Proenkephalin-processing Enzymes in Chromaffin Granules

A Model for Neuropeptide Biosynthesis[a]

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NOVEL PROHORMONE-PROCESSING ENZYMES

Peptide hormones and neurotransmitters are first synthesized as inactive protein precursors that are cleaved by a series of proteolytic steps at paired basic residues, and sometimes at single arginine sites, to generate the smaller biologically active neuropeptides.[1,2] The goal of this research was to determine the proteases required for converting prohormones to active neuropeptides. The prohormone-processing enzymes are crucial for the physiologic functions of active peptides in the neuroendocrine system. However, at the time that this research was started, the proteases involved in the prohormone-processing pathway were relatively unknown.

In our studies, processing of proenkephalin (PE) to enkephalin opioid peptides in the adrenal medulla has been used as a model prohormone-processing system. Enkephalin peptides in the nervous system are active analgesics; in the periphery, the actions of enkephalin peptides include modulation of immune-cell functions. Because the prohormone-processing enzymes are primarily located within secretory vesicles, chromaffin granules of bovine adrenal medulla provide a rich source of

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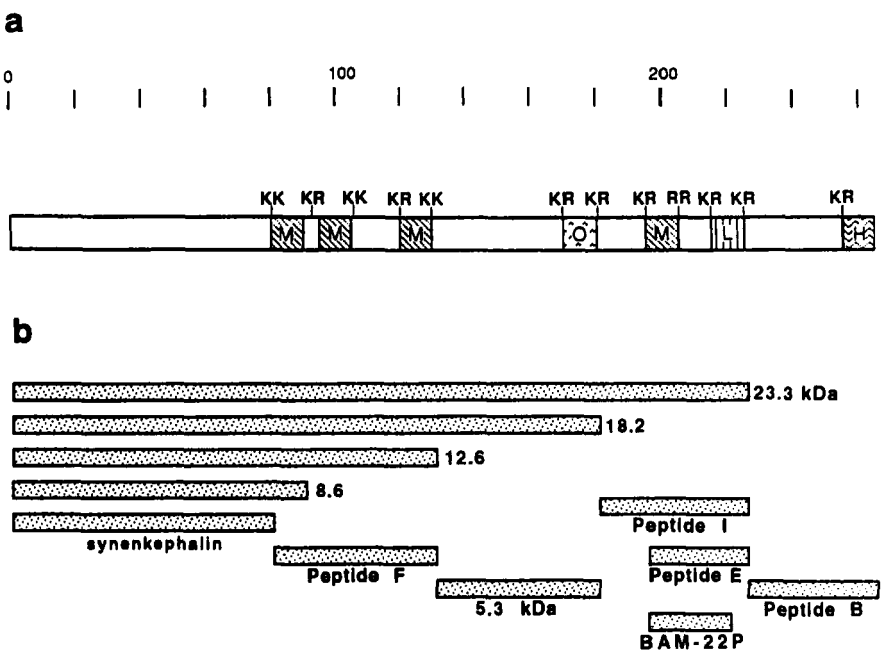

FIGURE 1. *Proenkephalin (PE) and processing products in adrenal medulla.* **a**: PE is schematically illustrated showing the active peptides met-enkephalin (M), met-enkephalin-Arg^6-Gly^7-Leu^8 (O), (leu)enkephalin (L), and met-enkephalin-Arg^6-Phe^7 (H). **b**: PE products in bovine adrenal medulla. Illustration of PE products identified in bovine adrenal medulla.[18-20]

homogenous secretory vesicles that can be isolated with high yield. A further advantage of our approach towards identifying relevant processing enzymes was to use full-length recombinant enkephalin precursor as substrate.[3,4] Thus, if a protease cleaves prohormones but not peptide substrates, full-length prohormone substrate would insure its detection.

Importantly, use of enkephalin precursor and chromaffin granules as a model secretory vesicle system has resulted in our discovery of novel prohormone processing enzymes, as follows: (a) the novel "prohormone thiol protease" (PTP),[2] (b) the subtilisin-like PC1/3 and PC2 proteases (PC=prohormone convertase),[5] and (c) the 70 kDa aspartic proteinase.[6] The success of this research has relied on the use of interdisciplinary approaches in protein/peptide biochemistry, enzymology, molecular biology, and cell biology. These studies have elucidated several enzymatic components involved in the biochemical processing pathway for converting prohormones to active neuropeptides.

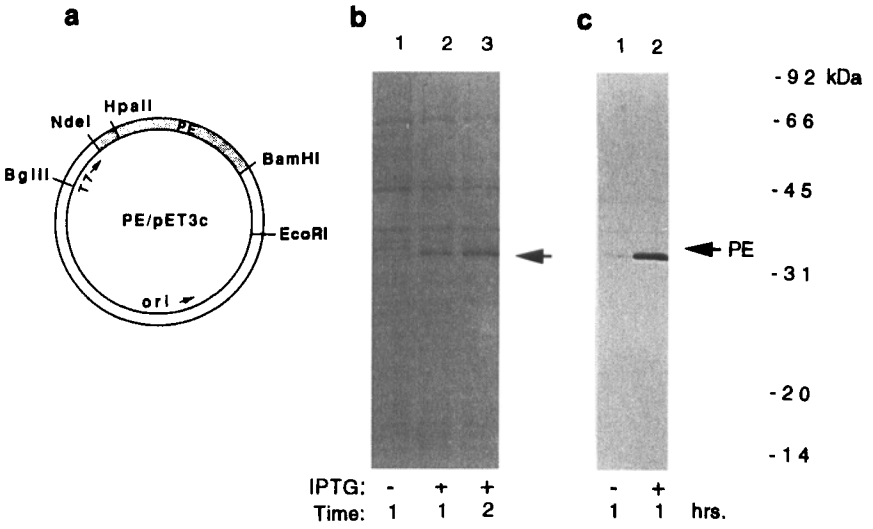

FIGURE 2. Expression of PE in *E. coli*. **a**: PE was expressed using the pET3c/PE expression construct, as illustrated.[21] **b**: Induction of PE was detected by SDS-PAGE gels stained with Coomassie blue. The arrow indicates IPTG (isopropyl-1-thio-β-D-galactopyranoside) induction of a new band corresponding in molecular size to PE. **c**: Western blots of PE expression. Immunoblots with the PE-18 monoclonal antibody (gift from Dr. B. Spruce)[20] demonstrate expression of recombinant PE (indicated by arrow).

IN VITRO IDENTIFICATION OF PROENKEPHALIN PROCESSING ENZYMES

Prohormone Thiol Protease

Our studies have identified the PTP as the major proteolytic activity responsible for PE processing in chromaffin granules.[4,7] PTP converts recombinant enkephalin precursor to intermediates and final met-enkephalin peptide products that are known to be produced *in vivo* (FIG. 1). PTP possesses correct cleavage specificity for cleavage at the paired basic residues (Lys-Arg, Arg-Arg-, Lys-Lys, Arg-Lys) and also cleaves monobasic Arg that is sometimes used as a processing site.[8–10] Cleavage at these basic residues results in production of the final product, met-enkephalin. PTP is active at the intragranular pH of 5.5,[4] indicating that it would be fully active *in vivo* in the intragranular acidic environment. Importantly, biochemical characteristics and peptide microsequencing of PTP indicate that it is a novel cysteine protease.[4,8–11]

Detailed analysis of PTP processing of authentic PE was examined with recombinant PE obtained by high level expression in *E. coli* using the pET3c expression vector (FIG. 2).[11] With *in vitro* PE concentrations at estimated *in vivo* PE levels of approximately 10^{-5} to 10^{-4} M, *in vitro* assays demonstrated that PTP converted purified recombinant PE (12 μM) to intermediates that represent those present *in vivo* (FIG. 3). PTP converted PE to 22.5, 21.7, 12.5, and 11.0 kDa intermediates that represented

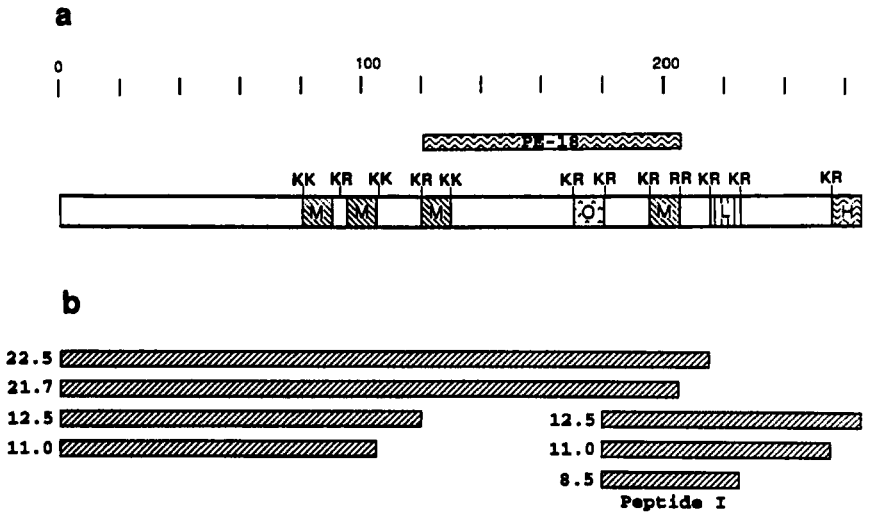

FIGURE 3. Proenkephalin products generated by the PTP. **a**: Immunoblots used the PE-18 monoclonal antibody that recognizes the mid-region of PE. **b**: *In vitro* PE products generated by PTP are illustrated. Processing products were predicted based on molecular size, reactivity with the PE-18 monoclonal antibody, and peptide microsequencing.[11]

TABLE 1. PTP Cleavage of Peptide-MCA Substrates[a]

Substrate	Proteolytic Activity μmol AMC[b]/h/mg −APM	+APM
Z-Phe-Arg-MCA	1,176	1,915
Bz-Arg-MCA	7	24
Boc-Gln-Gly-Arg-MCA	6	84
Bz-Val-Leu-Lys-MCA	695	885
Z-Arg-Arg-MCA	16	49
Boc-Gln-Arg-Arg-MCA	15	118
Boc-Gly-Arg-Arg-MCA	7	42
Z-Arg-Val-Arg-Arg-MCA	19	50
Boc-Gly-Lys-Arg-MCA	21	73
Boc-Glu-Lys-Lys-MCA	7	26

[a]PTP activity was measured with peptide-MCA substrates (25 μM) in the absence and presence of aminopeptidase M (APM). Greater apparent proteolytic activity in the presence of APM indicates PTP cleavage at the COOH-terminal side of the pair and between or at the NH_2-terminal side of the pair.

[b]aminomethylcoumarin.

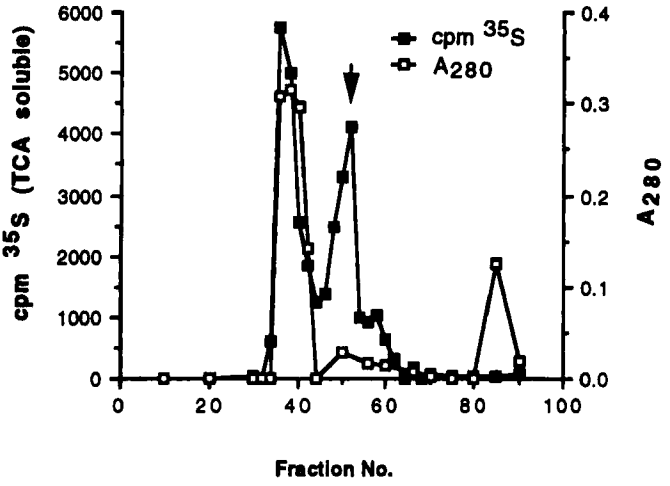

FIGURE 4. Sephacryl S200 chromatography of a soluble extract from chromaffin granules. The soluble fraction of chromaffin granules was subjected to Sephacryl S200 chromatography following concanavalin A affinity chromatography.[5,6] Enkephalin precursor-cleaving activity (■) is expressed as TCA (trichloroacetic acid) soluble [^{35}S]Met (cpm) generated by an aliquot (5 μL) of each column fraction. Absorbance at 280 (□) was also measured. The arrow indicates the 70 kDa peak of activity.

NH_2-terminal fragments of PE, as assessed by peptide microsequencing; differences in masses of the 22.5, 21.7, 12.5, and 11.0 kDa products reflect PTP processing of PE within the COOH-terminal region of PE. Products of 12.5, 11.0, and 8.5 kDa were generated by cleavage between Lys-Arg at the COOH-terminus of met-enkephalin-Arg^6-Gly^7-Leu^8. The 8.5 kDa product may represent peptide I, which is present in adrenal medulla. Cleavage studies of recombinant PE (FIG. 3) and with peptide-3-methylcoumarinamide (MCA) substrates (TABLE 1) both indicated that PTP cleaves at three sites of the pair of basic residues: (1) at the NH_2-terminal side of the pair, (2) between the two basic residues of the pair, and (3) at the COOH-terminal side of the pair. Most of the PTP cleavages occurred between the two basic residues and on the NH_2-temrinal side of the pair. By contrast, the PC1/3 and PC2 proteases demonstrate cleavage primarily at the COOH-terminal side of the pair.[5]

Kinetic studies indicated that PTP has a $K_{m(app)}$ value of 18.6 μM PE and $V_{max(app)}$ of 1.98 mmol/h/mg.[11] These kinetic constants are consistent with estimated intragranular levels of PE and PE-derived products. These results demonstrating PTP conversion of PE to intermediates resembling those *in vivo*, and kinetics that are compatible with *in vivo* processing of PE, implicate a role for PTP in PE processing.

Endogenous PC1/3 and PC2

The field of prohormone processing has recently given much attention to the subtilisin-like PC1/3 and PC2 proteases[13–15] that possess homology to the yeast Kex2

TABLE 2. PC1/3 and PC2 Cleavage of Peptide-MCA Substrates[a]

Peptide-MCA Substrate	Activity, nmol AMC/h/m			
	PC1/3		PC2	
	−APM	+APM	−APM	+APM
Boc-Arg-Val-Arg-Arg-MCA	351	345	762	747
pGlu-Arg-Thr-Lys-Arg-MCA	342	329	683	658
Boc-Gly-Lys-Arg-MCA	208	300	515	552
Boc-Phe-Val-Arg-MCA	144	144	416	416
Boc-Gln-Arg-Arg-MCA	125	192	345	364
Boc-Gly-Arg-Arg-MCA	116	118	336	360
Boc-Val-Leu-Lys-MCA	144	188	272	333
H-Arg-Gln-Arg-Arg-MCA	25	461	123	577
Boc-Glu-Lys-Lys-MCA	32	51	81	104
Boc-Gln-Gly-Arg-MCA	2	29	6	36

[a]Activity of PC1/3 and PC2 was measured with peptide-MCA substrates (100 μM) in the absence and presence of aminopeptidase M (APM). The lack of change in apparent proteolytic activity with APM indicates cleavage by PC1/3 and PC2 on the COOH-terminal side of the paired basic residues.

processing protease. These mammalian proteases were cloned by polymerase chain reaction PCR based on predicted active-site homology to the yeast Kex2 protease that processes pro-α-mating factor.[15] Although many laboratories have studied recombinant PC1/3 and PC2,[12–14] endogenous PC1/3 and PC2 that are naturally expressed *in vivo* have only been recently characterized. Many laboratories found tissue PC1/3 and PC2 to be difficult to purify. However, we have successfully purified high amounts of native PC1/3 and PC2;[5] characterization of these enzymes indicated that they indeed possess appropriate properties as candidate prohormone-processing enzymes.

During large-scale purification of PTP, a new peak of enkephalin precursor activity appeared on the Sephacryl S200 column (FIG. 4). This new 70 kDa peak was found to be represented by the prohormone convertases PC1/3 and PC2,[5] as well as by the 70 kDa aspartic proteinase (described in the next section).[6] PC1/3 and PC2 were purified from chromaffin granules by Concanavalin A-Sepharose, Sephacryl S-200, pepstatin A-agarose, and anti-PC1/3 or anti-PC2 immunoaffinity resins. PC1/3 and PC2 were monitored during purification by measuring proteolytic activities with [^{35}S]enkephalin precursor and Boc-Arg-Val-Arg-Arg-MCA substrates, and by following PC1/3 and PC2 immunoreactivity. Purified PC1/3 and PC2 each show a molecular weight of 66 kDa. PC1/3 and PC2 cleaved paired basic and monobasic sites within peptide-MCA substrates, with Boc-Arg-Val-Arg-Arg-MCA and pGlu-Arg-Thr-Lys-Arg-MCA as the most effectively cleaved peptides tested (TABLE 2). PC1/3 and PC2 showed pH optima of 6.5 and 7.0, respectively. Kinetic studies indicated apparent K_m values for hydrolysis of Boc-Arg-Val-Arg-Arg-MCA as 66 and 40 μM, with V_{max} values of 255 and 353 nmol/h/mg for PC1/3 and PC2, respectively. Inhibition by EGTA and activation by Ca^{2+} indicated PC1/3 and PC2 as Ca^{2+}-dependent proteases.

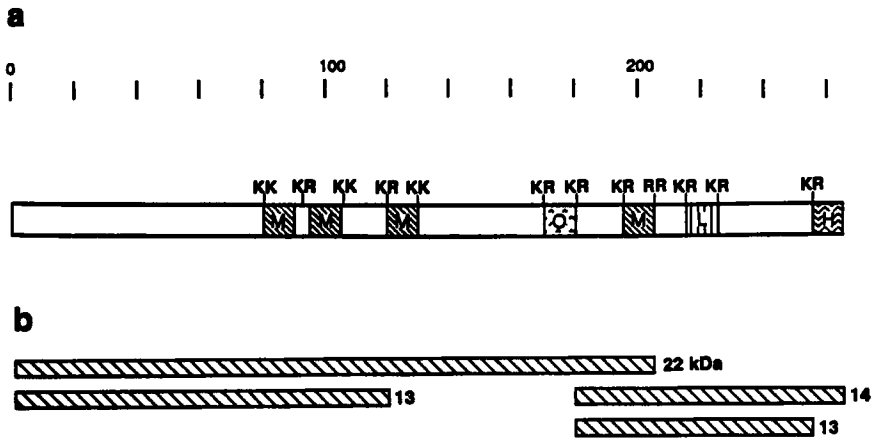

FIGURE 5. Processing of PE by the 70 kDa aspartic proteinase (preliminary results). **a**: PE structure. PE is illustrated showing the active opioid peptides met-enkephalin (M), met-enkephalin-Arg^6-Gly^7-Leu^8 (O), leu-enkephalin (L), and met-enkephalin-Arg^6-Phe^7 (H). **b**: Predicted PE products. PE products generated by the 70 kDa aspartic proteinase were identified based on NH_2-terminal peptide microsequencing of PE products, molecular size of products of SDS-PAGE, and theoretical molecular sizes of PE fragments from the known primary sequence.[22]

In addition, PC enzymes were activated by DTT and inhibited by thiol-blocking reagents, *p*-hydroxymercuribenzoate and mercuric chloride. These results illustrate the properties of endogenous PC1/3 and PC2 as prohormone-processing enzymes.

Of particular interest was the finding that PE was cleaved very slowly by PC1/3 and PC2 (unpublished observations),[16] but PE was readily cleaved by the PTP.[11] In fact, PTP was 10,000 times more efficient (based on V_{max}) in cleaving PE than PC1/3 or PC2.[11] Clearly, the prohormone-processing field must consider PTP as an important primary-processing enzyme. Future enzyme-antisense expression (using antisense oligonucleotides or antisense cDNAs) in chromaffin cells that process PE will be needed to compare the relative roles of PTP, PC1/3, and PC2 in the synthesis of met-enkephalin.

70 kDa Aspartic Proteinase

Studies of pro-opiomelanocortin (POMC) processing activity in pituitary secretory vesicles have indicated a 70 kDa aspartic proteinase as a primary POMC-converting enzyme (PCE).[17] PCE cleaves POMC at paired basic residues to generate appropriate ACTH and β-endorphin-related peptide products. Although purification of this pituitary aspartic proteinase was reported 10 years ago, it has not yet been cloned. The low amounts of enzyme in pituitary have precluded successful peptide microsequencing for molecular cloning.

During these studies of PE processing, we found that chromaffin granules (CG) are a highly enriched source of the 70 kDa aspartic proteinase that processes PE and

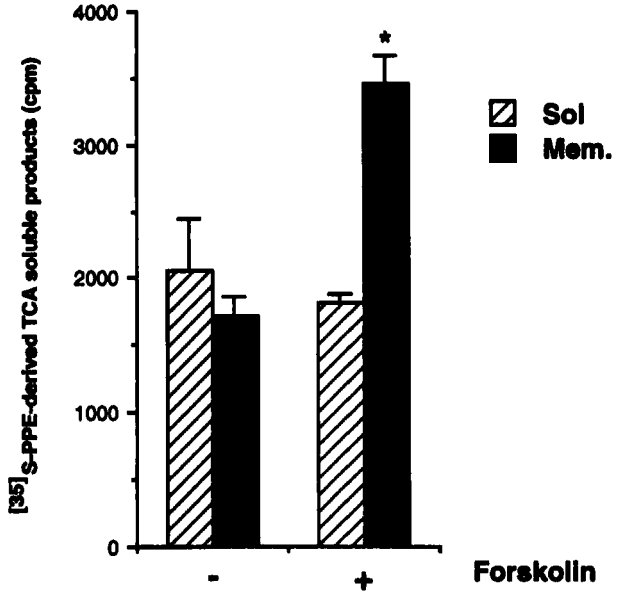

FIGURE 6. Elevated enkephalin precursor-cleaving activity in chromaffin granules. After 72 hours of forskolin treatment of chromaffin cells, the membrane and soluble components of chromaffin granules were isolated. Enkephalin precursor cleaving activities in membrane and soluble granule components from control and forskolin-treated cells were measured as described,[7] and expressed as $\bar{x} \pm$ SD. The asterisk (*) indicates statistically significant; $p < 0.005$ (Student's t test).

POMC.[6] Of significance is that we can purify 50 times more of the aspartic proteinase (100-200 μg) from chromaffin granules than can be achieved from pituitary. The 70 kDa aspartic proteinase showed optimum activity at pH 5.5. It was potently inhibited by pepstatin A, a selective aspartic proteinase inhibitor, but not by inhibitors of serine, cysteine, or metalloproteinases. Lack of inhibition by Val-D-Leu-Pro-Phe-Val-D-Leu, an inhibitor of pepsin, cathepsin D, and cathepsin E, distinguishes the CG aspartic proteinase from classical members of the aspartic proteinase family. The CG aspartic proteinase cleaved recombinant PE between the Lys_{172}-Arg_{173} pair located at the COOH terminus of met-enkephalin-Arg^6-Gly^7-Leu^8 (FIG. 5). The importance of full-length prohormone as substrate was demonstrated by the enzyme's ability to hydrolyze [^{35}S]PE and [^{35}S]POMC, and its inability to cleave tri- and tetrapeptide substrates. These results provide evidence for the role of an aspartic proteinase in PE and prohormone processing.

Our success in purifying high amounts of the 70 kDa aspartic proteinase will now allow peptide microsequencing and molecular cloning to be achieved. Development of cDNA and antibody reagents will facilitate progress in analyzing the role of this aspartic proteinase in prohormone processing.

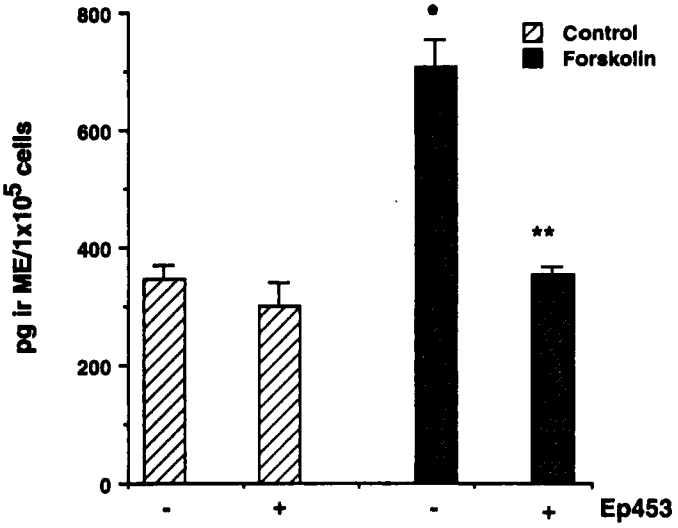

FIGURE 7. Blockade of the forskolin-stimulated rise in cellular met-enkephalin by a cysteine protease inhibitor of the PTP. Chromaffin cells were incubated with the cysteine protease inhibitor Ep453 24 hours prior to addition of forskolin. After continued incubation with Ep453 and forskolin for 72 hours, cells were harvested as acid extracts, and met-enkephalin was measured by radioimmunoassay, as described.[7] Met-enkephalin levels (picograms of peptide/ 1×10^5 cells) are expressed as $\bar{x} \pm$ SD. One asterisk (*) indicates $p < 0.001$, and two asterisks (**) indicate $p < 0.001$, as measures of statistical significance. ME = met-enkephalin.

CELLULAR ANALYSIS OF PTP AS A PRIMARY PE PROCESSING ENZYME

Identification *in vitro* of PTP as the major PE-processing enzyme in chromaffin granules led to the prediction that, during stimulation of met-enkephalin production in chromaffin cells, PTP may be coregulated. Therefore, cellular analyses of cAMP regulation of both PTP and met-enkephalin levels, and evaluation of PTP in elevated PE processing using a cysteine protease inhibitor of PTP were conducted.

In these studies,[7] PTP was examined during elevation of cellular met-enkephalin by forskolin, a direct activator of adenylate cyclase that produces cAMP. Treatment of chromaffin cells with forskolin for 72 hours increased enkephalin precursor-cleaving activity in isolated chromaffin granules by 170-180% over controls (100%) (FIG. 6). The elevated activity was inhibited by E-64c, a potent inhibitor of PTP and cysteine proteases; however, the activity was not inhibited by serine or aspartic protease inhibitors. The elevated activity was identified as PTP based on immunoprecipitation by anti-PTP immunoglobulins. Stimulation of PTP synthesis was involved in the forskolin-induced increase in PTP activity, as demonstrated by a ten-fold increase in [^{35}S]PTP pulse labeling in forskolin-treated chromaffin cells, as well as by Western blots of PTP.

TABLE 3. Relative Levels of PTP, PC1/3, PC2, and 70 kDa Aspartic Proteinase Purified from the Soluble Fraction of Chromaffin Granules[a]

Protease	Amount of Enzyme Purified from 600 Adrenal Glands	Percent Yield
PTP	1-2 μg	35%
PC1/3	100 μg	0.5-1%
PC2	500 μg	5%
70 kDa aspartic proteinase	250 μg	9%

[a]Results for recovery of purified proenkephalin-processing enzymes from chromaffin granules were compiled from reports of the purification of PTP,[4] PC1/3 and PC2,[6] and the 70 kDa aspartic proteinase.[5]

Importantly, the forskolin-mediated rise in cellular (Met)enkephalin levels was completely blocked when cells were preincubated with the cysteine protease inhibitor, Ep453 (FIG. 7), which is known to be converted by intracellular esterases to the more effective inhibitor, E-64c. Both E-64c and Ep453 inhibit PTP, with E-64c being more potent.[9,10] These results demonstrate a regulatory role for PTP in PE processing in chromaffin cells, and indicate that met-enkephalin formation and PTP are both controlled by cAMP.

TABLE 4. Cleavage of Recombinant Prohormone by Chromaffin Granule-Processing Proteases[a]

	Prohormones		
Proteases	PE	pNPY	POMC
PTP	Yes	Yes	Yes
	(10 min)	(1 h)	(6 h)
PC1/3	?	No	No
	(24 h)	(24 h)	(24 h)
PC2	?	No	No
	(24 h)	(24 h)	(24 h)
70 kDa aspartic proteinase	Yes	No	Yes
	(6, 24 h)	(24 h)	(6, 24 h)

[a]Recombinant prohormones proenkephalin (PE), proneuropeptide Y (pNPY), and pro-opiomelanocortin (POMC) were expressed in *E. coli* and purified. Prohormones (at 10^{-5} M) were incubated with purified PTP (1 ng), PC1/3 (1 μg), PC2 (1 μg), or 70 kDa aspartic proteinase (1 μg) for different time periods at 37° C. The degree of prohormone cleavage was assessed by densitometry of SDS-PAGE gels of processing assays.

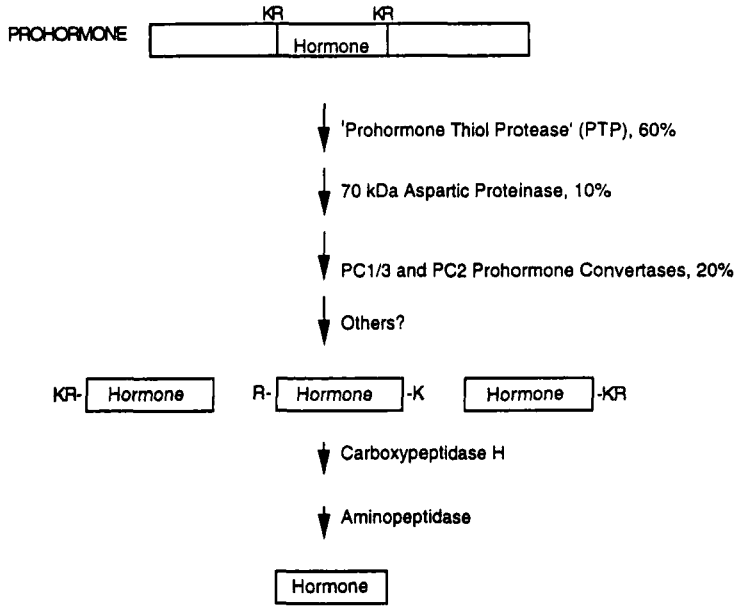

FIGURE 8. Proenkephalin-processing enzymes in chromaffin granules: a model for prohormone processing. A hypothetical prohormone is indicated, containing one copy of the peptide hormone. Candidate prohormone-processing enzymes found in chromaffin granules for PE processing are indicated in a hypothesized prohormone pathway involving several endoproteases and exoproteases. The percent of each protease that contributes to total enkephalin precursor-cleaving activity in chromaffin granules is indicated. It is noted that proportions of relative processing enzyme activities may vary depending on the prohormone substrate and cell type. K=Lys; R=Arg; PC=prohormone convertase.

DIFFERENTIAL LEVELS OF PROCESSING ENZYMES AND PRECURSOR SELECTIVITY

An interesting observation from the studies on PE-processing enzymes in chromaffin granules (as shown in FIG. 8) is that the primary processing protease, PTP, is the least abundant protease (when considering micrograms of enzyme protein) compared to the PC and aspartic proteinases (TABLE 3). When yields of purified proteases are compared from the same number of bovine adrenal glands, purification from six hundred glands provides 1-2 micrograms of PTP,[4] whereas several hundred micrograms, 100-500 μg, of purified PC1/3,[5] PC2,[5] and 70 kDa aspartic proteinase[6] are obtained. Thus, the high-specific activity of PTP for PE compared to the other candidate processing enzymes accounts for results indicating PTP as the primary PE-processing enzyme in chromaffin granules.

The efficient processing of PE by PTP, and lesser processing by PC1/3, PC2, and the 70 kDa aspartic proteinase leads to the important question of whether processing enzymes possess selectivity for particular prohormone substrates. Although prohor-

mones possess identical paired basic residue cleavage sites, their primary structures differ. Thus, although the processing proteases recognize the dibasic residue-cleavage sites, it was not known if each protease prefers certain precursor substrates.

Our laboratory has discovered that the processing proteases possess preference for prohormone substrates. Comparison of the ability of PTP, PC1/3, PC2, and the 70 kDa aspartic proteinase to cleave recombinant prohormones PE, POMC, and proneuropeptide Y indicated that clear substrate preferences exist (TABLE 4). For example, PE was cleaved most readily by PTP, was somewhat cleaved by the 70 kDa aspartic protease, and cleavage by PC1/3 and PC2 could not be detected. By contrast, processing of POMC was most efficiently accomplished by the 70 kDa aspartic proteinase. Thus, distinct proteases may be responsible as primary processing enzymes for different prohormones.

SUMMARY AND RELEVANCE TO MEDICINE

Our discovery of precursor preference of processing enzymes indicates possible development of future drugs that target specific proteases uniquely associated with processing of a particular prohormone. For example, selective processing of PE by the PTP suggests that future evaluation of modulation of PTP through central nervous system drug reagents may modify the endogenous analgesic effects of the enkephalins. With respect to blood pressure, neuropeptide Y (NPY) that is released from sympathetic nerve terminals is a strong vasoconstrictor. Our finding that only PTP (not PC1/3, PC2, or the aspartic proteinase) possesses the ability to convert pro-NPY to NPY suggests that investigation of inhibitors of peripheral PTP in blood pressure regulation should be initiated. Overall, elucidation of the proteolytic components required in prohormone processing will provide insights into the molecular mechanisms of human disease.

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