Endothelin stimulates phosphatidylinositol hydrolysis and DNA synthesis in brain capillary endothelial cells

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Endothelin-1 (ET-1) is a novel vasoconstricting and cardiotonic peptide that is synthesized by the vascular endothelium. Bovine aortic endothelial cells which secrete ET *in vitro* lack membrane receptor sites for the peptide. Endothelial cells from rat brain microvessels that do not secrete ET *in vitro* express large amounts of high-affinity receptors for ^{125}I-labelled ET-1 (K_d 0.8 nM). The ET receptor is recognized by sarafotoxin S6b and the different ET peptides with the following order of potency: ET-1 (K_d 0.5 nM) ≈ ET-2 (K_d 0.7 nM) > sarafotoxin S6b (K_d 27 nM) > ET-3 (K_d 450 nM). This structure–activity relationship is different from those found in vascular smooth muscle cells, renal cells and cardiac cells. ET-1 stimulates DNA synthesis in brain capillary endothelial cells. It is more potent than basic fibroblast growth factor. The action of ET on endothelial cells from microvessels involves phosphatidylinositol hydrolysis and intracellular Ca^{2+} mobilization. These observations suggest that brain endothelial cells might be an important target for ET.

INTRODUCTION

Endothelin-1 (ET-1) is a powerful vasoconstricting [1] and cardiotonic [2,3] peptide. In vascular smooth muscle cells (VSMC), it stimulates phosphatidylinositol hydrolysis and mobilizes Ca^{2+} from intracellular stores [4–6]. In addition, ET-1 activates a non-selective, Ca^{2+}-permeable, cationic channel which depolarizes cells and triggers the opening of voltage-dependent L-type Ca^{2+} channels [4]. It is for this reason that a large part of the vasoconstricting action of ET-1 is prevented by blockers of L-type Ca^{2+} channels [1]. Different isoforms of endothelins are now recognized. These are human/porcine ET-1 [1], human ET-2 [7] and rat ET-3 [8]. ETs also appear to have striking similarities with sarafotoxin S6b (SRTX), a neurotoxin isolated from the venom of the snake *Atractaspis engaddensis*, which has potent vasoconstricting and cardiotonic properties [9–11].

In this paper we show that bovine aortic endothelial cells, that produce ET-like material *in vitro*, are not a target for ETs. Conversely, endothelial cells from rat brain microvessels, that do not produce ET *in vitro*, have high-affinity membrane receptors for ET-1. We define the properties of interaction of this receptor with the different ETs and SRTX and show that ET acts as a mitogen for brain capillary endothelial cells and that its action involves phosphatidylinositol hydrolysis.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, M199 medium, Ham F12 medium and fetal bovine serum were from Gibco. *myo*-[2-^{3}H]Inositol (19 Ci/mmol), [*methyl*-^{3}H]-thymidine (25 Ci/mmol) and [3-[^{125}I]iodotyrosine] porcine ET-1 (2000 Ci/mmol) were from Amersham. Synthetic ET-1, ET-2 and ET-3 were purchased from Scientific Marketing Associates (London, U.K.). Synthetic SRTX was from Peninsula (St. Helens, Merseyside, U.K.). Dilutions of the peptides were freshly prepared using 0.01 % bovine serum albumin solutions. Bradykinin and ATP were from the Sigma Chemical Co. Indo-1/AM was from Calbiochem. Recombinant basic fibroblast growth factor was from Boehringer. Verapamil was from Knoll AG.

The standard solution for incubating cells was Earle's salt solution (140 mM-NaCl/5 mM-KCl/0.8 mM-$MgSO_4$/1.8 mM-$CaCl_2$/5 mM-glucose, buffered at pH 7.4 with 25 mM-Hepes/Tris).

Cell cultures

Endothelial cells were isolated from rat brain microvessels as described previously [12]. The B7 clone was grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, 200 units of penicillin/ml and 50 μg of streptomycin/ml. Cells were subcultured once a week. They retained their properties of interaction with ET for at least 50 passages. Cells at passages 5–20 were used in this study.

The thoracic aorta was isolated from 2-month-old calves and suspended in a Ca^{2+}-free Earle's solution supplemented with antibiotics. The aorta was cut open to expose the intima and washed with a Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline to remove blood cells. Endothelial cells were scraped out with a scalpel and incubated in the same medium with 0.1 % collagenase (Worthington CLSII) for 15 min. Cells were collected by centrifugation and seeded at a density of 5×10^5 cells/cm^2 using M199 culture medium supplemented with 20 % fetal calf serum, 2 mM-glutamine, 200 units of penicillin/ml and 50 μg of streptomycin/ml. Under these conditions, confluence was reached after 1 week of culture. The results presented in this paper were obtained with cells at passages 4–6.

Abbreviations used: ET, endothelin; SRTX, sarafotoxin S6b; BCEC, brain capillary endothelial cells; VSMC, vascular smooth muscle cells; EC_{50}, concn. giving 50 % of maximal effect.

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^{125}I-ET-1 binding experiments

Monolayers of endothelial cells in 12-well Corning plates were incubated at 37 °C in an Earle's salt solution supplemented with ^{125}I-ET-1 and the desired concentration of ET-2, ET-3, SRTX or unlabelled ET-1. After 45 min of equilibration at 37 °C, which was sufficient to reach equilibrium, cells were washed rapidly four times with ice-cold 0.1 M-$MgCl_2$, harvested into 0.1 M-NaOH and counted for radioactivity.

Receptor assay for ET

Washed membranes from brain capillary endothelial cells (BCEC) were prepared as described previously [12]. The reaction mixture contained 10 fmol of membrane-associated ET receptor, 250 μl of Earle's salt solution, 70 pM-^{125}I-ET-1 and 250 μl of serial dilutions of conditioned culture supernatants. Dilutions were prepared using unconditioned medium. Samples were incubated for 1 h at 37 °C and filtered under reduced pressure on to 0.22 μm Sartorius filters. After rinsing twice with 5 ml of ice-cold 0.1 M-$MgCl_2$, filters were counted for radioactivity. Calibration curves were obtained using known amounts of ET-1 and unconditioned culture medium. The detection limit of the assay was < 0.1 pmol/tube. This assay is 25 times more sensitive than that of previously described radioimmunoassays [13].

[^{3}H]Thymidine incorporation by endothelial cells

Cells were starved of serum for 48 h and incubated further in a mixture of Dulbecco's modified Eagle's medium/Ham F12 medium (50:50, v/v) supplemented with 3 μM-thymidine, 2 μCi of [^{3}H]thymidine/ml and the desired concentrations of fetal bovine serum, ET-1 or basic fibroblast growth factor. After 24 h of incubation, cells were washed twice with an Earle's salt solution, fixed for 10 min with ice-cold 5% trichloroacetic acid and rinsed with ice-cold ethanol. After digestion into 0.1 M-NaOH, the cell-associated radioactivity was counted.

Production of inositol phosphates

Monolayers of endothelial cells in 6-well Falcon plates were labelled with [^{3}H]inositol (2 μCi/ml) for 18 h in complete culture medium. After washing with an Earle's salt solution, cells were incubated at 37 °C for 10 min in a modified Earle's salt solution containing 100 mM-NaCl and 40 mM-LiCl and then stimulated with ET-1 or SRTX. The radioactivity incorporated into the different inositol phosphates [14] or into total inositol phosphates [4] was determined.

Intracellular Ca^{2+} measurements

Cells were loaded for 2 h at 37 °C with 5 μM-indo-1/AM in complete culture medium and then dissociated gently from the dishes, resuspended in complete medium and kept in the dark at room temperature until use. After incubation into the appropriate solution, the indo-1 fluorescence was assayed by flow cytometry using an ATC 3000 cell sorter (Odam-Brucker) [15]. U.v. excitation was from an argon laser at 251–364 nm. Blue (490–500nm) and violet (400–410 nm) band pass filters were used to collect indo-1 fluorescence emission, and the ratio of indo-1 violet/blue fluorescence was calculated digitally in real time for each individual cell. Cells were analysed at a flow rate of 500–1000 cells/s. Low-Ca^{2+} solutions were obtained by using appropriate concentrations of EGTA and $CaCl_2$.

Miscellaneous

Proteins were determined by the Bradford Coomassie Blue method [16] using reagents supplied by Bio-Rad.

Means ± S.E.M. given throughout.

RESULTS

Fig. 1(*a*) presents a typical Scatchard plot for the specific ^{125}I-ET-1 binding to BCEC. It shows that ^{125}I-ET-1 recognized a single family of binding sites with an equilibrium dissociation constant (K_d) of 0.8±0.2 nM (n = 5) and a maximum binding capacity of 400±95 fmol/mg of protein (n = 5). Unlabelled ET-1, ET-2, SRTX and ET-3 prevented ^{125}I-ET-1 binding to brain endothelial cells with $K_{0.5}$ values of 0.6 nM, 0.8 nM, 30 nM and 500 nM respectively (Fig. 1*b*). K_d values are given by: $K_{0.5} = K_d\{1+[^{125}\text{I-ET-1}]/K_d(^{125}\text{I-ET-1})\}$ where [^{125}I-ET-1] is the concentration of ^{125}I-ET-1 used (80 pM) and K_d (^{125}I-ET-1) is the dissociation constant of the ^{125}I-ET-1 receptor complex determined in direct binding experiments (0.8 nM). K_d values were estimated as 0.5 nM, 0.7 nM, 27 nM and 450 nM for ET-1, ET-2, SRTX and ET-3 respectively. ^{125}I-ET-1 binding experiments were also performed under the same conditions using bovine aortic endothelial cells, but no specific ^{125}I-ET-1 binding component could be detected (results not shown).

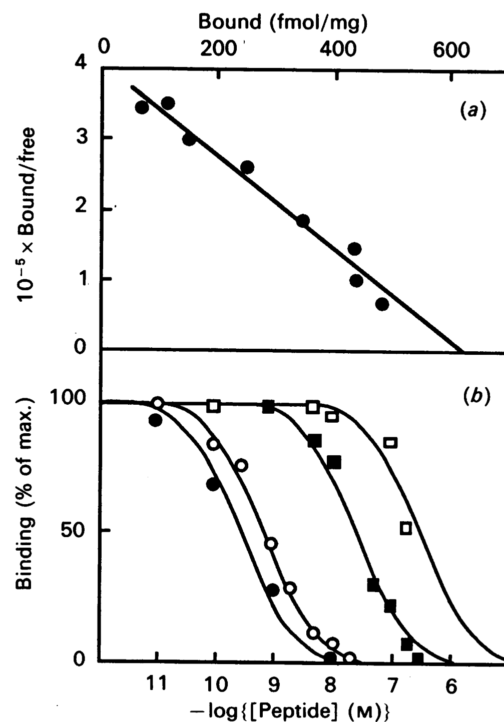

Fig. 1. Properties of the ET-1 receptor in BCEC

(*a*) Scatchard plot for specific ^{125}I-ET-1 binding to BCEC. The K_d was 0.7 nM, and maximum binding capacity was 600 fmol/mg of protein. Non-specific binding was less than 5% of total binding. (*b*) Competitions between ^{125}I-ET-1 and unlabelled ET-1 (●), ET-2 (○), SRTX (■) and ET-3 (□) for specific ^{125}I-ET-1 binding to BCEC. The concentration of ^{125}I-ET-1 was 80 pM.

Washed BCEC membranes were used in a receptor assay for measuring the production of ET-like material by endothelial cells. Fig. 2 shows that, as expected, serial dilutions of culture supernatants that have been conditioned for 5 days by monolayers of bovine aortic endothelial cells prevented ^{125}I-ET-1 binding. The concentration of ET-1-like material was 11.6 ± 0.9 nM ($n = 5$). In contrast, culture media that had been conditioned for the same period of time by BCEC were unable to prevent ^{125}I-ET-1 binding to washed membranes (Fig. 2), indicating that BCEC did not produce ET *in vitro*.

ET-1 promoted [^{3}H]thymidine incorporation by BCEC (Fig. 3*a*). Its action was additive to that of serum and it was greater than the action of basic fibroblast growth factor, a well known growth factor for endothelial cells [17]. Both in the absence and in the presence of 0.1% serum, the EC_{50} (concn. giving 50% of maximal effect) for the mitogenic action of ET-1 was 2–5 nM (Fig. 3*b*). Since BCEC do not secrete ET *in vitro* (Fig. 2), the observed effect is not part of an autocrine mechanism.

The addition of 200 nM-ET-1 induced a time-dependent increase in the [^{3}H]inositol radioactivity associated with InsP_3 (Fig. 4*a*), InsP_2 and InsP_1 (Fig. 4*b*). InsP_3 levels rose rapidly to reach a maximum after 10 s, and then decreased to the initial level within 1 min (Fig. 4*a*). InsP_2 levels remained constant after 1 min, whereas those of InsP_1 increased with time for at least 10 min (Fig. 4*b*). The EC_{50} value for ET-1 action on total inositol phosphate formation was 2 nM (Fig. 4*c*). SRTX also increased phosphatidylinositol turnover in BCEC with an EC_{50} value of 20 nM (Fig. 4*c*).

It has been shown that InsP_3 induces the release of Ca^{2+} from an intracellular pool in many cell types [18]. To demonstrate that ET-stimulated InsP_3 production in BCEC resulted in the mobilization of intracellular Ca^{2+} stores, we measured cytosolic Ca^{2+} levels in indo-1-loaded cells. Fig. 5(*a*) shows that ET-1 produced dose-dependent increases in $[Ca^{2+}]_i$. In the presence of 50 nM external Ca^{2+}, Ca^{2+} transients were of smaller size and shorter duration (Fig. 5*b*). Verapamil (10 μM), a specific inhibitor of L-type Ca^{2+} channels, had no effect on the time course or amplitude of the transients induced by ET-1, implying that these channels were not involved. Very similar Ca^{2+} transients were obtained with ET-2, ET-3 and SRTX (results not shown). Fig. 5(*c*) presents the dose–response curves for endothelin and SRTX actions on intracellular Ca^{2+} concentration. EC_{50} values were 5 nM, 10 nM, 100 nM and 500 nM for ET-1, ET-2, SRTX and ET-3 respectively. The latter values assumed that ET-3 and SRTX were full agonists of phospholipase C. Peak Ca^{2+} levels observed 20 s after the addition of 200 nM-ET-2, -SRTX and -ET-3 were 89.7 ± 4.3% ($n = 6$), 43.6 ± 7.3% ($n = 9$) and 24.0 ± 11.8% ($n = 3$) of the size of Ca^{2+} transients induced by 200 nM-ET-1.

Intracellular Ca^{2+} measurements were performed with bovine aortic endothelial cells that lack high-affinity ^{125}I-ET-1 binding sites. As expected, no action of the different

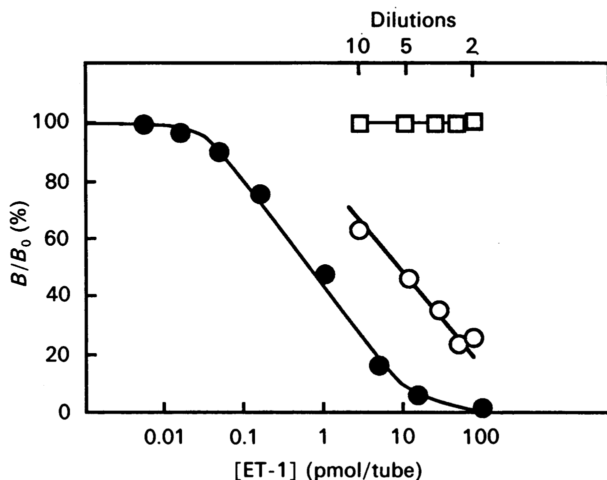

Fig. 2. Receptor assay for ET-like material in culture supernatants

The dilution curves for culture medium that had been conditioned for 5 days by monolayers of bovine aortic endothelial cells (○) and BCEC (□) are compared with that of standard ET-1 (●).

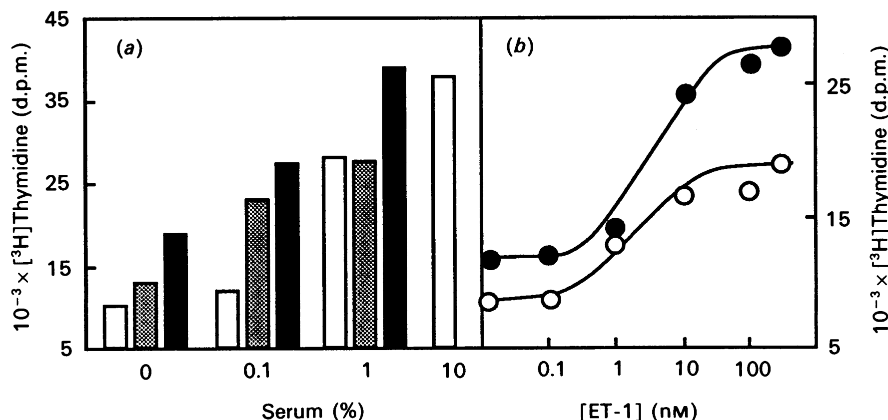

Fig. 3. Mitogenic activity of ET-1

(*a*) Compared actions of ET-1, basic fibroblast growth factor and serum on [^{3}H]thymidine incorporation by BCEC. Control experiments are shown by □. ▨, Experiments performed in the presence of 25 ng of basic fibroblast growth factor/ml; ■, experiments performed in the presence of 400 nM-ET-1. (*b*) Dose–response curves for ET-1 action on [^{3}H]thymidine incorporation by BCEC. Experiments were performed in the absence of serum (○) or in the presence of 0.1% fetal bovine serum (●).

ET peptides could be detected. We confirmed that, as previously described [19,20], the aortic endothelial cells used responded to bradykinin and ATP by large intracellular Ca^{2+} transients.

DISCUSSION

Endothelin is synthesized by endothelial cells from peripheral vessels such as porcine aorta, bovine carotid and pulmonary arteries [1,21] and bovine pulmonary microvessels [22]. Using a sensitive receptor assay for ET, we show here that bovine aortic endothelial cells secrete ET-like material *in vitro* but that rat BCEC are unable to do so. It seems therefore that ET secretion is not a general property of all vascular beds and that it might be restricted to peripheral vessels. We also show that bovine aortic endothelial cells do not have receptor sites for ET-1. This observation agrees with preliminary autoradiographic studies showing that, in the rabbit aorta, ^{125}I-ET-1-binding sites are preferentially localized towards the adventitial surface of the vessel wall [23].

Brain capillary endothelial cells express a single family of high-affinity receptor sites for ET-1. The K_d value of the ET-1 receptor complex is 0.8 nM, similar to the value found in VSMC [24,25]. The number of binding sites is 400 fmol/mg of protein, i.e about 250000 sites/cell. This value is 10 and 100 times higher than the corresponding values found in VSMC [24,25] and in mesangial cells [26] respectively, but similar to the value found in renal papillary and glomerular membranes [27]. The receptor

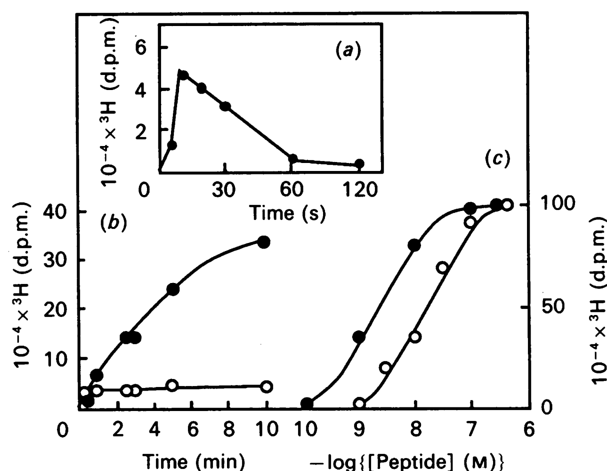

Fig. 4. Endothelin activates phosphatidylinositol hydrolysis in BCEC

(*a*) Time course of appearance of [^{3}H]inositol-labelled InsP_3 after the addition of 200 nM-ET-1. A time-independent basal radioactivity of 5000 d.p.m. was subtracted from all data. (*b*) Time course of appearance of [^{3}H]inositol-labelled InsP_1 (●) and InsP_2 (○) after the addition of 200 nM-ET-1. Time-independent basal radioactivities of 3000 d.p.m. (InsP_1) and of 3500 d.p.m. (InsP_2) were subtracted from experimental data. (*c*) Dose–response curves for ET-1 (●) and SRTX (○) stimulation of total [^{3}H]inositol phosphate formation. Time of exposure to the peptides was 5 min.

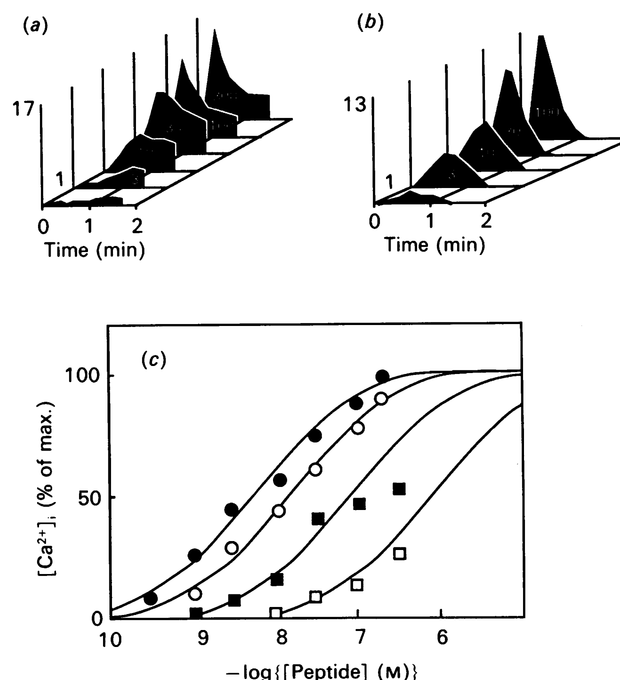

Fig. 5. Endothelins induce intracellular Ca^{2+} transients in BCEC

(*a*) and (*b*) Dose-dependence for the action of ET-1 on intracellular Ca^{2+} levels in BCEC. Experiments were performed at 1.8 mM-Ca^{2+} (*a*) or at 50 nM external Ca^{2+} (*b*). Concentrations used (in nM) are indicated. All experiments were performed on the same batch of cells. Relative fluorescence units are indicated by the vertical bars. (*c*) Dose–response curves for ET-1 (●), ET-2 (○), SRTX (■) and ET-3 (□) actions on intracellular Ca^{2+} levels. The size of the peak of the Ca^{2+} transient was measured at 1.8 mM external Ca^{2+}. Data have been normalized to the size of the peak Ca^{2+} transient induced by 200 nM-ET-1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
ET-1	C	S	C	S	S	L	M	D	K	E	C	V	Y	F	C	H	L	D	I	I	W
ET-2	C	S	C	S	S	W	L	D	K	E	C	V	Y	F	C	H	L	D	I	I	W
SRTX	C	S	C	K	D	M	T	D	K	E	C	L	Y	F	C	H	Q	D	V	I	W
ET-3	C	T	C	F	T	Y	K	D	K	E	C	V	Y	Y	C	H	L	D	I	I	W

Fig. 6. Amino acid sequences of ET-1, ET-2, SRTX and ET-3

Sequences are ordered according to the relative K_d values for the ET-1 receptor of BCEC. Residues that are not found in ET-1 are outlined. The single letter code for amino acids is used.

site for ET-1 in BCEC recognizes all peptides of the ET family with the following order of potency: ET-1 (K_d 0.5 nM) ≈ ET-2 (K_d 0.7 nM) > SRTX (K_d 27 nM) > ET-3 (K_d 450 nM). This profile is clearly distinct from that of ET receptors that have been characterized in other cell types. The structure–activity relationship for vasoconstricting activities *in vitro* and pressor activities *in vivo* is: ET-1 ≈ ET-2 ≈ SRTX ≫ ET-3 [11,28–30]. In renal papillary and glomerular membranes, ET-1, ET-3 and SRTX are equally potent in inhibiting ^{125}I-ET-1 binding [27]. Finally, in the chick heart, two types of ET receptor have been defined [31]. One is specific for ET-1 and ET-2 (ET-1 = ET-2 > ET-3 ≫ SRTX). The other is more specific for ET-3 (ET-3 > ET-1 = ET-2 > SRTX). ET-3 is also more active as a vasodilator than ET-1 in the perfused rat mesentery [32]. All these results taken together could suggest the existence of distinct subtypes of ET receptor. Fig. 6 compares the amino acid sequences of the three endothelins and of SRTX. Structure–activity relationships suggest that the amino acid substitutions localized in a short 4-amino-acid residue stretch in the *N*-terminal portion of the peptides are probably important for determining binding affinities to BCEC. This part of the molecule is thought to form a loop structure that is constrained by the conserved 1–15 and 3–11 disulphide bridges [33].

ET-1 is mitogenic for BCEC. Its potency is similar to or higher than that of basic fibroblast growth factor, a well-known growth factor for vascular endothelial cells [17]. ET-1 acts as a poor mitogen for 3T3 fibroblasts [35], mesangial cells [36] and VSMC [37,38]. One reason for the greater mitogenic effect of ET-1 in BCEC could be that BCEC express 10–100 times higher levels of ET-1 receptors than VSMC or mesangial cells. The mitogenic action of endothelin on BCEC seems to involve phosphatidylinositol hydrolysis and Ca^{2+} mobilization from intracellular stores. ET-1 and SRTX activate phospholipase C in BCEC (Fig. 4). ET-1 has previously been reported to stimulate phosphatidylinositol hydrolysis in VSMC [4,14,34], fibroblasts [14,35], rat atria [3] and mesangial cells [36]. The same action was reported for SRTX in rat atria and brain [10,11]. These observations suggest that ET receptors of VSMC, atrial cells and BCEC that are pharmacologically distinct are all coupled to phosphoinositide hydrolysis. Endothelins produced large intracellular Ca^{2+} transients in BCEC which are partly dependent on external Ca^{2+}. The order of potency of the different endothelins for inducing intracellular Ca^{2+} transients is ET-1 ≈ ET-2 > SRTX > ET-3 (Fig. 5*c*). This is identical to the order of the relative affinities of the different endothelins for the ET-1 receptor as determined in ^{125}I-ET-1 binding experiments (Fig. 1*b*). The fact that identical EC_{50} values (2–5 nM) were observed for ET-1-induced phospholipase C activity, intracellular Ca^{2+} transients and [^{3}H]thymidine incorporation suggests that phospholipase C activation and Ca^{2+} mobilization are involved in the mitogenic action of ET-1.

This work was supported by the Centre National de la Recherche Scientifique, the Fondation sur les Maladies Vasculaires, the Fondation pour la Recherche Médicale and the Association pour la Recherche sur le Cancer. We thank Mrs. N. Boyer, V. Friend and M. T. Ravier for expert technical assistance and Professor M. Lazdunski for support and helpful discussions.

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Received 1 August 1989/19 October 1989; accepted 27 October 1989