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# Failure of BQ123, a more potent antagonist of sarafotoxin 6b than of endothelin-1, to distinguish between these agonists in binding experiments

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**1** In homogenates of human saphenous vein, [$^{125}$I]-ET-1 and [$^{125}$I]-S6b each labelled a single population of high affinity binding sites with $K_D$ values of $0.64 \pm 0.11$ nM and $0.55 \pm 0.08$ nM respectively. Hill slopes were close to one. However, the density of receptors labelled by [$^{125}$I]-ET-1 was significantly greater than that by [$^{125}$I]-S6b ($187.6 \pm 23.0$ compared to $91.7 \pm 23.6$ fmol mg$^{-1}$ protein, $P < 0.02$).

**2** BQ123, an $ET_A$-selective antagonist, inhibited specific [$^{125}$I]-ET-1 and [$^{125}$I]-S6b binding with equal affinity. BQ123 competed in a biphasic manner for both [$^{125}$I]-ET-1 (0.1 nM) and [$^{125}$I]-S6b (0.1 nM) with $ET_A$ $K_D$ values of $0.55 \pm 0.17$ nM and $0.52 \pm 0.02$ nM and $ET_B$ $K_D$ values of $14.4 \pm 2.60$ $\mu$M and $11.2 \pm 0.31$ $\mu$M respectively. S6b monophasically inhibited 0.1 nM [$^{125}$I]-ET-1 ($K_D$ $1.16 \pm 0.9$ nM) but competed for 0.25 nM [$^{125}$I]-ET-1 in a bipahasic manner ($K_D$ high affinity site $1.99 \pm 0.84$ nM, $K_D$ low affinity site $0.68 \pm 0.63$ $\mu$M, ratio 67% : 33%).

**3** BQ123 antagonized the vasoconstrictor responses of ET-1 with a $pK_B$ value of 6.47 whereas BQ123 exhibited 50 fold higher affinity against S6b-mediated vasoconstriction with a $pK_B$ value of 8.18. Regression slopes were $0.80 \pm 0.13$ and $1.08 \pm 0.11$ respectively.

**4** In desensitization experiments, S6b (300 nM) did not contract preparations which were no longer responsive to ET-1 whereas a small contraction to ET-1 (300 nM) was obtained in preparations rendered unresponsive to S6b.

**5** Medial sections of non-diseased human aorta, which express only $ET_A$ receptors, were used to compare dissociation rates of the two agonists. The time course for the dissociation of [$^{125}$I]-ET-1 and [$^{125}$I]-S6b was similar with 20–30% of each ligand dissociating at 4 h.

**6** These data suggest that whilst BQ123, in common with other endothelin antagonists, is a much more potent blocker of S6b contractile responses than of ET-1 contractile responses, this is not reflected by the equal affinity of BQ123 determined in competition binding experiments against both [$^{125}$I]-ET-1 and [$^{125}$I]-S6b. This discrepancy in antagonist potency is probably not due to a marked difference in the rate of dissociation of [$^{125}$I]-ET-1 and [$^{125}$I]-S6b from endothelin receptors. One possible explanation is that ET-1 is activating an additional population of receptors which may have lower affinity for BQ123. This is suggested by the discrepancy in receptor density identified by [$^{125}$I]-ET-1 and [$^{125}$I]-S6b.

**Keywords:** BQ123; [$^{125}$I]-ET-1; [$^{125}$I]-S6b; endothelin receptor subtypes; human saphenous vein; saturation and competition binding; *in vitro* pharmacology; antagonist potency

## Introduction

Endothelin (ET) receptors are currently defined by the order of potency of the three ET peptide isoforms such that at the $ET_A$ receptor ET-1 and ET-2 have equal affinity and are more potent than ET-3, whereas the $ET_B$ receptor does not distinguish among the three peptides. This pattern of agonist preference facilitated the identification of expressed gene products as either $ET_A$ (Arai *et al.*, 1990) or $ET_B$ receptors (Sakurai *et al.*, 1990) and remains crucial in attributing an ET-mediated event to one of the two receptor subtypes. A second family of peptides, the sarafotoxins, has been described which possesses striking sequence homology to the endothelin peptides although they are of non-mammalian origin (Kloog *et al.*, 1988). Sarafotoxin 6b (S6b), like ET-1, does not distinguish between the two endothelin receptor subtypes whereas sarafotoxin 6c (S6c) is a selective $ET_B$ agonist (Williams *et al.*, 1991).

The availability of selective endothelin receptor agonists and antagonists initially allowed for a more rigorous classification of the two receptors, particularly in terms of antagonist selectivity and potencies. Recently, however, the use of these compounds has led to reports of ET-mediated responses that do not appear to fit the current receptor classification and has led to speculation that further subtypes of endothelin receptors exist (for review see Bax & Saxena, 1994). Particularly in *in vitro* studies, ET responses have been observed with a pharmacological profile which suggests that they are not mediated either by the $ET_A$ receptor or by the $ET_B$ receptor as these are identified at present. The most frequently described 'atypical' response is that antagonists such as BQ123 and FR139317, in preparations containing predominantly $ET_A$ receptors, are more potent against ET-3 or S6b than against ET-1 itself. This has been observed in both vascular and non-vascular smooth muscle preparations such as the rat aorta (Summer *et al.*, 1992), goat cerebral artery (Salom *et al.*, 1993), human saphenous vein (Bax *et al.*, 1993a), human coronary artery (Godfraind, 1993), human umbilical artery (Bodelsson & Stjernquist, 1993), human omental vein (Riezebos *et al.*, 1994) and rat vas deferens (Eglezos *et al.*, 1993). One explanation for these results is that ET-1 mediates its effects through a different receptor subtype from either ET-3 or S6b. It is also possible that in addition to a common binding site, ET-1 is able to activate a further receptor population that is insensitive to available antagonists. Interestingly we are not aware of any report demonstrating that ET-1 and S6b can be blocked with equal potency by any of the available antagonists. Thus these apparently aberrant observations are actually typical.

We have previously reported that BQ123 competes for iodinated ET-1 in thin sections of human tissues with subnanomolar affinity (Karet *et al.*, 1993; Davenport *et al.*, 1995).

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We have also demonstrated that BQ123 blocks ET-1 mediated vasoconstriction, in human blood vessels (Maguire *et al.*, 1994; Maguire & Davenport, 1995) with $pA_2$ values comparable to those reported by others in animal $ET_A$ *in vitro* preparations (Ihara *et al.*, 1992; Moreland *et al.*, 1992; Sumner *et al.*, 1992; Hay *et al.*, 1993). Using human saphenous vein, the aim of the present work was firstly to determine by saturation binding analysis whether or not [$^{125}$I]-ET-1 and [$^{125}$I]-S6b identify identical receptor populations and to show if there is a discrepancy between the ability of BQ123 to compete for each of the two radiolabelled peptides. This was compared to the potency of BQ123 as an antagonist of ET-1 and S6b-mediated vasoconstriction in human isolated saphenous veins. Finally, dissociation binding experiments were carried out to attempt to address the observed anomalies.

A preliminary account of this work was presented to the British Pharmacological Society (Maguire *et al.*, 1995).

## Methods

### *Tissue collection*

Human saphenous vein was obtained from 198 individuals (145 male, 53 female; 39–83 years old) receiving coronary artery bypass grafts and non-diseased human aorta from four patients undergoing heart transplantation. Tissue was collected with local ethical approval. These blood vessels were either frozen immediately in liquid nitrogen and stored at −70°C until required for homogenate preparation or, for *in vitro* experiments, collected in oxygenated Krebs solution and kept refrigerated until use.

### *Saphenous vein homogenate preparation*

Tissue homogenates were prepared in four separate batches. Saphenous veins were dissected free of silk ligatures and connective tissue and homogenized for 1 min using a Polytron (Kinematica A.G., Lucerne, Switzerland) in ice cold Tris-HCl buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM EGTA, 100,000 iu ml$^{-1}$ aprotinin, pH 7.4). The homogenate was centrifuged (1000 *g*, 1 min, 4°C), the pellet discarded and the supernatant further purified by centrifugation at 40,000 *g* for 30 min (4°C). The resulting pellet was resuspended in Tris-HCl buffer and subjected to two additional centrifugation steps (40,000 *g*, 30 min, 4°C) with the final pellet resuspended in HEPES buffer (50 mM HEPES, 5 mM $MgCl_2$. 0.3% bovine serum albumin, 4°C, pH 7.4). The concentration of protein was determined (Biorad Laboratories Ltd., Hemelhempstead, Herts., U.K.) and the homogenate diluted to give a final concentration of 6 mg protein ml$^{-1}$. Aliquots were then stored at −70°C until required.

### *Saturation and competition binding assays*

Saphenous vein homogenates were thawed, centrifuged (20,000 *g* 10 min, 4°C) and suspended in HEPES buffer (23°C). For saturation experiments, homogenate aliquots (final assay concentration 2 mg ml$^{-1}$) were added to tubes containing increasing concentrations (2 pM–2 nM) of either [$^{125}$I]-ET-1 or [$^{125}$I]-S6b (2000 Ci mmol$^{-1}$, Amersham International, Bucks., U.K.). Non-specific binding was defined by addition of 1 μM ET-1 or 1 μM S6b respectively. To allow a direct comparison of maximum receptor densities, saturation experiments with the two radioligands were carried out simultaneously on the same homogenate preparation. Values of affinity ($K_D$) and receptor density ($B_{max}$ expressed as fmol bound mg$^{-1}$ protein) were determined by analysis of the data pooled from five separate experiments using EBDA (McPherson, 1983) and the non-linear iterative curve fitting programme LIGAND (Munson & Rodbard, 1980).

For competition experiments, homogenate aliquots (final assay concentration 2 mg ml$^{-1}$) were added to tubes containing 0.1 nM of either [$^{125}$I]-ET-1 or [$^{125}$I]-S6b (2000 Ci mmol$^{-1}$, Amersham International, Bucks, U.K.) and increasing concentrations (20 pM–100 μM) of BQ123. Non-specific binding was determined by the inclusion of 1 μM ET-1 or 1 μM S6b respectively. The tubes were vortexed, incubated for 2 h at 23°C, after which they were centrifuged (20,000 *g*, 10 min, 4°C) and the pellets washed and cetrifuged in ice-cold Tris-HCl buffer (20,000 *g*, 10 min). The final pellets were counted for $^{125}$I content. The data were again analysed using EBDA and LIGAND to obtain values of $K_D$ and $B_{max}$. To clarify whether or not the two agonists share a common binding site, additional competition experiments were carried out with S6b (2 pM–10 μM) against either 0.1 nM or 0.25 nM [$^{125}$I]-ET-1.

### In vitro *experiments*

Segments of human saphenous vein were cut into rings, 2–3 mm in width, and the endothelium removed by rubbing the luminal surface of the ring gently with a blunt metal seeker. Preparations were transferred to organ baths containing oxygenated Krebs-Henseleit solution maintained at 37°C and allowed to equilibrate for 90 min. Contractile responses were measured isometrically (F30 force transducers, Hugo Sachs Elektronik, March-Hugstetten, Germany) and recorded on a Graphtec chart recorder (Linton Instrumentation, Diss, Norfolk, U.K.). Control responses were obtained to 50 mM KCl, the preparations were then washed and allowed to recover for a further 60 min. BQ123 (10 nM–10 μM) or vehicle (control) was added to the bathing medium and the saphenous veins incubated for 30 min. Cumulative concentration-response curves were then constructed to either ET-1 or S6b (0.1 nM–1 μM). When addition of a higher concentration of agonist elicited no further contractile response 50 mM KCl was added to determine the maximum possible contractile response for each preparation. Agonist responses were subsequently expressed as a percent of this KCl response. Data for Schild analysis (Arunlakshana & Schild, 1959) were derived from the graphs of agonist concentration ($log_{10}$) plotted against response (% KCl response) in the absence and presence of increasing concentrations of BQ123. If the slopes of the resulting Schild regressions were not significantly different from one, the slope was constrained to one to determine the value of $pK_B$ for BQ123 against each of the agonists used.

In a number of experiments cumulative concentration-response curves were constructed to ET-1 until addition of a higher concentration produced no further contraction. At this point S6b (300 nM) was added to the bath and any response recorded. The reverse experiment was also performed with the concentration-response curve to S6b followed by addition of ET-1 (300 nM).

### *Cross-desensitization experiments*

Rings of saphenous vein were exposed to a high concentration (300 nM) of either ET-1 or S6b. Each response was allowed to decline, without washing, and return to baseline. Two further additions were made, although no response was usually obtained. At this point S6b (300 nM) was added to preparations not responsive to ET-1 and ET-1 (300 nM) was added to preparations that no longer responded to S6b.

### *Dissociation studies*

Endothelin receptors on smooth muscle cells of non-diseased human aorta appear to be of the $ET_A$ subtype (Bacon & Davenport, 1996) with a small number of $ET_B$ receptors detected only in atherosclerotic aortic tissue (Davenport *et al.*, 1995). We used medial sections of human aorta which show no macroscopic evidence of disease to address whether the observed discrepancy in sensitivity of ET-1 and S6b to BQ123 is due to a different rate of dissociation from the $ET_A$ receptor. Sections (10 μm) of the media of human aorta were cut using a cryostat and mounted on gelatine-coated microscope slides.

Tissue sections were incubated with 1 nM [$^{125}$I]-ET-1 or [$^{125}$I]-S6b for 2 h (23°C), rinsed briefly in ice-cold Tris-HCl buffer and then placed in an excess (250 ml) of HEPES buffer (23°C) for increasing time periods up to 4 h. Non-specific binding was determined by the inclusion of 1 $\mu$M unlabelled ET-1 or S6b respectively. Sections were then counted for $^{125}$I content.

### *Materials*

ET-1 and sarafotoxin 6b were purchased from Novabiochem (Nottingham, U.K.). Stock solutions ($10^{-4}$ M) of these peptides were dissolved in 0.1% acetic acid and kept at −20°C. BQ123 (*cyclo* (D-Trp-D-Asp-L-Pro-D-Val-L-Leu)) was synthesized by solid phase t-Boc chemistry and dissolved in dimethylsulphoxide to give a stock concentration of $10^{-3}$ M.

### *Statistics*

All values are expressed as mean ± s.e. mean unless otherwise stated, and $n$ values refer to the number of individuals from whom tissue was obtained. Individual saturation and competition binding experiments were analysed with EBDA. The resulting data files were co-analysed with LIGAND to obtain final estimates and errors for dissociation constant ($K_D$) and receptor density ($B_{max}$). Using the Shapiro and Francia probability plot correlation test there was no evidence of non-normality for values of $B_{max}$ determined by saturation analysis. These data were subsequently compared by Student's two-tailed $t$ test with a significance level of $P<0.05$. In competition experiments the presence of one, two or three sites was determined using the $F$-ratio test in LIGAND. The model adopted was that which provided the significantly best fit $P<0.05$).

## Results

### *Saturation experiments*

[$^{125}$I]-ET-1 and [$^{125}$I]-S6b bound with subnanomolar affinity to a single population of receptors (or more than one receptor population for which the ligands had equal affinity) in human saphenous vein. The pooled $K_D$ values derived by LIGAND analysis were 0.64 ± 0.11 nM ($n=5$) for [$^{125}$I]-ET-1 and 0.55 ± 0.08 nM ($n=5$) for [$^{125}$I]-S6b. Hill slopes were 0.93 ± 0.02 and 0.97 ± 0.01 respectively. However, [$^{125}$I]-ET-1 consistently labelled a significantly higher density of receptors ($B_{max}$ 187.6 ± 23.0 fmol mg$^{-1}$ protein) than [$^{125}$I]-S6b ($B_{max}$ 91.7 ± 23.6 fmol mg$^{-1}$ protein) ($P<0.02$, Student's two-tailed $t$ test) (Figure 1).

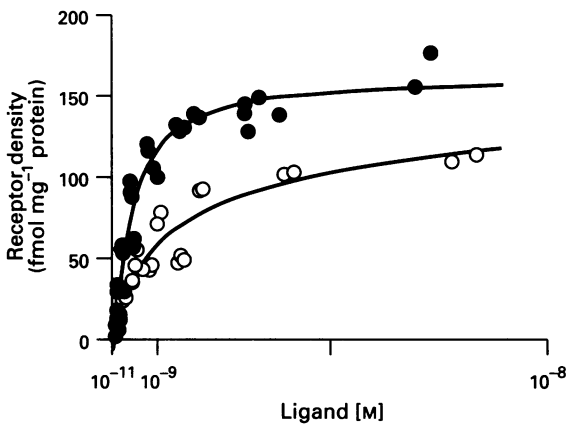


**Figure 1** Saturation binding curves for [$^{125}$I]-ET-1 (●) and [$^{125}$I]-S6b (○). Increasing concentrations of each radioligand (2 pM − 2 nM) were incubated with homogenates of human saphenous vein for 2 h at 23°C. Data points are individual values from 5 experiments.

### *Competition experiments*

Similar $K_D$ values were obtained for [$^{125}$I]-ET-1 and [$^{125}$I]-S6b and therefore the same concentration of each (0.1 nM) was chosen for use in the competition experiments. This particular concentration of each radioligand was calculated from the law of mass action to label approximately 30 − 35% of the total endothelin binding sites.

The $ET_A$-selective antagonist, BQ123, inhibited the specific binding of 0.1 nM [$^{125}$I]-ET-1 and [$^{125}$I]-S6b in a biphasic manner (Figure 2a and b). For each radioligand, BQ123 competed for at least 85% of sites with subnanomolar affinity and with micromolar affinity for the remaining 15% (Table 1). The two sites would appear to correspond to the $ET_A$ and $ET_B$ receptors which we have previously shown to be present in this tissue (Davenport *et al.*, 1995). The competition curves for BQ123 against [$^{125}$I]-ET-1 and [$^{125}$I]-S6b were essentially the same, with comparable $K_D$ values obtained for BQ123 at both receptor subtypes for each of the radioligands.

Sarafotoxin 6b (2 pM − 10 $\mu$M) completely inhibited specifically bound 0.1 nM [$^{125}$I]-ET-1 with a $K_D$ value of 1.16 ± 0.19 nM (Figure 2c). A one site fit was preferred over the two or three site models, indicating that the two agonists are identifying identical receptor populations at this concentration of [$^{125}$I]-ET-1. However, at the higher concentration of [$^{125}$I]-ET-1 (0.25 nM) a two site fit was preferred (Figure 2d). S6b competed for 67% of the binding sites with a $K_D$ of 1.99 ± 0.84 nM and for the remaining 33% of sites with a $K_D$ of 0.68 ± 0.63 $\mu$M ($n=3$).

### *The potency of BQ123 as an antagonist of ET-1- and S6b-mediated vasoconstriction compared in human saphenous vein*

ET-1 and S6b potently contracted human isolated saphenous vein with $EC_{50}$ values of 3.67 nM (2.32 − 5.79 nM, $n=15$) and 6.99 nM (3.49 − 14.0 nM, $n=11$) respectively (geometric mean with 95% confidence intervals). The maximum contractile response to each peptide, expressed as a percentage of that obtained to 50 mM KCl was 90 ± 2.38 for ET-1 and 90 ± 2.46 for S6b.

Increasing concentrations of BQ123 produced progressive rightward shifts of the ET-1 and S6b concentration-response curves in segments of human isolated saphenous vein. However, the range of concentrations of BQ123 required was different in each case. Micromolar concentrations (0.3 − 10 $\mu$M) of BQ123 were required to antagonize the constrictor effects of ET-1 with only nanomolar concentrations (10 − 300 nM) required to block the effects of S6b. BQ123 appeared to act in a competitive manner against both agonists; concentration-response curves to ET-1 and S6b were displaced in a parallel manner without any reduction in the maximum response (Figure 3a and b). Slopes of the Schild regressions were 0.80 ± 0.13 for antagonism of ET-1 and 1.08 ± 0.11 for the antagonisms of S6b. These were not significantly different from one ($P>0.05$) therefore constraining them to unity yielded $pK_B$ values for BQ123 of 6.47 ($\equiv K_B$ 339 nM) and 8.18 (6.6 nM) for the antagonism of ET-1 and S6b respectively (Figure 3c).

Following an ET-1 concentration-curve, 300 nM S6b was unable to elicit further contractile response; however, a small contraction was obtained to 300 nM ET-1 after a maximum response to S6b (Figure 4).

### *Desensitization experiments*

Exposure of tissue to a high concentration of either ET-1 or S6b rendered the preparations unresponsive to repeated additions of the same agonist. However, whereas addition of S6b to rings not responding to ET-1 did not elicit any response, a small contraction was obtained with ET-1 in preparations not responding to S6b (Figure 5).

*Dissociation rates for [$^{125}$I]-ET-1 and [$^{125}$I]-S6b in human aorta*

Approximately 20% of specific [$^{125}$I]-ET-1 binding had dissociated from endothelin receptors in sections of human aorta after 20 min, with no further loss over the next 220 min. [$^{125}$I]-S6b dissociated by a similar amount after 20 min but then continued to dissociate slowly such that approximately 25–30% had been lost at 4 h (Figure 6). Significantly more [$^{125}$I]-S6b than [$^{125}$I]-ET-1 had dissociated at 240 min ($P<0.01$, Student's two-tailed $t$ test).

**Table 1** Inhibition of 0.1 nM [$^{125}$I]-ET-1 and 0.1 nM [$^{125}$I]-S6b binding by BQ123

| | $ET_AK_D$ (nM) | $ET_BK_D$ (μM) | $ET_A$:$ET_B$ |
|---|---|---|---|
| [$^{125}$I]-ET-1 | 0.55±0.17 | 14.4±2.6 | 85:15 |
| [$^{125}$I]-S6b | 0.52±0.02 | 11.2±0.3 | 88:12 |

Data were analysed using the iterative, non-linear curve fitting programme LIGAND. Values are the co-analysed mean±s.e.mean from three separate experiments.

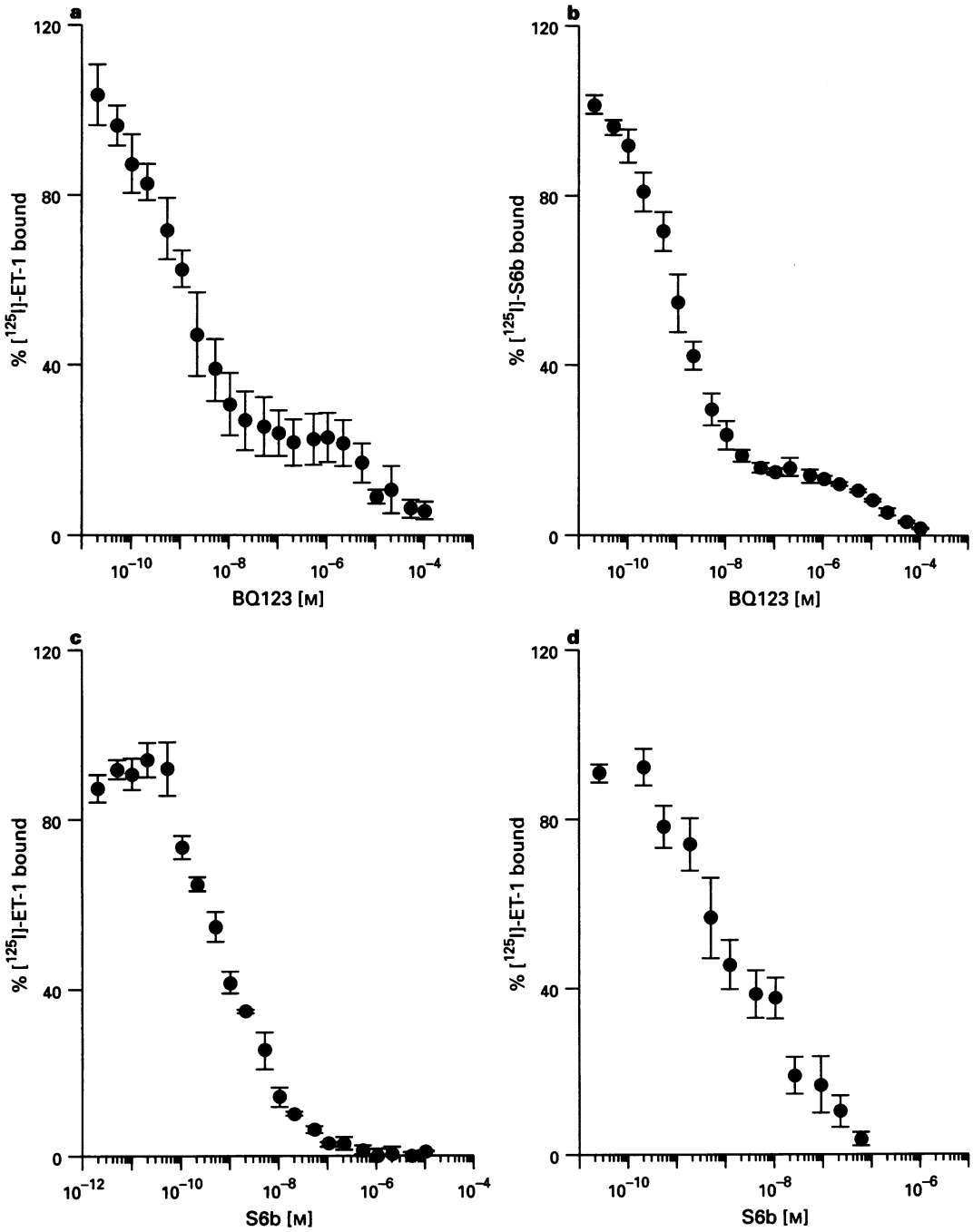


**Figure 2** Competition curves for (a) BQ123 (20 pM–100 μM) against 0.1 nM [$^{125}$I]-ET-1; (b) BQ123 (20 pM–100 μM) against 0.1 nM [$^{125}$I]-S6b; (c) S6b (2 pM–10 μM) against 0.1 nM [$^{125}$I]-ET-1; and (d) S6b (20 pM–1 μM) against 0.25 nM [$^{125}$I]-ET-1 in human saphenous vein. Data points are the mean±s.e. mean of three experiments.

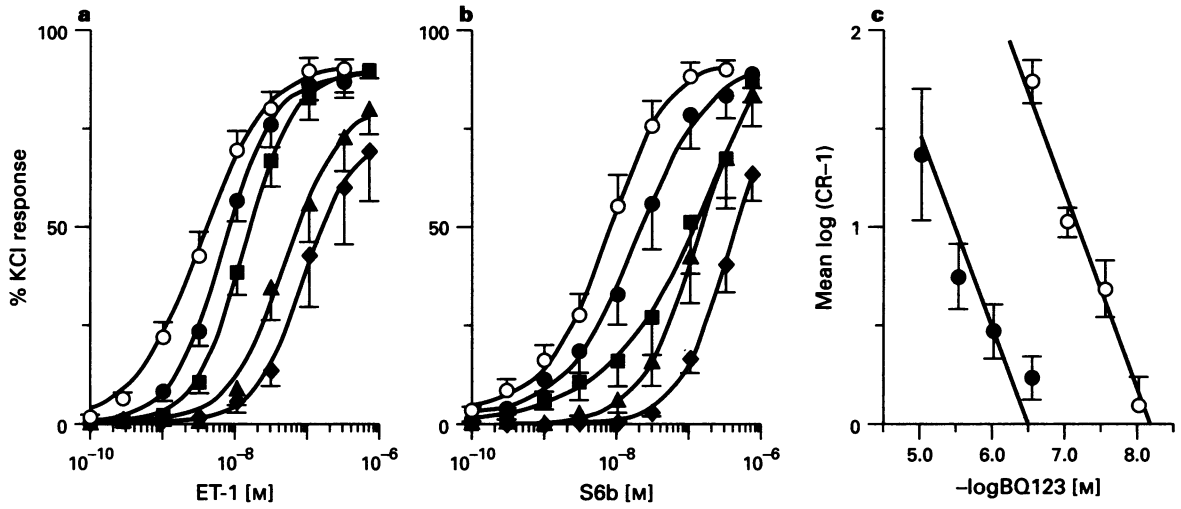


**Figure 3** Antagonism of ET-1- and S6b-mediated vasoconstriction by BQ123 in human isolated saphenous vein. (a) ET-1 concentration-response curves in the absence (○) and presence of 0.3 (●), 1.0 (■), 3 $\mu$M (▲) and 10 $\mu$M (◆) BQ123; (b) S6b concentration-response curves in the absence (○) and presence of 10 (●), 30 (■), 100 nM (▲) and 300 nM (◆) BQ123; (c) Arunlakshana-Schild plot for the antagonism of ET-1 (●) and S6b (○) by BQ123 (10 nM – 10 $\mu$M). Slopes of the Schild regressions are constrained to unity. Data points are the mean ± s.e.mean ($n$ = 6–8).

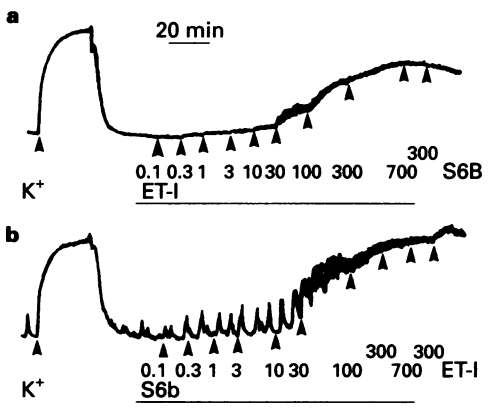


**Figure 4** Cumulative concentration-response curves to (a) ET-1 (0.1 – 700 nM) followed by 300 nM S6b and to (b) S6b (0.1 – 700 nM) followed by 300 nM ET-1 in human isolated saphenous vein. The trace is representative of three experiments.

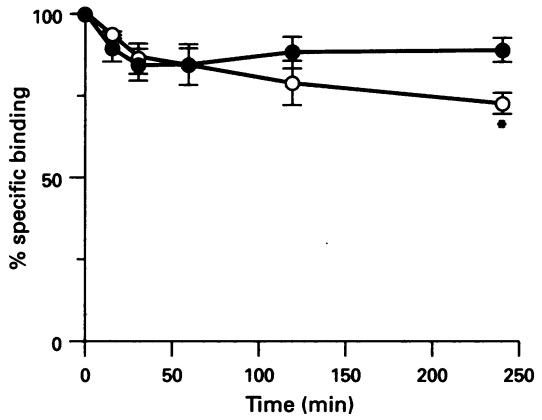


**Figure 6** Time course for the dissociation of 1 nM [$^{125}$I]-ET-1 (●) and 1 nM [$^{125}$I]-S6b (○) from sections of human aorta at 23°C. Tissue sections were incubated with each radioligand for 2 h and then washed in an excess of HEPES buffer for differing time periods up to 4 h. Data points are the mean ± s.e. mean ($n$ = 4). *Significantly different ($P$ < 0.05, Student's two-tailed $t$ test).

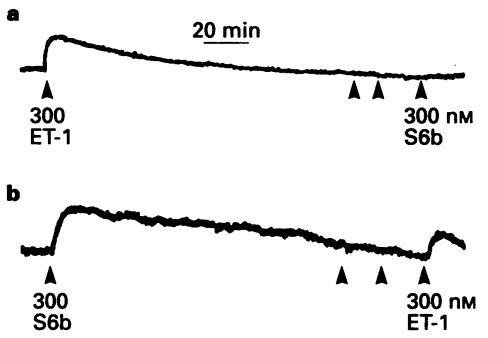


**Figure 5** Effect of (a) 300 nM S6b on a saphenous vein preparation desensitized to ET-1 by three consecutive additions of 300 nM ET-1 and (b) the effect of 300 nM ET-1 on a preparation no longer responsive to S6b after three additions of 300 nM S6b. The agonist additions are made without washing the preparations at any time during the experiment. This trace is representative of three experiments.

## Discussion

Since the discovery that the sarafotoxins possess striking sequence homology to the endothelins (Kloog *et al.*, 1988) much evidence has been published to suggest that these peptides mediate their effects through a heterogenous receptor population (see reviews by Sokolovsky, 1992; 1994). However, despite results from biochemical (for example Kloog *et al.*, 1989; Galron *et al.*, 1990) and pharmacological experiments (Warner *et al.*, 1993; Sudjarwo *et al.*, 1994; Yoneyama *et al.*, 1995) which suggest the presence of up to four endothelin receptors, only two, the $ET_A$ and the $ET_B$, have been cloned from mammalian sources.

The distribution pattern of high affinity binding sites for the endothelins and sarafotoxins was found to be very similar in human, porcine and rat tissues (Davenport *et al.*, 1991), suggesting a common receptor system. However, recent reports that endothelin antagonists are less effective against ET-1 than against other members of the ET/sarafotoxin family implies that the peptides mediate their actions through different receptors and that the current classification of endothelin receptors may require reappraisal (Bax & Saxena, 1994).

Our aim was to investigate more closely the receptors through which ET-1 and S6b produce vasoconstriction in the human saphenous vein. From saturation analysis, the Hill slopes for both [$^{125}$I]-ET-1 and [$^{125}$I]-S6b were close to one, suggesting that each was binding to a single population of receptors or to heterogenous receptor populations with equal affinity. The latter is most likely as we have previously shown that the smooth muscle layer of human saphenous vein expresses mRNA encoding both the $ET_A$ and $ET_B$ receptors, present in the ratio of 85% : 15% (Davenport *et al.*, 1995) and these peptides are not thought to distinguish between the two subtypes. It was therefore of considerable interest to us that ET-1 bound to a significantly higher density of receptors than S6b. This implies the presence of an additional ET-1 receptor population that is either not sensitive, or is somehow inaccessible, to S6b, at least over this concentration-range (up to 2 nM). Only a very few other studies have made a direct comparison of saturation data for [$^{125}$I]-ET-1 and [$^{125}$I]-S6b. Jones & Hiley (1990) also reported a higher density of ET-1 sites than of S6b sites in rat cerebellum and in a comparative study on normotensive and spontaneously hypertensive animals, Gulati & Rebello (1992) observed that ET-1 labelled more receptors than S6b in spinal cord, in both animal groups, although this difference did not extend to other brain regions.

Whilst several receptors may exist for which the endothelins and sarafotoxins have differing affinities, ET-1 and S6b must have at least one binding site in common since we were able to show that S6b completely inhibits specifically bound [$^{125}$I]-ET-1 (0.1 nM). This experiment confirms observations made by several groups in which ET-1 and S6b were each able to compete for the specific binding of the other (Ambar *et al.*, 1989; Bousso-Mittler *et al.*, 1989; Gu *et al.*, 1989; Kloog *et al.*, 1989; Martin *et al.*, 1989).

Similarly, we observed that the $ET_A$-selective antagonist, BQ123, inhibited [$^{125}$I]-ET-1 and [$^{125}$I]-S6b in a biphasic manner with equal subnanomolar affinity for about 85% ($ET_A$ receptors) of the total and micromolar affinity for the remaining binding sites (the $ET_B$ receptors). Thus in these experiments, BQ123 was unable to distinguish between the two radiolabelled peptides, suggesting that at this particular concentration (0.1 nM) they delineate identical populations of $ET_A$ and $ET_B$ receptors. From the saturation data it is clear that the discrepancy in $B_{max}$ values is more apparent at the higher concentrations of the labelled peptides. Indeed using a higher concentration of [$^{125}$I]-ET-1, S6b competed with nanomolar affinity for the majority of the sites but micromolar affinity for the remaining 30%. This suggests that ET-1 is binding to two receptor populations which can be distinguished by S6b. The BQ123 competition data we obtained here are comparable to data previously published for the inhibition of [$^{125}$I]-ET-1 from medial (smooth muscle layer) sections of human coronary artery. Messenger RNA encoding both $ET_A$ and $ET_B$ receptors is also present in coronary artery smooth muscle and BQ123 competed in a biphasic manner with subnanomolar affinity for 87% of binding sites ($ET_A$) and micromolar affinity for the remaining 13% ($ET_B$) (Davenport *et al.*, 1993). However this contrasts with data reported by Bax and colleagues (1993b) also in human coronary artery. In their autoradiographic study BQ123 competed in a monophasic manner for 30 pM [$^{125}$I]-ET-1, presumably suggesting only the presence of $ET_A$ receptors, but a two site competition curve was preferred for the inhibition of [$^{125}$I]-S6b. In this latter case BQ123 was 100 times more potent at the high affinity site than the low affinity site and each site comprised 50% of the total. In our hands BQ123 exhibits several thousand fold selectivity for $ET_A$ receptors over $ET_B$ receptors and as stated previously the ratio of $ET_A$ : $ET_B$ receptors in human coronary artery we find is 85 : 15 and not 50 : 50. We cannot explain the discrepancies observed although they may result from the different experimental methods employed by the two groups: homogenate binding in one and quantitative autoradiography in the other.

Although both $ET_A$ and $ET_B$ receptors are present in the smooth muscle layer of human saphenous vein, endothelin contractile responses *in vitro* appear to be mediated mainly via the $ET_A$ receptor (Davenport & Maguire, 1994; Maguire & Davenport, 1995; White *et al.*, 1994). Presumably the density of $ET_B$ receptors in this tissue is too low for their activation to contribute significantly to vasoconstriction. In functional experiments, in common with many other groups, we found that BQ123 was a much more potent antagonist of S6b-mediated vasoconstriction than of ET-1. Indeed 50 times more BQ123 was required to block ET-1 than S6b responses. The $pA_2$ value for blockade of ET-1 was consistent with that reported in a wide range of vascular preparations containing $ET_A$ receptors, both animal and human. However the $pK_B$ value obtained for the antagonism of S6b by BQ123 (8.18; $K_B = 6.6$ nM) shows much better correlation to the subnanomolar affinity exhibited by BQ123 for $ET_A$ receptors in the competition experiments. Considering the results we have obtained in the binding experiments, one explanation for our functional data is that, in addition to $ET_B$ receptors, which we do not generally detect in *in vitro* contractile experiments, two other endothelin receptor populations are present, one sensitive to ET-1 and S6b, with high affinity for BQ123 (the $ET_A$ receptor) and a second site sensitive to ET-1, with low or no affinity for S6b and BQ123 (an 'ET-1' site). From the data presented here it is difficult to determine whether this is truly a separate binding site or reflects an ability of ET-1 to bind to the known ET receptors in a manner different from that of other agonists and antagonists. For example if more than one ET-1 molecule could bind to each endothelin receptor this would result in an apparently higher $B_{max}$ value for ET-1 than for S6b. Whatever the explanation, the $pK_B$ values obtained for antagonism of ET-1 responses by BQ123 (and other antagonists) may be a composite value for antagonism of ET-1 acting at more than one site, for which BQ123 has differing affinities. The potency of antagonists determined against S6b (and probably ET-3), we feel, may more accurately describe the classical $ET_A$ receptor.

One other report on ET-1/S6b receptors in human saphenous vein (Bax *et al.*, 1993a) describes very similar data, in that ET-1 was much less sensitive to BQ123 than was S6b, but the authors' interpretation of the data is somewhat different from our own. They observed that BQ123 at 0.1 and 1 $\mu$M was ineffective against ET-1 (in our own experiments some effect of BQ123 against ET-1 was seen at 0.3 $\mu$M); however, BQ123 caused biphasic inhibition of S6b, antagonizing concentrations of S6b greater, but not lower, than 1 nM. Our explanation of these results is that S6b is acting at both $ET_A$ (BQ123-sensitive) and a low density of $ET_B$ (BQ123-insensitive) receptors, which in this case do appear to contribute in a small way to the observed constrictor response. If as we suggest ET-1 produces contraction, not only through these two, but also through an additional 'ET-1 site', BQ123 will appear much less effective than expected because although the $ET_A$ receptors are blocked ET-1 will still have access to this other constrictor site.

In the presence of a maximal concentration of ET-1 no further contractile reponse was elicited at any concentration of S6b; however, ET-1 was found to produce a further small degree of contraction in the presence of a maximal response to S6b. The former observation confirms that ET-1 and S6b share a common binding site, the latter observation again suggests ET-1 has access to an additional binding site; activation of both these binding sites producing vasoconstriction. Similarly, in the cross-desensitization experiments, S6b was without effect on an ET-1 desensitized preparation, but ET-1 was able to contract preparations which no longer responded to S6b. Comparable results have been reported in human coronary artery, although the authors concluded that ET-1 and S6b mediate their vasoconstrictor effects through completely distinct receptors which they suggested are subtypes of the $ET_A$ receptor distinguished by their differing affinities for BQ123 (Bax *et al.*, 1994).

Whilst desensitization experiments may imply the presence of more than one receptor an equally plausible explanation is that the two agonists may be binding to a single receptor for which they exhibit differing efficacy. Thus manipulation of

receptor density, for example by desensitization, may be sufficient to abolish the response to an agonist with low efficacy but leave a sufficient density of viable receptors to enable an agonist with higher efficacy to elicit a response. In the present study S6b has higher affinity than ET-1 in the saturation experiments but is always less potent than ET-1 in functional experiments (Maguire and Davenport, 1995), this strongly implies that S6b has lower efficacy than ET-1 at endothelin receptors in human saphenous vein. We have observed that the response to ET-1 elicited in a preparation desensitized to S6b can be reduced dramatically (although not completely abolished) if the S6b is left in contact with the tissue for several hours before the ET-1 is added (not shown).

We considered the possibility that differences in the rates of dissociation of ET-1 and S6b may explain the differing abilities of BQ123 to antagonize their contractile responses, whilst competing with equal affinity against each in the competition studies. ET-1 is known to bind with high affinity to its receptor and to dissociate very slowly (<20% ater 24 h, Hirata *et al.*, 1988). If bound ET-1 does not dissociate (possibly due to rapid internalisation of the receptor-ET-1 complex), the opportunity for BQ123 to compete for that receptor is attenuated. In sections of non-diseased human aorta which express only $ET_A$ receptors, and were therefore used for clarity, the dissociation rate for ET-1 and S6b appeared to be equally slow. Although significantly more S6b had dissociated at the 4 h time point, it seems unlikely that this small difference could account for the observed discrepancy in antagonist potency. However, the binding experiments were carried out at room temperature and the functional experiments at 37°C, it may be that a much greater difference in agonist-receptor complex dissociation rates would be observed if these experiments could be repeated at the physiologically relevant temperature.

In conclusion, we have demonstrated that in human saphenous vein ET-1 and S6b bind to common receptor proteins which we believe are analogous to the cloned $ET_A$ and $ET_B$ receptors. However, ET-1 also appears to bind to an additional constrictor 'receptor' population, the presence of which may lead to underestimation of antagonist potencies when these are determined against ET-1 in functional assays. Further investigation to fully characterize these additional receptors is required. Understanding why functional responses to ET-1 are more difficult to antagonize than those to S6b would greatly assist in the development of potent therapeutic agents, since pertubations of the endothelin system are implicated in a number of human diseases such as cerebral vasospasm associated with subarachnoid haemorrhage.

## References


AMBAR, I., KLOOG, Y., SCHVARTZ, I., HAZUM, E. & SOKOLOVSKY, M. (1989). Competitive interaction between endothelin and sarafotoxin: binding and phosphoinositides hydrolysis in rat atria and brain. *Biochem. Biophys. Res. Commun.*, **158**, 195–201.

ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANASHI, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*, **348**, 730–732.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BACON, C.R. & DAVENPORT, A.P. (1996). Endothlin receptors in human coronary artery and aorta. *Br. J. Pharmacol.*, **117**, 986–992.

BAX, W.A., AGHAI, Z., VAN TRICHT, C.L.J., WASSENAAR, C. & SAXENA, P.R. (1994). Different endothelin receptors involved in the endothelin-1- and sarafotoxin S6b-induced contrations of the human isolated coronary artery. *Br. J. Pharmacol.*, **113**, 1471–1479.

BAX, W.A., BOS, E. & SAXENA, P.R. (1993a). Heterogeneity of endothelin/sarafotoxin receptors mediating contraction of the human isolated saphenous vein. *Eur. J. Pharmacol.*, **239**, 267–268.

BAX, W.A., BRUINVELS, A.T., VAN SUYLEN, R.-J., SAXENA, P.R. & HOYER, D. (1993b). Endothelin receptors in the human coronary artery, ventricle and atrium: A quantitative autoradiographic analysis. *Naunyn-Schmied. Arch. Pharmacol.*, **348**, 403–410.

BAX, W.A. & SAXENA, P.R. (1994). The current endothelin receptor classification: time for a reconsideration? *Trends Pharmacol. Sci.*, **15**, 379–386.

BODELSSON, G. & STJERNQUIST, M. (1993). Characterization of endothelin receptors and localization of $^{125}$I-endothelin-1 binding sites in human umbilical artery. *Eur. J. Pharmacol.*, **249**, 299–305.

BOUSSO-MITTLER, D., KLOOG, Y., WOLLBERG, Z., BDOLAH, A., KOCHVA, E. & SOKOLOVSKY, M. (1989). Functional endothelin/sarafotoxin receptors in the rat uterus. *Biochem. Biophys. Res. Commun.*, **162**, 952–957.

DAVENPORT, A.P. & MAGUIRE, J.J. (1994). Is endothelin-induced vasoconstriction mediated only by $ET_A$ receptors in humans? *Trends Pharmacol. Sci.*, **15**, 9–11.

DAVENPORT, A.P., MORTON, A.J. & BROWN, M.J. (1991). Localization of Endothelin-1 (ET-1), ET-2, and ET-3, mouse VIC, and sarafotoxin S6b binding sites in mammalian heart and kidney. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 7), S152–S155.

DAVENPORT, A.P., O'REILLY, G. & KUC, R.E. (1995). Endothelin $ET_A$ and $ET_B$ receptors are expressed by smooth muscle in the human vasculature: majority of the $ET_A$ subtype. *Br. J. Pharmacol.*, **114**, 1110–1116.

DAVENPORT, A.P., O'REILLY, G., MOLENAAR, P., MAGUIRE, J.J., KUC, R.E., SHARKEY, A., BACON, C.R. & FERRO, M.A. (1993). Human endothelin receptors characterized using reverse transcriptase-polymerase chain reaction, in situ hybridization, and subtype-selective ligands BQ123 and BQ3020: evidence for expression of $ET_B$ receptors in human vascular smooth muscle. *J. Cardiovasc. Pharmacol.*, **22** (Suppl. 8), S22–S25.

EGLEZOS, A., CUCCHI, P., PATACCHINI, R., QUARTARA, L., MAGGI, C.A. & MIZRAHI, J. (1993). Differential effects of BQ-123 against endothelin-1 and endothelin-3 on the rat vas deferens: evidence for an atypical endothelin receptor. *Br. J. Pharmacol.*, **109**, 736–738.

GALRON, R., BDOLAH, A., KLOOG, Y. & SOKOLOVSKY, M. (1990). Endothelin/sarafotoxin receptor induced phosphoinositide turnover: effects of pertussis and cholera toxins and of phorbal ester. *Biochem. Biophys. Res. Commun.*, **171**, 949–954.

GODFRAIND, T. (1993). Evidence for receptor heterogeneity of endothelin receptor distribution in human coronary artery. *Br. J. Pharmacol.*, **110**, 1201–1205.

GU, X.-H., CASLEY, D.J. & NAYLER, W.G. (1989). Sarafotoxin S6b displaces specifically bound [$^{125}$I]-endothelin. *Eur. J. Pharmacol.*, **162**, 509–510.

GULATI, A. & REBELLO, S. (1992). Characteristics of endothelin receptors in the central nervous system of spontaneously hypertensive rats. *Neuropharmacology*, **31**, 243–250.

HAY, D.W.P., LUTTMANN, M.A., HUBBARD, W.C. & UNDEM, B.J. (1993). Endothelin receptor subtypes in human and guinea-pig pulmonary tissues. *Br. J. Pharmacol.*, **110**, 1175–1183.

HIRATA, Y., YOSHIMI, H., YAKAICHI, S., YANAGISAWA, M. & MASAKI, T. (1988). Binding and receptor down-regulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *FEBS Lett.*, **239**, 13–17.

IHARA, M., NOGUCHI, K., SAEKI, T., FUKURODA, T., TSUCHIDA, S., KIMURA, S., FUKAMI, T., ISHIKAWA, K., NISHIKIBE, M. & YANO, M. (1992). Biological profiles of highly potent novel endothelin antagonists selective for the $ET_A$ receptor. *Life Sci.*, **50**, 247–255.

JONES, C.R. & HILEY, C.R. (1990). Heterogeneity of [$^{125}$I]sarafotoxin S6b binding in rat tissues. *Eur. J. Pharmacol.*, **183**, 1746.

KARET, F., KUC, R.E. & DAVENPORT, A.P. (1993). Novel ligands BQ123 and BQ3020 characterize endothelin receptor subtypes $ET_A$ and $ET_B$ in human kidney. *Kidney Int.*, **44**, 36–42.

KLOOG, Y., AMBAR, I., SOKOLOVSKY, M., KOCHVA, E., BDOLAH, A. & WOLLBERG, Z. (1988). Sarafotoxin, a novel vasoconstrictor peptide: phosphoinositide hydrolysis in rat heart and brain. *Science*, **242**, 268–270.

KLOOG, Y., BOUSSO-MITTLER, D., BDOLAH, A. & SOKOLOVSKY, M. (1989). Three apparent receptor subtypes for the endothelin/sarafotoxin family. *FEBS Lett.*, **253**, 199–202.

MCPHERSON, G. (1983). A pratical computer-based approach to the analysis of radioligand binding experiments. *Comput. Prog. Biomed.*, **17**, 107–114.

MAGUIRE, J.J. & DAVENPORT, A.P. (1995). $ET_A$ receptor-mediated constrictor responses to endothelin peptides in human blood vessels *in vitro*. *Br. J. Pharmacol.*, **115**, 191–197.

MAGUIRE, J.J., KUC, R.E., O'REILLY, G. & DAVENPORT, A.P. (1994). Vasoconstrictor endothelin receptors characterized in human renal artery and vein *in vitro*. *Br. J. Pharmacol.*, **113**, 49–54.

MAGUIRE, J.J., KUC, R.E., ROUS, B.A., FITZGERALD, F. & DAVENPORT, A.P. (1995). BQ123 inhibits [$^{125}$I]ET-1 and [$^{125}$I]S6b binding with subnanomolar affinity but is a more potent antagonist of S6b vasoconstriction in human isolated blood vessels. *Br. J. Pharmacol.*, **114**, 75P.

MARTIN, E.R., MARSDEN, P.A., BRENNER, B.M. & BALLERMANN, B.J. (1989). Identification and charaterization of endothelin binding sites in rat renal papillary and glomerular membranes. *Biochem. Biophys. Res. Commun.*, **162**, 130–137.

MORELAND, S., MCMULLEN, D.M., DELANEY, C.L., LEE, V.G. & HUNT, J.T. (1992). Venous smooth muscle contains vasoconstrictor ET(B)-like receptors. *Biochem. Biophys. Res. Commun.*, **184**, 100–106.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: a versatile computerized approach for the characterization of ligand binding systems. *Anal. Biochem.*, **107**, 220–239.

RIEZEBOS, J., WATTS, I.S. & VALLANCE, P.J.T. (1994). Endothelin receptors mediating functional responses in human small arteries and veins. *Br. J. Pharmacol.*, **111**, 609–615.

SAKURAI, T., YANAGISAWA, M., TAKUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*, **348**, 732–735.

SALOM, J.B., TORREGROSA, G., BARBERÁ, M.D., JOVER, T. & ALBORCH, E. (1993). Endothelin receptors mediating contraction in goat cerebral arteries. *Br. J. Pharmacol.*, **109**, 826–830.

SOKOLOVSKY, M. (1992). Endothelins and sarafotoxins: physiological regulation, receptor subtypes and transmembrane signalling. *Pharmacol. Ther.*, **54**, 129–149.

SOKOLOVSKY, M. (1994). Endothelins and sarafotoxins: receptor heterogeneity. *Int. J. Biochem.*, **26**, 335–340.

SUDJARWO, S.A., TANAKA, T., MATSUDA, Y., OKADA, T. & KARAKI, H. (1994). Subtypes of endothelin $ET_A$ and $ET_B$ receptors mediating venous smooth muscle contraction. *Biochem. Biophys. Res. Commun.*, **200**, 627–633.

SUMNER, M.J., CANNON, T.R., MUNDIN, J.W., WHITE, D.G. & WATTS, I.S. (1992). Endothelin $ET_A$ and $ET_B$ receptors mediate vascular smooth muscle contraction. *Br. J. Pharmacol.*, **107**, 858–860.

WARNER, T.D., ALLCOCK, G.H., CORDER, R. & VANE, J.R. (1993). Use of the endothelin antagonists BQ123 and PD142893 to reveal three endothelin receptors mediating smooth muscle contraction and the release of EDRF. *Br. J. Pharmacol.*, **110**, 777–782.

WHITE, D.G., GARRAT, H., MUNDIN, J.W., SUMNER, M.J., VALLANCE, P.J. & WATTS, I.S. (1994). Human saphenous vein contains both endothelin $ET_A$ and $ET_B$ contractile receptors. *Eur. J. Pharmacol.*, **257**, 307–310.

WILLIAMS, D.L. JR., JONES, K.L., PETTIBONE, D.J., LIS, E.V. & CLINESCHMIDT, B.V. (1991). Sarafotoxin 6c: an agonist which distinguishes between endothelin receptor subtypes. *Biochem. Biophys. Res. Commun.*, **175**, 556–561.

YONEYAMA, T., HORI, M., MAKATANI, M., YAMAMURA, T., TANAKA, T., MATSUDA, Y. & KARAKI, H. (1995). Subtypes of endothelin $ET_A$ and $ET_B$ receptors mediating tracheal smooth muscle contraction. *Biochem. Biophys. Res. Commun.*, **207**, 668–674.

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