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Characterization of endothelin receptors in the human umbilical artery and vein

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1 The aim of the present study was to characterize pharmacologically endothelin receptors that are present in human umbilical vessels.

2 Endothelin-1 (ET-1) and endothelin-2 (ET-2) are potent stimulants of both the human umbilical artery (pEC_{50} 7.9 and 7.5) and vein (pEC_{50} 8.1 and 8.0). Endothelin-3 (ET-3) is inactive on the artery but contracts the vein (pEC_{50} 7.6). IRL1620 is inactive in both vessels. The order of potency of agonists is suggestive of a typical ET_A receptor in the artery (ET-1 = ET-2 > > ET-3) and a mixture of ET_A and ET_B receptors in the vein (ET-1 = ET-2 $\geqslant$ ET-3).

3 The selective ET_A receptor antagonist, BQ123, competitively inhibits the effect of ET-1 in the human umbilical artery (pA_2 6.9), while in the vein, only a mixture of BQ123 and BQ788 (a selective ET_B antagonist) weakly displaces to the right the cumulative concentration-response curve to ET-1. Contractions induced by ET-3 in the vein are inhibited by BQ788 (pA_2 7.6), but not by BQ123.

4 Inhibition of Ca^{2+} channels by nifedipine (0.1 μM) is accompanied by a significant decrease of the maximal response to ET-1 by 40% in the artery and by 30% in the vein. The response of the vein to ET-3 is almost abolished by nifedipine.

5 The results indicate that: (i) endothelins contract the human isolated umbilical artery via stimulation of an ET_A receptor type; (ii) the contraction induced by ET-1 in the vein is mediated by both ET_A and ET_B receptors, while ET-3 stimulates the ET_B receptor; (iii) the contribution of Ca^{2+} channels to the contraction mediated by the ET_B receptor appears to be more important than to that mediated by the ET_A receptor.

Keywords: Endothelins; ET_A and ET_B receptors; human umbilical vessels; smooth muscle

Introduction

The 21-amino acid peptides of the endothelin family, endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), are the most potent vasoconstrictors known (Yanagisawa *et al.*, 1988). The actions of endothelins (ETs) are mediated by two receptors named ET_A and ET_B. These receptors have been cloned both in animals (Arai *et al.*, 1990; Sakurai *et al.*, 1990) and in man (Haendler *et al.*, 1992). At the vascular level, ETs cause endothelium-dependent vasodilatation via ET_B receptors (Douglas *et al.*, 1992) and prominent vasoconstriction by a direct action on smooth muscle cells via ET_A receptors (Webb, 1991). However, in some vascular tissues, contractile ET_B receptors localized in smooth muscle cells have been described (Moreland *et al.*, 1992; Sumner *et al.*, 1992). ET-1 has been reported to be secreted by human umbilical vein endothelial cells (Haegerstrand *et al.*, 1989; Hemsén *et al.*, 1991) as well as by vascular smooth muscle cells (Yu & Davenport, 1995). It has been suggested that ET-1 may be involved in the regulation of vascular tone in the foetomaternal region during pregnancy since it has been found to increase to high levels in plasma during delivery (Nisell *et al.*, 1990).

Although the pharmacology of ETs has been extensively studied in blood vessels of various animal species (for a recent review see Rubanyi & Polokoff, 1994), few studies have been performed in vessels of human origin (Hemsén *et al.*, 1991; Hay *et al.*, 1993; Riezebos *et al.*, 1994; Maguire & Davenport, 1995; Bacon & Davenport, 1996). The aim of the present study was to attempt a characterization of the endothelin receptors in the human umbilical artery (hUA) and vein (hUV) with the intention of developing reliable *in vitro* pharmacological assays for human native vascular receptors of endothelins.

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Methods

Tissue preparation

Segments of human umbilical vessels were prepared from 46 umbilical cords obtained from 23 to 40 year old women after spontaneous delivery at term. The cords were placed in cold (4°C) Krebs solution. The lapse of time between the delivery and the experiment was on average 5 h (range 1–12 h). In the laboratory, the middle segment of the cord, 7–8 cm in length, was placed in Krebs solution at room temperature and, within 30 min, the two arteries and the vein were dissected free of surrounding tissues and mechanically denuded of their endothelium. The arteries (hUA) were cut into ring segments 10 mm in length, and placed in 10 ml organs baths containing Krebs solution at 37°C and stretched to a resting tension of 4 g; the vein (hUV) was cut into strips, 20 mm in length, and preloaded with 2 g. Changes of tension induced by various agents (see below) were measured by Grass FT03 force transducers and recorded on a Linseis multichannel chart recorder (model 2005).

Agonist and antagonist experiments

After an equilibration period of 2–3 h, the preparations were contracted with KCl (100 mM). The absence of functional endothelium was assessed by the absence of relaxation to acetylcholine (ACh, 1 μM) in tissues precontracted by 5-hydroxytryptamine (5-HT 0.1 μM). About 30 min later, cumulative concentration-response curves (CRCs) to ETs were performed (0.5 log unit steps, only one curve in each tissue).

CRCs were measured for ET-1, ET-2 and ET-3 in order to determine a order of potency of agonists. IRL1620, which has been shown to be a selective ET_B agonist in animal tissues (Takai *et al.*, 1992) was also tested.

Affinities of antagonists were also determined. Both the selective ET_A, (BQ123, Ihara *et al.*, 1992) and ET_B (BQ788, Ish-

ikawa *et al.*, 1994) receptor antagonists were tested. The antagonists were left in contact with the tissue for 15 min before measuring, in their presence, the CRC of ET-1 (in the artery) and of both ET-1 and ET-3 (in the vein). Three concentrations (0.3, 1 and 3 μM) of BQ123 were tested in the artery to construct a Schild plot, while only two concentrations (0.1 and 1 μM) of BQ788 were tested in the vein to estimate antagonist affinity.

To investigate the role of L-type Ca^{2+} channels in the contractile response to ETs, some experiments were performed in the presence of nifedipine 0.1 μM applied 25 min before performing CRCs to ET-1 in the hUA, and to both ET-1 and ET-3 in the hUV.

Materials

ET-1, ET-2, ET-3 and IRL1620 (Suc-[Glu^9, Ala^{11,15}]-endothelin-1(8-21)) were purchased from American Peptide Company (Sunnyvale, CA, U.S.A.). Stock solutions (0.2 mM) were made in water (ET-1) or in dimethylsulphoxide 14% (ET-2, ET-3, IRL1620). BQ123 (*cyclo* (D-Trp-D-Asp-L-Pro-D-Val-L-Leu)) and BQ788 (N-*cis*-2, 6-dimethylpiperidinocarbonyl-L-methylleucyl-D-1-methoxy-carbonyl-tryptophanyl-D-norleucine) were from Peptides International (Louisville, Kentucky, U.S.A.) and were dissolved in 200 mM Na_2HPO_4(pH 7.0) at 1 mM (BQ123) and in pure dimethylsulphoxide at 10 mM (BQ788). Stock solutions (10 mM) of nifedipine (Sigma Chemical Co., St. Louis, MO, U.S.A.) were made in pure dimethylsulphoxide.

All stock solutions were kept at −20°C until use. All other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and E. Merck (Darmstadt, Germany). Krebs solution (gassed with 95% O_2 and 5% CO_2, pH 7.4) had the following composition (in mM): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10.

Data analysis and terminology

All the data are expressed as mean ± s.e. mean. Data were statistically analysed using Student's two-tailed *t* test via a software package (Tallarida & Murray, 1986): *P* values lower than 0.05 were considered to be significant.

The pharmacological terminology adopted in this paper is in line with the recent IUPHAR recommendations (Jenkinson *et al.*, 1995; Vanhoutte *et al.*, 1996).

The agonist apparent affinities are given as pEC_{50} = the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist. Antagonist affinities are given in terms of pA_2 = the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of agoinst needed to elicit the original submaximal response (Schild, 1947; Jenkinson *et al.*, 1995). pA_2 were obtained (a) for BQ123 from a Schild plot, according to Arunlakshana & Schild (1959) and (b) for BQ788 from the Gaddum-Schild equation:

$$pA_2 = \log_{10}[(\text{dose ratio} - 1)/\text{ antagonist conentration}],$$

where the slope is made equal to 1.

Results

All the experiments presented below were performed on hUA and hUV without functional endothelium. As shown in Figure 1 (a, b), ET-1 and the two homologues, ET-2 and ET-3, induce concentration-dependent contraction of the hUA and hUV. ET-1 and ET-2 had similar appparent affinities (pEC_{50} between 7.5 and 8.1; see Table 1) in the two vessels. In contrast, ET-3 was almost inactive in the hUA, whereas in the hUV, although significantly less potent, ET-3 evoked a similar maximal contraction to ET-1 and ET-2. The selective ET_B agonist, IRL1620 at concentrations up to 0.3 μM, was inactive in both vessels (Figure 1a, b).

Table 1 Pharmacological characterization of endothelin receptors in human umbilical vessels

	Human umbilical artery		*Human umbilical vein*	
Agonist	pEC_{50}	E_{max} (g)	pEC_{50}	E_{max} (g)
ET-1	7.9	3.3±0.3	8.1	7.1±0.5
ET-2	7.5	3.1±0.2	8.0	6.5±0.8
ET-3	inactive		7.6	7.0±0.6
IRL1620	inactive		inactive	
Antagonist	pA_2		pA_2	
BQ123 vs ET-1	6.9		inactive	
BQ788 vs ET-1	inactive		inactive	
BQ123 vs ET-3	–		inactive	
BQ788 vs ET-3	–		7.6	

pEC_{50}, the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist. E_{max}, the maximal possible effect that an agonist can elicit in a given tissue under particular conditions. pA_2, the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of agonist needed to elicit the original submaximal response.

A 15 min pretreatment with the selective ET_A receptor antagonist, BQ123 (10 μM) completely inhibited the ET-1-induced contraction in the hUA without significantly affecting the response of the hUV to the same agonist (Figure 1c, d). The selective ET_B receptor antagonist, BQ788 (1 μM) was inactive in both the hUA and hUV. However, the mixture of the two antagonists (BQ123 10 μM + BQ788 1 μM) was as active as BQ123 (10 μM) in the hUA, while in the hUV (where both antagonists were inactive when tested alone) the mixture of the two was able to displace to the right the CRCs to ET-1 by 0.6 log unit (Figure 1c, d). In the vein stimulated with ET-3, BQ788 was able to displace to the right the CRCs in a concentration-dependent manner. In contrast, BQ123 (10 μM) did not show any effect either when tested alone or in combination with BQ788 (1 μM). (Figure 2).

The antagonistic activity of BQ123 against ET-1 in the hUA was studied over a range of 3 concentrations to construct a Schild plot. The results of this set of experiments are shown in Figure 3. In the presence of increasing concentrations of BQ123, the CRCs to ET-1 were shifted to the right in a parallel fashion and there was no change in the maximal contraction induced by the agonist. The analysis of the Schild plot yielded a straight line with slope of 0.98, a pA_2 value of 6.93 and a correlation coefficient of 0.99.

Similarly, in the hUV, the CRC to ET-3 was shifted to the right and the maximal response was maintained in the presence of BQ788 (0.1 μM). The pA_2 value estimated from the Gaddum-Schild equation, assuming the slope of unity, was 7.6 (Table 1).

In another series of experiments, the contribution of dihydropyridine-sensitive Ca^{2+} channels to the contractile responses of hUA to ET-1 and of hUV to both ET-1 and ET-3 was assessed by use of nifedipine. Treatment with nifedipine (0.1 μM) for 25 min, reduced the contractile response of the hUA to ET-1 over a whole range of concentrations (Figure 4): the reduction averaged about 40%. In the hUV, the inhibitory effect of nifedipine was different when measured against ET-1 or ET-3; the contraction induced by ET-3 was strongly inhibited (by more than 70%), while the contraction to ET-1 was reduced by only 30%.

Discussion

A pharmacological characterization of the receptors mediating the contractile effects of endothelins in human umbilical vessels has been attempted by use of the classical criteria re-

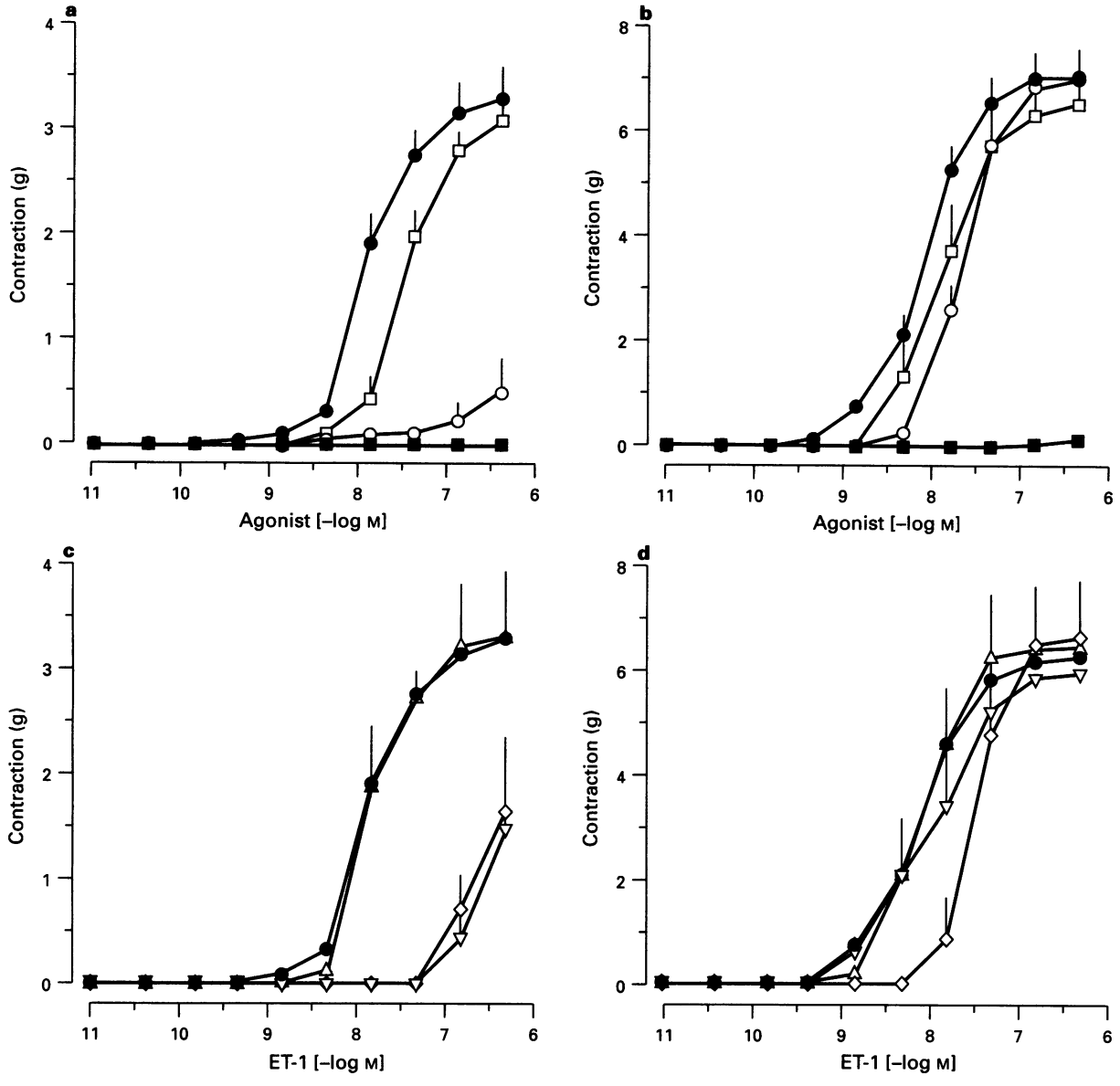

Figure 1 (a and c) Human umbilical artery; (b) and (d) human umbilicial vein. (a and b) Cumulative concentration-response curves to: ET-1 (●), ET-2 (□), ET-3 (○), IRL620 (■) in artery (a) and vein (b). (c and d) Effects of antagonists on the cumulative concentration-response curves to ET-1: control (●), BQ123 10 μM (▽), BQ788 1 μM (△), BQ123 10 μM + BQ788 1 μM (◇), in artery (c) and vein (d). Points are means with s.e. mean of at least 5 experiments.

commended by Schild, namely the order of potency of agonists and the affinities of competitive antagonists (Table 1). Active functional sites for ETs have been demonstrated in the endothelium and the smooth muscle of human and animal vessels (Webb, 1991; Douglas *et al.*, 1992; Moreland *et al.*, 1992; Sumner *et al.*, 1992). In the present study, the endothelium was removed, in order to investigate only the smooth muscle receptors that mediate the contractile effects of endothelins in the hUA and the hUV.

The results described above indicate that the contractions of the hUA in response to ETs is mediated by a ET_A receptor type, since: (i) the order of potency of agonists (ET-1 $\geq$ ET-2 > > ET-3) is suggestive of a typical ET_A receptor: indeed, ET-1 is more active than ET-3 by 1.5 log unit, a difference of affinity that is similar to that observed in pure ET_A systems, such as the rat aorta (1.9 log unit, Sumner *et al.*, 1992) and the rabbit carotid artery (1.7 log unit, Calò *et al.*, 1996) (see also recent results obtained by Bacon & Davenport, with binding assays in the ET_A receptors of the human aorta, where ET-1 (K_D 0.60 $\pm$ 0.20 nM) shows higher affinity than ET-3 (K_D 8.21 $\pm$ 1.62 nM)); (ii) BQ123, which has been shown to act as competitive antagonist of ET_A receptors in animals (Ihara *et al.*, 1992), inhibits the effect of ET-1 in a competitive manner (Schild plot in Figure 3) with a pA_2 value of 6.9 which is similar to that obtained in other human tissues (Maguire *et al.*, 1994; Maguire & Davenport, 1995) (see also results of binding experiments by Bacon & Davenport, 1996). These results are at variance with those reported by Bodelsson & Stjernquist (1993), who found BQ123 to be inactive against ET-1 in the hUA. The reason for this apparent discrepancy may be the low concentration of antagonist (0.1 μM) used by the Swedish authors, a concentration which is near to the pA_2 value (6.9) obtained in the present study.

In the hUV, the order of potency of agonists (ET-1 = ET-2 $\geq$ ET-3) suggests the presence of ET_B contractile receptors, since the difference of affinity between ET-1 and ET-3 is only 0.5 log unit. Part of the contractile effect may however be contributed by ET_A receptors, as already observed in animal vessels (e.g. the rabbit jugular vein, Calò *et al.*, 1996) and in the human coronary artery (Bacon & Davenport, 1996). In the present

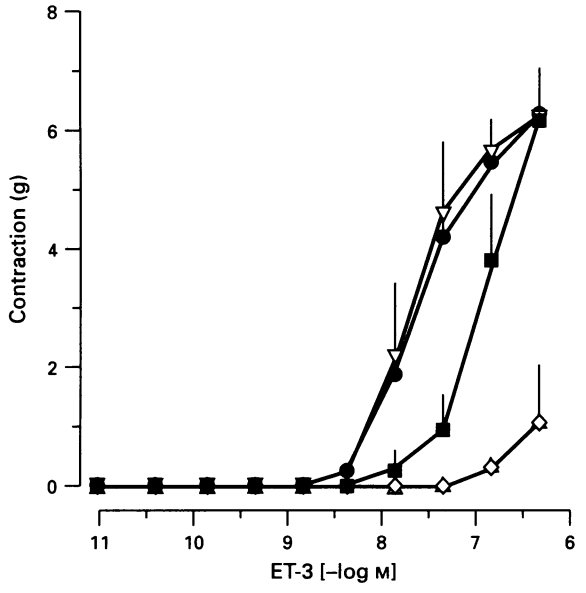

Figure 2 Human umbilical vein; effects of antagonists on the cumulative concentration-response curves to ET-3: control (●), BQ123 10 μM (▽), BQ788 0.1 μM (■), BQ788 1 μM (△), BQ123 10 μM + BQ788 1 μM (◇). Points are means with s.e. mean of at least 5 experiments.

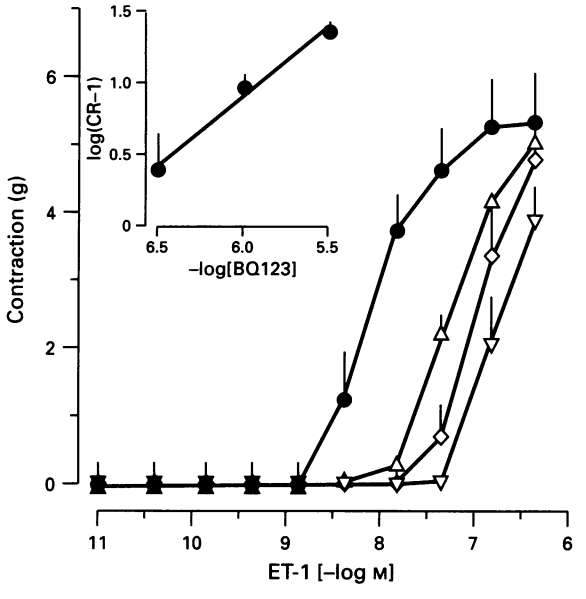

Figure 3 Human umbilical artery; effect of BQ123 on the cumulative concentration-response curves to ET-1: control (●), BQ123 0.3 μM (△), 1 μM (◇), 3 μM (▽). Corresponding Schild plot is inserted. Points are means with s.e. mean of at least 5 experiments.

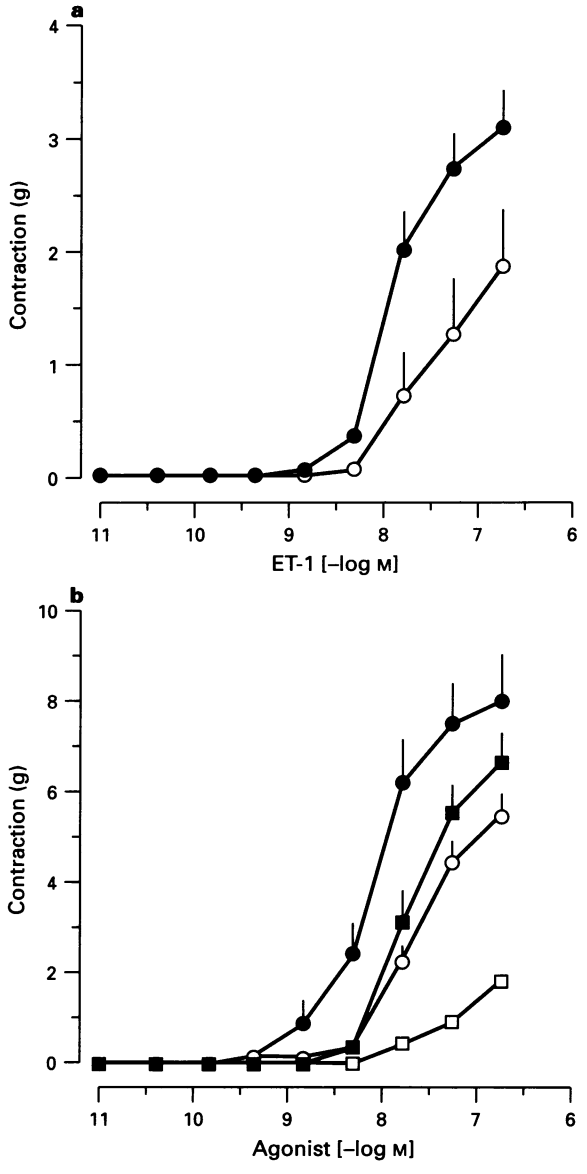

Figure 4 (a) Human umbilical artery; effects of nifedipine on the cumulative concentration-response curves to ET-1: control (●), nifedipine 0.1 μM (○). (b) Human umbilical vein; effects of nifedipine on the cumulative concentration-response curves to ET-1 and ET-3: ET-1 (●), nifedipine 0.1 μM vs ET-1 (○), ET-3 (■), nifedipine 0.1 μM vs ET-3 (□). Points are means with s.e. mean of at least 5 experiments.

study the ET_B receptor of the hUV has been characterized with ET-3, an agonist which shows very low potency at the ET_A receptor (Figure 1). The existence of ET_B receptors in the hUV could not be confirmed with IRL1620, a synthetic compound which has been shown to be selective for the ET_B receptor in animal tissues (Takai *et al.*, 1992; Calò *et al.*, 1996). The human ET_B receptor appears therefore to differ from the ET_B of other species because of its sensitivity to IRL1620. Other ET_B-selective compounds, such as BQ3020 as well as sarafotoxin 6c, were not used in the present experiment. These compounds have however been found to be less selective for the human than for the animal ET_B receptors by Bacon & Davenport (1996).

The results obtained with two antagonists, BQ123 (selective for ET_A) and BQ788 (selective for ET_B) support the above interpretation since: (i) the contraction of the hUV in response to ET-1 (the mixed ET_A and ET_B receptor stimulant) is not affected by BQ123 or BQ788 given alone; (ii) in the presence of the two antagonists the CRC of ET-1 is however displaced to the right by 0.6 log unit, suggesting that ET_A and ET_B receptors may contribute to the contractile effect of ET-1 in the hUV. When the tissue is stimulated by ET-3 (which is quite selective for the ET_B receptor), BQ123 is ineffective, while BQ788 antagonizes efficiently the effect of ET-3 (pA_2 7.6) The CRC to ET-3 measured in the presence of BQ788 (0.1 μM) is parallel to the control and this suggests that the antagonism is competitive.

As already shown by Bodelsson & Stjernquist (1993), the contraction induced by ET-1 in the hUA involves the activation of dihydropyridine-sensitive Ca^{2+} channels. This has been confirmed in the present study by showing that nifedipine reduces by 40% the response to ET-1 in this tissue (Figure 4). Moreover, the activation of Ca^{2+} channels by ET-1 in the hUA, clearly depends upon the occupation of ET_A receptors,

since the effect of ET-1 is completely abolished in the presence of high concentration of BQ123 (i.e. 10 μM). This rules out any direct stimulation of Ca^{2+} channels by ET-1. Nifedipine was also found to inhibit the myotropic effects of ETs in the hUV. The inhibitory effect of nifedipine is significantly stronger against ET-3 (−70%) than against ET-1 (−30%). This different sensitivity to nifedipine corroborates the idea that in the hUV ET-1 and ET-3-induced contractions depend on the activation of different endothelin receptor populations. Furthermore, since the effect of ET-3 depends exclusively on activation of ET_B receptors, the present findings suggest that the contribution of Ca^{2+} channel-activation to the contraction mediated by ET_B receptors is more important than to that mediated by ET_A receptors.

In conclusion, the results obtained in the present study indicate that the contractile effects of endothelins in the hUA result from the activation of ET_A receptors, while the contractions of the hUV are mediated in large part by ET_B functional sites. The human endothelin receptors are inhibited by selective antagonists such as BQ123 and BQ788, which (when given at high concentrations) are able to eliminate completely the myotropic effect of ET-1 in the hUA (BQ123) and that of ET-3 in the hUV (BQ788). ET_A and ET_B receptors are therefore the essential functional sites for endothelins in the two tissues and act in part by opening the dihydropyridine-sensitive Ca^{2+} channels.

The experimental data summarized above indicate also that the hUA and hUV are promising pharmacological preparations for studying the interactions of new chemicals with native human ET_A and ET_B receptors. The study of ET_B receptors in the hUV will however require the use of ET-3 (or other ET_B-selective compounds) as agonist and will also require the use of a selective ET_A receptor antagonist, in order to prevent the interference of the ET_A contractile site.

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