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Role of the neutral endopeptidase 24.11 in the conversion of big endothelins in guinea-pig lung parenchyma

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1 We have studied the conversion of big endothelin-1 (big ET-1), big endothelin-2 (big ET-2) and big endothelin-3 (big ET-3) and characterized the enzyme involved in the conversion of the three peptides in guinea-pig lung parenchyma (GPLP).

2 Endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) (10 nM to 100 nM) caused similar concentration-dependent contractions of strips of GPLP.

3 Big ET-1 and big ET-2 also elicited concentration-dependent contractions of GPLP strips. In contrast, big ET-3, up to a concentration of 100 nM, failed to induce a contraction of the GPLP.

4 Incubation of strips of GPLP with the dual endothelin converting enzyme (ECE) and neutral endopeptidase (NEP) inhibitor, phosphoramidon (10 μM), as well as two other NEP inhibitors thiorphan (10 μM) or SQ 28,603 (10 μM) decreased by 43% ($P< 0.05$), 42% ($P< 0.05$) and 40% ($P< 0.05$) the contractions induced by 30 nM of big ET-1 respectively. Captopril (10 μM), an angiotensin-converting enzyme inhibitor, had no effect on the contractions induced by big ET-1.

5 The incubation of strips of GPLP with phosphoramidon (10 μM), thiorphan (10 μM) or SQ 28,603 (10 μM) also decreased by 74% ($P< 0.05$), 34% and 50% ($P< 0.05$) the contractions induced by 30 nM big ET-2 respectively. As for the contractions induced by big ET-1, captopril (10 μM) had no effect on the concentration-dependent contractions induced by big ET-2.

6 Phosphoramidon (10 μM), thiorphan (10 μM) and SQ 28,603 (10 μM) significantly potentiated the contractions of strips of GPLP induced by both ET-1 (30 nM) and ET-3 (30 nM). However, the enzymatic inhibitors did not significantly affect the contractions induced by ET-2 (30 nM) in this tissue.

7 These results suggest that the effects of big ET-1 and big ET-2 result from the conversion to ET-1 and ET-2 by at least one enzyme sensitive to phosphoramidon, thiorphan and SQ 28,603. This enzyme corresponds possibly to EC 3.4.24.11 (NEP 24.11) and could also be responsible for the degradation of ETs in the GPLP.

Keywords: Endothelin; big endothelin; endothelin-converting enzyme; neutral endopeptidase; guinea-pig lung parenchyma

Introduction

Endothelin-1 (ET-1) is a 21-amino acid peptide isolated from porcine cultured endothelial cells (Yanagisawa *et al.*, 1988). Experimental data show that ET-1 has potent contractile activity on airway smooth muscle both *in vivo* and *in vitro* (Payne & Whittle 1988; Macquin-Mevier *et al.*, 1989; Maggi *et al.*, 1989; Filep *et al.*, 1991). Furthermore it has been suggested that ET-1 can participate in the development of bronchoconstriction in some pathophysiological states (Uchida *et al.*, 1988; Maggi *et al.*, 1989; Mattoli *et al.*, 1991; Springall *et al.*, 1991).

Two other isoforms of the 21-amino acid peptide, endothelin-2 (ET-2) and endothelin-3 (ET-3), have been characterized (Bloch *et al.*, 1989; Inoue *et al.*, 1989; Onda *et al.*, 1990). Based on sequence analysis of cDNA encoding for the precursor of endothelin (preproendothelin), it is suggested that ET-1 is generated from an inactive intermediate, named big ET-1, by a putative endothelin-converting enzyme (ECE) (Itoh *et al.*, 1988; Yanagisawa *et al.*, 1988). Recent studies strongly suggest that the ECE involved in the conversion of big ET-1 to ET-1 in endothelial cells is a membrane-bound phosphoramidon-sensitive metalloproteinase structurally related to neutral endopeptidase 24.11 (EC 3.4.24.11, NEP) (Matsumura *et al.*, 1990; Okada *et al.*, 1990; Shimada *et al.*, 1994; Xu *et al.*, 1994). Fawzi *et al.* (1994) and Shima *et al.* (1994) have shown that the guinea-pig lungs are a rich source of phosphoramidon-sensitive ECE activity. Since ET-2 and ET-3 are derived from big ET-2 and big ET-3 respectively (Bloch *et al.*, 1989; Ohkubo *et al.*, 1990), it was hypothesized that the biosynthetic pathway for ET-2 and ET-3 formation was similar to that of ET-1.

This led us to study the conversion of big ET-1, big ET-2 and big ET-3 in the guinea-pig lung parenchyma (GPLP) and to characterize the enzyme involved in the conversion of the three peptides. The effects of enzymatic inhibitors were also evaluated on the contractile response of ET-1, ET-2 and ET-3. A preliminary account of part of this work was presented to the XII[th] International Congress of Pharmacology (Lebel *et al.*, 1994).

Methods

Tissue preparation

Dunkin-Hartley guinea-pigs weighing 250–300 g were killed by cervical dislocation and rapidly bled. The lung parenchyma were excised and placed in Krebs-Henseleit buffer (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.18, $NaHCO_3$ 25, $MgSO_4.7H_2O$ 1.18 and glucose 11.1, pH 7.4). Strips of peripheral parenchyma were prepared from the anterior surface of the lobe as described before by Sirois *et al.* (1982). The tissues were mounted in 5 ml organ baths and a tension of 2 g was applied. Tissues were allowed to stabilize for 1 h in warm (37°C) and oxygenated (95% O_2:5% CO_2) Krebs-Henseleit solution with washing every 15 min. Contractile re-

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sponses were recorded isometrically on a Grass 7D polygraph using Grass model FTO3C force-displacement transducers.

Experimental protocol

Following an equilibration period of 60 min, tissues were contracted with two successive concentrations of histamine (4.5 μM) corresponding to 50% of the maximal contraction, with a 30 min washout period between contractions. The tissues were incubated for 50 min with phosphoramidon (10 μM), thiorphan (10 μM), SQ 28,603 (10 μM) (Abassi *et al.*, 1993), captopril (10 μM), a combination of phosphoramidon (10 μM) and thiorphan (10 μM) or the vehicle prior to and during the administration of peptides (10 nM to 100 nM). To avoid tachyphylaxis, each parenchymal strip was used for only one concentration of ETs or big ETs (Battistini *et al.*, 1994). Responses are expressed as a percentage of the contraction produced by 4.5 μM histamine.

Drugs

Endothelin-1 (ET-1) was synthesized in our laboratories. Endothelin-2 (ET-2), endothelin-3 (ET-3), big endothelin-1 (big ET-1) (human), big endothelin-2 (big ET-2) (1–37) (human) and big endothelin-3 (big ET-3) (human) were purchased from Peninsula Laboratories (Belmont, CA, U.S.A.), big ET-1 (22–38) was purchased from American Peptide Co. (Sunnyvale, U.S.A.). ETs and big ETs were dissolved in phosphate buffered saline. Phosphoramidon (Peptide Institute Inc., Osaka, Japan), captopril, thiorphan and histamine dihydrochloride (Sigma, St-Louis, U.S.A.) were dissolved in distilled water. Compound SQ 28,603 (N-[2-(mercaptomethyl)-1-oxo-3-phenylpropyl]-β-alanine) (Squibb Institute of Medical Research, Princeton, NJ, U.S.A.) was dissolved in $NaHCO_3$ 2.5% (Seymour *et al.*, 1991). All drugs were further diluted with Krebs-Henseleit solution when necessary. The vehicle used for dissolving SQ 28,603 at a final concentration of 0.001% was without effect on the response of the GPLP to histamine or big ET-1.

Statistical analysis

All values are expressed as mean ± s.e.mean. An unpaired, two-tailed, Mann-Whitney U test was used for statistical analysis of the data.

Results

Contractile activity of ETs and big ETs

ET-1, ET-2 and ET-3 elicited concentration-dependent contractions of strips of GPLP (10 nM to 100 nM) (Figure 1a). The c-terminal fragment of big ET-1, big ET-1 (22–38), was inactive at concentrations up to 0.3 μM (Figure 1a). The magnitude of the contractions was similar for the three peptides. Big ET-1 and big ET-2 also elicited concentration-dependent contractions of strips of GPLP (Figure 1b). However, at an intermediate concentration (30 nM), big ET-1 was twice as efficient as big ET-2 in contracting the GPLP (contractions of 66% and 38% respectively). In contrast, big ET-3, up to a concentration of 100 nM, failed to induce a contraction of GPLP (Figure 1b).

Effect of enzymatic inhibitors on the conversion of big ETs

Incubation of strips of GPLP with phosphoramidon, thiorphan and SQ 28,603 (10 μM), all inhibitors of metalloprotease, induced a significant reduction in the concentration-dependent contractions induced by big ET-1 (Figure 2a and b). The inhibition of the responses to selected concentrations of big ET-1 varied from 22% to 61% and maximal inhibitions were achieved with thiorphan (10 μM) at a low concentration

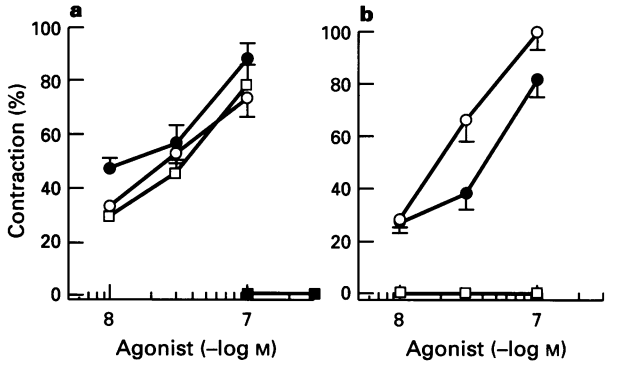

Figure 1 Effect of endothelin-1 (○), endothelin-2 (●), endothelin-3 (□) and big endothelin-1 (22–38) (■) (a) and big endothelin-1 (○), big endothelin-2 (●) and big endothelin-3 (□) (b) on the guinea-pig lung parenchyma. Results are expressed as a percentage of the response to histamine (4.5 μM). n = 4 to 7.

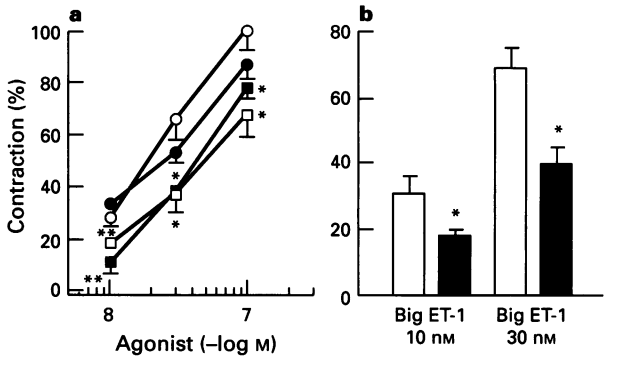

Figure 2 Effect of big endothelin-1 in the presence of vehicle (○), captopril (10 μM; ●), phosphoramidon (10 μM; □) and thiorphan (10 μM; ■) (a) and in the presence of vehicle (open columns) and SQ 28,603 (10 μM; solid columns) (b) on the guinea-pig lung parenchyma. Results are expressed as a percentage of the response to histamine (4.5 μM). n = 4 to 7. $^*P<0.05$, $^{**}P<0.01$.

(10 nM) of big ET-1. Phosphoramidon and thiorphan were equieffective in inhibiting the contractions induced by big ET-1 (10 nM to 100 nM). A combination of phosphoramidon (10 μM) and thiorphan (10 μM) did not displace further the concentration-response curve induced by big ET-1 (results not shown). Incubation of strips of GPLP with captopril (10 μM), an inhibitor of angiotensin I converting enzyme (ACE), did not affect the concentration-response curve induced by big ET-1 (Figure 2a).

Responses of the GPLP to big ET-2 (10 nM to 100 nM) were significantly reduced when they were incubated with 10 μM of each of the enzymatic inhibitors separately, except for captopril (Figure 3a and b). Furthermore, the combination of phosphoramidon and thiorphan did not further decrease the contractions of the tissues induced by big ET-2 (30 nM) (control: 38 ± 6; in presence of inhibitors: 16 ± 4%, n = 4 to 5, $P<0.05$). In another series of experiments, all inhibitors tested at a concentration of 1 μM against ET-1 and big ET-1 were found to be inactive (results not shown).

Effect of selected enzymatic inhibitors on the contractile activity of ETs

ET-1-induced contractions of GPLP were potentiated by treatment with selected concentrations of the enzymatic inhibitors, phosphoramidon, thiorphan and SQ 28,603 (Figure 4). Phosphoramidon (10 μM) increased the contractions induced by an intermediate concentration of ET-1 (30 nM) from 52% to 75% ($P<0.05$) which corresponds to a potentiation of

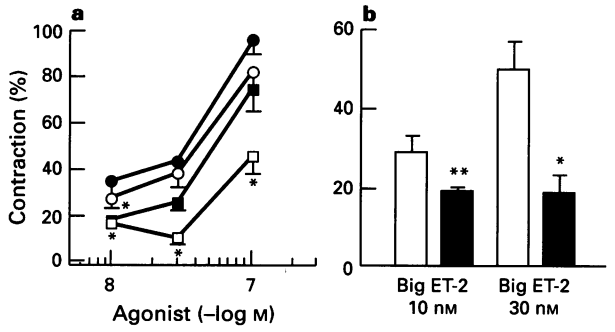

Figure 3 Effect of big endothelin-2 in the presence of vehicle (○), captopril (10 μM; ●), phosphoramidon (10 μM; □) and thiorphan (10 μM; ■) (a) and in the presence of vehicle (open columns) and SQ 28,603 (10 μM; solid columns) (b) on the guinea-pig lung parenchyma. Results are expressed as a percentage of the response to histamine (4.5 μM). n = 4 to 7. $^*P<0.05$, $^{**}P<0.01$.

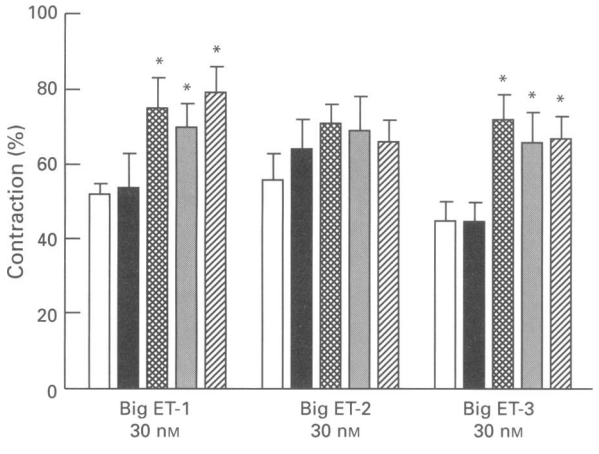

Figure 4 Effect of endothelin-1, endothelin-2 and endothelin-3 in the presence of vehicle (open columns), captopril (10 μM; solid columns), phosphoramidon (10 μM; cross-hatched columns), thiorphan (10 μM; stippled columns) and SQ 28,603 (10 μM; hatched columns) on the guinea-pig lung parenchyma. Results are expressed as a percentage of the response to histamine (4.5 μM). n = 4 to 7. $^*P<0.05$.

44%. Thiorphan and SQ 28,603 potentiated the contractions of strips of GPLP to ET-1 (30 nM) by 35% and 52% ($P<0.05$) respectively. Incubation of strips of GPLP with the same concentration of both phosphoramidon and thiorphan did not potentiated further the contractions induced by ET-1 (results not shown). Captopril (10 μM) did not affect the contraction of strips of GPLP to ET-1 (Figure 4).

The selected enzymatic inhibitors (10 μM) did not reduce the contractile response of strips of GPLP to ET-2 and 3 (Figure 4). On the contrary, the incubation of the tissues with the enzymatic inhibitors (except captopril) potentiated the contractions induced by ET-3 (Figure 4), phosphoramidon and thiorphan being equieffective in increasing the contractions of the tissues produced by ET-3 (30 nM). In another series of control experiments, phosphoramidon, thiorphan or SQ 28,603 (10 μM each) did not alter the response of the GPLP to histamine (4.5 μM; results not shown).

Discussion

Our results show that ET-1, ET-2 and ET-3 are equieffective in inducing concentration-dependent contractions of strips of GPLP. Since the three peptides were equieffective in producing contractions of GPLP, it is suggested that ET_B receptors mediate ET-induced contraction in these tissues. However Battistini *et al.* (1994) demonstrated that both ET_A and ET_B receptors mediated the ET-1-induced contractions of the GPLP but ET_B receptors appeared to be predominant. It was also demonstrated that ET_A receptors account for only 15% of the total endothelin-1 binding in the guinea-pig lung (Ihara *et al.*, 1992). In the guinea-pig lung trachea both ET_A and ET_B receptors were present and cooperated in mediating ET-1-induced contractions (Hay *et al.*, 1993; Battistini *et al.*, 1994; Inui *et al.*, 1994). ET_B receptors have also been shown to mediate the contraction induced by ETs in the guinea-pig lung bronchi (Hay *et al.*, 1993; Battistini *et al.*, 1994).

Big ET-1 and big ET-2 but not big ET-3 elicited concentration-dependent contractions of strips of GPLP. Interestingly, D'Orléans-Juste *et al.* (1991) demonstrated that unlike big ET-1, big ET-3 induced no pressor responses in anaesthetized guinea-pigs suggesting that the ECE activity may not convert big ET-3 to its active metabolite, ET-3. Recent studies also demonstrated that ETs, big ET-1 and big ET-2 are potent pressor agents in guinea-pigs (Pons *et al.*, 1991a,b; Gratton *et al.*, 1995). However, ETs, big ET-1 but not big ET-2 increased airway resistance. Furthermore, big ET-1 but not big ET-2 released thromboxane A_2 from guinea-pig perfused lung (Gratton *et al.*, 1995). The authors suggested the presence of two distinct phosphoramidon-sensitive ECEs in the guinea-pig, one responsible for the systemic conversion of big ETs which possesses the efficacy to convert big ET-1 and big ET-2 but not big ET-3 and another in the pulmonary vasculature, which is localized in the vicinity of the sites responsible for eicosanoid release and converts big ET-1 more readily than big ET-2. It has also been demonstrated that the ET-1 effects varied with the route of administration of the peptide. Intra-arterial administration of the peptide leads to ET-1 effects mediated via the generation of cyclo-oxygenase products. However, in aerosol administration, the peptide appears to act on airway smooth muscle cells through an indomethacin-insensitive process (Pons *et al.*, 1991c). It is of interest that big ET-1 produces a contractile response 50% greater than that observed with the mature peptide itself at 0.1 μM; this effect has yet to be explained. We can however exclude a contribution of the c-terminal fragment big ET-1 (22–38) as the latter is devoid of intrinsic activity.

In order to characterize the enzyme responsible for the conversion of big ET-1 and big ET-2 to ET-1 and ET-2 in GPLP, various enzymatic inhibitors of metallopeptidase were tested. Our results show that in strips of GPLP, the conversion of big ET-1 and big ET-2 was attenuated by phosphoramidon, thiorphan and SQ 28,603. Furthermore, the three enzymatic inhibitors were equieffective in inhibiting the conversion of big ET-1 and big ET-2. Many investigators have reported that the putative endothelin-converting enzyme (ECE) is a membrane-bound neutral proteinase sensitive to phosphoramidon (Matsumura *et al.*, 1990; Okada *et al.*, 1990; Shimada *et al.*, 1994). Recently, this enzyme has been shown to be selective for the conversion of big ET-1 to ET-1 compared to the conversion of big ET-3 to ET-3 in various *in vivo* and *in vitro* systems such as perfused rat and guinea-pig lungs, perfused rabbit kidney and in blood pressure monitoring of rat and guinea-pig (Télémaque *et al.*, 1993). Furthermore, the conversion of big ET-1 to ET-1 in guinea-pig upper bronchi is sensitive to phosphoramidon but not to thiorphan (Lebel *et al.*, 1995). Our results suggest that the enzyme responsible for the conversion of big ET-1 and big ET-2 to their active metabolites in the GPLP differs from the ECE previously reported in endothelial cells and other cell types.

NEP 24.11 is known to cleave a variety of biologically active peptides such as angiotensin, bradykinin and substance P (Gafford *et al.*, 1983; Shimamori *et al.*, 1986). On the other hand, Gafford *et al.* (1983) and Olins *et al.* (1989) have demonstrated that phosphoramidon and thiorphan are equipotent in inhibiting this enzyme. Murphy *et al.* (1993) have shown that purified NEP 24.11 from porcine lung membranes hydrolyses big ET-1. It has also been demonstrated that the ECE exhibits 58% amino acid homology with NEP 24.11 (Xu *et al.*, 1994). Sawamura *et al.* (1993) solubilized and partially purified

the ECE from the membrane fraction of porcine lung and found two distinct ECE activities. One enzyme is sensitive to phosphoramidon and is selective for the conversion of big ET-1 to ET-1. They proposed that this enzyme was similar to the ECE found in endothelial cells. The other enzyme converted big ET-1 and big ET-2 but not big ET-3. Furthermore, this enzyme was sensitive to phosphoramidon as well as thiorphan. They proposed that this enzyme was similar to NEP 24.11 with respect to the sensitivities to proteinase inhibitors. Shima *et al.* (1994) also found two distinct ECE activities in guinea-pig lung membrane fraction. One purified enzyme converted big ET-1 to ET-1 and was inhibited by phosphoramidon, but not by thiorphan or captopril. Another enzyme was inhibited by phosphoramidon and also by thiorphan. The authors suggested that the first enzyme is a unique metalloprotease that converts big ET-1 to ET-1 and the second one seems to be a neutral endopeptidase (EC 3.4.24.11) or a similar enzyme. Pons *et al.* (1992) demonstrated that intravenous (i.v.) injections of phosphoramidon markedly reduced the bronchoconstrictor responses evoked by big ET-1 (i.v. injection) in the anaesthetized and ventilated guinea-pigs. However, thiorphan also partially reduced the bronchoconstrictor responses induced by big ET-1. Since the conversion of big ET-1 and big ET-2 to their respective endothelins in the GPLP is sensitive to phosphoramidon, thiorphan and SQ 28,603, it is proposed that this enzyme corresponds to NEP 24.11 and since captopril did not affect the contraction induced by big ETs, we can exclude the possibility that the enzyme is the angiotensin-converting enzyme. Furthermore, the absence of a detectable response of GPLP to big ET-3 suggests that the enzyme involved in its conversion to an active peptide is not present in this airway preparation. This substrate specificity may be due to the difference in the amino acid sequence of the cleavage site of big ETs and the addition of 3 amino acids in the c-terminal portion of big ET-3 when compared to big ET-1 and 2. The cleavage site in both big ET-1 and big ET-2 is Trp^{21}-Val^{22}, whereas that in big ET-3 is Trp^{21}-Ile^{22}.

The effects of enzymatic inhibitors on the contractile response of ETs were tested in order to exclude the possibility that the reduction of the contractions of strips of GPLP to big ETs could be attributed to unspecific activities of enzymatic inhibitors with the ETs receptors. Our results indicated that phosphoramidon, thiorphan and SQ 28,603 potentiated the contractile responses induced by ET-1 and ET-3 which suggested that the two peptides are hydrolyzed by the NEP 24.11. Our results confirm previous reports suggesting that NEP (probably epithelium-derived) contributes to the metabolism of ET-1 in guinea-pig and human airways (Hay, 1989; Vijayaraghavan *et al.*, 1990; Noguchi *et al.*, 1991; Yamaguchi *et al.*, 1992; Di Maria *et al.*, 1992). These results strongly suggested that the effects of the enzymatic inhibitors were probably not mediated by interactions with ET receptors. Interestingly, Sokolovsky *et al.* (1990) have demonstrated that incubation of endothelins with kidney neutral endopeptidase resulted in the degradation with a loss of biochemical activity of the peptides. More recently, Murphy *et al.* (1993) have demonstrated that the enzyme which hydrolyzes ET-1 in the porcine lung membrane fraction is NEP 24.11. In contrast to the contractions evoked by ET-1 and ET-3, the contractile responses induced by ET-2 were not affected by the presence of the enzymatic inhibitors which confirmed our hypothesis that these substances did not interfere directly with the biological action of ET-2.

In conclusion, this study shows that big ET-1, big ET-2 but not big ET-3 elicited potent contractile responses of strips of GPLP. The effects of big ET-1 and big ET-2 on this tissue result from the conversion to ET-1 and ET-2 by at least one enzyme sensitive to phosphoramidon, thiorphan and SQ 28,603. Our results suggest that this enzyme corresponds possibly to NEP 24.11 but not to the known ECE. Furthermore, our results suggested that this enzyme could also be responsible for the degradation of ETs in the GPLP. However, the presence of the ECE in the GPLP cannot be excluded.

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