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Cleavage of recombinant proenkephalin and blockade mutants by prohormone convertases 1 and 2: an *in vitro* specificity study

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Abstract

Proenkephalin (PE) derived-peptides are thought to be generated predominantly through endoproteolytic cleavage by prohormone convertases 1 and 2 (PC1 and PC2). In order to compare cleavage site preferences of these convertases, we studied the processing of recombinant wild-type rat PE and of two mutant PEs by recombinant purified mouse PC1 and PC2. Western blot analyses of timed digestions showed that both mouse PC1 and PC2 were able to produce a variety of large and intermediate sized-peptides from wild-type PE as well as from the precursors mutated at initial blockade sites. PC2 exhibited a broader specificity against PE than PC1, generating a much greater number of peptide products. Mass spectrometric identification of cleavage products showed that PC2 appeared to be the principal enzyme involved in the generation of smaller active opioids. Both enzymes were able to cleave various KR- and KK-containing sites, but PC2 was also able to cleave efficiently at an RR-V site and a KK-M site not cleaved by PC1, suggesting the exclusion of large aliphatic residues at the P1′ position in PC1 cleavage. Alternative cleavage sites were readily chosen by convertases in blockade mutants, confirming *in vivo* results that cleavages do not follow an obligatory order. Furthermore, glycosylated PE was less efficiently processed by PC2, indicating that glycosylation may serve as a mechanism to hinder processing.

Keywords: MALDI-TOF peptide, PC1, PC2, proenkephalin, prohormone convertase.

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Like many other peptide hormone and neuropeptide precursors, proenkephalin (PE) is synthesized as an inactive large form which requires post-translational endoproteolytic processing to produce biologically active peptides. Prohormone convertases (PCs) are largely responsible for these processing events, cleaving at specific dibasic recognition sequences. Specifically, PC1 and PC2, enzymes present exclusively in the regulated secretory pathway (reviewed in Steiner 1998), have been implicated in the processing of PE to its bioactive opioid peptides (i.e. Met-enk, Leu-enk, Met-enk-RF and Met-enk-RGL) (Mathis and Lindberg 1992; Johanning *et al*. 1996b). These enzymes share considerable homology within their catalytic domains, but exhibit differences in their specificity as well as in their biosynthetic sites of action (reviewed in Seidah and Chretien 1999).

PC2 appears to exhibit a broader specificity than PC1 on the processing of both POMC and PE when cleavage is examined in AtT-20 cells (Johanning *et al*. 1998); however, the molecular basis for recognition of cleavage sites by PC1 and PC2 which contributes to differential enzyme specificity is not yet known. Also unclear is the contribution of the cellular milieu to cleavage site specificity (Benjannet *et al*. 1992). In this study we have used recombinant enzymes and recombinant precursors combined with mass spectrometry in an effort to describe the specificity of each convertase in PE processing.

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Abbreviations used: Leu-enk, Leu-enkephalin; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Met-enk, met-enkephalin; Met-enk-RF, met-enkephalin-Arg-Phe; MOI, multiplicity of infection; mPC1, mouse prohormone convertase 1; mPC2, mouse prohormone convertase 2; PE, proenkephalin; RIA, radioimmunoassay.

It has been shown that prohormone precursors are processed in a defined order by PC1 and PC2, with certain cleavages preceding others (Zhou *et al*. 1993); similar ordered processing was observed with PE (Mathis and Lindberg 1992; Rostovtsev and Wilson 1994). This finding led to the hypothesis that sequential unfolding of precursors might be required to expose later sites to prohormone convertases. However, mutagenic blockade of the initial cleavage sites of PE to uncleavable residues did not result in the lack of ability to produce final peptide products in AtT-20 cells (Johanning *et al*. 1996a) or in chromaffin cells (Liu *et al*. 1996). Surprisingly, alternative initial cleavage sites were chosen, and in AtT-20 cells the final complement of active opioid units from mutated precursors actually exceeded that derived from wild-type PE (Johanning *et al*. 1996a). However the kinetics of conversion of mutated precursors in neuroendocrine cells were quite different than that of the wild-type precursor, with slower initial cleavages and more rapid final cleavages. These results suggest that mutated substrates could interact differently with convertases than do the wild-type precursors; alternatively, cell biological factors (alterations in folding or transport) could have accounted for the differences in processing rates between wild-type and mutant PEs. In order to investigate whether these mutated precursors represent better or worse substrates for convertases than do natural precursors, we have investigated the kinetics of processing of these precursors by recombinant convertases in a controlled *in vitro* system. Lastly, as PE can be found in both glycosylated and unglycosylated forms in neuroendocrine cells (Lindberg and Shaw 1992), we have investigated whether this post-translational modification contributes to the regulation of PE cleavage.

Materials and methods

Proenkephalin and cleavage site blockade mutants

Purified recombinant rat proenkephalins (PE), consisting of wild-type and two cleavage site blockade mutants, were used as substrates in this study. Mutagenesis of proenkephalin, construction of the expression vectors and production of the recombinant proteins have been described elsewhere (Mathis and Lindberg 1992; Johanning *et al*. 1996a). The first cleavage site mutation involved a change of Lys237 to His237 and Arg238 to Lys238, a change which blocks the initial processing steps of proenkephalin, and is here termed 'blockade mutant' (Johanning *et al*. 1996a). The other mutant contained alterations at two distinct processing sites, i.e. two additional substitutions, Lys210 to His210 and Arg211 to Lys211, superimposed upon the same mutations as the blockade mutant; this protein was termed 'double blockade mutant' (see Fig. 1).

Overexpression and purification of recombinant wild-type proenkephalin and blockade mutants

Wild-type recombinant rat PE and the double blockade mutant were overexpressed in CHO cells using the dihydrofolate reductase-coupled (DHFR) amplification method as described earlier (Lindberg *et al*. 1991). Wild-type PE and the blockade and double blockade mutants were purified as described previously (Lindberg *et al*. 1991) from conditioned media and a 4.6 × 25 mm Vydac semipreparative C4 column (Vydac, Hesperia, CA, USA). The recombinant proteins were eluted with a gradient from 16% to 100% buffer B in 120 min Buffer A was 0.1% trifluoroacetic acid (TFA) and buffer B was 80% acetonitrile in 0.1% TFA. Aliquots were assessed for purity on 15% acrylamide gels using Coomassie blue staining.

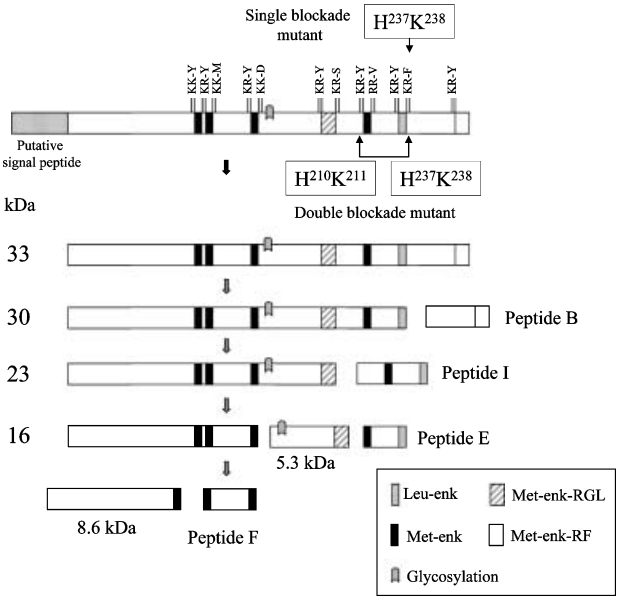

Fig. 1 Diagrammatic representation of proenkephalin and known cleavage sites for the generation of intermediate-sized and active-opioid peptides. Peptides known to be present in the bovine adrenal medulla (Udenfriend and Kilpatrick 1983) are shown below (rat and bovine PE have identical cleavage sites). Mutated sites of the recombinant blockade mutant and double blockade mutant are depicted by arrows.

Overexpression of mPC1 and mPC2

Recombinant PC1 and mPC2 were produced by the same method, i.e. overexpression in CHO cells using the DHFR method (Lindberg and Zhou 1995). PC2 CHO cells were stably supertransfected with cDNA encoding 21 kDa rat 7B2 (Lamango *et al*. 1996). In both cases, cells were grown in a Cellmax artificial capillary cell culture system (Cellco Inc., Germantown, MD, USA) (Johanning *et al*. 1998). PC1 and PC2 were, respectively, purified from conditioned media which was diluted 1 : 3 in Buffer A on separate 5 × 50 mm Protein-Pak anion-exchange columns (Waters Chromatography, Milford, MA, USA) as described; buffer A was 20 mM Bis-Tris, 0.1% Brij, pH = 6.5 and buffer B was 1 M sodium acetate, 20 mM bis-Tris, 0.1% Brij, pH = 6.5 as described (Johanning *et al*. 1998). For both PC1 and PC2, aliquots from fractions were assessed for purity on 8.8% acrylamide gels using Coomassie blue staining.

Recombinant blockade mutant expression in baculovirus-infected insect cells and purification

The overexpression of the blockade mutant in CHO cells did not reach satisfactory levels, and so this protein was expressed in a

baculovirus system. The blockade mutant insert was excised from the pCMV/blockade mutant-1 vector (Johanning *et al*. 1996a) by digestion with EcoR1 and BamH1 restriction enzymes. The fragment containing the PE blockade mutation (approximately 900 bp) was purified, electroeluted and ligated into the EcoR1 and BamH1 multiple cloning site of the pVL1392 Baculovirus transfer vector (Pharmingen, San Diego, CA, USA). Transfer vectors and BaculoGold™ DNA (Pharmingen) were cotransfected into Sf9 (*Spodoptera frugiperda*) cells using the procedure described in the instruction manual (Pharmingen). After successful transfection, viruses were plaque-purified using Agarplaque™ Plus Agarose (Pharmingen) in protein-free medium for 6–10 days until plaques were visible after staining with a 1 : 10 neutral red (Gibco BRL, Grand Island, NY, USA) solution (neutral red: PBS). Agarose plugs were collected and placed in a microcentrifuge tube with 1 mL TNM-FH serum-containing culture medium (Pharmingen) and virus eluted overnight at 4°C. Two hundred microliters of the centrifuged supernatant was used for the first viral stock. The procedure was repeated for the passage 2 stock. An end-point dilution assay was carried out to determine the titer, and amplification of the virus was performed three consecutive times using Sf9 cells grown in serum-free media. Sf9 cells were grown in Sf 900 II serum-free medium (Pharmingen) at a density of 1.4×10^7 cells/15 cm plate at a multiplicity of infection (MOI) of ~ 0.5 for this amplification step. Proteins were expressed in Hi Five™ (Pharmingen) cells in 2–4 15 cm plates (2.0×10^7 cells/plate) using the centrifuged supernatant from the second amplification step at a MOI ~4–5 for 1 h at 28°C. Cells were washed with fresh medium and cultured in a volume of 30 mL for 3 days. Media were collected, centrifuged and stored at −80°C until further use. Purification and assessment of purity of the recombinant blockade mutant protein was carried out as described above.

Proenkephalin and blockade mutant cleavage by PC1 and PC2

Time course digestion experiments of mutant and native PEs (1.8 μM final concentration) with either PC1 (0.08 μM final concentration, 0.22 μmol/h/mg specific activity) or PC2 (0.11 μM final concentration, 29 μmol/h/mg specific activity) were performed in a buffer containing 100 mM sodium acetate (pH = 5.5 for PC1, and pH = 5.0 for PC2), 5 mM $CaCl_2$ and 0.1% Brij (50 μL total volume). Enzymes were preincubated at the appropriate pHs and times (1 h for PC1 at pH. 5.5 and 20 min for PC2 at pH 5.0) (Johanning *et al*. 1998) for activation prior to the addition of recombinant prohormone substrates. The reaction mixtures with PC1 were incubated for 0, 1, 1.5, 2, 3, 5 and 8 h (the specific activity of PC1 is approximately 1/100th that of PC2). PC2-containing reactions were incubated for 0 min, 15 min, 1, 1.5, 2, 3, and 5 h. According to these results, further experiments on glycosylated and unglycosylated wild-type PEs were carried out at defined times (7.5 h and 45 min for PC1 and PC2, respectively) in order to observe the partially processed PE precursor for direct comparison of peptide products.

Western blotting analysis

For western analyses, convertase digestions were terminated with 1/10th volume of a 10× solution of Laemmli sample buffer (0.5 M Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol, 2% SDS and 0.06 mg/mL bromophenol blue). Samples were then boiled, and a tenth of the reaction mixture was subjected to electrophoresis on a 10–20% sodium dodecyl sulfate–polyacrylamide gradient gel (Bio-Rad, Hercules, CA, USA). Proteins were then transferred to nitrocellulose and subjected to western blotting as described previously (Johanning *et al*. 1996b). The antiserum used was raised against peptide F (Christie *et al*. 1984) and recognizes PE as well as PE cleavage products containing peptide F.

In an additional set of experiments, development of the blots was performed using alkaline phosphatase detection (for experiments with glycosylated and unglycosylated PE) or the Vistra™ ECF western blotting fluorescence system (Amersham, San Francisco, CA, USA) for chemiluminescence analyses (for the reminder of the analyses). Blots were imaged and scanned for quantitation of PE immediately after development of the membrane as described in the manufacturer's protocol using a Storm 840 (Molecular Dynamics, Sunnyvale, CA, USA) scanner and ImageQuant™ (Molecular Dynamics) as the software. In each case, total substrate within the PE band was scanned at each time point and the percentage disappearance of PE was calculated based on the initial total amount of substrate. All experiments were repeated 2–4 times.

Mass spectrometry of digestions

Mass spectrometry was used to identify digestion products undetected with other techniques such as those used above. Digestions of PE, blockade mutant or double blockade mutant (5.45 μM) were performed with either PC1 or PC2 (0.08 and 0.11 μM, respectively) as indicated previously, in a buffer containing 100 mM ammonium acetate (pH = 5.5 for PC1 and pH = 5.0 for PC2), 5 mM $CaCl_2$ and either 0.4% octyl glucoside or 0.1% CHAPS (total volume = 50 μL). All reactions were carried out in Eppendorf microcentrifuge tubes previously treated with a solution containing 50% acetonitrile and air-dried before use in the experiments to avoid interference of plastic polymers presence in the mass spectrometry analyses. Enzymes were preincubated as above prior to substrate addition. Digestions were carried for 15 h with PC1 and 120 min with PC2. Reactions were terminated by the addition of 100 μL of 100% acetonitrile, 0.1% TFA and immediately frozen in dry ice before MALDI analyses. Then, samples were vacuum-dried and resuspended in 100 μL of 100% acetonitrile and 5% formic acid. The process was repeated three times to remove the salts. Final samples were resuspended in 50 μL of the above solvent. Each sample was analyzed in two ranges with different matrices.

The spectra were acquired in linear mode on a PerSpective Voyager RP MALDI-TOF instrument. A total of 100–150 scans were averaged for each spectrum using an accelerating voltage of 27 kV, 75% grid voltage and 0.3% ion guide voltage. Alpha-cyano-4-hydroxycinnamic acid (CHCA) (Aldrich, Milwaukee, WI, USA) and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA) (Fluka, New York, NY, USA) were used as the matrices for protein and peptide analyses. They were made into saturated solutions in 50% ethanol and 1% acetic acid. For the acquisition of spectra from 500 to 10 000 amu, 2 μL of sample was mixed with 2 μL of CHCA solution in an eppendorf tube, and 2 μL of the mixture was loaded onto the MALDI plate. The calibration peptides for this mass range were Leu-enkephalin (M + 555.3) (synthetic) and bovine RNAse (M + 13 682) (ICN, Costa Mesa, CA, USA). For the acquisition of spectra from 8000 to 30 000 amu, 2 μL of sample was mixed with 2 μL of sinapinic acid solution in an Eppendorf tube, and 2 μL of

the mixture was loaded onto the MALDI plate. The calibration peptides for this range were Arg-insulin (M + 5963.8) (Sigma, St. Louis, MO, USA) and carbonic anhydrase (M + 29 026) (ICN, Costa Mesa, CA, USA). The masses of all the calibration peptides were verified using electrospray ionization on a LCQ (Finnigan) mass spectrometer.

The theoretical masses of all peptides derived from PE, the blockade mutant and the double blockade mutant were derived using the Sherpa program (University of Washington). In order to match the observed mass with the theoretical mass, an average error margin of 0.1% was given to the observed masses. There are several reasons for this: the MALDI instrument used was not equipped with delayed extraction, the peptide concentrations were below the detection sensitivity with reflectron mode within our instrument, and the mass range of detection was relatively broad. Keeping this in mind, only the major peptide ions were matched with the corresponding theoretical masses and the less abundant ions were ignored.

The masses of several peptides detected by MALDI in the mutant digests could not be recognized as PE-derived peptides (designated 'N/D'). As wild-type PE and mutants were expressed in different systems (CHO cells and baculovirus, respectively), these peptides may derive from minor contaminants which copurify with PEs produced via baculoviral systems.

Results

Figure 1 depicts a diagram of rat PE with its 12 cleavage sites as well as naturally occurring peptides resulting from the proteolytic cleavage of this substrate in the bovine adrenal medulla (Udenfriend and Kilpatrick 1983). The mutations present in the two blockade mutants are indicated. In a previous investigation (Johanning *et al*. 1996a) we found that these mutations at initial cleavage sites did not block precursor processing under *in vivo* conditions. Instead, processing of the mutants proceeded at alternative cleavage sites, albeit with different kinetics. Surprisingly, the final yield of mature enkephalins was better from the blockade mutants than from wild-type proenkephalin (Johanning *et al*. 1996a). We therefore investigated whether blockade mutants indeed represent better substrates for convertases, or whether cell biological factors account for the increased product yield seen *in vivo*. We also attempted to gain information on convertase specificity through precise identification of the products of digestion from each enzyme using mass spectrometry.

Digestion of wild-type PE with PC1 and PC2 generated a greater number of intermediate peptides than digestion of the blockade mutants

In order to investigate potential differences in the intermediates generated from wild-type and mutant PEs, we performed PC1 and PC2 digestions and analyzed the results using an antiserum which recognizes multiple molecular forms of peptide F (Christie *et al*. 1984). Figure 2 shows the results of digestions with PC1 (0.08 μM final concentration of convertase with 1.8 μM final concentration of PEs). One h

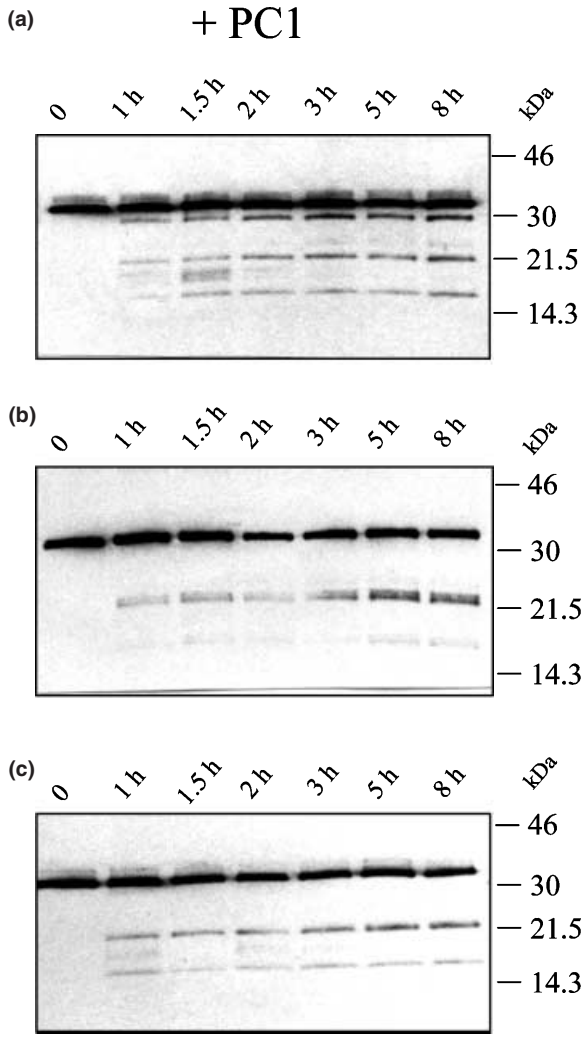

Fig. 2 Digestion of recombinant wild-type PE, blockade mutant and double blockade mutant by PC1. Western blotting of resulting digests of wild-type PE (a), blockade mutant (b) and double blockade mutant (c) by PC1 are shown. Recombinant precursors (PE, blockade mutant and double blockade mutant, 1.8 μM final concentration) were digested with preincubated PC1 (0.08 μM final concentration) for t = 0, 1, 1.5, 2, 3, 5, and 8 h at 37°C. Molecular masses of standards are indicated on the right.

of digestion of wild-type PE with PC1 (panel A) resulted in the production of four intermediate peptides with molecular masses of 30, 23, 18 and 16 kDa. Unlike wild-type PE, the single blockade mutant (Fig. 2b) yielded two doublets with Mrs of 23 and 16 kDa, and the double blockade mutant (Fig. 2c) yielded 23, 18 and 16 kDa peptide intermediates. At 90 min, an additional band appeared in the PE digestion below the 18 kDa species (Fig. 2a). At 3 h, another band with an M_r of 26 kDa appeared in digestions containing wild-type PE. Furthermore, the 30 and 26 kDa band is completely absent in both blockade mutant samples. These data support the idea that the initial removal of the 3.6 kDa peptide B unit

is effectively blocked in these mutants, and that in the double blockade mutant the removal of an amino-terminally extended form of peptide B is also prevented by the second mutation.

Wild-type PE and the blockade mutants (1.8 μM final concentration) were also subjected to PC2 digestion (0.11 μM final concentration) and aliquots of the samples subjected to western blotting using antiserum against peptide F as described above. Figure 3 shows that, similarly to PC1, PC2 was able to generate the 30, 23 and 16 kDa intermediate peptides from PE (Fig. 3a). By contrast, cleavage of the blockade mutants resulted only in the production of two intermediate-size peptides with masses of 23 and 16 kDa (Fig. 3b,c).

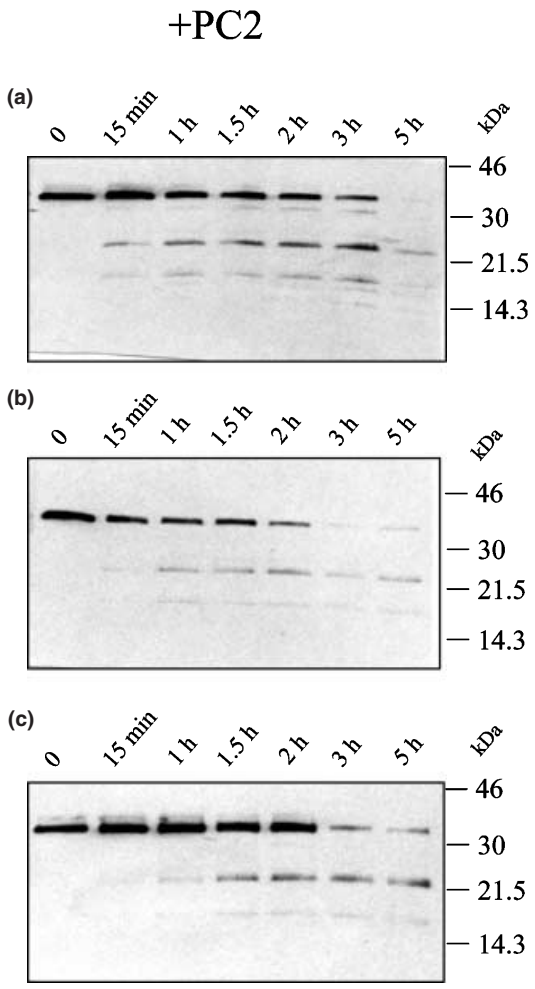

Fig. 3 Digestion of recombinant wild-type PE, blockade mutant and double blockade mutant by PC2. Western blotting of resulting digests of wild-type PE (a), blockade mutant (b) and double blockade mutant (c) by PC2 are shown. Recombinant precursors (PE, blockade mutant and double blockade mutant, 1.8 μM final concentration) were digested with preincubated PC2 (0.11 μM final concentration) for $t = 0$, 15, 60, 90, 120 min, 3, and 5 h at 37°C. Molecular masses of standards are indicated on the right.

Although PC1 seems to be primarily involved in the generation of intermediate-sized peptides from full-length PE *in vivo* (Johanning *et al*. 1996a; Johanning *et al*. 1996b), when PC1 and PC2 digests were compared, it was clear that PC2 was able to act efficiently on precursors without prior PC1 action.

Rates of cleavage of wild-type and mutant PEs by convertases

Western blots of the digestions of wild-type and blockade mutant PEs were quantitated in order to determine the rate of substrate disappearance using chemiluminescence. In preliminary experiments, substrate disappearance was determined to be linear as a function of time using this procedure. The total amounts of digests loaded on the gels and transferred to nitrocellulose membranes were less than those shown in the blots in Figs 2 and 3 to avoid maximal signal saturation in the bands corresponding to precursor and therefore misleading results of substrate disappearance over time. Cleavage efficiency was calculated at each time point based on 100% precursor at $t = 0$ h. Figure 4 summarizes these quantitations; 50% disappearance is indicated in each graph.

Overall, digestion of wild-type and mutant PEs by PC1 resulted in similar cleavage rates (Fig. 4) with a half-life of precursor disappearance of about 2 h for all proteins. PE cleavage by PC2 exhibited half-lives between 39 and 80 min for wild-type and mutant proteins; the single blockade mutant was more rapidly digested by PC2 than wild-type PE during the first 60 min, but the double blockade mutant was more slowly digested (Fig. 4). The rate of digestion by PC2 of all PEs seemed to slow at later time points, perhaps due to competition of PE cleavage by smaller fragments with increased affinity for the enzyme.

MALDI-TOF mass spectra of peptides generated from the digestion of wild-type and blockade mutant PEs

MALDI-TOF mass spectrometry was used to determine the molecular masses of the products resulting from PC1 and PC2 cleavage of wild-type and mutant PEs (Figs 5, 6 and 7). The peptide profiling covered in these analyses was from approximately 800–29 000 *m/z* (mass/charge) as we expected peptides ranging in mass from > 500 to < 27 500 Da. An area in the spectrum of the digests difficult to analyze was from > 900 to < 1800 *m/z* due to the presence of very small concentrations of Brij. This detergent was used to reconstitute the precursor proteins after lyophilization following purification. However, we were able to detect and identify certain peptides in this mass range. Arrows indicate in each case the identity of the peptides and corresponding precursor. A diagram of the precursor and products resulting from the cleavage is indicated below the spectra with the amino acid number starting from D25 (precursor without the signal peptide) to the carboxy terminal end of the protein, F269.

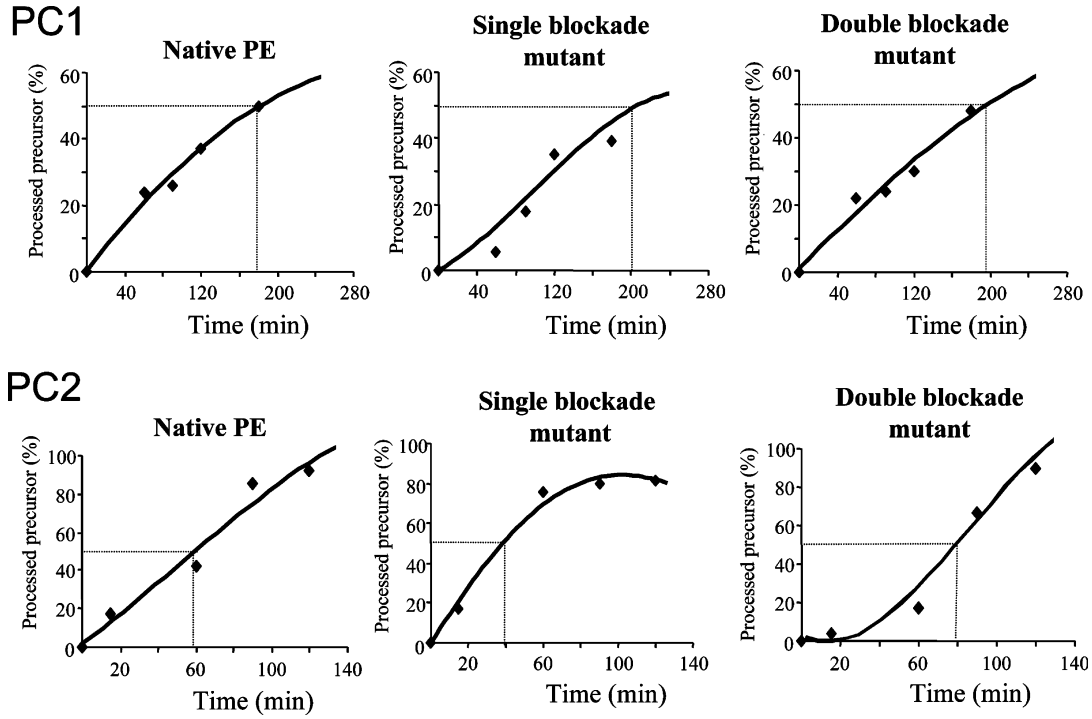

Fig. 4 Quantitation of the substrate disappearance of native PE and blockade precursors using chemiluminescence. Time 0 corresponds to 100% precursor. Fifty per cent (half-life) disappearance is indicated in each graph.

Figure 5 depicts the profiles generated by PC1 (Fig. 5a) and PC2 (B) digestions of wild-type PE. PC1 digestions were carried out for 15 h in order to be able to detect smaller size peptides resulting from the precursor cleavage. Figure 5(b) summarizes the results of PE digested with PC2 for 120 min after enzyme activation. The most striking feature of these digestions is the greater number of peptides generated by PC2 as compared to PC1. PC2 generated 13 peptide intermediates in the range of 2000–8000 *m/z* (Fig. 5a,b, middle panels), while PC1 generated three peptides in the same range. PC2 produced many of the same intermediate-sized PE-derived peptides as did PC1 (Fig. 5a,b, right-hand panels). However, there were many peptides generated only by PC2, such as D25-KR^{135} and many small fragments. Both PC2 and PC1 were capable of producing peptides B, I and F. Unlike PC1, PC2 did not generate intact peptide E (middle panels). PC2 produced all of the basic-residue extended penta- to octa-peptide active opioids (Fig. 5a,b, left-hand panels) while the only opioid produced by PC1 was Met-enk-KR. Leu-enk-KR was found only in PC2 but not in PC1 digests, suggesting that the lack of peptide E in PC2 digests could be due to further cleavage of this substrate by PC2.

Figure 6(a,b) depicts the mass spectra resulting from blockade mutant digestion by PC1 and PC2 under similar conditions. It is immediately clear that PC1 was unable to perform many of the PC2-mediated cleavages. Interestingly, two intermediate peptides (right panels) resulted from cleavage by both enzymes. These (S198-F269 and D25-R187) intermediate peptides were never present in digests of wild-type PE by PC1 or PC2. As with wild-type PE, there were far more peptides generated by cleavage of the blockade mutant by PC2 than with PC1 (Figs 6a and 7b, right-hand and middle panels). For mutant PEs, smaller peptides such as Met-enk-KR (Fig. 6b, left-hand panel) were abundantly present in samples digested with PC2 but were not present in digestions with PC1, in which range only detergent polymers were observed but no prominent peaks.

Similar results were obtained with the double blockade mutant. PC2-mediated digestion of this precursor resulted in a greater number of peptides when compared to PC1 digestion products (Fig. 7a,b). Two of the same intermediates found in blockade mutant digests were identified in the double blockade mutant digests (S198-F269 and D25-R187) but not in wild-type PE digests, suggesting effective mutational blockade. Three other intermediate peptides (Y107-K142, S198-R231 and M114-R135) appeared to be specific to PC2-cleaved double blockade mutant and were not produced by PC1 (Fig. 7b, middle panel). These peptides were also observed in wild-type PE samples digested with PC2 (Fig. 5b, middle panel), suggesting that PC2 exhibits preferential affinity for these sites both in the wild-type and mutant proteins. Few small opioids were identified in these digests; Met-enk-RF (Fig. 7b, left panel) was detected in double blockade mutant digests by PC2 but not by PC1.

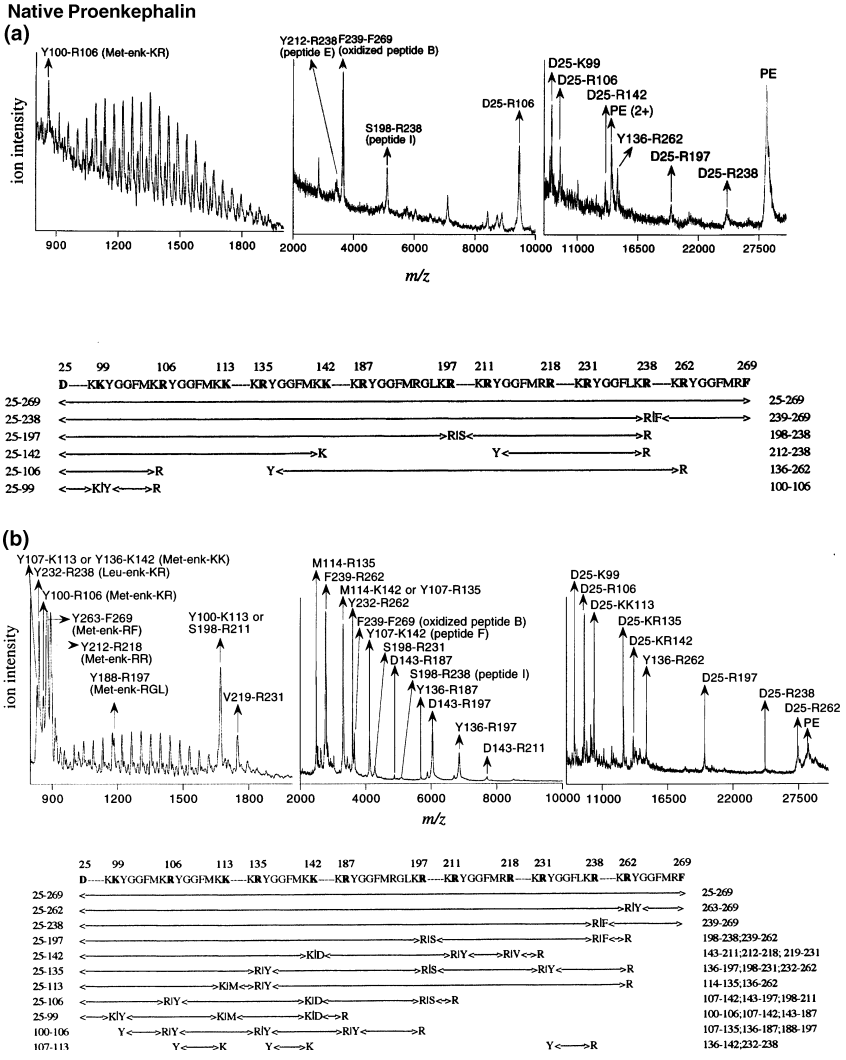

Fig. 5 MALDI-TOF MS spectra of wild-type PE digestions with PC1 and PC2. Wild-type PE (5.45 μM final concentration) was incubated with either PC1 (0.08 μM final concentration) (a) or PC2 (0.11 μM final concentration) (b). Spectra are representative of the digestions performed for 15 h with PC1 and 120 min with PC2. Peptides resulting from these cleavages are indicated by an arrow on top of the spectra. Sequences corresponding to the peptides generated by the PCs are indicated on the bottom.

In a separate set of experiments (spectra not shown), wild-type PE was digested first with PC1 for 8 h, at which time PC2 was added for additional 45 min. The spectra of peptides produced showed the same intermediates observed using PC1 alone but with the additional presence of Met-enk-RF as well as of Leu-enk-KR.

Both PC1 and PC2 can cleave glycosylated and unglycosylated PEs, although PC2 cleaves the glycosylated precursor inefficiently

Figure 8 depicts the proteolytic activity of PC1 and PC2 on glycosylated and unglycosylated PEs as assessed by western blotting experiments using antisera against peptide F; this antiserum detects amino and carboxy-terminally extended forms of this peptide. It should be noted that the only consensus sequence for glycosylation (N-Xaa-S/T) is N152-S153-S154, which occurs approximately midway in the molecule.

The data in Fig. 8 confirm that both enzymes can cleave the unglycosylated form of PE very efficiently. PC1 exhibited similar patterns of digestion of glycosylated and unglycosylated PEs (right panel) while PC2 cleaved the unglycosylated PE form much more efficiently than the glycosylated form; the products generated after 45 min of incubation of unglycosylated PE were more abundant than those obtained from the same amount of glycosylated PE (Fig. 8, middle panel). The 16 and 7 kDa peptides exhibited similar molecular weights during processing of both glycosylated and unglycosylated PEs, indicating that both bands correspond to PE-derived peptides which lack sugars (the 7 kDa band was observed only in PC2-treated samples after overdevelopment of the membranes). The molecular masses of 33, 30, 23 and 16 kDa observed in our experiments are in agreement with results obtained in previous studies carried out in transfected AtT-20 cells (Johanning *et al.* 1998). Furthermore, the band of approximately 7 kDa is likely to correspond to peptide F itself, which migrates at an anomalously high molecular weight due to its highly acidic character (Lindberg, unpublished observations). Thus, com-

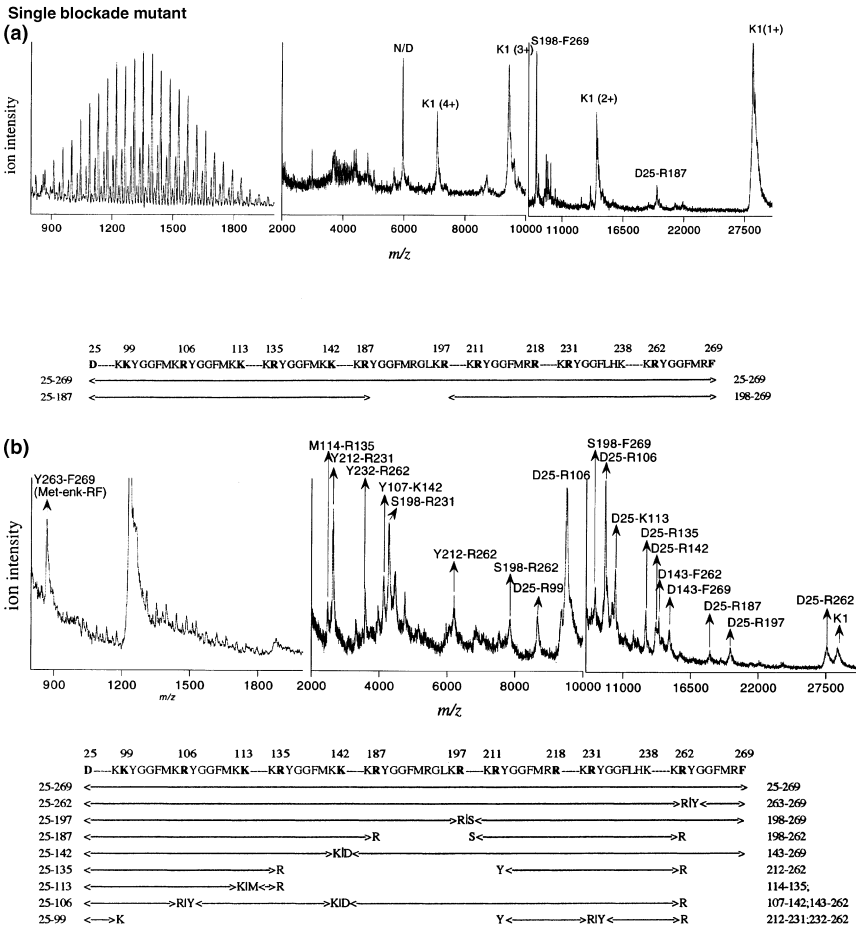

Fig. 6 MALDI-TOF MS spectra of single blockade mutant digestions with PC1 and PC2. K1 single blockade mutant (5.45 μM final concentration) was incubated with either PC1 (0.08 μM final concentration) (a) or PC2 (0.11 μM final concentration) (b). Spectra are representative of the digestions performed for 15 h with PC1 and 120 min with PC2. Peptides resulting from these cleavages are indicated by an arrow on top of the spectra. Sequences corresponding to these peptides generated by the PCs are indicated on the bottom. N/D, not determined peptides.

parison between glycosylated and unglycosylated peptides constitutes a useful tool to facilitate the identification of product peptides.

Discussion

Endoproteolytic processing of the complex precursor PE produces a spectrum of peptides that display a variety of biological activities as hormones and neurotransmitters, with or without opioid activity (reviewed in Hollt 1986). Intermediate enkephalin-containing peptides can themselves possess biological activities (Hollt 1986; Lembo *et al*. 2002) or can serve as substrates for the production of the penta- to octapeptide enkephalins. The endoproteolytic processing of this precursor is thought to be largely accomplished by the prohormone convertases PC1 and PC2 (Breslin *et al*. 1993; Johanning *et al*. 1998). In the present study, we have carried out *in vitro* assays to investigate the specificity of PC1 and PC2 on the processing of PE under controlled *in vitro* conditions. In order to better understand the molecular mechanisms followed by these convertases we have also evaluated whether cleavage site mutations of the PE molecule can modify processing.

Our mass spectrometry analysis of proenkephalin cleavage reveals information about cleavage site preference by the two enzymes. The most obvious preference we detected was the inability of PC1 to cleave at sites containing a large P1′ aliphatic residue, such as the KK-M site at 112–114 and the RR-V site at 217–219. While PC2 was able to cleave these sites in the present study, our previous work has demonstrated that internally quenched fluorogenic peptides containing these cleavage sites possess the worst K_{cat}/K_{ms} of a series of 11 proenkephalin-derived substrates (Johanning *et al*. 1998), indicating that PC2 also disfavors a large aliphatic P1′ residue. MALDI-TOF has been previously used to identify PE-derived peptides in bovine chromaffin granules (Goumon *et al*. 2000). In this study of adrenal tissue, which expresses PC1 and a small amount of PC2, almost complete usage of the double basic cleavage sites was observed. However, sites which remained uncleaved in this study included the two aliphatic P1′ sites mentioned above: KK-M and RR-V, supporting the idea that cleavage site analyses *in vitro* faithfully mimic cleavage site preferences *in vivo*. Indeed, we have previously noted in an analysis of over 60 cleavage sites that certain aliphatic residues (V, I, L, M, C) almost never appear in the P1′ position at sites known to be

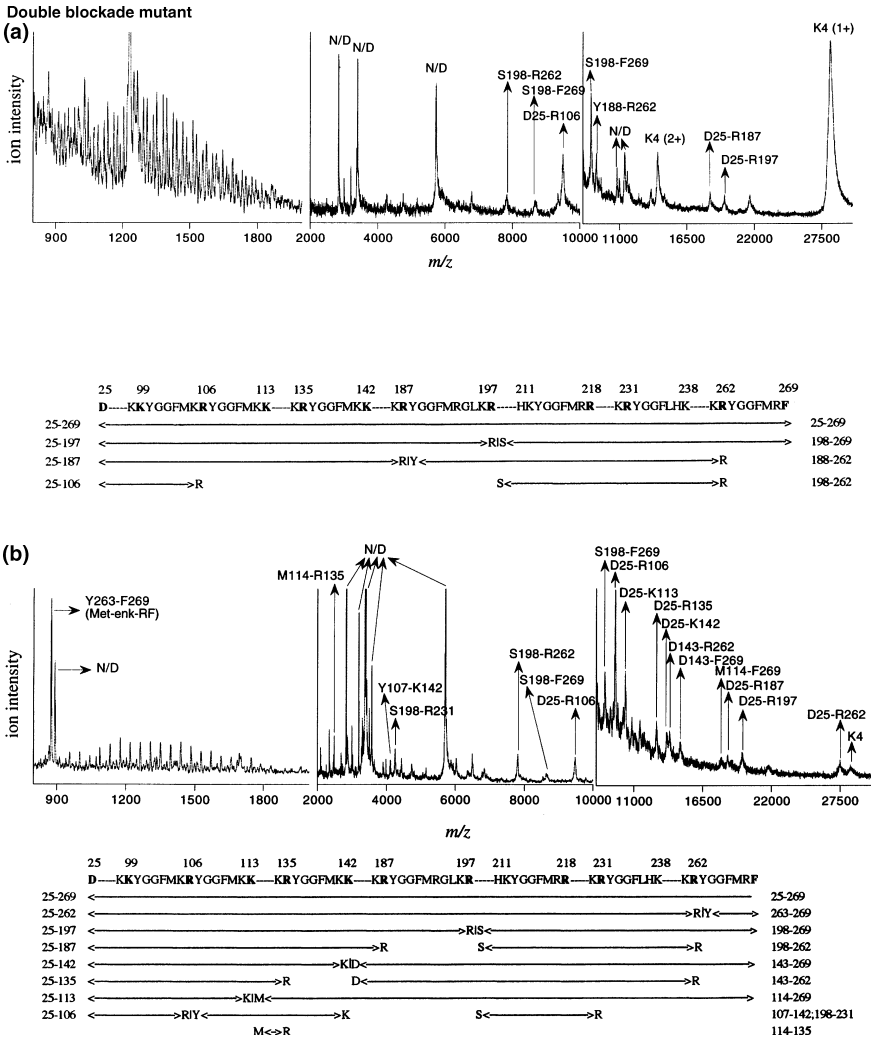

Fig. 7 MALDI-TOF MS spectra of double blockade mutant PE digestions with PC1 and PC2. K4 double blockage mutant (5.5 μM final concentration) was incubated with either PC1 (0.08 μM final concentration) (a) or PC2 (0.11 μM final concentration) (b). Spectra are representative of the digestions performed for 15 h with PC1 and 120 min with PC2. Peptides resulting from these cleavages are indicated by an arrow on top of the spectra. Sequences corresponding to the peptides generated by the PCs are indicated on the bottom. N/D, not determined peptides.

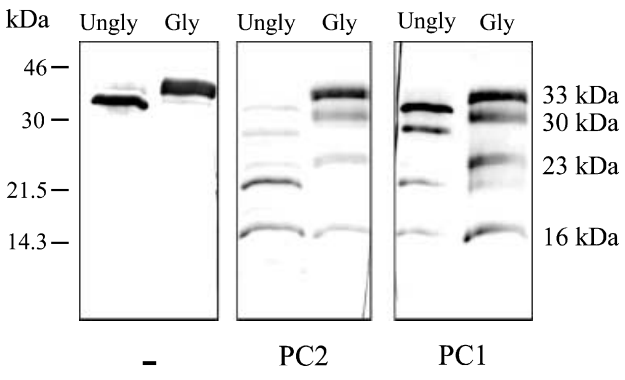

Fig. 8 Digestion of gycosylated and unglycosylated PEs by PC2 and PC1. Left-hand panel, western blotting of PEs. Middle panel, western blotting of digests of wild-type PE (1.8 μM final concentration) preincubated with PC2 (0.11 μM final concentration) for 45 min. Right-hand panel, western blotting of digests of wild-type PE (1.8 μM final concentration) with preincubated PC1 (0.11 μM final concentration) for 7.5 h. Molecular masses of bands detected are indicated on the right.

cleaved by convertases (Cameron *et al*. 2001). Also apparent from our mass spectrometry data is the fact that more of the KR-Y sites that flank enkephalin sequences are cleaved by PC2 than are cleaved by PC1, supporting the previously proposed idea that PC2 rather than PC1 is the major enzyme responsible for the production of active opioids (Johanning *et al*. 1998). There appeared to be little preference for types of dibasic residues: both KR and RR sites were cleaved by both enzymes. However, the KK-D site at residue 142, which lacks the unfavorable aliphatic P1′ residue, is not cleaved by PC1 but is efficiently cleaved by PC2, supporting the notion that KK sites in general may be preferentially cleaved by PC2. Lastly, our data also show that PC2 generates a greater number of total products than PC1, providing direct confirmation of the broader specificity of PC2 previously documented by analysis of cleavage of a large number of precursors both *in vitro* and *in vivo* (Cameron *et al*. 2001). It should be noted that processing *in vivo* could differ from that observed *in vitro* in that *in vivo* processing may be influenced

by the presence of other granular proteins such as granins and CPE. In fact, the presence of CPE has previously been associated with increased enzymatic activity of PC1 on other peptide precursors (i.e. prodynorphin; Day *et al*. 1998).

As regards the blockade mutants, it is clear that altering KR cleavage sites to His-Lys (HK) represents an effective means of blockade; for both enzymes, cleavage site usage was shifted to upstream available cleavage sites. However fewer products than expected were detected in mass spectrographic analyses of digestions of mutant PEs. While this result could potentially indicate differences in reactivity of mutated precursor or mutated intermediates with convertases, this was not borne out by kinetic studies of PE disappearance using western blotting. It should be pointed out that peptide F-immunoreactive products observed by western blotting represent only early cleavages, as smaller intermediates as well as penta- to octapeptide enkephalins are either poorly or not at all retained on blots. More work is required to investigate the efficiency of enkephalin liberation from mutant proteins by each convertase.

Peptide precursors are known to be cleaved in a semi-ordered fashion; for POMC, it has been suggested that PC1 first produces certain large intermediates and that PC2 then cleaves these intermediates to smaller products (reviewed in Mains and Eipper 2000). We and others have also noted order in the cleavage of PE, with the removal of peptide B occurring as a primary step (Mathis and Lindberg 1992; Rostovtsev and Wilson 1994). However, blockade of early cleavage sites did not appear to hinder cellular processing of PE (Johanning *et al*. 1996a; Liu *et al*. 1996), indicating that ordered cleavage is not obligatory. Similar results have been found in the processing of egg-laying hormone precursor, where cleavage site blockade resulted in an alternative proteolysis pattern using different cleavage sites (Jung *et al*. 1993). The western blotting and MALDI-TOF experiments presented here support the idea that alternative cleavage sites in mutants can be efficiently used by both convertases. In addition PC2 was able to cleave precursors without the previous action of PC1, suggesting that PC1 action is not required prior to PC2 action. These results are consistent with recent studies on the PC1 knockout mouse in which many PC1-mediated cleavages were successfully carried out by PC2 (Zhu *et al*. 2002).

Cleavage site preference in blockade mutants did not differ substantially from preference in the wild-type precursor, with one exception: MALDI-TOF analysis revealed that PC2 was able to cleave RR218-V219 in wild-type PE, but not in either mutant. PC1 was unable to cleave this site in any precursor. As noted above, this particular site appears to be more difficult to cleave due to the presence of the large P1′ aliphatic residue. This result showing the lack of cleavage of this site in mutants suggests that mutation of the cleavage sites might lead to a modification in the tertiary structure of the molecule that hinders processing at difficult sites. However, the similar rate of processing of all three precursors by PC1 indicates that any potential differences in structure do not affect reactivity with this enzyme; similarly no large changes in reactivity which could be ascribed to the mutations were noted using PC2 in the western blotting experiments. Collectively our results indicate that cleavage site mutations of PE do not affect the rate of PC1-mediated processing; PC2-mediated processing is only modestly if at all hindered. We conclude that the slowing of early processing of PE mutants seen in AtT-20 cells (Johanning *et al*. 1996a) must reflect cell biological factors rather than structural factors.

Many peptide hormone precursors, including proenkephalin (Watkinson and Dockray 1989) are subject to glycosylation (reviewed in Mains and Eipper 1984). PE can be found as glycosylated and unglycosylated forms in transfected AtT-20 cells (Mathis and Lindberg 1992) as well as in the neuronal cell line SKNMC, in which it is endogenously expressed (Lindberg and Shaw 1992). The question of whether glycosylation can affect proteolytic processing at neighboring sites is unclear; previous evidence suggests that glycosylation limits endoproteolytic access to a neighboring pair of basic residues (Loh and Gainer 1980; Birch *et al*. 1991). Our *in vitro* results confirm slower PC2-mediated processing of glycosylated vs. unglycosylated PE, while PC1-mediated processing was unaffected by the presence or absence of sugars. These results are perhaps not surprising given that the cleavage site nearest the glycosylation site is the KK-D site thought to be cleaved exclusively by PC2 (see Fig. 1).

In summary, our experiments using recombinant precursors and enzymes have confirmed the greater specificity of PC1 as opposed to PC2, and have provided information on cleavage site usage by these two enzymes which indicates that the P1′ residue is an important determinant of specificity. Mass spectrometric analysis of *in vitro* reactions containing recombinant peptide hormone precursors and enzymes represents a powerful method to investigate the complex cleavage events required to generate bioactive peptides under controlled conditions.

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References

Benjannet S., Reudelhuber T., Mercure C., Rondeau N., Chretien M. and Seidah N. G. (1992) Proprotein conversion is determined by a multiplicity of factors including convertase processing, substrate specificity, and intracellular environment: cell type-specific processing of human prorenin by the convertase PC1. *J. Biol. Chem.* **267**, 11417–11423.

Birch N. P., Estivariz F. E., Bennett H. P. and Loh Y. P. (1991) Differential glycosylation of N-POMC1-77 regulates the production of gamma 3-MSH by purified pro-opiomelanocortin converting

enzyme. A possible mechanism for tissue-specific processing. *FEBS Lett.* **290**, 191–194.

Breslin M. B., Lindberg I., Benjannet S., Mathis J. P., Lazure C. and Seidah N. G. (1993) Differential processing of proenkephalin by prohormone convertases 1(3) and 2 and furin. *J. Biol. Chem.* **268**, 27084–27093.

Cameron A., Apletalina E. V. and Lindberg I. (2001) The enzymology of PC1 and PC2. *The Enzymes* **XXII**, 291–331.

Christie D. L., Birch N. P., Aitken J. F., Harding D. R. and Hancock W. S. (1984) Antisera to synthetic peptides recognize high molecular weight enkephalin-containing proteins. *Biochem. Biophys. Res. Commun.* **120**, 650–656.

Day R., Lazure C., Basak A., Boudreault A., Limperis P., Dong W. and Lindberg I. (1998) Prodynorphin processing by proprotein convertase 2. Cleavage at single basic residues and enhanced processing in the presence of carboxypeptidase activity. *J. Biol. Chem.* **273**, 829–836.

Goumon Y., Lugardon K., Gadroy P., Strub J. M., Welters I. D., Stefano G. B., Aunis D. and Metz-Boutigue M. H. (2000) Processing of proenkephalin-A in bovine chromaffin cells. Identification of natural derived fragments by N-terminal sequencing and matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Biol. Chem.* **275**, 38355–38362.

Hollt V. (1986) Opioid peptide processing and receptor selectivity. *Annu. Rev. Pharmacol. Toxicol.* **26**, 59–77.

Johanning K., Mathis J. P. and Lindberg I. (1996a) Processing site blockade results in more efficient conversion of proenkephalin to active opioid peptides. *J. Biol. Chem.* **271**, 27871–27878.

Johanning K., Mathis J. P. and Lindberg I. (1996b) Role of PC2 in proenkephalin processing: antisense and overexpression studies. *J. Neurochem.* **66**, 898–907.

Johanning K., Juliano M. A., Juliano L., Lazure C., Lamango N. S., Steiner D. F. and Lindberg I. (1998) Specificity of prohormone convertase 2 on proenkephalin and proenkephalin-related substrates. *J. Biol. Chem.* **273**, 22672–22680.

Jung L. J., Kreiner T. and Scheller R. H. (1993) Expression of mutant ELH prohormones in AtT-20 cells: the relationship between prohormone processing and sorting. *J. Cell. Biol.* **121**, 11–21.

Lamango N. S., Zhu X. and Lindberg I. (1996) Purification and enzymatic characterization of recombinant prohormone convertase 2: stabilization of activity by 21 kDa 7B2. *Arch. Biochem. Biophys.* **330**, 238–250.

Lembo P. M., Grazzini E., Groblewski T., O'Donnell D., Roy M. O., Zhang J., Hoffert C., Cao J., Schmidt R., Pelletier M. *et al.* (2002) Proenkephalin A gene products activate a new family of sensory neuron – specific GPCRs. *Nat. Neurosci.* **5**, 201–209.

Lindberg I. and Shaw E. (1992) Posttranslational processing of proenkephalin in SK-N-MC cells: evidence for phosphorylation. *J. Neurochem.* **58**, 448–453.

Lindberg I. and Zhou Y. (1995) Overexpression of neuropeptide precursors and processing enzymes, in *Peptidases and Neuropeptide Processing* (Smith A. I., ed) pp. 94–108. Academic Press, San Diego.

Lindberg I., Shaw E., Finley J., Leone D. and Deininger P. (1991) Posttranslational modifications of rat proenkephalin overexpressed in Chinese hamster ovary cells. *Endocrinology* **128**, 1849–1856.

Liu F., Housley P. R. and Wilson S. P. (1996) Initial processing of human proenkephalin in bovine chromaffin cells. *J. Neurochem.* **67**, 1457–1462.

Loh Y. P. and Gainer H. (1980) Evidence that glycosylation of pro-opiocortin and ACTH influences their proteolysis by trypsin and blood proteases. *Mol. Cell. Endocrinol.* **20**, 35–44.

Mains R. E. and Eipper B. A. (1984) Post-translational processing of neuropeptide precursors. *NIDA Res. Monogr.* **54**, 168–183.

Mains R. E. and Eipper B. A. (2000) Proopiomelanocortin synthesis and cell-specific processing. *Handbook of Physiology, Section 7: the Endocrine System, Chapter V: POMC Synthesis and Cell-Specific Processing*, *IV: Coping with the environment: neural and endocrine mechanisms*, in Handbook of Physiology, Vol IV, (McEwen B. S., ed), pp. 85–101. Oxford University Press, London.

Mathis J. and Lindberg I. (1992) Posttranslational processing of proenkephalin in AtT-20 cells: evidence for cleavage at a Lys-Lys site. *Endocrinology* **131**, 2287–2296.

Rostovtsev A. P. and Wilson S. P. (1994) Processing of proenkephalin in bovine chromaffin cells occurs in two phases. *Mol. Cell. Endocrinol.* **101**, 277–285.

Seidah N. G. and Chretien M. (1999) Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain. Res.* **848**, 45–62.

Steiner D. F. (1998) The proprotein convertases. *Curr. Opin. Chem. Biol.* **2**, 31–39.

Udenfriend S. and Kilpatrick D. L. (1983) Biochemistry of the enkephalins and enkephalin-containing peptides. *Arch. Biochem. Biophys.* **221**, 309–323.

Watkinson A. and Dockray G. J. (1989) Identification and characterization of N-glycosylated and phosphorylated variants of proenkephalin A-derived peptides in bovine adrenal medulla, spinal cord and ileum. *Regul. Pept.* **26**, 313–322.

Zhou A., Bloomquist B. T. and Mains R. E. (1993) The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in a strict temporal order during proopiomelanocortin biosynthetic processing. *J. Biol. Chem.* **268**, 1763–1769.

Zhu X., Orci L., Carroll R., Norrbom C., Ravazzola M. and Steiner D. F. (2002) Severe block in processing of proinsulin to insulin accompanied by elevation of des-64,65 proinsulin intermediates in islets of mice lacking prohormone convertase 1/3. *Proc. Natl Acad. Sci. USA* **99**, 10299–10304.