Journal of Peptide Science
J. Peptide Sci. **6**: 251–263 (2000)

REVIEW

Soluble Neutral Metallopeptidases: Physiological Regulators of Peptide Action

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Received 28 February 2000
Accepted 16 March 2000

Abstract: Classically, the pre- and post-secretory processing of peptide signals appears to be mediated primarily by subtilisin-like peptidases in secretory vesicles and/or membrane-associated neutral endopeptidases in the extracellular environment. This article presents both biochemical and physiological evidence to support a role for soluble neutral metallopeptidases in the mediation of cell-to-cell communication by the selective generation and termination of peptide signals. These soluble peptidases have been implicated in the normal and disease-state processing of peptides involved in neurological, endocrine and cardiovascular functions. In this context, specific inhibitors of these enzymes could selectively modulate peptide levels and thus have considerable therapeutic potential. The aim of this review is to discuss the design and development of specific inhibitors of soluble neutral metallopeptidases that have been instrumental in identifying the roles of these enzymes. It will also review the evidence and present a case for the involvement of soluble neutral metallopeptidases in the regulation of peptide signalling in both central nervous system (CNS) and peripheral tissues. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: angiotensin; bradykinin; EC 3.4.24.15; EC 3.4.24.16; Gn-RH; inhibitor design; metallopeptidases; neurotensin; peptides

INTRODUCTION

The generation of bioactive peptide signals from larger inactive precursors involves a series of highly ordered events mediated by specific 'processing peptidases'. These processing events can occur within the cell, at the cell surface, in the extracellular environment and within the circulation. The intracellular processing of peptidases is thought to occur primarily within the secretory pathway, and appears largely mediated by members of the subtilisin family of serine proteases (reviewed in Reference [1]). At the cell surface, however, it is thought that membrane-associated enzymes are the prime mediators of peptide processing events. The membrane-associated thermolysin-like metallopeptidases have been shown to play a vital role in the regulation of cardiovascular and endocrine functions. The important physiological role of these mammalian ectoenzymes in both the generation and termination of peptide signals is well established. For example, angiotensin-converting enzyme (ACE) is involved in the metabolism of two major vasoactive peptides. ACE converts angiotensin I to the potent vasopressor angiotensin II [2] and inactivates the vasodilatory peptide bradykinin [3]. Endothelin-converting enzyme (ECE) catalyzes the final step in the biosynthesis of the vasoconstrictor endothelins [4] and neutral endopeptidase (NEP) inactivates the circulating hypotensive cardiac hormone, atrial naturietic factor [5].

Interestingly, over the last 10 years, evidence has started to accumulate to support a physiological involvement for the soluble neutral metallopeptidases in the regulation of both central and

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peripheral peptide activity [6]. In this context, endopeptidase EC 3.4.24.15 (EP24.15) and endopeptidase EC 3.4.24.16 (EP24.16) are soluble, neutral zinc metallopeptidases within the family that have a His-Glu-X-X-His (HEXXH) zinc binding motif. Both enzymes are true peptidases and only cleave short (<20 amino acids) substrates. EP24.15 preferentially cleaves bonds on the carboxyl side of hydrophobic amino acids, with an additional preference towards an aromatic or basic residue in the S_1 position or the P_1 and P_2 positions [7] (the nomenclature of Schechter and Berger). Residues upstream and downstream of the scissile bond influence specificity, for example, a Phe or Tyr in the P_3 position greatly increases the binding affinity for the substrate and rate of reaction [8]. EP24.16 on the other hand, exhibits a unique specificity for a proline in the S_2 and S_2' subsites [9].

Distinction between EP24.15 and EP24.16 is very difficult, as the enzymes display very similar characteristics in terms of substrate specificity and general physicochemical properties. The most striking similarity, as well as the distinguishing feature, between these two enzymes is their substrate specificities. *In vitro*, both enzymes hydrolyse identically a number of naturally occurring peptides, including gonadotrophin-releasing hormone (GnRH), bradykinin, dynorphin$_{(1-8)}$ and substance P (Figure 1). However, EP24.16 characteristically hydrolyses neurotensin (NT) at the Pro^{10}-Gly^{11} bond, while

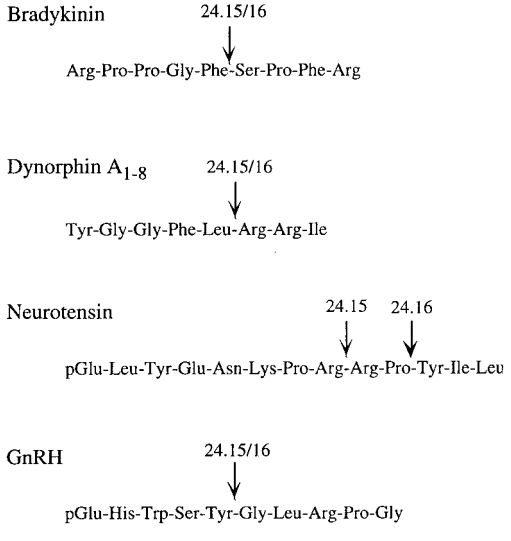

Figure 1 The cleavage sites in the naturally occurring peptides, bradykinin, dynorphin A, NT and GnRH by EC 3.4.24.14 (24.15) and EC 3.4.24.16 (24.16) (marked with arrows).

EP24.15 cleaves exclusively at the Arg^8-Arg^9 bond. In addition, only EP24.16 hydrolyses the acetylated neurotensin fragment Ac-NT$_{8-13}$ [6].

As would be expected, metal chelating agents such as EDTA and 1,10-phenanthroline significantly inhibit both enzymes. Inhibitors of other metallopeptidases, including phosphoramidon, thiorphan (NEP inhibitor) and captopril (ACE inhibitor), have little effect. An important difference between these enzymes is the effect of thiol reducing agents such as DTT. Like other metallopeptidases, EP24.16 is inhibited by such agents, reflecting the thiophilicity of the zinc atom. EP24.15, although inhibited at high thiol concentrations, is uniquely activated at low (<10 mM) concentrations. This activation has been shown to reflect the conversion of an inactive multimer to an active monomer via the disruption of intermolecular disulphide bridges [10]. This mechanism may represent a means by which changes in cellular redox potential or the action of physiological thiol:disulphide exchangers may regulate EP24.15 activity.

Given that the two enzyme activities share similar properties, it was not surprising to discover that their amino acid sequences are 60% homologous [11,12] (Figure 2). Despite the presence of several putative *N*-glycosylation sites, there are no indications for glycosylation of either enzyme. Furthermore, although hydrophobicity plots of their sequences show no marked hydrophobic regions suggestive of transmembrane domains, both enzymes have been shown to be capable of membrane association. Studies suggest EP24.16 is genuinely membrane bound [13] while EP24.15 has been demonstrated to be present on the outer plasma membrane of cells [14], although the mechanism of association is unclear. The sequence of the EP24.16 precursor contains a 24 amino acid, *N*-terminal putative mitochondrial targeting sequence, which is lacking in EP24.15. Furthermore, although EP24.15 and EP24.16 lack a typical signal peptide, both enzymes appear to be secreted using an as yet unknown, non-classical secretory pathway [13,15,16].

Subcellular fractionation studies have consistently revealed that EP24.15 activity is predominantly soluble (80%), with the remaining activity being both nuclear and membrane-associated; however, reports for the subcellular localization of EP24.16 vary. While EP24.16 activity has been shown to be cytoplasmic, mitochondrial and membrane-associated, the relative distribution seems to

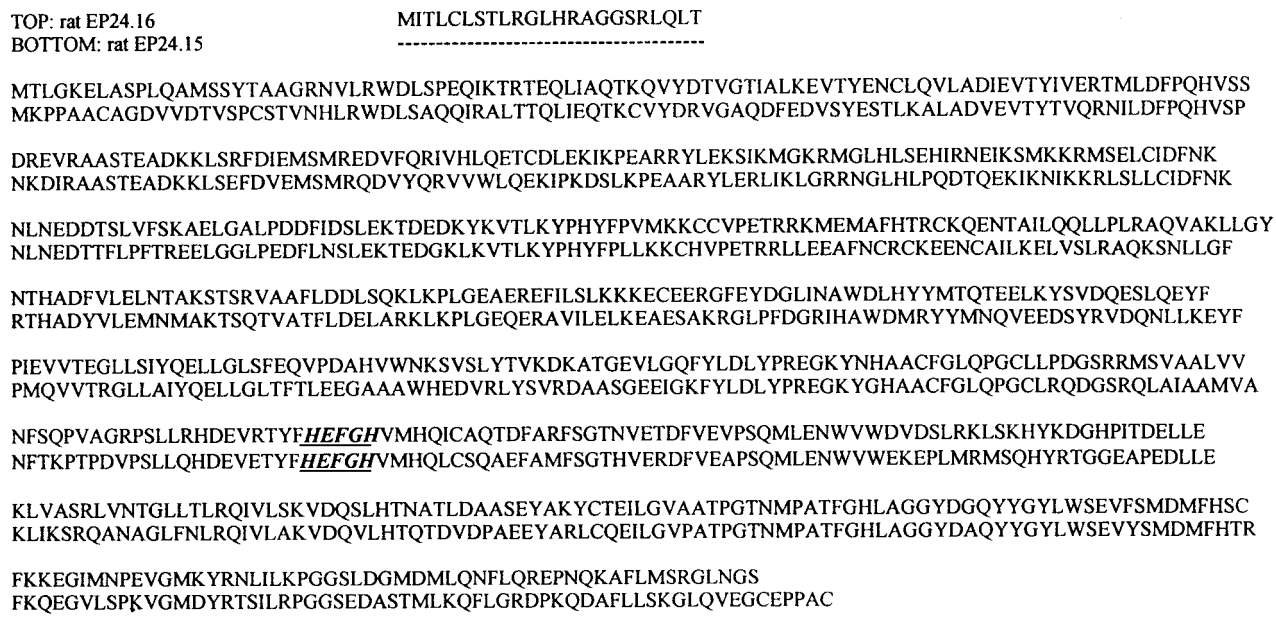

Figure 2 The comparison of primary amino acid sequence between rat EC 3.4.24.15 and rat EC 3.4.24.16. The conserved zinc-binding motif in both peptidases is underlined.

depend on the cell type examined. For example, in astrocytes, EP24.16 is distributed throughout cytoplasm, whereas in neurons, it is mainly associated with the plasma membrane and intracellular membrane-bound organelles [13]. Both EP24.15 and EP24.16 are of similar molecular weight (75–80 kDa) and exhibit almost identical chromatographic behavior. To date, the only method available to successfully separate the two activities is chromatography on hydroxyapaptite, where EP24.16 is eluted at a phosphate concentration significantly greater than EP24.15 [17,18].

Both enzymes are widely distributed in cells and tissues throughout the body. High levels of EP24.15 have been localized, both catalytically and immunohistochemically, to the brain, pituitary and testis, with lower levels in other tissues, such as the liver, kidney, spleen and lung [8]. Within the central nervous system (CNS), high concentrations of EP24.16 are localized to the olfactory bulb and tubercle, cinguate cortex, medial striatum and globus pallidus [19]. The distribution of EP24.15 and EP24.16 in areas rich in neuropeptide content is consistent with a role for these enzymes in the processing/metabolism of bioactive peptides.

Indeed, both EP24.15 and EP24.16 satisfy criteria, as proposed by Turner and Barnes [20], for classification as neuropeptide-inactivating enzymes (neuropeptidases). First, they have been located in the extracellular space, where interaction with peptide substrates can occur. Second, they are active at physiological pH. Third, hydrolysis products are biologically inactive at their receptors and finally, metabolically stable antagonists of peptides such as somatostatin and GnRH are resistant to degradation by these enzymes [6].

Although the precise physiological roles of EP24.15 and EP24.15 have yet to be defined, their ubiquitous distribution and range of substrates, together with the information we will present in this review, provide strong evidence to support an important role for these enzymes in the extracellular processing of peptide signals.

DESIGN OF THERMOLYSIN-LIKE METALLOPEPTIDASE INHIBITORS

Potent and selective synthetic inhibitors are powerful tools for defining both *in vitro* and *in vivo* the physiological contributions of a particular enzyme. Furthermore, since several zinc metallopeptidases have been identified as playing important biological roles in the activation and termination of peptide signals, inhibitors of these enzymes have considerable potential as therapeutic agents. Indeed, inhibitors of ACE are powerful antihypertensive agents and inhibitors of NEP have both antinociceptive and antihypertensive properties.

Among the family of zinc metallopeptidases, detailed structural information on the interactions between substrates/inhibitors and the active site is available only for thermolysin and carboxypeptidase A. Based on this information and given that members of this family share similar critical components in their active sites, a general strategy has been adopted for the design of competitive inhibitors of zinc metallopeptidases. Crucial to the efficacy of these inhibitors is the presence of a sequence, which fulfils the binding requirements of the substrate recognition site and the presence of a functional group, which acts as a ligand for the zinc atom present in the active site [21]. The latter can be achieved by the use of one of several metal co-ordinating groups, including carboxyalkyls, thiols, hydroxymates and phospho-groups. Peptides fulfilling the binding requirements of thermolysin and containing one of these zinc-coordinating groups have been shown to act as potent competitive inhibitors of this enzyme [22,23]. Similar potent and specific inhibitors of ACE [24,25], NEP [26,27] and, more recently, ECE [28,29], have been developed.

N-carboxyalkyl dipeptides have emerged as an important class of zinc metallopeptidase inhibitors. Their effect presumptively results from their similarity to the proposed transition state complex. These inhibitors are prepared by the reductive coupling of α-ketoacids or esters to the amino terminus of suitably protected dipeptides, creating a new asymmetric center [25]. The resultant diastereomers can then be separated by chiral chromatography. *N*-carboxyalkyl peptide derivatives have been shown to be potent inhibitors of thermolysin [30] and NEP [27,31] and such derivatives of proline-containing dipeptides (such as enalaprilat) act as potent inhibitors of ACE [25].

Phosphoramidon, a natural phospho-containing substance, is known to behave as a potent inhibitor of thermolysin, NEP [32,33] and ECE [34]. Another attractive approach to the development of zinc metallopeptidase inhibitors is, therefore, the synthesis of phosphate containing analogs of peptide substrates. Thermolysin, carboxypeptidase A and bacterial collagenases have been shown to be potently inhibited by synthetic substrate analogs, in which the scissile peptide bond is replaced by a tetrahedral phosphonamide moiety [35–37]. These inhibitors act as transition state analogs of the equivalent peptide substrate [36]. The major limitation to the phosphonamide derivative, however, is the chemical instability of the phosphonamide (P–N) bond. The P–N bond can be easily cleaved depending on both the pH of the medium and on the size and sequence of the phosphonamide peptide [36,38]. Since the replacement of the P–N bond by a phosphonate group results in a dramatic loss of potency [37], it has been postulated that the NH group of the phosphonamide bond forms hydrogen bonds within the active site. However, Yiotakis *et al.* [39] found that the replacement of the NH by CH_2, i.e. a phosphinic group, only marginally reduced potency, thus proving a viable alternative for overcoming the lability problem. Recently, phosphinic peptides have been successfully designed as potent and selective inhibitors of EP24.16 and EP24.15 (as discussed below).

Development of Selective Inhibitors of EP24.16

To establish the contribution of EP24.16 to the physiological inactivation of NT, Checler and colleagues have pursued the design and synthesis of potent and selective inhibitors of the enzyme. Preliminary experiments indicated that the degradation of tritiated NT could be blocked by dipeptides (Pro-Xaa), which mimicked the Pro-Tyr bond of NT; the bond hydrolyzed by EP24.16. Among the series of dipeptides generated, the most potent inhibitory effect was elicited by Pro-Ile with a K_i of approximately 90 μM. Importantly, Pro-Ile displayed a rather exclusive inhibition for EP24.16, not inhibiting other metallopeptidases such as EP24.15, NEP and ACE at a concentration of 5 mM [40]. However, the relatively low potency of the inhibitor in addition to its poor solubility at high concentrations precluded the use of this inhibitor for *in vivo* experiments.

Such limitations were not shared by the phosphonamide peptide, *N*-(phenylethylphosphonyl)-Gly-L-Pro-L-aminohexanoic acid (phosphodiepryl 03), a potent inhibitor of *Clostridium histolyticum* collagenases [37]. This inhibitor resembles the Leu-Gly-Pro sequence of the substrate Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp, which is cleaved at the Leu-Gly bond by both EP24.16 and EP24.15. As such, phosphodiepryl 03 was examined as a putative inhibitor of both enzymes [41]. Phosphodiepryl 03 potently blocked EP24.16 activity, displaying an affinity for the enzyme in the subnanomolar range, while inhibition of EP24.15 was 10-fold less potent. Furthermore, phosphodiepryl 03 did not affect other zinc metallopeptidases, at 1000-fold higher concentrations than the K_i the value for EP24.16 [41]. Subsequent work with phosphonamide peptide inhibitors

by the same group yielded the potent mixed inhibitor of EP24.16 and EP24.15, *N*-(2-(2-naphthyl)-ethylphosphonyl - glycyl - prolyl - norleucine) (phosphodiepryl 08). This new inhibitor inhibited both enzymes identically with a K_i of 0.4 nM [42].

More recently, Jiracek *et al.* [9] took a novel systematic approach to inhibitor design, based on combinatorial chemistry of phosphinic peptides. As for the phosphonamide group in phosphodiepryl 03 and phosphodiepryl 08, the phosphinic group is used as a surrogate of the scissile bond; however, it is far more chemically stable than the former. The most potent inhibitor developed in this study was Pro-L-Phe-$\psi(PO_2CH_2)$Gly-Pro (phosphodiepryl 33) (ψ indicates that the peptide bond has been modified, and the formula of the group that has replaced this peptide bond is in parentheses), which displays a K_i value of 4 nM. Unlike previous inhibitors, this compound is able to discriminate between EP24.16 and EP24.15 (2×10^3 times less potent) based on the unique specificity of the EP24.16 S_2 and S'_2 subsites for proline. Provided it is stable *in vivo*, this inhibitor should permit both the investigation of the function of EP24.16 and the reassessment of the relative roles of EP24.16 and EP24.15 in the pathophysiology of disease.

Development of Selective Inhibitors of EP24.15

Following their identification and characterization of EP24.15 [7], Orlowski and colleagues focused on the design of potent active site inhibitors to define the role of this enzyme in neuropeptide metabolism *in vivo*. This group generated a series of *N*-carboxymethyl derivatives containing a free carboxylate group capable of coordinating with the zinc in the active site of EP24.15 [43]. These substrate-based peptide derivatives were chosen as such peptide analogs had been shown previously to act as potent inhibitors of several zinc metallopeptidases, fulfilling the binding requirements of the substrate recognition site. The inhibitors were synthesized by reductive amidation of alanyl-alanyl-phenylalanyl-*para*-aminobenzoate (Ala-Ala-Phe-*p*-AB) with selected α-keto acids and aldehydes. This peptide was chosen as previous work by this group [7] had demonstrated that the phenylalanyl residue is important for substrate binding (apparently interacting with the hydrophobic pocket of the S'_3 subsite) and that the two Ala residues are required for binding to the S'_1 and S'_2 subsites. The compound *N*-[1-carboxy-2-phenyethyl]-Ala-Ala-Phe-*p*-AB produced the strongest competitive inhibition of EP24.15, with a K_i of 1.94 μM. The importance of the carboxylate moiety in coordinating with the active site zinc was confirmed as the deletion of this group resulted in decreased inhibitory potency by almost three orders of magnitude. A similar effect was produced when the inhibitor chain length was shortened by one alanine residue, highlighting the importance of interactions at both the S_1 and S'_3 subsites and with the catalytic zinc [43].

This research team subsequently showed that the replacement of the *N*-carboxymethyl group with an *N*-carboxyphenylpropyl group increased inhibitor potency by more than three orders of magnitude and thus synthesized and tested a number of substrate related *N*-(1-carboxy-3-phenylpropyl) peptide derivatives. The most potent of these derivatives, *N*-[1-(*RS*)carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP[Tyr]), inhibited EP24.15 in a competitive manner, with a K_i of 16 nM. A slight decrease in inhibition was seen when the Tyr was replaced by a Phe residue ($K_i = 27$ nM). By contrast, a pronounced decrease in potency (more than 20-fold) was produced when the Tyr residue was replaced by an Ala, further confirming the requirement for a bulky or hydrophobic residue in the P_1 position of substrates of EP24.15. Furthermore, the need for an Ala residue interacting with the S'_1 subsite was supported by the increase in K_i, by a factor of 65, when the Ala was changed to a Gly residue [44] (Figure 3(A)).

This inhibitor and its phenylalanine substituted analog (cFP[Phe]) initially proved valuable tools and were used in many studies on the pharmacological effects associated with the inhibition of EP24.15. These inhibitors were shown to be relatively EP24.15 specific, only weakly inhibiting NEP (K_i 35 μM) [43]. More recently, cFP[Phe] has been shown to inhibit EP24.16, albeit 10–100-fold less potently than EP24.15 [45]. Despite the finding that cFP-[Phe] had no intrinsic inhibitory activity on ACE [46], a previous study had reported that cFP[Phe] was indeed a potent inhibitor of ACE *in vitro*, inhibiting the conversion of Ang I to Ang II by pulmonary ACE to a similar extent as an ACE specific inhibitor [47]. This proved a point of interest. Subsequent *in vitro* [48] and *in vivo* [49] studies demonstrated that NEP, a common contaminant in ACE preparations, cleaved this inhibitor to cFP-Ala-Ala and Phe-pAB, as shown in Figure 4; the former having ACE inhibiting activity with a structure similar to that of enalaprilat [46,48].

To date, few other inhibitors of EP24.15 have been reported, although one compound,

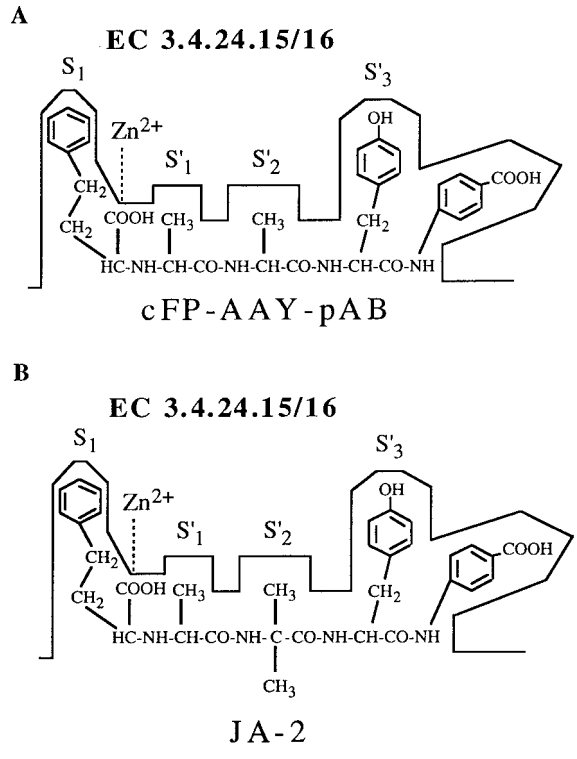

Figure 3 A schematic representation of EC 3.4.24.15 and EC 3.4.24.16 binding to the specific inhibitor cFP-AAY-pAB (A) and JA-2 (B) modified from Orlowski *et al.* [44] (reproduced with permission).

N-[(2*R*,4*S*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl) - 4 - thiazolidinecarbonyl] - L - phenyalanine (SA898), has been described [50]. This inhibitor, incorporating a sulfhydryl moiety, which is postulated to chelate to the active site zinc, was found to be a more potent and competitive inhibitor than cFP[Phe] (K_i of 9.1 nM). However, SA898 also significantly inhibited ACE, and to some extent NEP, thus limiting the utility of this compound. To date the most potent and selective inhibitors of EP24.15 are phosphinic peptides developed using a combinatorial approach by Jiracek *et al.* [51]. These compounds are Z-$_{(L,D)}$Pheψ(PO_2CH_2)$_{(L,D)}$Ala-Lys-Met (phosophodiepryl 21), which exhibits a K_i value of 0.12 nM and is approximately 2×10^3 times more potent against EP24.15 than EP24.16 and Z-$_{(L,D)}$Pheψ(PO_2CH_2)$_{(L,D)}$-Ala–Arg-Phe ($K_i = 0.16$ nM) and which is more than 3×10^3 times more potent against EP24.15 than EP24.16. The marked difference in inhibitor potency towards these two enzymes is due to a clear preference of EP24.15 for an arginine or a lysine residue in the P'_2 position, while these two residues are less well tolerated in this position by EP24.16. Furthermore, Z-$_{(L,D)}$Pheψ(PO_2CH_2)$_{(L,D)}$Ala-Arg-Phe, at 1 μM, does not inhibit the activity of several other zinc metallopeptidases including ACE and NEP.

PHYSIOLOGICAL ROLES OF EP24.15 AND EP24.16

Termination of Peptide Signals

Several lines of evidence have established that EP24.16 and EP24.15 are prime contributors towards the physiological termination of the neurotensinergic signal. Indeed, EP24.16 was initially referred to as neurotensin degrading neutral metalloendopeptidase or neurolysin based on its ability to cleave NT at Pro^{10}-Tyr^{11}, generating the biologically inert fragments NT(1–10) and NT(11–13) [52]. These two degradation products were ubiquitously detected during a study of NT metabolism by various tissues and cell cultures of both central and peripheral origin [51,53]. Furthermore, synthetic neurotensin analogues in which an aromatic D-amino acid was substituted at position 11, totally resisted proteolysis by rat brain tissue, purified EP24.16 and *in vivo* following intracerebroventricular administration in rat [54], establishing the Pro^{10}-Tyr^{11} bond as the prominent target for physiological inactivation. The use of a specific antiserum against EP24.16 established that the distribution of EP24.16 paralleled that of NT receptors [55] and in addition, that EP24.15 co-localizes with NT receptors in pure, differentiated cultured neurons from mouse embryo [56].

More direct evidence for the involvement of EP24.16 in the physiological inactivation of NT is provided by the development of specific inhibitors directed against the enzyme (as described earlier). Pro-Ile significantly enhanced the recovery of intravenously infused NT, and in turn decreased the formation of the NT(1–10) fragment in the ileum of the anaethetized dog [57]. However, the low affinity and poor solubility of Pro-Ile precludes its use when bolus (high concentration) administration is required. By contrast, the high potency and hydrophilicity of the phosphonamide peptides make them potentially more efficient pharmacological tools. Phosphodiepryl 08 virtually abolished NT degradation by pure cultured neurones from mouse embryos and greatly potentiated NT-induced antinociception in the mouse hot plate test [58]. Given that phosphodiepryl 08 behaves as a potent and

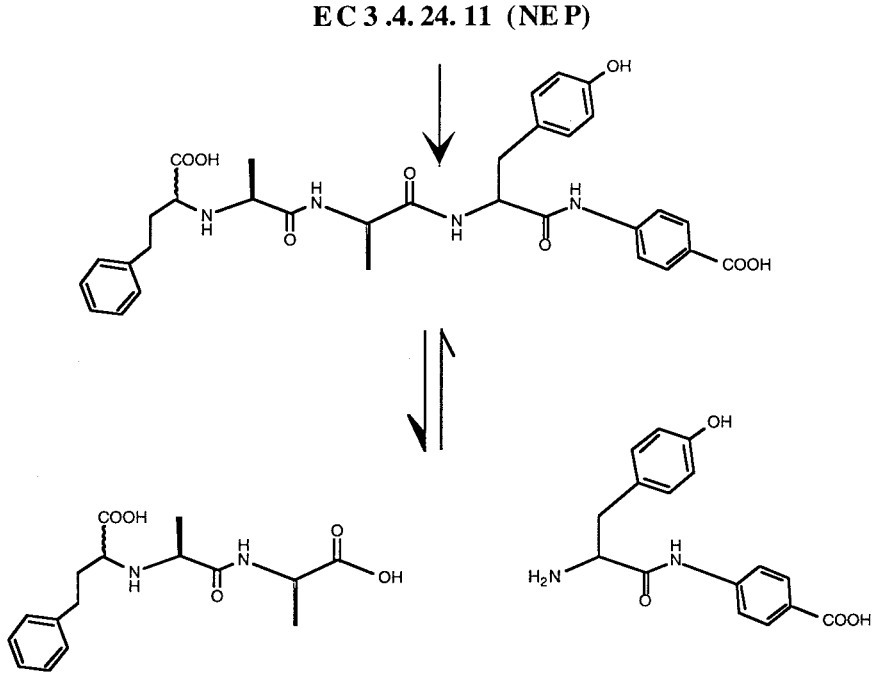

Figure 4 This figure shows the cleavage of cFP-AAY-pAB by EC 3.4.24.11 (between the Ala-Tyr bond), the *N*-terminal product of this cleavage being a potent ACE inhibitor.

selective mixed inhibitor of EP24.16 and EP24.15, this finding supported a role for one or both enzymes in the central termination of NT. The potent and fully selective inhibitors, phosphodiepryl 33 and phosphodiepryl 21, enabled delineation of the respective contributions of these enzymes. The role of EP24.16 in NT degradation was confirmed by phosphodiepryl 33, which significantly potentiated neurotensin-induced analgesia, dose-dependently inhibited the formation of NT(1–10) and concomitantly protected neurotensin from degradation by cultured neurones [58]. In addition, EP24.15 has also been implicated in the central inactivation of NT. Phosphodiepryl 21 greatly potentiated, in a dose-dependent manner, the neurotensin-induced analgesia in hot plate tested mice [59].

Moreover, previous studies in rat hypothalamic slices have identified EP24.15 as the activity responsible for the cleavage of the Arg^{8}-Arg^{9} bond of NT [60]. In addition, the use of Pro-Ile and cFP[Phe] significantly reduced the formation of the degradation products resulting from cleavage at the Pro^{10}-Tyr^{11} and Arg^{8}-Arg^{9} bonds, suggesting EP24.16 and EP24.15 are responsible for the cleavage of NT in these cells. Phosphoramidon and captopril had no effect on degradation by astrocytes [61]. Most interestingly, the neuroleptic drug haloperidol (a dopamine receptor antagonist) was shown to greatly reduce NT degradation in intact brain slices, the likely consequence of inhibiting a specific processing enzyme, identified as EP24.15 [62]. NT co-localizes and is released in many brain regions with dopamine and alterations in its concentration have been proposed to account for some of the pathophysiology associated with the neuropsychiatric disorder, schizophrenia. Decreased NT immunoreactivity in cerebrospinal fluid (CSF) has been observed in certain subpopulations of schizophrenic patients [63,64] and the subnormal concentrations of NT in the CSF schizophrenics return to normal levels following neuroleptic treatment [65]. Thus, EP24.15 may play a role in the pathophysiologic manifestations of schizophrenia via the inactivation of neurotensin.

EP24.15 has been shown to degrade cortical somatostation (SRIF 1–14) [8] and participate in the degradation of somatostatin by cultured neuronal and glial cells [66]. EP24.15 and EP24.16 were also identified as the main somatostatin-degrading activities purified from both rat and pig brain [6]. Furthermore, somatostatin degradation by cultivated rat cortical astrocytes occurred at the Phe^{6}-Phe^{7} and Thr^{10}-Phe^{11} bonds, consistent with cleavage by EP24.16 and EP24.15. Addition of

ProIle and cFP[Phe] significantly reduced the formation of these fragments. The expression of the enzyme by astrocytes was demonstrated by the chromatographic separation of solubilized cell preparations [61]. Given that the experimental lowering of SRIF 1–14 has been associated with severe memory impairment [67], EP24.15 and/or EP24.16 may contribute to the symptomatology of the neurodegenerative disorder, Alzheimer's disease. Furthermore, since astrocytes embrace neuronal synapses, the site of neuropeptide release, EP24.15 and EP24.16 on the surface of, and secreted by astrocytes, would be strategically placed to significantly contribute to the inactivation of neuropeptides, such as neurotensin and somatostatin, in the brain.

EP24.15 may be of importance in the central regulation of reproductive function. GnRH is released from the median eminence (ME) of the hypothalamus directly into the hypophysioportal blood system and carried to the anterior pituitary, where it stimulates the release of lutenizing hormone (LH) and follicle stimulating hormone (FSH) into the circulation. Several lines of evidence support a role for EP24.15 in modulating the GnRH signal to the pituitary. EP24.15 activity has been shown to be much higher in the rat ME, anterior pituitary and the preoptic area of the hypothalamus compared with other brain regions [68]; the latter being the major site of GnRH synthesis [69]. These tissues, and in particular the ME, also exhibited high levels of EP24.15 in the ovine hypothalamic–pituitary axis [70]. In addition, EP24.15 has been detected in hypophysial portal blood and its immunoreactivity detected in regions of the ME where both GnRH axons and hypophysial portal vessels are present [71], suggesting that EP24.15 is secreted within the ME and thus is perfectly placed to regulate the GnRH signal.

EP24.15 degrades GnRH at the Tyr^5-Gly^6 bond [7] and has been shown to be the primary enzyme responsible for the hydrolysis of GnRH at this bond in hypothalamic and pituitary preparations and also in the anterior pituitary cell line, AtT20 [72]. Furthermore, *in vivo* studies in rats have demonstrated that GnRH is extensively hydrolysed by EP24.15, since cFP[Phe] and not inhibitors of either NEP or ACE, greatly slowed the degradation of GnRH administered by either intravenous or intracerebroventricular routes. The increased half-life of the exogenously administered GnRH was to an extent typical of 'superactive' analogs of GnRH [73,74], which are rendered resistant to enzymatic degradation by the introduction of a D-amino acid in position 6. In addition, steroid-induced LH increase in ovariectomized rats was augmented by EP24.15 inhibition [71]. Further supporting a role for the enzyme in the regulation of GnRH is data suggesting that EP24.15 is under gonadal steroid control [69].

EP24.15 may also play a role in the peripheral (non-CNS) regulation of peptide metabolism. Evidence suggests a role for the enzyme in the control of the pressor response in mammals. *In vitro*, EP24.15 efficiently cleaves the Phe^5-Ser^6 bond of the vasodilatory peptide bradykinin [7]. The intravenous infusion of cFP into normotensive rats was shown to produce an immediate and marked fall in mean arterial pressure, which could be almost abolished by pre-administration of a kinin receptor antagonist. In addition, cFP also potentiated the bradykinin-induced vasopressor response [75]. These data, however, remain somewhat controversial since the breakdown product of cFP is a potent inhibitor of ACE (Figure 4), whose role in blood pressure regulation through the cleavage of bradykinin at the Phe^5-Ser^6 and Pro^7-Phe^8 bonds, has been well documented. Recent work in our laboratory has led to the development of new inhibitor of EP24.15 (Figure 3(B)), which is similar to cFP in terms of potency and specificity, but is resistant to proteolytic degradation [76]. The intravenous administration of this inhibitor potentiated bradykinin-induced hypotension without affecting the hypertensive effects of exogenous angiotensin I and angiotensin II [77]. This exciting finding suggests a role for EP24.15 in the metabolism of circulating bradykinin and hence in the regulation of blood pressure.

Generation of Peptide Signals

Peptide hormones may be cleaved into alternate, biologically active, smaller fragments, having quite distinct properties to the parent molecule. For example, it has been demonstrated that the endogenous breakdown product of GnRH, GnRH(1–5), can function as an *N*-methyl-D-aspartate (NMDA) receptor antagonist, selectively inhibiting the GnRH secretion evoked by NMDA [78]. Given that the action of EP24.15 on GnRH generates the GnRH(1–5) fragment, one may speculate on an important role for EP24.15 in the regulation of the glutamate induced GnRH release at these receptors.

EP24.15 rapidly converts several enkephalin-containing peptides, such as dynorphin $A_{(1-8)}$,

α- and β-neoendorphin, Met-enkephalin-Arg-Gly-Leu (MERGL) and metamorphamide, into the corresponding enkephalins Leu- and Met-enkephalin [8,79,80]. The inhibition of EP24.15 by cFP[Phe] has the effect of prolonging the action of dynorphin $A_{(1-8)}$ and MERGL at the κ- and δ-opioid receptors, respectively [81]. Opioid receptors are involved in the transmission, modulation and sensation of pain, and as such, the action of EP24.15 on opioid precursors implicates the enzyme in the modulation of nociception. Studies have shown that the central pre-treatment of rats with cFP[Phe] results in a significant and dose-dependent increase in antinociception on the tail flick and jump tests, without affecting basal nociceptive thresholds. Endogenous opioid peptides also appear, in part, to mediate the feedback regulation of LH release indirectly through the suppression of hypothalamic GnRH release. The systemic administration of naloxone has been shown to result in a massive release of LH [82] and following gonadectomy, the release of LH from the anterior pituitary is blocked by β-endorphin and dynorphin-like peptides and stimulated by Leu-enkephalin [83]. Thus, in addition to a role in pain perception, EP24.15 may also contribute to gonadotrophin release through the catabolism of opioids.

Finally, there is accumulating evidence suggesting that angiotensin (1–7) plays an important role in the renin–angiotensin system, acting in either an identical or opposite fashion to angiotensin II [84]. EP24.15 can directly convert angiotensin I to angiotensin (1–7). In vascular smooth muscle cells, the conversion of angiotensin I to angiotensin (1–7) is reduced by 90% in response to cFP[Phe]. Specific inhibitors of other enzymes known to degrade angiotensin I, ACE, NEP and prolyl endopeptidase, neither inhibited the generation of angiotensin (1–7) nor altered the metabolism of angiotensin I [85]. In addition, simultaneous measurements of angiotensin peptides in sheep plasma found no differences in peptide levels in arterial and jugular plasma. Angiotensin peptide levels in hypophysial-portal plasma were similar to those of jugular plasma, with the exception of Ang (1–7), the levels of which were 5-fold higher in hypophysial-portal plasma, and Ang I, for which the levels in hypophysial-portal plasma were 46% of jugular levels [86]. Angiotensin peptides are present in low abundance in the ovine ME, suggesting that they are not secreted into the hypophysial-portal circulation. However, the high levels of EP24.15 in the ME would be consistent with the processing of Ang I delivered to the ME by arterial blood leading to the increased levels of Ang (1–7) in the hypophysial-portal plasma. Hence, EP24.15 may contribute to the regulation of blood pressure, endothelial function and/or natriuresis through the processing of angiotensin I to angiotensin (1–7).

SUMMARY

In conclusion, taken together we believe that the information presented in this review supports a role for the participation of EP24.15 and EP24.16 in both the central and peripheral modulation of a number of biologically important peptides, including NT, GnRH, somatostatin, various peptides with opioid activity and finally, the vasoactive peptide bradykinin. Furthermore, there is also a body of evidence supporting a role for EP 24.15 in other proteolytic events such as the processing of the Alzheimer's disease β-amyloid protein [87] and presentation of major histocompatibility complex (MHC) class I molecules [88]. The development of potent highly specific and stable inhibitors of these enzymes will allow us to further assign the relative contribution of these peptidases to the physiological regulation of peptide delivery. In addition, like ACE, ECE and NEP, these soluble neutral metallopeptidases may ultimately represent another important target for therapeutic intervention, particularly in the design of inhibitors, which may have antinociceptive, and/or antihypertensive properties.

Acknowledgements

This work was supported by the Australian National Health & Medical Research Council. The authors gratefully acknowledge Dr R.A. Lew for critically evaluating the manuscript.

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