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Pharmacological evidence for the presence of three distinct functional endothelin receptor subtypes in the rabbit lateral saphenous vein

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1 Contraction of the rabbit isolated saphenous vein is mediated by a heterogeneous endothelin (ET) receptor population. This study has characterized these receptor subtypes by use of several pharmacologically distinct ET receptor agonists and antagonists.

2 ET-1, ET-3, sarafotoxin S6c (STXc) and [Ala^{3,11}]ET-1 produced biphasic, concentration-dependent contractions of the saphenous vein, responses which were best fitted by a two-site model comprised of a high (pM) and a low (nM) affinity site. In contrast, IRL 1620 only recognized one of these sites. $ET_{(16-21)}$ was devoid of contractile activity. ET-1, ET-3 and STXc were equipotent at the high affinity site (pD_2s of 12.0 ± 0.2, 12.2 ± 0.2 and 12.3 ± 0.3) indicating that this site had the characteristics of an ET_B receptor. In contrast, the low affinity site had the functional characteristics of an ET_C receptor since the pD_2s for ET-3 (10.2 ± 0.3) and STXc (10.6 ± 0.3) were significantly greater than that for ET-1 (9.1 ± 0.1). These contractile responses were insensitive to BQ-123, confirming that ET_A receptors were not involved in mediating this effect.

3 SB 209670 differentially antagonized the high affinity phases of the isopeptide concentration-response curves in a fashion dependent on the competing agonist: relative to the K_B obtained against STXc (0.15 nM). SB 209670 was 10 fold less potent when ET-1 was used as the competing agonist. This differential effect was not evident at the low affinity site ($K_B = 38$ nM). SB 209670 produced parallel, concentration-dependent rightward shifts in the concentration-response curve to STXc Ro 47-0203 was approximately 1 to 2 orders of magnitude less potent than SB 209670 at inhibiting the high affinity component of the concentration-response curve to STXc, whereas BQ-788 and Ro 46-2005 were approximately 3 orders of magnitude less potent than SB 209670. In addition to RES-701 and BQ-123, the high affinity site was insensitive to PD 142893 suggesting that it may represent an ET_{B2} receptor. Ro 47-0203 and SB 209670 were equipotent at inhibiting the low affinity component of the STXc concentration-response curve. Although Ro 46-2005, BQ-788, PD 142893 and RES-701 produced significant antagonism at the low affinity site, they were at least ten fold less potent than SB 209670.

4 ET-1, ET-3 and STXc produced endothelium-dependent vasorelaxation in the precontracted saphenous vein. Antagonist IC_{50}s were approximated as being: SB 209670, 3 nM; BQ-788 and RES 701, 300 nM; Ro 46-2005 and PD 142893, 3 μM; BQ-123, $\geqslant 10$ μM, consistent with vasorelaxation being mediated by an ET_{B1} receptor.

5 In summary, three pharmacologically distinct ET receptor subtypes have been identified in the rabbit saphenous vein. Two contractile receptors are present on the vascular smooth muscle, a high affinity site with the characteristics of an ET_{B2} receptor and a distinct lower affinity site with the characteristics of an ET_C receptor. In addition, an ET_{B1} receptor is present on the endothelium which mediates the vasodilator actions of this peptide family.

Keywords: Endothelin receptors; saphenous vein; SB 209670; Ro 47-0203; bosentan; Ro 46-2005; PD 142893; BQ-123; BQ-788; RES-701

Introduction

Two major subtypes of mammalian endothelin (ET) receptor have been cloned, the ET_A receptor, which exhibits selectivity for ET-1 over ET-3 and sarafotoxin S6c (STXc), and the ET_B receptor, which is non-isopeptide selective (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Masaki *et al.*, 1994). Although a putative ET_C receptor (selective for ET-3 over ET-1) has been cloned from the dermal melanophores of *Xenopus laevis* (Karne *et al.*, 1993), a mammalian homologue has not been identified. Initial structure-activity studies suggested that the primary role of the ET_A receptor was to mediate smooth muscle contraction and that this response was modulated indirectly by ET_B receptor-mediated nitric oxide release (Warner *et al.*, 1989; Douglas & Hiley, 1990; 1991a; Emori *et al.*, 1990). Subsequently, however, ET_B receptors have also been shown to mediate vasoconstriction (Douglas & Hiley, 1991a,b; Bigaud & Pelton, 1992; Clozel *et al.*, 1992; Harrison *et al.*, 1992; Panek *et al.*, 1992; Sumner *et al.*, 1992; James *et al.*, 1993; Gardiner *et al.*, 1994; Godfraind, 1994a,b; Seo *et al.*, 1994; Teerlink *et al.*, 1994). It has now been proposed that multiple ET_B receptors exist within the mammalian cardiovascular system (Warner *et al.*, 1993a,b; Douglas *et al.*, 1994b; Ohlstein *et al.*, 1994a). Warner *et al.* (1993a,b) have described the presence of 'ET_{B1}' and 'ET_{B2}' receptors which are distinguished by their anatomical location (endothelium *versus* smooth muscle), function (vasodilatation *versus* vasoconstriction) and pharmacological profile (sensitivity to PD 142893).

ET_B receptor-mediated vasoconstriction is of particular importance in the control of vascular tone in low pressure conduit vessels (Moreland *et al.*, 1994) such as those found in the venous and pulmonary circulations. One such vessel, the saphenous vein, is extremely sensitive to the vasoconstrictor actions of the ET isopeptides, responses mediated by a

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heterogeneous receptor population (Komori & Vanhoutte, 1989; Miller *et al.*, 1989; Berti *et al.*, 1990; Moreland *et al.*, 1992; Webb *et al.*, 1993; Gray *et al.*, 1994). Until recently it has only been possible to characterize this heterogeneous receptor population by use of agonists (Auguet *et al.*, 1993; Bax *et al.*, 1993; White *et al.*, 1994). However, several novel ET receptor antagonists have been described recently. In addition to the well characterized ET_A-selective antagonist, BQ-123 (Ihara *et al.*, 1991; Moreland, 1994) and the $ET_{A/B}$ antagonist PD 142893 (Doherty *et al.*, 1993), such antagonists include the nonpeptide $ET_{A/B}$ receptor antagonists SB 209670 (Ohlstein *et al.*, 1994b), Ro 47-0203 (bosentan) and Ro 46-2005 (Clozel *et al.*, 1993; 1994) and several peptidic ET_B-selective antagonists including BQ-788 and RES-701 (Ishikawa *et al.*, 1994; Tanaka *et al.*, 1994). Of these antagonists, SB 209670 is the most potent, exhibiting K_is at the human cloned ET_A and ET_B receptors of 0.4 and 18 nM (Nambi *et al.*, 1994b). The use of this antagonist in experimental disease models has identified a role for ET-1 in the aetiology of a variety of pathological processes including hypertension, renal failure, restenosis and cerebral vasospasm and neurodegeneration (Brooks *et al.*, 1994; Douglas *et al.*, 1994a,b, 1995; Ohlstein *et al.*, 1994a,b; Willette *et al.*, 1994). In the present study these ET receptor antagonists have been used to characterize the receptors responsible for mediating the constrictor and dilator actions of the ET isopeptides in the rabbit saphenous vein.

Methods

The lateral saphenous vein was excised from New Zealand White male rabbits (2–3 kg) following sodium pentobarbitone overdose (100 mg kg^{-1} via the central ear vein), cleaned of adherent tissue and cut into 4 mm rings. Rings were suspended under optimal resting tension (5 mN) in 10 ml organ baths (37 ± 1°C, aerated wtih 95% O_2/5% CO_2) containing Krebs-bicarbonate solution (mM): NaCl 112, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, dextrose 11 and indomethacin 0.01. Changes in isometric tension were recorded with Grass FTO3c force-displacement transducers linked to Beckman R-611 dynographs. Where appropriate, the endothelium was removed by rubbing the intimal surface of vessel with a stainless steel rod. Endothelium-intact and -denuded tissues were defined as those which, when exposed to acetylcholine (1 μM) in the presence of tone induced by noradrenaline (1 μM), responded with a relaxant response of either > 50% or < 10% relaxation, respectively. Tissues were allowed a 90 min equilibration period prior to any experimentation.

ET isopeptide-induced vasoconstriction in the rabbit isolated saphenous vein

Thirty min following the addition of vehicle, concentration-response curves to either ET-1, ET-3, STXc, [$Ala^{3,11}$]ET-1, IRL 1620 or $ET_{(16-21)}$ were constructed in the endothelium-intact tissues. Responses were expressed as a percentage of the contractile response elicited by 60 mM KCl determined at the beginning of each experiment. An individual ring preparation was used once to construct a single concentration-response curve. Concentration-response curves were analysed by fitting the experimental data to one of the following logistic equations by nonlinear least squares regression (Douglas *et al.*, 1994c):

$$\text{(i) One site fit } R = \frac{R_{max} \cdot C^n}{EC_{50}{}^n + C^n}$$

where R is the response, C the concentration of agonist, EC_{50} the concentration of agonist required to produce a half maximal response, n the Hill coefficient and R_{max} the maximal response or:

$$\text{(ii) Two site fit } R = \frac{R_{maxH} \cdot C^n{}_H}{EC_{50H}{}^n{}_H + C^n{}_H} + \frac{R_{maxL} \cdot C^n{}_L}{EC_{50H}{}^n{}_H + C^n{}_H}$$

where R is the response, C the concentration of agonist, EC_{50H} and EC_{50L} the concentration of agonist required to produce a half maximal response at a high and low affinity site, n_H and n_L the Hill coefficients at a high and low affinity site and R_{maxH} and R_{maxL} the maximal responses at a high and low affinity site, respectively.

The contractile actions of ET-1, ET-3 and STXc were also assessed in endothelium-denuded tissues in the presence either of vehicle, SB 209670 (1 μM) or BQ-123 (10 μM). In order to perform a more accurate determination of SB 209670 potency by Schild analysis, cumulative concentration-response curves were constructed to STXc either in the presence of vehicle or in the presence of increasing concentrations of SB 209670. So that the potency of a series of chemically and pharmacologically diverse ET receptor antagonists could be compared with SB 209670, cumulative concentration-response curves were also constructed to STXc in the presence of 10 μM Ro 46-2005, Ro 47-0203, PD 142893, BQ-788 or RES-701.

ET isopeptide-induced vasorelaxation in the rabbit isolated saphenous vein

Following a 90 min equilibration period, endothelium-intact saphenous vein rings were precontracted with 1 μM noradrenaline. Once a sustained contraction had been obtained, tissues were exposed to a single concentration of either ET-1, ET-3 or STXc in order to determine the degree of peptide-induced vasorelaxation (expressed as a percentage reversal of the induced tone).

In a separate series of experiments, tissues were precontracted with noradrenaline and exposed to 1 nM ET-3. Once the ensuing 'control' relaxant response had been recorded, the tissue was washed and subsequently incubated with either vehicle or SB 209670, BQ-788, Ro 46-2005, RES 701, PD 142893 or BQ-123 for 30 min period. Tissues were then recontracted with noradrenaline and exposed to a 'secondary' administration of ET-3. The degree of antagonist-induced inhibition was determined by comparing the degree of relaxation obtained to the 'control' and 'secondary' additions of 1 nM ET-3.

Materials

All solutions were made freshly each day and stored on ice in a light tight container. ET-1, ET-3, STXc, IRL 1620 (Namby *et al.*, 1994a) (Suc[Glu9;Ala11,15]-endothelin-1(8-21)) $ET_{(16-21)}$, [$Ala^{3,11}$]ET-1 and BQ-123 (cyclo(D-Trp-D-Asp-Pro-D-Val-Leu); Ihara *et al.*, 1991) were from American Peptide Co. (Santa Clara, CA, U.S.A.). SB 209670 (+)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid; Ohlstein *et al.*, 1994b), PD 142893 (Ac-D-Dip-Leu-Asp-Ile-Ile-Trp; Doherty *et al.*, 1993), BQ-788 (N-*cis*-2,6-dimethylpiperidinocarbonyl-L-γ-MeLeu-D-Trp (1-CO_2Me) -D-Nle; Ishikawa *et al.*, 1994), Ro 46-2005 (4-tert-butyl-N-[6-(2-hydroxy)-ethoxy))-5-(3-methoxyphenoxy)pyrimidin-4-yl]-benzene sulphonamide) and Ro 47-0203 (4-tert-butyl-N-[6-(2-hydroxy)-ethoxy)-5-(2-methoxyphenoxy)-2,2′-bipyrimidin-4-yl]-benzene sulphonamide) (Clozel *et al.*, 1993; 1994) were synthesized in the Department of Medicinal Chemistry, SmithKline Beecham. RES-701 (Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp; Tanaka *et al.*, 1994) was the generous gift of Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). All antagonists solution were made using dimethylsulphoxide (DMSO) as the diluent. DMSO, acetylcholine bromide, noradrenaline bitartrate (stock solution in 1 mg ml^{-1} ascorbate)

and indomethacin were from Sigma Chemical Co. (St Louis, MO, U.S.A.). All other reagents were analytical grade.

Statistics

Values are expressed as the mean ± s.e.mean and n represents the number of tissues studied in a particular group. Statistical comparisons were made by analysis of variance (ANOVA; Fisher's protected least squares difference) where $P<0.05$ was accepted as being significant.

Results

ET isopeptide-induced vasoconstriction in the rabbit isolated saphenous vein

ET-1, ET-3 and STXc produced concentration-dependent contractions of the endothelium-intact saphenous vein over a broad concentration-range of approximately 5 log units (Figure 1). The concentration-response curves generated were biphasic and best fitted to a two site model consisting of a high (pM) and low (nM) affinity site (Table 1). Removal of the endothelium did not have a significant effect on these contractile actions (Table 1). The contractile potencies of ET-3 and STXc at the high affinity site were not significantly different from that of ET-1. In contrast, however, STXc and ET-3 were significantly more potent than ET-1 at the low affinity site. Relative to ET-1, ET-3 and STXc were full agonists at the high affinity site and partial agonists at the low affinity site. Furthermore, relative to the low affinity site, the high affinity site appeared to be a 'lower efficacy site', contributing approximately 40% towards the overall contractile response to ET-1. The concentration-response curve for the contractile actions of [Ala^{3,11}]ET-1 was also biphasic and, similarly, was best fitted to a two site model (Figure 1). This analogue was 87 and 12 fold less potent than ET-1 at the high and low affinity sites, respectively (Table 1), but differed from ET-3 and STXc, in that, relative to ET-1, it was a full agonist at both sites. In contrast, the concentration-response curve generated to IRL 1620 was obtained over a much narrower concentration-range of approximately 3 log units

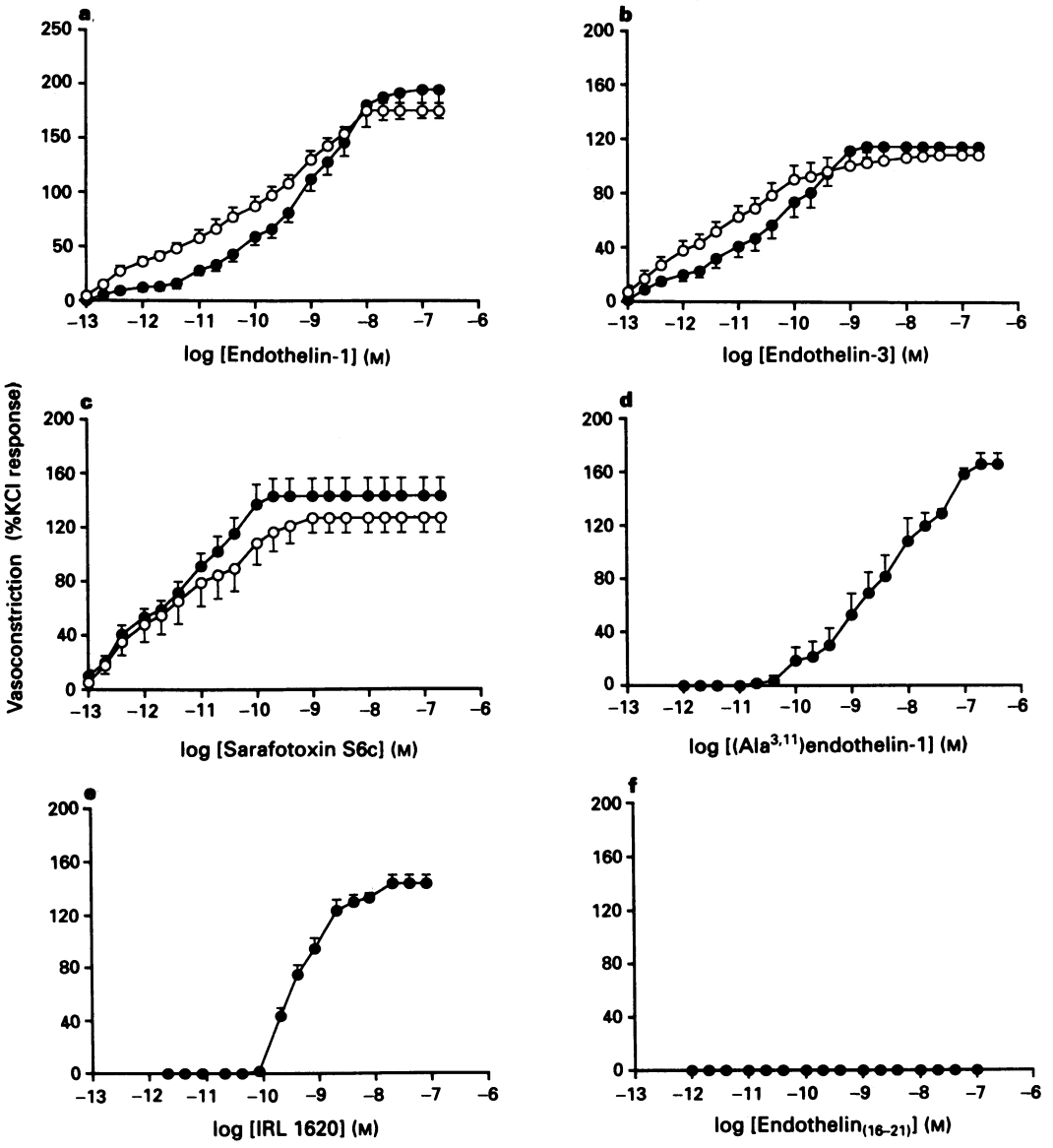

Figure 1 Cumulative concentration-response curves obtained in endothelium-intact (●) and endothelium-denuded (○) rabbit isolated saphenous vein rings in response to the addition of either (a) ET-1 ($n=7$ and 8, respectively), (b) ET-3 ($n=9$ and 8, respectively), (c) STXc (both $n=5$), (d) [Ala^{3,11}]ET-1 ($n=3$), (e) IRL 1620 ($n=3$) or (f) the C-terminal hexapeptide ET$_{(16-21)}$ ($n=3$). Values are the mean ± s.e.mean and n represents the number of tissues studied in a particular group. The corresponding pD_2 and R_{max} values are shown in Table 1. For abbreviations, see text.

(Figure 1) and, consequently, was best fitted to a one site model (Table 1). $ET_{(16-21)}$ was devoid of contractile activity (Figure 1, Table 1).

Effect of specific ET isopeptide agonists on antagonistic actions of 1 μM SB 209670 and 10 μM BQ-123 in the endothelium-denuded rabbit isolated saphenous vein

The contractile actions of ET-1, ET-3 and STXc were not significantly altered by BQ-123 (Figure 2, Table 2). In contrast, SB 209670 (1 μM) inhibited both components of the biphasic concentration-response curve to STXc producing parallel, 6918 and 28 fold rightward displacements of the high and low affinity components of this curve, respectively (Figure 2, Table 2). SB 209670 also inhibited the high and low affinity components of biphasic concentration-response curve to both ET-1 and ET-3 (Figure 2, Table 2) producing parallel, 646 and 22 fold rightward displacements in the high and low affinity components of the ET-1-response curve and 1259 and 74 fold rightward displacements in the high and low affinity components of the ET-3-response curve, respectively. Thus, SB 209670 differentially antagonized the high affinity component of the concentration-response curve dependent on the agonist used (a similar differential effect did not occur at the low affinity site).

Effect of SB 209670 on STXc-induced contraction of the endothelium-denuded rabbit isolated saphenous vein

SB 209670 (100 nM–3 μM) produced parallel, concentration-dependent rightward shifts in both components of biphasic

Table 1 Curve fitted parameters obtained for the contractile actions of some endothelin (ET) isopeptides in the rabbit isolated saphenous vein

	High affinity site		*Low affinity site*		
Agonist	pD_2	R_{max} (%KCl)	pD_2	R_{max} (%KCl)	n
ET-1					
Endothelium intact	11.27 ± 0.32	50 ± 11	8.87 ± 0.09	154 ± 30	7
Endothelium denuded	11.98 ± 0.25	74 ± 13	9.08 ± 0.14	105 ± 11	8
STXc					
Endothelium intact	12.44 ± 0.21*	64 ± 9	10.70 ± 0.14***	74 ± 15*	5
Endothelium denuded	12.35 ± 0.26	53 ± 9	10.63 ± 0.26**	74 ± 10*	5
ET-3					
Endothelium intact	11.60 ± 0.22	51 ± 9	9.84 ± 0.14***	65 ± 7**	9
Endothelium denuded	12.19 ± 0.21	54 ± 8	10.23 ± 0.32**	56 ± 9**	8
IRL 1620					
Endothelium intact	–	–	9.27 ± 0.16	142 ± 6	3
Endothelium denuded	ND	ND	ND	ND	–
[Ala^{3,11}]ET-1					
Endothelium intact	9.33 ± 0.59***	76 ± 37	7.79 ± 0.55***	95 ± 31	3
Endothelium denuded	ND	ND	ND	ND	–
$ET_{(16-21)}$					
Endothelium intact	<7	–	<7	–	3
Endothelium denuded	ND	ND	ND	ND	–

Values are the mean ± s.e.mean; *n* represents the number of animals used in a particular group. Parameters are derived by fitting the concentration-response data (Figure 1) to a logistic equation (see Methods). Statistical comparisons: $*P<0.05$, $**P<0.01$ and $***P<0.001$ (ANOVA; Fisher's Protected least squares difference) compared to values obtained to ET-1 in corresponding endothelium-intact or -denuded tissue. Endothelial denudation did not alter the concentration-response curves to ET-1, ET-3 or STXc. Unlike ET-1, ET-3, STXc and [Ala^{3,11}]ET-1, IRL 1620 was shown to fit best a one-site model. ND = values not determined in endothelium-denuded tissues. $ET_{(16-21)}$ (≤ 0.1 μM) was devoid of contractile activity in endothelium-intact tissues.

Table 2 Relative potencies of SB 209670 and BQ-123 at inhibiting endothelin (ET) isopeptide-induced contraction of the endothelium-denuded rabbit isolated saphenous vein

	High affinity site			*Low affinity site*			
Agonist	pD_2	K_B (nM)	pK_B	pD_2	K_B (nM)	pK_B	n
ET-1							
Control	11.98 ± 0.24			9.08 ± 0.14			8
BQ-123 10 μM	12.13 ± 0.23	NS	NS	8.30 ± 0.50	NS	NS	5
SB 209670 1 μM	9.17 ± 0.20***	1.55	8.81	7.74 ± 0.32**	48	7.32	5
ET-3							
Control	12.19 ± 0.21			10.23 ± 0.32			9
BQ-123 10 μM	12.05 ± 0.25	NS	NS	10.14 ± 0.27	NS	NS	7
SB 209670 1 μM	9.09 ± 0.10***	0.79	9.01	8.36 ± 0.07***	14	7.87	8
STXc							
Control	12.35 ± 0.26			10.63 ± 0.26			5
BQ-123 10 μM	12.35 ± 0.24	NS	NS	11.19 ± 0.42	NS	NS	4
SB 209670 1 μM	8.51 ± 0.11***	0.15	9.84	9.19 ± 0.17**	38	7.42	5

Values are the mean ± s.e.mean; *n* represents the number of animals used in a particular group. Parameters are derived by fitting the concentration-response data (Figure 2) to a logistic equation (see Methods). Statistical comparisons: $**P<0.01$ and $***P<0.001$ (ANOVA; Fisher's Protected least squares difference) compared to values obtained to a particular agonist in corresponding control tissues (which are from Table 1 and are included for ease of comparison). Since 10 μM BQ-123 failed to produce a significant inhibition of the agonist-induced contractile responses (NS), no K_B (or, therefore, pK_B) value could be determined.

concentration-response curve to STXc (Figure 3, Table 3). SB 209670 did not significantly alter the maximal contractile effects of STXc at either the high or the low affinity site, indicative of competitive antagonism. This was confirmed by Schild analysis where SB 209670 antagonized both the high and low affinity components of the biphasic concentration-response curve to STXc with regression slopes of 1.13 and 1.06, respectively (r^2 values of 0.97 and 0.99, respectively). SB 209670 was determined as having pA_2 values of 7.88 and 7.24 at the high and low affinity sites, respectively.

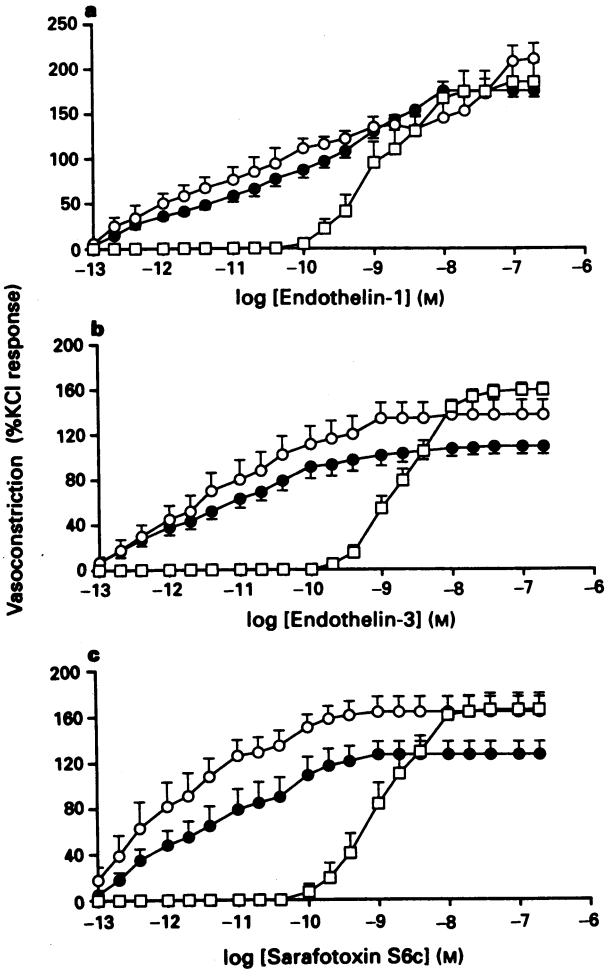

Figure 2 Cumulative concentration-response curves obtained in endothelium-denuded rabbit isolated saphenous vein rings in response to the addition of (a) ET-1, (b) ET-3 and (c) STXc in the presence of either vehicle (●; $n = 8$, 9 and 5, respectively), 10 μM BQ-123 (○; $n = 5$, 7 and 4, respectively) or 1 μM SB 209670 (□; $n = 5$, 8 and 5, respectively). Values are the mean ± s.e.mean and n represents the number of tissues studied in a particular group. Control values obtained to ET-1, ET-3 and STXc in the presence of vehicle are from Figure 1 and are included for ease of comparison. The corresponding pD_2 values determined for each agonist in the presence or absence of antagonist are shown in Table 2 along with the corresponding antagonist K_B and pK_B values. For abbreviations, see text.

Effect of 10 μM Ro 46-2005, Ro 47-0203, PD 142893, BQ-788 and RES-701 on the contractile actions of STXc in the endothelium-denuded rabbit isolated saphenous vein

Ro 47-0203, Ro 46-2005 and BQ-788, all at 10 μM concentrations, produced significant 832, 36 and 91 fold shifts in the

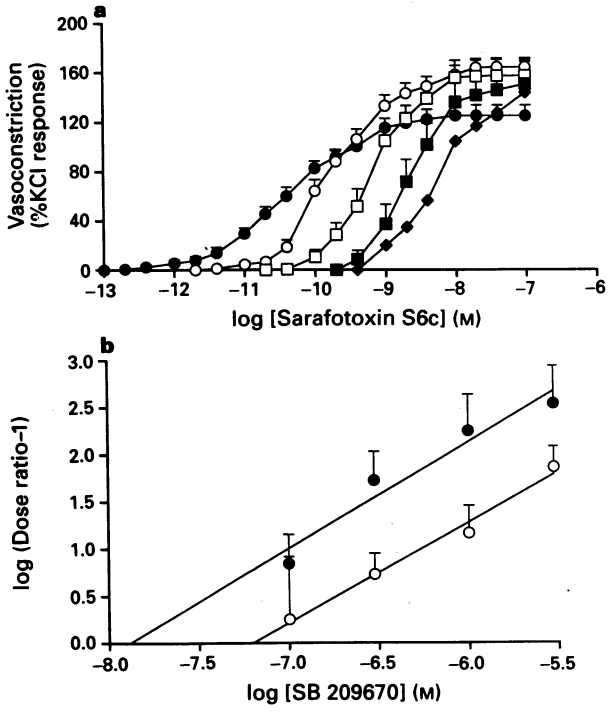

Figure 3 Panel (a) demonstrates the concentration-dependent inhibition by SB 209670 of the contractile responses observed in endothelium-denuded rabbit isolated saphenous vein rings in response to the addition of STXc; (●; $n = 6$) represents control responses, obtained in the presence of vehicle. Responses obtained in the presence of SB 209670 are as follows: 100 nM (○; $n = 6$); 300 nM (□; $n = 6$); 1 μM (■; $n = 6$); 3 μM (◆; $n = 6$). The corresponding pD_2 and R_{max} values obtained in the presence or absence of SB 209670 are shown in Table 3. Panel (b) represents the Schild analysis of these data which demonstrates that SB 209670 is a potent, competitive functional antagonist of both the high (○; $pA_2 = 7.88$; regression slope = 1.13; $r^2 = 0.97$) and low (●; $pA_2 = 7.24$; regression slope = 1.06; $r^2 = 0.99$) affinity components of the biphasic contractile response to STXc. Values are the mean ± s.e.mean and n represents the number of tissues studied in a particular group. For abbreviations, see text.

Table 3 Effect of SB 209670 on the STXc-induced contraction of the endothelium-denuded rabbit isolated saphenous vein

	High affinity site		*Low affinity site*		
	pD_2	R_{max} (%KCl)	pD_2	R_{max} (%KCl)	n
[SB 209670]					
Control	11.04 ± 0.33	76 ± 17	9.60 ± 0.24	50 ± 17	6
0.1 μM	10.09 ± 0.11**	73 ± 13	9.13 ± 0.25	92 ± 15	6
0.3 μM	9.30 ± 0.12***	96 ± 16	8.75 ± 0.19**	61 ± 15	6
1 μM	8.78 ± 0.23***	71 ± 9	8.38 ± 0.09***	80 ± 13	6
3 μM	8.50 ± 0.17***	97 ± 6	7.71 ± 0.22***	49 ± 10	6

Values are the mean ± s.e.mean; n represents the number of animals used in a particular group. Parameters are derived by fitting the concentration-response data (Figure 3) to a logistic equation (see Methods). Statistical comparisons: $^{**}P < 0.01$ and $^{***}P < 0.001$ (ANOVA; Fisher's Protected least squares difference) compared to values obtained to STXc in the absence of antagonist ('Control').

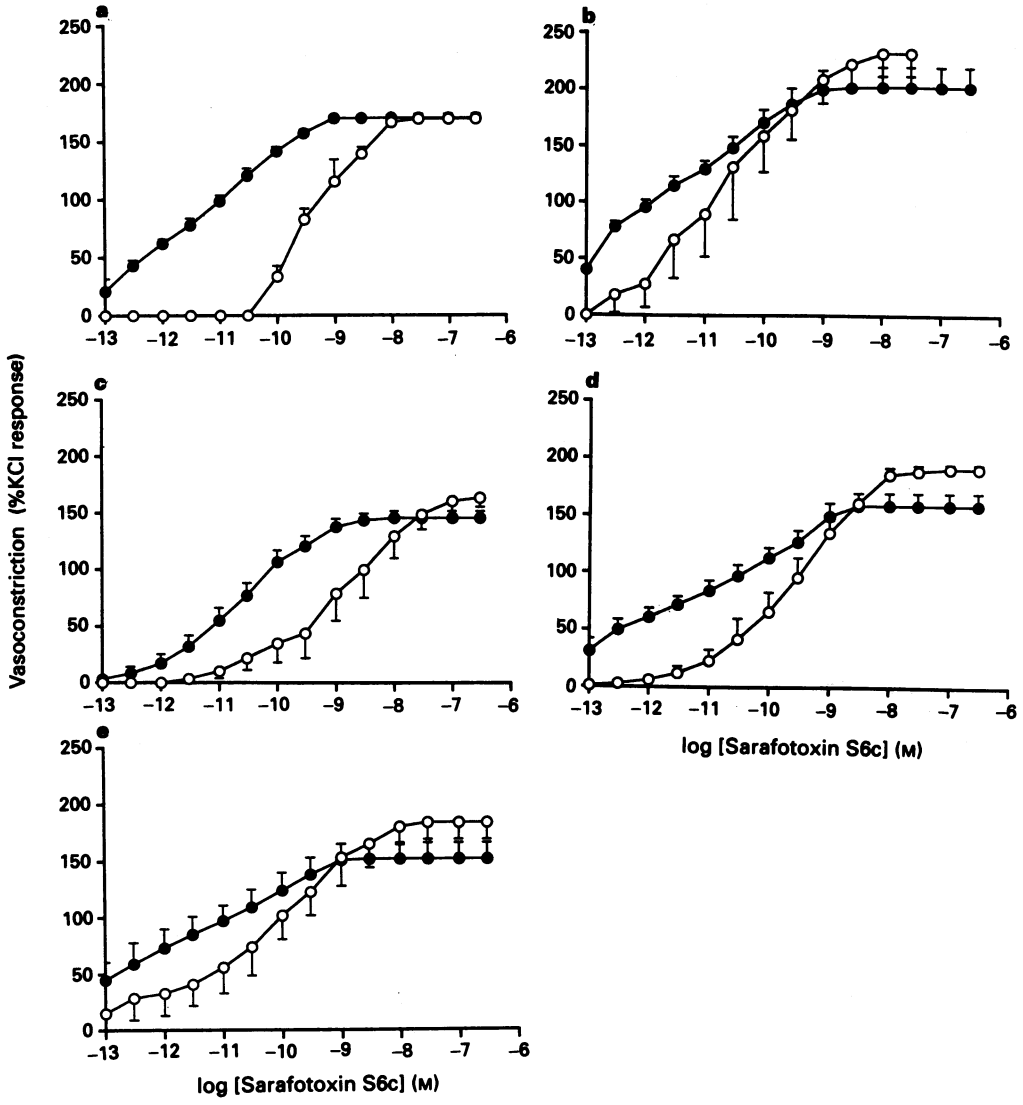

Figure 4 Cumulative concentration-response curves obtained to STXc in endothelium-denuded rabbit isolated saphenous vein rings in the presence of either (●) vehicle or (○) 10 μM concentrations of (a) Ro 47-0203 (both $n = 3$), (b) Ro 46-2005 ($n = 6$ and 3, respectively), (c) BQ-788 (both $n = 8$), (d) PD 142893 (both $n = 6$) and (e) RES-701 (both $n = 4$). Values are the mean ± s.e.mean and n represents the number of tissues studied in a particular group. The corresponding pD_2 values determined for STXc in the presence or absence of each individual antagonist are shown in Table 4 along with the corresponding antagonist K_B and pK_B values. For abbreviations, see text.

Table 4 Effect of various endothelin (ET) receptor antagonists at inhibiting the STXc-induced contraction of the endothelium-denuded rabbit isolated saphenous vein

	High affinity site				*Low affinity site*				
Antagonist	pD_2	K_B (nM)	pK_B	*Rank order*	pD_2	K_B (nM)	pK_B	*Rank order*	n
Control	12.70 ± 0.20				10.61 ± 0.19				3
Ro 47-0203 10 μM	9.78 ± 0.09***	14	7.85	97	8.38 ± 0.17***	62	7.21	2	3
Control	11.28 ± 0.26				9.74 ± 0.25				8
BQ-788 10 μM	9.32 ± 0.44**	110	6.96	758	8.54 ± 0.33*	673	6.17	18	8
Control	12.82 ± 0.08				10.42 ± 0.09				6
Ro 46-2005 10 μM	11.26 ± 0.48**	283	6.55	1,950	9.40 ± 0.43*	585	6.23	15	3
Control	12.66 ± 0.38				10.15 ± 0.29				6
PD 142893 10 μM	11.28 ± 0.71	NS	NS	69,183	9.08 ± 0.20*	1,710	5.77	45	6
Control	12.64 ± 0.38				10.11 ± 0.14				4
RES-701 10 μM	11.71 ± 0.68	NS	NS	69,183	9.24 ± 0.11**	2,023	5.69	54	4
Control	12.35 ± 0.26				10.63 ± 0.26				5
BQ-123 10 μM	12.35 ± 0.24	NS	NS	69,183	11.19 ± 0.42	NS	NS	263	4

Values are the mean ± s.e.mean; n represents the number of animals used in a particular group. Parameters are derived by fitting the concentration-response data (Figure 4) to a logistic equation (see Methods). Statistical comparisons: *$P<0.05$, **$P<0.01$ and ***$P<0.001$ (ANOVA; Fisher's Protected least squares difference) compared to corresponding control values obtained to STXc in the absence of antagonist ('Control'). Where a 10 μM concentration of antagonist failed to produce a significant inhibition of the contractile actions of STXc (NS), no K_B (and, therefore, pK_B) value could be determined. The rank order of antagonist potency was approximated based on the pK_B values determined in this Table (using a 10 μM concentration of antagonist) and those determined for SB 209670 against STXc (using a 1 μM concentration, Table 2).

high affinity portion of the STXc concentration-response curve (Figure 4). Thus, based on the K_B values determined for SB 209670, Ro 47-0203 was 1 to 2 orders of magnitude less potent than SB 209670 at this site, whereas Ro 46-2005 and BQ-788 were 3 orders of magnitude less potent (Table 4). However, 10 μM concentrations of PD 142893 and RES-701 failed to inhibit this portion of the concentration-response curve to STXc. In contrast, 10 μM concentrations of Ro 47-0203, Ro 46-2005, BQ-788, PD 142893 and RES-701 caused significant rightward shifts in the low affinity portion of the biphasic STXc concentration-response curve (170, 10, 16, 12 and 7 fold shifts, respectively). Therefore, Ro 47-0203 was equipotent with SB 209670 at this low affinity site, whereas Ro 46-2005, BQ-788, PD 142893 and RES-701 were 1 to 2 orders of magnitude less potent. SB 209670, BQ-788, Ro 47-0203 and Ro 46-2005 appeared to be 1 to 2 orders of magnitude less potent at the low affinity site relative to the high affinity site.

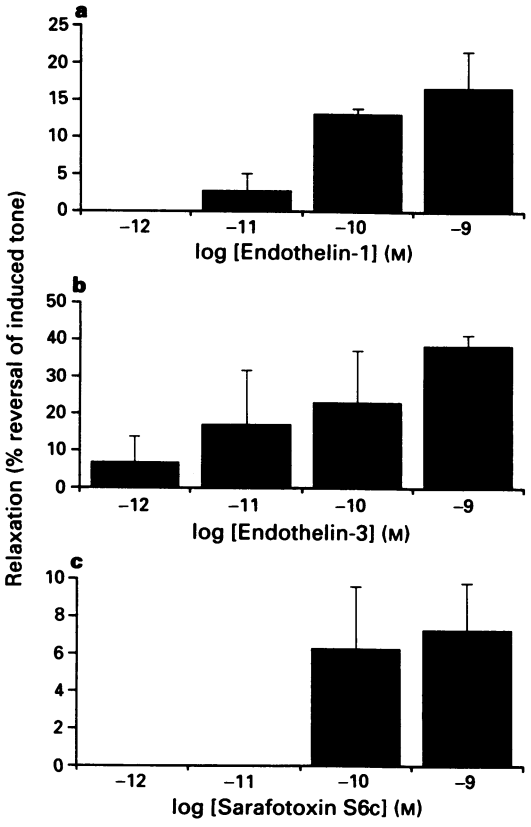

Figure 5 Concentration-dependent relaxant responses obtained in precontracted (1 μM noradrenaline) rabbit isolated saphenous vein rings with endothelium-intact following the addition of single concentration of either (a) ET-1 ($n = 3$), (b) ET-3 ($n = 7$) or (c) STXc ($n = 3$). Values are the mean ± s.e.mean and n represents the number of tissues studied in a particular group.

Vasodilator actions of the ET isopeptides

ET-3 produced concentration-dependent vasorelaxation in saphenous vein preparations precontracted with noradrenaline (Figure 5), an action dependent on the presence of a functional endothelium (Figure 6). At concentrations greater than 1 nM, the relaxant response to ET-3 was overwhelmed by a concomitant contractile response. The relaxant responses to ET-1 and STXc were qualitatively smaller than those obtained with ET-3 (Figures 5 and 7). As with ET-3, such responses were also superceded by vasoconstriction at agonist concentrations of greater than 1 nM (such that sigmoidal concentration-response curves, and, therefore, a rank order of relaxant potency, could not be determined).

Effect of ET receptor antagonists on the vasodilator actions of ET-3

SB 209670 was the most potent antagonist studied at inhibiting the relaxant actions of ET-3 (Figures 8 and 9) with significant inhibition being observed at antagonist concentrations as low as 1 nM. BQ-788 and RES-701 were also effective at inhibiting this response at concentrations of 1 μM, whereas Ro 46-2005 and PD 142893 only produced a significant (albeit virtually complete) inhibition at 10 μM concentrations. Although BQ-123 (10 μM) also produced significant antagonism of ET-3-induced relaxation, inhibition was incomplete. Since tissues were used to study the effects of a single concentration of antagonist against a single concentration of agonist, concentration-response curves could not

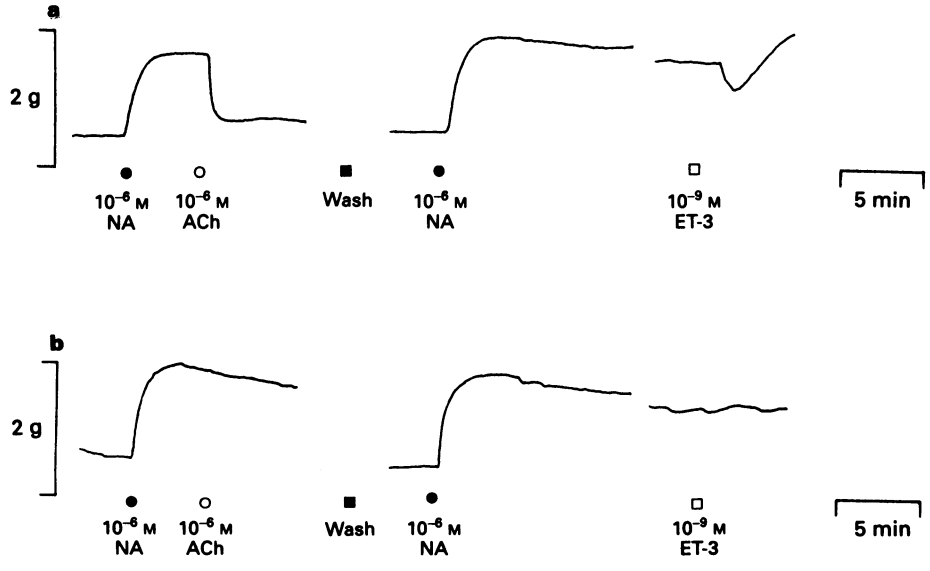

Figure 6 Representative experimental trace illustrating that, in rabbit isolated saphenous vein rings preconstricted with 1 μM noradrenaline (●; 10^{-6} M NA), the vasorelaxant actions of either 1 μM acetylcholine (○; 10^{-6} M ACh) or 1 nM ET-3 (□; 10^{-9} M) are obtained only in the presence of an intact functional endothelium (a). Panel (b) shows that when the endothelium is physically destroyed by gently rubbing the intimal surface of the saphenous vein with a stainless steel rod, the vasorelaxant activity of both acetylcholine and ET-3 are abolished. (■; Wash) represents where the tissue was washed in between exposure to acetylcholine and ET-3.

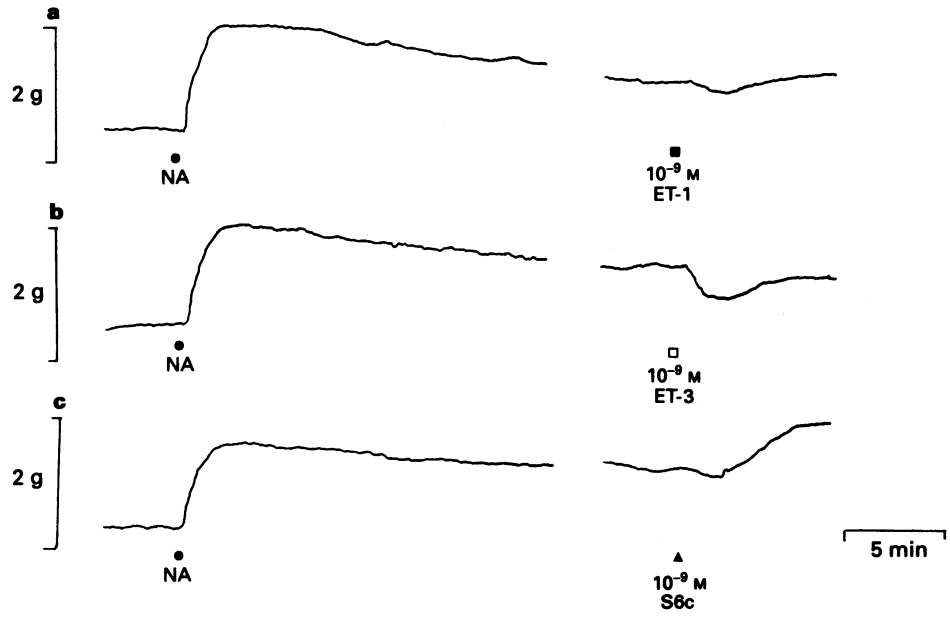

Figure 7 Representative experimental trace comparing the vasorelaxant activities of 1 nM concentrations of (a) endothelin-1 (ET-1; ■; 10^{-9} M), (b) endothelin-3 (ET-3, □; 10^{-9} M) and (c) sarafotoxin S6c (S6c, ▲; 10^{-9} M) in rabbit isolated, endothelium-intact saphenous vein rings preconstricted with 1 μM noradrenaline (●; NA).

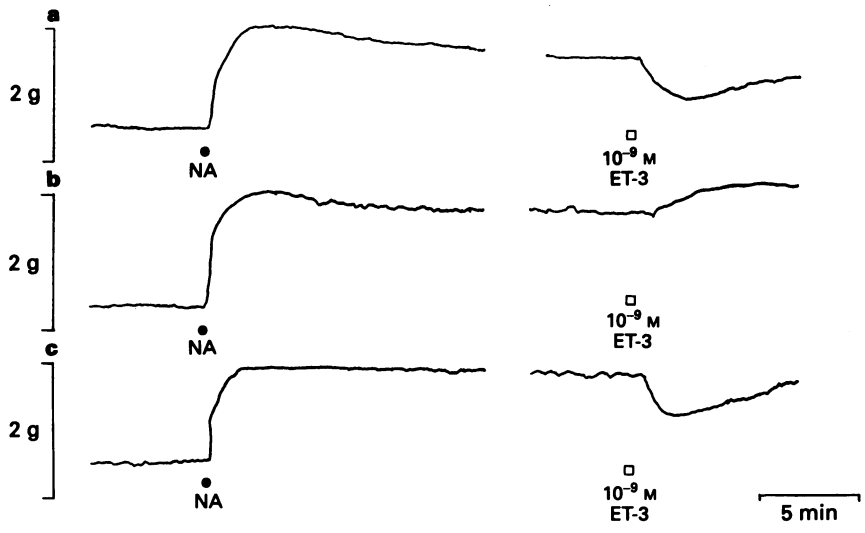

Figure 8 Representative experimental trace comparing the vasorelaxant activities of 1 nM concentrations of endothelin-3 (ET-3, □; 10^{-9} M) in rabbit isolated, endothelium-intact saphenous vein rings preconstricted with 1 μM noradrenaline (●; NA) in (a) control tissues (pretreated with vehicle) or those pretreated with either (b) 0.1 μM SB 209670 or (c) 10 μM BQ-123. The figure demonstrates that SB 209670 completely abolishes the vasorelaxant actions of ET-3, a response that is relatively insensitive to the ET_A-selective ET receptor antagonist, BQ-123 (see Figure 9).

be fitted to a logistic equation. Nevertheless, IC_{50}s were approximated as being: SB 209670, 3 nM; BQ-788 and RES 701, 300 nM; Ro 46-2005 and PD 142893, 3 μM; BQ-123, $\geqslant$ 10 μM.

Discussion

Contraction of the rabbit saphenous vein is mediated by a heterogeneous population of ET receptors (Auguet *et al.*, 1993; Bax *et al.*, 1993; Webb *et al.*, 1993; White *et al.*, 1994). In accord with Gray *et al.* (1994), the present study demonstrated that ET-1, ET-3, STXc produced biphasic contractions of the rabbit saphenous vein over a broad concentration-range, suggestive of negative cooperativity. Curve-fitting identified two distinct sites of high (pM) and low (nM) affinity. Significant contractile responses could be detected with agonist concentrations as low as 0.2 pM, a value similar to that required for ET-1 to elicit a positive inotropic response in rabbit papillary muscle (Kasai *et al.*, 1994). The rank order of contractile potency at the high affinity site was consistent with this being an ET_B receptor-mediated response (ET-1 = ET-3). In contrast, however, based on the definitions adopted by the IUPHAR Committee on Receptor Nomenclature (Masaki *et al.*, 1994), the low affinity site had the characteristics of an ET_C receptor since ET-3 and STXc were 14 and 35 fold more potent than ET-1 at this site. This is in accord with previous reports where STXc was a more potent vasoconstrictor than ET-1 in rabbit and human saphenous veins and pulmonary arteries (Moreland *et al.*, 1992; LaDoucer *et al.*, 1993; White *et al.*, 1994).

Further support for the hypothesis that contraction of the rabbit saphenous vein is mediated by two non-ET_A receptors comes from the observation that neither component of the

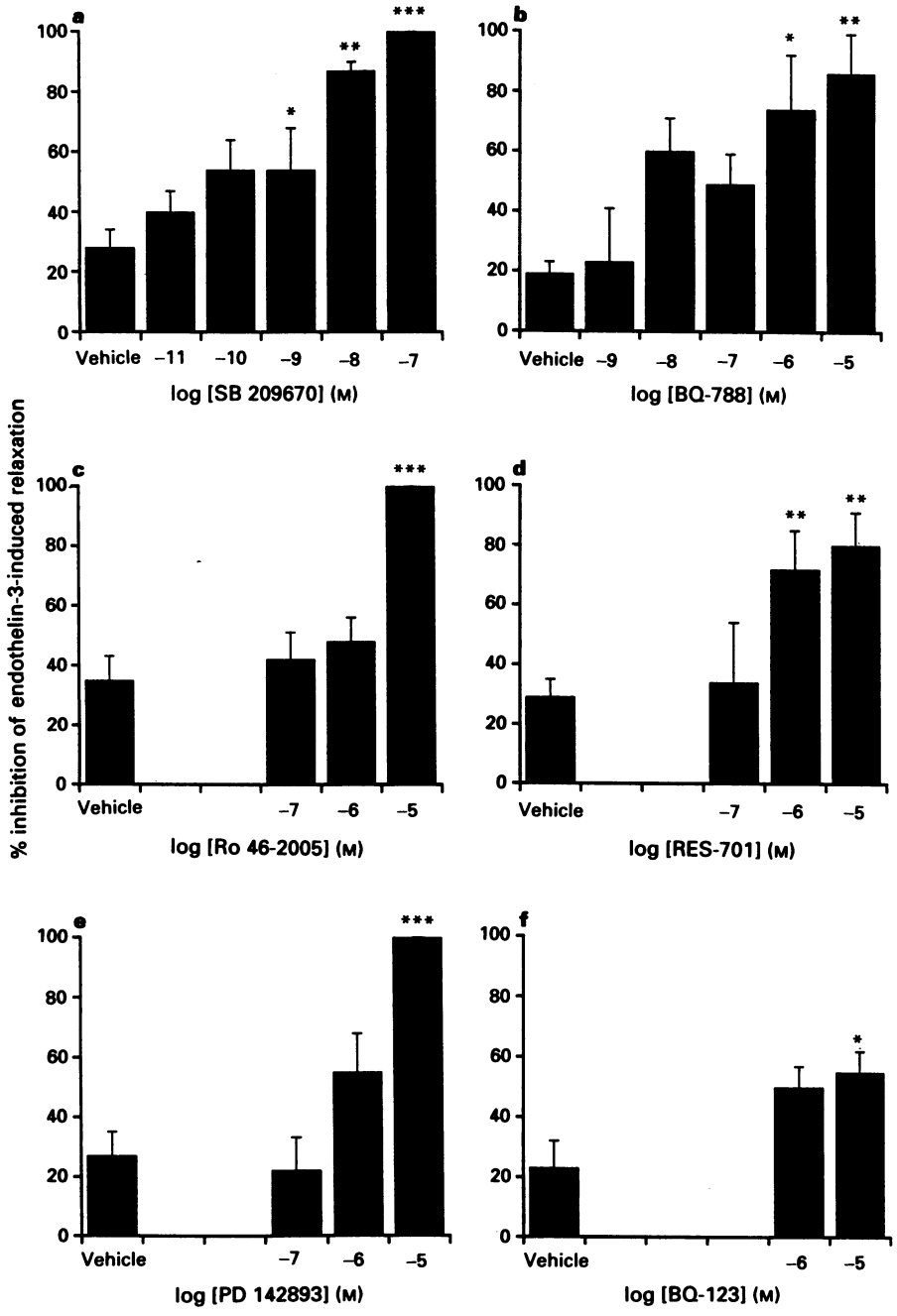

Figure 9 Inhibitory action of ET receptor antagonists on the vasodilator responses obtained to 1 nM ET-3 in endothelium-intact isolated saphenous vein rings of rabbit preconstricted with 1 μM noradrenaline. Responses were obtained in the presence of (a) SB 209670 (10 pM–100 nM; $n = 5$), (b) BQ-788 (1 nM–10 μM; $n = 3$), (c) Ro 46-2005 (0.1–10 μM; $n = 4$), (d) RES-701 (0.1–10 μM; $n = 6$), (e) PD 142893 (0.1–10 μM; $n = 5$) or (f) BQ-123 (1–10 μM; $n = 4$). Values are the mean ± s.e.mean and n represents the number of tissues studied in a particular group and statistical significance: $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for values obtained in the presence of antagonist compared to control values obtained in the presence of vehicle alone (ANOVA; Fisher's protected least squares difference). For abbreviations, see text.

biphasic contractile response to ET-1, ET-3 or STXc is inhibited by BQ-123. Similar findings have been reported by Moreland *et al.* (1992, 1994) and Sudjarwo *et al.* (1993) where ET-induced contractions of rabbit, monkey and porcine isolated saphenous veins were insensitive to the ET_A-selective antagonists, BQ-123 and FR 139317. Indeed, whilst several investigators have demonstrated weak inhibition of peptide-induced contraction of isolated saphenous veins by BQ-123, the pA_2 determined for this antagonist (approximately 6) is not consistent with this being the result of ET_A receptor occupation (Auguet *et al.*, 1992; Bax *et al.*, 1993; White *et al.*, 1994). The present findings are, in part, consistent with radioligand binding studies where [^{125}I]-ET-3 recognizes a high and low affinity form of an ET_B receptor in the rabbit saphenous vein (Webb *et al.*, 1993; Gray *et al.*, 1994). However, radioligand binding studies clearly demonstrate the presence of BQ-123-sensitive [^{125}I]-ET-1 binding sites and ET_A receptor mRNA in this tissue (Webb *et al.*, 1993; Gray *et al.*, 1994). Indeed, Gray *et al.* (1994) have demonstrated that 20% of the contractile response to the ET isopeptides was mediated by a BQ-123-sensitive, low affinity receptor. The reason for the disparity between the current data and the findings of Gray *et al.* (1994) is unclear but may reflect differences in the strains of rabbit used (Burgandy *versus* New Zealand White) or, alternatively, may be a function of the segment of saphenous vein studied (lateral *versus* medial

saphenous vein). Subtle differences in the precise anatomical origin of the vein may be of pharmacological significance since the relative contribution of specific ET receptor subtypes to peptide-induced vasoconstriction varies as one progresses along a vascular tree (Godfraind, 1994a,b; MacLean *et al.*, 1994). Unfortunately, the precise anatomical origin of the saphenous veins used by Gray *et al.* (1994) and Webb *et al.* (1994) was not stated.

Multiple functional ET_B receptors are present within the mammalian cardiovascular system: Warner *et al.* (1993a,b) have identified an ET_{B1} receptor (linked to endothelium-dependent vasodilatation in the rat mesentery) and an ET_{B2} receptor (which mediates contraction of the rat stomach strip and rabbit pulmonary artery). In addition to their anatomical and functional distinctions, the ET_{B1} and ET_{B2} receptors differ pharmacologically since only the ET_{B1} receptor is sensitive to PD 142893 (Warner *et al.*, 1993a,b). Since the high affinity site described in this study is insensitive PD 142893, it may correspond to an ET_{B2} subtype. This hypothesis is supported by the observation that [$Ala^{3,11}$]-ET-1, a peptide which is inactive at the ET_{B1} (Douglas & Hiley, 1990; 1991a; Topouzis *et al.*, 1991), recognizes the high affinity site in the saphenous vein. The contractile ET_{B2} and ET_C receptors were further distinguished by use of a variety of ET receptor antagonists. SB 209670 was a potent, competitive antagonist at both the high and low affinity sites and was one to two orders of magnitude and equipotent with Ro 47-0203 at these two sites, respectively. In addition, both Ro 46-2005 and BQ-788 inhibited the high and low affinity sites. In contrast, however, not only was the ET_{B2} receptor insensitive to BQ-123 and PD 142893, but it was also insensitive to RES-701. This contrasts the low affinity ET_C site which, although insensitive to BQ-123, was inhibited by both PD 142893 and RES-701.

In accord with Poli *et al.* (1991), $ET_{(16-21)}$ was ineffective as a vasoconstrictor in this tissue. Thus, in contrast to the original reports by Maggi *et al.* (1989), and in support of subsequent radioligand binding studies (Tschirhart *et al.*, 1991; Buchan *et al.*, 1994), $ET_{(16-21)}$ does not act as an agonist at the ET receptors responsible for mediating contraction of the rabbit saphenous vein.

SB 209670 differentially antagonized the high (but not the low) affinity phase of the concentration-response curve to ET-1, ET-3 and STXc in a fashion dependent on the competing agonist. Whilst this phenomenon contradicts conventional drug-receptor theory, a similar finding has been reported for SB 209670 in the rat pulmonary artery (ET_{B2} receptor-mediated contraction; Ohlstein *et al.*, 1994a) and has also been observed with BQ-123 and PD 142893 in numerous vascular preparations (Bax *et al.*, 1993; 1994; Warner *et al.*, 1993a; Kasai *et al.*, 1994; Smith *et al.*, 1994). The mechanism underlying this type of phenomenon is unknown, although it has been suggested that ET-3 and STXc interact with an additional site present on ET receptors which is inaccessible to ET-1 (Hiley *et al.*, 1992; Smith *et al.*, 1994).

The endothelium attenuates the contractile actions of the ET isopeptides by stimulating the release of nitric oxide (Warner *et al.*, 1989; Douglas & Hiley, 1990; 1991a). In the present study, both components of the biphasic concentration-response curves to ET-1, ET-3 and STXc were shifted by approximately 3 fold following endothelial destruction, consistent with previous observations made in human, canine and rabbit saphenous veins (Berti *et al.*, 1990; Poli *et al.*, 1991; Simonet *et al.*, 1992; Auguet *et al.*, 1993; Akar *et al.*, 1994). Indeed, in the presence of active tone, ET-3 produced endothelium-dependent vasorelaxation in this tissue. Vasorelaxation was also observed with ET-1 and STXc, although, in accord with Auguet *et al.* (1993), such responses were quantitatively less pronounced than those to ET-3. Based on the threshold concentration of peptide required to elicit a dilator response, vasorelaxation appeared to be mediated by a non-ET_A receptor subtype. Indeed, relaxation was insensitive to 1 μM BQ-123. Antagonist IC_{50}s were approximated as being: SB 209670, 3 nM; BQ-788 and RES 701, 300 nM; Ro 46-2005 and PD 142893, 3 μM; BQ-123, $\geqslant$ 10 μM. Since ET-3-induced vasorelaxation was antagonized by PD 142893, this response appeared to be mediated by an ET_{B1} receptor.

In summary, three pharmacologically distinct, functional ET receptor subtypes have been identified in the rabbit saphenous vein. Two contractile receptors are present on the vascular smooth muscle: a high affinity, low efficacy ET_{B2} receptor (i.e. STXc = ET-3 = ET-1; SB 209670 > Ro 47-0203 > BQ-788 > Ro 46-2005; insensitive to PD 142893, RES-701 and BQ-123) and a distinct lower affinity, high efficacy ET_C receptor (i.e. STXc $\geqslant$ ET-3 > ET-1; SB 209670 $\geqslant$ Ro 47-0203 > Ro 46-2005 and BQ-788 > RES 701 and PD 142893; insensitive to BQ-123). In addition, an ET_{B1} receptor is present on the endothelium which mediates the relaxant actions of the ET isopeptides (i.e. ET-3 $\geqslant$ ET-1 $\geqslant$ STXc; SB 209670 $\geqslant$ BQ-788 = RES 701 $\geqslant$ Ro 46-2005 and PD 142893; insensitive to BQ-123). However, although the available pharmacological data suggest the presence heterogeneous, functional receptor subtypes in this tissue, confirmation by conventional protein purification/molecular cloning is necessary before their existence can be firmly established. It is appropriate to note, however, that low stringency homology screening has only been able to identify mRNA transcripts in the rabbit saphenous vein which appear to correspond to the known cloned ET_A and ET_B receptors (Webb *et al.*, 1993). If novel mRNA species are present in this tissue, they are likely to either (i) differ markedly from those encoding for the known ET_A and ET_B receptors or (ii) be so similar to the known cloned receptors that it is readily mistaken for a known subtype during homology screening. To this end, it is interesting that a single nucleotide point mutation of the codon for residue Tyr^{129} present in the wild type human cloned ET_A receptor (e.g. from TAT [Tyr^{129}] to TTT [Phe^{129}]) can dramatically alter the pharmacological characteristics of the mutant receptor such that, essentially, it resembles an ET_B subtype (Lee *et al.*, 1994). Alternatively, it is conceivable that the high and low affinity sites linked to saphenous vein contraction are derived from a single gene product, differing only by the extent to which they are subject to post translational modification (e.g. proteolytic maturation or glycosylation).

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