFS-elicited contractions, in the presence and absence of SRTX-c, were compared by a paired Student's t-test. Concentration-response curves of SRTX-c in carbachol precontracted muscles in each experimental condition were evaluated with one-way repeated measures ANOVA. Effects of each dose of drug in different experimental conditions were tested by one-way ANOVA. When significant differences were detected by any of the ANOVA test, the Student-Newman-Keuls test was selected to perform multiple comparisons. $P<0.05$ value was accepted as significant.

Results

Effect of ET_B stimulation on the carbachol-elicited contraction of the iris sphincter muscle

Active tension of the iris sphincter muscle preparations elicited by the addition of carbachol (10^{-6} M) to the bath was quite stable, not significantly different between the different protocols and similar in control and test contractions, averaging 2.99±0.10 mN.

Addition of SRTX-c to the precontracted iris sphincter muscle promoted 19.1±2.6 % decrease of the active tension, while the control contraction decreased only by 8.3±2.0 % over the same period of time ($p<0.05$) (Fig. 1; upper panel). ET_B receptor stimulation by ET-1 in presence of BQ-123 decreased active tension by 16.4±1.8 %, while the control contraction decreased only by 9.02±2.8 % over the same period of time ($p<0.05$) (Fig. 1; lower panel). On the contrary, selective ET_{B1} receptor stimulation either by IRL-1620 or by SRTX-c in presence of BQ-788 (Fig. 2) did not promote a decline in active tension significantly different from the control contraction in the presence of the vehicle alone.

To test the influence of prostaglandins and NO on ET_B-induced relaxation of carbachol-induced contraction of the iris sphincter muscle, increasing concentrations of SRTX-c were tested in the presence of indomethacin or L-nitro-L-arginine (L-NA). While indomethacin completely inhibited the relaxing effect of SRTX-c (Fig. 3; upper panel), L-NA attenuated such effect only at concentrations of SRTX-c above 10^{-7} M.

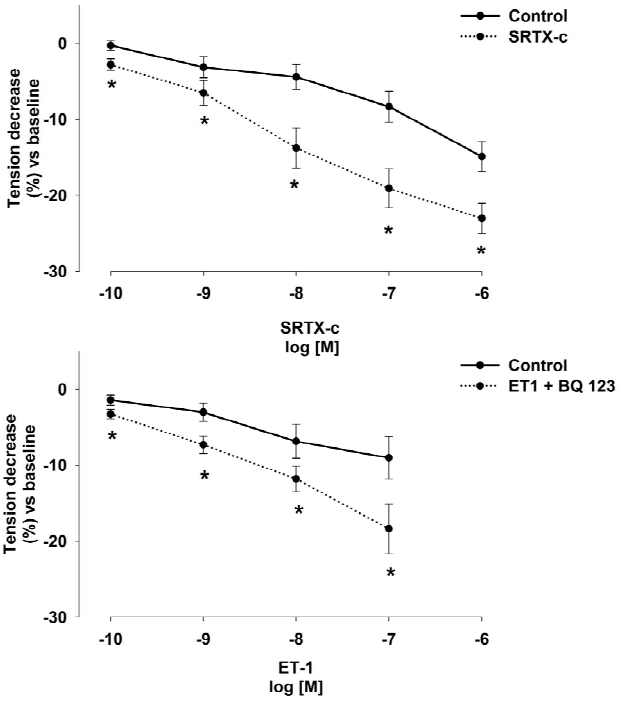

Fig. 1. Concentration-response curves of SRTX-c (10^{-10}–10^{-6} M; upper panel) and ET-1 (10^{-10}–10^{-7} M) in the presence of BQ-123 (10^{-5} M; lower panel) elicited tension decrease in carbachol-precontracted iris sphincter muscles. Control lines refer to recordings in presence of vehicle alone. $p<0.05$: * vs. control.

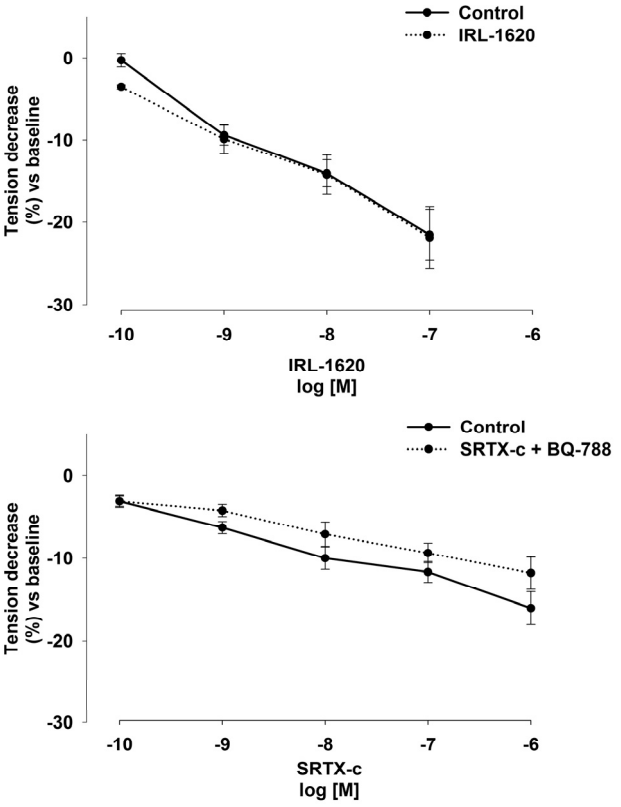

Fig. 2. Concentration-response curves of SRTX-c (10^{-9}–10^{-6} M) in presence of BQ-788 (10^{-5} M; upper panel) and IRL-1620 (10^{-10}–10^{-7} M; lower panel) elicited tension decrease in carbachol-precontracted iris sphincter muscles. Control lines refer to recordings in presence of vehicle alone. $p<0.05$: * vs. control.

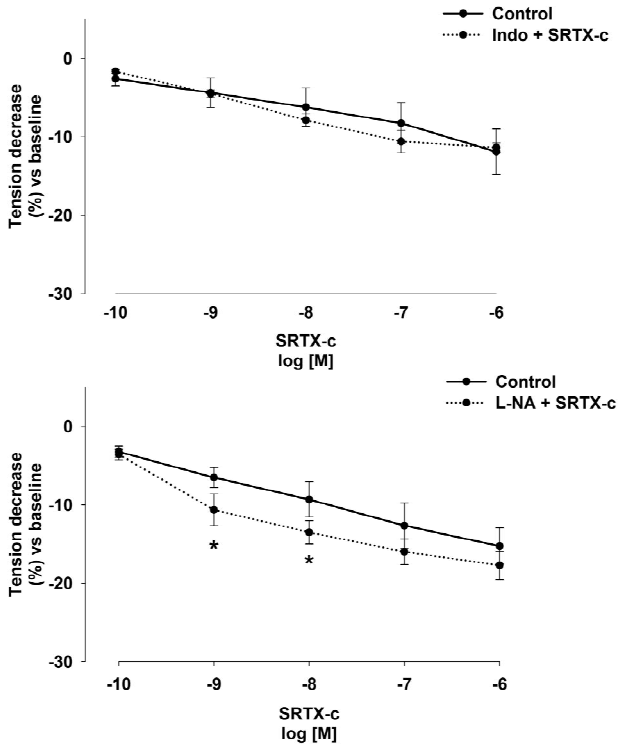

Fig. 3. Concentration-response curves of SRTX-c (10^{-9}–10^{-6} M) in presence of indomethacin (upper panel; 10^{-5} M) or L-nitro-L-arginine (lower panel; 10^{-5} M) elicited tension decrease in carbachol-precontracted iris sphincter muscles. Control lines refer to recordings in presence of vehicle alone. $p<0.05$: * vs. control.

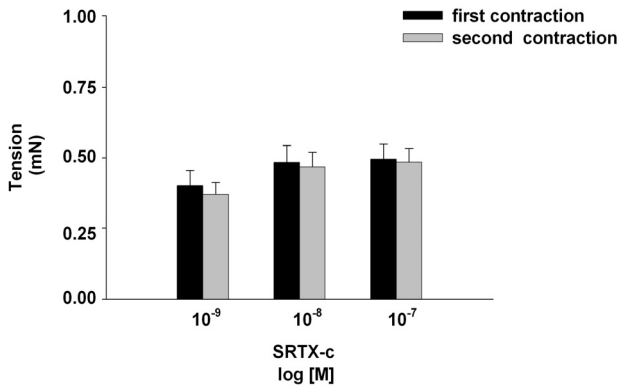

Fig. 4. Tension development in response to two consecutive groups of contractions elicited by EFS in the absence or presence of SRTX-c (10^{-9}–10^{-7} M).

Effect of ET_B stimulation on the EFS-elicited contraction

Active tension of the iris sphincter muscle preparations elicited by the EFS was quite stable not significantly differing between the different protocols. The active tension was similar in control and test contractions, averaging 0.45±0.02 mN. The presence of SRTX-c in the bath (10^{-9}–10^{-7} M) did not promote any change in muscle tension, elicited by the electric field stimulation (Fig. 4).

Table 1. Effects of ET_B stimulation and its subcellular pathway in the iris sphincter muscle.

	Δ Tension (% vs. control)	**p<0.05**
SRTX-c	–10.8 ± 2.03 %	vs. control
ET1+BQ123	–9.35 ± 1.79 %	vs. control
IRL-1620	–0.91 ± 5.45 %	vs. SRTX-c; ET-1
SRTX-c + BQ-788	2.30 ± 2.04 %	vs. SRTX-c; ET-1
SRTX-c + LNA	–4.46 ± 2.28 %	vs. SRTX-c
SRTX-c + Indo	–2.55 ± 3.00 %	vs. SRTX-c

Data are means ± S.E.M.

Discussion

The present study described the relaxation of the precontracted iris sphincter muscle, which was promoted by ET_B receptor stimulation. Interestingly, this effect is mediated by the ET_{B2} receptor subtype through prostaglandins and NO release.

In the rat, ET_A receptor stimulation contracts the iris sphincter muscle and potentiates its electric field-elicited contraction (Shinkai *et al.* 1994). In the same experimental preparation, Shinkai-Goromaru *et al.* (1997) reported a 140 % increase of electric field stimulation-developed tension, in response to ET_B receptor stimulation by SRTX-c. Under these conditions, ET_B receptor stimulation increased acetylcholine release in the prejunctional site of the cholinergic synapses. This finding is quite relevant as ET-3, which has a similar affinity for ET_A and ET_B receptors, is more abundant in the iris than ET-1 (Shinkai-Goromaru *et al.* 1997, Fernandez-Durango *et al.* 2003). On the contrary, in our study, ET_B receptor stimulation promoted relaxation of the carbachol-precontracted iris sphincter muscle. This was observed in response to either SRTX-c or ET-1 in presence of BQ-123, an ET_A receptor blocker. This observation suggests that the effects of ET_B stimulation are distinct in carbachol- and EFS-elicited contractions. However, in our study the latter was not affected by SRTX-c (10^{-9}–10^{-7}M), suggesting that, contrary to the rat, in the rabbit ET_B receptor stimulation does not increase acetylcholine release.

The presence of two functionally distinct ET_B receptors (ET_{B1} and ET_{B2}) in the muscle and endothelium was initially described in the swine pulmonary vein (Sudjarwo *et al.* 1993). Later, additional evidence on the existence of two ET_B receptor subtypes was reported in the rabbit venous saphenous muscle (Nishiyama *et al.* 1995),

in the rabbit tracheal smooth muscle (Yoneyama *et al.* 1995), in the rabbit basilar artery (Zuccarello *et al.* 1999), in the guinea pig ileum (Miasiro *et al.* 1999) and in the rabbit heart (Leite-Moreira and Bras-Silva 2004). The ET_{B2} receptor subtype promotes contraction of the swine pulmonary vein (Sudjarwo *et al.* 1993), the rabbit saphenous vein (Nishiyama *et al.* 1995), the rabbit basilar artery (Zuccarello *et al.* 1999) and in the rabbit tracheal smooth muscle (Yoneyama *et al.* 1995). It has a biphasic effect, i.e. relaxation followed by contraction, in the guinea pig ileum (Miasiro *et al.* 1998, 1999), and increases myocardial inotropy in the rabbit heart (Leite-Moreira and Bras-Silva 2004). SRTX-c acts preferentially on the ET_{B2} receptor subtype, while IRL-1620 selectively stimulates the ET_{B1} subtype (Karaki *et al.* 1994a,b, Sudjarwo *et al.* 1993, 1994, Yoneyama *et al.* 1995). In our study, we observed that SRTX-c, but not IRL-1620, relaxed the carbachol-precontracted muscle, an effect that was blocked by BQ-788. These findings suggest that the receptor subtype involved in ET_B-induced iris sphincter relaxation is the ET_{B2}. The effect of SRTX-c on ET_{B2}-induced rabbit vein contraction was also previously shown to be inhibited by BQ-788 (Karaki *et al.* 1994a). Interestingly, however, the iris sphincter was the first muscle where a relaxing instead of a contracting effect was described in response to ET_{B2} receptor stimulation.

The relaxing effect of ET_{B2} stimulation was dependent of prostaglandins and NO. These agents also mediate the negative inotropic (Leite-Moreira and Bras-Silva 2004) and the venous vasodilatory (De Nucci *et al.* 1988, Filep *et al.* 1991, Hirata *et al.* 1993) effects induced by ET_{B1} receptor stimulation. Prostaglandins and their receptors are widely distributed in the ocular tissues, including the iris sphincter muscle. The EP_2 receptor is the most abundant in rabbit (Csukas *et al.* 1992, Bhattacherjee *et al.* 1993), mouse and human iris (Biswas *et al.* 2004). In human eyes, FP, DP and EP receptors were localized in the ciliary body and iris, being particularly involved in the intraocular pressure regulation (Davis and Sharif 1999, Sharif *et al.* 2000, 2004). FP-receptors agonists promoted phospho-inositide (PI) hydrolysis, mitogen-activated protein kinase (MAPK) activation and myosin light chain phosphorylation causing Ca^{2+} mobilization and iris contraction (Ansari *et al.* 2004, Sharif *et al.* 2008). On the other side, EP_2, EP_4 and DP receptor activation produces intracellular cAMP accumulation and muscle relaxation (Abdel-Latif 2001). In our preparation, prostaglandins released by ET_{B2} stimulation partially reversed the carbachol-induced contraction. Further investigations are needed to indentify the subcellular pathway involved in this effect.

The blockade of endogenous NO production also inhibited SRTX-c induced relaxation. However, in contrast to prostaglandins that mediated relaxation over the entire concentration-response curve, NO dependence was only evident for the concentrations of SRTX-c higher than 10^{-7} M. In the bovine iris muscle, a non-adrenergic, non-cholinergic system (Pianka *et al.* 2000) was shown to promote a NO-dependent relaxation. NO release can occur directly from nitrergic neurons (Wiencke *et al.* 1994) or in response to substances such as adrenomedullin that promote its synthesis (Uchikawa *et al.* 2005).

This relaxing effect adds to other ET_B receptor-mediated effects in ocular tissues, such as the relaxation of the bovine ciliary muscle (Kamikawatoko *et al.* 1995) and the ocular hypotensive effect (Haque *et al.* 1995). In conjunction with ET-3 levels in the iris and ciliary tissues and with ET-1 levels in exfoliation syndrome patients (Koliakos *et al.* 2004) our results highlight the importance of endothelin (and ET_B pathway) as a regulator of the iris muscles.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

SRTX-c, sarafotoxin s6c; ET, endothelin; ET_A, endothelin receptor type A; ET_B, endothelin receptor type B; COX, cyclooxygenase; NO, nitric oxide

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