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COMMENTARY

Molecular Pharmacology of Endothelin Converting Enzymes

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ABSTRACT. A critical processing step in endothelin biosynthesis is the conversion of the intermediate "big endothelin" to its biologically active product catalysed by endothelin converting enzyme (ECE). In this commentary we discuss critically the cellular location, structure, and activity of the isoforms of ECE. The current evidence supporting a metallopeptidase ECE as the physiological regulator of endothelin production is described. Its sensitivity to inhibition by the fungal metabolite phosphoramidon and subsequent cloning of the enzyme indicate it to be a type II integral membrane protein homologous with neutral endopeptidase-24.11 (E-24.11), the major neuropeptide-degrading ectoenzyme in brain and other tissues. Unlike E-24.11, however, ECE exists as a disulphide-linked dimer of subunit M_r 120–130 kDa and is not inhibited by other E-24.11 inhibitors such as thiorphan. Alternative splicing produces two forms of ECE with distinct N-terminal tails. These isoforms of ECE-1 show similar specificity converting big endothelin-1 (ET-1) to ET-1 but big ET-2 and big ET-3 are converted much less efficiently. This suggests that additional forms of ECE remain to be isolated. Immunocytochemical studies indicate a predominant cell-surface location for ECE-1, like E-24.11. This is consistent with the conversion of exogenous big ET-1 when administered *in vivo* and the inhibition of this event by phosphoramidon. However, mature ET-1 can be detected in intracellular vesicles in endothelial cells, suggesting that some processing occurs in the constitutive secretory pathway. This may be mediated by ECE-2, a recently cloned member of the E-24.11/ECE family which has an acidic pH optimum. Selective inhibitors of ECE may have therapeutic applications in cardiovascular and renal medicine. BIOCHEM PHARMACOL 51;2:91–102, 1996.

KEY WORDS. endothelin; endothelin converting enzyme; metallopeptidase; endopeptidase-24.11; phosphoramidon; thiorphan; endothelium

A number of regulatory peptides are critical to the maintenance of vascular tone, including the angiotensins, bradykinin, and ANP†. Membrane-associated metallopeptidases play key roles in the post-secretory processing and metabolism of vasoactive peptides. Examples include ACE, E-24.11 (neutral endopeptidase), and several aminopeptidases. Inhibitors of ACE are important anti-hypertensive agents and E-24.11 inhibitors may well have a role to play in cardiovascular medicine [1, 2]. The endothelins comprise a new family of potent vasoconstrictor peptides that may regulate vascular tone and blood pressure [3, 4]. They have been implicated in the pathophysiology of cardiovascular as well as renal and respiratory diseases. Endothelin was originally identified in 1988 as a vasoconstrictor compound in the culture supernatant of porcine aortic endothelial cells and was shown to be a 21-amino acid peptide [5]. Subsequently, three distinct endothelin genes have been identified encoding three closely related peptides, ET-1, ET-2, and ET-3 [6].

Endothelins are synthesized constitutively by vascular endothelial or smooth muscle cells and appear to act as locally produced peptide hormones in a paracrine fashion [4]. They have been found in extravascular tissue including lung, pancreas and spleen, as well as in brain. ET-1 may play a role in vascular modelling and regulation of cell proliferation through its mitogenic activity, e.g. mediating cardiac growth and hypertrophy [7]. ET also acts on liver [8] where it can cause a sustained increase in portal pressure, increased glycogenolysis, and changes in liver O_2 consumption. Disruption of the ET-1 or ET-3 genes and mutations in the ET-B receptor gene have suggested an important role for the endothelin peptides as morphogens in a variety of developmental processes in humans and mice [9–12]. In particular, a mutation in the ET-B receptor gene has been implicated in Hirschsprung's disease, which is thought to be a developmental defect in innervation of the colon [12]. The biosynthesis of the endothelins involves a novel, perhaps unique, enzyme processing step catalysed by "ECE" [5]. Current evidence implicates one or more phosphoramidon-sensitive membrane-associated metallopeptidases in the production of the biologically active peptide [13]. Molecular cloning of metallopeptidase ECE has established it as a member of the E-24.11 family [14–17]. ECE inhibitors may complement the use of ET receptor antagonists in therapy and could be of benefit in a range of disorders, such as hypertension, congestive heart failure, and stroke, and of various dis-

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† *Abbreviations:* ACE, angiotensin converting enzyme; ANP, atrial natriuretic peptide; CTF, C-terminal fragment (of big endothelin-1); E-24.11, endopeptidase-24.11; ECE, endothelin converting enzyme; ET-1, ET-2, and ET-3, endothelin-1, -2, and -3, NEP, neutral endopeptidase; PR, phosphoramidon; and RIA, radioimmunoassay.

eases of the kidney [18, 19]. Recently, the role of endothelin receptor antagonists was reviewed extensively [20], and the present commentary will focus on the biochemistry and molecular pharmacology of the ECEs.

BIOSYNTHESIS OF ENDOTHELINS

Like many biologically active peptides, the endothelins are initially synthesized as a much larger precursor, prepro-ET, of approximately 200 amino acid residues [5]. After removal of the signal peptide early in biosynthesis, the propeptide is cleaved at pairs of basic amino acids to generate the intermediate big ET whose vasoconstrictor activity is approximately two orders of magnitude less than that of ET itself. This processing step is probably carried out by furin [21], a prohormone convertase of the constitutive secretory pathway. The conversion of big ET-1 to ET-1 and its CTF occurs via a unique processing event that involves cleavage at the Trp^{21}-Val^{22} bond, catalysed by ECE [5]. These processing events are summarized in Fig. 1. The conversion of big ET-2 to ET-2 also involves cleavage of a Trp-Val bond, but in big ET-3 the scissile bond is Trp-Ile. There is evidence supporting both an intracellular location for ECE and a plasma membrane location where it could act as an ectoenzyme in a post-secretory processing role [15, 22–24]. There are precedents for such post-secretory actions in hormone processing, e.g. in angiotensin, ANP, and glucagon [25] processing as well as in the processing of members of the pancreatic polypeptide family [26, 27]. The cell biology of ET processing, however, remains a controversial area but is critical to understanding how endothelin levels may be regulated. It is likely that isoforms of ECE exist with distinct specificities for big ET-1, -2, and -3.

NATURE OF THE ENDOTHELIN CONVERTING ENZYME

Since the discovery of ET in 1988, a variety of enzyme activities has been proposed as the physiological ECE, including serine, thiol, metallo or aspartic proteinases. The primary specificity of ECE was assumed initially to be the tryptophanyl residue in the P_1 position, implicating a chymotrypsin-like cleavage in the processing [5]. Chymotrypsin itself, however, will only cleave the Trp^{21}-Val^{22} bond following cleavage of the Tyr^{31}-Gly^{32} bond of big ET-1 [28]. Furthermore, chymotrypsin readily hydrolyses mature ET [28]. Wypij *et al.* [29] have identified a chymostatin-sensitive serine protease in rat lung able to process extracellular big ET-1 to ET-1. This activity was identified as mast cell chymase I, and the induced degranulation of mast cells was shown to increase significantly the processing of big ET-1 in a perfused rat lung preparation. There is no evidence to date that this activity might be responsible for normal big ET processing.

For a period, aspartic proteases were considered as possible ECE species. Initially, pepsin was shown to convert big ET to a product with potent constricting activity on isolated rat

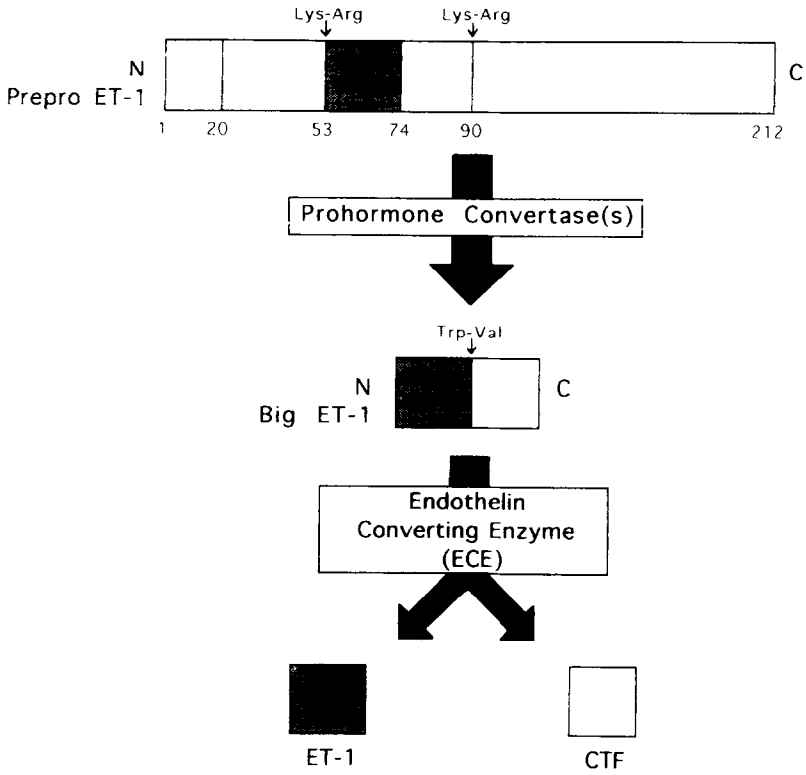

FIG. 1. Outline of the steps involved in the processing of the human preproendothelin-1 precursor to mature endothelin-1 and its C-terminal fragment. Note that the intermediate step involving the hydrolysis of "big ET" to ET catalysed by ECE is unique to this pathway.

aorta, and HPLC separation of the products confirmed that the correct cleavage had taken place [30]. Subsequently, Matsumura and colleagues [31] reported the detection of a converting activity with the characteristics of an aspartic protease in cultured porcine aortic endothelial cells. The activity was optimal at pH 4 and completely inhibited by pepstatin. A series of reports then followed describing both membrane and cytosolic aspartic protease converting activities in different cells and tissues [32–36]. These included a cathepsin D-like enzyme in bovine adrenal medulla and cultured bovine aortic endothelial cells [33, 34]. Cathepsin D itself was shown to cleave big ET at the critical Trp^{21}-Val^{22} bond but also hydrolysed the Asp^{18}-Ile^{19} bond, resulting in a truncated, inactive form of ET [37–39]. In contrast to cathepsin D, cathepsin E was reported to cleave big ET exclusively at the relevant bond to generate ET-1 and its C-terminal fragment [39]. In a subsequent, detailed kinetic study, cathepsin E was shown to bind all three endothelin precursors efficiently and cleave them to generate the endothelin products ET-1, ET-2, and ET-3 [40]. The K_m values for big ET-1, big ET-2, and big ET-3 were comparable (8–20 μM) and k_{cat} values were virtually identical (0.10–0.14 sec^{-1}), although the pH optimum for conversion of big ET-3 was somewhat lower. However, the use of cathepsin E inhibitors *in vivo* failed to provide confirmatory evidence for a physiological role for this enzyme in ET production [41]. Furthermore, incubation of vascular endothelial cells with pepstatin neither altered the ratio of ET-1 to big ET-1 nor the secretion of either peptide [42]. A further problem with a role for aspartic proteases in the processing pathway is the localization of the cathepsins in the acidic milieu of lysosomes, which is not consistent with the processing of the ET precursor through the constitutive secretory pathway. A thiol protease has also been reported to convert big ET to ET in primary aortic endothelial cells, but this activity was cytosolic in location, again inconsistent with an involvement in the secretory pathway [43]. Others have also identified a thiol protease mediating the conversion [44]. The plethora of activities that have been described in endothelial and other cells capable of catalysing big ET conversion illustrates the problem inherent in identifying a physiologically relevant converting enzyme. The genuine ECE must be located in the correct cellular compartments with the appropriate topology and specificity to mediate ET production exclusively and efficiently. A careful evaluation of the cell biology of ET processing could have avoided the proliferation of reports of irrelevant activities. The subsequent discussion in this commentary will deal exclusively with metalloprotease forms of ECE.

A PHYSIOLOGICALLY RELEVANT METALLOPROTEASE ECE

The key to identification of a metalloprotease ECE was the use of the compound PR [(*N*-α-rhamnopyranosyl-oxyhydroxyphosphinyl)-Leu-Trp], originally identified as a potent inhibitor of the bacterial zinc protease thermolysin [45] and subsequently as an equally potent (K_i = 2 nM) and specific inhibitor

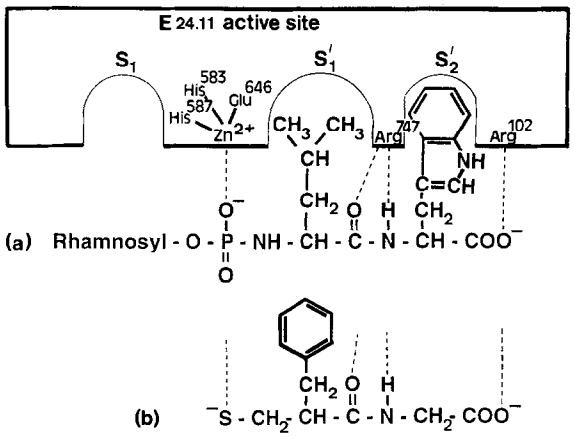

FIG. 2. Postulated mode of binding of the inhibitors phosphoramidon (a), and thiorphan (b), to the active site of endopeptidase-24.11. Active site residues identified by site-directed mutagenesis are indicated.

of the mammalian plasma membrane zinc peptidase, E-24.11 [46]. Note, however, that PR is not a general zinc-peptidase inhibitor, having no effect, for example, on the matrix metalloproteinases or on ACE. The postulated mode of binding of PR to E-24.11 is shown in Fig. 2, together with critical residues in the active site of the enzyme that have been identified by site-directed mutagenesis [1, 2]. A number of other potent inhibitors of E-24.11 have been synthesized [see, for example, Refs. 1 and 2] of which the best described is thiorphan (2-benzyl-3-mercaptopropionylglycine), also shown in Fig. 2 [47]. Numerous studies have now shown that PR at micromolar concentrations is able to inhibit exogenous big ET conversion *in vivo* and also *in vitro* in cultured cells. Thus, PR has been shown to inhibit the release of ET-1 from endothelial cells and to alter the ET-1:big ET-1 ratio in medium and intracellularly in a manner consistent with inhibition of conversion of big ET to ET [42, 48–51]. Administration of PR *in vivo* has also been shown to inhibit big ET conversion and the big ET-1 pressor response, but not the pressor response obtained with ET-1 [41, 52–54]. The majority of studies have failed to show an effect of other E-24.11 inhibitors (particularly thiorphan) on big ET conversion *in vivo* or *in vitro* [see, for example, Refs. 41 and 55]. However, McMahon *et al.* [53] have reported inhibition of the big ET pressor response by thiorphan, although PR was substantially more potent. The reported differences in thiorphan effects *in vivo* may reflect dosage differences since partially purified porcine lung ECE is inhibited by thiorphan with an I_{50} of 200 μM (Murphy LJ and Turner AJ, unpublished observations), whereas the K_i for E-24.11 is 2.3 nM [1]. All these observations have raised the possibility that ECE may be structurally and catalytically similar to E-24.11 but with diminished sensitivities to PR and thiorphan. Since PR is a metabolically stable, phosphorylated sugar derivative and unlikely to enter cells at an appreciable rate, it also raised the possibility that the conversion of big ET to ET may take place, at least in part, at the extracellular face of the plasma membrane, analogous to the production of the vasoconstrictor angiotensin II from angiotensin I [13].

E-24.11 AS A MODEL FOR ECE

The renal and intestinal brush border membranes have proved valuable model systems for the isolation and characterization of mammalian metallopeptidases [see, for example, Ref. 56]. The only endopeptidase in the renal brush border of pig and humans is the metallopeptidase E-24.11 (EC 3.4.24.11), which, like ACE, is a membrane glycoprotein existing as an ectoenzyme [56]. It therefore has the correct topology to process or metabolize released or circulating peptides. E-24.11 has a broad substrate specificity inactivating, among others, enkephalins (hence, its alternative nomenclature "enkephalinase"), tachykinins (e.g. substance P), cholecystokinin and atrial natriuretic peptides [2, 57, 58]. The enzyme is also often referred to as NEP, and its current approved enzyme nomenclature is "neprilysin." E-24.11 hydrolyses bonds on the amino side of hydrophobic residues, e.g. the Gly^3-Phe^4 bond of enkephalins [57, 58]. Therefore, it would be a candidate for cleavage of the Trp^{21}-Val^{22} bond of big ET at the extracellular face of the plasma membrane. The PR sensitivity of ET conversion both *in vivo* and *in vitro* therefore suggests a similarity with mammalian E-24.11 and bacterial thermolysin.

IS E-24.11 AN ENDOTHELIN CONVERTING ENZYME?

A number of reports have shown that E-24.11 degrades ET-1 efficiently [for example, Refs. 59 and 60], and this has tended to discount E-24.11 as a physiological ECE activity generating ET-1 from big ET. Indeed, a recent report [61] purported to show that recombinant E-24.11 was unable to generate ET-1 from big ET. Our own studies refute that conclusion and show that immunoreactive ET-1 and the C-terminal fragment are generated from big ET by purified E-24.11 [62]. Thermolysin converts big ET to ET-1 even more efficiently and indeed may be a more useful model for ECE action than is E-24.11 [62], particularly since crystal structures are available for thermolysin–inhibitor complexes [63, 64]. Although the ET-1 product from big ET-1 cleavage is degraded further by both thermolysin and E-24.11, significant steady-state levels of ET-1 remain detectable. This observation has important implications for much of the published literature on ECE. Any PR-sensitive "ECE" activity detected in membrane preparations could be attributable, at least in part, to E-24.11. Unless E-24.11 activity is selectively blocked by thiorphan (which does not inhibit ECE significantly at 10 μM), measurements of ET-1 generation will reflect the combined activities of several PR-sensitive enzymes. ECE can also be separated effectively from E-24.11 by immunoaffinity chromatography [65] or by isoelectric focusing [66].

MOLECULAR CHARACTERIZATION OF METALLOPROTEASE ECE ACTIVITIES

One of the first reports of an endothelin converting activity optimal at neutral pH was made by Ohnaka *et al.* [67], who identified such an enzyme in cultured bovine aortic endothelial cells. This activity was membrane-associated, inhibited by the metal chelators EDTA and EGTA, and insensitive to inhibitors of other classes of proteases. Okada *et al.* [68], however, first established the presence in endothelial cells of a membrane-bound PR-sensitive, thiorphan-insensitive metalloprotease, implicating an E-24.11-like activity that was distinct from E-24.11 itself. This activity was particularly sensitive to pH, with a sharp pH optimum around pH 7.0 [68]. Numerous other studies have subsequently reported similar findings of an endothelial phosphoramidon-sensitive metalloprotease ECE activity, both in the cytosol and in membrane fractions [see, for example, Refs. 50 and 69–71]. The significance of the cytosolic form and whether it is derived from the membrane form are still unknown. Evidence has also been presented for the presence of a membrane-bound phosphoramidon-sensitive ECE in smooth muscle cells [72], a conclusion previously reached in Ref. 73. Human polymorphonuclear leukocytes also appear to possess a PR-sensitive ECE [74]. It took a further 3 years before the appearance of the first reports of purification of ECE to homogeneity, a consequence of the relatively low specific activity of ECE in endothelial and other cell lines and in tissue preparations. Purification was also hindered by the lack of a suitably sensitive and rapid assay for the enzyme. Small fluorogenic peptides mimicking the region around the scissile bond are not hydrolysed by ECE, and assays have generally depended on the more time-consuming measurement of ET-1 production from big ET-1, monitoring this product by HPLC, RIA or enzyme immunoassay, or scintillation proximity assay. Initial purification of ECE to homogeneity was reported from rat lung [75] and from porcine aortic endothelium [76].

The rat enzyme was purified some 6300-fold from a lung "microsomal" preparation following solubilization with Triton X-100 [75]. The highly glycosylated nature of ECE was exploited in the use of lectin (wheat germ agglutinin) chromatography, followed by zinc-chelating Sepharose and Blue B-agarose. The purified protein was apparently homogeneous on reducing SDS–PAGE with an apparent M_r of 130,000, whereas E-24.11 migrates as a protein of M_r 90,000–100,000. Gel filtration of purified ECE on Superose 6 indicated a protein of M_r 170,000–190,000. ECE exhibits a number of properties consistent with it being an amphipathic integral transmembrane protein. In particular, the enzyme from porcine lung partitions predominantly into the detergent-phase after phase-separation in Triton X-114 (Murphy LJ and Turner AJ, unpublished observations), a characteristic typical of an integral membrane protein [77, 78]. It is not released from the membrane by phosphatidylinositol-specific phospholipase C, indicating that it is not a glycolipid-anchored membrane protein (Murphy LJ and Turner AJ, unpublished observations), unlike two other zinc membrane peptidases: aminopeptidase P and membrane dipeptidase [79, 80].

The purified, porcine aortic endothelium ECE exhibits characteristics similar to the rat lung enzyme, a purification factor of 12,000-fold being required to achieve homogeneity [76]. An apparent M_r of 120,000 was observed for the protein on reducing SDS–PAGE and 131,000 on sucrose density gra-

dient centrifugation. More recently, bovine adrenal cortex has been shown to be a relatively good source of ECE activity [15] from which it has been purified approximately 3000-fold as a glycoprotein of M_r 126,000 on reducing SDS–PAGE, by adopting a procedure similar to that described by Takahashi *et al.* [75].

Another useful source of ECE has derived from the use of transformed endothelial cell lines. The human cell line EA.hy926, is a hybrid resulting from fusion of primary human umbilical vein endothelial cells with a continuous human epithelial cell line (A549), originally derived from a human lung carcinoma [81]. EA.hy926 cells exhibit sustained expression of many differentiated functions of the epithelium, although they do not express ACE [82], unlike the parent endothelial cell line. The EA.hy926 line expresses ET-1 mRNA and has been shown to secrete big ET-1 and ET-1 into the culture medium, in a phosphoramidon-sensitive manner [83]. This cell line contains a metallopeptidase ECE that, on subcellular fractionation, appears to be associated predominantly with a plasma membrane fraction as an ectoenzyme [24, 66]. We have shown by phase-separation in Triton X-114 that ECE, in EA.hy926 cells, is an integral, transmembrane protein and can be co-immunoprecipitated from membranes by an antibody to aminopeptidase N, another integral membrane protein ([82]; Barnes K, Murphy LJ and Turner AJ, unpublished observations), again suggesting that a proportion of ECE is associated with the plasma membrane, but none of the above data excludes the possibility that some ECE activity is associated with vesicular or other intracellular membrane compartments. Intriguingly, the partially purified ECE from EA.hy926 cells could be renatured and assayed after non-reducing SDS–PAGE and was shown to migrate as a protein of M_r 280 kDa [24], consistent with a M_r of 250 kDa observed after gel filtration [24, 66]. In these cells, therefore, ECE is apparently twice the size of that reported in porcine aortic endothelium and lung. Schmidt *et al.* [17] have purified ECE from a membrane preparation of a permanent bovine endothelial cell line, FBHE. The cells were first incubated in the presence of 10^{-4} M PR for 48 hr before harvesting, a procedure that increased the specific activity of ECE 10-fold, hence considerably aiding purification. This represents the first demonstration that ECE may be an inducible enzyme. These and other authors have also established unequivocally by SDS–PAGE under reducing and non-reducing conditions that bovine endothelial ECE exists as a disulphide-linked dimer, each subunit being of M_r 120,000 [17, 84]. Covalent association of subunits is relatively uncommon among the membrane peptidase, although meprin and membrane dipeptidase are similarly organized [85–87]. It is interesting that E-24.11, when subjected to gel filtration, migrates as a dimer held together by non-covalent interactions between the globular domains [88] and a number of other brush border membrane hydrolases behave similarly [89]. Endothelial ACE, on the other hand, is monomeric but is internally duplicated with two active sites and hence can be thought of in functional terms as a pseudodimer [90]. Danielsen [91] has recently discussed the oligomeric assembly of plasma membrane glycoproteins.

MOLECULAR CLONING OF ECE

The purification of ECE by several groups in 1993 rapidly led to the molecular cloning and sequencing of the enzyme. Partial amino acid sequence data obtained for the rat lung enzyme were used to derive probes for screening a vascular endothelial cell cDNA library [14]. A similar strategy was used for cloning of the bovine ECE cDNA after purification of the enzyme from adrenal cortex [15]. Subsequently, the bovine aortic endothelial [16, 17] and human placental [17] ECE cDNAs have been obtained. All five predicted protein sequences are strikingly similar apart from some differences in the N-terminal region in two [15, 16] of the three bovine sequences that have been reported. Figure 3 indicates the predicted topology of the protein. Like E-24.11, ECE is a type II integral membrane protein with a short N-terminal cytoplasmic tail, a transmembrane hydrophobic domain that represents the uncleaved signal peptide, and a large putative extracellular domain containing the catalytic site and the motif HEXXH typical of many zinc peptidases, e.g. E-24.11 and ACE [10, 92–94]. ECE-1 is a highly glycosylated protein (approximately 33% carbohydrate) with 10 putative N-linked glycosylation sites and shows significant homologies, particularly in the C-terminal region, to both E-24.11 and the erythrocyte blood group antigen Kell [95, 96]. All three proteins also contain a cluster of four conserved cysteine residues in a 32 amino acid sequence immediately following the predicted transmembrane domain, a structural feature also seen in the brush border hydrolases, sucrase-isomaltase and γ-glutamyl transpeptidase [97, 98]. The N-terminal cytoplasmic domain of bovine ECE reported by Schmidt *et al.* [17] is highly similar (>92%) to the rat and human sequences [14, 17]. The lack of homology in the N-terminal 32 amino acids of the other two reported bovine ECE sequences [15, 16] might indicate an isoform of ECE but is attributed by Schmidt *et al.* [17] to the misinterpretation of incorporated intronic sequences, hence not reflecting the true amino acid sequence in this short region. However, it is unlikely that two independent groups [15, 16] have made identical cloning errors. Thus, it is likely that distinct subunits of ECE do exist, differing substantially only in their cytoplasmic domains, perhaps as a result of differential splicing. The "short" form (51–52 residues in the cytoplasmic domain) originally reported by Shimada *et al.* [14] and subsequently by Schmidt *et al.* [17] we designate the α subunit and the "longer" form (56 cytoplasmic residues) [15, 16] the β subunit. It is conceivable that both homo- and hetero-dimers of ECE (αα, αβ, ββ) may occur and differ in targeting, localization and some other properties. All forms of ECE show >95% homology in amino acid sequence in their putative extracellular domains.

In addition to the conserved HEXXH motif, some additional essential catalytic residues are conserved between E-24.11 and ECE. Site-directed mutagenesis has shown the presence in the E-24.11 active site of two residues, Arg^{102} and Arg^{747}, involved in substrate binding (Fig. 2) [99, 100]. Arg^{102} is conserved in ECE, but Arg^{747} is replaced in ECE by a glutamyl residue whose importance for substrate binding remains to be established. The third zinc ligand in E-24.11, Glu^{646} [101], is also conserved in ECE. The erythrocyte Kell protein

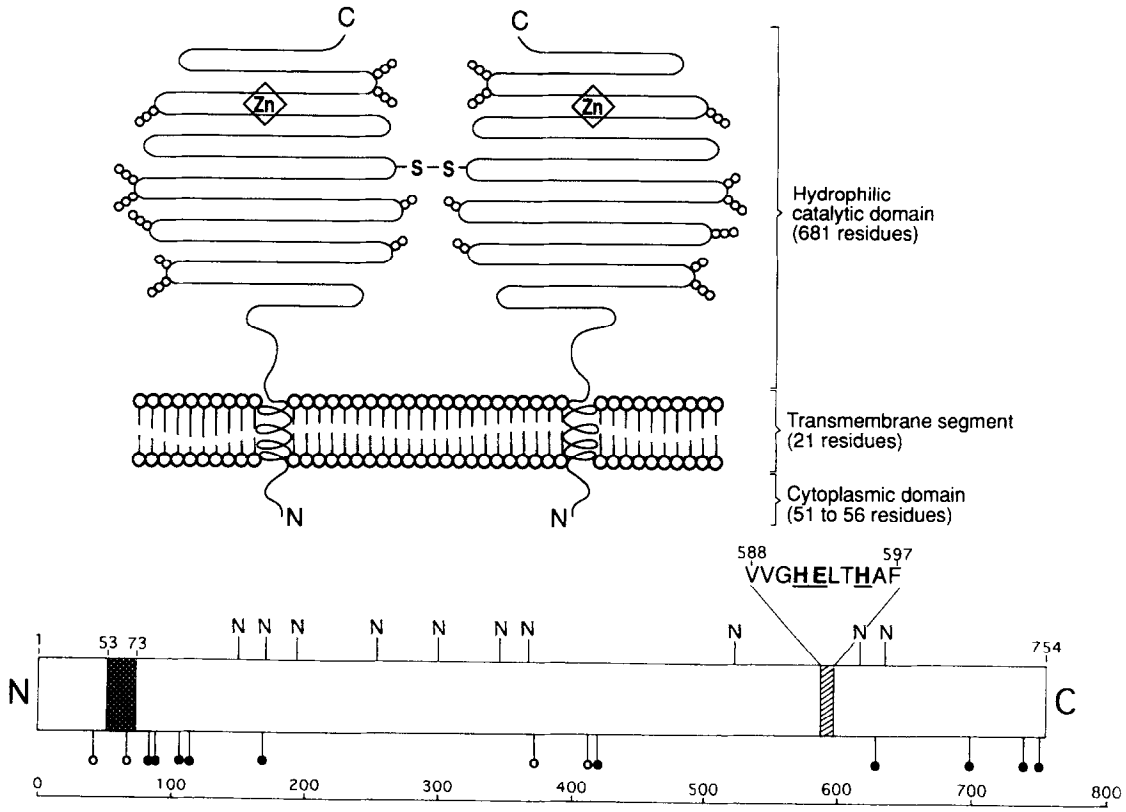

FIG. 3. Top panel; Proposed topology of ECE as a dimeric type II integral membrane protein based on the predicted protein sequence. The ten putative glycosylation sites in the protein are indicated in the extracellular domain. The dimeric structure of ECE is proposed in refs. 17 and 84; the precise location and number of disulphide bonds are unknown; Bottom panel; Domain structure of rat endothelial ECE indicating the presence of the amphipathic membrane-spanning domain (hatched box), the zinc motif (striped box), putative N-linked glycosylation sites (indicated by N), and cysteine residues (●, conserved between ECE and E-24.11; ○, non-conserved).

[95] retains zinc ligands equivalent to those in ECE and E-24.11, but Arg^{102} and Arg^{747} are not conserved. No peptidase or proteinase activity has yet been reported for Kell, and its functional significance on the surface of erythrocytes is unknown. E-24.11 is also expressed on some blood cells, constitutively on neutrophils [102] and at a high level on the lymphocyte cell surface in the majority of acute lymphoblastic leukaemias where it is often referred to as the "common acute lymphoblastic leukaemia antigen" (CALLA) or CD10 [103–105]. It is presumed to play a role in the differentiation or maturation of haemopoietic cells by hydrolysis of as yet unidentified immunoregulatory peptides.

Analysis of the tissue distribution of ECE mRNA by Northern blot analysis [14–17] reveals abundant expression of the protein in lung, pancreas, placenta, adrenal gland, ovary and testis. *In situ* hybridization analysis has confirmed the presence of ECE mRNA in vascular endothelial cells in various tissues including heart, lung, liver, brain, pancreas, kidney and adrenal [15]. Thus, ECE is relatively broadly distributed, consistent with the wide distribution of preproET-1 and in contrast with the more restricted distribution of E-24.11, which is highly abundant in the renal and intestinal brush borders and some cells of the immune system but found at very much lower levels in other tissues [see, for example, Ref. 106]. The high levels of ECE mRNA in lung and placenta are consistent with these being endothelium-rich tissues, but the abundance of ECE mRNA in pancreas [17] may reflect additional processing roles for the enzyme. It is worth commenting that ECE, E-24.11 as well as ACE are all expressed in cells of the male and female reproductive tracts, although their physiological functions at these locations are unknown. In the case of ECE, it may relate to the developmental roles of the endothelin peptides.

SUBSTRATE SPECIFICITY AND INHIBITION OF ECE

Studies on ECE from various cell lines and tissues reveal some distinctive features of its specificity. In all cases, the conversion of big endothelin substrate is limited to the production of endothelin, and no subsequent degradation occurs. No other biologically active peptides or their precursors have yet been identified as substrates for ECE. There are marked differences reported in the ability of purified, or cloned and expressed, ECE to process the three different big ET isoforms. The ECE activity partially purified from EA.hy926 cells was reported to process human big ET-1 much more efficiently than human big ET-2 (V_{max}/K_m = 0.09 relative to big ET-1); there was no appreciable activity detectable towards big ET-3 [24]. Most of

the cloned and expressed ECE proteins showed a similar specificity, converting big ET-2 poorly and failing to convert big ET-3 [14, 17]. However, bovine endothelial ECE cDNA described by Xu *et al.* [15], when expressed in CHO cells, processed all three big endothelin substrates, with the conversion of big ET-1 ≫ big ET-3 > big ET-2 [15]. This could reflect a genuine specificity difference. Nevertheless, most data now support the occurrence of isozymes of ECE, in particular a form with preference for the processing of big ET-3. Smooth muscle cells may also possess a distinct form of ECE. With the successful cloning of ECE, other members of the ECE family should now be identified rapidly. Indeed, in anticipation of such discoveries, Xu *et al.* [15] referred to their initial cloned ECE species as ECE-1, and this nomenclature has been adopted by others [17, 66].

Other key features of ECE specificity have emerged as a result of detailed studies on the purified enzymes. Some of these are highlighted in Fig. 4. The N-terminal disulphide-loop structure in big ET-1 [i.e. big ET-1 (1–15)] appears to hinder conversion since truncated forms have substantially higher specific activity, big ET-1 (16–37) showing about 3-fold greater activity [76]. A minimum sequence of IIW on the amino side of the scissile bond is all that is required for efficient cleavage [107], and replacement of the valyl residue in the P_1' position by phenylalanine enhances cleavage substantially [76]. The expression of mutant preproendothelins in *Xenopus* oocytes has also shown that there is no strict requirement for Trp-Val at the processing site [108]. C-terminal extension of the substrate sequence is essential, and comparison of C-terminally truncated peptides has shown that the C-terminal region at residues 32–37 appears to play an important part in the recognition of big ET-1 by endothelial ECE since big ET-1 (1–31) is not cleaved by ECE [15, 76, 107]. The peptide [Phe^{22}] big ET-1 (18–34) exhibits 12-fold higher specific activity than big ET-1 itself and may be a useful synthetic substrate for assay of ECE [76]. The key determinant of specificity between big ET-1, big ET-2 and big ET-3 appears to reside in residues 27–29, being, respectively, HVV (big ET-1), QTA (big ET-2) and QTV (big ET-3). The scissile bond in big ET-3 is W-I rather than W-V, and the C-terminal sequence in big ET-3 is completely different from big ET-1 and big ET-2, whereas there is moderate conservation in big ET-1 and big ET-2. The K_m of purified ECE for big ET-1 is reported to be in the range of 0.2 μM (rat lung [75]) to 23 μM (recombinant human enzyme [17]).

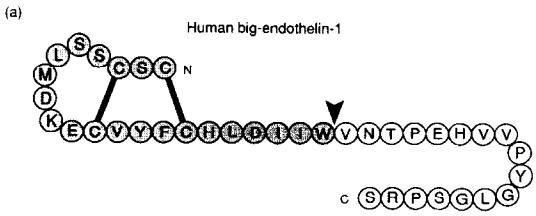

FIG. 4. (a) Sequence of human big ET-1; the peptide bond hydrolysed by ECE is indicated by the arrow. The shaded N-terminal residues correspond to ET-1. (b) Partial sequences of big ET-1, big ET-2, and big ET-3 critical to the specificity of hydrolysis by porcine aortic endothelial ECE. The synthetic peptide [Phe^{22}]big ET-1 (18–34) is hydrolysed approximately 12 times more efficiently than big ET-1 itself [74]. Note that the large N-terminal, disulphide-bonded region of ET-1 is not essential for efficient catalysis, but an extended C-terminal domain is critical. The one-letter code for amino acids is used; a = amide.

There are limited reports on requirements for efficient inhibition of ECE. PR is several orders of magnitude less potent on ECE than on E-24.11. A K_i of 3.5 μM for PR was estimated for the ECE from EA.hy926 cells [24]. I_{50} values for PR range from 0.35 μM (rat lung ECE [14]) to 0.8 μM (porcine aortic endothelial ECE [76]). Thiorphan is reported to inhibit ECE weakly (I_{50} > 100 μM) or not at all. Other potent E-24.11 inhibitors, including kelatorphan, SQ28603, SCH 32516, and SCH 39370, exhibit no significant inhibitory effects on porcine lung ECE at concentrations of up to 100 μM (Murphy LJ and Turner AJ, unpublished observations). Features that are important for selectivity of ECE over E-24.11 include the introduction of a P_1 unit (which is lacking in phosphoramidon and thiorphan), and the prototype compound, 3-(1-naphthyl)-1-phosphonopropyl-L-leucyl-L-tryptophan, represents one of the most potent ECE inhibitors reported in the literature (I_{50} = 0.26 μM) and shows 400-fold greater ECE/E-24.11 selectivity than does PR [109]. Because of the much wider substrate specificity of E-24.11 compared with ECE, it may prove difficult to eliminate E-24.11 inhibitory activity from any ECE inhibitor. However, a potent, dual inhibitor of ECE and E-24.11 that could simultaneously maintain ANP levels and inhibit ET-1 production may be beneficial. De Lombaert *et al.* [110] have reported recently a pharmacological profile of such a dual inhibitor (CGS 26303), which displays an I_{50} of 0.1 nM for E-24.11 and 1.1 μM for ECE.

CELL BIOLOGY OF ENDOTHELIN PROCESSING

The sequence similarities of ECE with E-24.11 and Kell imply that ECE possesses a similar topology and location and should exist, therefore, as an ectoenzyme converting big ET-1 to ET-1 at the extracellular face of the plasma membrane. Immunocytochemical studies of ECE-1 localization in endothelial cells support this conclusion [84]. The ability of PR to inhibit conversion of exogenously administered big ET-1 to ET-1 is also consistent with this interpretation [52–55, 111]. This latter study [111] also demonstrated that the removal of big ET-1 from the circulation is not dependent on its prior conversion to ET-1. Since ECE has also been identified on smooth muscle cells [72, 73], the conversion event could occur either immediately upon secretion of big ET-1 from endothelial cells or at the surface of smooth muscle cells in the vicinity of endothelin receptors. The observation that removal of the endothelium does not affect the vasoconstrictor activity of big ET-1 in the rat mesenteric artery, nor its inhibition by PR, provides some

support for the latter concept [112]. Endogenous big ET, however, may be processed by a different route from the exogenously provided precursor, and other data are supportive of intracellular conversion of big ET-1 to ET-1. Thus, the detailed cell biology of endothelin processing remains controversial. Processing of proendothelin to big ET presumably begins in the constitutive secretory pathway in endothelial cells, but little else is known with any certitude. Both big ET and fully processed ET appear to be present within the cell, and we have detected immunoreactive ET-1 in a vesicular fraction within bovine endothelial cells [113]. In an attempt to identify the location of big ET processing, Xu *et al.* [15] conducted a set of double-transfection studies. These authors concluded that a portion of expressed ECE-1 is located as an ectoenzyme at the cell surface where it can cleave exogenous big ET, albeit inefficiently. The majority of the ECE activity was suggested to be located intracellularly in a membrane-bound compartment, possibly the Golgi, where efficient conversion occurs because of co-localization and concentration of enzyme and substrate. The apparent I_{50} for phosphoramidon inhibition of endogenous big ET conversion by intact cells was only approximately 100 μM in this system, whereas for exogenous conversion, or with purified ECE, the I_{50} was approximately 1 μM. Thus, PR has only limited access to the intracellular compartment, consistent with its structure as a phosphorylated sugar derivative. One caveat remains for this set of data: over-expression of ECE-1 cDNA in a cell line may result in an accumulation of ECE-1 protein in the Golgi [84]. Other data, however, also support an intracellular location for some of the endogenous processing of big ET-1. Most studies of cultured cells [for example, Refs. 48 and 49] demonstrate the release of mainly ET-1 rather than its precursor, and high concentrations (100 μM) of PR are required to inhibit this intracellular conversion [48]. The demonstration that ECE-1 protein can be induced by culturing in the presence of high (100 μM) concentrations of PR [17] also suggests that the induction is mediated by inhibition of intracellular rather than cell-surface ECE. It is possible that the same ECE protein mediates both intracellular and cell-surface conversions, although the presence of more than one enzyme in the cell cannot be excluded. A recently cloned ECE-2, homologous with ECE-1 and E-24.11 but with an acidic pH optimum, may have a role to play in intravesicular processing [114]. If ECE-1 is located partly in the Golgi, as suggested in Ref. 15, this is in marked contrast to the location of E-24.11, implying that distinct targeting signals are present within the two proteins, ECE-1 possessing a signal for Golgi retention absent from E-24.11. Construction of chimeric E-24.11/ECE proteins may help to identify any such signals that may reside in the distinct cytoplasmic domains of the putative α and β subunits of ECE.

One possibility that has not been considered to date is that caveolae may be a site of endothelin processing. Caveolae are plasma membrane invaginations that can close off to form non-clathrin-coated vesicles [115, 116]. These vesicles are extremely abundant in endothelial and smooth muscle cells and contain high concentrations of a coat protein, caveolin, and many GPI-anchored membrane proteins [117]. ACE has been reported to be localized to the plasma membrane and to caveolae in pig aortic endothelial cells in culture [118], and the endothelin ET_A receptor clusters in caveolae on binding its ligand and can be co-immunoprecipitated with caveolin [119]. Since procedures are now available for the isolation of caveolae [120, 121], this question can also be readily addressed.

UNANSWERED QUESTIONS

In the last 12 months very striking advances have been made in our understanding of the novel processing event that occurs in the production of mature endothelin. ECE has been defined as a phosphoramidon-sensitive, integral membrane metalloproteinase belonging to the E-24.11/Kell family. Since E-24.11 may play an important role in ET degradation *in vivo* [122], it is intriguing that these two evolutionarily related proteins play consecutive roles in ET metabolism. Phosphoramidon-sensitive, E-24.11-related proteins have been found in diverse species including mammals, insects [123, 124], *Aplysia* [125], and *Mytilus edulis* [126]. It will be fascinating to know whether ECE is a much more recently evolved protein reflecting its highly restricted physiological role. In addition to the problems discussed above on the precise location of endothelin production, many questions remain to be resolved. How many ECE species are there and, on a wider issue, how many further members of the mammalian E-24.11/ECE/Kell gene family remain to be discovered? What endogenous factors regulate the expression of ECE? What are the locations and functions of other ECE isoforms? Is there any role for any of the non-metalloprotease ECE activities that have been reported in either normal or pathological situations? What is the significance of the intermediate production of big ET in endothelin processing? Does big ET itself have some physiological role? Does ECE hydrolyse any other known regulatory peptide, implying a wider physiological role for the enzyme? This question is particularly important in relation to the use of ECE inhibitors in clinical medicine. What are the structural features that permit a very broad substrate specificity for E-24.11, yet a highly restricted one for ECE? Unfortunately, the very highly glycosylated and amphipathic nature of ECE is likely to render any detailed structural determination of the protein impracticable at present, particularly since there has been no report of successful crystallization of the less glycosylated E-24.11. However, site-directed mutagenesis and construction of chimaeric enzymes may again help to clarify specificity features. If earlier reports of a soluble form of PR-sensitive ECE can be confirmed [for example, Ref. 50], then this species may be more amenable to a detailed structural study. And, finally, are ECE inhibitors going to prove of value therapeutically, and for what disease conditions? The discovery of potent, non-peptide, endothelin receptor antagonists [127] has already taken some of the limelight away from ECE. Selective and potent ECE inhibitors are now needed to address many of the above questions.

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Note added in proof: An additional human ECE cDNA sequence has now been reported (Shimada K, Matsushita Y, Wakabayashi K, Takahashi M, Matsubara A, Iijima Y and Tanzawa K, Cloning and functional expression of human endothelin-converting enzyme cDNA. *Biochem Biophys Res. Commun,* **207:** 807–812, 1995). This provides further support for the concept of "short" and "long" forms of ECE subunits. This human clone predicts 56 cytoplasmic residues that show 77% homology with the larger bovine forms [14, 16] but no significant homology to the shorter rat, human, and bovine forms in the first 32 amino acids.

References

1. Roques BP, Zinc metallopeptidases: Active site structure and design of selective and mixed inhibitors: New approaches in the search for analgesics and anti-hypertensives. *Biochem Soc Trans* **21:** 678–685, 1993.
2. Roques BP, Noble F, Dauge V, Fournié-Zaluski M-C and Beaumont A, Neutral endopeptidase 24.11: Structure, inhibition and experimental and clinical pharmacology. *Pharmacol Rev* **45:** 87–146, 1993.
3. Masaki T and Yanagisawa M, Endothelins. *Essays Biochem* **27:** 79–89, 1992.
4. Kennedy RL, Haynes WG and Webb DJ, Endothelins as regulators of growth and function in endocrine tissues. *Clin Endocrinol (Oxf)* **39:** 259–265, 1993.
5. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayishi Y, Mitsui Y, Yazaki Y, Goto K and Masaki T, A novel potent, vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332:** 411–415, 1988.
6. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K and Masaki T, The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci USA* **86:** 2863–2867, 1989.
7. Shubeita HE, McDonough PM, Harris AN, Knowlton KU, Glembotski CC, Brown JH and Chien KR, Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes. A paracrine mechanism for myocardial cell hypertrophy. *J Biol Chem* **265:** 20555–20562, 1990.
8. Gandhi CR, Stephenson K and Olson MS, Endothelin, a potent peptide agonist in the liver. *J Biol Chem* **265:** 17432–17435, 1990.
9. Kurihara Y, Kurihara H, Suzuki H, Kodama T, Maemura K, Nagai R, Oda H, Kuwaki T, Cao W, Kamada N, Jishage K, Ouchi Y, Azuma S, Toyoda Y, Ishikawa T, Kumada M and Yazaki Y, Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* **368:** 703–710, 1994.
10. Baynash AG, Hosoda K, Giaid A, Richardson JA, Emoto N, Hammer RE and Yanagisawa M, Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell* **79:** 1277–1285, 1994.
11. Hosoda K, Hammer RE, Richardson JA, Baynash AG, Cheung JC, Giaid A and Yanagisawa M, Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell* **79:** 1267–1276, 1994.
12. Puffenberger EG, Hosoda K, Washington SS, Nakao K, deWit D, Yanagisawa M and Chakravarti A, A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell* **79:** 1257–1266, 1994.
13. Turner AJ, Endothelin-converting enzyme and other families of metalloendopeptidases. *Biochem Soc Trans* **21:** 697–701, 1993.
14. Shimada K, Takahashi M and Tanzawa K, Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. *J Biol Chem* **269:** 18275–18278, 1994.
15. Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D and Yanagisawa M, ECE-1: A membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* **78:** 473–485, 1994.
16. Ikura T, Sawamura T, Shiraki T, Hosokawa H, Kido T, Hoshikawa H, Shimada K, Tanzawa K, Kobayashi S, Miwa S and Masaki T, cDNA cloning and expression of bovine endothelin converting enzyme. *Biochem Biophys Res Commun* **203:** 1417–1422, 1994.
17. Schmidt M, Kröger B, Jacob E, Seulberger H, Subkowski T, Otter R, Meyer T, Schmalzing G and Hillen H, Molecular characterization of human and bovine endothelin converting enzyme (ECE-1). *FEBS Lett* **356:** 238–243, 1994.
18. Simonson MS and Dunn MJ, The molecular mechanisms of cardiovascular and renal regulation by endothelin peptides. *J Lab Clin Med* **119:** 622–639, 1992.
19. Kohan DE, Endothelins in the kidney: Physiology and pathophysiology. *Am J Kidney Dis* **22:** 493–510, 1993.
20. Warner TD, Battistini D, Doherty AM and Corder R, Endothelin receptor antagonists: Actions and rationale for their development. *Biochem Pharmacol* **48:** 625–635, 1994.
21. Laporte S, Denault J-B, D'Orléans-Juste P and Leduc R, Presence of furin mRNA in cultured bovine endothelial cells and possible involvement of furin in the processing of the endothelin precursor. *J Cardiovasc Pharmacol* **22** (Suppl 8): S7–S10, 1993.
22. Corder R, Harrison VJ, Khan N, Änggård EE and Vane JR, Effects of phosphoramidon in endothelial cell cultures on the endogenous synthesis of endothelin-1 and on conversion of exogenous big endothelin-1 to endothelin-1. *J Cardiovasc Pharmacol* **22** (Suppl 8): S73–S76, 1993.
23. Gui G, Xu D, Emoto N and Yanagisawa M, Intracellular localization of membrane-bound endothelin-converting enzyme from rat lung. *J Cardiovasc Pharmacol* **22** (Suppl 8): S53–S56, 1993.
24. Waxman L, Doshi KP, Gaul SL, Wang S, Bednar RA and Stern AM, Identification and characterization of endothelin converting activity from EAHY 926 cells: Evidence for the physiologically relevant human enzyme. *Arch Biochem Biophys* **308:** 240–253, 1994.
25. Blache P, Kervran A, Le-Nguyen D and Bataille D, Miniglucagon production from glucagon: An extracellular processing of a hormone used as a prohormone, *Biochimie* **76:** 295–299, 1994.
26. Medeiros MS and Turner AJ, Processing and metabolism of peptide-YY: Pivotal roles of dipeptidylpeptidase-IV, aminopeptidase-P and endopeptidase-24.11. *Endocrinology* **134:** 2088–2094, 1994.
27. Medeiros MS and Turner AJ, Post-secretory processing of regulatory peptides: The pancreatic polypeptide family as a model example. *Biochimie* **76:** 283–287, 1994.
28. Takaoka M, Miyata Y, Takenobu Y, Ikegawa R, Matsumura Y and Morimoto S, Mode of cleavage of pig big endothelin-1 by chymotrypsin. Production and degradation of mature endothelin-1. *Biochem J* **270:** 541–544, 1990.
29. Wypij DM, Nichols JS, Novak PJ, Stacy DL, Berman J and Wiseman JS, Role of mast cell chymase in the extracellular processing of big endothelin-1 to endothelin-1 in the perfused rat lung. *Biochem Pharmacol* **43:** 845–853, 1992.
30. Takaoka M, Takenobu Y, Miyata Y, Ikegawa R, Matsumura Y and Morimoto S, Pepsin, an aspartic protease, converts porcine big endothelin-1 to 21-residue endothelin. *Biochem Biophys Res Commun* **166:** 436–442, 1990.
31. Matsumura Y, Ikegawa R, Takaoka M and Morimoto S, Con-

version of porcine big endothelin to endothelin by an extract from the porcine aortic endothelial cells. *Biochem Biophys Res Commun* **167:** 203–210, 1990.
32. Ikegawa R, Matsumura Y, Takaoka M and Morimoto S, Evidence for pepstatin-sensitive conversion of porcine big endothelin-1 to endothelin-1 by the endothelial cell extract. *Biochem Biophys Res Commun* **167:** 860–866, 1990.
33. Sawamura T, Kimura S, Shinmi O, Sugita Y, Kobayashi M, Mitsui Y, Yanagisawa M, Goto K and Masaki T, Characterization of endothelin converting enzyme activities in soluble fraction of bovine cultured endothelial cells. *Biochem Biophys Res Commun* **169:** 1138–1144, 1990.
34. Sawamura T, Kimura S, Shinmi O, Sugita Y, Yanagisawa M, Goto K and Masaki T, Purification and characterization of putative endothelin converting enzyme in bovine adrenal medulla: Evidence for a cathepsin D-like enzyme. *Biochem Biophys Res Commun* **168:** 1230–1236, 1990.
35. Wu-Wong JR, Budzik GP, Devine EM and Opgenorth TJ, Characterization of endothelin converting enzyme in rat lung. *Biochem Biophys Res Commun* **171:** 1291–1296, 1990.
36. Knap AK, Soriano A, Savage P, Del Grande D and Shetty SS, Identification of a novel aspartyl endothelin converting enzyme in porcine aortic endothelial cells. *Biochem Mol Biol Int* **29:** 739–745, 1993.
37. Sawamura T, Shinmi O, Kishi N, Sugita Y, Yanagisawa M, Goto K, Masaki T and Kimura S, Analysis of big endothelin-1 digestion by cathepsin D. *Biochem Biophys Res Commun* **172:** 883–889, 1990.
38. Takaoka M, Hukumori Y, Shiragami K, Ikegawa R, Matsumura Y and Morimoto S, Proteolytic processing of porcine big endothelin-1 catalyzed by cathepsin D. *Biochem Biophys Res Commun* **173:** 1218–1223, 1990.
39. Lees WE, Kalinka S, Meech J, Capper SJ, Cook ND and Kay J, Generation of human endothelin by cathepsin E. *FEBS Lett* **273:** 99–102, 1990.
40. Robinson PS, Lees WE, Kay J and Cook ND, Kinetic parameters for the generation of endothelin-1, -2 and -3 by human cathepsin E. *Biochem J* **284:** 407–409, 1992.
41. Bird JE, Waldron TL, Little DK, Asaad MM, Dorso CR, DiDonato G and Norman JA, The effects of novel cathepsin E inhibitors on the big endothelin pressor response in conscious rats. *Biochem Biophys Res Commun* **182:** 224–231, 1992.
42. Shields PP, Gonzales TA, Charles D, Gilligan JP and Stern W, Accumulation of pepstatin in cultured endothelial cells and its effect on endothelial processing. *Biochem Biophys Res Commun* **177:** 1006–1012, 1991.
43. Deng Y, Savage P, Shetty SS, Martin LL and Jeng AY, Identification and partial purification of a thiol endothelin-converting enzyme from porcine aortic endothelial cells. *J Biochem (Tokyo)* **111:** 346–351, 1992.
44. Knap AK and Wigg AM, A comparison of endothelin-converting enzyme activity from primary aortic endothelial cells with activities in cultured human cell lines. *J Cardiovasc Pharmacol* **22** (Suppl 8): S90–S93, 1993.
45. Suda H, Aoyagi T, Takeuchi T and Umezawa H, A thermolysin inhibitor produced by actinomycetes: Phosphoramidon. *J Antibiotics (Tokyo)* **26:** 621–623, 1973.
46. Kenny AJ, Proteinases associated with cell membranes. In: *Proteinases in Mammalian Cells and Tissues* (Ed. Barrett AJ), pp. 393–444. Elsevier/North-Holland Biomedical Press, Amsterdam, 1977.
47. Roques BP, Fournié-Zaluski MC, Soroca E, Lecomte JM, Malfroy B, Llorens C and Schwartz JC, The enkephalinase inhibitor thiorphan shows anti-nociceptive activity in mice. *Nature* **288:** 286–288, 1980.
48. Ikegawa R, Matsumura Y, Tsukahara Y, Takaoka M and Morimoto S, Phosphoramidon, a metalloproteinase inhibitor, suppresses the secretion of endothelin-1 from cultured endothelial cells by inhibiting a big endothelin-1 converting enzyme. *Biochem Biophys Res Commun* **171:** 669–675, 1990.
49. Sawamura T, Kasuya Y, Matsushita Y, Suzuki N, Shinmi O, Kishi N, Sugita Y, Yanagisawa M, Goto K, Masaki T and Kimura S, Phosphoramidon inhibits the intracellular conversion of big endothelin-1 to endothelin-1 in cultured endothelial cells. *Biochem Biophys Res Commun* **174:** 779–784, 1991.
50. Takada J, Okada K, Ikenaga T, Matsuyama K and Yano M, Phosphoramidon-sensitive endothelin converting enzyme in the cytosol of cultured bovine endothelial cells. *Biochem Biophys Res Commun* **176:** 860–865, 1991.
51. Takaoka M, Shiragami K, Fujino K, Miki K, Miyake Y, Yasuda M, Wang GF, Hisaki K, Matsumura Y and Morimoto S, Phosphoramidon-sensitive endothelin converting enzyme in rat lung. *Biochem Int* **25:** 697–704, 1991.
52. Shinyama H, Uchida T, Kido H, Hayashi K, Watanabe M, Matsumura Y, Ikegawa R, Takaoka M and Morimoto S, Phosphoramidon inhibits the conversion of intracisternally administered big endothelin-1 to endothelin-1. *Biochem Biophys Res Commun* **178:** 24–30, 1991.
53. McMahon EG, Palomo MA, Moore WM, McDonald JF and Stern MK, 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 USA* **88:** 703–707, 1991.
54. Matsumura Y, Hisaki K, Takaoka M and Morimoto S, Phosphoramidon, a metalloproteinase inhibitor, suppresses the hypertensive effect of big endothelin-1. *Eur J Pharmacol* **185:** 103–106, 1990.
55. Pollock DM and Opgenorth TJ, Evidence for metalloprotease involvement in the *in vivo* effects of big endothelin-1. *Am J Physiol* **261:** R257–R263, 1991.
56. Kenny AJ, Stephenson SL and Turner AJ, Cell surface peptidases. In: *Mammalian Ectoenzymes* (Eds. Kenny AJ and Turner AJ), pp. 169–210. Elsevier, Amsterdam, 1987.
57. Turner AJ, Endopeptidase-24.11 and neuropeptide metabolism. In: *Neuropeptides and Their Peptidases* (Ed. Turner AJ), pp. 183–201. Ellis Horwood, Chichester, 1987.
58. Matsas R, Fulcher IS, Kenny AJ and Turner AJ, Substance P and [Leu]enkephalin are hydrolyzed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli. *Proc Natl Acad Sci USA* **80:** 3111–3115, 1983.
59. Sokolovsky M, Galron R, Kloog Y, Bdolah A, Indig FE, Blumberg S and Fleminger G, Endothelins are more sensitive than sarafotoxins to neutral endopeptidase: Possible physiological significance. *Proc Natl Acad Sci USA* **87:** 4702–4706, 1990.
60. Vijayaraghavan J, Scicli AG, Carretero OA, Slaughter C, Moomaw C and Hersh LB, The hydrolysis of endothelins by neutral endopeptidase 24.11 (enkephalinase). *J Biol Chem* **265:** 14150–14155, 1990.
61. Abassi ZA, Golomb E, Bridenbaugh R and Keiser HR, Metabolism of endothelin-1 and big endothelin-1 by recombinant neutral endopeptidase EC 3.4.24.11. *Br J Pharmacol* **109:** 1024–1028, 1993.
62. Murphy LJ, Corder R, Mallet AI and Turner AJ, Generation by the phosphoramidon-sensitive peptidases, endopeptidase-24.11 and thermolysin, of endothelin-1 and C-terminal fragment from big endothelin-1. *Br J Pharmacol* **113:** 137–142, 1994.
63. Weaver LH, Kester WR and Matthews BW, A crystallographic study of the complex of phosphoramidon with thermolysin. A model for the presumed catalytic transition state and for the binding of extended substrates. *J Mol Biol* **114:** 119–132, 1977.
64. Monzingo AF and Matthews BW, Binding of *N*-carboxymethyl dipeptide inhibitors to thermolysin determined by X-ray crystallography: A novel class of transition state analogues for zinc peptidases. *Biochemistry* **23:** 5724–5729, 1984.
65. Murphy LJ, Greenhough KJ and Turner AJ, Processing and

metabolism of endothelin peptides by porcine lung membranes. *J Cardiovasc Pharmacol* **22** (Suppl 8): S94–S97, 1993.
66. Corder R, Khan N and Harrison VJ, A simple method for isolating human endothelin converting enzyme free from contamination by neutral endopeptidase 24.11. *Biochem Biophys Res Commun* **207:** 355–362, 1995.
67. Ohnaka K, Takayanagi R, Yamauchi T, Okazaki H, Ohashi M, Umeda F and Nawata H, Identification and characterization of endothelin converting activity in cultured bovine endothelial cells. *Biochem Biophys Res Commun* **168:** 1128–1136, 1990.
68. Okada K, Miyazaki Y, Takada J, Matsuyama K, Yamaki T and Yano M, Conversion of big endothelin-1 by membrane-bound metalloendopeptidase in cultured bovine endothelial cells. *Biochem Biophys Res Commun* **171:** 1192–1198, 1990.
69. Matsumura Y, Ikegawa R, Tsukahara Y, Takaoka M and Morimoto S, Conversion of big endothelin-1 to endothelin-1 by two types of metalloproteinases derived from porcine aortic endothelial cells. *FEBS Lett* **272:** 166–170, 1990.
70. Matsumura Y, Ikegawa R, Tsukahara Y, Takaoka M and Morimoto S, *N*-Ethylmaleimide differentiates endothelin converting activity by two types of metalloproteinases derived from vascular endothelial cells. *Biochem Biophys Res Commun* **178:** 531–538, 1991.
71. Devine EM, Marselle CA, Pederson TM, Wu-Wong JR, Budzik GP, Dillon TP and Opgenorth TJ, Identification of a neutral metalloendopeptidase endothelin converting enzyme from various cultured endothelial cell lines. *FASEB J* **5:** A1417, 1991.
72. Hioki Y, Okada K, Ito H, Matsuyama K and Yano M, Endothelin converting enzyme of bovine carotid artery smooth muscle cells. *Biochem Biophys Res Commun* **174:** 446–451, 1991.
73. Fukuroda T, Noguchi K, Tsuchida S, Nishikibe M, Ikemoto F, Okada K and Yano M, Inhibition of biological actions of big endothelin-1 by phosphoramidon. *Biochem Biophys Res Commun* **172:** 390–395, 1990.
74. Sessa WC, Kaw S, Hecker M and Vane JR, The biosynthesis of endothelin-1 by human polymorphonuclear leukocytes. *Biochem Biophys Res Commun* **174:** 613–618, 1991.
75. Takahashi M, Matsushita Y, Iijima Y and Tanzawa K, Purification and characterization of endothelin-converting enzyme from rat lung. *J Biol Chem* **268:** 21394–21398, 1993.
76. Ohnaka K, Takayanagi R, Nishikawa M, Haji M and Nawata H, Purification and characterization of a phosphoramidon-sensitive endothelin-converting enzyme in porcine aortic endothelium. *J Biol Chem* **268:** 26759–26766, 1993.
77. Bordier C, Phase separation of integral membrane proteins in Triton X-114 solution. *J Biol Chem* **256:** 1604–1607, 1981.
78. Turner AJ, Purification of membrane-associated proteins. *Methods Mol Cell Biol* **4:** 205–211, 1994.
79. Hooper NM and Turner AJ, Ectoenzymes of the kidney microvillar membrane: Aminopeptidase P is anchored by a glycosylphosphatidylinositol moiety. *FEBS Lett* **229:** 340–344, 1988.
80. Hooper NM, Low MG and Turner AJ, Renal dipeptidase is one of the membrane proteins released by phosphatidylinositol-specific phospholipase C. *Biochem J* **244:** 465–469, 1987.
81. Edgell C-JS, McDonald CC and Graham JB, Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci USA* **80:** 3734–3737, 1983.
82. Greenhough K, Walkden BJ, Murphy LJ, McLaren HE, Barnes K and Turner AJ, Membrane peptidase activity of a human endothelial cell line (EA.hy926). *Biochem Soc Trans* **22:** 454S, 1994.
83. Saijonmaa O, Nyman T, Hohenthal U and Fyhrquist F, Endothelin-1 is expressed and released by a human endothelial hybrid line (EA.hy926). *Biochem Biophys Res Commun* **181:** 529–536, 1991.
84. Takahashi M, Fukuda K, Shimada K, Barnes K, Turner AJ, Ikeda M, Koike H, Yamamoto Y and Tanzawa K, Localization of rat endothelin-converting enzyme to vascular endothelial cells and some secretory cells. *Biochem J* **311:** 657–665, 1995.
85. Marchand P, Tang J and Bond JS, Membrane association and oligomeric organization of the α and β subunits of mouse meprin A. *J Biol Chem* **269:** 15388–15393, 1994.
86. Gorbea CM, Beynon RJ and Bond JS, Meprins: Activation, subunit interactions, and anchoring. In: *Mammalian Brush Border Membrane Proteins II* (Eds. Lentze MJ, Naim HY and Grand RJ), pp. 89–97. Georg Thieme Verlag, Stuttgart, 1994.
87. Littlewood GM, Hooper NM and Turner AJ, Ectoenzymes of the kidney microvillar membrane. Affinity purification, characterization and localization of the phospholipase C-solubilized form of renal dipeptidase. *Biochem J* **257:** 361–367, 1989.
88. Fulcher IS and Kenny AJ, Proteins of the kidney microvillar membrane. The amphipathic forms of endopeptidase purified from pig kidneys. *Biochem J* **211:** 743–753, 1983.
89. Kenny AJ and Maroux S, Topology of microvillar membrane hydrolases of kidney and intestine. *Physiol Rev* **62:** 91–128, 1982.
90. Soubrier F, Alhenc-Gelas F, Hubert C, Allegrini J, John M, Tregear G and Corvol P, Two putative active centers in human angiotensin-I converting enzyme revealed by molecular cloning. *Proc Natl Acad Sci USA* **85:** 9386–9390, 1988.
91. Danielsen EM, Dimeric assembly of brush border enzymes. In: *Mammalian Brush Border Membrane Proteins II* (Eds. Lentze MJ, Naim HY and Grand RJ), pp. 236–239. Georg Thieme Verlag, Stuttgart, 1994.
92. Jongeneel CV, Bouvier J and Bairoch A, A unique signature identifies a family of zinc-dependent metallopeptidases. *FEBS Lett* **242:** 211–214, 1989.
93. Jiang W and Bond JS, Families of metalloendopeptidases and their relationships. *FEBS Lett* **312:** 110–114, 1992.
94. Rawlings ND and Barrett AJ, Evolutionary families of peptidases. *Biochem J* **290:** 205–218, 1993.
95. Lee S, Zambas ED, Marsh WL and Redman CM, Molecular cloning and primary structure of Kell blood group protein. *Proc Natl Acad Sci USA* **88:** 6353–6357, 1991.
96. King M-J, Blood group antigens on human erythrocytes—distribution, structure and possible functions. *Biochim Biophys Acta* **1197:** 15–44, 1994.
97. Hunziker W, Spiess M, Semenza G and Lodish HF, The sucrase—isomaltase complex: Primary structure, membrane-orientation, and evolution of a stalked, intrinsic brush border protein. *Cell* **46:** 227–234, 1986.
98. Laperche Y, Bulle F, Aissani T, Chobert M-N, Aggerbeck M, Hanoune J and Guellaen G, Molecular cloning and nucleotide sequence of rat kidney γ-glutamyl transpeptidase cDNA. *Proc Natl Acad Sci USA* **83:** 937–941, 1986.
99. Bateman RC Jr, Jackson D, Slaughter CA, Unnithan S, Chai YG, Moomaw C and Hersh LB, Identification of the active-site arginine in rat neutral endopeptidase 24.11 (enkephalinase) as arginine 102 and analysis of a glutamine 102 mutant. *J Biol Chem* **264:** 6151–6157, 1989.
100. Beaumont A, Le Moual H, Boileau G, Crine P and Roques BP, Evidence that both arginine 102 and arginine 747 are involved in substrate binding to neutral endopeptidase (EC 3.4.24.11). *J Biol Chem* **266:** 214–220, 1991.
101. Le Moual H, Devault A, Roques BP, Crine P and Boileau G, Identification of glutamic acid 646 as a zinc-coordinating residue in endopeptidase 24.11. *J Biol Chem* **266:** 15670–15674, 1991.
102. Connelly JC, Skidgel RA, Schulz WW, Johnson AR and Erdös EG, Neutral endopeptidase 24.11 in human neutrophils: Cleavage of chemotactic peptide. *Proc Natl Acad Sci USA* **82:** 8737–8741, 1985.
103. LeTarte M, Vera S, Tran R, Addis JB, Omizuka RJ, Quackenbush EJ, Jongeneel CV and McInnis RR, Common acute lym-

phoblastic leukemia antigen is identical to neutral endopeptidase. *J Exp Med* **168:** 1247–1253, 1988.
104. Shipp MA, Vijayaraghavan J, Schmidt EV, Masteller EL, D'Adamio L, Hersh LB and Reinherz EL, Common acute lymphoblastic leukemia antigen (CALLA) is active neutral endopeptidase 24.11 ("enkephalinase"): Direct evidence by cDNA transfection analysis. *Proc Natl Acad Sci USA* **86:** 297–301, 1989.
105. Turner AJ, Membrane peptidases of the nervous and immune systems. *Adv Neuroimmunol* **3:** 163–170, 1993.
106. Howell S, Murray H, Scott CS, Turner AJ and Kenny AJ, A highly sensitive e.l.i.s.a. for endopeptidase-24.11, the common acute-lymphoblastic-leukaemia antigen (CALLA, CD-10), applicable to material of porcine and human origin. *Biochem J* **278:** 417–421, 1991.
107. Okada K, Arai Y, Hata M, Matsuyama K and Yano M, Big endothelin-1 structure important for specific processing by endothelin-converting enzyme of bovine endothelial cells. *Eur J Biochem* **218:** 493–498, 1993.
108. Fabbrini MS, Vitale A, Pedrazzini E, Nitti G, Zamai M, Tamburin M, Caiolfa VR, Patrono C and Benatti L, *In vivo* expression of mutant preproendothelins: Hierarchy of processing events but no strict requirement of Trp-Val at the processing site. *Proc Natl Acad Sci USA* **90:** 3923–3927, 1993.
109. Fukami T, Hayama T, Amano Y, Nakamura Y, Arai Y, Matsuyama K, Yano M and Ishikawa K, Aminophosphonate endothelin converting enzyme inhibitors: Potency enhancing and selectivity improving modifications of phosphoramidon. *Bioorg Med Chem Lett* **4:** 1257–1262, 1994.
110. De Lombaert S, Ghai RD, Jeng AY, Trapani AJ and Webb RL, Pharmacological profile of a non-peptidic dual inhibitor of neutral endopeptidase 24.11 and endothelin-converting enzyme. *Biochem Biophys Res Commun* **204:** 407–412, 1994.
111. Corder R and Vane JR, Radioimmunoassay evidence that the pressor effect of big endothelin-1 is due to local conversion to endothelin-1. *Biochem Pharmacol* **49:** 375–380, 1995.
112. Hisaki K, Matsumura Y, Nishiguchi S, Fujita K, Takaoka M and Morimoto S, Endothelium independent pressor effect of big endothelin-1 and its inhibition by phosphoramidon in rat mesenteric artery. *Eur J Pharmacol* **241:** 75–81, 1993.
113. Harrison VJ, Barnes K, Turner AJ, Wood E, Corder R and Vane JR, Identification of endothelin 1 and big endothelin 1 in secretory vesicles isolated from bovine aortic endothelial cells. *Proc Natl Acad Sci USA* **92:** 6344–6348, 1995.
114. Emoto N and Yanagisawa M, Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. *J Biol Chem* **270:** 15262–15268, 1995.
115. Rothberg KG, Heuser JE, Donzell WC, Ying Y-S, Glenney JR and Anderson RGW, Caveolin, a protein component of caveolae membrane coats. *Cell* **68:** 673–682, 1992.
116. Anderson RGW, Potocytosis of small molecules and ions by caveolae. *Trends Cell Biol* **3:** 69–71, 1993.
117. Turner AJ, PIG-tailed membrane proteins. *Essays Biochem* **28:** 113–127, 1994.
118. Ryan JW, Day AR, Schulz DR, Ryan US, Chung A, Marlborough DI and Dorer FE, Localization of angiotensin converting enzyme (kininase II). I. Preparation of antibody hemeoctapeptide conjugates. *Tissue Cell* **8:** 111–124, 1976.
119. Chun M, Liyanage UK, Lisanti MP and Lodish HF, Signal transduction of a G protein-coupled receptor in caveolae: Colocalization of endothelin and its receptor with caveolin. *Proc Natl Acad Sci USA* **91:** 11728–11732, 1994.
120. Chang W-J, Ying Y-s, Rothberg KG, Hooper NM, Turner AJ, Gambliel HA, De Gunzburg J, Mumby SM, Gilman AG and Anderson RGW, Purification and characterization of smooth muscle cell caveolae. *J Cell Biol* **126:** 127–138, 1994.
121. Lisanti MP, Scherer PE, Vidugiriene J, Tang Z, Hermanowski-Vosatka A, Tu YH, Cook RF and Sargiacomo M, Characterization of caveolin-rich domains isolated from an endothelial rich source: Implications for human disease. *J Cell Biol* **126:** 111–126, 1994.
122. Abassi ZA, Tate J, Golomb E and Keiser H, Role of neutral endopeptidase in the metabolism of endothelin. *Hypertension* **20:** 89–95, 1992.
123. Isaac RE, Neuropeptide-degrading endopeptidase activity of locust (*Schistocerca gregaria*) synaptic membranes. *Biochem J* **255:** 843–847, 1988.
124. Lamango NS and Isaac RE, Identification of an insect neuropeptide-degrading endopeptidase. *Insect Biochem Mol Biol* **22:** 777–783, 1993.
125. Bawab W, Aloyz RS, Crine P, Roques BP and DesGroseillers L, Identification and characterization of a neutral endopeptidase activity in *Aplysia californica*. *Biochem J* **296:** 459–465, 1993.
126. Shipp MA, Stefano GB, D'Adamio L, Switzer SN, Howard FD, Sinisterra J, Scharrer B and Reinherz EL, Down-regulation of enkephalin-mediated inflammatory responses by CD10/neutral endopeptidase 24.11. *Nature* **347:** 394–396, 1990.
127. Clozel M, Breu V, Burri K, Cassal J-M, Fischli W, Gray GA, Hirth G, Löffler B-M, Müller M, Neidhart W and Ramuz H, Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. *Nature* **365:** 759–761, 1993.