Contractile activity of endothelin precursors in the isolated gallbladder of the guinea-pig: presence of an endothelin-converting enzyme

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1 We have compared the activities of the endothelin precursors (human big ET-1₁₋₃₈, porcine big ET-1₁₋₃₉, big ET-2₁₋₃₈ and big ET-3₁₋₄₁ amide) and their respective mature 21 amino acid peptides as contractors of isolated gallbladder strips of the guinea-pig. We have also used different protease inhibitors and/or epithelial cell removal to investigate the nature and the location of the endothelin-converting enzyme (ECE) activity responsible for the conversion of porcine big ET-1₁₋₃₉ in this isolated preparation. In addition, we have conducted binding studies to investigate whether porcine big ET-1₁₋₃₉ interacts directly with ET receptors.

2 ET-1, ET-2 and ET-3 were equipotent at causing 50% of the contractions of the isolated strips (22.9 ± 6.8 , 39.5 ± 9.9 and 35.9 ± 3.7 ($\times 10^{-9}$ M), respectively), as estimated with contractions to 3×10^{-7} M taken as 100%. Big ET-1₁₋₃₈ was equipotent to ET-1 while big ET-1₁₋₃₉ was one fifth as potent as ET-1, and big ET-2₁₋₃₈ was one fifth as potent as ET-2. Precursors of ET-1 and ET-2 induced similar contractions at 3×10^{-7} M. Conversely, the contraction induced by big ET-3₁₋₄₁ amide (3×10^{-7} M) was only 14% of that induced by the same concentration of ET-3 ($287 \pm 37\%$ of $5 \mu\text{M}$ histamine).

3 The kinetics of the responses induced by single additions of 3×10^{-7} M of the endothelin isopeptides were compared. A single addition of 3×10^{-7} M ET-1, ET-2 or ET-3 produced contractions that reached a steady state in 28.2 ± 4.2 , 21.1 ± 1.3 and 24.0 ± 3.8 min, respectively and took 2.7 ± 0.4 , 2.1 ± 0.1 and 1.6 ± 0.1 min to reach half of this steady-state response.

4 Contractions induced by 3×10^{-7} M big ET-1₁₋₃₈ or big ET-1₁₋₃₉ reached a plateau in 38.5 ± 3.6 and 35.6 ± 3.3 min, respectively, and half of these responses were attained in 12.0 ± 2.5 and 7.1 ± 1.1 min. Thus, these contractions developed more slowly than those induced by ET-1. Contractions induced by 3×10^{-7} M big ET-2₁₋₃₈ were also much slower to develop than those to ET-2, for these took 49 ± 2 min to reach plateau and 19.4 ± 2.1 min to attain half that response. Contractions induced by 3×10^{-7} M big ET-3₁₋₄₁ amide took 50.2 ± 3.7 min to reach a plateau and 27.3 ± 3.0 min to reach half of this response.

5 Phosphoramidon (0.1 , 1 and 3×10^{-4} M) inhibited contractions induced by big ET-1₁₋₃₉. For instance, the contractions induced by 3×10^{-7} M big ET-1₁₋₃₉ were inhibited by 10^{-4} M or 3×10^{-4} M of phosphoramidon by $62.8 \pm 6.7\%$ or $74.5 \pm 4.6\%$, respectively. Similarly, contractions induced by ET-2₁₋₃₈ were inhibited by $91.3 \pm 5.4\%$ and the small response induced by big ET-3₁₋₄₁ amide was abolished by 3×10^{-4} M phosphoramidon. Conversely, the neutral endopeptidase (EC 24.11) inhibitor DL-thiorphan (3×10^{-4} M) had no effect. Captopril (10^{-5} M), pepstatin A (10^{-5} M), phenylmethylsulphonylfluoride (PMSF, 10^{-3} M), aprotinin (10^{-5} M), E-64 (10^{-5} M), cystatin (10^{-6} M), leupeptin (10^{-4} M), chymostatin (10^{-4} M), or bestatin (10^{-5} M) did not inhibit but rather increased to a similar, but small degree the contractions induced by 3 to 30×10^{-9} M big ET-1₁₋₃₉. Only captopril (10^{-5} M) or leupeptin (10^{-4} M) increased the contraction induced by 3×10^{-7} M big ET-1₁₋₃₉. Phosphoramidon (10^{-4} M), pepstatin (10^{-5} M) or PMSF (10^{-3} M) did not affect contractions induced by ET-1.

6 Removal of the epithelium increased by 70% the size of the contraction induced by $5 \mu\text{M}$ histamine (1.08 ± 0.05 g; $n = 160$ to 1.84 ± 0.14 g; $n = 12$) but did not affect, in absolute terms, the contraction induced by ET-1 (as a % of the response to histamine, these responses were, of course, apparently depressed). Epithelium removal did, however, increase the size of the contractions induced by 3 to 30×10^{-9} M big ET-1₁₋₃₉ which was very similar to the effect of the protease inhibitors.

7 In competition binding studies on membranes prepared from the guinea-pig gallbladder, 10^{-11} M ET-1 inhibited by $76.9 \pm 3.1\%$ the binding of [¹²⁵I]-ET-1 while porcine big ET-1₁₋₃₉ caused no inhibition (0.7 ± 3.0 ; $n = 3$). ET-1 (10^{-6} M) inhibited binding by $95.7 \pm 1.1\%$ ($n = 3$) while at this much higher concentration, big ET-1₁₋₃₉ inhibited binding by only $16.8 \pm 4.2\%$ ($n = 3$). This clearly suggests that big ET-1₁₋₃₉ does not bind directly to ET receptors.

8 Thus, a phosphoramidon-sensitive endothelin-converting enzyme (ECE), different from neutral endopeptidase (NEP; EC 24.11) and not located on the epithelium, converts big ET-1 into ET-1 in the gallbladder of the guinea-pig. This ECE appears to act preferentially on big ET-1 or big ET-2 over big ET-3.

Keywords: Endothelins; big-endothelins; endothelin-converting enzyme (ECE); guinea-pig gallbladder; protease inhibitors; phosphoramidon

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Introduction

Endothelin-1 (ET-1) is a 21 amino acid peptide, first isolated from the culture medium of aortic endothelial cells (Yanagisawa *et al.*, 1988). ET-1 and the related peptides ET-2, ET-3 and the sarafotoxins (SX6a, SX6b, SX6c, SX6d) cause vasoconstriction, vasodilatation, mitogenesis and various renal, cardiac and endocrine effects (see Huggins *et al.*, 1993). ET/SX peptides are also potent contractors of non-vascular smooth muscle preparations such as airways (Battistini *et al.*, 1992, 1994c; Hay *et al.*, 1993), the urinary bladder (Maggi *et al.*, 1989b; Saenz de Tejada *et al.*, 1992; Traish *et al.*, 1992), the uterus (Bousso-Mittler *et al.*, 1989; Svane *et al.*, 1993; Rae *et al.*, 1993a,b), the stomach fundus (Wood *et al.*, 1992; Warner *et al.*, 1993a), the ileum (Maggi *et al.*, 1989a; Wollberg *et al.*, 1991; Guimaraes & Rae, 1992; Miasiro *et al.*, 1993; Warner *et al.*, 1993b), the colon (Lin & Lee, 1990; Takahashi *et al.*, 1990) and the gallbladder (Maggi *et al.*, 1990; Moumami *et al.*, 1992; Battistini *et al.*, 1994b). In this last preparation, ET-1 causes a slowly-developing contraction (EC_{50} : 10 nM) and is 20 and 40 times more potent than carbachol and histamine, respectively, although 5 times less potent than cholecystokinin (Moumami *et al.*, 1992). The response to ET-1 is not affected by tetrodotoxin, atropine or ω -conotoxin, but is reduced by nicardipine, diltiazem or removal of calcium (Moumami *et al.*, 1992). We have recently identified two receptors, an ET_B receptor and an additional ET receptor not conforming to the established ET_A/ET_B receptor subtype classification, that mediate contractions to the endothelins in isolated gallbladder strips of the guinea-pig (Battistini *et al.*, 1994b). Interestingly, human gallbladder epithelial cells in primary culture secrete endothelin, both intrahepatic and extrahepatic bile duct epithelial cells appear to contain big ET-1 and the release of ET is increased by physiological concentrations of cholecystokinin (Housset *et al.*, 1993). Thus, ET-1 is produced locally, and by a paracrine route could play a role in choledochal motility and gallbladder contraction. Alteration of this pathway could be associated with the formation of gallstones. It is known that ET-1 is formed by a putative endothelin converting enzyme (ECE) from a 38 (human) or 39 (porcine)-amino acid precursor by cleavage between residues Trp²¹ and Val²² (Yanagisawa *et al.*, 1988; Inoue *et al.*, 1989). A similar process may well form the other two endothelins, ET-2 and ET-3, from their precursors big ET-2₁₋₃₇, big ET-2₁₋₃₈ or big ET-3_{1-41 amide} (Bloch *et al.*, 1989; 1991; Onda *et al.*, 1990). Numerous candidates have been proposed as the ECE for the conversion of big ET-1, but a membrane-bound phosphoramidon-sensitive neutral metalloprotease is the one most studied (see Opegenorth *et al.*, 1992). Such an enzyme has been purified from the rat lung (Takahashi *et al.*, 1993) and the membrane fraction of porcine aortic endothelium (Ohnaka *et al.*, 1993) and cloned from rat endothelial cells (Shimada *et al.*, 1994).

Here, we have compared mechanical responses to human big ET-1₁₋₃₈, porcine big ET-1₁₋₃₉, big ET-2₁₋₃₈, big ET-3_{1-41 amide}, ET-1, ET-2 and ET-3 to assess the ECE activity in the gallbladder. We have also used several protease inhibitors and removal of the epithelium to characterize the nature and location of the ECE involved in the conversion of porcine big ET-1₁₋₃₉. In addition, receptor binding studies were employed to demonstrate whether this precursor could bind directly to ET receptors.

A preliminary account of some of these data was presented at the Spring meeting of the British Pharmacological Society (Battistini *et al.*, 1994a).

Methods

Preparations of the tissues for organ bath experiments

Male Hartley guinea-pigs (300–400 g) were killed by cervical dislocation. The gallbladder was rapidly excised, placed in

warmed (37°C) and oxygenated (95% O₂: 5% CO₂) Krebs-Henseleit solution (comparison, mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.18, NaHCO₃ 25, MgSO₄·7H₂O 1.18 and glucose 5.6, pH 7.4) and trimmed free of connective tissue and fat. Each gallbladder was opened along its longitudinal axis and two muscle strips were prepared by cutting parallel to the long axis. The strips were mounted in 10 ml organ baths under tensions of 1.0 g and connected to Grass FT.03 force-displacement isometric transducers coupled to a Grass 79D pen recorder (Grass Instrument Co., Quincy, MA, U.S.A.). Following an equilibration period (60 min), tissues were contracted 3 times with histamine (5×10^{-6} M) with a 30 min washout period between contractions.

Experimental protocols for organ bath experiments

In the first set of experiments, concentration-response curves (0.001 to 3×10^{-7} M) to ET-1, ET-2, ET-3, human big ET-1₁₋₃₈, porcine big ET-1₁₋₃₉, big ET-2₁₋₃₈ and big ET-3_{1-41 amide} were constructed in the isolated strips. Concentrations of any endothelin peptides higher than 3×10^{-7} M were not tested because of the cost of the peptide. In a second set of experiments, the tissues were incubated with one of several protease inhibitors (phosphoramidon (10^{-5} M, 10^{-4} M, 3×10^{-4} M), an inhibitor of some metalloendopeptidases; 1,10-phenanthroline (10^{-4} M), an inhibitor of metalloproteases (chelator); DL-thiorphan (3×10^{-4} M), an inhibitor of metalloprotease; captopril (10^{-4} M), an angiotensin-converting enzyme inhibitor; pepstatin A (10^{-5} M), an inhibitor of some aspartic proteases (like cathepsin D, pepsin, renin); PMSF (10^{-3} M), an irreversible inhibitor of serine protease; aprotinin (10^{-6} M), a serine protease inhibitor; E-64 (10^{-5} M), a cysteine protease inhibitor; cystatin (10^{-6} M), a cysteine protease inhibitor leupeptin (10^{-4} M), a trypsin-like serine and cysteine protease inhibitor; chymostatin (10^{-4} M), a chymotrypsin-like and cysteine protease inhibitor; or, bestatin (10^{-5} M), an inhibitor of aminopeptidases) for 30 min before addition of cumulative concentrations of either big ET-1₁₋₃₉ or ET-1. The concentrations of inhibitors used in the present set of experiments was based on effective concentrations reported by Beynon & Salvesen (1989). In a third set of experiments, a single addition of either 3×10^{-7} M ET-1, ET-2, ET-3, big ET-1₁₋₃₈, big ET-1₁₋₃₉, big ET-2₁₋₃₈ or big ET-3_{1-41 amide} was given to equilibrated preparations. In a final set of experiments, the epithelium was gently removed by rubbing with a wet cotton swab and concentration-dependent response curves (0.001 to 3×10^{-7} M) to ET-1 or porcine big ET-1₁₋₃₉ were constructed. Removal of the epithelium was assessed by light microscopical examinations. Responses to agonists, in the presence or absence of inhibitors or the epithelium, were not compared in paired tissues. In all experiments, only one response or response curve to a single agonist was recorded in each strip. The contractile responses to each agonist were standardized by comparison to the contraction induced by 5×10^{-6} M histamine.

Preparation of membranes for competition binding studies

Gallbladders were removed as before, minced to small pieces and homogenized for 10 min (Polytron homogenizer) in 10 volumes of ice-cold assay buffer (Tris-HCl 50 mM, EDTA 1 mM, phenylmethylsulphonylfluoride (PMSF) $100 \mu\text{g ml}^{-1}$, captopril 10 μM , pepstatin 1 μM , at pH 7.4). The homogenate was centrifuged (Beckman, model GS-6R) for 15 min at 1,500 g (4°C), the supernatant collected and centrifuged (Beckman, model Optima L80) for 60 min at 100,000 g (4°C). The pellet formed was resuspended in 3 ml of ice-cold assay buffer (as above) and its protein content determined by the method of Bradford (1976). The membranes were stored at -70°C until used.

Competition binding assays

Membranes (20 µg of protein) were incubated in binding buffer (EDTA 1 µM, PMSF 100 µg ml⁻¹, MgCl₂ 1 mM, CaCl₂ 100 µM, pepstatin 1 µM, captopril 10 µM, Tris-HCl 50 mM, BSA 0.1%, at pH 7.4) with [¹²⁵I]-ET-1 (2000 Ci mmol⁻¹) in the presence of 10⁻¹¹ M or 10⁻⁶ M ET-1 or big ET-1₁₋₃₉, in the presence or absence of phosphoramidon (100 µM). After 240 min at room temperature, the binding was stopped by filtering the incubate through a Whatman glass filter followed by washing with 3 × 4 ml of ice-cold binding buffer. The amount of radioactivity present on the filter was measured in a gamma counter (Hydrogamma 16, Innotron, U.K.) for 60 s.

Agonists and peptidase inhibitors

ET-1 was synthesized at the INRS-Santé (Montréal, P.Q., Canada). ET-2, ET-3, big ET-1₁₋₃₉, big ET-1₁₋₃₈, big ET-2₁₋₃₈ and big ET-3_{1-41 amide} were obtained from Peptide Institute, Inc. (Scientific Marketing Associates, Barnet, Herts, U.K.). All peptidase inhibitors (see Table 2) and histamine hydrochloride were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). All agonists were dissolved in distilled water and stored at -20°C in aliquots of 5 × 10⁻⁵ M. Further dilutions were made in Krebs buffer. Peptidase inhibitors were dissolved in water, methanol or dimethylsulphoxide (DMSO), according to their solubility (Beynon & Salvesen; 1989). The final concentrations of vehicle in the baths were ≤ 0.1%.

Statistical analysis

All values in the text and tables are expressed as mean ± s.e.mean. Statistical analysis of the data was performed with an unpaired, two-tailed, Mann-Whitney U test and *P* < 0.01 to 0.05 were considered significant. Although full concentration-response curves could not be obtained, the relative potencies of the agonists was estimated by taking the response to 3 × 10⁻⁷ M to be 100%. Significance between concentration-response curves (10⁻¹⁰ to 3 × 10⁻⁷ M) induced by big ET-1₁₋₃₉ in the presence or absence of a protease inhibitor was calculated by a two-way, with replication, analysis of variance using the Pharmacological Calculation System (v. 4.2).

Results

Effect of big endothelins

ET-1, ET-2 and ET-3 (10⁻¹⁰ to 3 × 10⁻⁷ M) were equipotent in causing slowly-developing concentration-dependent contractions of the isolated gallbladder strips and produced similar increases in tension at 3 × 10⁻⁷ M (Battistini *et al.*, 1994a,b). Human big ET-1₁₋₃₈ also caused concentration-dependent contractions of the preparations which were similar to those induced by ET-1, while porcine big ET-1₁₋₃₉ was one fifth as potent as ET-1 (Figure 1a). Big ET-2₁₋₃₈ was one fifth as potent as ET-2 (Figure 1b) while big ET-3_{1-41 amide} (3 × 10⁻⁷ M) induced 40 ± 15% of the response induced by 5 × 10⁻⁶ M histamine, which was equivalent to only 14% of the concentration induced by ET-3 (3 × 10⁻⁷ M) (Figure 1c).

Comparative effects of a single addition of endothelin precursors or corresponding mature peptides

Single addition of ET-1, ET-2 or ET-3 (3 × 10⁻⁷ M) caused long-lasting contractions that took 21 to 28 min to reach plateau but less than 3 min to reach half-maximum values (Figure 2a; Table 1). Big ET-1₁₋₃₈ and big ET-1₁₋₃₉ (3 × 10⁻⁷ M) induced much more slowly developing contrac-

tions that took 36 and 39 min to reach a plateau. Half of the responses were reached within 8 and 12 min (Figure 2a-b; Table 1). Big ET-2₁₋₃₈ (3 × 10⁻⁷ M) induced an even slower developing contraction than big ET-1 (Figure 2c; Table 1). The response to big ET-3_{1-41 amide} was small and took more than 27 min to reach half the level achieved at 50 min (Figure 2d; Table 1).

Figure 1 Contractions of strips of the isolated gallbladder of the guinea-pig induced by (a) endothelin-1 (ET-1) (○), big ET-1₁₋₃₈ (●) or big ET-1₁₋₃₉ (■); (b) ET-2 (○) or big ET-2₁₋₃₈ (●); (c) ET-3 (○) or big ET-3_{1-41 amide} (●); *n* = 5-12. Results are expressed as the percentage of the response to histamine at 5 × 10⁻⁶ M, and are means with s.e.mean. *P < 0.05 between agonists (compared by an unpaired, two-tailed, Mann-Whitney U test).

Effects of protease inhibitors on the contractile activity of big ET-1₁₋₃₉

Contractions induced by big ET-1₁₋₃₉ were inhibited by 1,10-phenanthroline (10^{-4} M) and concentration-dependently by phosphoramidon (10^{-5} to 3×10^{-4} M) (Table 2). They were not affected, however, by DL-thiorphan (3×10^{-4} M) (Table

2). Captopril (10^{-5} M), pepstatin A (10^{-5} M), PMSF (10^{-3} M), aprotinin (10^{-5} M), E-64 (10^{-5} M), cystatin (10^{-6} M), leupeptin (10^{-4} M), chymostatin (10^{-4} M) or bestatin (10^{-5} M) potentiated the contractions, especially those big ET-1₁₋₃₉ at concentrations of 3×10^{-9} to 3×10^{-8} M, such that they were similar to the responses induced by ET-1 (Table 2).

Figure 2 Representative tracings of contractions of strips of the isolated gallbladder of the guinea-pig induced by a single addition of 3×10^{-7} M endothelin-1 (ET-1), ET-2, ET-3, big ET-1₁₋₃₈, big ET-1₁₋₃₉, big ET-2₁₋₃₈ or big ET-3₁₋₄₁ amide. The first arrow is for the contraction induced by addition of histamine (5×10^{-6} M).

Table 1 Effect of a single addition of 3×10^{-7} M endothelin-1 (ET-1), ET-2, ET-3 or their respective precursors in isolated gallbladder strips of the guinea-pig

Agonists	n	Contraction (% 5 μ M histamine)		t_{max} (min)		$t_{1/2}$ (min)	
ET-1	5	254.7 ± 36.4	Control	28.2 ± 4.2	Control	2.7 ± 0.4	Control
ET-2	3	237.2 ± 14.7	NS	21.1 ± 1.3	NS	2.1 ± 0.1	NS
ET-3	8	151.9 ± 9.6	*	24.0 ± 3.8	NS	1.6 ± 0.1	*
big ET-1 ₁₋₃₉	3	285.2 ± 45.2	NS	38.5 ± 3.6	NS	12.0 ± 2.5	*
big ET-1 ₁₋₃₈	8	220.5 ± 19.9	NS	35.6 ± 3.3	NS	7.1 ± 1.1	*
big ET-2 ₁₋₃₈	8	133.6 ± 10.0	†	49.0 ± 2.0	*†	19.4 ± 2.1	*†
big ET-3 ₁₋₄₁ amide	5	32.4 ± 7.7	†	50.2 ± 3.7	*†	27.3 ± 3.0	*†

* $P < 0.05$, compared to ET-1. † $P < 0.05$, compared to the respective mature peptide. NS, non-significant as compared to ET-1.

Table 2 Effect of various protease inhibitors on contractions of isolated gallbladder strips of the guinea-pig induced by big ET-1₁₋₃₉

Inhibitors	Concentration (μM)	n	Concentrations at 3 × 10 ⁻⁷ M (% 5 μM histamine)	EC ₅₀ ¹ (nM)	Shift of the C/R curve (x-fold)
Control	—	11	196 ± 20	—	1.0
Captopril	1	6	346 ± 45	<i>P</i> < 0.05	21.6 ± 3.3
Pepstatin A	10	5	266 ± 52	NS	17.4 ± 3.4
PMSF	1 mM	5	254 ± 30	NS	33.5 ± 7.8
Aprotinin	1	7	240 ± 33	NS	31.5 ± 7.9
E-64	10	6	222 ± 19	NS	26.0 ± 7.7
Cystatin	1	5	189 ± 22	NS	52.9 ± 34.0
Leupeptin	100	6	288 ± 19	<i>P</i> < 0.05	20.3 ± 1.8
Chymostatin	100	4	236 ± 49	NS	48.5 ± 23.4
Bestatin	10	6	197 ± 29	NS	24.9 ± 5.6
1,10-Phenanthroline	100	4	113 ± 12	<i>P</i> < 0.05	247.5 ± 120.8
DL-Thiorphan	300	6	193 ± 15	NS	69.4 ± 15.6
Phosphoramidon	300	3	50 ± 7	<i>P</i> < 0.01	469.2 ± 80.8
	100	5	73 ± 19	<i>P</i> < 0.01	241.1 ± 51.2
	10	3	197 ± 25	NS	108.6 ± 18.3

All values are mean ± s.e.mean.

¹The apparent EC₅₀ is an estimate of the effective concentration causing 50% of the response induced by 3 × 10⁻⁷ M of big ET-1₁₋₃₉. *P* < 0.01; *P* < 0.05; statistically different. NS, non-significant as compared to control.

Effects of phosphoramidon on the contractile activity of big ET-2₁₋₃₈ or big ET-3₁₋₄₁ amide

Contractions induced by big ET-2₁₋₃₈ (188.0 ± 35.7% of 5 μM histamine at 3 × 10⁻⁷ M; *n* = 7) were inhibited by 3 × 10⁻⁴ M phosphoramidon (16.3 ± 10.4% of 5 μM histamine at 3 × 10⁻⁷ M; *n* = 3). Similarly, the small response to big ET-3₁₋₄₁ amide (39.5 ± 14.6%; *n* = 6) was abolished (3.2 ± 3.2; *n* = 3).

Effects of phosphoramidon, 1, 10-phenanthroline, PMSF or pepstatin on contractions induced by ET-1

Phosphoramidon (10⁻⁴ M; *n* = 3), pepstatin A (10⁻⁵ M; *n* = 5) or PMSF (10⁻³ M; *n* = 6) did not affect contractions induced by ET-1 whereas 1, 10-phenanthroline (10⁻⁴ M) inhibited by 40% the contraction induced by 3 × 10⁻⁷ M ET-1 (*n* = 6; *P* < 0.05) and shifted the ET-1 concentration-response curve to the right. Protease inhibitors (up to 3 × 10⁻⁴ M) or their vehicles did not affect the basal tone of isolated gallbladder strips or contractions induced by 5 × 10⁻⁶ M histamine (1.08 ± 0.05 g; *n* = 160).

Effects of epithelium removal on contractions induced by histamine, ET-1 or porcine big ET-1₁₋₃₉

Removal of the epithelium increased by 70% the contraction induced by 5 × 10⁻⁶ M histamine (1.08 ± 0.05 g, *n* = 160 to 1.84 ± 0.14 g, *n* = 12). Consequently, the calculated responses to either ET-1 or porcine big ET-1₁₋₃₉ were attenuated. However, expressed as the direct response (g), removal of the epithelium did not affect the contractions induced by ET-1 (Figure 3a). Conversely, it increased the contractions induced by 3 to 30 × 10⁻⁹ M big ET-1₁₋₃₉ (Figure 3b).

Competition binding studies

Total binding of [¹²⁵I]-ET-1 was 39.8 ± 2.5% of total counts (*n* = 16). ET-1 (10⁻¹¹ M) caused a 76.9 ± 3.1% inhibition of total binding while porcine big ET-1₁₋₃₉ (10⁻¹¹ M) caused no inhibition (0.7 ± 3.0; *n* = 3). A concentration of 10⁻⁶ M ET-1 inhibited [¹²⁵I]-ET-1 binding further (95.7 ± 1.1%, *n* = 3), while at the same concentrations, big ET-1₁₋₃₉ caused only a 16.8 ± 4.2% inhibition (*n* = 3). The presence of phosphoramidon (100 μM) did not affect the binding of either peptide.

Figure 3 Contractions of strips of the isolated gallbladder of the guinea-pig induced by (a) endothelin-1 (ET-1) (*n* = 6-7) or (b) big ET-1₁₋₃₉ (*n* = 6-11), in the presence (○) or absence (●) of the epithelium. Results are expressed in grams and are means with s.e.mean *P < 0.05 (compared by an unpaired, two-tailed, Mann-Whitney U test).

Discussion

Here we demonstrate that the gallbladder of the guinea-pig contains ECE activity. Human and porcine big ET-1 caused similar concentration-dependent contractions to those produced by ET-1, although these responses developed more slowly. In addition, the response to porcine big ET-1₁₋₃₉ was inhibited by the endopeptidase inhibitor, phosphoramidon, which is known to inhibit ECE and NEP, but not by thiorphan which inhibits NEP but not ECE. Contractions induced by big ET-2₁₋₃₈ or big ET-3₁₋₄₁ amide were also sensitive to phosphoramidon. The ECE activity in this tissue resembles, therefore, the phosphoramidon-sensitive ECE described in endothelial cells, vascular smooth muscle cells and within the circulation (see Opgenorth *et al.*, 1992).

Human big ET-1₁₋₃₈ was equipotent with ET-1 while porcine big ET-1₁₋₃₉ was one fifth as potent as ET-1 in causing concentration-dependent contractions. Comparable observations have been made *in vivo* where these ET-1 precursors have similar potencies to ET-1 as pressor agents (Kashiwabara *et al.*, 1989; Fukuroda *et al.*, 1990; Matsumura *et al.*, 1990; D'Orleans-Juste *et al.*, 1990; 1991). Conversely, ET-1 is much more potent than big ET-1 at contracting isolated vascular preparations such as the rat pulmonary artery (Hirata *et al.*, 1990), the rat thoracic aorta (Kashiwabara *et al.*, 1989; Nishikori *et al.*, 1991), or the porcine coronary artery (Kimura *et al.*, 1989). Despite their equal potency in our experiments, both ET-1 precursors caused more slowly developing contractions than did ET-1 which may be attributed to the requirement for initial enzyme-dependent conversion. A similar observation was reported by Fukuroda *et al.* (1990) who found that a single addition of ET-1 (3 nM) in isolated porcine coronary arteries caused a response that took 15 min to reach half of the peak contraction whereas the response to big ET-1₁₋₃₈ (30 nM) took 35 min to reach half of the peak. Thus, the gallbladder contains an ECE activity which converts human or porcine big ET-1 and big ET-2₁₋₃₈ such that contractions induced by these precursors are similar to those induced by the mature peptides. These contractile effects are not explained by a direct effect on endothelin receptors, for binding assays demonstrated that big ET-1 displaced [¹²⁵I]-ET-1 only very poorly, as has been noted before (Hirata *et al.*, 1990). The most interesting aspect of this study is that, unlike isolated vascular preparations where big ET-1 is almost always 10–100 times less potent than ET-1, big ET-1 is equipotent to ET-1 in the guinea-pig gallbladder. This is similar to the human bronchus (Advenier *et al.*, 1992), the rat uterus (Rae *et al.*, 1993a, b) and the rat vas deferens (Télémaque & D'Orleans-Juste, 1991). This is highly suggestive that these non-vascular preparations are particularly rich in ECE, supporting the idea of extra-vascular roles for endothelin.

In the present study, big ET-2₁₋₃₈ was found to be approximately equipotent to porcine big ET-1₁₋₃₉ in producing contractions, and the similar speed of these contractions was suggestive of similar rate of conversion. Big ET-3, on the other hand was almost entirely without effect. Thus, the phosphoramidon-sensitive ECE activity present in the guinea-pig gallbladder is selective for big ET-1 or big ET-2 over big ET-3 as is the case in cultured bovine endothelial cells (Okada *et al.*, 1990; Takada *et al.*, 1991), in rat or human brain (Warner *et al.*, 1992a, b), in rat microvessels (Lawrence & Brain, 1993), in the circulation of the guinea-pig (D'Orleans-Juste *et al.*, 1991), in guinea-pig lungs (D'Orleans-Juste *et al.*, 1991) and in the rat vas deferens (Télémaque & D'Orleans-Juste, 1991). Conversely, it has been shown that big ET-3 is converted to its active

metabolite by a phosphoramidon-sensitive ECE in porcine cultured aortic endothelial cells (Matsumura *et al.*, 1992), and that big ET-3 produces a phosphoramidon-sensitive elevation in blood pressure in anaesthetized rats (Matsumura *et al.*, 1993; Pollock *et al.*, 1993). Thus, there may be varied phosphoramidon-sensitive or insensitive ECEs in different tissues and species. However, as we found that contractions of isolated gallbladder strips caused by big ET-1₁₋₃₉ were not inhibited by inhibitors of angiotensin-converting enzyme or aspartic, serine or cysteine proteases, we may exclude these enzymes as ECEs in this preparation. Similarly, the ECE present in endothelial cells is not inhibited by other endopeptidase inhibitors such as thiorphan or captopril (Matsumura *et al.*, 1990; Okada *et al.*, 1990). The relationship between the ECE in the gallbladder and that in endothelial cells may well become easier to define now that a phosphoramidon-sensitive ECE has been purified from rat lung (Takahashi *et al.*, 1993) and the membrane fraction of porcine aortic endothelium (Ohnaka *et al.*, 1993), and cloned in rat endothelial cells (Shimada *et al.*, 1994).

Contractions induced by big ET-1₁₋₃₉ were potentiated, albeit to a small degree, by the protease inhibitors, such that contractions induced by porcine big ET-1₁₋₃₉ became similar to those induced by ET-1. Most probably, this is due to the prevention of nonspecific degradation of the peptidic precursor and/or its mature peptide, i.e. ET-1, following the slow conversion. The peptidase inhibitors did not affect the contractions induced by ET-1. This may well be because ET-1 induces contraction very rapidly and in this short time is less susceptible to degradation. The nature of the following responses suggest that much of these metabolic processes are epithelially localised, as contractions induced by big ET-1₁₋₃₉ were potentiated in a manner similar to those in the presence of protease inhibitors, while contractions induced by ET-1 were not affected. These experiments also demonstrated that the ECE activity is not associated with the epithelium and that the conversion may take place on the underlying smooth muscle cells.

In conclusion, the guinea-pig gallbladder is a rich source of a phosphoramidon-sensitive ECE that catalyzes selectively the conversion of big ET-1 to ET-1, as observed in cultured endothelial cells (Ohnaka *et al.*, 1990). This is a most interesting enzyme because phosphoramidon interferes with the pharmacological effects of exogenous big-ET-1 *in vivo* (Okada *et al.*, 1990; Matsumura *et al.*, 1990; Ikegawa *et al.*, 1990), decreases the size of the infarct following myocardial ischaemia reperfusion (Grover *et al.*, 1993) and reduces cerebral vasospasm after subarachnoid haemorrhage (Matsumura *et al.*, 1991). Thus, the study of ECE may lead to the development of specific inhibitors which could be effective agents in blocking the production of ET-1 and so the deleterious effects of ETs in many pathogenic states (see Battistini *et al.*, 1993; Warner *et al.*, 1994). Isolated gallbladder strips of the guinea-pig could therefore provide a useful bioassay to test novel ECE inhibitors, for this preparation has an ECE activity similar to that in endothelial or vascular smooth muscle cells.

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References

- ADVENIER, C., LAGENTE, V., ZHANG, Y. & NALINE, E. (1992). Contractile activity of big endothelin-1 on the human isolated bronchus. *Br. J. Pharmacol.*, **106**, 883–887.
- BATTISTINI, B., D'ORLEANS-JUSTE, P. & SIROIS, P. (1992). Les endothélines et le poumon. *Méd. Sci.*, **8**, 679–687.

- BATTISTINI, B., D'ORLEANS-JUSTE, P. & SIROIS, P. (1993). Endothelins in physiopathology: circulating plasma levels and presence in other biological fluids. *Lab. Invest.*, **68**, 600–628.
- BATTISTINI, B., O'DONNELL, L.J.D., WARNER, T.D., FOURNIER, A., FARTHING, M.J.G. & VANE, J.R. (1994a). Characterization of an endothelin-converting enzyme activity in the isolated gallbladder of the guinea-pig. *Br. J. Pharmacol.*, **112**, 493P.
- BATTISTINI, B., O'DONNELL, L.J.D., WARNER, T.D., FOURNIER, A., FARTHING, M.J.G. & VANE, J.R. (1994b). Characterization of endothelin (ET) receptor subtypes in the isolated gallbladder of the guinea-pig: evidence for an additional ET receptor subtype. *Br. J. Pharmacol.*, **112**, 1244–1250.
- BATTISTINI, B., WARNER, T.D., FOURNIER, A. & VANE, J.R. (1994c). ET_B receptor subtypes mediate the contraction of isolated bronchi from guinea-pig. *Br. J. Pharmacol.*, **111**, 1009–1016.
- BEYNON, R.J., SALVESEN, G. (1989). Commercially available protease inhibitors. Appendix III. In *Proteolytic Enzymes: A Practical Approach*. ed. Beyond, R.J. & Bond, J.S. pp. 241–249. Oxford: IRL Press, Oxford Univ. Press.
- BLOCH, K.D., EDDY, R.L., SHOWS, T.B. & QUERTERMOUS, T. (1989). cDNA cloning and chromosomal assignment of the gene encoding ET-3. *J. Biol. Chem.*, **264**, 18156–18161.
- BLOCH, K.D., HONG, C.C., EDDY, R.L., SHOWS, T.B. & QUERTERMOUS, T. (1991). cDNA cloning and chromosomal assignment of the endothelin 2 gene: vasoactive intestinal contractor peptide is rat endothelin 2. *Genomics*, **10**, 236–242.
- 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.
- BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of protein dye binding. *Anal. Biochem.*, **72**, 248–254.
- D'ORLEANS-JUSTE, P., LIDBURY, P.S., WARNER, T.D. & VANE, J.R. (1990). Intravascular big-endothelin increases circulating levels of endothelin-1 and prostanoids in the rabbit. *Biochem. Pharmacol.*, **39**, R21–R22.
- D'ORLEANS-JUSTE, P., TELEMAQUE, S. & CLAING, A. (1991). Different pharmacological profiles of big-endothelin-3 and big-endothelin-1 *in vivo* and *in vitro*. *Br. J. Pharmacol.*, **104**, 440–444.
- FUKURODA, T., NOGUCHI, K., TSUCHIDA, S., NISHIKIBE, M., IKEMOTO, F., OKADA, K. & YANO, M. (1990). Inhibition of biological actions of big endothelin-1 by phosphoramidon. *Biochem. Biophys. Res. Commun.*, **172**, 390–395.
- GROVER, G.J., SLEPH, P.G., FOX, M. & TRIPPODO, N.C. (1993). Role of endothelin-1 and big endothelin-1 in modulating coronary vascular tone, contractile function and severity of ischemia in rat hearts. *J. Pharmacol. Exp. Ther.*, **263**, 1074–1082.
- GUIMARAES, C.L. & RAE, G.A. (1992). Dual effects of endothelins -1, -2 and -3 on guinea-pig field-stimulated ileum: possible mediation by two receptors coupled to pertussis toxin-insensitive mechanisms. *J. Pharmacol. Exp. Ther.*, **261**, 1253–1259.
- HAY, D.W.P., HENRY, P.J. & GOLDIE, R.G. (1993). Endothelin and the respiratory system. *Trends Pharmacol. Sci.*, **14**, 29–32.
- HIRATA, Y., KANNO, K., WATANABE, T.X., KUMAGAYE, S.-I., NAKAJIMA, K., KIMURA, T., SAKAKIBARA, S. & MARUMO, F. (1990). Receptor binding and vasoconstrictor activity of big endothelin. *Eur. J. Pharmacol.*, **176**, 225–228.
- HOUSSET, C., CARAYON, A., HOUSSET, B., HANNOUNE, L. & POUPON, R. (1993). Endothelin-1 secretion by human gallbladder epithelial cells in primary culture. *Lab. Invest.*, **69**, 750–755.
- HUGGINS, J.P., PELTON, J.T. & MILLER, R.C. (1993). The structure and specificity of endothelin receptors: their importance in physiology and medicine. *Pharmacol. Ther.*, **59**, 55–123.
- IKEGAWA, R., MATSUMURA, Y., TSUKAHARA, Y., TAKAOKA, M. & MORIMOTO, S. (1990). Phosphoramidon, a metalloproteinase inhibitor, suppresses the secretion of endothelin-1 from cultured endothelial cells by inhibiting a big endothelin-1 converting enzyme. *Biochem. Biophys. Res. Commun.*, **171**, 669–675.
- INOUE, A., YANAGISAWA, M., KIMURA, S., KASUYA, Y., MIYAUCHI, T., GOTO, K. & MASAKI, T. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 2863–2867.
- KASHIWABARA, T., INAGAKI, Y., OHATA, H., IWAMATSU, A., NOMIZU, M., MORITA, A. & NISHIKORI, K. (1989). Putative precursors of endothelin have less vasoconstrictor activity *in vitro* but a potent pressor activity *in vivo*. *FEBS Lett.*, **247**, 73–76.
- KIMURA, S., KASUYA, Y., SAWAMURA, T., SHINMI, O., SUGITA, Y., YANAGISAWA, M., GOTO, K. & MASAKI, T. (1989). Conversion of big endothelin-1 to 21 residue endothelin-1 is essential for expression of full vasoconstrictor activity: structure-activity relationships of big endothelin-1. *J. Cardiovasc. Pharmacol.*, **13**, S5–S7.
- LAWRENCE, E. & BRAIN, S.D. (1993). Big endothelin-1 and big endothelin-3 are constrictor agents in the microvasculature: evidence for the local phosphoramidon-sensitive conversion of big endothelin-1. *Eur. J. Pharmacol.*, **233**, 243–250.
- LIN, W.W. & LEE, C.Y. (1990). Biphasic effects of endothelin in the guinea-pig ileum. *Eur. J. Pharmacol.*, **176**, 57–62.
- MAGGI, C.A., GIULIANI, S., PATACCHINI, R., BARBANTI, G., TURINI, D. & MELI, A. (1990). Contractile responses of the human urinary bladder, renal pelvis and renal artery to endothelins and sarafotoxin S6b. *Gen. Pharmacol.*, **21**, 247–249.
- MAGGI, C.A., GIULIANI, S., PATACCHINI, R., ROVERO, P., GIACHETTI, A. & MELI, A. (1989a). The activity of peptides of the endothelin family in various mammalian smooth muscle preparations. *Eur. J. Pharmacol.*, **174**, 23–31.
- MAGGI, C.A., GIULIANI, S., PATACCHINI, R., TURINI, D., BARBANTI, G., GIACHETTI, A. & MELI, A. (1989b). Multiple sources of calcium for contraction of the human urinary bladder muscle. *Br. J. Pharmacol.*, **98**, 1021–1031.
- MATSUMURA, Y., FUJITA, K., TAKAOKA, M. & MORIMOTO, S. (1993). Big endothelin-3-induced hypertension and its inhibition by phosphoramidon in anaesthetized rats. *Eur. J. Pharmacol.*, **230**, 89–93.
- MATSUMURA, Y., HISAKI, K., TAKAOKA, M. & MORIMOTO, S. (1990). Phosphoramidon, a metalloproteinase inhibitor, suppresses the hypertensive effect of big endothelin-1. *Eur. J. Pharmacol.*, **185**, 103–106.
- MATSUMURA, Y., IKEGAWA, R., SUZUKI, Y., TAKAOKA, M., UCHIDA, T., KIDO, H., SHINYAMA, H., HAYASHI, K., WATANABE, M. & MORIMOTO, S. (1991). Phosphoramidon prevents cerebral vasospasm following subarachnoid hemorrhage in dogs: The relationship to endothelin-1 levels in the cerebrospinal fluid. *Life Sci.*, **49**, 841–848.
- MATSUMURA, Y., TSUKAHARA, Y., KUNINOBU, K., TAKAOKA, M. & MORIMOTO, S. (1992). Phosphoramidon-sensitive endothelin converting enzyme in vascular endothelial cells converts big endothelin-1 and big endothelin-3 to their mature form. *FEBS Lett.*, **305**, 86–90.
- MIASIRO, N., PAIVA, A.C.M., IHARA, M. & YANO, M. (1993). Two receptor subtypes are involved in the contractile component of the guinea-pig ileum responses to endothelins. *J. Cardiovasc. Pharmacol.*, **22**, S211–S213.
- MOUMMI, C., GULLIKSON, G.W. & GAGINELLA, T. (1992). Effect of endothelin-1 on guinea-pig gallbladder smooth muscle *in vitro*. *J. Pharmacol. Exp. Ther.*, **260**, 549–553.
- NISHIKORI, K., AKIYAMA, J.H., INAGAKI, Y., OHTA, H., KASHIWABARA, T., IWAMATSU, A., NOMIZU, M. & MORITA, A. (1991). Receptor binding affinity and biological activity of C-terminal elongated forms of endothelin-1. *Neurochem. Int.*, **18**, 535–539.
- OHNAKA, K., TAKAYANAGI, R., NISHIKAWA, M., HAJI, M. & NAWATA, H. (1993). Purification and characterization of a phosphoramidon-sensitive endothelin-converting enzyme in porcine aortic endothelium. *J. Biol. Chem.*, **268**, 26759–26766.
- OHNAKA, K., TAKAYANAGI, R., YAMAUCHI, T., OKAZAKI, H., OHASHI, M., UMEDA, F. & NAWATA, H. (1990). Identification and characterization of endothelin converting activity in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.*, **168**, 1128–1136.
- OKADA, K., MIYAZAKI, Y., TAKADA, J., MATSUYAMA, K., YAMAKI, T. & YANO, M. (1990). Conversion of big endothelin-1 by membrane-bound metalloendopeptidase in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.*, **171**, 1192–1198.
- ONDA, H., OHKUBO, S., OGI, K., KOSAKA, T., KIMURA, C., MATSUMOTO, H., SUZUKI, N. & FUJINO, M. (1990). One of the endothelin gene family, endothelin 3 gene, is expressed in the placenta. *FEBS Lett.*, **261**, 327–330.
- OPGENORTH, T.J., WU-WONG, J.R. & SHIOSAKI, K. (1992). Endothelin-converting enzymes. *FASEB J.*, **6**, 2653–2659.

- POLLOCK, D.M., DIVISH, B.J., MILICIC, I., NOVOSAD, E.I., BURREN, N.S. & OPGENORTH, T.J. (1993). *In vivo* characterization of a phosphoramidon-sensitive endothelin-converting enzyme in the rat. *Eur. J. Pharmacol.*, **231**, 459–464.
- RAE, G.A., CALIXTO, J.B. & D'ORLEANS-JUSTE, P. (1993a). Conversion of big endothelin-1 in rat uterus causes contraction mediated by ET_A receptors. *J. Cardiovasc. Pharmacol.*, **22**, S192–S195.
- RAE, G.A., CALIXTO, J.B. & D'ORLEANS-JUSTE, P. (1993b). Big-endothelin-1 contracts rat isolated uterus via a phosphoramidon-sensitive endothelin ET_A receptor-mediated mechanism. *Eur. J. Pharmacol.*, **240**, 113–119.
- SAENZ DE TEJADA, I., MUELLER, J.D., DE LAS MORENAS, A., MACHADO, M., MORELAND, R.B., KRANE, R.J., WOLFE, H.J. & TRAISH, A.M. (1992). Endothelin in the urinary bladder. I. Synthesis of endothelin-1 by epithelia, smooth muscle and fibroblasts suggest autocrine and paracrine cellular regulation. *J. Urology*, **148**, 1290–1298.
- SHIMADA, K., TAKAHASHI, M. & TANZAWA, K. (1994). Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. *J. Biol. Chem.*, **269**, 18275–18278.
- SVANE, D., LARSSON, B., ALM, P., ANDERSON, K.-E. & FORMAN, A. (1993). Endothelin-1: Immunocytochemistry, localization of binding sites, and contractile effects in human uteroplacental smooth muscle. *Am. J. Obstet. Gynecol.*, **168**, 233–241.
- TAKADA, J., OKADA, K., IKANAGA, T., MATSUYAMA, K. & YANO, M. (1991). Phosphoramidon-sensitive ECE in the cytosol of cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.*, **176**, 860–865.
- TAKAHASHI, K., JONES, P., KANSE, S.M., LAM, H.J., SPOKES, R.A., GHATEL, M.A. & BLOOM, S.R. (1990). Endothelin in the gastrointestinal tract: presence of endothelin-like immunoreactivity, endothelin-1 messenger RNA, endothelin receptors, and pharmacological effect. *Gastroenterol.*, **99**, 1660–1667.
- TAKAHASHI, M., MATSUSHITA, Y., IJIMA, Y. & TANZAWA, K. (1993). Purification and characterization of endothelin-converting enzyme from rat lung. *J. Biol. Chem.*, **268**, 21394–21398.
- TÉLÉMAQUE, S. & D'ORLEANS-JUSTE, P. (1991). Presence of phosphoramidon-sensitive endothelin-converting enzyme which converts big-endothelin-1, but not big-endothelin-3, in the rat vas deferens. *Naunyn-Schmied. Arch. Pharmacol.*, **344**, 505–507.
- TRAISH, A., MORAN, E., KRANE, R.J. & SAENZ DE TEJADA, I. (1992). Endothelin in the urinary bladder. II. Characterization of endothelin receptor subtypes. *J. Urology*, **148**, 1299–1306.
- WARNER, T.D., ALLCOCK, G.H., CORDER, R. & VANE, J.R. (1993a). Use of the endothelin antagonists BQ-123 and PD 142893 to reveal three endothelin receptors mediating smooth muscle contraction and the release of EDRF. *Br. J. Pharmacol.*, **110**, 777–782.
- WARNER, T.D., ALLCOCK, G.H., MICKLEY, E.J. & VANE, J.R. (1993b). Characterization of endothelin receptors mediating the effects of the endothelin/sarafotoxin peptides on autonomic neurotransmission in the rat vas deferens and guinea-pig ileum. *Br. J. Pharmacol.*, **110**, 783–789.
- WARNER, T.D., BATTISTINI, B., DOHERTY, A.M. & CORDER, R. (1994). Endothelin receptor antagonists: actions and rationale for their development. *Biochem. Pharmacol.*, **48**, 625–635.
- WARNER, T.D., BUDZIK, G.P., MATSUOTO, T., MITCHELL, J.A., FORSTERMANN, U. & MURAD, F. (1992a). Regional differences in endothelin converting enzyme activity in rat brain: inhibition by phosphoramidon and EDTA. *Br. J. Pharmacol.*, **106**, 948–952.
- WARNER, T.D., SCHMIDT, H.H.W., KUK, J., MITCHELL, J.A. & MURAD, F. (1992b). Human brain contains a metalloprotease that converts big endothelin-1 to endothelin-1 and is inhibited by phosphoramidon and EDTA. *Br. J. Pharmacol.*, **106**, 505–506.
- WOLLBERG, Z., BDOLAH, A., GALRON, R., SOKOLOVSKY, M. & KOCHVA, E. (1991). Contractile effects and binding properties of endothelins/sarafotoxins in the guinea-pig ileum. *Eur. J. Pharmacol.*, **198**, 31–36.
- WOOD, J.G., YAN, Z.Y. & CHEUNG, L.Y. (1992). Relative potency of endothelin analogues on changes in gastric vascular resistance. *Am. J. Physiol.*, **262**, G977–G982.
- YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

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