Specificity of Prohormone Convertase 2 on Proenkephalin and Proenkephalin-related Substrates*

(Received for publication, March 18, 1998, and in revised form, June 17, 1998)

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In the central and peripheral nervous systems, the neuropeptide precursor proenkephalin must be endoproteolytically cleaved by enzymes known as prohormone convertases 1 and 2 (PC1 and PC2) to generate opioid-active enkephalins. In this study, we have investigated the specificity of recombinant mouse PC2 for proenkephalin-related internally quenched (IQ) peptides, for methylcoumarin amide-based fluorogenic peptides, and for recombinant rat proenkephalin. IQ peptides exhibited specificity constants (kcat/Km) between 9.4 × 106 M−1 s−1 (Abz-Val-Pro-Arg-Met-Glu-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Glu-EDDnp; where Abz is ortho-aminobenzoic acid and EDDnp is N-(2,4-dinitrophenyl)ethylenediamine) and 0.24 × 104 M−1 s−1 (Abz-Tyr-Gly-Gly-Phe-Met-Arg-Val-Gly-Pro-Glu-EDDnp), with the peptide B to Met-enk-Arg-Phe cleavage preferred (Met-enk is met-enkephalin). Fluorogenic substrates with P1, P2, and P4 basic amino acids were hydrolyzed with specificity constants ranging between 2.0 × 107 M−1 s−1 (Ac-Orn-Ser-Lys-Arg-MCA; where MCA is methylcoumarin amide) and 1.8 × 104 M−1 s−1 (<Glu-Arg-Thr-Lys-MCA; where <Glu is pyrog glutamic acid). Substrates containing only a single basic residue were not appreciably hydrolyzed, and substrates lacking a P4 Arg exhibited kcat of less than 0.05 s−1. Substitution of ornithine for Lys at the P4 position did not significantly affect the kcat but increased the Km 2-fold. Data from both sets of fluorogenic substrates supported the contribution of a P4 Arg to PC2 preference. Analysis of proenkephalin reaction products using immunoblotting and gel permeation chromatography demonstrated that PC2 can directly cleave proenkephalin and that the generation of small opioid peptides from intermediates is mediated almost entirely by PC2 rather than by PC1. These results are in accord with the analysis of PC2 knock-out brains, in which the amounts of three mature enkephalins were depleted by more than three-quarters.

Prohormones and proenkeptides are synthesized as inactive large precursors that are proteolytically cleaved during intracellular transport to generate active peptide forms for extracellular release (1, 2). Pairs of basic amino acid residues such as Lys-Arg and Arg-Arg- and to a certain extent Lys-Lys and Arg-Lys- have been recognized as consensus sites of proteolytic cleavage (1, 2). Primary and secondary structures in proproteins are thought to be important for enzyme recognition and selectivity of cleavage (1–3). The involvement of prohormone convertases 1 and 2 (PC1 and PC2), serine proteinases expressed in neuroendocrine tissues, in precursor cleavage has been widely accepted (1, 2) although other enzymes have recently also been reported to be involved in opioid peptide precursor cleavage (4).

Previous reports from our laboratory using cell-based systems have provided support for the idea that PC1 is the chief enzyme responsible for the production of small, bioactive opioid peptides (5, 6). Detection of peptides generated in PE-expressing AtT-20 cells (7), which contain high quantities of endogenous PC1 (8), consists mainly of 3–18-kDa enkephalin-containing peptides, although some mature enkephalins such as Met-enk-Arg-Phe and Met-enk are also present (7). In contrast, the major PE products in PC2-containing AtT-20/PE cells, as well as in PC2-expressing Rin-PE cells, are the fully processed opioid peptides Met-enk-Arg-Phe, Met-enk-Arg-Gly-Leu, Met-enk, and Leu-enk (6, 7). In agreement with the notion that PC2 expression is correlated with more complete processing of PE, antisense experiments have shown that PC2 is largely responsible for the processing of PE into smaller opioid peptides in Rin cells (6). These data imply that PC2 can cleave at a wider range of sites within PE than PC1; however, the structural factors that differentiate PC1 from PC2 cleavage sites remain unclear.

Since the PCs have only recently become available in recombinant form (9–12), there is limited information on their reaction and kinetics with natural substrates. Examples of studies on the cleavage of naturally occurring peptides by recombinant

* This work was supported in part by National Institutes of Health Grant DA05084 (to L. L.) and by MRC Grant PG-11474 from the Medical Research Council of Canada (to C. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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† The abbreviations used are: PC1, prohormone convertase 1; PC2, prohormone convertase 2; IQ, internally quenched; Abz, ortho-aminobenzoic acid; MCA, methylcoumarin amide; EDDnp, N-(2,4-dinitrophenyl)ethylenediamine; HPGPC, high pressure gel permeation chromatography; Leu-enk, Leu-enkephalin; MCA, methylcoumarin amide; <Glu, pyrog glutamic acid; Boc, t-butoxycarbonyl; Met-enk, met-enkephalin; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-hydroxypropyl-1,3-diol; PE, proenkephalin; RIA, radioimmunoassay; MALDITOF, mass spectroscopy laser desorption ionization-time of flight; HPLC, high pressure liquid chromatography.
PC1 includes the cleavage of proopiomedullin (13), α1-antitrypsin (14) prothrombin-releasing hormone by PC1 (15), prodynorphin (16), and progluca (14). Studies of recombinant PC2 cleavage on natural substrates include proglucagon (18, 19), cholecystokinin-33 (20), and prodynorphin (21). Comparative work on both enzymes includes studies on the cleavage of proenkephalin (22) and proinsulin (23, 24). Taken together, this work supported the idea that both prohormone convertases prefer paired basic cleavage sites containing a P4 basic residue and can cleave at single basic residues given the presence of additional amino-terminal basic residues. However, in general, work with natural substrates has not revealed additional preferences for residues surrounding the cleavage site. Synthetic fluorogenic and internally quenched peptides and metalloprotease amide (MCA)-containing substrates have been used to determine kinetic parameters of subtilisin-like enzymes and to explore the structural features surrounding the dibasic cleavage sites that contribute to enzyme specificity (25, 26). In this study, we have investigated the kinetic properties of PC2 against proenkephalin-related internally quenched substrates and a series of fluorogenic peptide substrates. In addition, we examined the hydrolysis of recombinant PE in vitro by PC1 and PC2. Finally, we have used PC2 knock-out mice (27) to confirm the involvement of this enzyme in the natural processing of PE.

**MATERIALS AND METHODS**

*Internally Quenched Fluorescent Peptide Substrates—* To determine the specificity of PC2 for proenkephalin-derived peptides, internally quenched Abz-peptide-EDDnp substrates (where Abz is ortho-aminobenzoic acid and EDDnp is N-(2, 4-dinitrophenyl)ethylenediamine) were synthesized and purified as described previously (28, 29). The molecular weight and purity of synthesized peptides were checked by mass spectroscopy laser desorption ionization-fragmentation of (MALDI-TOF) using ToSpec-E from Micromass. In these inmolecularly quenched peptides the Abz group is attached to the amino terminus and the EDDnp moiety to a carboxyl-terminal glutamine, necessary for the solid-phase peptide synthesis (29). A total of 11 substrates, 11–12 amino acids in length, was synthesized corresponding to the sequences surrounding the dibasic cleavage sites that contribute to enzyme specificity (25, 26). In this study, we have investigated the kinetic properties of PC2 against proenkephalin-related internally quenched substrates and a series of fluorogenic peptides. In addition, we examined the hydrolysis of recombinant PE in vitro by PC1 and PC2. Finally, we have used PC2 knock-out mice (27) to confirm the involvement of this enzyme in the natural processing of PE.

**Purification of Recombinant mPC2—** Recombinant mPC2 was overexpressed in Chinese hamster ovary cells using the dihydrofolate reductase-coupled amplification method previously described (30); cells were subsequently stably supertransfected with cDNAs encoding 21-kDa rat 7B2 (12). Cells were grown in a Cellmax artificial capillary cell culture system (Cellco, Germantown, MD). PC2 was purified from 20 ml of conditioned medium (in which the primary proteins present were the 71- and 75-kDa proenzyme forms) diluted 1:3 in buffer A on a 5 × 50-mm Protein-Pak anion-exchange column (Waters Chromatography, Milford, MA) using a step gradient from 0 to 35% B in 175 min at a flow rate of 0.25 ml/min, followed by a further gradient to 100% B in 50 min at 0.50 ml/min. 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. Two-mL fractions were collected and assessed for purity by SDS-PAGE and Coomassie staining.

**Purification of Recombinant Proenkephalin and PC1—** Recombinant rat PE was overexpressed in Chinese hamster ovary cells using the dihydrofolate-reductase-coupled amplification method (30). Recombinant PE was purified from the conditioned medium essentially as described previously (30) using a 4.6 × 25-mm Yvdac semi-preparative C4 column (Yvdac, fractionation, CA) by elution with 86% acetonitrile in 0.1% trifluoroacetic acid. Recombinant PC1 was produced by the same method, but cells were grown in a Cellmax artificial capillary cell culture system as for PC2 (Cellco, Germantown, MD). PC1 was purified from 20 ml of conditioned medium diluted 13 in buffer A on a 5 × 50-mm Protein-Pak anion-exchange column (Waters Chromatography, Milford, MA) using a gradient from 0 to 100% B in 120 min. The buffers used were identical to those employed for PC2. The flow rate was 0.50 ml/min, and 1-mL fractions were collected. PC1 fractions were assessed for purity as above.

**kcat/Km Determinations of Internally Quenched Peptides—** The actual concentration of each substrate was determined by spectrophotometry (absorbance at 365 nm; extinction coefficient, 17, 300 M−1 cm−1). A stock solution of 1 mM substrate was prepared in dimethyl sulfoxide. Internally quenched substrates (final dilutions of 2 μM and 200 nM) were subjected to digestion by recombinant mouse PC2 at 37 °C in a buffer containing 100 mM sodium acetate, pH 5.0, 5 mM calcium chloride, and 0.1% Brij in a total volume of either 1 ml or 250 μl. Cuvettes and buffer were kept at 37 °C prior to the addition of substrate. Recombinant pro-PC2 was diluted 1:3 with the above buffer and incubated for 20 min at 37 °C to obtain the 66-kDa autoactivated form of this enzyme (12). The specific activity of the preparation under saturating substrate concentrations was 29 μmol/h/mg. Substrate was added to reaction buffer in the cuvette, and the cuvette was placed in the thermostated fluorometer (37 °C) for equilibration. Abs fluorescence of each substrate was measured with a Perkin-Elmer fluorometer (excitation = 320 nm; emission = 420 nm) at time 0 and was recorded at various points after the addition of PC2 (36 nm final concentration). These reaction conditions represent pseudo first-order conditions. Fluorescence data were fitted to a first-order curve by nonlinear regression (one phase exponential decay) using GraphPad version 2.0 (ISI Software, CA) as shown in Equation 1.

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Y = \text{Span} e^{-k \cdot t} + \text{plateau} \quad \text{(Eq. 1)}
\]

where Y is the amplitude of the fluorescence change; k is the apparent first-order rate constant; and plateau is the fluorescence at the end point of the reaction. The resultant apparent first-order rate constants were divided by the moles of enzyme (as calculated from the protein concentration and assuming a molecular mass of 66 kDa for activated PC2). These experiments were always performed in duplicate and, for the three best substrates of each group, at different substrate concentrations. In addition, six IQ substrates (Fig. 1, 1, 2, 5, 7, 9, and 10) were subjected to PC1 digestion under the same pseudo first-order rate conditions (2 μM substrate). PC1 (115 nm final) was preincubated in 100 mM sodium acetate, pH 5.5, 5 mM calcium chloride, 0.1% Brij for 1 h in order to attain linear cleavage rates (9). The enzyme was then added to the substrate as described above for PC2 digestions in a total volume of 1 ml.

To verify cleavage at the dibasic pair, each Abz-peptide-EDDnp substrate (100 μM) was incubated overnight with PC2 (5 μM) in the same reaction mixture as described above for subsequent isolation of the product by HPLC and was identified by mass spectroscopy (MALDI-TOF).

**Hydrolysis of Methylcoumarin Amide (MCA) Substrate Series—** Custom synthesis of Cbz-Arb-Ser-Lys-lys-MCA (where Cbz is benzoyloxy-carbonyl) was performed by Enzyme Systems Products (Dublin, CA) and verified by amino acid composition. The 7B2 hT1-21 peptide was synthesized by LSUMC Core Laboratories. Boc-Gly-Arg-lys-MCA, Boc-Glu-Arg-Thr-Lys-Arg-MCA, Boc-Val-Pro-Arg-MCA, Boc-Val-Leu-Lys-MCA, and <Glu-Arb-Thr-Lys-MCA were purchased from Peptides International, Inc. (Louisville, KY). All other fluorogenic substrates were synthesized at the Clinical Research Institute of Montreal, Canada, as described elsewhere (31). A series of MCA-based fluorogenic substrates was tested for hydrolysis rates by PC2 (3.7 to 260 ng, depending on the rate of hydrolysis) in 100 mM sodium acetate buffer, pH 5.0, containing 5 mM calcium chloride, 0.1% Brij, and 100 mM 25-kDa 7B2 (7B2 hT1-21 peptide) was subjected to digestion with recombinant PC2 (5 nM) in the presence of 100 mM sodium acetate buffer, pH 5.0, 5 mM calcium chloride, 0.1% Brij for 1 h in order to attain linear cleavage rates (9). The enzyme was then added to the substrate as described above for PC2 digestions in a total volume of 1 ml.

**Fluorogenic Peptide Enzyme Assays—** PC assays were performed in duplicate at 37 °C in a buffer consisting of 100 mM sodium acetate, 5 mM calcium chloride, and 0.1% Brij (final concentrations); the pH of the sodium acetate buffer in PC-containing reactions was 5.5, and the pH of PC2-containing reactions was 5.0. Activity was estimated using the fluorogenic substrate <Glu-Arb-Thr-Lys-MCA. For kcat/Km determinations, pro-PC2 was preincubated in assay buffer for 20 min at 37 °C for conversion to the 66-kDa active form (12). The reactions were initiated by the addition of substrate. All reactions were carried out in a 50-μl total reaction volume in a polypropylene microtiter plate as described previously (12). Free amino methyl coumarin was measured as...
with a Cambridge Technology microtiter plate fluorometer (Watertown, MA) at excitation = 380 nm and emission = 460 nm.

Proenkephalin Cleavage by PC1 and PC2—For the time course digestion experiment PC1 and PC2 (0.24 µg; 55 and 72 nm final concentrations, respectively) were preincubated at the appropriate pH values (1 h for PC1 at pH 5.5 and 20 min for PC2 at pH 5.0) as described above in a 50-µl reaction volume; recombinant rat PE (1.25 µg; 90 µM final concentration) was then added. The reaction mixtures were incubated for 0, 10, 30, 60, 90 and 120 min, at which time 1/10 volume of a 10 fold concentration was then added. The reaction mixtures were incubated at high substrate concentrations, and Michaelis-Menten kinetics cannot be applied (41). To avoid these complications, pseudo

radiometry was added. Samples were then boiled, and a tenth of the reaction mixture was subjected to electrophoresis on a 10–20% SDS-polyacrylamide gradient gel (Bio-Rad). Proteins were then transferred to nitrocellulose and subjected to Western blotting as described previously (6). The antiserum used was raised against peptide F (32) and recognizes proenkephalin as well as PE cleavage products containing peptide F. Chromaffin granule total protein (about 20 µg) was subjected to electrophoresis to compare the pattern of natural cleavage products to those resulting from the digestion with PC1 and PC2. All experiments were repeated at least twice.

Radioimmunoaassay and Size Separation of PE Digestion Products—PE (450 nM final concentration) was incubated with preincubated PC2 (40 nM) in 100 µl acetate buffer, pH 5.0, at 37 °C for 30 min. The reaction mixture was then acidified with trifluoroacetic acid (0.1% final concentration) and frozen until HPGPC, performed as described previously (33). HPGPC fractions were collected into polypropylene tubes to which 5 µl of bovine serum albumin had been added as carrier. Duplicate aliquots of each fraction were subjected to enkephalin radioimmunoaasay as described previously (34), either untreated or following treatment with trypsin and carboxypeptidase B (35) to reveal cryptic enkephalin sequences and to remove carboxyl-terminal basic amino acids that interfere with immunoreactivity. Enkephalin antisera were used against Met-enk-Ary-Gly-Leu (JAS; Ref. 36), Met-enk (RB4; Ref. 37), and Leu-enk (38). PE digestions with PC2 and RIAs were carried out at least twice with similar results.

High Pressure Gel Permeation Analysis of Enkephalins in PC2 Knock-out and Control Mouse Brain—Frozen brains from PC2-knockout and wild-type control mice were thawed and immediately homogenized in 5 volumes of ice-cold 1 n acetic acid, 20 mM HCl, and 0.1% β-mercaptoethanol. After centrifugation at 10,000 × g for 30 min, half of the supernatant was lyophilized and resuspended in 250 µl of 32% acetonitrile containing 0.1% trifluoroacetic acid for gel permeation analysis. High pressure gel permeation was carried out as described previously (6) except that the flow rate was 0.40 ml/min, and 0.4 ml was collected per fraction. Aliquots of fractions were assayed for the various enkephalins by RIA, in certain cases following tryptic digestion and carboxypeptidase B trimming as described previously (35), and the results were reported per fraction. The experiment was repeated once with a separate set of brains.

RESULTS

Fig. 1 is a diagrammatic representation of rat PE that depicts the different cleavage sites and peptides known to result from proteolytic cleavage of this precursor molecule (39). The numbers above the PE structure indicate the 11–12-residue sequences with considerable variability surrounding the Lys-

first-order reaction kinetics were applied to measure the specificity constants of internally quenched peptides for PC2, since under conditions where the substrate concentration is far below the K_m, substrate inhibition is not likely to occur. Fig. 2 depicts the progression of the digestion of the short peptide B-based sequence (internally quenched substrate J (IQ 1), see Fig. 1) by PC2. The curve was fitted using nonlinear regression according to Equation 1 under “Materials and Methods.”

Eleven internally quenched fluorogenic substrates containing amino acid sequences around proenkephalin cleavage sites were subjected to kinetic analysis to determine PC2 preference (Table I). The results are given from the highest to the lowest k_cat/K_m. Specificity constants represent the average of replicate hydrolyses performed on different occasions. MALDI-TOF and HPLC were performed on cleaved IQ substrates to positively identify cleavage products. In all cases, the HPLC and the mass spectroscopy results confirmed that PC2 cleaved at the paired basic site and not at other positions.

A 20-fold difference in specificity constants for the various sites within PE was observed using the IQ substrate series (Table I). A 2-fold difference in IQ 1 versus IQs 8 and 10 was observed, indicating that when the P’ residues are constant, PC2 prefers the P residues of peptide B to other sites. These values were fairly constant upon replication; there was an approximately 6% margin of error between replicate determinations for the k_cat/K_m values of IQ 1 and between 2 and 10% errors for IQs 8, 10, and 5. There was little difference in specificity constants among IQs 8, 10, and 5, which contain sequences with considerable variability surrounding the Lys-

\[
\text{Putative} \quad \text{signal} \quad \text{peptide}
\]

\[
\begin{align*}
23 \text{ kDa} & \quad \text{Peptide B} \\
18 \text{ kDa} & \quad \text{Peptide I} \\
8.6 \text{ kDa} & \quad \text{Peptide F} \\
5.3 \text{ kDa} & \quad \text{Peptide E}
\end{align*}
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Fig. 1. Structure of rat proenkephalin and known sites of endoproteolytic cleavage. Intermediate-sized peptides and active enkephalins (Met-enk-Ary-Gly-Leu, Met-enk-Ary-Phe, Met-enk, and Leu-enk) have been isolated from bovine adrenal medulla (39). The numbers above each cleavage site represent the internally quenched peptides that were synthesized for this study.

Determination of Specificity Constants (k_cat/K_m) of PC2 for PE-derived Internally Quenched Fluorogenic Substrates—Whereas internally quenched peptide substrate series represent an effective means to determine enzyme preference at various subsites (25, 29, 40, 41), intermolecular quenching limits the substrate concentrations that can be employed in a given experiment to less than 20 µM, making the use of these substrates for direct K_m determinations problematic. In addition, several internally quenched peptides have been found to exhibit substrate inhibition, in which initial velocity decreases at high substrate concentrations, and Michaelis-Menten kinetics cannot be applied (41). To avoid these complications, pseudo
Specificity of PC2 on Proenkephalin

Arg cleavage sites; these data indicate a fairly wide specificity for PC2.

PC2 exhibited a preference for Lys-Arg over Lys-Lys pairs for cleavage, as evidenced by an at least 10-fold difference between the IQ substrates bearing a Lys-Arg site (IQs 1, 8, 10, and 5) compared with those with a Lys-Lys (IQs 6, 11, 9, and 4) cleavage site. The only Arg-Arg-containing substrate tested (IQ 7) compared with those with a Lys-Lys (IQs 6, 11, 9, and 4) cleavage, as evidenced by an at least 10-fold difference between PC2 and PC1-containing cell lines (7, 33), we believed would represent the idea that, contrary to furin and PC1 (24), PC2 does not appear to play a particularly crucial role in determining specificity. A 20-fold decrease in PC2 preference was observed when Arg was substituted for Ala at positions P6 and P7 (IQs 14 and 15

We compared the sequences within proenkephalin with the synthetic fluorogenic substrate (pGlu-Arg-Thr-Lys-Arg-MCA) commonly used for PC activity determinations. Surprisingly, this short fluorogenic peptide was found to represent a comparably good substrate for PC2 (Table I). PC2 was able to cleave this substrate with a $k_{cat}/K_m$ of $1.90 \times 10^2$ M$^{-1}$ s$^{-1}$, comparable to the specificity constants obtained with the much longer IQ substrates 8, 10, and 5.

To confirm the above kinetic values, independent experiments were carried out using a different substrate concentration (200 nM) with the three most preferred substrates and the same enzyme concentration. Specificity constants derived from these independent experiments resulted in very similar values (within 2–6%; data not shown). In another series of experiments, IQ substrates 1, 8, and 5 were used with PC2 from another purification (36 nM). The specificity constants from these experiments were slightly lower (data not shown), but the order of substrate preference was retained. The discrepancy in specificity constants was attributable to the lower specific activity of this particular PC2 preparation.

PC2 Prefers Arg at Positions P4 and P5 in the Peptide B IQ Substrates—Since the peptide B-based IQ substrate was apparently the most preferred, we synthesized a series of IQ substrates with the same sequence but substituting Arg and Ala residues at various positions (Table II). Among the Arg-containing substrates, the greatest difference in $k_{cat}/K_m$ from the original peptide B-based IQ substrate (IQ 1) was observed with IQ 12, which contained an Arg at P5 (Table I); the specificity constant of this particular substrate was twice that of IQ 1. The substrate containing Arg at P4 (IQ 13) also exhibited an appreciably increased $k_{cat}/K_m$ compared with other IQ substrates. A substitution of Ala at P5 (IQ 18) resulted in a decrease in PC2 preference compared with an Arg substitution at this position (IQ 12). Nonetheless, when IQ 18 (Table II) was compared with IQ 1, which contains a P5 Glu (Table I), there was no appreciable decrease in specificity constants, indicating that an amino acid switch from Glu to Ala at P5 is not detrimental. Slightly decreased specificity constants were observed when Arg was substituted for Ala at positions P6 and P7 (IQs 14 and 15 versus IQs 19 and 20). However, the P4 position appears to play a particularly crucial role in determining specificity. A 20-fold decrease in PC2 preference was observed when Ala was substituted for Arg at P4 (IQ 22 versus IQ 19).

In addition to the Ala scan, we also tested the effect of size (Table I) on the peptide B IQ substrate (pGlu-Arg-Thr-Lys-Arg-MCA) commonly used for PC activity determinations. Surprisingly, this short fluorogenic peptide was found to represent a comparably good substrate for PC2 (Table I). PC2 was able to cleave this substrate with a $k_{cat}/K_m$ of $1.90 \times 10^2$ M$^{-1}$ s$^{-1}$, comparable to the specificity constants obtained with the much longer IQ substrates 8, 10, and 5.

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To confirm the above kinetic values, independent experiments were carried out using a different substrate concentration (200 nM) with the three most preferred substrates and the same enzyme concentration. Specificity constants derived from these independent experiments resulted in very similar values (within 2–6%; data not shown). In another series of experiments, IQ substrates 1, 8, and 5 were used with PC2 from another purification (36 nM). The specificity constants from these experiments were slightly lower (data not shown), but the order of substrate preference was retained. The discrepancy in specificity constants was attributable to the lower specific activity of this particular PC2 preparation.
5, 7, 9, and 10 were subjected to specificity analysis with PC1 under the same pseudo first-order reaction rate conditions. Due to the much lower catalytic rate exhibited by PC1 (0.25 μmol/h/mg) compared with PC2 (29 μmol/h/mg), higher PC1 enzyme concentrations (115 nM) and longer times of incubation were used in these experiments. \( k_{cat}/K_m \) values of PC1 for IQs 5 and 2 were \( 6.4 \times 10^3 \) and \( 1.8 \times 10^3 \), respectively, but no change in the initial fluorescence value was observed for the other IQ substrates tested (IQs 1, 7, 9, and 10) over a period of several hours, indicating that they did not serve as substrates. In fact, the specificity constants of IQs 2 and 5 for cleavage by PC1 were fairly low; these results indicate relatively low PC1 preference for these substrates compared with PC2, potentially due to the low affinity of PC1 for substrates containing a P’ bulky hydrophobic residue (26).

**Synthetic Fluorogenic Substrates: Basic Residues at Positions P1, P2, and P4 Are Preferred by PC2**—Fig. 3 depicts Lineweaver-Burk analysis of various fluorogenic substrates by PC2. Note that extremely long times of incubation (18 h) were required to observe significant hydrolysis of substrates lacking other basic residues such as Boc-Val-Pro-Arg-MCA. These analyses, from which the kinetic values shown in Table III were derived, can be used to infer binding interactions between the substrate side groups and the PC2 subsites. The presence of basic residues at positions P1, P2, and P4 resulted in substrates with at least 34-fold higher \( k_{cat}/K_m \) values than substrates lacking any of these basic residues (Table III). Data obtained using substrates 6–8 revealed that a P4 basic residue was more important in ensuring a lower \( K_m \) than a P2 basic, although this effect could also be due to the varying substrate length. Substrate 2, which differed from all the other substrates containing three basic residues in possessing a Pro residue at the P3 position, displayed almost 3-fold higher \( k_{cat} \) than the next best substrate. Substrate 5, with the unnatural amino acid Orn at P4, exhibited a higher \( K_m \) than substrate 3 with a Lys at the same position.

A preference for an Arg residue at the P1 position over Lys may exist since substrate 10, unlike substrate 9, was not appreciably hydrolyzed. Except for substrate 1, whose 3-fold higher specificity constant is attributable to its 3-fold lower \( K_m \), the specificity of PC2 for substrates 3, 4, and 5 (which displayed comparable \( k_{cat} \) values to substrate 1) was influenced more by \( V_{max} \) than by \( K_m \). There was a higher degree of tolerance at the P3 position than at all the other positions examined.

**PC2 Does Not Require Prior Action of PC1 in Processing Proenkephalin (PE) in Vitro**—In order to investigate the processing of recombinant PE by the recombinant prohormone convertases, purified PC1 (0.24 µg, final concentration 72 nM) and PC2 (0.24 µg, 55 nM) were incubated with recombinant PE (1.25 µg, 0.91 µM) for either 0, 10, 30, 60, 90, or 120 min; aliquots of reaction mixtures were then subjected to Western blotting (Fig. 4). The 1st lane in each panel shows the band corresponding to PE at zero time. Thirty minutes later, peptides with apparent molecular masses of 30, 23, and 16 kDa were observed in both digestion reactions (Fig. 4, A and B). These experiments showed that in roughly equal enzyme concentrations, PC2 was much more efficient than PC1 in cleaving PE. After 60 min of incubation, 26- and 18-kDa peptides were present as additional PC2 digestion products. At 90 min of digestion, PE was more than 50% digested in PC2-containing samples. By using comparable amounts of enzyme, 2 h after the incubation period PE was completely processed by PC2, unlike PC1-containing samples that were less than 50% digested at this time. Chromaffin granule protein was immunoblotted along with these digests (Fig. 4A, 7th lane) in order to compare the natural pattern of processing with that resulting from the

**in vitro experiments.** Except for the intermediate with an approximate molecular mass of 23 kDa, the remaining peptides possessed slightly different molecular masses. This might be due to the fact that in vitro, but not in vivo, these peptides would be expected to contain dibasic residue extensions. It should be noted that the blotting method will only detect larger intermediate peptides resulting from PE digestion, as smaller peptides such as peptide F would be expected to wash off of the blot during the procedure. Indeed, the disappearance of peptide F-immunoreactive fragments over time represents indirect evidence that further cleavage into smaller peptides occurred with PC2 but not PC1-digested PE.
PC2-mediated Cleavage of Proenkephalin Results in the Formation of Small Enkephalins—High pressure gel permeation chromatography was used to separate the reaction products of proenkephalin with PC2; RIs of Met-enk-Arg-Phe, Met-enk, and Leu-enk were used to identify enkephalin-containing peptides (Fig. 5). PC2 was able to produce low molecular weight enkephalins (500–1000 Da) including Met-enk-Arg-Phe, Leu-enk, and Met-enk, unlike PC1 which could only generate intermediate-sized enkephalins of 3 kDa and larger (42).²

PC2-Knock-out Mouse Brain Is Greatly Depleted of Enkephalins—Fig. 6 shows the profile of immunoreactive enkephalins in the brains of wild-type or PC2-knock-out animals. It should be noted that the small enkephalins resulting from recombinant PE cleavage exhibited slightly different elution times when compared with the in vivo knock-out experiments, since the presence of the salts in the reaction mixture tends to affect the elution profile.³ The Fig. 6A depicts Met-enk-Arg-Phe-immunoreactive peptides; the amount of mature Met-enk-Arg-Phe, usually the predominant immunoreactive peptide in brain (43, 44) is severely diminished in the PC2-knock-out animal, with a concomitant increase in the amount of the immediate precursor, peptide B. Digestion of precursors with trypsin and carboxypeptidase B was required to liberate the cryptic immunoreactivity in intermediates prior to assay for Leu-enk (Fig. 6B) and Met-enk (Fig. 6C). Again, the amount of mature enkephalins was severely depleted in the knock-out animals, and the Met-enk assay showed increased levels of Met-enk-containing precursors.

DISCUSSION

There is a large body of evidence implicating PC1 and PC2 in prohormone and proneuropeptide processing (reviewed in Refs.1 and 2). However, little information is available in relation to the specificity of each of these enzymes as well as to the kinetic properties of PCs on natural substrates. In the present study we have attempted to gain information on the preferences of PC2 at various substrate subsites as well as to examine the contribution of each prohormone convertase to the cleavage of proenkephalin and proenkephalin-related peptides.

Eleven internally quenched peptides (from P7 or P6 to P5) with PE cleavage site sequences were subjected to kinetic analysis to determine specificity constants of these IQ substrates. PC2 showed a great preference for the peptide B dodecapeptide sequence (IQ 1, Table I) however, unlike PC1, most other sites were cleaved with reasonable efficiency, supporting the wider specificity of PC2 as opposed to PC1. The preference of PC2 for the peptide B cleavage to Met-enk-Arg-Phe is in agreement with our in vivo experiments in PE-transfected AtT-20 cells (6). In these studies, cells coexpressing PC2 produced greater quantities of mature PE-derived peptides such as Met-enk-Arg-Phe and Met-enk-Arg-Gly-Leu compared with control cells expressing only PC1. Similarly, Rin cells in which PC2 synthesis was blocked through antisense expression exhibited diminished production of active enkephalins (6). Taken together with our previous in vivo results (6), the in vitro specificity data presented here provide further support for the role of PC2 in the production of small opioid-active enkephalins.

In support of the preference of PC2 for peptide B, in vitro experiments have demonstrated efficient cleavage of synthetic peptide B by PC2 as assessed by an HPLC peptide cleavage assay (\(K_m = 89 \mu M; k_{cat} = 0.47 s^{-1}\)).² These results showing relatively high \(K_m\) values of PC2 with putative natural peptide substrates are in agreement with recent work of others using our recombinant PC2, such as the conversion of CCK33 to CCK8 with a \(K_m\) of 105 \(\mu M\) (20) and the conversion of pro-NPY with a \(K_m\) of 69 \(\mu M\) (45). It is interesting to note that, contrary to PC1 (24), PC2 exhibits neither a higher turnover nor a better affin-
ity for larger peptide substrates, potentially indicating that the minimal substrate-binding site in PC2 may not be as extensive as that in PC1.

We then performed specificity studies with internally quenched peptides based upon peptide B but containing substitutions of either an Arg or an Ala at different positions. Although most of these substituted peptides showed respectable \( k_{cat}/K_m \) values of greater than \( 1 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \), a dramatic improvement in PC2 preference was observed when Arg was substituted at either P5 or P4, an unexpected result since none of the natural proenkephalin peptides possess either P4 or P5 basic residues. In similar kinetic studies with pro-parathyroid hormone-related internally quenched substrates cleaved by furin and PC1 (46), a P4 Arg-containing sequence (GSRKRR-SVSE) was preferred by these enzymes by 3–5-fold. Recent data obtained from peptide combinatorial library screening in our laboratory support a preference for Arg at P4 for both PC1 and PC2.4

IQ substrate series are invaluable in examining PC2 specificity and sequence preferences, but when used under pseudo first-order reaction conditions they cannot yield the individual contributions of the \( K_m \) and the \( V_{max} \) to the specificity constant. We examined these parameters using a series of fluorogenic substrates which when cleaved yield the highly fluorescent

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product amino methyl coumarin. The specificity constants for the hydrolysis of various fluorogenic substrates by PC2 (Table III) distinguish three classes of substrates as follows: those with three basic residues at the P1, P2, and P4 subites; those with two basic residues, an Arg at P1 and another at one of the other preferred basic residue-binding sites (P2 or P4); and the last group, only slightly hydrolyzed, which had only a single basic residue occupying the P1 subsite. These results are in complete accord with our IQ substrate data in which the presence of a dibasic pair (i.e., Lys-Arg) at P1 and P2 and an Arg at P4 resulted in the highest specificity constant for PC2 of all of the IQ substrates studied. This pattern is also in agreement with other kinetic studies on the mammalian convertases that demonstrated that for maximal hydrolyses rates by PC1 (17, 31) and by furin (31, 46, 47), these three subsites must be occupied by basic residues. Non-kinetic surveys of various fluorogenic substrates using PC1 and furin revealed a similar trend (9, 11, 17, 48). Substrate phage display experiments using furin demonstrated a requirement for a P1 basic residue in conjunction with a P2 and/or a P4 basic residue (49). This requirement for a P4 basic residue appears to be restricted to the mammalian enzymes, as studies with the yeast proteinase were shown an effect neither on nor on by a P4 basic residue (50). It should be noted that most PC1 and PC2 substrates do not contain a P4 basic residue; therefore, this preference may exist for the benefit of other reactions, such as autocatalysis of proenzyme cleavage, which is thought in the case of pro-PC2 to occur at two tetrabasic sequences within the proregion (51, 52).

The specificity pattern displayed by the mammalian proprotein convertases has been attributed to the presence of negatively charged binding pockets that interact with positively charged substrate side groups (53, 54); however, the mutational studies that would confirm this in the case of the PCs have not yet been performed. For PC2, the importance of charge versus length of the side chain in enzyme binding was demonstrated by the fluorogenic substrate containing a substitution of Orn at the P4 position, which exhibited a 3-fold increased compared to similar substrates with a P4 Arg or Lys. This weaker binding may be attributable to the shorter side group of Orn and could indicate that a negative charge located deep inside the S4 subsite of the enzyme is important for effective electrostatic interaction. Interestingly, when Orn versus Arg and Lys substitution studies were performed using human PC1 and furin, a P4 Orn also had a marked deleterious effect on PC1 (18-fold drop in compared with Arg, attributable mainly to an effect on ) and an even greater effect on furin (280-fold reduction in , due mainly to ) (31). Since our results with PC2 resemble PC1 more than furin, the P4 subsite of PC2 may be more similar to that of PC1 than to that of furin.

The P3 position within the substrate appears to represent a position where more tolerance is generally allowed, for kex2 (41, 50), PC1 (31) and furin (49), although acidic residues in this position are discriminated against by furin (31). Our results with PC2 support the idea that substitution at the P3 position resulted in relatively mild effects on the kinetics of substrate hydrolysis. Apart from MCA substrate 2, which contained a P3 Pro and displayed a 3-fold higher than the other substrates, various substitutions at this position did not reveal any significant side group preferences, and this conclusion was further supported by the IQ substrate data. The P3 Pro preference for PC2 appears to be unusual for this family of enzymes; Pro was not selected in a furin phage display study at this position (49), and in a quenched fluorescent peptide study a P3 Pro decreased rather than increased the specificity constants of furin and PC1 (31).

Our IQ substrate data indicate that substitutions of Arg at P6 and P7 were well accepted by PC2 compared with Ala at this position, indicating that positively charged residues at these positions neither improved nor were detrimental for PC2 recognition. These positions may therefore not be as important to determining PC2 specificity as the P1 to P4 residues; however, other substitutions will have to be tested to substantiate this conclusion. Similar results were observed in experiments with other internally quenched peptides in which PC1 tolerated considerable variety at the P6 position (46).

In order to examine the cleavage of a known physiological precursor with PC2, we analyzed the reaction of recombinant PE with PC1 and PC2 using Western blotting and RIA. These studies showed that PC2 was quite capable of cleaving PE without prior action of PC1, although this in all likelihood does not represent the physiological reaction in tissues expressing both enzymes, since PC1 may be available for catalytic action earlier in the secretory pathway than PC2 (55). Assays of size-separated immunoreactive enkephalins showed that whereas PC1 was unable to cleave peptide B to a Met-enk-Arg-Phe, PC2 readily performed this cleavage, and a major peak corresponding to this heptapeptide was observed. Overall, in agreement with the natural profile of enkephalin-containing peptides in tissues (34, 39, 44), distinct groups of peptides with characteristic sizes were generated by each enzyme; PC1 generated larger intermediates, and PC2 generated small peptides. However, some discrepancies were observed, such as the absolute inability of PC1 to perform the cleavage of either the IQ substrate containing the peptide B sequence (Table I) or the cleavage of synthetic peptide B to Met-enk-Arg-Phe in vitro compared with the known presence of this heptapeptide in AT-20 cells, which are thought to express only PC1 (55). The reasons for these discrepancies are not clear at present but may have to do with the presence of very small quantities of PC2 in AT-20 cells (56) or cellular contributions to substrate selectivity.

We also analyzed the contribution of PC2 to total PE processing in vivo by performing enkephalin RIAs on wild-type and PC2-knock-out mouse brain extracts. Taken together with previous in vivo and in vitro results (6, 33) and the findings presented here, these data support the notion that the predominant enzyme responsible for the production of the opioid-active mature penta- to octapeptide enkephalins in normal brain is PC2. The fact that no obvious diminution of brain function occurred in these animals in the face of such severe depletion of opioids indicates the potential presence of surplus functional neuropeptide in normal animals (27), potentially similar to the extreme depletion of dopamine required for the observation of Parkinson’s-like symptoms. Alternatively, developmental compensation could have occurred in the PC2 knock-out animals to employ alternative neurotransmitters to compensate for the loss of PC2.

There is disagreement as to the primary enzymes involved in the physiological cleavage of proenkephalin. Whereas a recent report has implicated an enzyme known as prohormone thiol protease as the principal enzyme involved in PE cleavage (4), the involvement of PC1 and PC2 in peptide hormone processing has been widely accepted (1, 2). The present investigation provides strong support for the idea that PC2, which our data show possesses the necessary specificity to play a primary role in the physiological processing of PE-derived intermediates, actually does so in vivo.

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Acknowledgments—We are grateful to Francois Jean for the synthesis of the peptidyl-MCA substrates. We thank Joelle Finley for assistance with the bioassay evaluations, Elizabeth Guerra for help with the radioimmunoassays, and Nathan Rockwell for advice in setting up the IQ substrate experiments.

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