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Use of the endothelin antagonists BQ-123 and PD 142893 to reveal three endothelin receptors mediating smooth muscle contraction and the release of EDRF

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1 We have compared the receptors mediating the contractions of rings of rat thoracic aorta or rabbit pulmonary artery and rat stomach strips in response to the endothelin/sarafotoxin (ET/SX) family of peptides and to those mediating endothelium-dependent vasodilatations within the isolated perfused mesentery of the rat. To discriminate ET_A receptors from ET_B receptors we have used the criteria that ET-1 is more active than SX6c on ET_A receptors, and that the ET/SX peptides are equiactive on ET_B receptors. We have also assessed the effects of the ET_A receptor-selective antagonist BQ-123, and the non-selective ET receptor antagonist PD 142893 on the responses of each preparation to the ET/SX peptides.

2 ET-1-induced constrictions of the rat thoracic aorta (EC_{50} 3×10^{-10} M), a prototypic ET_A receptor-mediated response, or isolated perfused mesentery of the rat were antagonized by BQ-123 (10^{-5} M) or PD 142893 (10^{-5} M). SX6c did not constrict either the rat isolated perfused mesentery or the rat thoracic aorta. Thus, ET_A receptors mediate these constrictions.

3 ET-1 and SX6c were approximately equipotent in constricting rabbit pulmonary artery rings (EC_{50}s $3-6 \times 10^{-10}$ M). Neither BQ-123 (10^{-5} M) nor PD 142893 antagonized the contractions induced by ET-1. These effects suggest mediation by ET_B receptors but PD 142893 (10^{-5} M) did give a 3 fold antagonism of constrictions induced by SX6c.

4 SX6c was more potent than ET-1 in contracting the rat stomach strip (threshold concentrations 10^{-10} and 3×10^{-10} M). Contractions to ET-1 or SX6c were unaffected by BQ-123 (10^{-5} M), again indicative of ET_B receptor-mediated events. PD 142893 (10^{-5} M) was ineffective against ET-1 but produced a 3 fold antagonism of SX6c.

5 In the rat isolated perfused mesentery ET-1 or SX6c (0.3–300 pmol) were equipotent in producing dose-related vasodilatations that were unaffected by BQ-123 (10^{-6} M), indicative of an ET_B receptor-mediated response. In contrast to the other ET_B-mediated responses, PD 142893 (10^{-6} M) strongly antagonized these vasodilatations.

6 Thus, ET_A receptors mediate constrictions of the rat thoracic aorta and rat isolated perfused mesentery whereas ET_B receptors mediate constrictions of the rabbit pulmonary artery and rat stomach strip and endothelium-dependent dilatations within the mesentery. However, within the group of ET_B receptor-mediated responses, endothelium-dependent vasodilatations are sensitive to PD 142893, whereas contractions of the isolated smooth muscle preparations are not. Thus, the receptor present on the endothelium responsible for the release of nitric oxide in response to the ET/SX peptides is most probably different from that present on smooth muscle that mediates BQ-123-insensitive contractions.

Keywords: Endothelin-1; endothelin receptors; endothelium-dependent relaxations

Introduction

The endothelins constitute a family of three structurally very closely related peptides (Inoue *et al.*, 1989) that may well be expressed in all mammalian species for the genes for endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) are present for instance in human, porcine, rat and murine tissues (Inoue *et al.*, 1989; Saida *et al.*, 1989). Among non-mammalian species the endothelins are closely related to the sarafotoxins, which are present in the venom of *Atractaspis engaddensis* (Kloog *et al.*, 1988; Bdolah *et al.*, 1989). The effects of these peptides are considered to be mediated by two receptors. The ET_A receptor has several hundred fold more affinity for binding ET-1, ET-2 or SX6b than ET-3 or SX6c whereas the ET_B receptor binds the ET/SX peptides with equal affinity (Ambar *et al.*, 1989; Arai *et al.*, 1990; Sakurai *et al.*, 1990; Saeki *et al.*, 1991; Williams *et al.*, 1991; Clozel *et al.*, 1992). Contractions of different isolated smooth muscle preparations may be mediated by either ET_A or ET_B receptors (Harrison *et al.*, 1992; Hay, 1992; Moreland *et al.*, 1992). In addition, the ET_B receptor is present on endothelial and other cells and mediates the release of endothelium-derived relaxing factor/nitric oxide (EDRF/NO) (Warner *et al.*, 1989; Ishii *et al.*, 1991). However, there have also been reports of an endothelial receptor that selectively responds to ET-3 (Emori *et al.*, 1990) and mediates a prolonged release of EDRF/NO (Warner *et al.*, 1992b). Thus, the classification of receptors as ET_A and ET_B may not describe all the existing receptor subtypes. BQ-123 (cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-)) is a selective ET_A receptor antagonist (Ihara *et al.*, 1992) and PD 142893 (Ac-(3,3-D-diphenylalanyl)-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp trifluoroacetate) is a non-selective ET_A/ET_B receptor antagonist (Hingorani *et al.*, 1992; LaDouceur *et al.*, 1992; Cody *et al.*, 1992). Using these antagonists and ET-1 and SX6c as agonists we have compared the receptors mediating the constrictor responses of the rat aorta (a prototypic ET_A receptor mediated response, Maggi *et al.*, 1989) that is antagonized by BQ-123 (pA_2 6.93, Sumner *et al.*, 1992), the rabbit pulmonary artery (a prototypic ET_b receptor mediated response; Maggi *et al.*, 1990; Panek *et al.*, 1992) and the rat stomach strip, and the vasodilatations of the rat mesentery (a

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prototypic endothelium-endothelin receptor mediated response; Warner *et al.*, 1989).

Some of these data have been presented to the British Pharmacological Society (Warner *et al.*, 1992a; 1993).

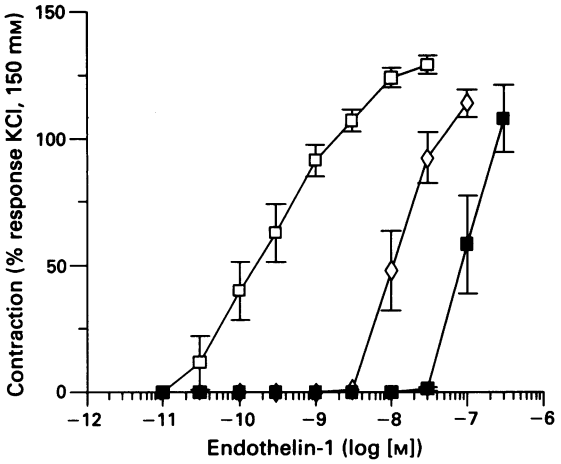

Figure 1 Effects of BQ-123 and PD 142893 on the contractile responses of the rat thoracic aorta to endothelin-1 (ET-1). Data are presented as the contractions of rings of rat thoracic aorta to ET-1 expressed as % of the contraction to KCl (150 mM). Control (□); BQ-123 (10^{-5} M), (■); PD 142893 (10^{-5} M), (◇). Results are calculated as the mean (± s.e.mean) ($n \geq 6$).

Methods

Organ bath experiments

Male Wistar rats (250–400 g) or New Zealand white rabbits were killed with thiopentone sodium (Sagatal, 120 mg kg^{-1}, i.p. or i.v.). Strips prepared from the fundus of the rat stomach (resting tension 2 g) and rings of rat aorta with the endothelium removed (2–3 mm, resting tension 1 g) and the first branches of the rabbit pulmonary artery (3 mm, resting tension 1 g) were set up in isolated organ baths containing 10 ml of Krebs buffer. Responses of the tissues were detected by isometric transducers (Hugo Sachs Electronik, Germany) and displayed on a chart recorder (Graphtec WR3101). The bathing Krebs solution, which was gassed with 95% O_2:5% CO_2 at a temperature of 37°C, contained bacitracin (3 mg l^{-1}), bovine serum albumin (50 mg l^{-1}), indomethacin (5×10^{-6} M), thiorphan (10^{-6} M), captopril (10^{-6} M) and bestatin (10^{-6} M) to exclude indirect effects induced by the production of prostanoids and to minimize the possible local metabolism by tissue enzymes (Maggi *et al.*, 1989). In a range of different isolated tissues we have found that this can produce up to a 1 log unit decrease in the threshold concentration of ET-1 (data not shown, Maggi *et al.*, 1990). After an equilibration period of 1 h the tissues were contracted by 0.15 M KCl. After wash out the tissues were incubated with either BQ-123 (10^{-5} M), PD 142893 (10^{-5} M) or vehicle for 20 min before being exposed to cumulative concentrations of ET-1 (10^{-11} to 3×10^{-7} M). Each tissue was used for only one curve. Contractile responses to the ET/SX peptides were standardized

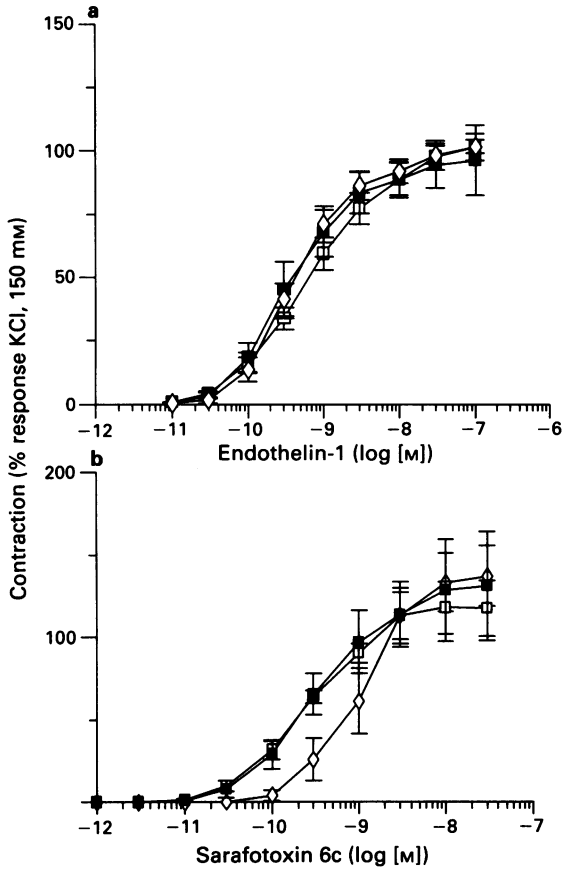

Figure 2 (a) Effects of BQ-123 and PD 142893 on the contractile responses of the rabbit pulmonary artery to endothelin-1 (ET-1). Data are presented as the contractions of rings of rabbit pulmonary artery to ET-1 expressed as % of the contraction to KCl (150 mM). (b) Effects of BQ-123 and PD 124893 on the contractile responses of the rabbit pulmonary artery to sarafotoxin 6c (SX6c). Control (□); BQ-123 (10^{-5} M), (■); PD 142893 (10^{-5} M), (◇). Results are calculated as the mean (± s.e.mean) ($n \geq 6$).

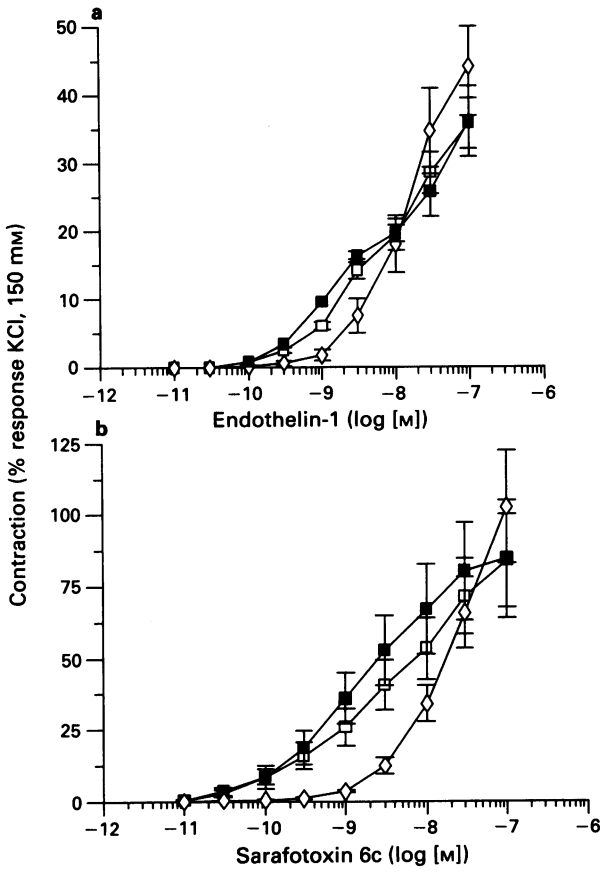

Figure 3 (a) Effects of BQ-123 and PD 142893 on the contractile responses of the rat stomach strip to endothelin-1 (ET-1). (b) Effects of BQ-123 and PD 142893 on the contractile responses of the rat stomach strip to sarafotoxin 6c (SX6c). Data are presented as the contractions of the tissues to ET-1 expressed as % of the contraction to KCl (150 mM). Control (□); BQ-123 (10^{-5} M), (■); PD 142893 (10^{-5} M), (◇). Results are calculated as the mean (± s.e.mean) ($n \geq 6$).

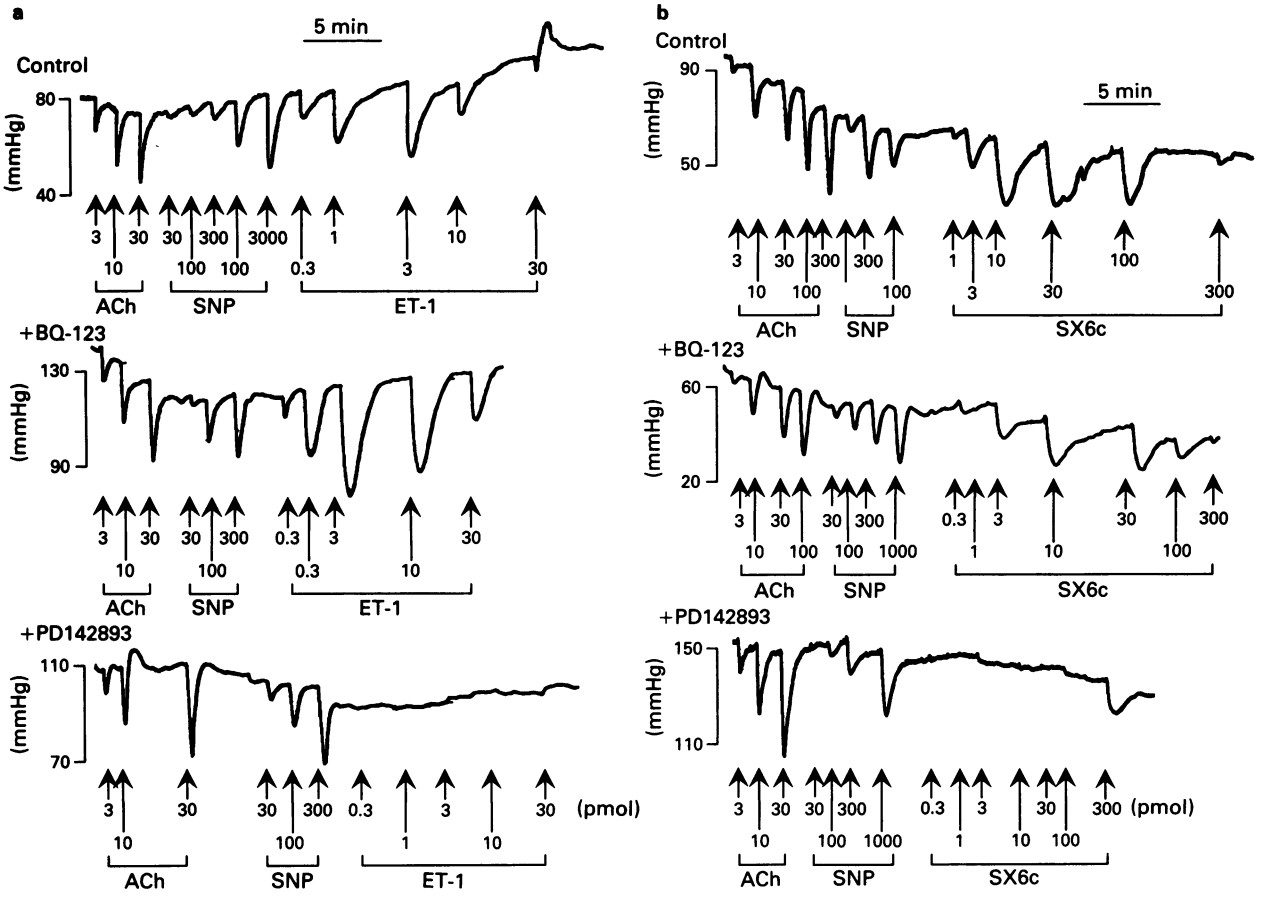

Figure 4 Representative traces of the effects of BQ-123 and PD 142893 on the vasodilatations of the rat mesentery in response to bolus injections of endothelin-1 (ET-1, 0.3–30 pmol) or sarafotoxin 6c (SX6c, 0.3–300 pmol). (a) Vasodilatations of the rat mesentery to ET-1. Upper panel, control; middle panel, in the presence of BQ-123 (10^{-6} M); lower panel, in the presence of PD 142893 (10^{-6} M). (b) Vasodilatations of the rat mesentery to SX6c. Upper panel, control; middle panel, in the presence of BQ-123 (10^{-6} M); lower panel, in the presence of PD 142893 (10^{-6} M). Tone was introduced into the preparations by the infusion of methoxamine (5×10^{-5}–1×10^{-4} M). ACh – acetylcholine (3–300 pmol); SNP – sodium nitroprusside (30–3000 pmol). Each trace is representative of at least 4 experiments.

by comparison to the KCl contraction. In some experiments we confirmed the removal of the endothelial cells from preparations by the lack of relaxation in response to acetylcholine (10^{-6} M).

Isolated perfused mesentery of the rat

The isolated perfused mesentery of the rat was prepared by the method of McGregor (1965). Male albino Wistar rats were anaesthetized with pentobarbitone sodium (120 mg kg^{-1}, i.p.), the abdomen opened and the ileocolic and colic branches of the superior mesenteric artery ligated. The superior mesenteric artery was cannulated and the superior mesenteric vascular bed perfused via the artery for 5 min (2 ml min^{-1}) with Krebs buffer containing heparin (100 u ml^{-1}). The animal was then killed by cutting the diaphragm. The intestine was separated from the mesentery and the preparation supported on a petri-dish and the arteries perfused at a constant flow of 5 ml min^{-1} with warmed (37°C) and gassed (95% O_2:5% CO_2) Krebs buffer containing indomethacin (5×10^{-6} M) to block cyclo-oxygenase activity. Changes in perfusion pressure were measured with a transducer approximately 15 cm from the tip of the arterial cannula (Warner *et al.*, 1989). After an equilibration period of 30 min the mesenteric vessels were constricted by methoxamine (5×10^{-5} M). Vehicle, BQ-123 (10^{-6} M) or PD 142893 (10^{-6} M) was then infused for 10 min before and continued during, injection of drugs in volumes of 1–3 μl to minimize any injection artefacts.

Materials

The Krebs buffer had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.17, $CaCl_2$. $6H_2O$ 2.5, $NaHCO_3$ 25 and glucose 5.6. BQ-123 and PD 142893 were synthesized by Parke-Davis, MI, U.S.A. ET-1 and SX6c were purchased from Peptide Institute (Osaka, Japan). BQ-123 and the ET/SX peptides were reconstituted in 0.9% w/v saline containing 1% w/v bovine serum albumin and 10 mM sodium bicarbonate. PD 142893 was reconstituted in dimethylsulphoxide (DMSO, 10^{-3} M) and added directly to the organ baths or further diluted into buffer (as for BQ-123 and the ET/SX peptides) for infusion into the rat mesentery. Bovine serum albumin, bacitracin, thiorphan, captopril, bestatin, methoxamine, phenylephrine, sodium nitroprusside and acetylcholine were obtained from Sigma Chemical Co. (Poole, Dorset) and dissolved in either distilled water or saline. Indomethacin (from Sigma) was made as a stock solution in sodium bicarbonate (5%). The salts for the Krebs solution were obtained from BDH (Lutterworth, Leics.).

Statistics

Statistical differences between points were determined by an unpaired two tail Student's t test and between concentration-response curves by two-way analysis of variance. $P<0.05$ was taken as significant.

Results

Rat thoracic aorta (RTA)

Control constrictions of the RTA induced by KCl were 1.2 ± 0.1 g ($n = 50$). ET-1 caused contractions of the RTA with a threshold concentration of 3×10^{-11} M and an EC_{50} of 3×10^{-10} M (Figure 1a). In the presence of BQ-123 (10^{-5} M) or PD 142893 (10^{-5} M) the concentration of ET-1 to produce 75% of the response to KCl (150 mM) was increased from 5×10^{-10} to 1.5×10^{-7} M or 2×10^{-8} M, respectively (Figure 1a, $P < 0.05$ for each). SX6c (up to 10^{-7} M, $n = 4$) did not constrict the RTA.

Rabbit pulmonary artery (RbPA)

Control constrictions of the RbPA induced by KCl were 2.1 ± 0.1 g ($n = 50$). ET-1 caused concentration-dependent contractions of the RbPA (EC_{50}, 6×10^{-10} M) which were unaffected by BQ-123 or PD 142893 (Figure 2a, $P > 0.05$ for each). Contractions induced by SX6c (EC_{50}, 3×10^{-10} M) were antagonized by PD 142893 (EC_{50}, 10^{-9} M, $P < 0.05$) but not by BQ-123 (Figure 2b).

Rat stomach strip (RSS)

Control constrictions of the RSS induced by KCl were 2.4 ± 0.1 g ($n = 50$). SX6c was more potent than ET-1 in contracting the RSS (threshold concentrations, 10^{-10} M and 3×10^{-9} M; concentrations to produce 30% of the response to KCl (150 mM) 7×10^{-10} and 5×10^{-8} M, Figures 3a and 3b). Contractions induced by ET-1 were unaffected by either BQ-123 or PD 142893 (Figure 3a, $P > 0.05$ for each), whereas contractions induced by SX6c (Figure 3b) were antagonized by PD 142893 (concentration to produce 30% of the response to KCl (150 mM) 8×10^{-9} M, $P < 0.05$), although not by BQ-123 (Figure 3b).

Rat isolated perfused mesentery (RMes)

Methoxamine (5×10^{-5} M) increased the perfusion pressure of the RMes by 91 ± 6 mmHg ($n = 36$). In preconstricted preparations ET-1 (Figure 4a) or SX6c (0.3–300 pmol, Figure 4b) produced dose-related vasodilatations. For ET-1 the higher doses caused short-lived vasodilatations followed by prolonged vasoconstrictions. SX6c produced only vasodilatations without secondary vasoconstrictions, although doses of greater than 30 pmol produced progressively smaller vasodilatations (Figure 4b) possibly due to receptor desensitization. BQ-123 (10^{-6} M) had no effect on either the magnitude or the duration of the vasodilatations caused by ET-1 (Figures 4a, 5a and 5b) or SX6c (Figures 4b, 6a and 6b). Infusion of PD 142893 (10^{-6} M) significantly decreased both the magnitude and the duration of the responses to ET-1

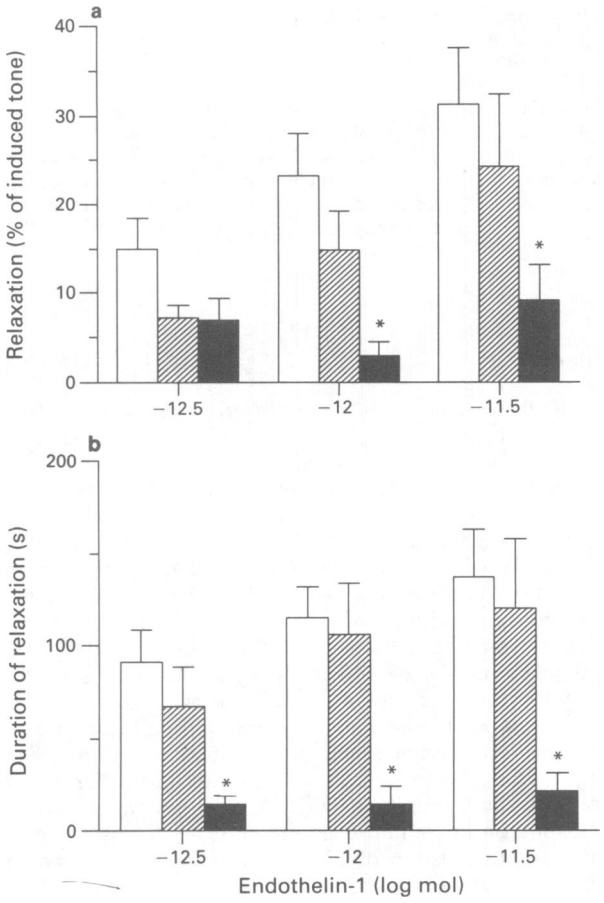

Figure 5 Effect of BQ-123 or PD 142893 on vasodilatations in response to endothelin-1 (ET-1) in the rat mesentery. (a) Vasodilatations in response to bolus injections of ET-1 expressed as % relaxation of tone induced by infusion of methoxamine (5×10^{-5}–1×10^{-4} M). (b) Duration of vasodilatations in response to bolus injections of ET-1. Control, open columns; BQ-123 (10^{-6} M), hatched columns; PD 142893 (10^{-6} M), solid columns. Each column represents the mean ± s.e.mean of at least 4 determinations.

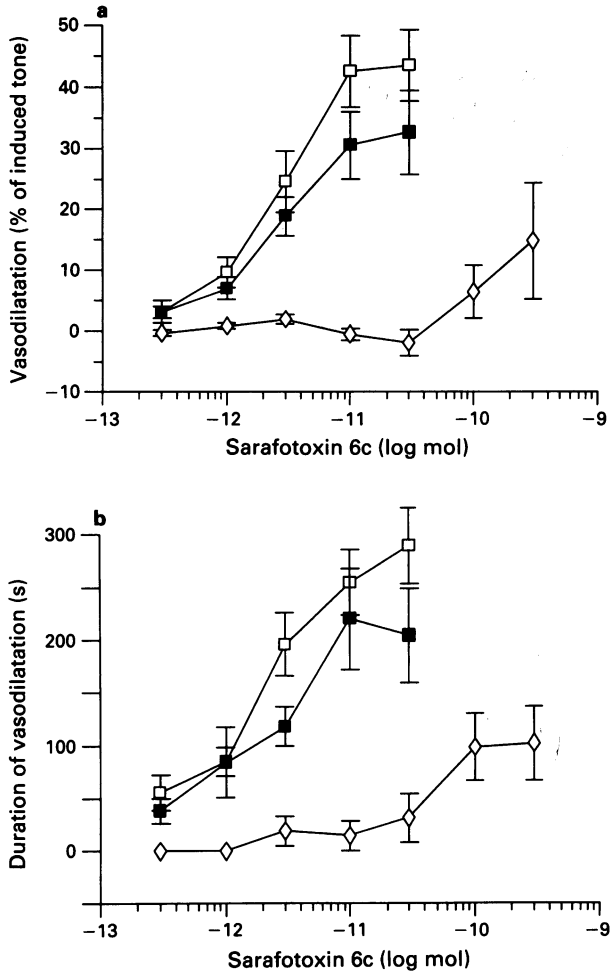

Figure 6 Effect of BQ-123 or PD 142893 on vasodilatations in response to sarafotoxin 6c (SX6c) in the rat mesentery. (a) Vasodilatations in response to bolus injections of SX6c expressed as % relaxation of tone induced by infusion of methoxamine (5×10^{-5}–1×10^{-4} M). (b) Duration of vasodilatations in response to bolus injections of SX6c. Control (□); BQ-123 (10^{-6} M), (■); PD 142893 (10^{-6} M) (◇). Results are calculated as the mean (± s.e.mean) ($n \geq 6$).

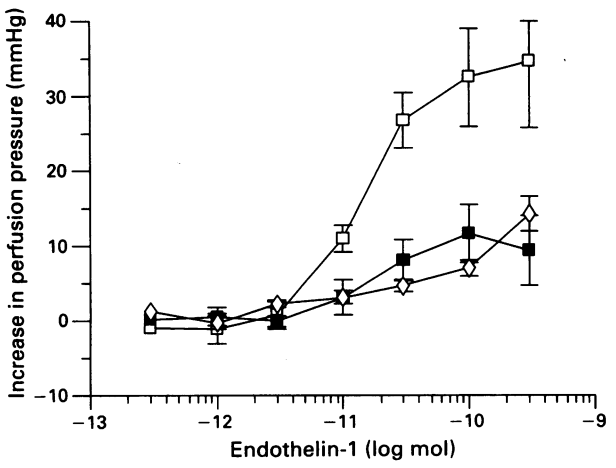

Figure 7 Effect of BQ-123 or PD 142893 on vasoconstriction in response to endothelin-1 (ET-1) in the rat mesentery. Data are expressed as the maximum increase in perfusion pressure following bolus injection of ET-1. Control (□); BQ-123 (10^{-6} M) (■); PD 142893 (10^{-6} M) (◇). Results are calculated as the mean (± s.e.mean) ($n \geqslant 6$).

(Figures 4a, 5a and 5b) or SX6c (Figures 4b, 6a and 6b), causing, for instance, a rightwards shift of 2 orders of magnitude in the dose-response curve to SX6c. BQ-123 and PD 142893 both strongly antagonized the secondary vasoconstriction induced by ET-1 (Figures 4a, 4b and 7). BQ-123 and PD 142893 did not affect endothelium-dependent vasodilatations in response to acetylcholine, or endothelium-independent vasodilatations in response to sodium nitroprusside (Figures 4a and 4b, $n \geqslant 8$ for each).

Discussion

Here we have compared the activities of the two endothelin receptor antagonists, BQ-123 and PD 142893, on the responses to ET-1 and SX6c of isolated vascular and non-vascular preparations. Our data indicate that the division ET_A and ET_B may not be sufficient to describe all endothelin-receptor subtypes, for the ET_B receptors present on smooth muscle are functionally distinct from those present on the endothelium.

The effects of ET-1 that are mediated via ET_A receptors are sensitive to blockade by BQ-123, whereas those that are mediated via ET_B receptors are BQ-123-insensitive (Ihara *et al.*, 1992; Hay, 1992; Moreland *et al.*, 1992; Sumner *et al.*, 1992). For instance, BQ-123 antagonizes the constrictions of the guinea-pig aorta induced by ET-1 but not the guinea-pig bronchus, and so these responses have been described as ET_A and ET_B receptor-mediated, respectively (Hay, 1992). We found that BQ-123 effectively antagonized constrictions of the RTA (Sumner *et al.*, 1992) and RMes induced by ET-1, but not those of the RbPA or RSS. Thus, constrictions of the RTA and RMes induced by ET-1 represent ET_A receptor-mediated events, whereas the contractions of the RbPA and RSS as well as the vasodilatations of the RMes are ET_B receptor-mediated events. This would correlate with the relative potencies of the other ET/SX peptides because we also observed that SX6c was very much less potent than ET-1 as a constrictor of the RTA (Panek *et al.*, 1992) or RMes, typical of an ET_A receptor-mediated response, whereas in the RbPA (Maggi *et al.*, 1989; Panek *et al.*, 1992) or RSS (Spokes *et al.*, 1989) SX6c was equipotent with, or even more potent than ET-1, typical of ET_B receptor-mediated responses.

Thus, the relative activities of the ET/SX peptides and the effects of BQ-123 are consistent with a division of ET-receptors into two groups ET_A and ET_B. However, our experiments using PD 142893 are not consistent with this idea. PD 142893 antagonized ET_A-mediated responses, i.e. constrictions of the RTA or RMes, in agreement with previous reports (Cody *et al.*, 1992). However, PD 142893 revealed differences in those effects of ET-1 that were insensitive to BQ-123, in that it antagonized effectively the vasodilatations of the RMes induced by ET-1, but it had no effect on ET-1-induced constrictions of the RbPA or RSS. PD 142893 slightly inhibited constrictions of the RbPA or RSS induced by SX6c, causing approximately three fold shifts in the SX6c concentration-response curves. In comparison PD 142893, at a ten times lower concentration, caused a 100 fold shift in the vasodilator response curve to SX6c in the RMes. This suggests that the ET_B receptors consist of two populations. Although all appear to be equally sensitive to the ET/SX peptides and insensitive to the effects of BQ-123, some such as those mediating the release of EDRF/NO are blocked more readily by PD 142893. These apparent differences between ET_B receptors may correspond with recent reports that different populations of high- and low-affinity ET_B receptors are present in brain (Sokolovsky *et al.*, 1992). As low stringency Southern blot analysis of human genomic DNA revealed only two genes, most probably corresponding to the ET_A and ET_B receptors (Sakamoto *et al.*, 1991), this third receptor type may represent a post-translational modification of the ET_B receptor, or a novel, unidentified receptor with low sequence similarities to the other two receptors.

Our finding that neither BQ-123 nor PD 142893 antagonized the effects of ET-1 on the RbPA is consistent with data derived from receptor binding assays (Panek *et al.*, 1992) and confirms earlier reports that ET_A receptors are not uniformly expressed on vascular smooth muscle (Harrison *et al.*, 1992; Moreland *et al.*, 1992). It is also in agreement with our observations that BQ-123 does not fully antagonize the pressor effects of the ET/SX peptides in the ganglion-blocked rat (McMurdo *et al.*, 1993) nor the vasoconstrictions in response to the ET/SX peptides in all vascular beds in the anaesthetized rat (Cristol *et al.*, 1993).

In conclusion, our results suggest that there are at least three ET receptor types. Constrictions of the RTA and RMes appear to be mediated by ET_A receptors, as defined both by the selective effects of the ET/SX peptides and by antagonism with BQ-123. Endothelium-dependent vasodilatations of the RMes and contractions of the RbPA and RSS fit the profile of effects mediated by ET_B receptors, as defined by the non-selectivity of the ET/SX peptides and the lack of antagonism by BQ-123. However, the difference in sensitivity of these ET_B receptor-mediated responses to PD 142893 distinguish the release of EDRF/NO and the constrictions of the isolated smooth muscle preparations as being mediated by different receptor types.

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