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SUBTYPES OF ENDOTHELIN ETA AND ETB RECEPTORS MEDIATING VENOUS SMOOTH MUSCLE CONTRACTION

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Summary: In rabbit saphenous vein, endothelin (ET)-1 and ET-3 induced sustained contractions whereas the selective agonists of the ET_B receptor, sarafotoxin S6c (STXc) and IRL 1620, induced transient contractions. In the presence of an ET_A antagonist, BQ-123, contractions induced by ET-1, STXc and IRL 1620 did not change whereas ET-3 induced only transient contraction. The ET_B antagonists, RES-701-1 and IRL 1038, only weakly antagonized the effects of these stimulants. In the muscle pretreated with STXc, neither STXc nor IRL 1620 was effective whereas ET-3 induced sustained contraction at higher concentrations than ET-1. In the muscle pretreated with STXc, BQ-123 weakly antagonized the effect of ET-1 and abolished the effect of ET-3. These results suggest that there are two types of ET receptors; less tachyphylactic and isopeptide-selective ET_A receptor, and tachyphylactic and isopeptide-nonselective ET_B receptor. The ET_A receptors may be further classified as a BQ-123-sensitive ET_{A1} and a BQ-123-insensitive ET_{A2} subtypes. The ET_B receptors may also be subclassified as the ET_{B1} and ET_{B2} subtypes based on the sensitivity to the ET_B antagonists.

Endothelins (ETs) bind to two types of receptor; ET_A and ET_B (1). In arteries, the contractile effects of ETs are mediated by the ET_A receptor which is more effectively activated by ET-1 than ET-3 (1) and is inhibited by BQ-123 (2) or FR139317 (3). In vascular endothelium, ET-1 and ET-3 activate the ET_B receptor with equal efficacy (2) to release nitric oxide and this receptor is inhibited by IRL 1038 (4) or RES-701-1 (5). In the veins, ET-1, ET-3 and the ET_B-selective agonists, IRL 1620 (6,7) and sarafotoxin S6c (STXc), induced contractions at similar concentrations and BQ-123 did not inhibit these contractions, suggesting the involvement of the ET_B receptor (8,9). However, IRL 1038 did not inhibit these contractions (8). In the ileum, ETs induced relaxation by activating the ET_B receptor which is not sensitive to IRL 1038 (10).

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Another ET_B antagonist, RES-701-1, showed similar effects to IRL 1038 (5). From these results, it was proposed that there are two subtypes of ET_B receptors; the ET_{B1} receptor which is sensitive to conventional ET_B antagonists and the ET_{B2} receptor which is not sensitive to these antagonists (5,7,8,10). In the present experiments, we further characterized the ET receptor subtypes in the smooth muscle of rabbit saphenous vein.

Materials and Methods

Male, New Zealand white rabbits were killed under anaesthesia and the saphenous vein was removed and cut into rings of 2-3 mm width. The endothelium was removed by gently rubbing the intimal surface with a glass rod moistened with physiological salt solution which contained (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, ethylenediamine tetraacetic acid (EDTA) 0.01, and glucose 5.5. High K^+ solution was made by substituting 72.7 mM NaCl with equimolar KCl. These solutions were saturated with 95% O_2 and 5% CO_2 mixture at 37°C to maintain the pH at 7.4.

The force of contraction was recorded isometrically. Muscle preparations were attached to a holder under a resting force of 10 mN and equilibrated for 60-90 min. During this period, high K^+ was repeatedly applied until the peak force was reproducible. The concentrations of stimulants to induce a half maximum contraction (EC_{50}) was calculated from the cumulative concentration-response curves. Results are expressed as mean ± S.E.M. Student's *t* test was used for the statistical analysis of the results. $P<0.05$ was considered to be significant.

ET-1, ET-3 and STXc were purchased from the Peptide Institute (Osaka, Japan). BQ-123 (2), IRL 1038 (8,10) and IRL 1620 (6,7) were synthesized in our laboratories. RES-701-1 (cyclic [Gly^1-Asp^9] [Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp]) (5,11) was isolated from *Streptomyces sp.*

Results and Discussion

Fig. 1 shows the effects of cumulative addition of ET-1, ET-3 and the selective ET_B agonists, STXc and IRL 1620. All of these stimulants induced graded contraction. The EC_{50} for ET-1 (38.9 pM) was similar to that for STXc (87.1 pM) and the EC_{50} for ET-3 (933 pM) was similar to that for IRL 1620 (1,740 pM) (Table 1), supporting the suggestion that the contractile effects of ETs are mediated by the isopeptide-nonselective ET_B receptor (8,9).

Since the concentration-response curves for STXc (Fig. 1) and IRL 1620 (data not shown) were bell-shaped, the effects of single application of a high concentration (300 nM) of ET-1,

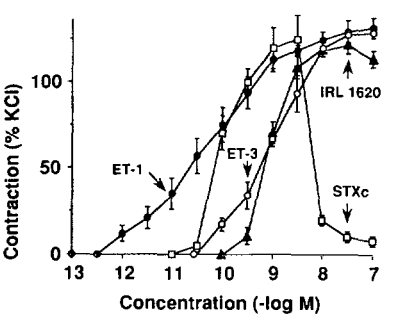


Figure 1. The contractile effects of ET-1 (●), ET-3 (○), STXc (□) and IRL 1620 (▲). Each point represents the mean of 4-8 experiments. S.E.M. are shown by vertical bars.

Table 1. EC_{50} values for the contractile effects of endothelin-1, endothelin-3, sarafotoxin S6c and IRL 1620 in the rabbit saphenous vein

Conditions	EC_{50} -log M ± S.E.M.	EC_{50} pM	n
Endothelin-1 (control)	10.41 ± 0.13	38.9	8
+ 3 μM BQ-123	10.32 ± 0.04	47.9	4
+10 μM RES-701-1	9.71 ± 0.18*	195	4
+ 3 μM IRL 1038	9.86 ± 0.06*	138	4
+ 300 nM STXc[a)]	9.12 ± 0.08*	759	4
+ 300 nM STXc[a)] + 3 μM BQ-123	8.63 ± 0.03*	2,340	4
+ 300 nM ET-3[a)]	9.10 ± 0.07*	794	4
+ 300 nM ET-3[a)] + 3 μM BQ-123	8.28 ± 0.03*	5,250	4
Endothelin-3 (control)	9.03 ± 0.01	933	8
+ 3 μM BQ-123	8.90 ± 0.06	1,260	4
+10 μM RES-701-1	8.53 ± 0.18*	2,950	4
+ 3 μM IRL 1038	8.59 ± 0.03*	2,570	4
+ 3 μM BQ-123 + 3 μM IRL 1038	8.50 ± 0.05*	3,160	4
+ 300 nM STXc[a)c)]	7.29 ± 0.13*	51,300	4
+ 300 nM STXc[a)] + 3 μM IRL 1038[c)]	7.34 ± 0.04*	45,700	4
+ 300 nM STXc[a)] + 3 μM BQ-123	> 6.5* [b)]	>300,000	4
+ 300 nM ET-3[a)c)]	7.31 ± 0.05*	49,000	4
+ 300 nM ET-3[a)] + 3 μM BQ-123	> 6.5* [b)]	>300,000	4
Sarafotoxin S6c (control)[c)]	10.06 ± 0.08	87.1	8
+ 3 μM BQ-123[c)]	10.00 ± 0.08	100	4
+10 μM RES-701-1[c)]	9.68 ± 0.05*	209	4
+ 3 μM IRL 1038[c)]	9.56 ± 0.07*	275	4
+ 300 nM STXc[a)]	>> 6.50* [b)]	>300,000	4
+ 300 nM ET-3[a)]	>> 6.50* [b)]	>300,000	4
IRL 1620 (control)[c)]	8.76 ± 0.11	1,740	4
+ 10μM RES-701-1	7.53 + 0.13*	29,500	
+ 300 nM STXc[a)]	> 5.50* [b)]	>3,000,000	4

[a)]: The ET_B receptor was desensitized by the pretreatment with 300 nM STXc or 300 nM ET-3 (see Fig. 2). [b)]: No effect at the concentrations up to 300 nM for ET-3 and STXc and up to 3 μM for IRL 1620. [c)]: Bell-shaped concentration-response curve. *: Significantly different from respective control with $P<0.05$. n: number of experiments.

ET-3 or STXc were examined. As shown in Fig. 2, ET-1 and ET-3 induced sustained contractions whereas STXc induced only a transient contraction. IRL 1620 (3 μM) also induced transient contraction. In contrast, lower concentrations of these stimulants induced sustained contractions. After the contraction induced by 300 nM STXc returned to the resting level, muscle was washed with normal solution for 30 min and then STXc was added again. However, the second application of STXc was ineffective at inducing contraction. In the STXc-pretreated muscle, in contrast, ET-1, ET-3 (Fig. 2), 72.7 mM KCl and 1 μM norepinephrine (data not shown) still induced sustained contractions. Since it has been shown that ET_B receptor is strongly tachyphylactic (12), it is suggested that high concentrations of STXc and IRL 1620 selectively activated the ET_B receptor and then desensitized this receptor, resulting in a transient contraction (Fig. 2) or a bell-shaped concentration-response curve (Fig. 1).

Figs. 3, 4 and 5 show the effects of antagonists. Contractions induced by ET-1 and STXc were not affected by 3 μM BQ-123. The same concentration of BQ-123 has been shown to induced 100- to 1,000-fold increase in the EC_{50} for the contractile effect of ET-1 (mediated by

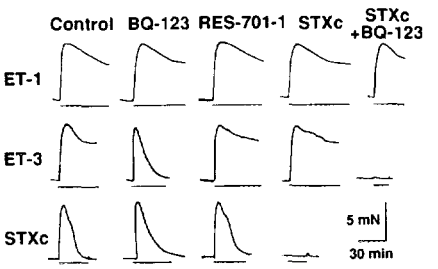
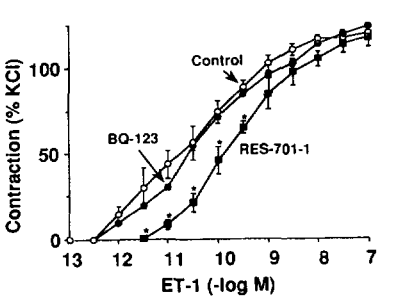


Figure 2. The contractile effects of ET-1 (upper row), ET-3 (middle row) and STXc (lower row) at a concentration of 300 nM. The first column: control contractions. BQ-123 (3 μM; second and fifth columns) and RES-701-1 (10 μM; third column) were added 30 min before the addition of stimulant. Pretreatment with STXc (fourth and fifth columns) was done by treating the muscle with 300 nM STXc for 30 min followed by a 30 min wash. Contraction induced by ET-3, but not ET-1, was reversed by washing. Traced from a result typical of 4 experiments.

the ET_A receptor) in arteries (2,8). In the presence of BQ-123, however, higher concentrations of ET-3 ($\geq$ 30 nM) inhibited its own contraction, making a bell-shaped concentration-response curve (Fig. 4) and single application of 300 nM ET-3 induced only a transient contraction (Fig. 2). Washing the ET-3, the second application of ET-3 (in the presence of BQ-123) was ineffective (data not shown). These results suggest that ET-3 activates both the tachyphylactic ET_B receptor and the BQ-123-sensitive ET_A receptor.

As shown in Figs. 3-5 and Table 1, 10 μM RES-701-1 and 3 μM IRL 1038 weakly antagonized the effect of ET-1, ET-3 and STXc, increasing the EC_{50} by only 3- to 5-fold. It has been shown that the same concentrations of these antagonists almost completely inhibit the ET_B-mediated, endothelium-dependent relaxation in arteries (4,5). Combined application of 3 μM BQ-123 and 3 μM IRL 1038 showed similar effect to that of IRL 1038 alone on the EC_{50} for ET-3, suggesting that activation of the ET_A receptor is not responsible for the weak inhibitory effects of the ET_B antagonists. In contrast, desensitization of the ET_B receptor showed strong



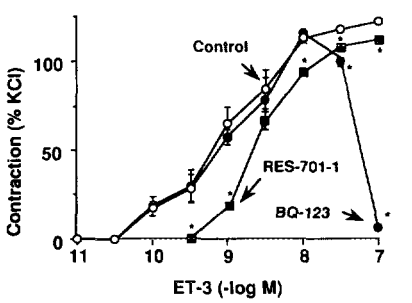


Figure 3. The contractile effects of ET-1 in the absence of antagonists (○), or in the presence of 3 μM BQ-123 (●) or 10 μM RES-701-1 (■). Antagonists were added 30 min before the addition of stimulant. Each point represents the mean of 4 to 8 experiments. S.E.M. are shown by vertical bars. *: Significantly different from the point in the absence of antagonist with $P<0.05$.

Figure 4. The contractile effects of ET-3. See Fig. 3 for explanation.

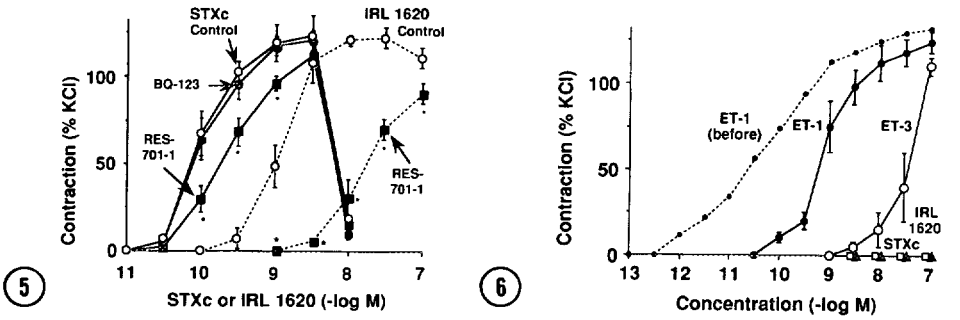


Figure 5. The contractile effects of STXc and IRL 1620. See Fig. 3 for explanation.

Figure 6. The contractile effects of ET-1 (●), ET-3 (○), STXc (□) and IRL 1620 (▲) in the muscle pretreated with 300 nM STXc. The curve for ET-1 before the STXc-treatment is also shown [ET-1 (before)]. See Fig. 3 for explanation.

antagonizing effects increasing the EC_{50} at least 20-fold. These results support the suggestion that the tachyphylactic ET_B receptors in veins can be subclassified into the ET_{B1} subtype which is sensitive to conventional ET_B antagonists and the ET_{B2} subtype which is not sensitive to these antagonists (5,7,8,10).

Fig. 6 and Table 1 show the effects of desensitization of the ET_B receptor by pretreatment with STXc or ET-3. In the ET_B-desensitized muscles, ET-1 was approximately 60 times more potent than ET-3 to induce contraction whereas STXc and IRL 1620 were ineffective. These contractions were antagonized by BQ-123 but not by RES-701-1 or IRL 1038, suggesting the involvement of the ET_A receptor. Interestingly, however, 3 μM BQ-123 completely inhibited the effect of 100 nM ET-3 although it only weakly antagonized the effect of ET-1. A higher concentration of BQ-123 (10 μM) did not show additional inhibitory effect (data not shown). These results suggest that the receptors activated by ET-1 and ET-3 are different. In the human saphenous vein pretreated with BQ-123, it has been shown that ET-1 was more effective than ET-3 to induce contraction (13). In rat vas deferens pretreated with BQ-123, it has also been shown that ET-1 was more effective than STXc to enhance the contraction induced by electrical stimulation (14). From these results, we suggest that there is a BQ-123-insensitive subtype of isopeptide-selective ET_A receptor (ET_{A2}) in addition to the BQ-123-sensitive ET_{A1} receptor.

Based on these observations, effects of ETs and their analogues are explained as follows (Table 2). Since desensitization of the ET_B receptor showed the effect similar to, but stronger than the ET_B antagonists, ET-1 may activate both the ET_{B1} and ET_{B2} receptors. After the ET_B desensitization, higher concentrations of ET-1 induced contraction which was only weakly antagonized by BQ-123, suggesting that ET-1 activates both the ET_{A1} and ET_{A2} receptors at higher concentrations than to activate the ET_B receptor. Since ET-3 induced only transient contraction in the presence of BQ-123, and since BQ-123 did not change the EC_{50} for ET-3, ET-3 seems to activate the tachyphylactic ET_B receptor at lower concentrations and also activates the BQ-123-sensitive ET_{A1} receptor (but not the BQ-123-insensitive ET_{A2} receptor) at the higher concentrations. Desensitization of the ET_B receptor more strongly antagonized the effect of ET-3 than the ET_B antagonists, suggesting that ET-3 activates both the ET_{B1} and ET_{B2}

Table 2. Pharmacological classification of the endothelin receptors

	ET_{A1}	ET_{A2}	ET_{B1}	ET_{B2}
Tachyphylaxis:	-	-	++	++
Agonistic action:				
Endothelin-1	++	++	++	++
Endothelin-3	+	-	++	++
Sarafotoxin S6c	-	-	++	++
IRL 1620	-	-	++	+
Antagonistic action:				
BQ-123	++	-	-	-
RES-701-1	-	-	++	-
IRL 1038	-	-	++	-

++: strong. +: weak. -: no.

receptors. Since the ET_B desensitization abolished the effects of STXc and IRL 1620, and since the ET_B antagonists showed weaker effects than the ET_B desensitization, STXc and IRL 1620 seemed to be the selective ET_B agonists activating both the ET_{B1} and ET_{B2} receptors. Since the ET_B antagonist relatively strongly inhibited the effect of IRL 1620, IRL 1620 may relatively selectively activates the ET_{B1} receptor and this may be the reason why IRL 1620 was less potent than STXc to induce contraction.

In conclusion, it is suggested that there are two types of ET receptors mediating contraction in the rabbit saphenous vein; less tachyphylactic, isopeptide-selective ET_A receptor, and tachyphylactic, isopeptide-nonselective ET_B receptor. Binding assays have also showed that there are ET_A and ET_B receptors in rabbit saphenous vein (15). We further suggest that the ET_A receptors may be classified into BQ-123-sensitive ET_{A1} and BQ-123-insensitive ET_{A2} subtypes. The ET_B receptor has been subclassified as the ET_{B1} and ET_{B2} subtypes based on the sensitivity to conventional ET_B antagonists (5,7,8,10) and we have confirmed this.

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References

1. Sakurai, T., Yanagisawa, M. and Masaki, T. (1992) Trends Pharmacol. Sci. *13*, 103-108.
2. Ihara, M., Noguchi, K., Saeki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukami, T., Ishikawa, K., Nishikibe, M. and Yano, M. (1992) Life Sci. *50*, 247-255.
3. Sogabe, K., Nirei, H., Shobo, M., Nomoto, M., Ao, S., Notsu, Y. and Ono, T. (1993) J. Pharmacol. Exp. Ther. *264*, 1040-1046.
4. Karaki, H., Sudjarwo, S. A., Hori, M., Sakata, K., Urade, Y., Takai, M. and Okada, T. (1993) Eur. J. Pharmacol. *231*, 371-374.
5. Karaki, H., Sudjarwo, S. A., Hori, M., Tanaka, T. and Matsuda, Y. (1994) Jpn. J. Pharmacol. *64 (suppl.), in press.*
6. Karaki, H., Sudjarwo, S. A., Hori, M., Takai, M., Urade, Y. and Okada, T. (1993) Br. J. Pharmacol. *109*, 486-490.
7. James, A. F., Urade, Y., Webb, R. L., Karaki, H., Umemura, I., Fujitani, Y., Oda, K., Okada, T., Lappe, R. W. and Takai, M. (1993) Cardiovasc. Drug Rev. *11*, 253-270.

8. Sudjarwo, S. A., Hori, M., Takai, M., Urade, Y., Okada, T. and Karaki, H. (1993) Life Sci. *53*, 431-437.
9. Moreland, S., McMullen, D. M., Delaney, C. L., Lee, V. G. and Hunt, J. T. (1992) Biochem. Biophys. Res. Commun. *184*, 100-106.
10. Hori, M., Sudjarwo, S. A., Oda, K., Urade Y. and Karaki, H. (1994) Life Sci., *54*, 645-652.
11. Matsuda, Y., Tanaka, T., Morishita, Y., Nozawa, M., Ohno, T. and Yamada, K. (1993) Circulation *88 (part 2)*, I-281.
12. Henry, P. J. (1993) Br. J. Pharmacol. *110*, 435-441.
13. Bax, W. A., Bos E. and Saxena, P. (1993) Eur. J. Pharmacol. *239*, 267-268.
14. Eglezos, A., Cucchi, P., Patacchini, R., Quartara, L., Maggi, C. A. and Mizrahi, J. (1993) Br. J. Pharmacol. *109*, 736-738.
15. Webb., M. L., Liu, E. C. K., Monshizadegan, H., Chao, C.-C., Lynch, J., Fisher, S. M. and Rose, P. M. (1993) Mol. Pharmacol. *44*, 959-965.