Regional differences in endothelin converting enzyme activity in rat brain: inhibition by phosphoramidon and EDTA

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1 It has been demonstrated previously that conversion of big endothelin-1 (bET-1) to endothelin-1 (ET-1) is inhibited *in vitro* and *in vivo* by phosphoramidon. In addition, ET-1 binding sites and mRNA have been shown within the brain. Here we expand upon our previous observation that rat brain contains phosphoramidon-inhibitable endothelin converting enzyme (ECE) and show that this activity is not uniformly distributed throughout the brain.

2 ECE activity was detected by a bioassay which depended upon the 10,000 fold difference in potency between bET-1 and ET-1 as stimulants of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) accumulation in kidney epithelial (PK_1) cells of the pig. Data were confirmed by specific enzyme-linked immunosorbent assay (ELISA) employing antibody directed against ET-1/3$_{(17-21)}$.

3 Following homogenization of the whole brain and ultracentrifugation the $100,000 \, g$ pellet contained greater than 4 times more ECE activity than the cytosol. Washing of the pellet with KCl (1 M) and extraction with the detergent CHAPS (20 mM) revealed a phosphoramidon-inhibitable ECE within the residual particulate fraction (nominally classified as the cytoskeletal fraction). Phosphoramidon (IC_{50}, approx. 5 μM) or EDTA inhibited the conversion of bET-1 to ET-1 by the cytoskeletal fraction of rat brain by more than 60%.

4 Following dissection of rat brain into olfactory bulb, cerebral cortex, striatum, hippocampus, cerebellum, midbrain (including thalamus), hypothalamus and medulla oblongata (including pons) the greatest ECE was detected in the hypothalamus and medulla oblongata. After fractionation, the ECE-activities in the cytoskeletal fractions prepared from the hypothalamus or medulla oblongata were inhibited concentration-dependently by phosphoramidon or EDTA, with maximum inhibitions of $>80\%$ and $>70\%$, respectively.

5 These data show that rat brain contains a phosphoramidon- and EDTA-inhibitable ECE which may be similar to that present in endothelial cells. The localization of this enzyme correlates with published reports of immunoreactive-ET-1, ET-1-binding sites, and messenger RNA for ET-1 in the rat brain, and suggests the presence of the entire synthetic pathway for ET-1.

Keywords: Endothelin-1, big endothelin-1; endothelin-converting enzyme; phosphoramidon; EDTA; metalloprotease; endothelin-3; big endothelin-3

Introduction

The biosynthesis of endothelin-1 (ET-1, Yanagisawa *et al.*, 1988) involves the cleavage of its immediate precursor, big ET-1 (bET-1) by a specific endothelin converting enzyme (ECE). Although various candidates have been proposed for this ECE the most likely is a phosphoramidon-inhibitable activity that has been detected in endothelial cells (Ohnaka *et al.*, 1990; Okada *et al.*, 1990; Warner *et al.*, 1992) and vascular smooth muscle cells (Matsumura *et al.*, 1991a). This conclusion is strengthened by the observations that phosphoramidon both decreases the release of ET-1 from cultured endothelial cells (Ikegawa *et al.*, 1990) and diminishes the physiological responses to bET-1 *in vivo* (Matsumura *et al.*, 1991b; Shinyama *et al.*, 1991), and *in vitro* (Hisaki *et al.*, 1991; Télémaque & D'Orleans-Juste, 1991).

Various groups have provided evidence that endothelin has a role within the brain. For instance, there is a selective distribution of endothelin-binding sites (Koseki *et al.*, 1989) and the presence of ET mRNA (MacCumber *et al.*, 1990), particularly in the hypothalamus (Lee *et al.*, 1990), and further it has been suggested that endothelin has an impor-

tant role in the posterior pituitary system (Yoshizawa *et al.*, 1990). Centrally administered endothelin has also been shown to affect motor activity (Lecci *et al.*, 1990) and arterial blood pressure and blood flows (Hashim & Tadepalli, 1991). In addition, phosphoramidon has been shown to block the vasoconstrictor effects of bET-1 on the basilar artery, *in vivo*, and to inhibit the effects of centrally administered bET-1 (Hashim & Tadepalli, 1991). We have previously reported that rat brain contains a phosphoramidon-inhibitable ECE (Warner *et al.*, 1992). Here we show the further characterization of this enzyme and describe the regional differences in the distribution of ECE activity within the rat brain. The enzyme activity was detected both by enzyme-linked immunosorbent assay (ELISA) and bioassay, using the elevation in guanosine 3′ : 5′-cyclic monophosphate (cyclic GMP) induced by ET-1 in kidney epithelial (PK_1) cells of the pig (Warner *et al.*, 1992). The use of the bioassay gives an assurance that the ELISA is detecting biologically active ET-1.

Methods

Preparation of rat brains

Rats (male, Sprague-Dawley, 250–400 g) were rendered unconscious with CO_2 and decapitated. The brains were

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rapidly removed and frozen in liquid N_2. In further experiments brains were allowed to thaw at 4°C to a half-frozen state and dissected into eight different regions (Förstermann et al., 1990) according to the method of Glowinski & Iversen (1966). The regions were olfactory bulb, cerebral cortex, striatum, hippocampus, cerebellum, midbrain (including thalamus), hypothalamus and medulla oblongata (including pons). Following dissection the regions were once again frozen in liquid N_2.

Preparation of subcellular fractions

Rat brains and brain regions were thawed in 5 volumes of buffer A (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES) 50 mM, NaCl 100 mM, pH 7.4) and homogenized on ice by use of a glass tissue grinder with a Teflon pestle. The homogenates were then centrifuged at 100,000 g for 1 h. Subsequent fractions were prepared as described previously (Warner et al., 1992). Briefly, the pellet fraction was washed once with buffer A, and resuspended in buffer A by homogenization. In some experiments the particulate fraction was washed with 1 M KCl (in buffer A) for 5 min at 4°C. After centrifugation (100,000 g, for 30 min), the pellet was resuspended in buffer A. The particulate fraction was then treated with the detergent 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulphonate (CHAPS, 20 mM) for 20 min at 4°C. After centrifugation (100,000 g, 30 min), the supernatant was collected (CHAPS-solubilized pellet fraction) and the pellet resuspended in buffer A (nominally classified as the cytoskeletal fraction). All fractions were assayed for ECE activity. Protein was determined with the Bradford reagent (Bio-Rad) with bovine serum albumin used as the standard. Aliquots of the subcellular fractions were stored at −70°C.

Assay of endothelin converting enzyme (ECE) activity

The activity of ECE within rat brain fractions was quantified by bioassay and selective ELISA (Warner et al., 1992). Briefly, rat brain protein (22.5 μg protein) was incubated (37°C, 20–180 min), at pH 7.4 in the presence of bET-1 (135 pmol) and any test inhibitors. All samples were incubated in duplicate in the absence and presence of phosphoramidon (100 μM) to permit the calculation of the metalloprotease-ECE activity in each. At the end of this time a mixture of protease/peptidase inhibitors (phenylmethylsulphonyl fluoride 1 mM, leupeptin 2 μM, pepstatin A 1 μM) and EDTA (1 mM) was added to each sample, to a total volume of 62.5 μl. The incubates were than rapidly frozen in liquid N_2 and stored (max. 2 h) at −20°C. To quantitate the amounts of ET-1 in each fraction the samples were rapidly thawed and 12.5 μl of each was bioassayed on PK_1 cells (as described below), the remaining 50 μl was assayed by ELISA. Each ECE/bET-1 combination was assayed in triplicate in each experiment. In some experiments big endothelin-3 (bET-3) was used as a substrate instead of bET-1 and the production of endothelin-3 (ET-3) measured.

Bioassay with LLC-PK$_l$ cells

PK_1 cells (ATCC CL 101) were grown to confluency in 12-well plates (well diameter, 35 mm) containing Medium 199 with 10% foetal bovine serum, 2 mM L-glutamine, 100 u ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin at 37°C under an atmosphere of 95% air: 5% CO_2. Prior to experimentation, the culture medium was removed and the cells were washed twice with 1 ml of Locke solution (pH 7.4) of the following composition (mM): NaCl 154.0, KCl 5.6, CaCl$_2$ 2.0, MgCl$_2$ 1.0, NaHCO$_3$ 3.6, glucose 5.6, HEPES 10.0 and 3-isobutyl-1-methylxanthine (IBMX) 0.3. Cells were equilibrated for 20 min in 0.5 ml of the same buffer. Experiments were conducted at 37°C with gentle shaking. After exposing the cells to either ET-1/3 standards or ECE/bET-1/3 mixtures for 4 min, the incubation buffer was removed and reactions were

stopped by adding 1 ml of ice-cold sodium acetate buffer (50 mM, pH 4.0). Then, samples were quickly frozen with liquid N_2 (Ishii et al., 1991). After thawing the frozen samples at room temperature, the content of cyclic GMP in the cells was determined by radioimmunoassay (Steiner et al., 1972). The concentration of ET-1/3 present in the sample was then calculated from the levels of cyclic GMP produced in the PK_1 cells compared to the levels produced by known concentrations of ET-1/3 (Warner et al., 1992).

Endothelin ELISA

The amounts of ET-1/3 formed in incubates of bET-1/3 and the test protein fractions were also measured by specific ELISA with antibody directed against the C-terminal fragment of ET-1/3 (ET-1/3$_{(17-21)}$) with approximately 0.01% cross reactivity with bET-1/3. Binding was detected by peroxidase-labelled goat anti-rabbit IgG antibody using o-phenylenediamine-2HCl as a substrate. The optical density of each well was measured at 490 nm with a microplate reader (Bio-Tek model EL311).

Chemicals

3-Isobutyl-1-methyl-xanthine (IBMX) and N-(α-rhamno-pyra-nosyl-oxy-hydroxy-phosphinyl)-Leu-Trp (phosphoramidon) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Big ET-1 (big endothelin, human) big endo-thelin-3 (human), ET-1 and ET-3 were obtained from Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.). All other chemicals and reagents used were of the highest quality available.

Statistics

Results are shown as mean ± s.e.mean for n experiments. Student's unpaired t test was used to determine the significance of differences and a P value of less than 0.05 was taken as significant.

Results

Localization of phosphoramidon-inhibitable ECE

Fractionation of the rat brain homogenate revealed four times more ECE activity within the 100,000 g pellet than the 100,000 g cytosol (Figure 1a), as measured by either bioassay or ELISA. Treatment of the pellet by washing with KCl (1 M, 5 min) and extraction with the detergent CHAPS (20 mM, 20 min) resulted in the greater part of the phosphoramidon-inhibitable activity remaining within the particulate fraction (cytoskeletal fraction, Figure 1b). In the presence of phosphoramidon (100 μM) this activity was decreased by 70.7 ± 10.1% (n = 7), as assayed by PK_1 cells, or 52.2 ± 9.8% (n = 4), as assayed by ELISA. Similarly, EDTA (1 mM) inhibited the ECE activity by 74.8 ± 14.2 (n = 5, PK_1 cells) or 47.2 ± 10.2% (n = 3, ELISA). In either assay phosphoramidon inhibited the ECE activity with an IC$_{50}$ of approx. 5 μM (Figure 2). In one experiment employing bET-1 and bET-3 as substrates for the brain fractions, conversion of only bET-1 and not of bET-3 was detected.

Regional distribution of phosphoramidon-inhibitable ECE

At 1 h but not 3 h, inhibition of the formation of immunoreactive (ir)-ET-1/3$_{(17-21)}$ by a mixture of phosphoramidon (100 μM) and EDTA (1 mM) was seen in homogenates prepared from the hypothalamus (29 ± 4%, n = 3) or the medulla oblongata (28 ± 75, n = 3). By ELISA there was a reduction by 84–99% (n = 3 for each) in the formation of ir-ET-1/3$_{(17-21)}$, in the presence of the protease and peptidase inhibitors (phenylmethylsulphonyl fluoride
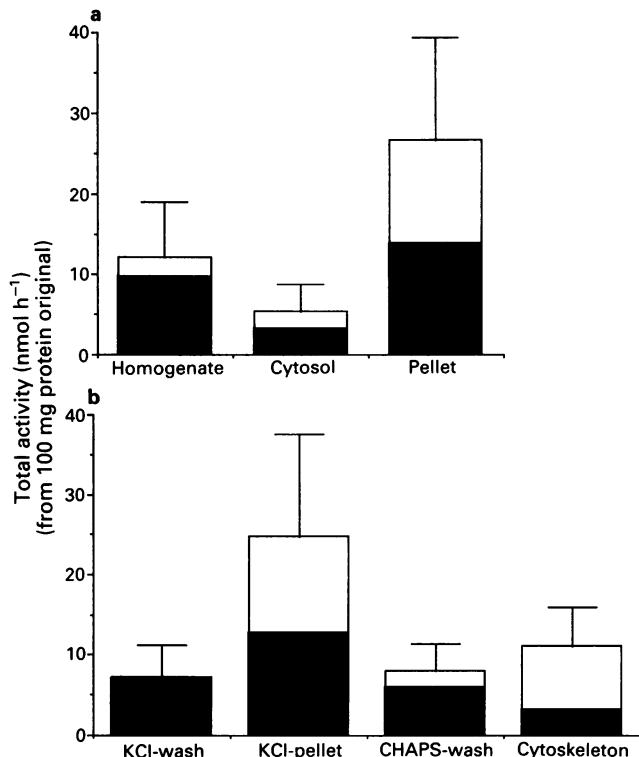
Figure 1 Localization of endothelin converting enzyme (ECE) activity in fractions prepared from the rat brain. The figure represents pooled data from 5 experiments employing both PK₁-cell bioassay and ELISA, each done in triplicate, on 3 different preparations. Results are expressed as the total activities in fractions prepared from homogenates containing 100 mg total protein. Sections within each column indicate the proportion of activity inhibited (open columns) and not inhibited (filled columns) by phosphoramidon (100 µM). (a) ECE activity in homogenate, and 100,000 *g* cytosol and pellet. (b) ECE activity in extracts of the 100,000 *g* pellet.

1 mM, leupeptin 2 µM, pepsatin A 1 µM) and EDTA (1 mM). Addition of N-ethylmaleimide (1 mM) inhibited all enzymatic activity as assessed by ELISA or bioassay ($n = 3$, for each brain region).

The cytoskeletal fraction of each region was tested by PK₁-cell bioassay and ELISA. The extent of phosphoramidon-inhibitable ECE in each region was, hypothalamus (83 ± 2% by ELISA; 80 ± 7% by PK₁), medulla oblongata (72 ± 2%; 82 ± 6%), midbrain (65 ± 7%; 61 ± 5%), olfactory bulb (0 ± 0%; 38 ± 10%), cerebral cortex (27 ± 5%; 46 ± 12%), striatum (25 ± 13%; 32 ± 17%), cerebellum (28 ± 9%; 51 ± 1%) and hippocampus (33 ± 12%; 61 ± 24%) (Figure 3). EDTA (1 mM) caused similar inhibitions. The activity of both the hypothalamus (Figure 4a) and the medulla oblongata (Figure 4b) was inhibited in a concentration-dependent manner by phosphoramidon and EDTA. In the presence of the peptidase and protease inhibitors and EDTA no production of ir-ET-1/3₍₁₇₋₂₁₎ was detected in any of the brain regions or brain fractions (20–60 min, $n = 3$ for each).

Figure 3 Detection of endothelin converting enzyme (ECE) activity in rat brain regions by ELISA or bioassay. The cytoskeletal fraction (22.5 µg protein) from different rat brain regions was incubated with bET-1 (135 pmol) for 60 min alone ($n = 3$, open columns) or in the presence of phosphoramidon (100 µM, $n = 3$, hatched columns) or EDTA (1 nM, $n = 3$, stippled columns). For comparison, data are shown from three PK₁-cell bioassay and ELISA experiments carried out in parallel: (a) cerebral cortex; (b) hypothalamus. S.e.mean shown by error bars.
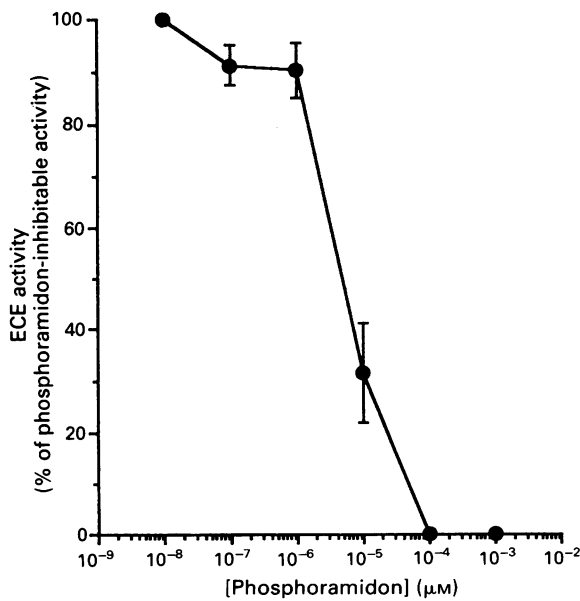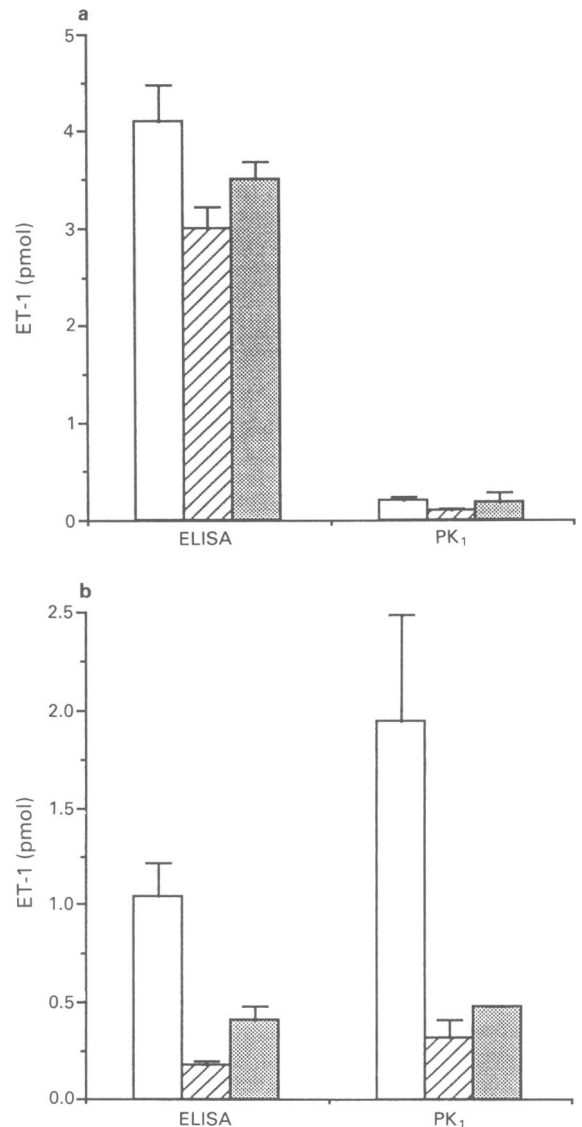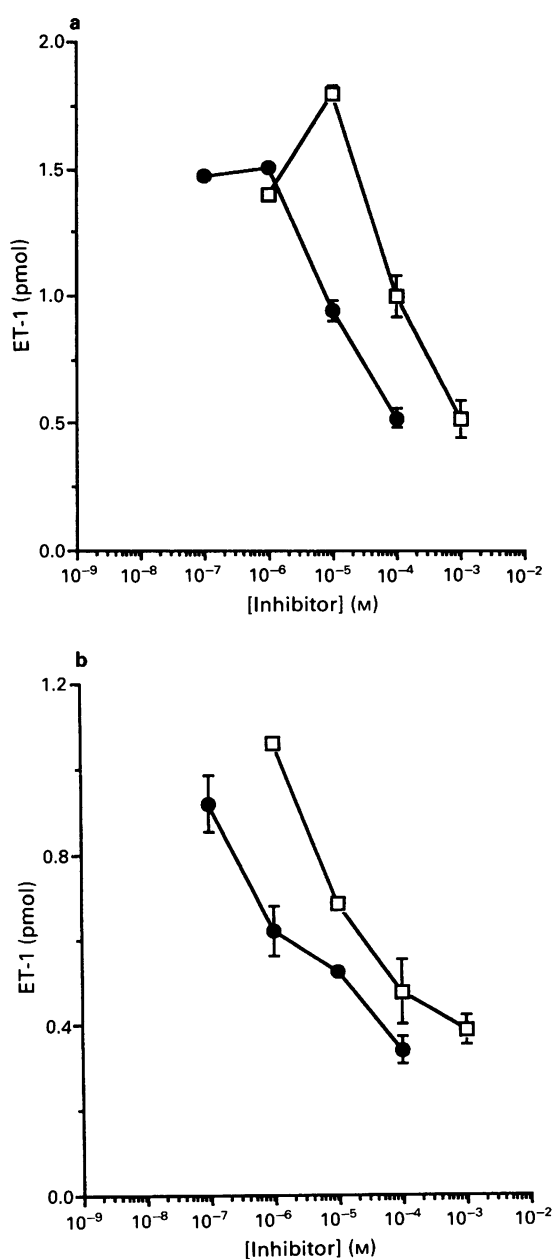

Figure 2 Inhibition of endothelin converting enzyme (ECE) activity by phosphoramidon. Rat brain cytoskeletal fraction (22.5 µg protein) was incubated with bET-1 (135 pmol) for 20 min in the presence of phosphoramidon (0.01–100 µM). Each point with a vertical bar represents the mean ± s.e. of the mean. Data are pooled from three PK₁-cell bioassay and ELISA experiments, each done in triplicate.

a

ET-1 (pmol)

[Inhibitor] (M)

b

ET-1 (pmol)

[Inhibitor] (M)

Figure 4 Inhibition of endothelin converting enzyme (ECE) in hypothalamus and medulla oblongata by phosphoramidon or EDTA. The cytoskeletal fraction (22.5 μg protein) from the hypothalalmus or the medulla oblongata was incubated with bET-1 (135 pmol) and either phosphoramidon (0.1–100 μM, $n = 3$, ●) or EDTA (1 μM–1 mM, $n = 3$, □). Data was obtained from ELISA experiments. (a) Hypothalamus; (b) medulla oblongata. s.e.mean shown by error bars.

No phosphoramidon-inhibitable conversion of bET-3 to ET-3 was detected in any of the brain regions ($n = 3$ for each).

Discussion

Our data show that rat brain contains a metalloprotease-ECE activity which is inhibitable by phosphoramidon or EDTA. This suggests that this enzyme is closely related to that present in endothelial cells (Ohnaka et al., 1990; Okada et al., 1990), and to the enzyme responsible for the conversion of exogenous bET-1 to ET-1 in the circulation (Matsumura et al., 1991b). However, unlike the activity in endothelial cells the ECE in brain was largely particulate and was only poorly extractable by detergent. In endothelial cells

there is considerably more ECE activity within the cytosol (Takada et al., 1991; Warner et al., 1992), and the activity present in the particulate fraction can be solubilised with detergent (Okada et al., 1990; Warner et al., 1992). Thus, the brain ECE may be located in sites other than the brain endothelium, consistent with the previous findings of endothelin receptors, ET-1-stimulated inositol phosphate turnover, ET-1-promoted mitogenesis, ET-1 messenger RNA, and ET-1 immunoreactivity with brain cells (Koseki et al., 1989; Crawford et al., 1990; MacCumber et al., 1990; Yoshizawa et al., 1990).

In our further experiments to localize the ECE-activity within the rat brain we found that, as in homogenates of whole brain, only very poor phosphoramidon-inhibitable activities were detectable in homogenates of the brain regions. This is probably explained by the presence of other activities masking the phosphoramidon-inhibitable one. This was not revealed, however, by N-ethylmaleimide, which has been reported to discriminate ECE in the particulate fraction of endothelial cells (Matsumura et al., 1991c). Indeed, in these experiments we could not detect any formation of ET-1 by bioassay, or of ir-ET-1$_{(17-21)}$ by ELISA. Thus, the particulate enzyme present in the brain may be different from that in the endothelial cell.

Significant ECE-activities were detected in the cytoskeletal fractions prepared from the hypothalamus and medulla oblongata. Of the remaining regions the highest activity was detected in the midbrain, while the other regions all expressed lower and insignificant activities forming less than 0.2 pmol ET-1 per incubate, as detected by bioassay (data not shown). There was a good correlation in the amount of activity detected in the hypothalamus (Figure 3) and medulla oblongata by ELISA and bioassay, whereas in incubates using protein prepared from other regions the correlation was not so good (Figure 3). For instance, in incubates containing protein prepared from the cerebral cortex, a high level of ir-ET-1$_{(17-21)}$ was detected by ELISA, but no significant activity was detected by bioassay. This was most probably due to the ELISA detecting fragments of bET-1 that were not biologically active. It may also be possible that these regions very rapidly metabolize ET-1 once it is formed, leaving fragments detectable by ELISA but not by bioassay. These suggestions may also explain why in all experiments we found that the ELISA showed either phosphoramidon or EDTA to inhibit the ECE-activity less than did parallel bioassay.

Interestingly, we were not successful in detecting the conversion of bET-3 to ET-3 in any of the rat brain fractions or regions, although it has been reported that rat brain contains more ET-3 than ET-1 (Takahashi et al., 1991). This was not due to the inability of our assays to detect ET-3 since ET-1$_{(17-21)}$ and ET-3$_{(17-21)}$ have the same amino acid sequence, and the PK$_1$-cell bioassay is equally sensitive to ET-1 and ET-3 (Ishii et al., 1991). The explanation may lie in the fact that the assay employed here was optimised to detect the conversion of bET-1 to ET-1 by the neutral metalloprotease characterized in endothelial cells. It may not be so good for detecting the conversion of bET-3 to ET-3. These results do suggest, however, that the phosphoramidon-inhibitable ECE is selective for bET-1. Due to the close similarity in structure between ET-1 and ET-3 (Inoue et al., 1989) this suggests the possibility of a very strict requirement for binding to the active site of the enzyme.

In our experiments we assayed all samples in duplicate in the absence and presence of phosphoramidon. The difference in ET-1 amounts between the two samples gave a true measure of the ET-1 formation. This protocol provided a control within the bioassay and ELISA for non-specific effects of protein and bET-1 in the assays. The ET-1 thus measured could therefore be taken as an indication of the selective ET-1 formation by the ECE-metalloprotease. The finding that no ir-ET-1$_{(17-21)}$ or ET-1 was detected by ELISA or bioassay after the incubation of brain fractions with bET-

1 and a cocktail of protease/peptidase inhibitors shows that bET-1, at the concentrations used here, did not cross react with the antibody and that the fractions *per se* did not contain detectable amounts of endogenous ET-1.

The localization of phosphoramidon-inhibitable ECE which we describe here correlates well with the reported sites of irET-1 and mRNA for ET-1 in the paraventricular and supraoptic nuclei in the hypothalamus (Yoshizawa *et al.*, 1990), in the thalamus, midbrain, pons, medulla and cerebellum (Yoshimi *et al.*, 1991) and of autoradiographed determinations of the binding sites for ET-1 which are located mainly in the hypothalamic and thalamic areas (Koseki *et al.*, 1989). It also correlates with studies in man which have shown the highest density of cells containing ET-1 mRNA to

be in the hypothalamus (Lee *et al.*, 1990). This correlation strongly suggests that the formation of ET-1 in nervous tissue is dependent upon the activity of an ECE which is the same as, or closely related to, the metalloprotease responsible for the processing of bET-1 in endothelial cells. Taken together these data demonstrate the presence of the entire pathway required for the production and effects of ET-1 within the brain.

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