Br. J. Pharmacol. (1993), **110**, 331–337

Comparison of the cardiovascular and neural activity of endothelin-1, -2, -3 and respective proendothelins: effects of phosphoramidon and thiorphan

[1]Giovan G. Mattera, Anthony Eglezos, Anna Rita Renzetti & [2]Jacques Mizrahi

Pharmacology Department, Laboratori Guidotti SpA, Via Livornese 402, San Piero a Grado, 56122, Pisa, Italy

1 In the anaesthetized, ganglion-blocked rat, intravenous boluses of endothelin-1, endothelin-2 and endothelin-3 induced a transient hypotensive effect followed by a potent long lasting pressor response ($ED_{50\,mmHg}$: 0.72 ± 0.05, 1.8 ± 0.2 and 2.7 ± 0.3 nmol kg^{-1}, respectively). The maximal effect for the three peptides was of a similar order of magnitude (ΔMAP: 84 to 89 mmHg). Neither of these effects was influenced by phosphoramidon or thiorphan (10 mg kg^{-1}, i.v.).

2 Intravenously administered big-endothelin-1 and -2 induced a transient (1–2 min) hypotension followed by a potent long lasting (>25 min) vasopressor effect ($ED_{50\,mmHg}$: 1.8 ± 0.2 and 6.7 ± 0.4 nmol kg^{-1}, respectively), with a similar maximal activity (Δ MAP: 85 ± 4 and 81 ± 2.4 mmHg, respectively). The onset of the big-endothelin-1 vasopressor effect was more rapid (5–6 min) than that of big-endothelin-2 (10–13 min). Big-endothelin-3 was found to induce only a potent, long lasting (>35 min) hypertension, with a maximal effect of 75 ± 4.6 mmHg at 10 nmol kg^{-1} and an $ED_{50\,mmHg}$ of 6.5 ± 0.4 nmol kg^{-1}. The onset of this effect was much slower (20–25 min) than that of the other proendothelins. Pressor responses induced by big-endothelin-1, -2 and -3 (3, 15 and 10 nmol kg^{-1}, respectively) were markedly reduced (60, 80 and 100%) in the presence of phosphoramidon (10 mg kg^{-1}, i.v.). Thiorphan (10 mg kg^{-1}, i.v.) did not inhibit the effects of big-endothelin-1, -2 and -3.

3 In the electrically stimulated rat vas deferens, endothelin-1 and -2 were found to be equipotent enhancers of the twitch response ($EC_{100\%}$: 4.0 ± 0.4 nM and 7.9 ± 4.8 nM, respectively), both about 3–4 fold as active as endothelin-3 ($EC_{100\%}$: 19 ± 2.5 nM). Endothelin-1 and -3 showed a comparable maximal stimulatory effect (E_{max}: 296 ± 30 and 262 ± 24%) while endothelin-2 was less active (E_{max}: 194 ± 30%).

4 Big-endothelin-1 and -2 were potent enhancers of the twitch reponse too ($EC_{100\%}$: 10.0 ± 2.6 nM and 21.6 ± 3.2 nM, respectively), with a comparable maximal stimulatory effect (E_{max}: 254 ± 22 and 264 ± 24%). Big-endothelin-3 was found to be less potent ($EC_{100\%}$: 275 ± 21 nM), but retained a marked potentiating effect (E_{max}: 200 ± 38%). Phosphoramidon, but not thiorphan, concentration-dependently (10 and 100 μM) reduced big-endothelin-1 (58 and 86% respectively) and big-endothelin-2 (21 and 56%) -mediated responses. Conversely, the big-endothelin-3 effect was reduced by phosphoramidon only at 100 μM (− 70%), while thiorphan acts concentration-dependently (31 and 71% at 10 and 100 μM respectively); thus, in the rat vas deferens, big-endothelin-1 and -2 were as potent as their corresponding endothelins, while big-endothelin-3 was about 20 times less potent than endothelin-3.

5 The increasing effect of endothelin-2 (194 ± 30% over baseline) was significantly enhanced by either 10 μM phosphoramidon (277 ± 42%) or thiorphan (318 ± 15%). The endothelin-1 and endothelin-3-mediated twitch enhancement was not affected by the two protease inhibitors (10 μM).

6 These results suggest that *in vivo* big-endothelin-1, -2 and -3, are processed through a similar phosphoramidon-sensitive enzymatic pathway although with different apparent affinity. This enzymatic process is probably attributable to a neutral endoprotease, distinct from neutral-endopeptidase 24.11 (NEP). On the other hand, a NEP-like enzymatic activity may be involved, in the rat vas deferens, in the activation of big-endothelin-3 to endothelin-3 and in the metabolism of endothelin-2, but not of endothelin-1 or endothelin-3.

Keywords: Endothelins; proendothelins; phosphoramidon, thiorphan; mean arterial pressure; rat vas deferens

Introduction

Endothelin-1, endothelin-2 and endothelin-3 are members of the endothelin peptide family, which have a distinct distribution and represent the agonists for a related family of endothelin receptors, namely ET_A and ET_B (Kloog & Sokolovsky, 1989; Arai *et al.*, 1990; Sakurai *et al.*, 1990). These receptors are distributed in peripheral tissues (Power *et al.*, 1989) and the central nervous system (Jones *et al.*, 1989). Endothelin-1 is one the most potent vasopressors known (Yanagisawa *et al.*, 1988) and it is now widely accepted that endothelin-1 exerts its activities not only as a potent constrictor of the vascular smooth muscle but also as a modulator of the release of circulating hormones from kidney, atria and adrenal glands (Miller *et al.*, 1989; Jaffer *et al.*, 1990; Gomez-Sanchez *et al.*, 1990). Further, endothelin-1 was shown to be a modulator of neuronal activity (Yanagisawa & Masaki, 1989), inducing a potent facilitation of the nerve-mediated twitch response at the post-junctional level on the rat vas deferens (Maggi *et al.*, 1989; Télémaque & D'Orléans-Juste, 1991; Hiley *et al.*, 1989) and inhibiting the twitch response in the guinea-pig vas deferens (Wiklund *et al.*, 1990) by a pre-junctional mechanism; these observations underline the possible role of the endothelins as neuromodulator peptides.

Endothelin-1 is derived from proendothelin (big-endothelin-1) by a putative endothelin converting enzyme (ECE) that cleaves the 38-mer at the bond between Trp^{21}-Val^{22} (Yanag-

[1] Author for correspondence.
[2] Present address: Italfarmaco, S.p.A., Via dei Lavoratori, 54 20092 Cinisello Balsamo, Milano, Italy.

isawa et al., 1988). Since big-endothelin-1 has only 1/100th of the rat aorta contractile activity of endothelin-1 (Kashiwabara et al., 1989), inhibition of the ECE should effectively block the biological activities involving conversion of big-endothelin-1 to endothelin-1. This was the case in functional studies *in vitro* (D'Orléans-Juste et al., 1991a,b; Hisaki et al., 1991) and *in vivo* (Fukuroda et al., 1990; Matsumura et al., 1990b; Pollock & Opgenorth, 1991; McMahon et al., 1991; Pons et al., 1991; Mattera et al., 1992a,b). Further, ECE has been described as a phosphoramidon-sensitive neutral endopeptidase distinct from the thiorphan/phosphoramidon-sensitive neutral endopeptidase (NEP 24.11).

Haemodynamic effects of big-endothelin-2 have been described by Gardiner et al. (1992a). These effects were similar and phosphoramidon-sensitive, like those of big-endothelin-1, though less potent.

On the other hand, big-endothelin-3 was previously described as a very poor substrate for ECE (Okada et al., 1990, 1991; Takada et al., 1991; D'Orléans-Juste et al., 1991a; Mattera et al., 1992b), suggesting that the enzymatic cleavage of the proendothelins was facilitated when Trp^{21}-Val^{22} bond is present. More recently these data have been contradicted by observations *in vitro* (Matsumura et al., 1992) and *in vivo* (Gardiner et al., 1992a,b). Matsumura and coworkers have found, in membranes from cultured vascular endothelial cell, a phosphoramidon-sensitive conversion of big-endothelin-3 to its active form, while Gardiner and coworkers, in conscious Long Evans rats, have found that big-endothelin-3 exerts clear pressor and vasoconstrictor effects which are big-endothelin-1-like and phosphoramidon-sensitive.

Endothelin-1, -2 and -3 were all described as good substrates for NEP 24.11 (Vijayaraghavan et al., 1990). On the other hand, McKay et al. (1992) reported that the metabolism of the endothelins is apparently compound and species-specific, since only the endothelin-3 mediated contractile effect was potentiated by phosphoramidon in rabbit and man, but not in canine bronchus, while endothelin-1 remained unaffected. To our knowledge *in vivo* data to support these findings have not been reported so far.

In the present study we have investigated the biological effects of the endothelins and their precursors, in the presence and in the absence of phosphoramidon and thiorphan, in two systems representative of two different actions of endothelins: one *in vivo* model, for the cardiovascular effects, and one *in vitro* model, for the neuromodulatory effects.

Methods

In vivo: *pressure changes in anaesthetized, ganglion-blocked rat*

All experiments were carried out on male Sprague-Dawley rats (220–250 g, Charles River, Italy), fasted overnight, and anaesthetized with ethyl urethane (1.25 g kg^{-1}, i.m.). Rats were placed on a heating pad to maintain a constant body temperature (37 ± 0.5°C). Both femoral veins were catheterized (PE-50) for infusion of the ganglion-blocking agent and for protease inhibitor administration. Catheters (PE-50) were implanted in the left carotid artery and right jugular vein for monitoring arterial pressure and for injection of peptides, respectively. The trachea was cannulated to allow free breathing. Mean arterial pressure (MAP) was measured with a Bentley Trantec 800 pressure transducer connected with a pre-amplifer (BM614, Biomedica Mangoni) and recorded on an Astromed MT 9500 polygraph. Following a 20 min post-operative recovery period, ganglion-blockade was produced in rats with a constant infusion of pentolinium (0.1 mg^{-1} kg^{-1} min^{-1}) throughout the experiment.

Rat treatment protocols were as follows: three groups of rats received 5–6 i.v. doses of big-endothelin-1 (0.1, 0.3, 1.0, 3.0, 5.0 and 10.0 nmol kg^{-1}, $n = 6$), big-endothelin-2 (0.1, 0.3, 1.0, 3.0, 10 and 15 nmol kg^{-1}, $n = 4$) or big-endothelin-3 (0.3, 1.0, 3.0 and 10 nmol kg^{-1}, $n = 4$). Another three groups of rats were used for cumulative-dose response curves to endothelin-1 (0.03, 0.1, 0.3, 0.5, 1.0 and 2.0 nmol kg^{-1}, $n = 6$), endothelin-2 (0.4, 0.8, 1.8, 2.5, 3.0 and 5 nmol kg^{-1}, $n = 4$) or endothelin-3 (0.1, 0.3, 1, 3, 5 and 9 nmol kg^{-1}, $n = 5$). All the doses are expressed as actual dose injected. Cumulative dose-response curves were constructed by administering the next dose when the effect of the preceding one had reached a stable response, for at least 5 min, or the response started to fall.

To determine the effect of the protease inhibitors on the pressor responses induced by big-endothelin-1 (3 nmol kg^{-1}), big-endothelin-2 (15 nmol kg^{-1}), big-endothelin-3 (10 nmol kg^{-1}), endothelin-1 (1 nmol kg^{-1}), endothelin-2 (1.5 nmol kg^{-1}) and endothelin-3 (4 nmol kg^{-1}); phosphoramidon (Pho) and thiorphan (Thi), both at 10 mg kg^{-1} or vehicle (saline or 0.5% dimethylsulphoxide (DMSO), respectively) were administered (0.5 ml kg^{-1}) 5 min prior to peptide challenge.

In vitro: *electrically-stimulated rat vas deferens*

Male Sprague Dawley rats (250–300 g, Charles Rivers, Italy) were killed by cervical dislocation and the *vasa deferentia pars prostatica* (RVD) were rapidly removed, cleaned and placed in tissue baths containing warm (37°C), oxygenated (95% O_2, 5% CO_2) Krebs solution of the following composition (mM): NaCl 118, KCl 4.69, $MgSO_4$ 1.18, KH_2PO_4 1.20, glucose 11, $NaHCO_3$ 25 and $CaCl_2$ 2.52.

Activity was recorded along the longitudinal axis of RVD (1.5 cm) with an isotonic transducer (Ugo Basile, Italy) under a resting tension of 0.5 g. The tissues were electrically stimulated submaximally (10 V, 0.25 ms pulse width, 200 ms pulse interval, 5 s trains every 60 s) by means of platinum electrodes connected to a digital stimulator (3T Biomedica Mangoni).

Following a 60–90 min equilibration period, cumulative concentration-response curves ($n = 4$–6) for big-endothelin-1 (0.1, 1, 5, 10, 50, 100 and 500 nM), big-endothelin-2 (0.1, 1, 5, 10, 50 and 100 nM) and big-endothelin-3 (0.01, 0.05, 0.1, 0.2, 0.35, 0.5, 0.75 and 1 μM) and their corresponding endothelins (0.1, 1, 5, 10, 50 and 100 nM; $n = 7$–11) were constructed. Only one curve was carried out in each tissue. When studying the effects of peptidase inhibitors (Pho and Thi 10 and 100 μM), tissues were incubated for 30 min in the presence of the test substance, followed by cumulative addition of one of the three endothelins (0.1, 1, 5, 10, 50 and 100 nM, $n = 3$), or a single dose of the proendothelin (big-endothelin-1 and -2: 100 nM; big-endothelin-3: 500 nM, $n = 3$).

Drugs

Human isoforms of proendothelins and endothelins were used. Peptides were purchased from the Peptide Institute (Osaka, Japan). Stock solutions of peptides (0.1 mM), prepared in isotonic saline or 0.1% acetic acid (big-endothelin-3) for *in vivo* experiments and in water for *in vitro* experiments (all peptides), were stored at − 20°C and thawed only once, immediately prior to use. Stock solutions were tested for purity by high performance liquid chromatography (h.p.l.c.) analysis (using a 5 μm Vydac C18 column, with ultraviolet detection at 215 nm). In all cases a single peak corresponding to each peptide was detected.

Protease inhibitors were obtained from Novabiochem (Laufelfingen, Switzerland) or Sigma Chemicals (St. Louis, Mo, U.S.A.) and dissolved either in DMSO (0.5%) or isotonic saline.

Data analysis

In vitro and *in vivo*, results are presented as mean ± s.e.mean. $EC_{100\%}$ (the concentration of peptide that induces a 100% increase of twitch response over basal response) or $ED_{50\,mmHg}$ (the dose of peptide that induces an increase of 50 mmHg of

MAP) values were calculated using the Macintosh Allfit Programme version 1.0. E_{max} respresents the maximal effect induced by the highest concentration or dose tested for each peptide. Comparison between means was carried out by Student's unpaired t-test. A value of $P<0.05$ was taken as significant.

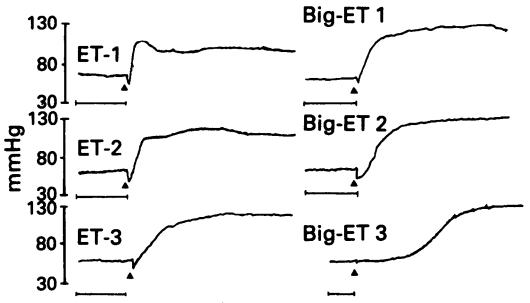

Figure 1 Typical tracings representing the mean arterial pressure (MAP) effects of intravenous endothelins (ET-1: 1 nmol kg^{-1}; ET-2; 1.5 nmol kg^{-1} and ET-3: 4 nmol kg^{-1}) and related proendothelins (Big-ET1: 3 nmol kg^{-1}; Big-ET2 15 nmol kg^{-1} and Big-ET3: 10 nmol kg^{-1}) in anaesthetized ganglion-blocked rats. Animals received peptide as a bolus at the time indicated by black arrow points; the horizontal bars represent 5 min in each case.

Results

In vivo: *MAP changes in the anaesthetized ganglion-blocked rat*

Dose-response curves Basal MAP of the ganglion-blocked rats was 57 ± 1 mmHg (n = 30). Endothelins induced a transient (1–3 min) fall in systemic pressure followed by a long lasting (>25 min) rise of MAP (Figure 1). The hypotensive effect was a dose-dependent and a maximum fall of − 31 ± 2 and − 25 ± 2 mmHg at 2 and 3 nmol kg^{-1} for endothelin-1 and -2 respectively, was reached (Figure 2a). Endothelin-3 also induced a significant hypotension at an intermediate dose (− 13 ± 2 mmHg at 3 nmol kg^{-1}), whereas the highest dose (9 nmol kg^{-1}) was devoid of any depressor activity (Figure 2a).

The endothelin-1-induced vasopressor effect had an $EC_{50\,mmHg}$ of 0.7 ± 0.05 nmol kg^{-1}. Endothelin-2 and -3 were about 2 and 4 fold less active than endothelin-1, with ED_{50mmHg} values of 1.8 ± 0.2 and 2.7 ± 0.3 nmol kg^{-1}, respectively (Figure 2b). The E_{max} obtained at the highest doses tested of each peptide, was of similar magnitude (ΔMAP 84 ± 4.2; 84 ± 3.8 and 89 ± 4.7 mmHg above basal values for endothelin-1, -2 and -3, respectively).

Big-endothelin-1 and -2 induced a rapid, short-lived (1–3 min) hypotensive effect (Figure 1) which was consistently observed in all animals tested and was found to be dose-dependent with a maximum fall of − 18 ± 2.8 and − 31 ± 9 mmHg respectively (Figure 3a). Big-endothelin-3 also showed a hypotensive effect, but without a clear dose-dependency and only in experiments to determine cumulative dose-response curves. The maximal pressure fall was of − 7 ± 2.8 mmHg at a dose of 10 nmol kg^{-1} (Figure 3a).

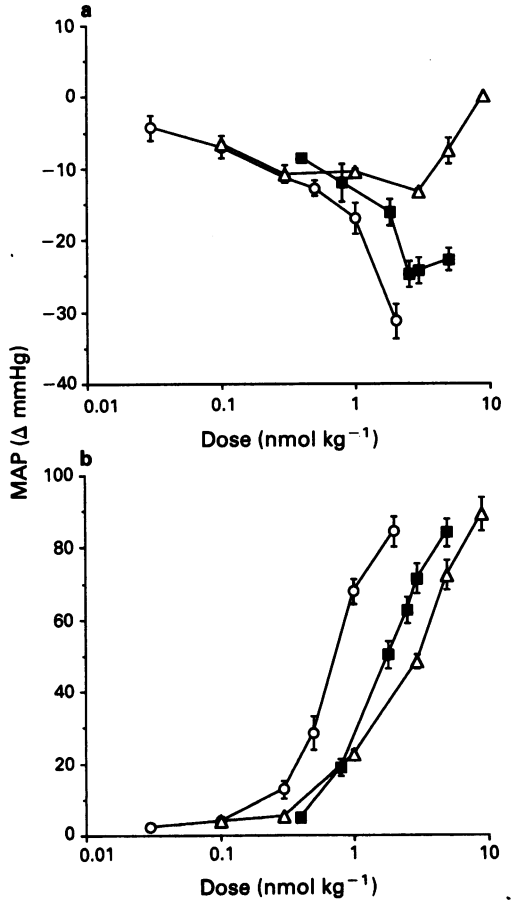

Figure 2 Dose-response curves to endothelin-1 (○), endothelin-2 (■) and endothelin-3 (△), showing hypotensive (a) and hypertensive (b) phases of mean arterial pressure (MAP), in anaesthetized ganglion-blocked rat. Values are mean ± s.e.mean; n = 4–6.

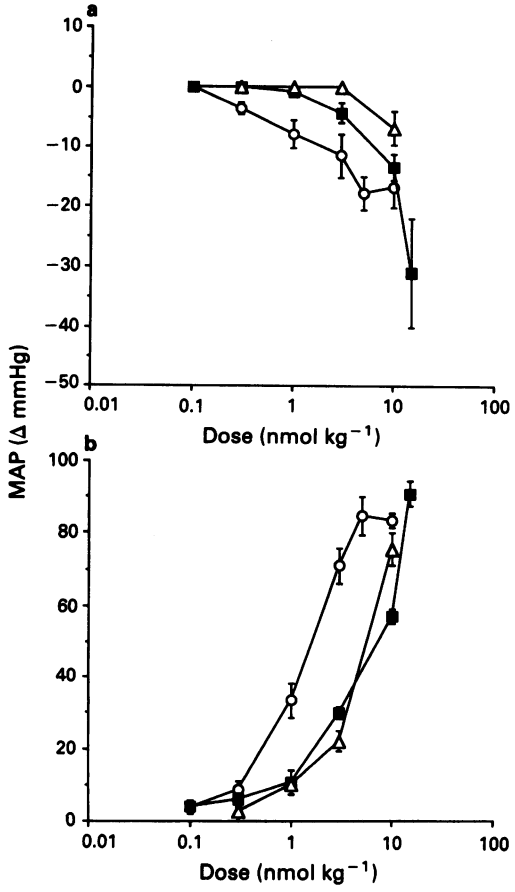

Figure 3 Dose-response curves to big-endothelin-1 (○), big-endothelin-2 (■) and big-endothelin-3 (△), showing hypotensive (a) and hypertensive (b) phases of mean arterial pressure (MAP), in anaesthetized ganglion-blocked rat. Values are mean ± s.e.mean; n = 3–6.

Proendothelins induced a potent long lasting (25–35 min) vasopressor effect (Figure 1). Big-endothelin-1 was more active than big-endothelin-2 ($ED_{50\,mmHg}$: 1.8 ± 0.2 and 6.7 ± 0.4 nmol kg^{-1} respectively) while having similar E_{max} (ΔMAP: 85 ± 4 and 91 ± 2.4 mmHg). The effect of big-endothelin-2 was slower in onset than that of big-endothelin-1 (10–13 *vs* 5–6 min). Although the dose-response curve for big-endothelin-3 was incomplete, this peptide showed an activity close to that of big-endothelin-2, in the range of the tested doses, with an $ED_{50\,mmHg}$ 6.5 ± 0.4 nmol kg^{-1} for an E_{max} of 75 ± 4.6 mmHg at 10 nmol kg^{-1} (Figure 3b). In our model the pressor response of big-endothelin-3 showed the longest latency (25–30 min).

Single-dose: effects of phosphoramidon and thiorphan Pho and Thi were found to be devoid of any significant effect *per se*.

The transient hypotensive effects of 3 nmol kg^{-1} big-endothelin-1 and 15 nmol kg^{-1} big-endothelin-2 were not significantly affected by a 10 mg kg^{-1} bolus of Pho (ΔMAP: − 5.0 ± 1.3 and − 12.0 ± 4.2 mmHg with Pho versus − 4.3 ± 0.9 and − 9.0 ± 2.6 mmHg without Pho, respectively). In this condition big-endothelin-3 (10 nmol kg^{-1}) did not produce any hypotension. On the contrary, the vasopressor response induced by the three proendothelins was reduced from 60 ± 1.7 to 25 ± 3.7 mmHg ($P<0.001$ *vs* control), from 59 ± 3.1 to 11.7 ± 2.8 mmHg ($P<0.001$ *vs* control) and from 69 ± 7.5 to 1.5 ± 1.5 mmHg ($P<0.001$ *vs* control) respectively (Figure 4a).

Pho had no significant effect on either endothelin-1 (1 nmol kg^{-1}, ΔMAP: 49 ± 1.9 *vs* 52 ± 2.2 mmHg), endothelin-2 (1.5 nmol kg^{-1}, ΔMAP: 49 ± 2.9 *vs* 46 ± 3.2 mmHg), or endothelin-3 (4 nmol kg^{-1}, ΔMAP: 59 ± 2.9 *vs* 67 ± 1.8 mmHg) -induced pressor response (Figure 4b). The transient vasodepressor effects induced by either endothelin-1 (ΔMAP: − 11.0 ± 1.7 *vs* − 12.0 ± 1.3 mmHg), endothelin-2 (ΔMAP: − 11.0 ± 1.5 *vs* − 11.0 ± 0.6 mmHg) or endothelin-3 (Δ MAP: − 9.0 ± 2.4 *vs* − 10.0 ± 1.4 mmHg) were not changed in the presence of Pho.

Thi, under the same conditions, was inactive against the effects of the three endothelins and related proendothelins (data not shown).

In vitro: *electrically-stimulated rat vas deferens*

Effect of endothelins on rat vas deferens twitch response Electrical stimulation before addition of peptides induced a stable contractile response (12 ± 3 mm). The twitch response to electrical stimulation was not affected by enzyme inhibitors and, in the presence or absence of peptides, it could be abolished by tetrodotoxin (1 μM) indicating its neural origin (data not shown).

Endothelin-1, -2 and -3 increased, concentration-dependently, the RVD twitch response to electrical stimulation; the threshold concentration of the endothelins that significantly enhanced the twitch response was between 0.1 and 1 nM (Figure 5a). Endothelin-1 and -2 were equipotent ($EC_{100\%}$: 4.0 ± 0.4 and 7.9 ± 4.8 nM, respectively), both being three–four fold more active than endothelin-3 ($EC_{100\%}$; 19 ± 2.5 nM). All three peptides induced a maximum stimulatory

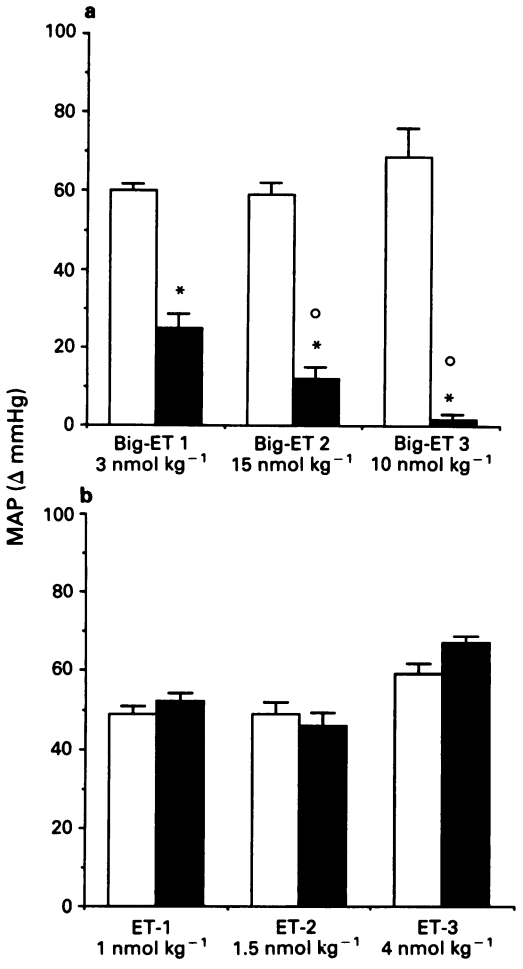

Figure 4 Effects of (a) big-endothelin-1 (Big-ET 1: 3 nmol kg^{-1}), big-endothelin-2 (Big-ET 2: 15 nmol kg^{-1}) and big-endothelin-3 (Big-ET 3: 10 nmol kg^{-1}), or (b) endothelin-1 (ET-1: 1 nmol kg^{-1}), endothelin-2 (ET-2: 1.5 nmol kg^{-1}) and endothelin-3 (ET-3: 4 nmol kg^{-1}), in the absence (open columns) or in the presence (closed columns) of phosphoramidon (10 mg kg^{-1}), on mean arterial pressure (MAP) in anaesthetized, ganglion-blocked rat. Values are mean ± s.e.mean; $n = 6$. *$P<0.05$ unpaired t test vs control; °$P<0.05$ unpaired t test vs big-ET-1.

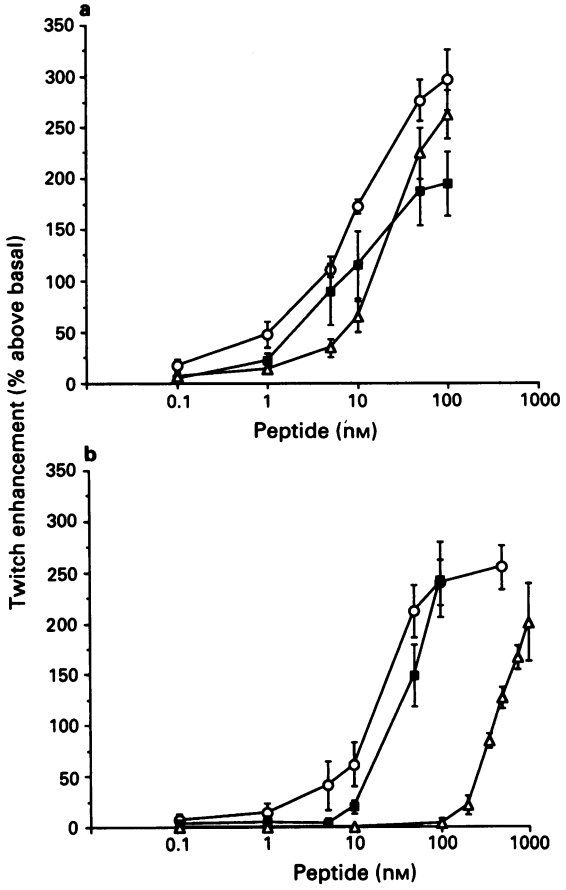

Figure 5 Concentration-response curves to (a) endothelin-1 (○), endothelin-2 (■) and endothelin-3 (△), and (b) related proendothelins on electrically stimulated twitch response of rat vas deferens. Results are presented as % above basal. Each point represents the mean ± s.e.mean of 4–11 experiments.

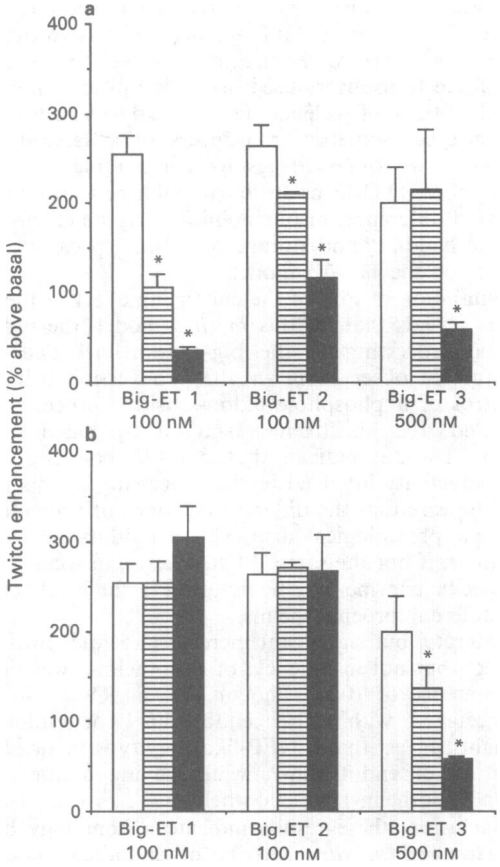

Figure 6 Effects of (a) phosphoramidon or (b) thiorphan at concentrations of 10 μM (hatched columns) or 100 μM (closed columns) on the enhancement of the twitch response of the electrically stimulated vas deferens of the rat induced by big-endothelin-1 (Big-ET 1, 100 nM), big-endothelin-2 (Big-ET 2, 100 nM) and big-endothelin-3 (Big-ET 3, 500 nM). Results are presented as % above basal. Each column represents the mean ± s.e.mean of at least 3 determinations. *$P<0.05$, unpaired t test.

effect at 100 nM: endothelin-1 and -3 were equally effective (296 ± 30 and 262 ± 24% above baseline, respectively), while endothelin-2 was less active, with a maximal stimulation of 194 ± 30%. Endothelins dose-dependently increased RVD basal tone (data not shown).

Effect of the proendothelins on the rat vas deferens twitch response Big-endothelin-1 enhanced (E_{max}: 254 ± 21.5%) RVD twitch response in a concentration-dependent manner ($EC_{100\%}$: 10.0 ± 2.6 nM), with a threshold concentration around 1 nM (Figure 5b). In our model, big-endothelin-1 was only two times less effective than endothelin-1. Big-endothelin-2 was also an effective enhancer of the twitch response with a threshold around 10 nM and with an $EC_{100\%}$ value of 21.6 ± 3.2 nM and an E_{max} of 264 ± 24%, being three times less potent than endothelin-2. Big-endothelin-3 enhanced the RVD twitch response with a threshold around 200 nM and an $EC_{100\%}$ of 275.3 ± 20.7 nM and an E_{max} of 200 ± 38% (Figure 6b). Big-endothelin-3 was therefore at least 20 times less potent than its related endothelin. Proendothelins enhance basal tone too, but only at higher doses (data not shown).

Effects of phosphoramidon and thiorphan Both Pho and Thi up to 100 μM had no effect on electrical induced twitch response.

In the presence of either Pho or Thi (10 μM), the effects of endothelin-1 or -3 remained unchanged, whereas a significant potentiation of the E_{max} of endothelin-2, to 277 ± 42% and 318 ± 15% respectively, was observed (Table 1). Pho inhibited significantly and concentration-dependently the twitch enhancement mediated by both big-endothelin-1 and -2 (Figure 6a). Thi, under the same conditions was totally ineffective (Figure 6b). Conversely Thi (10 and 100 μM) was able to reduce big-endothelin-3 twitch response enhancement (31 ± 14 and 71 ± 5%, respectively; $P<0.05$). In contrast, Pho was found effective ony at 100 μM (70 ± 12%, $P<0.05$).

Table 1 Effect of phosphoramidon (Pho) and thiorphan (Thi) on endothelin-induced facilitation of the twitch response in the electrically stimulated vas deferens of the rat

		$EC_{100\%}$ (nM)	E_{max} (% above basal)
Endothelin-1		4.0 ± 0.4	296 ± 30
	Pho	5.2 ± 0.7	249 ± 25
	Thi	3.5 ± 0.8	298 ± 14
Endothelin-2		7.9 ± 4.8	194 ± 30
	Pho	10.5 ± 0.1	277 ± 42*
	Thi	5.1 ± 2.0	318 ± 15*
Endothelin-3		19.2 ± 2.5	262 ± 24
	Pho	22.5 ± 1.1	252 ± 16
	Thi	17.9 ± 3.1	220 ± 11

E_{max} = maximal effect obtained at the highest concentration tested for each peptide (see methods); Pho = phosphoramidon 10 μM; Thi = thiorphan 10 μM.
*$P<0.05$, unpaired Student's t test.

Discussion

The *in vivo* results of the present work show that endothelins, in the anaesthetized ganglion-blocked rat, induce a rapid, profound and transient hypotension followed by a long lasting hypertensive effect confirming the data previously obtained in a number of different models (Inoue *et al.*, 1989; Douglas & Hiley, 1990; Le Monnier de Gouville & Cavero, 1991; Randall, 1991; Mattera *et al.*, 1992a,b). The hypotensive effect induced by endothelin-1 and 2 has a similar overall profile, whereas that of endothelin-3 appears to be weaker, and is probably subject to rapid tachyphylaxis. The hypertensive effect is dose-dependent and characterized by a similar onset time. The rank order of potency is: endothelin-1 > endothelin-2 > endothelin-3. The E_{max} of the three endothelins is very similar, a finding in contrast with the results reported by Inoue *et al.* (1989), who described a smaller maximal response for endothelin-3.

Although less potent than the respective endothelins, big-endothelin-1 and -2 retain similar pressor activity in terms of maximal effect. The time to reach the maximum increase in blood pressure, for each dose, was about the same for endothelin-1 and big-endothelin-1, in accordance with previously reported findings (Kashiwabara *et al.*, 1989; Douglas *et al.*, 1991), while the onset of big-endothelin-2 effect is definitely slower.

In our *in vivo* model, big-endothelin-3 shows a clear vasopressor activity characterized by the slowest onset among all peptides tested and not preceded by a dose-dependent hypotensive effect. These data suggest that the presence of a Trp^{21}-Ile^{22} bond, instead of the Trp^{21}-Val^{22} one, in big-endothelin-3, may reduce the affinity for ECE, and decrease the velocity of enzymatic conversion. This reduction may not permit a rapid achievement of an efficacious concentration of active peptide at receptor sites, thus producing the observed slow onset. These results are in accordance with recent biochemical (Matsamura *et al.*, 1992) and functional (Gardiner *et al.*, 1992a,b), studies, but in sharp contrast to a number of previous studies (Okada *et al.*, 1990, 1991; Télémaque & D'Orléans-Juste, 1991; D'Orléans-Juste *et al.*, 1991a,b; Mattera *et al.*, 1992a; Takada *et al.*, 1992) in which

no vasopressor activity had been shown for big-endothelin-3. At the moment no exhaustive explanation can be given for this important discrepancy, but it may be supposed that difference in the source of the peptide and in its handling (i.e. different way of solubilization in either buffer saline or acetic acid, storage, etc.) plays an important role. Moreover species-specificity of big-endothelin cleavage could be advocated in some instances (e.g. D'Orléans-Juste *et al.*, 1991a,b, used guinea-pigs instead of rats), as well as interference originating from the pharmacological manipulation of animals (anaesthetics, ganglion-blocking agents). In order to obtain a better defined picture of the physiologically important roles of big-endothelin-3 a careful study seems to be necessary to explore these hypotheses systematically. However, the data produced in the present work seem to reinforce the concept that the *in vivo* biological actions of the proendothelins are mediated through the corresponding endothelins.

Conversion of the proendothelins to their active forms is generally agreed to involve a neutral Pho-sensitive metalloendopeptidase (Matsumura *et al.*, 1990a,b; 1991; LeMonnier de Gouville & Cavero, 1991; Yano *et al.*, 1991; Mattera *et al.*, 1992a,b; Gardiner *et al.*, 1992a). Accordingly, in this study, Pho significantly reduced the responses to the three proendothelins. The following rank order of inhibition: big-endothelin-3 > big-endothelin-2 > big-endothelin-1 was found. Such strong inhibition of the conversion of big-endothelin-3 by Pho, as well as the difference in the behaviour of big-endothelin-3 compared to that of big-endothelin-1 and -2 (slower onset of the hypertensive effect and the absence of the early hypotension) pointed out before, might suggest that the big-endothelin-1 and -2 are more efficiently converted than big-endothelin-3.

Thi was unable to block proendothelin-induced pressor responses, in contrast with a previous study (McMahon *et al.*, 1991) that showed a weak, but dose-dependent inhibition of porcine big-endothelin-1 pressor response by Thi. Most likely this discrepancy is related to the lower dose used in this work and/or to the different peptide isoform (human *vs* porcine).

Finally, the sensitiveness to Pho and insensitiveness to Thi of proendothelin-induced pressor response support the concept that all three proendothelins are converted by the same enzyme *in vivo*. On the other hand, neither Pho nor Thi have any significant influence on the pressor responses to endothelin-1, -2 or -3. This finding does not support the involvement of a NEP-like activity in the metabolism of the endothelins *in vivo*, as has been described *in vitro* (Vijayaraghavan *et al.*, 1990; Fagny *et al.*, 1991).

As regards the *in vitro* model, both proendothelins and endothelins have been shown to be potent enhancers of the twitch response to electrical stimulation, with potency values in agreement with previous studies (Maggi *et al.*, 1989; Télémaque & D'Orléans-Juste, 1991). However, in contrast to Télémaque & D'Orléans-Juste (1991), in our experimental conditions we observed that: (a) all peptides exerted a concentration-related increase of the basal tone and (b) big-endothelin-3 behaved as a full agonist similarly to the related endothelin. The first difference may be due to the different type of force transducers used: isotonic in our study, isometric in the study of Télémaque & D'Orléans-Juste. In fact, when we used isometric transducers in a separate set of experiments, we obtained results comparable to those of Télémaque & D'Orléans-Juste (unpublished observations). The second difference, in our opinion, may be chiefly related to peptide handling and storage or animal species as already discussed for the *in vivo* model.

The inhibition of Pho of the enhancing effect of the proendothelins reveals that, in this *in vitro* model, the profile for enzymatic processing of the big-endothelin-1 and -2 was similar to that observed *in vivo*, the conversion being under the control of a phosphoramidon-sensitive process. On the other hand, the sensitiveness showed by big-endothelin-3 activity to Thi may indicate that in RVD there is a different enzymatic activity involved in the processing of this peptide. Thus it appears that the different activities of proendothelins in different physiological districts are modulated essentially by a similar, if not the same, ECE, even if, in some cases, an organ-specific enzyme may be involved in the local activation of the different proendothelins.

A moderate, but significant increase (1.5 fold) in the maximal effect, but not in potency, of endothelin-2 was observed in the presence of 10 μM Pho or Thi, in RVD. No similar effect occurred with either endothelin-1 or endothelin-3. This might suggest that a NEP-like activity is involved in the degradation of endothelin-2, while having minimal or no effect on endothelin-1 or endothelin-3.

In conclusion, these results indicate a homology between the *in vivo* and *in vitro* activity of endothelin converting enzyme. This enzymatic activity is probably not NEP 24.11, as indicated by the inconsistent activity of thiorphan. Further, the previously described selectivity of endothelin converting enzyme for the conversion of big-endothelin-1 is more likely to be related to the selectivity for the Trp^{21}-Val^{22} bond, a bond also present in big-endothelin-2, but not in big-endothelin-3 (Trp^{21}-Ile^{22}). Moreover, in the rat vas deferens another enzyme, different from vascular ECE, may be involved in the big-endothelin-3 enzymatic processing. The degradation of the endothelins is possibly tissue- and isopeptide-dependent, since only endothelin-2 was susceptible to thiorphan or phosphoramidon *in vitro*, while *in vivo* these neutral protease inhibitors seem to be totally inactive.

This work was supported by IMI grant No 55202. The authors thank Mr M. Bachi and Mr M. Guelfi for their excellent technical assistance and express their gratitude to Drs M. Criscuoli, G. D'Agostino and A. Subissi for their support and suggestions during the preparation of this manuscript.
Laboratori Guidotti SpA is part of A. Menarini Pharmaceuticals, Firenze, Italy.

References

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

D'ORLÉANS-JUSTE, P., TÉLÉMAQUE, S. & CLAING, A. (1991a). Different pharmacological profiles of big-endothelin-3 and big-endothelin-1 in vivo and also in vitro through a phosphoramidon-sensitive conversion to endothelin-1. *Br. J. Pharmacol.*, **104**, 440–444.

D'ORLÉANS-JUSTE, P., LINDBURY, P.S., TÉLÉMAQUE, S., WARNER, T.D. & VANE, J.R. (1991b). Human big-endothelin releases prostacyclin in vivo and in vitro. *J. Cardiovasc. Pharmacol.*, **17** (Suppl 7), S251–S255.

DOUGLAS, S.A. & HILEY, C.R. (1990). Responses to endothelin-1, human proendothelin (1-38) and porcine proendothelin (1-39) in the rat on intravenous administration and in the blood perfused mesentery. *Neurochem. Int.*, **4**, 445–454.

FAGNY, C., MICHEL, A., LEONARD, I., BERKENBOOM, G., FONTAINE, J. & DESCHODT-LANCKMAN, M. (1991). In vitro degradation of endothelin-1 by endopeptidase 24.11 (enkephalinase). *Peptides*, **12**, 773–778.

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.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1992a). Inhibition by phosphoramidon of the regional haemodynamic effects of proendothelin-2 and -3 in conscious rats. *Br. J. Pharmacol.*, **107**, 584–590.

GARDINER, S.M., KEMP, P.A., COMPTON, A.M. & BENNETT, T. (1992b). Coeliac haemodynamic effects of endothelin-1, endothelin-3, proendothelin-1[1-38] and proendothelin-3[1-41] in conscious rats. *Br. J. Pharmacol.*, **106**, 483–488.

GOMEZ-SANCHEZ, C.E., COZZA, E.N., FOECKLING, M.F., CHIOU, S. & FERRIS, M.W. (1990). Endothelin receptor subtypes and stimulation of aldosterone secretion. *Hypertension*, **15**, 744–747.

HILEY, C.R., PELTON, J.T. & MILLER, R.C. (1989). Effects of endothelin on field stimulated rat vas deferens and guinea pig ileum. *Br. J. Pharmacol.*, **96**, 104P.

HISAKI, K., MATSUMURA, Y., IKEGAWA, R., NISHIGUCHI, S., HASASHI, K., TAKAOKA, M. & MOROMOTO, S. (1991). Evidence for Phosphoramidon-sensitive conversion of big endothelin-1 to endothelin-1 in isolated rat mesenteric artery. *Biochem. Biophys. Res. Commun.*, **177**, 1127–1132.

INOUE, A., YANAGISAWA, M., KIMURA, S., KASUYA, Y., MIYAUCHI, K., 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.

JAFFER, F.E., KNAUSS, C., POPTIC, E. & ABBOUD, H.E. (1990). Endothelin stimulates PDGF secretion in cultured human mesangial cells. *Kidney Int.*, **38**, 1193–1198.

JONES, C.R., HILEY, C., PELTON, J.T. & MOHR, M. (1989). Autoradiographic visualization of the binding sites for [I^{125}] endothelin in rat and human brain. *Neurosci. Lett.*, **97**, 276–279.

KASHIWABARA, T., INAGAKI, Y., OHTA, H., IWAMATSU, A., NOMIZU, M., MORITA, A. & NISHIKORI, K. (1989). Putative precursors of endothelin have less vasoconstrictor activity in vitro but a potent pressor effect in vivo. *FEBS Lett.*, **247**, 73–76.

KLOOG, Y. & SOKOLOVSKY, M. (1989). Similarities in mode and sites of action of sarafotoxins and endothelins. *Trends Pharmacol. Sci.*, **10**, 212–214.

LE MONNIER DE GOUVILLE, A.C. & CAVERO, I. (1991). Differential pharmacological profile of endothelin-1 and its precursor, big endothelin. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 7), S362–365.

MAGGI, C.A., GIULIANI, S., PATACCHINI, R., ROVERO, P., GIACHETTI, A. & MELI, A. (1989). The activity of peptides of the endothelin family in various mammalian smooth muscle preparations. *Eur. J. Pharmacol.*, **174**, 23–31.

MATSUMURA, Y., IKEGAWA, R., TAKAOKA, M. & MORIMOTO, S. (1990a). Conversion of porcine big endothelin to endothelin by an extract from the porcine aortic endothelial cells. *Biochem. Biophys Res. Commun.*, **167**, 203–210.

MATSUMURA, Y., HISAKI, K., TAKAOKA, M. & MORIMOTO, S. (1990b). Phosphoramidon, a metalloproteinase inhibitor, suppresses the hypertensive effect of big endothelin-1. *Eur. J. Pharmacol.*, **185**, 103–106.

MATSUMURA, Y., IKEGAWA, R., TSUKAHARA, Y., TAKAOKA, M. & MORIMOTO, S. (1991). n-Ethylmaleimide differentiates endothelin converting activity by two types of metalloproteinases derived from vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **178**, 531–538.

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.

MATTERA, C.G., EGLEZOS, A., CUCCHI, P., RENZETTI, A.R. & MISRAHI, J. (1992a). Phosphoramidon-sensitive conversion of human proendothelin in vivo: effect on pressure and plasma prostacyclin. *Br. J. Pharmacol.*, **107**, 161P.

MATTERA, C.G., RENZETTI, A.R. & MIZRAHI, J. (1992b). Metalloprotease dependent conversion of human proendothelin in vivo. *Pharmacol. Res.*, **26** (Suppl. 1), 159.

MCKAY, K.O., BLACK, J.L. & ARMOUR, C.L. (1992). Phosphoramidon potentiates the contractile response to endothelin-3, but not endothelin-1 in isolated airway tissue. *Br. J. Pharmacol.*, **105**, 929–932.

MCMAHON, E.G., PALOMO, M.A., MOORE, W.M., MCDONALD, J.F. & STERN, M.K. (1991). Phosphoramidon blocks the pressor activity of porcine big endothelin 1(1-39) in vivo and conversion of big endothelin 1 (1-39) to endothelin 1 (1-21) in vitro. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 703–707.

MILLER, V.M., REDFIELD, M.M. & BURNETT, J.C. (1989). Integrated cardiac, renal and endocrine action of endothelin. *J. Clin. Invest.*, **83**, 317–320.

OKADA, K., MIYAZAKI, J., TAKADA, J., ARAI, Y., 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.

OKADA, K., TAKADA, J., ARAI, Y., MATSUYAMA, K. & YANO, M. (1991). Importance of the C-terminal region of Big-endothelin-1 for specific conversion by phosphoramidon-sensitive endothelin converting enzyme. *Biochem. Biophys. Res. Commun.*, **180**, 1019–1023.

POLLOCK, D.M. & OPGENORTH, T.J. (1991). Evidence for metalloprotease involvement in the in vivo effects of big Endothelin 1. *Am J. Physiol,*. **261**, R257–R263.

PONS, F., TOUVAY, C., LAGENTE, V., MENCIA-HUERTA, J.M. & BRAQUET, P. (1991). Bronchopulmonary and pressor activities of endothelin-1 (ET-1), ET-2, ET-3 and Big ET-1 in the Guinea Pig. *J. Cardiovasc. Pharmacol,*. **17** (Suppl. 7), S326–S328.

POWER, R.F., WHARTON, J., SALAS, S.P., KANSE, S., GHATEI, M., BLOOM, S.R. & POLAK, J.M. (1989). Autoradiographic localisation of endothelin binding sites in human and porcine coronary arteries. *Eur. J. Pharmacol.*, **160**, 199–200.

RANDALL, M.D. (1991). Vascular activities of the endothelins. *Pharmacol. Ther.*, **50**, 73–93.

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

TAKADA, J., OKADA, K., IKEGAWA, T., MATSUYAMA, K. & YANO, M. (1991). Phosphoramidon-sensitive endothelin-converting enzyme in the cytosol of cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.*, **176**, 860–865.

TÉLÉMAQUE, S. & D'ORLÉANS-JUSTE, P. (1991). Presence of a 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**, 500–507.

VIJAYARAGHAVAN, J., SCICLI, A.G., CARRETTERO, O.A., SLAUGHTER, C., MOOMAW, C. & HERSH, L.B. (1990). The hydrolysis of endothelins by neutral endopeptidase 24.11 (enkefalinase). *J. Biol. Chem.*, **265**, 14150–14155.

WIKLUND, N.P., OHLEN, A., WIKLUND, C.U., HEDQVIST, P. & GUSTAFSSON, L.E. (1990). Endothelin modulation of neuroeffector transmission in rat and guinea pig vas deferens. *Eur. J. Pharmacol.*, **185**, 25–33.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TONOBE, Y., KOBAYASHI, M., MITSUI, Y,. YAZAKI, Y,. GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **32**, 411–415.

YANAGISAWA, M. & MASAKI, T. (1989). Endothelin, a novel endothelium-derived peptide. *Biochem. Pharmacol.*, **38**, 1877–1883.

YANO, M., OKADA, K., TAKADA, J,. HIOKI, Y., MATSUYAMA, K., FUKURODA, T., NOGUCHI, K., NISHIKIBE, M. & IKEMOTO, F. (1991). Endothelin-converting enzyme and its in vitro and in vivo inhibition. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 7), S26–S28.

(Received January 11, 1993
Revised April 14, 1993
Accepted April 19, 1993)