Different endothelin receptors involved in endothelin-1- and sarafotoxin S6B-induced contractions of the human isolated coronary artery

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1 Endothelin receptors, that mediate contraction of the human isolated coronary artery, were characterized by use of a number of agonists and antagonists. Contraction induced by the non-selective agonists, endothelin (ET)-1 and sarafotoxin S6b, was compared in endothelium-intact and endothelium-denuded ring segments. The effects of ET-1 and BQ-123 (an ET_A receptor antagonist) were investigated both in ring segments and in spirally cut strips. Lastly, the effect of phosphoramidon was studied on contraction induced by big-ET-1.

2 The order of agonist potency (pD₂) in endothelium-intact coronary artery ring segments was: ET-1 (8.27) ≈ sarafotoxin S6b (8.16) > big-ET-1 (<7.1) ≈ ET-3 (<6.9). [Ala^{1,3,11,15}]ET-1 (ET_B receptor agonist) caused significant contraction only at 1 μM, whereas 0.3 μM big-ET-3 had no effect. Removal of the endothelium in ring segments did not affect the contractile response to ET-1 or to sarafotoxin S6b.

3 After a full concentration-response curve had been obtained to ET-1 or sarafotoxin S6b, further contractions of the endothelium-intact coronary artery segments could only be achieved by applying ET-1 in segments exposed to sarafotoxin S6b, and not the reverse.

4 BQ-123 (0.1 μM) antagonized contractions of endothelium-intact ring segments induced by sarafotoxin S6b (pK_B 7.86). Only 10 μM BQ-123 antagonized contractions induced by ET-1 (pK_B 5.75). FR139317 was also more potent against sarafotoxin S6b (pK_B 8.24–8.47) than against ET-1 (pK_B 6.11). [Ala^{1,3,11,15}]ET-1 (1 μM) had no effect on the contractile response to ET-1 or to sarafotoxin S6b.

5 In strip preparations with intact endothelium, the pD₂ of ET-1 increased to 9.04 ± 0.16 (vs. 8.50 ± 0.07 in rings), and BQ-123 (1 μM) caused a rightward shift of the ET-1 induced concentration-response curve (pK_B 6.62 vs. 5.75 in rings).

6 Contractile responses to big-ET-1 of endothelium-intact coronary artery segments were attenuated in the presence of phosphoramidon (100 μM), indicating conversion of big-ET-1 to ET-1 within the coronary artery segment.

7 The present study indicates that ET-1 and sarafotoxin S6b contract the human isolated coronary artery via different receptors, which can probably be best characterized as subtypes of the ET_A receptor. Furthermore, it is demonstrated that the type of preparation (ring or strip) may affect the potency of ET-1 as an agonist and of BQ-123 as an antagonist.

Keywords: Human coronary artery; ET receptor subtypes; endothelin-1; sarafotoxin S6b; [Ala^{1,3,11,15}]ET-1; BQ-123; FR139317; big-ET-1; phosphoramidon

Introduction

The cloning of endothelin ET_A (Arai *et al.*, 1990) and ET_B (Sakurai *et al.*, 1990) receptors, and the subsequent development of relatively selective receptor agonists and antagonists, have stimulated the characterization of functional receptors, mediating vascular smooth muscle contraction to endothelin isopeptides. For instance, ET_A receptors have been shown to mediate contractile responses in the rat aorta (Sumner *et al.*, 1992), whereas ET_B receptors were found to mediate contraction of the rabbit saphenous vein (Moreland *et al.*, 1992). Other smooth muscle preparations were found to contract via a mixed receptor population, or via non-ET_A, non-ET_B receptors (e.g. the pig coronary artery: Harrison *et al.*, 1992; Schoeffter & Randrianisoa, 1993). In particular, the putative ET_A receptor antagonist BQ-123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp-]; Ihara *et al.*, 1992a) was found to discriminate between contractions induced by endothelin-1 (ET-1) and contractions induced by ET-3 or sarafotoxin S6b in rat aorta (Sumner *et al.*, 1992), rat vas deferens (Eglezos *et al.*, 1993; Warner *et al.*, 1993) and goat cerebral artery (Salom *et al.*,

1993). Also in the human saphenous vein it was found that BQ-123 antagonized sarafotoxin S6b-induced contractions with higher affinity than ET-1-induced contractions (Bax *et al.*, 1993a). Similar data were obtained in the human umbilical artery (Bodelsson & Stjernquist, 1993). These apparently heterogeneous receptor populations could not be readily characterized as 'typical' ET_A or ET_B receptors.

In the human isolated coronary artery, Godfraind (1993) observed differential effects of BQ-123 against ET-1 and ET-3-induced contractile responses. Furthermore, the nature of the contractile endothelin receptors varied between proximal and distal coronary artery segments; distal segments were more sensitive to both the agonist effect of ET-1 (as was also observed by Chester *et al.*, 1992) and the antagonist effect of BQ-123. It was therefore concluded that distal parts of the artery contract via ET_A receptors only, but that other receptors are involved in the contractile response of proximal segments (Godfraind, 1993). Using quantitative receptor autoradiography in the human coronary artery, it was previously shown that [¹²⁵I]-ET-1 labelled a homogeneous receptor population in the media of proximal segments of the human coronary artery. [¹²⁵I]-sarafotoxin S6b labelled a

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similar receptor, but also labelled a non-ET_A, non-ET_B receptor with relatively high affinity for BQ-123 and [Ala^{1,3,11,15}]ET-1 (ET_B receptor selective: Saeki *et al.*, 1991) (Bax *et al.*, 1993b).

In the present study, we have further characterized endothelin receptors that mediate contraction of the human isolated coronary artery, using BQ-123 and FR139317 (ET_A receptor selective: Sogabe *et al.*, 1992) as antagonists. We also examined whether the response to ET-1 or to sarafotoxin S6b is altered in the presence or absence of functionally intact endothelium (De Nucci *et al.*, 1988). We hypothesized that part of the differences found by Chester *et al.* (1992) and Godfraind (1993), between large proximal and small distal coronary artery segments, could be due to facilitated diffusion of the large peptide molecules to the receptors in distal coronary artery segments, rather than to a different receptor profile. To verify this hypothesis we compared the effect of ET-1 and BQ-123 on ring segments and on spirally cut coronary artery strips. Lastly, we investigated whether local conversion of big-ET-1 to ET-1 (Yanagisawa *et al.*, 1988; Okada *et al.*, 1990) is involved in the contractile response to big-ET-1. A part of this study was presented to the joint meeting of the British Pharmacological Society and the Società Italiana Farmacologia (Bax *et al.*, 1994).

Methods

Tissue preparation and experimental procedure

The right epicardial coronary artery was obtained from 43 hearts of organ donors, who had died of non-cardiac disorders less than 24 h before the tissue was taken to the laboratory (25 cerebrovascular accident, 16 polytrauma, 2 cerebral hypoxia; 24 male, 19 female; age 1–54 years). The hearts were provided by the Rotterdam Heart Valve Bank (Bio Implant Services Foundation/Eurotransplant Foundation) after removal of the aortic and pulmonary valves for homograft valve transplantation. The study was approved by the Joint Medical Ethics Committee of the Erasmus University Rotterdam and the University Hospital Rotterdam 'Dijkzigt'. The hearts were stored at 0–4°C in commonly used sterile organ protecting solutions (University of Wisconsin (UW) solution, HTK-Bretschneider solution, or EuroCollins solution, see Dreikorn, 1992), immediately following circulatory arrest. After arrival in the laboratory, the right coronary artery was removed and placed in a cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25 and glucose 8.3; pH 7.4. Segments 2 (mid segment) and 3 (distal segment: AHA Committee Report, 1975) of the coronary artery were cut into rings with similar diameter and approximately 4 mm in length. The rings were suspended on stainless steel hooks in 15 ml organ baths containing the Krebs bicarbonate solution, aerated with 95% O₂ and 5% CO₂ and maintained at 37°C. Vessel segments containing macroscopically visible atherosclerotic lesions were not used. In some ring segments, the endothelium was removed with a cotton swab and a watchmakers' forceps. However, if not mentioned otherwise, results refer to ring segments with intact endothelium. For the comparison of ring and strip segments, we made two such pairs from adjacent portions of the coronary artery with intact endothelium. The 4 mm rings were prepared as described above, while the strips were cut in a spiral manner (diameter 1 mm) and attached to silk threads.

All segments were allowed to equilibrate for at least 30 min and were washed every 15 min. Changes in tension were recorded with a Harvard isometric transducer. Preparations were stretched to a stable tension of approximately 20 mN. The tissue was exposed to K⁺ (30 mM) twice. Subsequently, the functional integrity of the endothelium was verified by observing relaxation to substance P (1 nM) after precontrac-

tion with prostaglandin F_{2α} (PGF_{2α}, 1 μM). After washout, the tissue was exposed to 100 mM K⁺ to determine the maximal contractile response to K⁺, which was expressed as mN. The tissue was allowed to equilibrate in the Krebs solution for a period of 30 min.

Determination of agonist and antagonist potency

After this period of equilibration, some segments were treated with vehicle only (controls), whereas others were incubated with an antagonist for 30 min. A cumulative concentration-response curve was then obtained on every segment. Responses were expressed as a percentage of the contractile response to 100 mM K⁺. Curves obtained in the presence of a receptor antagonist were compared to the control curves. All curves were obtained in a paired, parallel experimental set-up. Some concentration-response curves were obtained in duplicate or triplicate. These curves were averaged and considered as a single curve in further analysis.

Analysis of data

Curves were analysed by means of a computerized curve fitting technique (De Lean *et al.*, 1978) to obtain E_{max} (maximal response), and pD₂ (–log of the molar concentration of an agonist needed to reach half of its maximal effect, i.e. –logEC₅₀) values, which were averaged for the respective agonists. For antagonists against ET-1 or sarafotoxin S6b, a pK_B value was calculated, assuming the nature of antagonism to be competitive, using the equation described by Furchgott (1972):

$$pK_B = -\log[B] + \log\{([A_2]/[A_1]) - 1\},$$

where [B] is the antagonist concentration and [A₁] and [A₂] are respectively, the EC₅₀ values of the agonist in the absence and presence of the antagonist. For calculation of pK_B values, only paired experiments were taken into account, pK_B values are given only when a more than 2 fold shift of the EC₅₀ was obtained. All data are presented as mean ± s.e. mean. pD₂ and E_{max} of agonist-induced contractions without or in the presence of an antagonist were compared by Student's paired *t* test. Mean contractile responses were compared by Analysis of Variance (ANOVA) followed by Student's *t* test, for paired data where appropriate. A correlation coefficient, *r*, was calculated according to Pearson. A *P* value less than 0.05 was assumed to denote a significant difference.

Compounds

The compounds used in this study were purchased from the sources indicated: Prostaglandin F_{2α} (tris salt), substance P acetate and phosphoramidon: Sigma Chemical Co. (St. Louis, U.S.A.); ET-1: Saxon Biochemicals GMBH (Hannover, Germany); ET-1, sarafotoxin S6b, FR139317 and BQ-123 (sodium salt): Neosystem S.A. (Strasbourg, France); sarafotoxin S6b, ET-3, big-ET-1 and BQ-123 (sodium salt): Novabiochem AG (Läufelfingen, Switzerland); sarafotoxin S6b, big-ET-3, and [Ala^{1,3,11,15}]ET-1: Peninsula Laboratories (Belmont, U.S.A.). It should be noted that due to non-availability of the compounds and/or cost factors, we had to order ET-1, sarafotoxin S6b, and BQ-123 from more than one source. Although direct comparisons were not made, the compounds obtained from different sources did not differ in potency. Stock solutions for [Ala^{1,3,11,15}]ET-1, and ET-1 (Saxon) were prepared in 0.1% acetic acid. Other compounds were dissolved in water.

Results

Basic properties of the preparations

All ring segments, when endothelium was left intact, relaxed to 1 nM substance P after precontraction to 1 μ M PGF_{2 α} ($73 \pm 5\%$ of the contractile response to PGF_{2 α} ; $n = 43$). This is in accordance with previous findings with substance P in the human isolated coronary artery obtained from patients undergoing cardiac transplantation (Bossaller *et al.*, 1987). K⁺, 100 mM, caused a mean contractile response of 45 ± 3 mN ($n = 43$). Where the endothelium had been removed from the ring segments, the relaxant response to substance P was virtually abolished ($2 \pm 1\%$ of the contractile response to 1 μ M PGF_{2 α} ; $n = 13$), and the contractile response to potassium was decreased from 37 ± 5 mN to 21 ± 3 mN. In strip preparations ($n = 5$) the relaxant response to substance P was $31 \pm 12\%$ of the contractile response to 1 μ M PGF_{2 α} , and the contractile response to 100 mM K⁺ was 20 ± 6 mN.

Effect of agonist peptides

In endothelium-intact coronary artery segments, sarafotoxin S6b, ET-1, big-ET-1, and ET-3 caused concentration-dependent contractions. [Ala^{1,3,11,15}]ET-1 caused a small contractile response at the highest concentration used (1 μ M), whereas big-ET-3 had no effect at 0.3 μ M (Figure 1). For big-ET-1, ET-3 and [Ala^{1,3,11,15}]ET-1 the highest concentration tested (1 μ M) may not have reached the maximal contractile response, which could affect the given approximate pD₂ and E_{max} values. The order of potency of the agonists tested was: ET-1 $\approx$ sarafotoxin S6b > big-ET-1 $\approx$ ET-3 > [Ala^{1,3,11,15}]ET-1 $\geq$ big-ET-3 (for E_{max} and pD₂, see Table 1). The contractile response to ET-1 or sarafotoxin S6b in ring segments was not affected by removing the endothelium (Figure 2). Comparison of the E_{max} of 21 paired vessel segments, in which a concentration-response curve to both ET-1 and sarafotoxin S6b was constructed, revealed that the E_{max} but not the pD₂ of sarafotoxin is slightly but significantly different from that of ET-1 ($105.7 \pm 4.9\%$ vs. $95.2 \pm 3.9\%$, respectively, $P = 0.01$). No age-related variation was observed for the pD₂ of ET-1 ($n = 37$) and sarafotoxin S6b ($n = 26$) (correlation age, pD₂: $r = -0.12$ and -0.05 , $P = 0.24$ and 0.40 , respectively). Only the E_{max} of ET-1, but not of sarafotoxin S6b, was found to be somewhat higher in younger patients (correlation age, E_{max}, ET-1: $r = -0.36$, $P = 0.02$; sarafotoxin S6b: $r = 0.07$, $P = 0.37$).

Figure 3 shows experimental recordings of responses obtained with ET-1 and sarafotoxin S6b in endothelium-intact coronary artery segments. The addition of 0.1 μ M sarafotoxin S6b at the end of a full concentration-response curve with ET-1 did not result in a further increase of tension (Figure 3a). However, in a parallel vessel segment, the addition of 0.1 μ M ET-1 after a full concentration-response curve to sarafotoxin S6b, caused further increase of tension (Figure 3b and also 3d). Figure 3c shows that the maximal contractile response to sarafotoxin S6b (and also to ET-1: not

Figure 1 Contractions of the endothelium-intact human isolated coronary artery to endothelin (ET) receptor agonists; (●) ET-1 ($n = 37$); (○) sarafotoxin S6b ($n = 26$); (◆) big-ET-1 ($n = 4$); (▼) ET-3 ($n = 4$); (△) [Ala^{1,3,11,15}]ET-1 ($n = 7$); (▲) big-ET-3 ($n = 3$). Agonist concentration refers to mol l⁻¹. pD₂ and E_{max} are given in Table 1. Where no error bar is visible, error falls within the limits of the symbol.

shown) slowly faded after the highest concentration of agonist has been added. The mean contraction (as a percentage of contraction induced by 100 mM K⁺), directly and 60 min after adding 0.3 μ M ET-1 or 0.3 μ M sarafotoxin S6b was: $84.0 \pm 5.2\%$ and $26.2 \pm 3.5\%$, respectively, for ET-1 ($n = 18$ segments from 10 hearts), and $90.7 \pm 10.0\%$ and $56.3 \pm 11.8\%$, respectively, for sarafotoxin S6b ($n = 10$ segments from 5 hearts). Sixty min after the maximal concentration of sarafotoxin S6b had been added (not shown in Figure 3), the subsequent addition of 0.03 μ M ET-1 resulted in an increase of contractile force (from $33.0 \pm 11.7\%$ to $81.1 \pm 6.0\%$ of contraction induced by 100 mM K⁺, $n = 5$), whereas the subsequent adding of 0.03 μ M sarafotoxin S6b had no further effect (from $66.2 \pm 3.2\%$ to $64.3 \pm 4.0\%$ of contraction induced by 100 mM K⁺, $n = 5$). When 0.1 μ M ET-1 was added after this failure of 0.03 μ M sarafotoxin S6b to produce further contraction, an increase of contractile force to $99.6 \pm 3.1\%$ of contraction induced by 100 mM K⁺ was observed ($n = 5$; $P < 0.01$). Sixty min after the maximal concentration of ET-1 had been added, subsequent addition of neither 0.03 μ M ET-1 nor 0.03 μ M sarafotoxin S6b resulted in a further increase of contraction (from $22.1 \pm 4.9\%$ to $22.6 \pm 5.0\%$ ($n = 10$) and $31.3 \pm 6.2\%$ to $32.1 \pm 6.1\%$ ($n = 8$) of contraction induced by 100 mM K⁺, respectively).

Antagonist effects

In endothelium-intact coronary artery segments 0.1 μ M BQ-123 antagonised contractions induced by sarafotoxin S6b resulting in a pK_B of 7.86 ± 0.08 ($n = 6$). This same concen-

Table 1 Potency (pD₂ ($-\log [EC_{50}, \text{mol l}^{-1}]$)) and maximal effect (E_{max}, expressed as a percentage of the contractile response to 100 mM K⁺) of endothelin receptor agonists

Agonist	Concentration-range studied	n	pD ₂	E _{max}
ET-1	30 pM–0.3 μ M	37	8.27 ± 0.06	$102.7 \pm 5.4\%$
Sarafotoxin S6b	30 pM–0.3 μ M	26	8.16 ± 0.05	$104.5 \pm 5.4\%$
Big-ET-1	0.1 nM–1 μ M	4	$< 7.1 \pm 0.02^1$	$> 124.3 \pm 12.4\%^1$
ET-3	0.1 nM–1 μ M	4	$< 6.9 \pm 0.1^1$	$> 84.4 \pm 9.6\%^1$
[Ala ^{1,3,11,15}]ET-1	0.1 nM–1 μ M	7	NC ¹	$> 11.4 \pm 3.6\%^1$
Big-ET-3	0.1 nM–0.3 μ M	3	No effect	

n, number of segments tested; NC, not calculated; ¹maximal effect possibly not reached at 1 μ M.

Figure 2 (a) Contractions of the human isolated coronary artery to endothelin-1 (ET-1) in endothelium-intact (●), or endothelium-denuded (Δ) ring segments ($n = 8$). (b) Contractions of the human isolated coronary artery to sarafotoxin S6b in endothelium-intact (○), or endothelium-denuded (Δ) ring segments ($n = 5$). The concentration of ET-1 and sarafotoxin S6b refers to mol l^{-1} . (c) Relaxant responses to 1 nM substance P in the same segments expressed as a percentage of precontraction with 1 μM prostaglandin F_{2α} (PGF_{2α}). E +, endothelium intact; E -, endothelium denuded. Where no error bar is visible, error falls within the limits of the symbol.

Figure 3 Experimental recordings of concentration-response curves, preceded by the reference contractile response to 100 mM K⁺, in four endothelium-intact coronary artery segments obtained from a 41 year old male (a and b) and a 25 year old female (c and d). (a) After reaching the E_{max} in a concentration-response curve to ET-1, the addition of 0.1 μM sarafotoxin S6b (arrow) did not cause an additional response. (b) After reaching the E_{max} in a concentration-response curve to sarafotoxin S6b, the addition of 0.1 μM ET-1 (arrow) caused an additional contractile response. (c) After reaching the E_{max} of a concentration-response curve to sarafotoxin (but also to ET-1: not shown) contraction slowly faded away (also a and b). (d) Addition of a concentration of sarafotoxin S6b, causing a submaximal effect in a parallel segment, resulted in a sustained contractile response. After subsequently completing the concentration-response curve in this particular segment, a further contractile response could be induced by addition of 0.1 μM ET-1 (arrow).

ration of BQ-123 caused a small increase of contraction induced by ET-1 ($n = 7$). Only 10 μM BQ-123 antagonized contractions induced by ET-1 (pK_B : 5.75 ± 0.18 ; $n = 4$). FR139317 was also more potent against sarafotoxin S6b than against ET-1. This resulted in pK_B values for FR139317 against sarafotoxin S6b of 8.47 ± 0.17 (3 nM) and 8.24 ± 0.08 (0.3 μM) (both $n = 5$). However, 3 μM FR139317 applied against ET-1-induced contractions resulted in a pK_B of 6.11 ± 0.16 ($n = 6$). The small contractile response induced by 1 μM [Ala^{1,3,11,15}]ET-1 faded during the 30 min incubation

when tested as an antagonist. [Ala^{1,3,11,15}]ET-1 (1 μM) did not affect the contractile response to ET-1 or to sarafotoxin S6b ($n = 6$) (Figure 4).

Comparison with strip preparations

ET-1 was more potent as an agonist on endothelium-intact strip preparations than on endothelium-intact ring segments (pD_2 : 9.04 ± 0.16 in strips vs. 8.50 ± 0.07 in rings; $n = 5$). The pK_B of BQ-123 (1 μM) against ET-1-induced contractions

Figure 4 Contractions of the endothelium-intact human isolated coronary artery to endothelin-1 (ET-1) (upper panels), and sarafotoxin S6b (lower panels) in the absence (control ET-1: ●; control sarafotoxin S6b: ○) or presence of the endothelin receptor antagonist BQ-123 ($n = 4-13$), FR139317 ($n = 5-6$), or [Ala^{1,3,11,15}]ET-1 ($n = 6$). Concentrations of receptor antagonists were 3 nM (◆), 0.03 μ M (□), 0.1 μ M (▽), 0.3 μ M (▼), 1 μ M (△), 3 μ M (▲) or 10 μ M (■). Concentrations of ET-1 and sarafotoxin S6b refer to mol l⁻¹, pK_B values are mentioned in the text. Where no error bar is visible, error falls within the limits of the symbol.

Figure 5 Contractions of the human isolated coronary artery to endothelin-1 (ET-1) in endothelium-intact ring preparations (closed symbols, $n = 12$) and endothelium-intact spiral strip preparations (open symbols, $n = 5$), in the absence (control, ● and ○) or presence of 1 μ M BQ-123 (■ and □). For pD₂ and pK_B values, see text. The concentration of ET-1 refers to mol l⁻¹. Where no error bar is visible, error falls within the limits of the symbol.

Figure 6 Contractions of the endothelium-intact human isolated coronary artery to big endothelin-1 (big-ET-1) in the absence (control, ◆) and presence of 100 μ M phosphoramidon (▼). $n = 4$; *, $P < 0.05$ vs. control response. The concentration of big-ET-1 refers to mol l⁻¹. Where no error bar is visible, error falls within the limits of the symbol.

of strips was 6.62 ± 0.18 (Figure 5) vs. 5.75 ± 0.18 in rings (10 μ M BQ-123).

Big-ET-1 and phosphoramidon

The concentration-response curve to big-ET-1 was remarkably steep in comparison with the other endothelin isopeptides (Figure 1). As shown in Figure 6, the pretreatment of the vessel segment with phosphoramidon (100 μ M) resulted in significant attenuation of the contractile responses and in a

non-parallel shift of the big ET-1-induced concentration-response curve.

Discussion

The main findings of the present study are the observed differences between contractions induced by ET-1 and sarafotoxin S6b. The ET_A receptor antagonists BQ-123 and FR139317 were clearly more potent against sarafotoxin S6b

than against ET-1. ET-1 and sarafotoxin S6b induced similar contractile responses in endothelium-intact and endothelium-denuded vessel segments. After a full concentration-response curve had been obtained to ET-1 or sarafotoxin S6b, further contractions of the endothelium-intact coronary artery segments could be achieved only by applying ET-1 in segments exposed to sarafotoxin S6b, and not in segments exposed to ET-1.

It is to be noted that most concentration-response curves were obtained in vessel segments in which the endothelium was left intact, due to the more frequent occurrence of spontaneous phasic oscillations in the absence of endothelium. These spontaneous rhythmic contractions would hinder detailed analysis of concentration-response curves (Kawasaki *et al.*, 1981). Destruction of the endothelium in a perfused mesenteric arterial bed of the rat increased the contractile response to ET-1, sarafotoxin S6b, ET-3 and big-ET-1 (Douglas & Hiley, 1990). We established for ET-1 and sarafotoxin S6b that removal of the endothelium in coronary artery segments in our model does not alter the contractile response (Figure 2). However, the possibility cannot be ruled out that contractile responses to the other agonists are modulated by the presence of endothelium.

Heterogeneity of receptors involved in the response to ET-1 and sarafotoxin S6b?

The present data supply evidence for heterogeneous mechanisms mediating the contractile response to ET-1 and sarafotoxin S6b. First, the ET_A receptor antagonists, BQ-123 and FR139317, had significantly different potencies against ET-1- and sarafotoxin S6b-induced contractile responses. Secondly, after a concentration-response curve ending with 0.3 μ M ET-1 or 0.3 μ M sarafotoxin S6b, further contractions could be achieved only with ET-1 in segments that had been exposed to sarafotoxin S6b and not in those exposed to ET-1. Also in view of the $E_{\max}$ of sarafotoxin S6b being slightly, but significantly, higher than that of ET-1, it is difficult to explain why ET-1, but not sarafotoxin S6b, caused further contraction of segments exposed to the other compound. Possibly, ET-1 caused desensitization of events beyond activation of the receptor for sarafotoxin S6b, without affecting this receptor itself. The latter was also suggested by Cardell *et al.* (1992), who made similar observations in the guinea-pig pulmonary artery, using ET-3 instead of sarafotoxin S6b. Hiley and co-workers (1992b) observed analogous cross-desensitization using ET-1 and ET-3, but suggested that ET-3 interacted with an ET-1-insensitive accessory binding site on the receptor used by ET-1 itself. This explanation of two agonists binding to different binding-sites on the same receptor also cannot be ruled out as the reason for the differences in antagonist potency observed against ET-1 and sarafotoxin S6b.

To characterize endothelin receptors in the present study, several complicating factors have to be kept in mind. First, we have no knowledge of possible differential metabolic- or uptake mechanisms of any of the endothelin receptor ligands. Indeed, contractile responses to high $E_{\max}$ concentrations of ET-1 or sarafotoxin S6b (with a magnitude of over 100% of the contractile response to 100 mM K⁺) fade away partially. However, this decline is apparently not due to metabolic breakdown, since in our study the contractile response to lower concentrations of the peptides was sustained (Figure 3). Secondly, one may argue that receptor pharmacodynamics are not conventionally straightforward in interactions with endothelin receptors. After activating the receptor in human and porcine smooth muscle cells, the endothelin receptor-ligand complex was found to be internalized and, thus, possibly unavailable for competition (Resink *et al.*, 1990). It has also been reported that an endothelin receptor antagonist like BQ-123 may act either as a competitive, or non-competitive antagonist (Hiley *et al.*, 1992a), depending

on whether or not equilibrium conditions are present (Vigne *et al.*, 1993). Lastly, the ligand-receptor kinetics for endothelins (e.g. different association/dissociation rates for different endothelin isopeptides, Galron *et al.*, 1991) could have implications for functional studies investigating the receptors involved. On the other hand, these conceivable, but yet theoretical pitfalls should not preclude comparison of the present observations with endothelin receptor characterizations in smooth muscle preparations as reported in previous studies. Furthermore, the involvement of heterogeneous receptors is supported by the observation of an additional non-ET_A, non-ET_B binding site in human coronary arteries labelled by [¹²⁵I]-sarafotoxin S6b, but not by [¹²⁵I]-ET-1, with particularly high affinity for BQ-123 (Bax *et al.*, 1993b). Also in support of the involvement of heterogeneous receptors are previous studies in human isolated saphenous veins in which BQ-123 biphasically inhibited sarafotoxin S6b-induced contractions. BQ-123 was more potent against high than against low concentrations of sarafotoxin S6b (Bax *et al.*, 1993a).

Are ET_A receptors involved in the contractile responses to ET-1 and sarafotoxin S6b?

Originally, ET_A receptors were differentiated from ET_B receptors by a high affinity of ET_A receptors for ET-1 compared to ET-3 (Arai *et al.*, 1990; Williams *et al.*, 1993). ET_B receptors, however, have similar affinity for ET-1 and ET-3 (Sakurai *et al.*, 1990; Williams *et al.*, 1993). Considering the difference in potency of ET-1 and ET-3 in the present study, it may be concluded that ET-1-induced responses in the present study are mediated by ET_A receptors. In addition, this order of potency virtually rules out the involvement of the putative ET_C receptor, which has higher affinity for ET-3 than for ET-1 (Masaki *et al.*, 1994). The presently observed agonist order of potency is similar to that in the rat thoracic aorta (Sumner *et al.*, 1992), the rabbit carotid artery (White *et al.*, 1993), and the guinea-pig aorta (Schoeffter & Randrianisoa, 1993). In all of these animal preparations, it was concluded that ET_A receptors mediate the contractile response. The fact that in the present study BQ-123 antagonized ET-1-induced contractions only at 10 μ M ($pK_B = 5.75$) would however argue against involvement of the ET_A receptor, since BQ-123 has submicromolar (Ihara *et al.*, 1992a; Williams *et al.*, 1993) to nanomolar (Molenaar *et al.*, 1993; Davenport *et al.*, 1993) affinity for ET_A receptors in binding studies. In our experiments 0.1 μ M BQ-123, if anything, caused a small potentiation of the contractile response. The pK_B value of BQ-123 against sarafotoxin S6b appears in better agreement with ET_A receptor involvement. It is however unlikely that sarafotoxin S6b activates an ET_A receptor, which is not activated by ET-1, since ET-1 has an even higher affinity for ET_A receptors than sarafotoxin S6b (Arai *et al.*, 1990).

The pK_B value (6.11) of FR139317 against ET-1 is in reasonable accordance with an ET_A receptor-mediated mechanism. Previously found pA_2 values for FR139317 include 6.0 in the rabbit femoral artery (Doherty *et al.*, 1993), 6.65 in the guinea-pig pulmonary artery (Cardell *et al.*, 1993) and 7.2 in the human coronary artery (Maguire *et al.*, 1994) and rabbit aorta (Sogabe *et al.*, 1993). A pK_B value of FR139317 as high as 8.2–8.5 against sarafotoxin S6b-induced responses (present results) has not yet been described for an ET_A receptor-mediated functional response in organ bath experiments and appears too high to point towards a 'typical' ET_A receptor-mediated mechanism.

Recent studies assessing the antagonist effects of BQ-123 against ET-1-induced contractions of human coronary artery rings (2 mm) report that, even at 0.1 μ M, BQ-123 antagonized contractile responses to ET-1, resulting in pA_2 values of 6.4–7.5 (Godfraind, 1993; Davenport *et al.*, 1993). Both these studies concluded that ET_A receptors mediate the contractile responses to ET-1. It may be hypothesized that the difference in the potency of BQ-123 in those studies and our study, and also the discrepancies within other studies

between large proximal and small distal coronary artery segments (Chester *et al.*, 1992; Godfraind, 1993), could be due to differential diffusion of the large peptide molecules to the receptors in these two types of arterial segments. Indeed, we found in the present study that the type of preparation (4 mm ring or 1 mm spiral strip) made a difference in the observed potency of both ET-1 as an agonist and BQ-123 as an antagonist (Figure 5). Although the influence of the applied resting tension on strips and rings cannot be excluded, the observed discrepancy may be due to different drug concentrations in the receptor compartment, caused by differences in the diffusion barrier of large molecules (Kenakin, 1993), such as the endothelins. This could play a role in the observed divergence found between proximal and distal human coronary artery segments (Chester *et al.*, 1992; Godfraind, 1993), possibly in addition to a different receptor profile. One may argue that partial endothelial damage of the strip preparation affected the present results. However, it must be kept in mind that even a *complete* removal of the endothelium in ring segments did not affect the contractile response to ET-1 or sarafotoxin S6b (Figure 2). Similar diffusion-related differences may also play a role in the recently observed 3 fold leftward shift of the ET-1 concentration-response curve of human vertebral arteries of infants, when compared to vertebral arteries of adults (0–2 years vs. 38–71 years) (Charpie *et al.*, 1994). Our data from human coronary arteries do not support such age-dependent differences in the response to ET-1 or sarafotoxin S6b.

The pK_B value of 6.62 ± 0.18 for BQ-123, obtained here in strip preparations against ET-1, would not seem out of line when compared to a pA_2 of 6.93 in the rat aorta (Sumner *et al.*, 1992), of 6.5 in the rabbit femoral artery (Doherty *et al.*, 1993), of 6.8 and 6.9 in the rabbit carotid artery and thoracic aorta, respectively (White *et al.*, 1993), and even compared to pA_2 values of 6.8–7.5 in the more distal parts of 2 mm human coronary artery rings (Godfraind, 1993). In all of these preparations the response was characterized as being mediated via ET_A receptors. Interestingly, Schoeffter & Randrianisoa (1993), using 3 mm ring preparations, found that ET-1-induced contractile responses of the porcine coronary artery were virtually insensitive to BQ-123 (pK_B : 5.21) and concluded that these contractions were mediated by non- ET_A receptors. In contrast, an earlier study in the porcine coronary artery, using spiral strips (diameter 1 mm), found an apparently ET_A receptor-linked pA_2 value of 7.4 (Ihara *et al.*, 1992a). Also in the porcine coronary artery, the agonist potency of ET-1 in the strip preparation was clearly higher (pD_2 : ≈ 10 ; Ihara *et al.*, 1992a) than in the ring preparation studied by Schoeffter & Randrianisoa (1993) (pD_2 : 8.3).

The presence of mRNA encoding for ET_A receptors has been established in smooth muscle cells of the human coronary artery (Winkles *et al.*, 1993; Davenport *et al.*, 1993). In a receptor autoradiography analysis of endothelin receptors in the human coronary artery, it was shown that 30 pM [125 I]-ET-1 labelled a homogeneous receptor population in the human coronary artery with similar characteristics to the ET_A receptors identified in atrium and ventricle (Bax *et al.*, 1993b). Also Davenport *et al.* (1994), studying binding displacement of 100 pM [125 I]-PD151242, concluded that ET_A receptors were present in the media of (atherosclerotic) human coronary artery.

Taking into account (i) the higher potency of ET-1 compared to ET-3, (ii) the pK_B value which we obtained (6.62 ± 0.18) for BQ-123 against ET-1 in strip preparations, (iii) the potency of FR139317 in coronary artery rings, and (iv) the previously obtained evidence from radioligand binding and molecular biology experiments for the presence of ET_A receptors in the human coronary artery, we have to conclude that contractile responses to ET-1 were most likely mediated via an ET_A receptor, despite a pK_B value of only 5.75 for BQ-123 against ET-1-induced responses in 4 mm coronary artery rings. We showed that the type of preparation can affect the potency of both ET-1 and BQ-123, which

may have significant implications for the use of these compounds in other studies involving endothelin receptor characterization. Considering the associated high pK_B values of BQ-123 and especially FR139317, we propose that contractions to sarafotoxin S6b are mediated via another subtype of ET_A receptors. A similar suggestion has been made by Salom *et al.* (1993) for the effects of sarafotoxin S6b in the goat cerebral artery. Possibly, the effects of ET-3 (also extremely potently antagonized by BQ-123) are mediated via the same receptor, not only in the human isolated coronary artery (Godfraind, 1993), but also in the human small omental vein (Riezebos *et al.*, 1994), in the rat vas deferens (Eglezos *et al.*, 1993; Warner *et al.*, 1993), and in the guinea-pig left atrium (Hatton *et al.*, 1994). Although the above mentioned complicating receptor dynamics or kinetics cannot entirely be ruled out, no evidence is available as yet for such mechanisms.

Are ET_B receptors involved in contractile responses?

The very small contractile response elicited by high concentrations of [$Ala^{1,3,11,15}$]ET-1 in the human isolated coronary artery would argue against the involvement of ET_B receptors. Davenport *et al.* (1993) found a complete lack of agonist effect in the human coronary artery of both [$Ala^{1,3,11,15}$]ET-1 and BQ-3020, another ET_B receptor selective agonist (Ihara *et al.*, 1992b). In the pig coronary artery [$Ala^{1,3,11,15}$]ET-1 acted as a partial agonist with a pD_2 of 7.4 (Schoeffter & Randrianisoa, 1993). Furthermore, [$Ala^{1,3,11,15}$]ET-1 induced phosphoinositol turnover in human ET_B receptor-transfected COS-7 cells (K_D 20 nM, Stavros *et al.*, 1993), and caused relaxation of the porcine pulmonary artery (ED_{40} 4.4 nM, Saeki *et al.*, 1991). To verify whether [$Ala^{1,3,11,15}$]ET-1 acted as a partial agonist in this preparation, concentration-response curves to both ET-1 and sarafotoxin S6b were constructed in the presence and absence of 1 μ M [$Ala^{1,3,11,15}$]ET-1, but no antagonism was observed (Figure 4). Thus, in the present study, no evidence was obtained to support functional involvement of contractile ET_B receptors. Also in our radioligand binding study, in which either 30 pM [125 I]-ET-1 or [125 I]-sarafotoxin S6b were used, no indication was found for the presence of ET_B receptors on the smooth muscle of the human coronary artery (Bax *et al.*, 1993b). However, in another investigation, where 100 pM [125 I]-ET-1 was used as a radioligand, it was concluded that a small proportion (13%) of ET_B receptors was present in the human coronary artery (Davenport *et al.*, 1993). The presence of mRNA encoding for ET_B receptors has been shown in human coronary artery smooth muscle cells (Davenport *et al.*, 1993; Winkles *et al.*, 1993). Possibly, these ET_B receptors do not play a major role in endothelin-induced vasoconstriction, but they may be involved in other yet unknown effects. Recently, however, it was shown in the human internal mammary artery and vein that ET-1 may induce contractions via a mixed ET_A/ET_B receptor population (Seo *et al.*, 1994).

Effects of big-ET-1 and phosphoramidon

The 21-amino acid peptide, ET-1, is formed after cleavage between Trp²¹ and Val²² of a 38 amino acid precursor peptide, big-ET-1 (Yanagisawa *et al.*, 1988; Okada *et al.*, 1990). The contractile response of big-ET-1 in isolated tissues may develop directly via binding of big-ET-1 to vascular smooth muscle receptors (Hirata *et al.*, 1990) as well as indirectly via ET-1 cleaved from big-ET-1 by the endothelin-converting-enzyme (ECE), which is reflected by the steep nature of the concentration-response curve. The activity of ECE was found to vary between different vascular beds (Auguet *et al.*, 1992), as shown by the ability of phosphoramidon, a metalloprotease inhibitor of ECE (Okada *et al.*, 1990), to inhibit the contractile response induced by big-ET-1. Since in the present study, pretreatment with 100 μ M phosphoramidon partially reduced the contractile response to big-ET-1, we conclude that ECE, present in the coronary artery, contributed

to the contractile response induced by big-ET-1. It has been suggested that ECE has substrate specificity for big-ET-1 compared to big-ET-3 (Okada *et al.*, 1990). Apart from this explanation, the absence of contractile effects to big-ET-3 in the present study could also result from the lower contractile potency of ET-3 compared to ET-1 (Table 1), combined with, possibly, a low affinity of big-ET-3 itself for vascular contractile receptors.

In conclusion, the present study showed that ET-1 and sarafotoxin S6b contract the human isolated coronary artery via different receptor mechanisms, which can probably be characterized best as subtypes of the ET_A receptor. Res-

ponses to ET-1 and sarafotoxin S6b are not modified by endothelial denudation. This study also showed that the type of preparation (ring of strip) may affect the potency of ET-1 as an agonist, and of BQ-123 as an antagonist. Finally, the contractile response to big-ET-1 is mediated in part by conversion to ET-1, taking place within the vascular wall of the human isolated coronary artery.

We would like to thank Dr Philippe Schoeffter (Sandoz Pharma Ltd., Basel, Switzerland) for many fruitful discussions. Supported by the Netherlands Heart Foundation, grant 89.252 and 93.146.

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(Received February 21, 1994

Revised July 4, 1994

Accepted August 23, 1994)