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The membranes of cultured rat brain astrocytes contain endothelin-converting enzyme activity

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Abstract

Both endothelins and their big-endothelin precursors were found capable of inducing the release of arachidonic acid from purified cultures of rat astrocytes. Their order of potency was as follows: big-endothelin-3 < big-endothelin-1 < endothelin-1 = endothelin-3. Mature endothelins induced the release of arachidonic acid in a rapid fashion. In contrast, much longer incubation times were required for big-endothelins to exert an effect, suggesting that their activity was dependent on their conversion. When big-endothelin-1 was added to the incubation medium of intact live astrocytes, it was converted into mature endothelin-1 in a time-dependent manner and the conversion was inhibited by phosphoramidon. This suggests that astrocytic endothelin-converting enzyme is (at least in part) an external membrane-bound metalloprotease. Some conversion of big-endothelin-3 into endothelin-3 also occurred. However, it was less efficient than the conversion of big-endothelin-1, which is compatible with the lower bioactivity of big-endothelin-3 vs. that of big-endothelin-1 in astrocytes.

Keywords: Endothelin; Big-endothelin; Endothelin-converting enzyme; Brain; Astrocyte; Phospholipase A_2

1. Introduction

Endothelins are members of a family comprising three isoforms called endothelin-1, -2 and -3 (Inoue et al., 1989). These peptides derive from three distinct but closely related genes that encode for preproendothelins. The processing of endothelins is of particular interest (Opgenorth et al., 1992). It is generally believed that proendothelins are cleaved intracellularly into an intermediary form called 'big-endothelins'. This peptide is then further cleaved into mature endothelin by the so-called 'endothelin-converting enzyme'. The predominant form of endothelin-converting enzyme described by most investigators is an external membrane-bound metalloprotease that is inhibited by phosphoramidon and displays strong substrate specificity for big-endothelin-1 (Opgenorth et al., 1992; Okada et al., 1990; Ahn et al., 1992; Ikegawa et al., 1991). However, some diversity may occur, since intracellular microsome-associated forms of endothelin-converting enzyme have been reported, both in rat lung cells (Gui et al., 1993) or in COS cells transfected with expression plasmids for cloned endothelin-converting enzyme (Xu et al., 1994). In addition, a small number of reports indicate that big-endothelin-3 may serve as a substrate to endothelin-converting enzyme in some preparations (Tsukahara et al., 1993; Matsumara et al., 1992; Xu et al., 1994).

Endothelins were initially identified in endothelial cells (Yanagisawa et al., 1988). Although these cells constitute the richest source of endothelins, these peptides are synthesized in other locations as well, including smooth muscle, brain, reproductive tissues and endocrine glands (Masaki, 1993). In brain, endothelin-1 and endothelin-3 have been found to be produced both by neurons (MacCumber et al., 1991) and by astrocytes (MacCumber et al., 1991; Ehrenreich et al., 1991, 1993). In addition, astrocytes exhibit high-affinity binding sites for endothelins, which induce a variety of potent responses in these cells, including the activation of protein kinase C and of phospholipase A_2, a rise in intracellular calcium and the induction of cell prolifer-

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ation (Supattapone et al., 1989; Tencé et al., 1992; Marin et al., 1991).

A variety of investigators have studied the conversion of big-endothelins by peripheral tissues either in vivo or in vitro (Opgenorth et al., 1992; Okada et al., 1990; Ahn et al., 1992; Ikegawa et al., 1991; Télémaque et al., 1992). However, very little attention has been paid to endothelin-converting enzyme activity within the brain. Warner et al. have reported the presence of endothelin-converting enzyme activity in brain homogenates, and suggested that this enzyme was different from that in endothelial cells on the basis of differences in solubility in detergents (Warner et al., 1992a,b). However, the brain enzyme preparations they used were only poorly inhibited by phosphoramidon (Warner et al., 1992a), suggesting that their preparation was contaminated by other non-specific peptidases, and making it difficult to study the properties of brain endothelin-converting enzyme in detail. This prompted us to examine whether endothelin-converting enzyme activity could be detected in highly purified cultures of brain cells that have been shown to produce mature endothelins, i.e. rat brain astrocytes.

2. Materials and methods

2.1. Cultures of astrocytes

Secondary cultures of rat brain type-1 astrocytes were prepared as described previously (Zahs et al., 1993; Deschepper and Picard, 1994). Briefly, diencephalon or frontal cortex were dissected out from the brains of 1-day-old rats, enzymatically dispersed with Dispase (Boehringer Mannheim) and plated in 100 × 200-mm dishes previously coated with poly-D-lysine. After 5 days of maintenance in culture medium (a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mix supplemented with 10% fetal calf serum and antibiotics), the cells growing on top of the confluent monolayer of type-1 astrocytes (mainly cells of the O-2A lineage and of microglial origin) were removed by thorough washing with PBS. The cells that remained attached to the plates were maintained for 3–7 additional days. When needed for experiments, these cells were trypsinized, replated in 24-well plates at a concentration of 50,000 cells/cm^2 and grown for 3 additional days. In order to avoid contamination of cells with non-specific proteases that are present in cell culture-grade trypsin preparations, cells were passaged only with solutions of crystallized trypsin (Sigma, St. Louis, MO).

2.2. Release of arachidonic acid

Monitoring of the release of tritiated arachidonic acid was performed as described previously (Tencé et al., 1992). Three days after replating astrocytes in 24-well plates, tritiated arachidonic acid (200 Ci/mmol, purchased from Du Pont Canada) was added to the culture medium (final concentration 1 μCi/ml). The cells were incubated in that medium overnight and then washed 2 times with warm Krebs phosphate buffer supplemented with fatty acid-free bovine serum albumin (1 mg/ml). The cells were then exposed for 15–30 min at 37°C to 200 μl of the same medium supplemented with adenosine deaminase (1 IU/ml) and containing various concentrations of the agonists. Incubation media were centrifuged for 10 min and radioactivity was measured in the supernatants by counting the samples in a beta counter in the presence of scintillation fluid. To normalize the results, cells from each well were sonicated in buffer, and DNA was quantitated in the presence of ethidium bromide by fluorescence as described previously (Deschepper and Picard, 1994). Values were expressed as cpm radioactivity/μg DNA. In some cases, all values were divided by the mean of the values from the control group and expressed as % of control. When dose-response experiments were performed, the experimental values were fitted to a nonlinear curve, using the logistic function and the SlideWritePlus program (Advanced Graphics Software, Carlsbad, CA) for IBM-compatible computers. In addition, the program was used to calculate the pD_2 values (defined as $-\log EC_{50}$).

2.3. Conversion of big-endothelins

Astrocytes were replated in 24-well plates exactly as described above. After 3 days in culture, the cells were washed twice with Krebs phosphate buffer supplemented with fatty acid-free bovine serum albumin (1 mg/ml). The cells were then incubated at 37°C for various lengths of time with 250 μl of the same buffer supplemented with big-endothelin-1 or big-endothelin-3 (10^{-7} M). The concentrations of endothelin-1 or endothelin-3 were subsequently measured in 100 μl aliquots of incubation medium by radioreceptor assay, as described below.

2.4. Radioreceptor assay for endothelin

The conditions for the radioreceptor assay were adapted from those described previously for endothelin-receptor binding assays (Thibault et al., 1994). Endothelin receptor-containing membranes were prepared from adult rat lungs as follows. The tissue was homogenized with a Polytron in buffer (12 ml/g of tissue) containing 15 mM $NaHCO_3$ (pH 8.3) and 1 mM phenylmethylsulfonylfluoride, and then centrifuged for 15 min at 1500 × g. The supernatant was saved, the pellet was resuspended in half the original volume, rehomogenized and centrifuged. Both supernatants

were pooled and centrifuged at 48000 × *g* for 30 min. The pellet was resuspended in 50 mM Tris pH 7.4, 1 mM phenylmethylsulfonylfluoride (10 ml/g of tissue) and recentrifuged at 48000 × *g* for 15 min. The last pellet was resuspended in 50 mM Tris pH 7.4, 1 mM phenylmethylsulfonylfluoride and stored in small aliquots at −80°C. Concentration of the protein in the aliquots was determined by the Bradford protein assay (Bradford, 1976).

For receptor binding, 10 μg of lung membrane proteins were incubated with 50 μl of ^{125}I-labeled endothelin-1 (25000 cpm), 100 μl of 2.5 × binding buffer (125 mM Tris pH 7.2, 125 U/ml aprotinin, 0.25% bacitracin, 12.5 mM $MgCl_2$ and 1.25% bovine serum albumin) and 100 μl of either cell incubation buffer or buffer containing 10^{-7} M of big-endothelin and various concentrations of endothelin. The tubes were incubated at room temperature for 90 min, then filtered on No. 34 glass filter (Schleicher and Schuell, Keene, NH) previously incubated in 5% dry skimmed milk to decrease nonspecific binding. The filters were further washed 3 times with 50 mM Tris pH 7.2, 150 mM NaCl and counted in a gamma counter.

3. Results

Application of endothelin-1 for 15 min on primary cultures of rat astrocytes induced the release of arachidonic acid in a dose-related fashion ($pD_2 = 8.7 \pm 0.12$) (Fig. 1, left panel), whereas big-endothelin-1 had very little effect when applied in the same time-frame. However, incubations of 30 min allowed big-endothelin-1 to increase the release of arachidonic acid, although the response was less sensitive ($pD_2 = 7.6 \pm 0.02$) than what was obtained with endothelin-1 ($pD_2 = 9.2 \pm 0.17$) (Fig. 1, right panel).

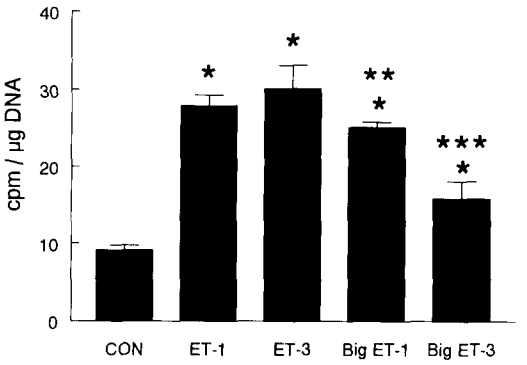

Fig. 2. Bar graph shows release of [^{3}H]arachidonic acid after 45 min exposure to 10^{-7} M endothelin-1, endothelin-3, big-endothelin-1 or big-endothelin-3. Values are mean ± S.E.M. ($n = 6$). $^*P < 0.01$ vs. control; $^{**}P < 0.05$ vs. endothelin-1 or endothelin-3; $^{***}P < 0.01$ vs. big endothelin-1. All groups were compared by one-way ANOVA followed by Newman-Keuls post-hoc test.

We compared the maximal responses obtained with a 45 min application of 10^{-7} M endothelin-1, endothelin-3, big-endothelin-1 and big-endothelin-3 (Fig. 2). All peptides induced a release of arachidonic acid that was significantly greater than control. Endothelin-1 was equipotent with endothelin-3, which is compatible with the presence of mostly endothelin B-type endothelin receptors on astrocytes (Couraud et al., 1991; Schwartz and Wilson, 1992). The big-endothelin-1 induced release of arachidonic acid was comparable in

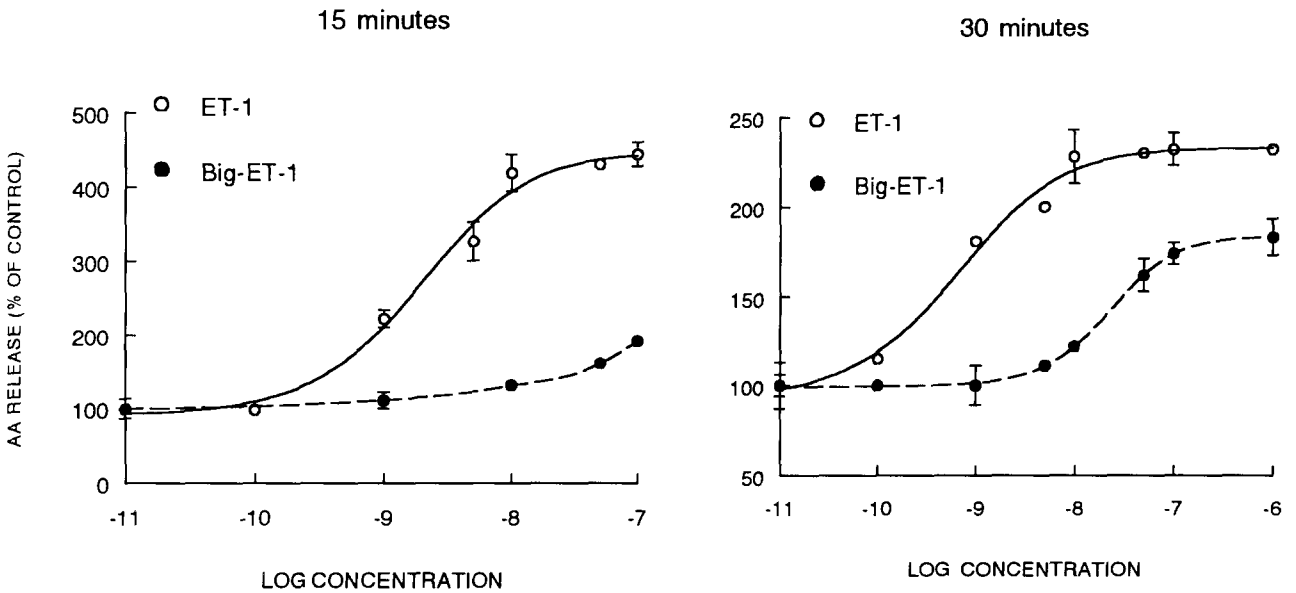

Fig. 1. *Left*. Line graphs show dose-response curves of [^{3}H]arachidonic acid release after 15 min exposure to increasing concentrations (mol) of endothelin-1 or big-endothelin-1. Each point represents mean ± S.E.M. ($n = 4$) and is calculated as percentage of control (with no peptide added to the medium). *Right*. Line graphs show similar dose response curves obtained after 30 min exposure to the same peptides.

magnitude but statistically lower to the release induced by endothelin-1 and endothelin-3. The effect obtained with big-endothelin-3 was significantly lower than what had been obtained with all other three peptides.

To test whether exogenously applied big-endothelins were converted into processed endothelins, astrocytes were incubated for various periods of time with 10^{-7} M big-endothelin-1 or -3 in the presence or absence of metalloprotease inhibitors. The amounts of mature endothelin-1 or -3 generated in the supernatants were subsequently measured by radioreceptor assay. This method was chosen because the interpretation of the results from radioimmunoassays are usually complicated by cross-reactivity between big-endothelins and mature endothelins, and that this problem is reportedly less prominent with receptor-based assays (Warner et al., 1992b; Takahashi et al., 1993). Endothelin-1 and endothelin-3 caused a dose-dependent displacement of [^{125}I]endothelin-1 from the lung receptor preparation, whereas 10^{-7} M big-endothelin-1 or -3 did not affect the binding. Therefore, standard curves were established by incorporating 10^{-7} M big-endothelin-1 (when endothelin-1 was used for displacement) or 10^{-7} M big-endothelin-3 (when endothelin-3 was used for displacement) to every tube. In this way, all possible cross-reactivity with big-endothelins was eliminated, and any displacement of [^{125}I]endothelin-1 could solely be accounted for by the presence of mature endothelin in the cell incubation medium. As shown in Fig. 3, such standard curves allowed the detection of endothelin-1 or endothelin-3 concentrations ranging from 0.1 to 10 nM (practically, this corresponds to a minimal detectable concentration of 62.5 pg per 100 μl sample).

When astrocytes were incubated with 10^{-7} M big-endothelin-1, endothelin-1 increased in the incubation

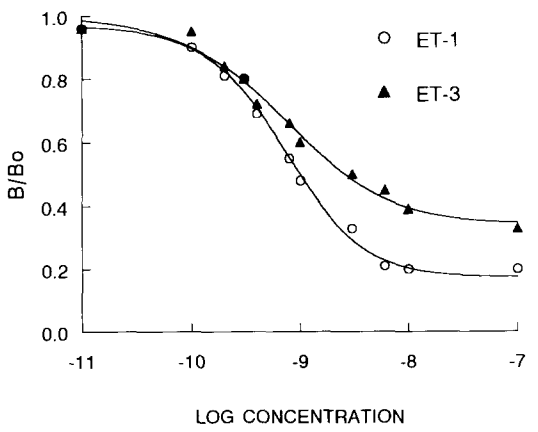

Fig. 3. Line graphs show displacement of [^{125}I]endothelin-1 from a lung membrane endothelin-receptor preparation by increasing concentrations of either endothelin-1 or endothelin-3, in the presence of 10^{-7} M big-endothelin-1 or big-endothelin-3, respectively.

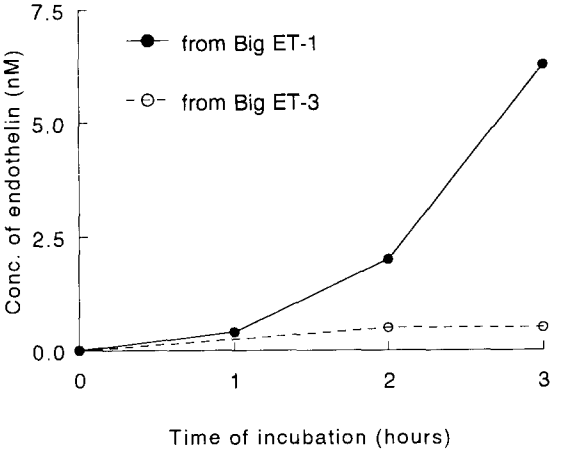

Fig. 4. Line graphs show the time course of appearance of endothelin-1 in medium containing 10^{-7} M big-endothelin-1 and incubated in the presence of astrocytes, and of endothelin-3 in medium containing 10^{-7} M big-endothelin-3. No endothelin was ever detected when the incubation was performed in the presence of 10^{-4} M phosphoramidon.

medium in a time-dependent fashion, and reached 6.5 nM after 3 h of incubation (Fig. 4). Addition of 10^{-4} M phosphoramidon to the incubation medium totally abolished all endothelin-1 generation. Likewise, addition of 10^{-7} M big-endothelin-3 caused an increase of endothelin-3 in the incubation medium (Fig. 4). The conversion appeared to occur much less efficiently than with endothelin-1, since only 0.5 nM of endothelin-3 were detected in the medium after 3 h of incubation. However, all generation of endothelin-3 was abolished by addition of 10^{-4} M phosphoramidon.

4. Discussion

Using several models, others investigators have shown that the pharmacological properties of big-endothelins are highly dependent on the presence of endothelin-converting enzyme and their accessibility to this enzyme (Télémaque et al., 1992; Kashiwabara et al., 1989; D'Orléans-Juste et al., 1991). Endothelins elicit a variety of responses in astrocytes, but the responsiveness of these cells to big-endothelins had not been tested to date. Since astrocytes produce endothelin-1 and endothelin-3 (MacCumber et al., 1991; Ehrenreich et al., 1991,1993), it is logical to assume that they would contain endothelin-converting enzyme activity. By investigating the pharmacological properties of big-endothelins on astrocytes, we investigated whether exogenous big-endothelins had access to endothelin-converting enzyme in these cells.

Others had reported that endothelin-1 and endothelin-3 induced the release of arachidonic acid from

mouse astrocyte cultures (Tencé et al., 1992). We report here that primary cultures of rat astrocytes show similar responses, and we used this system to test the bioactivity of big-endothelins on astrocytes. In contrast to mature endothelin-1 or endothelin-3, big-endothelin-1 was capable of inducing the release of arachidonic acid only when longer incubation times were used, suggesting that a conversion step might be required. After 45 min of incubation, the response obtained with 10^{-7} M big-endothelin-1 was almost as effective as that obtained with endothelin-1 or endothelin-3. Big-endothelin-3 was less effective than all three other peptides, suggesting that if conversion did occur, it was less efficient than the conversion of big-endothelin-1.

By measuring the abundance of endothelin-1 or -3 in the incubation medium of astrocytes after addition of big-endothelins, we tested directly whether big-endothelins were actually converted into mature endothelins. Conversion of big-endothelin-1 occurred in a time-dependent fashion, and the amounts of endothelin-1 generated were similar to what has been previously obtained with 30 μg of rat lung microsomal proteins, one of the richest known sources of endothelin-converting enzyme (Takahashi et al., 1993). In most cells containing endothelin-converting enzyme, at least part of the activity is present at the level of the plasma membrane. This is in agreement with the recent cloning and sequencing of the complementary DNA of endothelin-converting enzyme, demonstrating that it is an ectopeptidase presenting an anchoring hydrophobic sequence similar to that of other metalloproteases (Shimada et al., 1994; Xu et al., 1994). Likewise, the conversion of exogenous big-endothelin by live intact astrocytes indicates that these cells contain a membrane-bound form of endothelin-converting enzyme with an external active domain. In addition, 100% of the activity was inhibited by 10^{-4} M phosphoramidon, which is in agreement with other reports about the metalloprotease nature of the enzyme (Opgenorth et al., 1992; Télémaque et al., 1992; Okada et al., 1990; Ahn et al., 1992; Ikegawa et al., 1991; Takahashi et al., 1993; Ohnaka et al., 1993). Cultured astrocytes may therefore constitute a clean source for at least one form of brain endothelin-converting enzyme, since contamination with non-specific proteases appeared to be less of a problem than in rat brain homogenates (Warner et al., 1992a).

Results in the literature concerning the conversion of big-endothelin-3 are variable. Endothelin-converting enzyme has been found to exhibit selective substrate specificity for big-endothelin-1 in most systems, but conversion of big-endothelin-3 has been shown to occur in some circumstances (Tsukahara et al., 1993; Matsumara et al., 1992). Recently, two groups have succeeded in purifying endothelin-converting enzyme from different sources (Takahashi et al., 1993; Ohnaka et al., 1993). Purified endothelin-converting enzyme from porcine aortic endothelium was totally inactive on big-endothelin-3 (Ohnaka et al., 1993). In contrast, big-endothelin-3 was converted by purified endothelin-converting enzyme from rat lung, although not as efficiently as big-endothelin-1 (Takahashi et al., 1993). Recent results using recombinant endothelin-converting enzyme have yielded divergent results as well. Thus, COS cells transfected with rat endothelin-converting enzyme complementary DNA convert big-endothelin-1, but not big-endothelin-3 (Shimada et al., 1994). In contrast, CHO cells transfected with bovine endothelin-converting enzyme complementary DNA do convert big-endothelin-3, although not as efficiently as big-endothelin-1 (Xu et al., 1994). With our astrocyte cultures, only small amounts of endothelin-3 were generated when big-endothelin-3 was added to the incubation medium. However, this appeared to represent genuine conversion since all generation of endothelin-3 was blocked by 10^{-4} M phosphoramidon. This finding is also in agreement with the relative potencies of big-endothelin-1 and -3 on arachidonic acid release.

The recent clonig of endothelin-converting enzyme from rat and bovine endothelial cells made it possible to investigate the tissue-distibution of endothelin-converting enzyme mRNA by Nortern blot analysis (Shimada et al., 1994; Xu et al., 1994). However, only very little endothelin-converting enzyme mRNA was found in either rat or bovine brains. Two possibilities may account for this observation: (1) the brain form may be somewhat different from that found in vascular cells; and (2) endothelin-converting enzyme mRNA may be found only in certain brain cells. If so, cultured astrocytes may well represent a way to obtain an enriched population of cells expressing endothelin-converting enzyme.

A relatively recent finding in biology is that astrocytes have the capability of producing peptide precursors, including angiotensinogen, proenkephalin and prosomatostatin (Intebi et al., 1990; Shinoda et al., 1989; Melner et al., 1990). The significance of these properties is not always clear, since in many cases the precursors are secreted in their unprocessed form. In contrast, astrocytes have been shown to produce endothelins in their mature forms (MacCumber et al., 1991; Ehrenreich et al., 1991). This may be a consequence of the accessibility of endothelin-converting enzyme by virtue of its localization on the external side of the plasma membrane. As a corollary, it may provide astrocytes with the means to produce endothelins in the brain in a time- and endothelin-converting enzyme-dependent manner rather than in a pulsatile fashion. However, our findings do not exclude the possibility that endothelins may be processed intracellularly in astrocytes as well.

Acknowledgements

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