RESEARCH COMMUNICATION

Novel activity of endothelin-converting enzyme: hydrolysis of bradykinin

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Endothelin-converting enzyme (ECE) is the key enzyme in the production of the potent vasoconstrictor endothelin from its inactive precursor big endothelin. To date, no other physiological peptide substrate has been identified for ECE. Here, by using Chinese hamster ovary (CHO) cells transfected with rat ECE-1 cDNA, we have established that ECE can hydrolyse the vasodilator bradykinin. The hydrolysis of bradykinin by ECE is exclusively at the Pro^7–Phe^8 bond, producing bradykinin-(1–7) and bradykinin-(8–9). Hydrolysis is completely inhibited by 100 μM phosphoramidon and 200 μM EDTA, but only slightly by the specific neprilysin inhibitor thiorphan (100 μM). The ability of ECE to act as a peptidyl dipeptidase rather than an endopeptidase in hydrolysing bradykinin suggests a much broader specificity for the enzyme than previously recognized, which may lead to the design of new and specific inhibitors of ECE and to the identification of other potential physiological substrates.

INTRODUCTION

Endothelin-1 (ET-1) is a 21-amino-acid peptide originally identified as the most potent vasoconstrictor peptide produced by endothelial cells [1]. Three separate endothelin genes have been identified encoding three distinct endothelin peptides (ET-1, -2 and -3) [2]. Elevation of endothelin levels has been implicated in the pathogenesis of several diseases, including atherosclerosis [3], heart failure [4], fibrotic diseases [5] and pulmonary hypertension [6]. ET-1 is initially synthesized as a large precursor polypeptide (proendothelin-1), the processing of which occurs in two steps: firstly, cleavage in the constitutive secretory pathway by furin [7], producing the biologically inactive big ET-1 (38 or 39 amino acids, depending on species), followed by selective cleavage at the Trp^{21}–Val^{22} bond by endothelin-converting enzyme (ECE) to generate ET-1 and its C-terminal fragment (see [8] for a review). Two ECE genes have been cloned (*ECE-1* and *ECE-2*): both of the proteins show sequence similarity to neutral endopeptidase-24.11 (NEP; neprilysin, EC. 3.4.24.11) and with KELL and PEX proteins [9]. All contain the zinc-binding motif HExxH and are members of a subfamily of the M13 gluzincin peptidase family.

No other peptide substrate has yet been identified for ECE, except for some truncated sequences of big ET itself [10]. In the present study, we have examined various vasoregulatory peptides as possible ECE substrates, including ET-1, atrial natriuretic peptide (ANP), angiotensins I and II, and bradykinin (BK). Using Chinese hamster ovary (CHO) cells transfected with rat ECE-1 cDNA, we have shown that ECE-1 can hydrolyse BK with cleavage at the Pro^7–Phe^8 bond. ECE does not hydrolyse ET-1, consistent with previous observations [11]. No significant hydrolysis of ANP and angiotensins I and II, over background levels, was detected.

EXPERIMENTAL

Materials

CHO cells were from the European Collection of Cell Culture (Salisbury, Wiltshire, U.K.) Trypsin/EDTA, penicillin, streptomycin, non-essential amino acids, foetal-calf serum and transfection reagent (lipofectAmine) were purchased from Gibco–BRL (Paisley, U.K.). Phosphoramidon was obtained from the Peptide Institute (Osaka, Japan). The thimet oligopeptidase inhibitor *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]Ala-Ala-Phe-4-aminobenzoate (Cpp-AAF-4ab) was donated by Dr. A. I. Smith (Baker Medical Research Institute, Melbourne, Australia). The plasmid pcDL-SRα296/rECE (containing rat ECE-1 cDNA) and monoclonal antibody AEC27-121 were gifts from Dr. K. Tanzawa (Sankyo, Tokyo, Japan) [12]. The peptides big ET-1, ET-1 and the C-terminal fragment of big ET-1 were obtained from Cambridge Research Biochemicals (Northwich, Cheshire, U.K.). The synthetic peptide substrate for ECE, [Phe^{22}]big ET-1-(18–34) [13], was synthesized by the Multiple Sclerosis Peptide Laboratory (Oxford Brookes University, Oxford, U.K.). The enhanced chemiluminescence (ECL) detection kit was from Amersham International (Amersham, Bucks., U.K.). BK, BK-(1–7),ANP, angiotensin I, angiotensin II, Glasgow minimal essential medium, nucleosides, sodium pyruvate, phenylalanine and the neprilysin inhibitor thiorphan were purchased from Sigma (Poole, Dorset, U.K.). Phe-Arg was synthesized by Dr. J. Keen (University of Leeds, Leeds, U.K.). All other reagents were of analytical grade.

Cell growth and expression

CHO cells were cultured in Glasgow minimal essential medium, supplemented with 10% (v/v) foetal-calf serum (heat inactivated), 2 mM nucleosides, 10 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine/asparagine at 37 °C in 5% CO_2. The expression vector pcDL-SR296/rECE was used to transfect CHO cells. For transient expression, CHO cells were plated in 150 cm^2 flasks at approx. 2×10^6 cells. After 24 h growth, the cells were washed twice with OptI Mem and transfected (5 μg of DNA/flask) by using lipofectAmine as cationic lipid (DNA/lipid, 1:10, w/w). The cells were incubated for 3 h before adding Glasgow minimal essential medium containing 10% (v/v) foetal calf serum. At a time of

Abbreviations used: ACE, angiotensin-converting enzyme; ANP, atrial natriuretic peptide; BK, bradykinin; CHO, Chinese hamster ovary; Cpp-AAF-4ab, *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]Ala-Ala-Phe-4-aminobenzoate; ECE, endothelin-converting enzyme; ECL, enhanced chemiluminescence; ET, endothelin; NEP, neutral endopeptidase-24.11 (neprilysin).

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24 h after transfection, the medium was aspirated and the cells were replaced with fresh medium. After a further 24 h, the cells were harvested and membranes were prepared as previously described [14]. Isolated membranes were solubilized with 1 % (w/v) Triton X-100, then subjected to Western blotting or enzyme assay. Protein concentrations were determined using the bicinchoninic acid method [15].

SDS/PAGE and immunoblotting

PAGE was performed as described in [16], using a 5 % stacking and a 7.5 % separating gel. The gels were blotted [17] using a semi-dry blot, and the detection of protein was performed by using ECL (ECL kit, Amersham). Pre-stained molecular-mass standards were run in parallel.

Hydrolysis of vasoregulatory peptides by ECE-1

For the detection of peptide hydrolysis by rat ECE-1, solubilized transfected and non-transfected CHO cell membranes (10 μg of total protein) were preincubated in a total volume of 100 μl for 30 min in 100 mM Tris/HCl buffer, pH 7.0, containing Cpp-AAF-4ab to inhibit low levels of endogenous thimet oligopeptidase activity. Appropriate reaction mixtures contained 100 μM phosphoramidon to check the specificity of hydrolysis. The reaction was started by adding BK to 250 μM final concentration. After incubation for 30 min at 37 °C, samples were heated to 100 °C for 4 min and then centrifuged at 2000 ***g*** for 20 min at 4 °C. A similar procedure was employed for investigating the possible hydrolysis of angiotensin I, II and ANP. For the determination of K_m and V_{max}, the BK concentration was in the range 0.1–2.0 mM, and for determining the I_{50} of various inhibitors, the BK concentration was 0.25 mM. Reaction conditions were maintained such that hydrolysis was linear with respect to time and protein concentration. The assay of ECE activity when using big ET-1 or [Phe^{22}]big ET-1-(18–34) as substrate, and their product quantification, was as described in [13].

HPLC quantification of BK, BK-(1–7) and BK-(8–9)

BK, BK-(1–7) and BK-(8–9) (Phe-Arg) were separated on a reverse-phase μBondapak C_{18} column, using trifluoroacetic acid/acetonitrile/water as the mobile phase. All the separations were carried out at room temperature at a flow rate of 1.5 ml/min. Solvent A consisted of 0.02 % (v/v) trifluoroacetic acid in water, and solvent B consisted of 0.02 % (v/v) trifluoroacetic acid and 45 % (v/v) acetonitrile in water. Both solvents were filtered and degassed before use. BK, BK-(1–7) and BK-(8–9) were resolved and quantified by HPLC using a linear gradient of acetonitrile from 9 to 91 % in 0.02 % (v/v) trifluoroacetic acid, pH 2.5, for 20 min, followed by a 5 min wash at final conditions. Product detection was at 214 nm. The BK-(1–7) was used for kinetic quantification and was calibrated against an authentic standard. Similar procedures were used for the separation of ANP, angiotensins I and II, ET-1 and their products.

RESULTS AND DISCUSSION

Hydrolysis of BK by ECE

Immunoblotting of a membrane preparation (Figure 1) from ECE-1-transfected (lane 1) and mock-transfected (lane 2) CHO cells indicated that ECE-1 protein is only detected in the ECE-1-transfected cells. Incubation of ECE-1-transfected membranes with 250 μM BK for 30 min, followed by HPLC separation, revealed extensive hydrolysis of the peptide (Figure 2b). The primary attack on this peptide was at the Pro^{7}–Phe^{8} bond,

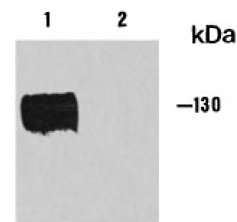

Figure 1 Immunoblotting of rat ECE-1 expressed in CHO cells

For details, see the Experimental section. The samples (8 μg of protein) were subjected to SDS/PAGE (7.5% acrylamide) and analysed by immunoblotting using monoclonal antibody AEC27-121. The lanes are as follows: 1, ECE-1-transfected cells; 2, non-transfected cells.

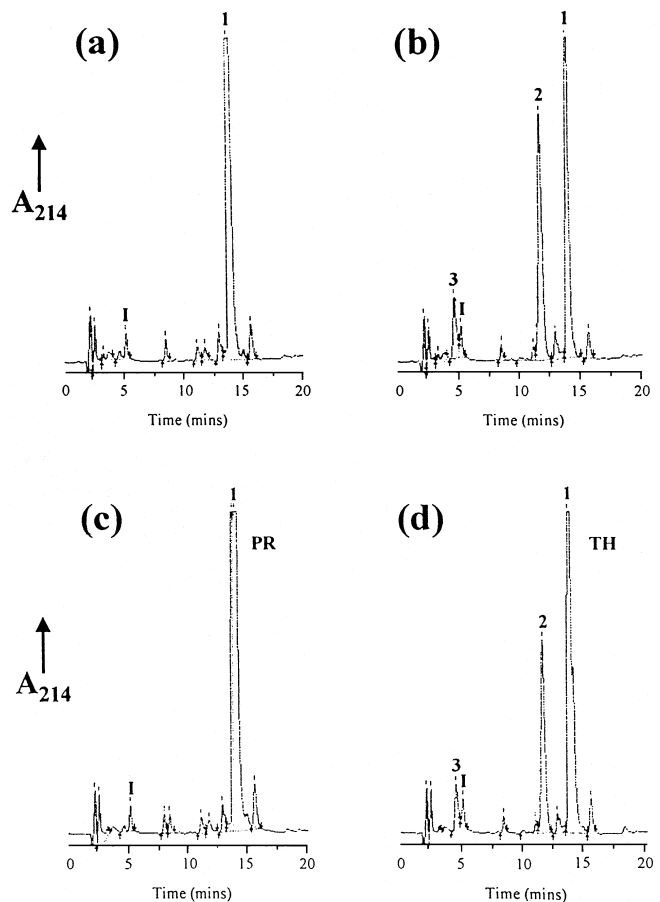

Figure 2 HPLC separation of BK and its hydrolysis products, BK-(1–7) and BK-(8–9), after incubation with membranes from ECE-1-transfected CHO cells

Solubilized CHO cell membranes (10 μg of total protein) were incubated with BK (250 μM) at 37 °C for 30 min. Samples were then processed, and substrates and products were separated by HPLC, as described in the Experimental section. Peaks eluted between 2 and 20 min, with their retention times in parentheses, as follows: 1, BK (14.4 min); 2, BK-(1–7) (12.2 min); 3, BK–(8–9) (4.8 min); I, Cpp-AAF-4ab (5.0 min); PR, phosphoramidon (14.6 min); TH, thiorphan (14.8 min). The four traces represent: (**a**) non-transfected CHO cells; (**b**) ECE-1-transfected CHO cells; (**c**) as (**b**) plus 100 μM phosphoramidon; (**d**) as (**b**) plus 100 μM thiorphan.

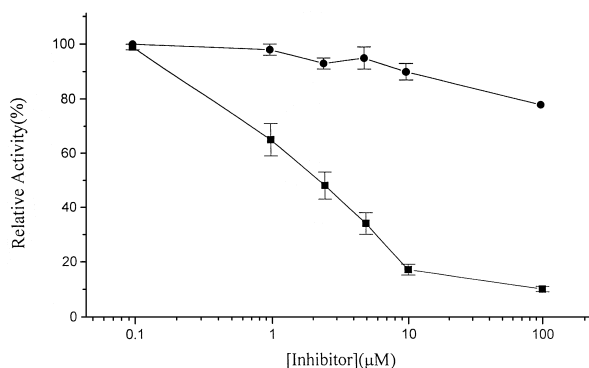

Figure 3 Inhibition by phosphoramidon and thiorphan of BK hydrolysis by ECE-1

For details, see the Experimental section. ECE-1-transfected CHO cell membranes (10 μg of protein) were incubated with BK at a concentration of 250 μM for 1 h in the presence or absence of inhibitor (concentration range of 0.1–100 μM). (■), phosphoramidon; (●), thiorphan. The S.D. is for four independent transfections, each performed in duplicate.

producing BK-(1–7) and BK-(8–9). The products were identified by co-elution with synthetic peptide, MS and amino acid composition (results not shown). No BK hydrolysis was detected in membranes from mock-transfected cells (Figure 2a). The hydrolysis of BK was almost completely inhibited by 100 μM phosphoramidon (Figure 2c) but only slightly by 100 μM thiorphan (Figure 2d). Using BK as substrate, phosphoramidon exhibits an I_{50} of 2.5 μM and thiorphan of > 200 μM (Figure 3), consistent with previous studies of ECE using big ET-1 as substrate [12]. Similar inhibition curves are observed when using big ET-1 or the big ET-1 analogue, [Phe^{22}]big ET-1-(18–34), as substrate (results not shown). Thus ECE hydrolyses BK at the Pro^7–Phe^8 bond, and therefore acts as a peptidyl dipeptidase in addition to its endopeptidase action on big ET-1. No hydrolysis of ET-1 was observed with ECE-1-transfected membranes on prolonged incubation up to 16 h, which is consistent with previous observations [11]. No significant hydrolysis of ANP or angiotensin I or II over background levels was detected (results not shown),

Hydrolysis of BK-(8–9) (Phe-Arg) by CHO cells

The BK-(8–9) peak (Figure 2b, peak 3) was also found to contain some free Phe, due to further hydrolysis of the Phe-Arg. This was not due to ECE activity, since it was not inhibitable by 100 μM phosphoramidon. Non-transfected CHO cells hydrolysed 100 μM Phe-Arg to the same extent as transfected cells, indicating an endogenous Phe-Arg-hydrolysing activity. This activity was inhibited by 100 μM EDTA but not by amastatin, bestatin, cilastatin or guanidinoethylmercaptosuccinic acid (inhibitors, respectively, of aminopeptidases, membrane dipeptidase and carboxypeptidase B).

Kinetic analysis of peptide hydrolysis by ECE-1

Table 1 shows kinetic comparisons of ECE, using three different substrates. The K_m for BK approaches 1 mM, several orders of magnitude higher than for circulating concentrations of the peptide. However, the V_{max}/K_m values for big ET-1 and BK are similar. The big ET-1 analogue {[Phe^{22}]big ET-1-(18–34)} is hydrolysed at approximately a 5-fold greater efficiency than the natural substrate, consistent with previous studies [10]. The I_{50} values for inhibition of ECE hydrolysis by phosphoramidon and thiorphan are broadly similar for all three substrates.

Previous studies on ECE have revealed some distinctive features of its specificity that contrast markedly with the very broad specificity of its homologue, NEP (see [9] for review). These include mutagenesis and expression of preproendothelin in heterologous cell lines [18], study of truncated sequences of big ET-1 [10], chemical modification [11] and molecular modelling of big ET-1 [19]. Thus conversion of big ET substrate is limited to the production of ET, and no further degradation occurs. There are even marked differences in the ability of ECE to process the three different big ET isoforms, the velocity of hydrolysis being: big ET-1 $>$ big ET-2 $\gg$ big ET-3. The secondary structure of big ET appears to be important for determining the rate of hydrolysis [11], and the N-terminal disulphide-loop region of big ET-1 (amino acids 1–15) appears to hinder conversion, since truncated forms lacking this loop region exhibit higher specific activities. For example, big ET-1-(16–37) shows approx. 3-fold greater rate of hydrolysis than big ET-1 itself [10]. The sequence of Ile^{19}-Ile^{20}-Trp^{21} on the amino side of the scissile bond (Trp^{21}–Val^{22}) is the minimum requirement for efficient cleavage of a truncated big ET analogue. Replacement of the valine residue in the P_1' position by phenylalanine enhances cleavage by an order of magnitude [10]. Based on these studies is our use of the analogue [Phe^{22}]big ET-1-(18–34) as a substrate for rapid and sensitive HPLC assay of ECE [13]. The expression of mutant preproendothelins in *Xenopus* oocytes has also shown that there is no strict requirement for Trp-Val at the processing site [18]. C-terminal extension of the substrate sequence appears to be essential, and comparison of C-terminally truncated peptides [10] has shown that the C-terminal region at residues 32–37 of big ET-1 plays an important part in recognition of big ET-1 by ECE, since big ET-1-(1–31) is not cleaved. In the light of these studies, therefore, it is remarkable that ECE cleaves the unrelated BK at a significant rate, thereby acting as a peptidyl dipeptidase rather

Table 1 Comparison of ECE specificity, using BK, [Phe^{22}]big ET-1-(18–34) or big ET-1 as substrate

The results are for four independent transfections, each performed in duplicate, and are expressed as means ± S.E.M. Values indicating mg of protein refer to Triton X-100-solubilized membrane protein and not pure enzyme.

Substrate	Specific activity (nmol·min⁻¹·mg⁻¹)	V_{max} (nmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max}/K_m (min^{-1}·mg^{-1}·ml)	I_{50} (μM)	
					PR	TH
Bradykinin	21.2 ± 1.4	38.8 ± 1.5	978 ± 18	0.04 ± 0.002	2.5 ± 0.1	> 200
[Phe^{22}]big ET-1-(18–34)	17.2 ± 1.3	37.6 ± 1.2	203 ± 13	0.19 ± 0.01	3.5 ± 0.1	200 ± 9
Big ET-1	0.17 ± 0.02	0.30 ± 0.01	7.6 ± 0.2	0.04 ± 0.002	8.5 ± 0.3	> 200

than an endopeptidase. The bond cleaved in BK, Pro–Phe, also implies that ECE can act as a post-proline cleaving enzyme and emphasizes the importance of a large hydrophobic group in the P_1' position of the substrate.

Hydrolysis of the Pro^7–Phe^8 bond of BK by ECE and other membrane metallopeptidases

Inactivation of BK by cleavage of the Pro^7–Phe^8 bond has been identified for at least three mammalian ectopeptidases, angiotensin-converting enzyme (ACE), NEP and now ECE [20]. Thus this further reinforces the fact that ECE and NEP are highly similar in their active sites [21]. The main differences between these enzymes in their processing of BK lies in the affinity of BK for each enzyme (approx. K_m values: ACE, 0.4 μM; NEP, 90 μM; ECE, 970 μM) and the occurrence of secondary hydrolysis sites. ECE hydrolyses exclusively the Pro^7–Phe^8 bond, whereas ACE cleaves consecutively the Pro^7–Phe^8 bond and the Phe^5–Ser^6 bond. NEP hydrolyses either at the Gly^4–Phe^5 or the Pro^7–Phe^8 bond. Although ACE (EC 3.4.15.1) is the principal contributor to BK metabolism *in vivo* [22], it will be interesting to determine, by using specific ECE inhibitors *in vitro* or *in vivo*, whether ECE may contribute locally to BK metabolism at the surface of endothelial cells where it is predominantly located.

General conclusions

The present study has established that ECE can hydrolyse the vasodilator peptide BK, in addition to its presumed substrate, big ET. The lack of sequence similarity in these peptides and the different nature of the hydrolysis (peptidyl dipeptidase compared with endopeptidase action) suggests that ECE may have a far broader specificity than previously recognized and may have additional physiological roles. This may relate to the subcellular location of ECE, which is found both at the plasma membrane as an ectoenzyme and intracellularly in a vesicle population and in the *trans*-Golgi network [23]. Intracellular ECE may play the predominant role in processing of big ET through the constitutive secretory pathway, whereas cell-surface ECE may additionally contribute to the metabolism of distinct circulating peptides yet to be identified. This observation has important consequences in terms of the design and application of ECE inhibitors in therapy. The precise structural requirements for substrate binding and catalysis by ECE remain to be defined fully. A primary requirement would appear to be a large hydrophobic residue in the S_1' site but with rather greater flexibility at the S_1 site.

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