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The Processing Proteases Prohormone Thiol Protease, PC1/3 and PC2, and 70-kDa Aspartic Proteinase Show Preferences among Proenkephalin, Proneuropeptide Y, and Proopiomelanocortin Substrates

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Proteases of cysteine, aspartic, and subtilisin classes have been indicated as candidate prohormone processing enzymes. The chromaffin granule proenkephalin processing proteases have been characterized as the novel cysteine protease prohormone thiol protease (PTP), a 70-kDa aspartic proteinase, and the subtilisin-like PC1/3 and PC2 enzymes. The goal of this study was to assess whether these processing proteases possess preference(s) for prohormone substrates. The recombinant prohormones proenkephalin, proneuropeptide Y (pro-NPY), and proopiomelanocortin (POMC) were expressed in *Escherichia coli* using the T7 expression system and purified for *in vitro* processing studies. Results indicated that the chromaffin granule processing proteases possess selectivity for particular prohormones. PTP preferred proenkephalin, with good cleavage of pro-NPY and slow processing of POMC. In contrast, the 70-kDa aspartic proteinase cleaved POMC most readily, with cleavage of proenkephalin and some processing of pro-NPY. PC1/3 and PC2 preferred POMC among the prohormones tested. Importantly, these results indicate that prohormone selectivity of processing proteases may be an important factor in predicting the primary and rate-limiting protease(s) required for processing a particular prohormone.
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Peptide hormones and neurotransmitters are synthesized as protein precursors that require proteolytic processing to liberate the biologically active neuropeptides (1–3). Prohormone processing occurs primarily within the secretory vesicle (1–3); therefore, the majority of the proteolytic processing enzymes should be present within the secretory vesicle organelle.

Our studies have investigated proenkephalin processing enzymes within bovine chromaffin granules as a model secretory vesicle system for prohormone processing. Utilizing recombinant enkephalin precursor as substrate, proteases of three different mechanistic classes were isolated and characterized with respect to proenkephalin processing. The novel cysteine protease known as prohormone thiol protease (PTP)[2] is a 33-kDa glycoprotein that represents the majority (60%) of total enkephalin precursor cleaving activity in chromaffin granules (1, 4–7). Other activities involved in proenkephalin processing were identified as the subtilisin-like PC1/3 and PC2 (PC, prohormone convertase) proteases of 68 kDa (9) and a 70-kDa aspartic proteinase (8). All of these processing proteases (4–9) possess specificity for cleavage at paired basic residue prohormone processing sites, are optimally active near the intragranular pH of 5.5–5.8, and are glycoproteins. The primary role for PTP in proenkephalin processing in chromaffin cells was demonstrated by increased PTP during cAMP-induced elevation of enkephalin peptide levels and by blockade of the cAMP-induced rise in enkephalin levels when cells were pretreated with a cysteine protease inhibitor of PTP (13).

Interestingly, comparison of the primary processing proteases in different secretory vesicle systems for proenkephalin, proopiomelanocortin (POMC), and proinsulin indicates that each prohormone appears to be primarily processed by a different protease(s). Whereas

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[2] Abbreviations used: PTP, prohormone thiol protease; PC, prohormone convertase; POMC, proopiomelanocortin; PCE, POMC-converting enzyme; pro-NPY, proneuropeptide Y; PE, proenkephalin; IPTG, isopropyl-β-thiogalactopyranoside; LPH, lipotropin hormone.

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proenkephalin appears to be mainly processed by the cysteine protease PTP in chromaffin granules (1, 4–9), POMC in secretory vesicles of pituitary intermediate lobe appears to be primarily processed by the aspartic proteinase POMC-converting enzyme (PCE) (14). PC2 was observed as a smaller POMC cleaving activity compared to PCE in pituitary secretory vesicles (15). In contrast, proinsulin processing in insulinoma granules involves the subtilisin-like proteases PC2 and PC1/3 (16, 17). These results suggest that processing of specific prohormones may involve distinct proteases of different mechanistic classes.

The unique primary sequences of prohormones raise the question of whether each prohormone may be preferentially processed by certain processing enzyme(s). Knowledge of possible precursor preference(s) of these proteases may provide inferences into cellular mechanisms of prohormone processing. Therefore, this study has compared the relative processing of proenkephalin, proneuropeptide Y (pro-NPY), and POMC by the candidate processing enzymes PTP, the 70-kDa aspartic proteinase, and the PC1/3 and PC2 subtilisin-like proteases. These proteases are colocalized with enkephalins (18) and NPY (19) in chromaffin granules of adrenal medulla and are also known to be present in pituitary (1–3; Hook *et al.,* manuscript in preparation) where POMC processing occurs. Results from this study suggest that each prohormone may be selectively processed by certain processing enzymes and that each processing protease demonstrates preference(s) for prohormone substrates.

EXPERIMENTAL PROCEDURES

Purification of PTP, 70-kDa aspartic proteinase, and PC1/3 and PC2 from bovine chromaffin granules. The cysteine, aspartic, and subtilisin-like processing proteases with enkephalin precursor cleaving activities were purified from bovine adrenal medullary chromaffin granules as previously described (4, 8, 9). Briefly, the soluble fraction of chromaffin granules was chromatographed on concanavalin A–Sepharose, which resulted in elution of enkephalin precursor cleaving activity (assayed as described previously with ^{35}S-(Met)–preproenkephalin substrate (4, 8, 9)) in the concanavalin A-bound fraction. Subsequent molecular sieving on a Sephacryl S200 column separated the peak of PTP (eluting at the void volume) from a peak of apparent M_r of 70 kDa that contains the aspartic and subtilisin proteases. PTP from the Sephacryl S200 step was purified by chromatofocusing followed by thiopropyl–Sepharose (4). The 70-kDa aspartic proteinase was purified by pepstatin A affinity chromatography of the 70-kDa peak obtained from the Sephacryl S200 column, as previously described (8). The unbound fractions from the pepstatin A column contain PC1/3 and PC2, as assessed by anti-PC1/3 and anti-PC2 immunoblots (9). Purification of PC1/3 and PC2 was achieved by immunoaffinity chromatography with anti-PC1/3 and anti-PC2 immunoaffinity columns, as previously described (9). Purification from chromaffin granules from 600 bovine adrenal glands provided approximately 1–3 μg PTP (4), 200 μg 70-kDa aspartic proteinase (8), 200 μg PC2 (9), and 100 μg PC1/3 (9). The greater degree of purification required to obtain purified PTP (88,000-fold) (4), compared to the PC proteases (50- to 100-fold) (9) or aspartic proteinase (350-fold) (8), indicates the very high specific activity of PTP which is present at much lower concentrations within the chromaffin granule compared to the other processing proteases. PTP's high specific activity (4) is one factor that accounts for PTP as the major enkephalin precursor cleaving activity in chromaffin granules.

Expression and purification of recombinant proenkephalin, pro-NPY, and POMC. Expression of prohormones (Fig. 1a) in *Escherichia coli* utilized the T7 expression system with the pET3c vector (Fig. 1b), as originally described by Studier *et al.* (12). Using the rat preproenkephalin cDNA (20), the DNA fragment encoding proenkephalin (PE) (with signal sequence deleted) was subcloned into *Nde*I and *Bam*HI sites of the pET3c vector, as described previously (7), to generate the PE/pET3c construct. Pro-NPY and POMC expression constructs were generated by PCR amplification of pro-NPY from the rat prepro-NPY cDNA (21) and by PCR amplification of POMC from the porcine prePOMC cDNA (22), with incorporation of *Nde*I and *Bam*HI sites at 5′- and 3′-ends, respectively. PCR of pro-NPY utilized the primers 5′-AAACATATGTACCCCTCCAAGCCG-3′ and 5′-AAAGGATCCCCATCACCACATGGAAG-3′; PCR of POMC utilized the primer 5′-AAACATATGTGGTGCTTGGAGAGCAGCCAGTGTCAG-3′. PCR were conducted as described by the manufacturer's protocol (Perkin–Elmer) in 100 μl containing 1 ng cDNA template and primers at 0.2 μM, with thermocycling consisting of 30 cycles of 1 min each at 94, 50, and 72°C. After digestion of PCR products with *Nde*I and *Bam*HI, ligation into *Nde*I and *Bam*HI sites of the pET3c vector, and amplification of expression constructs in DH5α *E. coli* cells, pro-NPY/pET3c and POMC/pET3c plasmid constructs were subjected to DNA sequencing (as previously described (23)) to confirm authentic pro-NPY and POMC nucleotide sequences generated by PCR.

Expression of prohormones in BL21(DE3) *E. coli* induced by IPTG (isopropyl-β-thiogalactopyranoside) was conducted as previously described for expression of proenkephalin (7). Pro-NPY expression was monitored by Coomassie blue protein staining of SDS–PAGE gels and anti-NPY immunoblots. POMC expression was detected by immunoblots with anti-*N*-POMC (24) (monoclonal antibody against *N*-POMC was a gift from Dr. P. Crine, University of Montreal) or anti-β-LPH (anti-β-lipotropin hormone serum was from the National Pituitary Agency, Gaithersburg, MD).

Purification of proenkephalin expressed in *E. coli* was performed by chromatography by DEAE–Sepharose, preparative SDS–PAGE, and reverse-phase HPLC, as described previously (7). Purification of pro-NPY expressed in *E. coli* was achieved by sonicating cells (from a 10-liter culture) in 50 mM Tris–HCl, pH 7.5 (buffer A), collecting solubilized pro-NPY by centrifugation (27,000*g* for 30 min), and purifying pro-NPY on DEAE–Sepharose and Superose-12 columns. Pro-NPY (monitored by SDS–PAGE gels and anti-NPY immunoblots) was eluted from the DEAE–Sepharose column by a 0–500 mM NaCl gradient (500 ml gradient) in buffer A; subsequent chromatography on the Superose-12 HR 10/30 FPLC column, with buffer A as eluant, resulted in purified pro-NPY.

Purification of POMC was conducted by resuspension of *E. coli* cells from a 5-liter culture in 50 mM Mops–HCl, pH 6.5, and 6.0 M urea (buffer B), sonication, and collection of solubilized POMC by centrifugation (27,000*g* for 30 min). Chromatography on DEAE–Sepharose with elution of POMC by a 0–500 mM NaCl gradient (400 ml) in buffer B, followed by preparative SDS–PAGE of POMC on a Bio-Rad Model 491 preparative electrophoresis cell (performed as described previously (7)), resulted in purification of POMC to apparent homogeneity.

Incubation of processing proteases with prohormones. Each processing enzyme was incubated with prohormones under established optimum buffer conditions reported for PTP (4, 7), 70-kDa aspartic proteinase (8), and PC1/3 and PC2 (9). Prohormones (at approximately 2–10 μM) were incubated with PTP (1 ng), 70-kDa aspartic proteinase (1 μg), PC1/3 (1 μg), or PC2 (1 μg) for 0 to 24 h at 37°C. The extent of proenkephalin, pro-NPY, and POMC processing was assessed by SDS–PAGE, electrophoretic transfer to PVDF membranes with amido black staining of precursors, and densitometry for

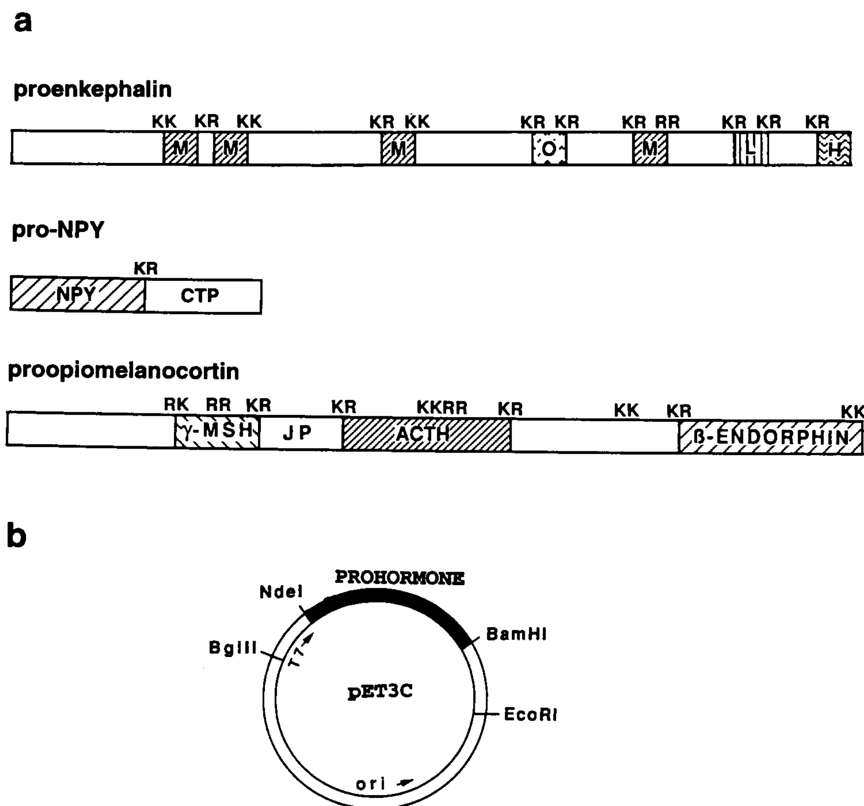

FIG. 1. Prohormone structures for expression. (a) Prohormone structures. Proenkephalin, proneuropeptide Y (pro-NPY), and proopiomelanocortin (POMC) prohormone structures are illustrated. Proenkephalin contains four copies of (Met)enkephalin (M), one copy of (Leu)enkephalin (L), and the enkephalin-related peptides known as the octapeptide (O) and heptapeptide (H). Pro-NPY contains NPY- and COOH-terminal peptide (CTP) sequences. POMC contains ACTH and β-endorphin-related bioactive peptides. (b) Expression with the pET3c vector. Prohormone cDNAs were subcloned at *Nde*I and *Bam*HI restriction sites of the pET3c vector (12), for expression in *E. coli.*

quantitation of prohormone remaining after incubation. Duplicate or triplicate assays varied by less than 10%.

These *in vitro* levels of processing enzymes were chosen because the proteases at these concentrations were previously shown to effectively cleave proenkephalin or peptide–MCA substrates (4–9). It is of interest to note that the high specific activity of PTP (4), compared to the 70-kDa aspartic proteinase (8) or the PC enzymes (9), accounts for efficient prohormone processing by lower levels of PTP.

RESULTS

Expression and purification of proenkephalin, pro-NPY, and POMC. The T7 expression system with the pET3c vector provides expression of high levels of prohormones in *E. coli* (Fig. 1). Pro-NPY and POMC expression in Bl21(DE3) *E. coli* cells was excellent (Fig. 2). Expression of proenkephalin also occurs at high levels, as reported (7). Expression of pro-NPY of 8 kDa occurred after 2 h incubation with IPTG, as detected by anti-NPY immunoblots (Fig. 2a). Expression of 35-kDa POMC occurred with 30 to 120 min incubation with IPTG, as detected by anti-*N*-POMC immunoblots (Fig. 2b). Apparently, two lower-molecular-weight bands of POMC immunoreactivity were detected, indicating some degradation of POMC during expression.

Purification of proenkephalin to apparent homogeneity, as visualized by SDS–PAGE (Fig. 3a), was achieved by DEAE–Sepharose, preparative gel electrophoresis, and HPLC, as described (7). Pro-NPY and

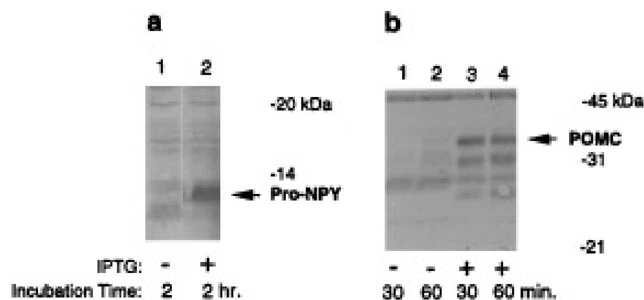

FIG. 2. Expression of Pro-NPY and POMC. (a) Expression of pro-NPY. IPTG-induced expression (2 h) of pro-NPY in BL21(DE3) cells is indicated by induction of the 8-kDa band that is immunoreactive with anti-NPY serum on immunoblots. (b) Expression of POMC. IPTG-induced expression of POMC (30 and 60 min) is indicated by induction of the 35-kDa band that is immunoreactive with anti-*N*-POMC monoclonal antibody on immunoblots. The anti-*N*-POMC antibody recognizes the N-terminal region of POMC (24).

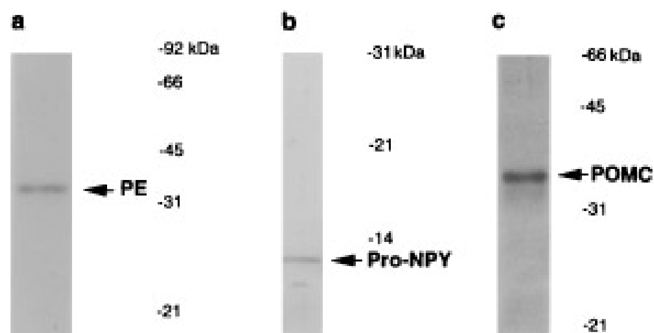

FIG. 3. Purified recombinant proenkephalin, pro-NPY, and POMC. The recombinant prohormones proenkephalin (3 μg) (a), pro-NPY (3 μg) (b), and POMC (4 μg) (c) were purified to apparent homogeneity, as indicated by single bands on SDS–PAGE gels stained for protein with Coomassie blue. Arrows show the indicated prohormones.

POMC were each purified by simple two-step purification procedures. Pro-NPY was purified by DEAE–Sepharose and Superose-12 FPLC columns. Elution of the DEAE–Sepharose column resulted in isolation of a pro-NPY peak at approximately 150 mM NaCl. Subsequent chromatography on a molecular sieving Superose-12 FPLC column resulted in a single band of 8-kDa pro-NPY, as indicated by SDS–PAGE (Fig. 3b). POMC was purified by DEAE–Sepharose and preparative gel electrophoresis. After collection of POMC from the unbound fractions of the DEAE–Sepharose column and preparative gel electrophoresis, SDS–PAGE indicated purification of 35-kDa POMC to apparent homogeneity (Fig. 3c).

Extent of prohormone processing by the chromaffin granule processing proteases PTP, 70-kDa aspartic proteinase, PC1/3, and PC2. *In vitro* incubation of prohormones and processing enzymes utilized prohormones in the micromolar range which is estimated near *in vivo* prohormone concentrations (1, 25, 26). Recombinant proenkephalin (10 μM) was incubated with chromaffin granule processing enzymes in time-course studies to compare relative processing of PE by different proteases (Fig. 4). PTP (1 ng) efficiently processed 50% of PE by 20 min incubation, with 80–90% of PE processed by 1–2 h (Fig. 4A). The 70-kDa aspartic proteinase (1 μg) also cleaved PE, with 50% cleavage of PE occurring after approximately 35 min incubation (Fig. 4B). PC1/3 and PC2 (approximately 1 μg of each enzyme) were incubated together with PE for up to 24 h. The PC enzymes demonstrated little cleavage of PE; even after 24 h incubation, less than 10% of PE was processed (Fig. 4C). The PC enzymes, however, demonstrated high activity with the peptide Boc-Arg-Val-Arg-Arg–MCA as substrate (9). These results suggest that PE is most readily cleaved by PTP, followed by the 70-kDa aspartic proteinase, and little cleavage by the PC enzymes was detected.

Relative processing of pro-NPY (4.6 μM) by the different proteases was also examined (with enzymes at the same concentrations as used for *in vitro* proenkephalin processing) (Fig. 5). Cleavage of pro-NPY by PTP (1 ng) was readily detected by 30 min incubation, with 50% of pro-NPY cleaved by 2.3 h (Fig. 5A). By 6 h, greater than 90% of pro-NPY was processed by PTP. In contrast, the 70-kDa aspartic proteinase and PC enzymes were poor in processing pro-NPY, compared to PTP (Fig. 5B). After 6 h incubation, the PC and aspartic

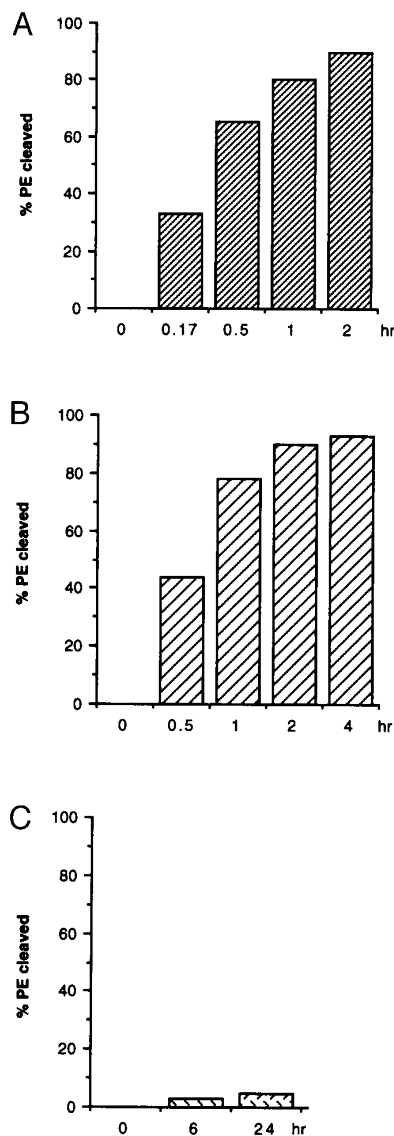

FIG. 4. Proenkephalin processing by PTP, 70-kDa aspartic proteinase, PC1/3, and PC2. Proenkephalin was incubated with PTP (1 ng) (A), 70-kDa aspartic proteinase (1 μg) (B), and PC1/3 plus PC2 (approximately 1 μg of each enzyme), indicated as PC1/2 (C), in time-course studies. Processing reactions were subjected to SDS–PAGE gels with Coomassie blue staining, and densitometry was performed to quantitate the extent of prohormone processed. Percentage PE cleaved by processing enzymes was calculated relative to controls consisting of PE incubated alone (without enzymes).

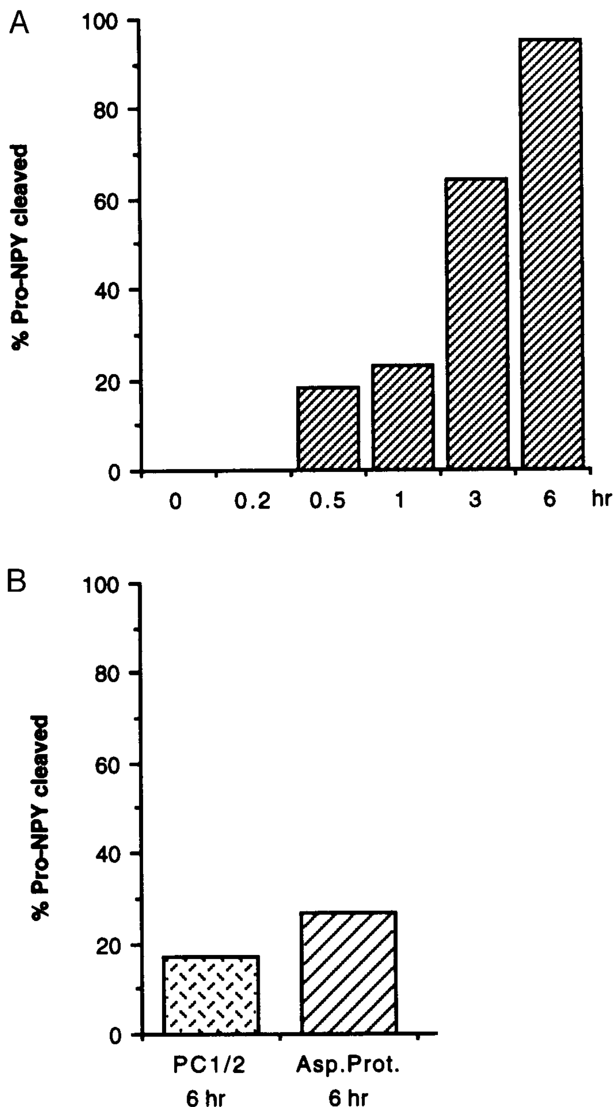

FIG. 5. Pro-NPY processing by PTP, 70-kDa aspartic proteinase, PC1/3, and PC2. Pro-NPY was incubated with PTP (1 ng) (A), 70-kDa aspartic proteinase (1 μg) (B), and PC1/3 plus PC2 (approximately 1 μg of each enzyme), indicated as PC1/2 (B), in time-course studies. The extent of prohormone processed was assessed by densitometry of SDS–PAGE gels of processing reactions. Percentage pro-NPY cleaved by proteases was determined relative to controls consisting of pro-NPY incubated alone (without enzymes).

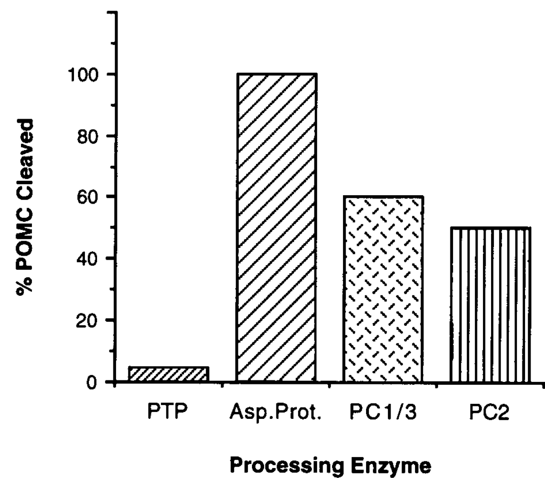

FIG. 6. POMC processing by PTP, 70-kDa aspartic proteinase, PC1/3, and PC2. POMC was incubated with PTP (1 ng), 70-kDa aspartic proteinase (1 μg), PC1/3 (1 μg), and PC2 (1 μg) for 24 h at 37°C. The extent of prohormone processed was assessed by densitometry of SDS–PAGE gels of processing reactions. Controls of POMC incubated without enzyme showed no processing.

proteases cleaved only 17 and 27% of pro-NPY, respectively.

Detection of POMC processing required longer incubation times than that observed for proenkephalin or pro-NPY processing. After 24 h incubation of POMC (2 μM) with each processing enzyme, it was evident that the 70-kDa aspartic proteinase cleaved POMC most readily (Fig. 6). Most of the POMC was completely cleaved by the 70-kDa aspartic proteinase after 24 h. The PC1/3 and PC2 enzymes (1 μg for each enzyme) cleaved approximately 60 and 50% of POMC, respectively, after 24 h. However, PTP incubated with POMC for 24 h resulted in a small degree (5%) of POMC cleaved.

Evidence for selective prohormone processing. Comparison of incubation times necessary for 50% processing of each prohormone (Table I) demonstrates that each prohormone was most readily cleaved by one protease. Proenkephalin was most readily cleaved by PTP, with some cleavage by the 70-kDa aspartic proteinase and a low degree of processing by the PC proteases. Pro-NPY was most efficiently cleaved by PTP, with much slower processing by the 70-kDa aspartic proteinase and the PC enzymes. POMC was most readily cleaved by the 70-kDa aspartic proteinase within 4 h

TABLE I

Incubation Times for 50% *in Vitro* Processing of Prohormones by Chromaffin Granule Processing Proteases

	Incubation time (h) for 50% processing			
Prohormone	PTP	70-kDa aspartic proteinase	PC1/3	PC1/2
Proenkephalin	0.3	0.6	>24	>24
Pro-NPY	2.3	11	17–18	17–18
POMC	>24	<4	20	24

Note. Proenkephalin (10 μM), pro-NPY (4.2 μM), and POMC (2 μM) were incubated with the processing enzymes PTP (1 ng/20 μl), 70-kDa aspartic proteinase (1 μg), or the PC1/3 (1 μg) or PC2 (1 μg) proteases. Time course studies (from Figs. 4–6) allowed estimation of the incubation time required for 50% of the prohormone to be processed.

TABLE II

Processing Rates of Prohormones by Chromaffin Granule Processing Proteases

Prohormone	Processing activity, μmol/h/mg enzyme			
	PTP	70-kDa aspartic proteinase	PC1/3	PC2
Proenkephalin	383	0.23	<0.001	<0.001
Pro-NPY	420	0.012	<0.005	<0.005
POMC	<10	1.4	0.28	0.24

Note. Conditions for *in vitro* incubation of prohormones and processing proteases were identical as described for Table I. Relative processing (μmol/h/mg enzyme) was estimated for each prohormone and protease.

incubation, with some processing by the PC enzymes detected by 20–24 h and a low degree of processing by PTP. However, when relative processing considers levels of enzyme (Table II), PTP appears to readily cleave POMC. The 70-kDa aspartic proteinase also processes POMC with an estimated rate of similar magnitude as PTP (Table II). PC1/3 and PC2 appear to process POMC more slowly.

Of particular interest was the finding that the four processing proteases demonstrated large differences in relative processing rates for the different prohormones (Table II). PTP processing of proenkephalin and pro-NPY were similar, at 383 and 420 μmol/h/mg enzyme, with lower processing of POMC at less than 10 μmol/h/mg. In contrast, the maximum rate of processing by the 70-kDa aspartic proteinase with POMC as substrate was approximately 400 times less than PTP's rate of processing (for proenkephalin and pro-NPY). In addition, the observed rate of PC1/3 and PC2 processing of POMC was approximately one-fourth of that observed for the 70-kDa aspartic proteinase. Comparison of PTP with PC enzymes in this study (Table II) illustrates PTP processing of proenkephalin at a rate approximately 80,000 to 380,000 times greater than that of PC1/3 or PC2. The relative roles of these proteases for *in vivo* prohormone processing will depend on their intracellular concentrations, and possible regulation by endogenous protease inhibitors (1, 27, 28), within secretory vesicles.

Prohormone products. The appropriate processing of these prohormones by PTP, 70-kDa aspartic proteinase, and the PC enzymes has been demonstrated to occur at paired basic residue sites to generate active neuropeptide products. Products generated from recombinant proenkephalin, pro-NPY, and POMC by the processing proteases are summarized (from several studies) in this section to illustrate the abilities of these enzymes to generate relevant neuropeptide products.

PTP *in vitro* converts recombinant proenkephalin to high-molecular-weight intermediates (4, 7) and (Met)-enkephalin (29) that represent the natural proenkephalin products *in vivo* (Fig. 7). Characterization of PTP's cleavage specificity indicates that it cleaves between and at the NH_2-terminal side of paired basic residue sites within the precursor (4–7). PTP also cleaves recombinant pro-NPY to generate a 4-kDa product representing the NH_2-terminal fragment of pro-NPY (30) (Fig. 7), which is compatible with production of NPY by cleavage at the Lys-Arg site separating NPY and the COOH-terminal peptide of pro-NPY (Fig. 1). PTP cleavage sites within POMC have not been determined due to the low level of POMC products that are not detected with current methods.

The 70-kDa aspartic proteinase cleaves proenkephalin between Lys and Arg to generate appropriate intermediates (Fig. 8). The chromaffin granule 70-kDa aspartic proteinase also converts POMC to ACTH- and β-endorphin-related products (31) (Fig. 8) that resemble those produced by the pituitary 70-kDa aspartic proteinase known as PCE (3, 14). Production of these POMC products is compatible with cleavage at paired basic residues. Cleavage of pro-NPY by the 70-kDa

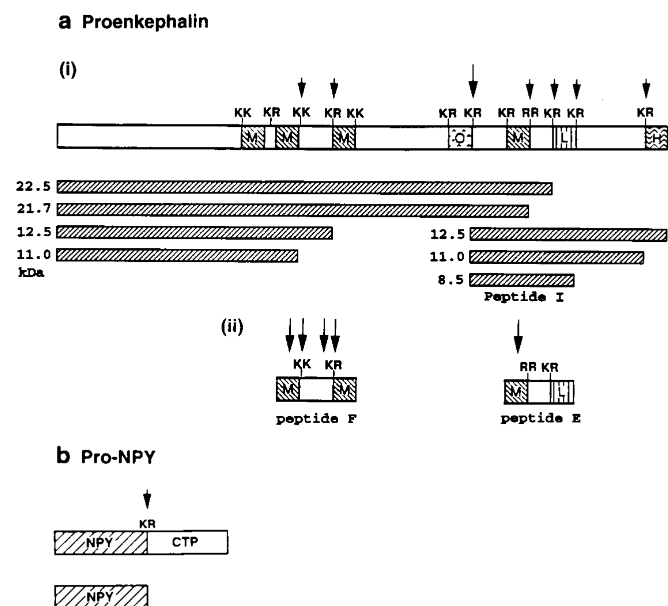

FIG. 7. PTP: Proenkephalin and POMC processing products. (a) Proenkephalin processing. (i) Recombinant proenkephalin (PE) was incubated with purified PTP, and products were identified based on peptide microsequencing, apparent M_r, and reactivity to a monoclonal antibody against the midregion of PE, as described (7, 29). Deduced cleavage sites are indicated by arrows; the cleavage site at the large arrow occurred between the Lys-Arg basic residues. (ii) Peptide F and peptide E were incubated with PTP, products were isolated by HPLC, and their identities were determined by peptide microsequencing, as described previously (29). Cleavage sites are indicated by arrows. (b) Pro-NPY processing. Recombinant pro-NPY was incubated with PTP, and the NH_2-terminal product was identified by peptide microsequencing and apparent M_r, as described (30). The arrow indicates the predicted cleavage site.

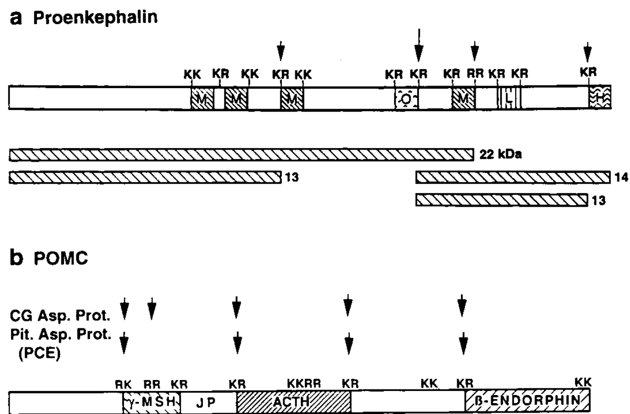

FIG. 8. Aspartic proteinase: Proenkephalin and POMC processing products. (a) Proenkephalin processing. Recombinant proenkephalin was incubated with purified 70-kDa aspartic proteinase from chromaffin granules, and products were identified based on peptide microsequencing and apparent M_r, as described (8). The deduced cleavage sites are indicated by arrows; the large arrow indicates cleavage between Lys-Arg residues. (b) POMC processing. Recombinant POMC was incubated with purified aspartic proteinase from chromaffin granules, and products were identified based on reactivity to anti-β-LPH (lipotropin hormone) and anti-*N*-POMC sera as described (31). Comparison of putative POMC cleavage sites for the chromaffin granule 70-kDa aspartic proteinase and the pituitary (pit) PCE (POMC-converting enzyme) is illustrated above the POMC precursor.

aspartic proteinase was not apparent; therefore, potential NPY-related products could not be detected.

PC1/3 and PC2 processing of POMC (32, 33) and proenkephalin (34) has been demonstrated by cotransfection of PC enzymes and prohormone cDNAs coexpressed in cell lines by the laboratories of Seidah and colleagues (32, 34) and Steiner and colleagues (33). PC1/3 appropriately cleaves at the COOH-terminal side of paired basic residues to generate ACTH and β-LPH (Fig. 9) (32, 33). PC2 demonstrates more extensive processing of POMC than PC1/3 (32, 33) to generate more fully processed products including α-MSH and β-endorphin (Fig. 9). PC1/3 and PC2 products have also been shown to appropriately process proenkephalin at paired basic residue sites (Fig. 9) (34).

Thus, while the candidate processing enzymes—PTP, 70-kDa aspartic proteinase, PC1/3, and PC2—are capable of appropriate prohormone processing, results from this study indicate that each processing protease clearly prefers certain prohormone substrates.

DISCUSSION

Demonstration of selective prohormone processing by particular proteases was achieved by comparing *in vitro* processing of different prohormones—proenkephalin, pro-NPY, and POMC—by the candidate processing proteases PTP, 70-kDa aspartic proteinase, and PC1/3 and PC2 subtilisin-like proteases. These *in vitro* studies utilized recombinant prohormones expressed and purified from *E. coli* and endogenous processing proteases purified from chromaffin granules of bovine adrenal medulla (4–9). Each prohormone was preferentially cleaved by particular prohormone processing enzyme(s). Importantly, each protease demonstrates preference for prohormone substrates.

Recombinant prohormones were successfully expressed in the T7 expression system (12) in *E. coli.* Low levels of prohormones *in vivo* preclude isolation of adequate amounts of prohormones for *in vitro* studies. Expression of abundant levels of prohormones allows their purification in two or three steps. Pro-NPY was purified by DEAE–Sepharose, followed by molecular sieving on a Superose-12 FPLC. POMC purification also utilized DEAE–Sepharose, as well as preparative gel electrophoresis. Proenkephalin expressed in *E. coli* can be purified by three steps involving ion exchange (DEAE–Sepharose), preparative gel electrophoresis, and reverse-phase HPLC (7). Recombinant prohormones have allowed *in vitro* studies to compare preferences of candidate processing enzymes for prohormone substrates.

Results indicated that each processing protease prefers one prohormone among proenkephalin, proneuropeptide Y, and POMC (Table II). The PTP readily cleaves proenkephalin and pro-NPY. PTP is capable of some cleavage of POMC, but at a relative rate of only 2–3% compared to that of proenkephalin or pro-NPY. In contrast, the 70-kDa aspartic proteinase prefers POMC over proenkephalin and pro-NPY. PC1/3 and PC2 show greatest activity with POMC. Overall, PTP demonstrates preference for proenkephalin and pro-

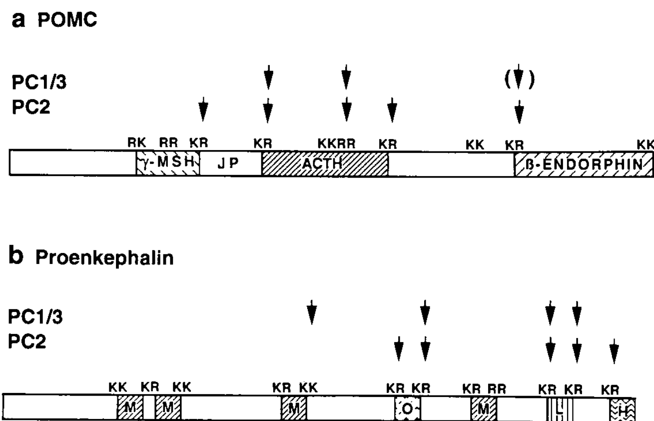

FIG. 9. PC1/3 and PC2: POMC and proenkephalin processing products. (a) POMC processing. PC1/3 and PC2 processing of POMC at paired basic residue cleavage sites (arrows) was determined in cotransfection studies of PC and POMC cDNAs coexpressed in cell lines by the laboratories of Seidah and colleagues (32) and Steiner and colleagues (33). (b) Proenkephalin processing. PC1/3 and PC2 processing of proenkephalin at the indicated paired basic residue cleavage sites (arrows) was determined in cotransfection studies of PC and PE cDNAs by the laboratory of Seidah and colleagues (34).

NPY, while the 70-kDa aspartic proteinase and PC enzymes prefer POMC. The colocalization of PTP activity with proenkephalin, pro-NPY, and their processing products in adrenal medulla is consistent with the proposed role for PTP in processing these precursors. Also, previous detection of a 70-kDa aspartic proteinase, known as PCE (14), and PC2 (15) activities in secretory vesicles of pituitary intermediate lobe is consistent with the proposed roles of aspartic and subtilisin proteases for POMC processing.

These findings indicate that determination of the primary processing proteases involved in processing a particular prohormone will require knowledge of the enzyme's prohormone preference. Among processing proteases colocalized with the prohormone and its peptide products, selectivity of the enzyme for the prohormone of interest, as well as *in vivo* enzyme levels, will be an important factor in prohormone processing. Knowledge of the precursor preferences of the processing proteases may allow prediction of rate-limiting, regulatory processing enzyme(s). Indeed, the preference of PTP, over the 70-kDa aspartic proteinase and PC enzymes, for proenkephalin processing suggests that PTP may be involved in the regulation of enkephalin peptide production. This hypothesis is substantiated in chromaffin cell studies showing that cAMP stimulation of proenkephalin processing and (Met)enkephalin levels involves increased PTP (13); moreover, the requirement for PTP in enkephalin production was supported by blockade of the cAMP-induced rise in enkephalin levels by a potent cysteine protease inhibitor of PTP (6, 13). It will be important in future studies to determine the functional role of selective processing proteases in the biosynthesis of peptide hormones and neurotransmitters.

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