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

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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 (k_{cat}/K_m) between 9.4×10^4 M^{-1} s^{-1} (Abz-Val-Pro-Arg-Met-Glu-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Gln-EDDnp; where Abz is *ortho*-aminobenzoic acid and EDDnp is *N*-(2,4-dinitrophenyl)ethylenediamine)) and 0.24×10^4 M^{-1} s^{-1} (Abz-Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-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 2.0×10^3 M^{-1} s^{-1} (Ac-Orn-Ser-Lys-Arg-MCA; where MCA is methylcoumarin amide) and 1.8×10^4 M^{-1} s^{-1} (<Glu-Arg-Thr-Lys-Arg-MCA; where <Glu is pyroglutamic acid). Substrates containing only a single basic residue were not appreciably hydrolyzed, and substrates lacking a P4 Arg exhibited k_{cat} of less than 0.05 s^{-1}. Substitution of ornithine for Lys at the P4 position did not significantly affect the k_{cat} but increased the K_m 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 proneuropeptides 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[1] 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 concerned with the generation of intermediate-sized peptides from proenkephalin (PE) and that PC2 is mostly responsible for the production of small, bioactive opioid peptides (5, 6). The profile 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

[1] 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, pyroglutamic acid; Boc, *t*-butoxycarbonyl; Met-enk, met-enkephalin; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; PE, proenkephalin; RIA, radioimmunoassay; MALDI-TOF, mass spectroscopy laser desorption ionization-time of flight; HPLC, high pressure liquid chromatography.

PC1 include the cleavage of proopiomelanocortin (13), anthrax toxin-protective antigen (14) pro-thyrotropin-releasing hormone by PC1 (15), prodynorphin (16), and proalbumin (17). Studies of recombinant PC2 cleavage on natural substrates include proglucagon (18, 19), cholecystokinin-33 (20), and prodynorphin (21). Comparative work on both enzymes includes reports on the cleavage of proneuropeptide Y (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 methylcoumarin 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 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.

MATERIALS AND METHODS

Internally Quenched Fluorescent Peptide Substrates—To determine the specificity of PC2 for proenkephalin-derived peptides, internally quenched Abz-peptidyl-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-time of flight (MALDI-TOF) using TofSpec-E from Micromass. In these intramolecularly 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 PE cleavage sites (Fig. 1). In addition, another set of 10 substrates was synthesized that corresponded to analogs of a 12-residue peptide B-like sequence (Abz-VPEMEKRYGGFMQ-EDDnp). In the latter peptides, Arg and Ala were substituted at various positions from P3 to P7 to determine the effect of sequence preference. To investigate the potential requirement for length, an extended peptide B-related substrate was synthesized (Abz-LPSDEEGESYSKEVPEMEKRYGGFMQ-EDDnp).

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 media (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 first 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 on 8.8% gels using Coomassie staining.

Purification of Recombinant Proenkephalin and PC1—Recombinant rat PE was overexpressed in Chinese hamster ovary cells using the dihydrofolate-reductase amplification method (30). Recombinant PE was purified from the conditioned medium essentially as described previously (30) using a 4.6 × 25-mm Vydac semi-preparative C4 column (Vydac, Hesperia, CA) by elution with 80% 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 1:3 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.

k_{cat}/K_m Determinations of Internally Quenched Peptides—The actual concentration of each substrate was determined using a spectrophotometer by measuring the EDDnp absorbance at 365 nm (EDDnp extinction coefficient, 17, 300 M^{-1}). A stock solution of 1 mM peptide 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. Abz fluorescence of each substrate was measured with a Perkin-Elmer fluorometer (λexcitation = 320 nm; λemission = 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.

$$Y = \text{Span } e^{-kt} + \text{plateau} \qquad \text{(Eq. 1)}$$

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 two 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-peptidyl-EDDnp substrate (100 μM) was incubated overnight with PC2 (5 nM) 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-Arg-Ser-Lys-Arg-MCA (where Cbz is benzyloxycarbonyl) was performed by Enzyme Systems Products (Dublin, CA) and verified by amino acid composition. The 7B2 hCT1-31 peptide was synthesized by LSUMC Core Laboratories. Boc-Gly-Arg-Arg-MCA, Boc-Gly-Lys-Arg-MCA, Boc-Val-Pro-Arg-MCA, Boc-Val-Leu-Lys-MCA, and <Glu-Arg-Thr-Lys-Arg-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 nM 21-kDa 7B2 (which stabilizes the enzyme; Ref. 12) at 37 °C. Various concentrations of the different peptides were used, as indicated in the figure legends, and the rates of hydrolysis were determined by fluorometry. The background fluorescence in each substrate at the start of incubation was subtracted from the final values. K_m and V_{max} values were obtained by applying Michaelis-Menten kinetics to the data using the Enzfitter program (Elsevier-Biosoft).

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 PC1-containing reactions was 5.5, and the pH of PC2-containing reactions was 5.0. Activity was estimated using the fluorogenic substrate <Glu-Arg-Thr-Lys-Arg-MCA. For k_{cat}/K_m 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

with a Cambridge Technology microtiter plate fluorometer (Watertown, MA) at λexcitation = 380 nm and λemission = 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; 0.91 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× solution of Laemmli sample buffer (0.5 M Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 10% glycerol, 2% SDS, and 0.06 mg/ml bromphenol blue) 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.

Radioimmunoassay and Size Separation of PE Digestion Products—PE (450 nM final concentration) was incubated with preincubated PC2 (40 nM) in 100 mM 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 μg of bovine serum albumin had been added as carrier. Duplicate aliquots of each fraction were subjected to enkephalin radioimmunoassay 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 used were raised against Met-enk-Arg-Phe (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 0.40 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 internally quenched peptides used for studies of PC2 specificity. As shown in the diagram, peptide B is an example of an intermediate that results from cleavage at a paired basic site and that serves as a substrate for generation of the opioid peptide Met-enk-Arg-Phe. Peptide E, an intermediate-sized peptide, can give rise to both Met-enk and Leu-enk.

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
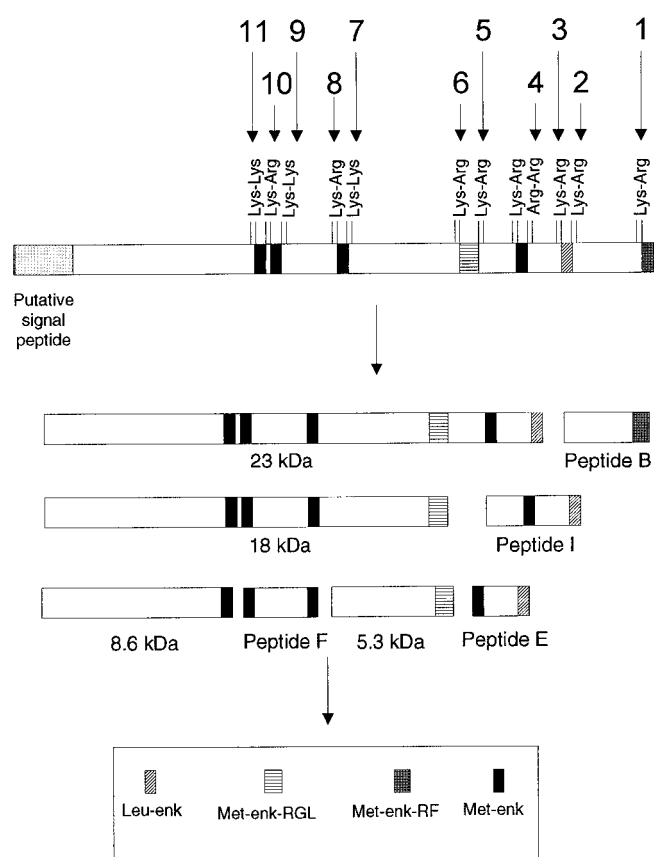

FIG. 1. **Structure of rat proenkephalin and known sites of endoproteolytic cleavage.** Intermediate-sized peptides and active enkephalins (Met-enk-Arg-Gly-Leu, Met-enk-Arg-Phe, Met-enk, and Leu-enk) have been isolated from bovine adrenal medulla (39). The *numbers above* each cleavage site represent the internally quenched peptides that were synthesized for this study.

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 *1* (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-
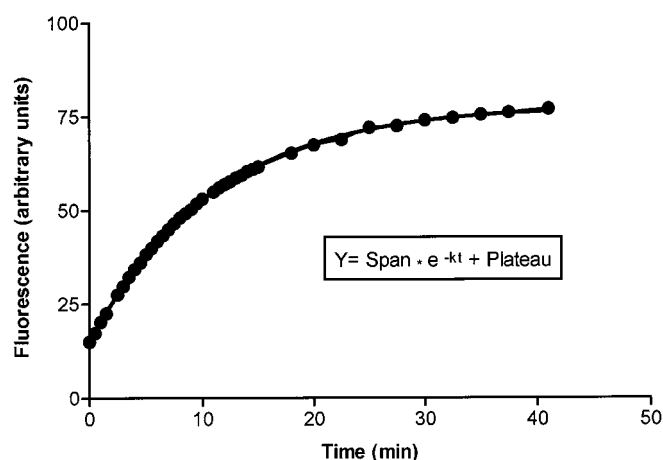
FIG. 2. **Graphical representation of the hydrolysis of an IQ substrate based on peptide B.** Time course of cleavage of Abz-VPEMEKRYGGMFMQ-EDDnp under pseudo first-order reaction conditions The *curve* was fitted with a nonlinear regression equation (see "Materials and Methods").

TABLE I
Kinetic properties of PC2 on internally quenched substrates and the fluorogenic peptide pGlu-Arg-Thr-Lys-Arg-MCA

Reactions were determined under pseudo-first order conditions, as described in the text. Numbers in the left column represent the positions assigned in the PE precursor as shown in Fig. 1. ↓ indicates cleavage site.

IQ	Peptide sequence, Abz-peptidyl-Q-EDDnp	k_{cat}/K_m
		$\times 10^4\ M^{-1}\ s^{-1}$
1	–VPEMEKR ↓ YGGFM–	4.59
8	–GEILAKR ↓ YGGFM–	2.17
10	–YGGFMKR ↓ YGGFM–	2.16
5	–FMRGLKR ↓ SPQLE–	2.13
3	–MDYQKR ↓ YGGFL–	1.11
7	–YGGFMKK ↓ DADEG–	1.03
2	–YGGFLKR ↓ FAESL–	0.94
6	–EDSTSKR ↓ YGGFM–	0.46
11	–SHLLAKK ↓ YGGFM–	0.39
9	–YGGFMKK ↓ MDELY–	0.33
4	–YGGFMRR ↓ VGRPE–	0.24
	pERTKR ↓ MCA	1.90

TABLE II
Kinetic properties of PC2 on internally quenched peptides containing Arg and Ala substitutions

The best substrate from Table I, the peptide B analog, was used as a base to systematically examine the effect of Ala and Arg substitutions. Results represent the mean of two independent replicate time course experiments performed at a substrate concentration of 2 μM. ↓ indicates the cleavage site.

IQ#	Peptide sequence Abz-Peptidyl-Q-EDDnp	k_{cat}/K_m
		$\times 10^4\ M^{-1}\ s^{-1}$
	—— ArgScanning ——	
12	–VPRMEKR ↓ YGGFM–	9.36
13	–VPEREKR ↓ YGGFM–	6.62
14	–RPEMEKR ↓ YGGFM–	5.40
15	–VREMEKR ↓ YGGFM–	4.52
16[a]	LPSDEEGESYSKEVPEMEKR ↓ YGGFM	3.58
17	–VPEMRKR ↓ YGGFM–	2.45
	—— Ala Scanning ——	
18	–VPAMEKR ↓ YGGFM–	4.51
19	–APEMEKR ↓ YGGFM–	3.05
20	–VAEMEKR ↓ YGGFM–	3.06
21	–VPEMAKR ↓ YGGFM–	3.28
22	–VPEAEKR ↓ YGGFM–	0.38

[a] Peptide 16 is not part of the Arg or Ala scanning series.

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 4) had the lowest k_{cat}/K_m, potentially indicating lesser preference by PC2. Interestingly, charges seemed to be well tolerated at the P3 and P5 positions (IQs 1 and 8).

We compared the sequences within proenkephalin with the synthetic fluorogenic substrate (<Glu-Arg-Thr-Lys-Arg-MCA) commonly used for PC activity determinations. Surprisingly, this short fluorogenic peptide was found to represent a comparatively good substrate for PC2 (Table I). PC2 was able to cleave this substrate with a k_{cat}/K_m of $1.90 \times 10^4\ M^{-1}\ s^{-1}$, 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 experi-

ments, 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 13).

In addition to the Ala scan, we also tested the effect of size preference using peptide B sequences. A 25-amino acid long peptide B-related substrate (IQ 16) was compared with smaller sized IQ substrates (it should be noted that natural peptide B is a 31-amino acid peptide). No appreciable increase in PC2 preference was observed when the longer IQ 16 (Table I) was compared with the shorter IQ 1 (Table II). These results support the idea that, contrary to furin and PC1 (24), PC2 does not exhibit a great preference for longer *versus* shorter peptides.

For PC1 studies, we tested several IQ substrates that, based upon our *in vivo* results on the cleavage of proenkephalin in PC1-containing cell lines (7, 33), we believed would represent good candidates for cleavage by this enzyme. IQ substrates 1, 2,

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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 6.4×10^3 and 1.8×10^3, 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. 4*A, 7th lane*) in order to compare the natural pattern of processing with that resulting from the
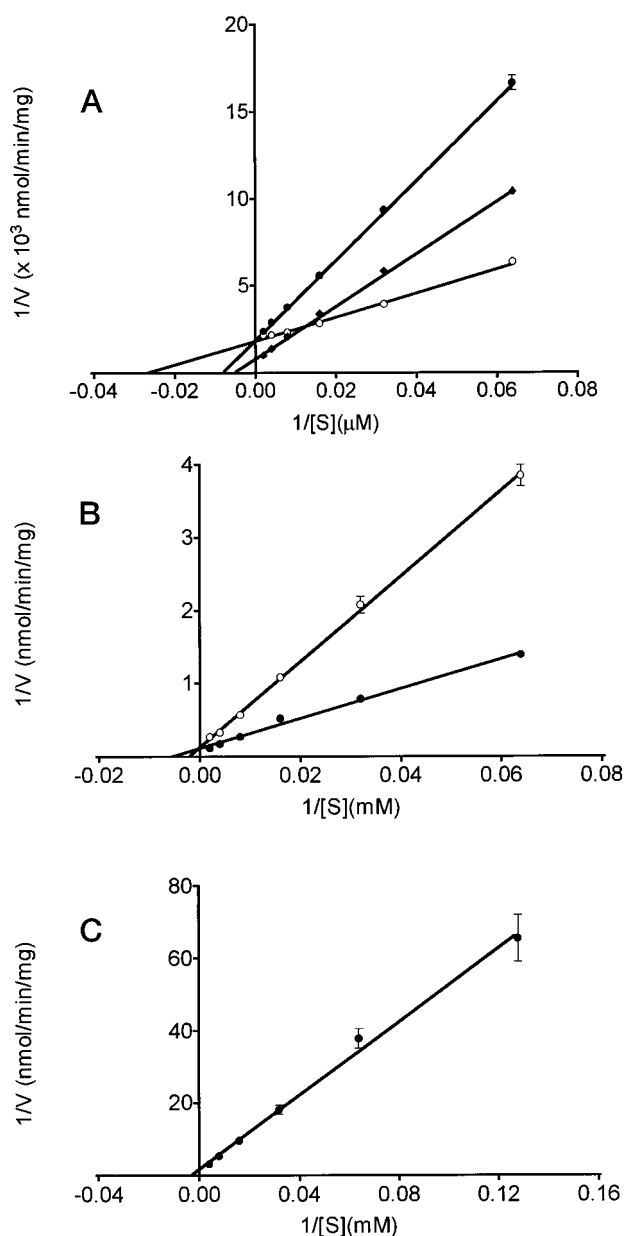

FIG. 3. **Lineweaver-Burk analysis of the cleavage of MCA fluorogenic peptides.** *A,* Cbz-Arg-Ser-Lys-Arg-MCA (●), <Glu-Arg-Thr-Lys-Arg-MCA (○), and Ac-Arg-Pro-Lys-Arg-MCA (♦) by 3.7 ng of PC2 after a 6.5 h of incubation; *B,* Ac-Arg-Phe-Ala-Arg-MCA by 260 ng of PC2 after 2 h of incubation (○); Boc-Gly-Lys-Arg-MCA (●) by 65 ng of PC2 after 18 h of incubation; and *C,* Boc-Val-Pro-Arg-MCA (●) by 65 ng of PC2 after 18 h of incubation. Purified pro-PC2 was preincubated in assay buffer for 1 h at 37 °C to activate the proenzyme and then incubated with various concentrations of substrate in the presence of 100 nM 21-kDa 7B2; initial hydrolysis rates were then measured. Individual data points represent the means ± S.E., $n = 3$.

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.

TABLE III
Kinetic properties of the hydrolysis of fluorogenic peptidyl MCA substrates

Concentrations of the enzyme and incubation times are as shown in Figure 3. Values represent the mean ± S.E. (N = 3).

	Peptide P5 P4 P3 P2 P1 PI	K_m	V_{max}	k_{cat}	k_{cat}/K_m
		μM	$nmol/min/mg$	s^{-1}	$M^{-1} s^{-1}$
1	pGlu-Arg-Thr-Lys-Arg-MCA	32 ± 3	510 ± 13	0.57 ± 0.01	17,800
2	Ac-Arg-Pro-Lys-Arg-MCA	260 ± 13	1500 ± 40	1.65 ± 0.04	6400
3	Ac-Lys-Ser-Lys-Arg-MCA	140 ± 22	660 ± 60	0.73 ± 0.07	5000
4	CBZ-Arg-Ser-Lys-Arg-MCA	110 ± 3	510 ± 4	0.57 ± 0.004	4800
5	Ac-Orn-Ser-Lys-Arg-MCA	330 ± 98	590 ± 110	0.66 ± 0.13	2000
6	Boc-Gly-Arg-Arg-MCA	510 ± 18	28 ± 6	0.03 ± 0.01	58
7	Boc-Gly-Lys-Arg-MCA	440 ± 36	17 ± 1	0.02 ± 0.001	46
8	Ac-Arg-Phe-Ala-Arg-MCA	270 ± 56	6 ± 0.5	0.01 ± 0.001	37
9	Boc-Val-Pro-Arg-MCA	1300 ± 110			
10	Boc-Val-Leu-Lys-MCA	NH[a]			

[a] NH, not hydrolyzed sufficiently for accurate determination of kinetic parameters.
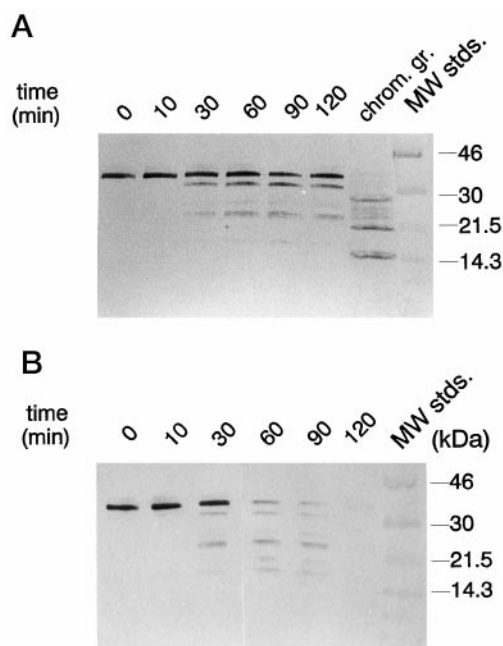

FIG. 4. **Processing of recombinant proenkephalin by PC1 and PC2: Western blotting.** PE (1.25 μg) was incubated with either preincubated PC1 (*A*) and PC2 (*B*), and reaction mixtures were subjected to Western blotting with antiserum against peptide F. Chromaffin granule protein (*chrom. gr*) was included in this blot. Molecular mass standards are indicated.

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; RIAs 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. 6*A* depicts Met-enk-Arg-Phe-im-

[2] K. Johanning and I. Lindberg, unpublished data.
[3] K. Johanning, unpublished data.

munoreactive 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. 6*B*) and Met-enk (Fig. 6*C*). 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 P'5) 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 μM; 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 CC8 with a K_m of 105 μM (20) and the conversion of pro-NPY with a K_m of 69 μM (45). It is interesting to note that, contrary to PC1 (24), PC2 exhibits neither a higher turnover nor a better affin-
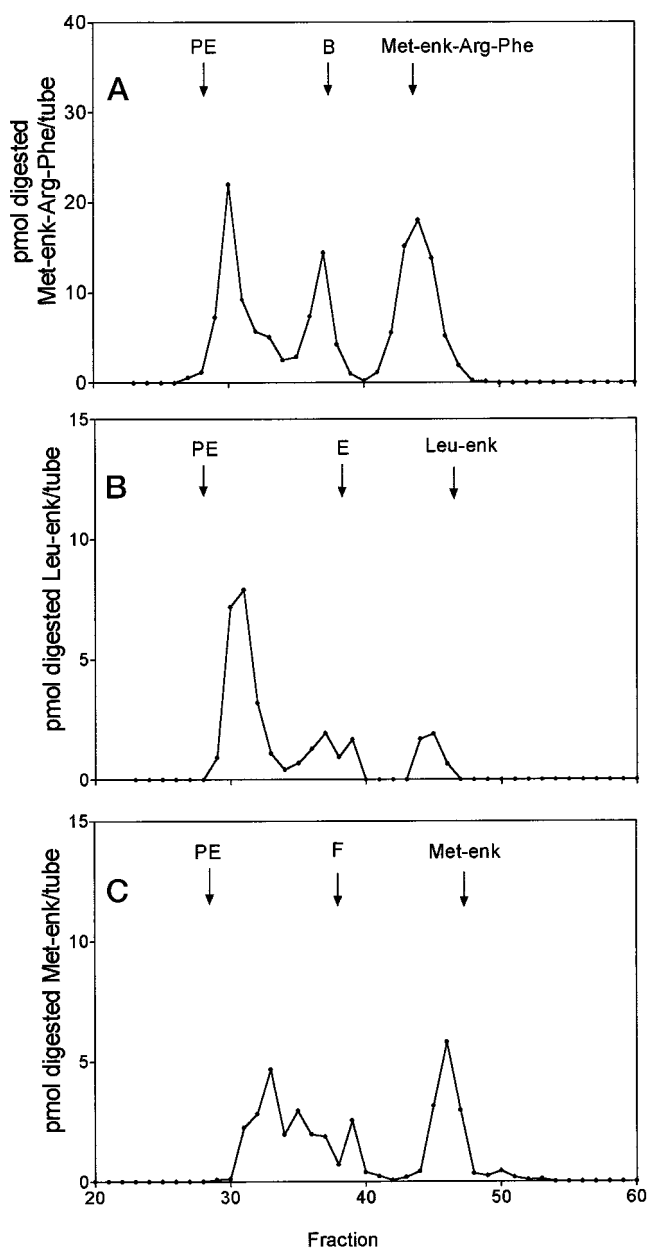
FIG. 5. *In vitro* **processing of recombinant proenkephalin by PC2: size separation and RIA analysis.** Following HPGPC of reaction mixtures, enkephalin radioimmunoassays were performed on aliquots of fractions. *B* and *C*, samples were digested with trypsin and carboxypeptidase B prior to RIA to reveal immunoreactivity within larger fragments. *A*, Met-enk-Arg-Phe; *B*, Leu-enkephalin; *C*, Met-enkephalin. *Arrows* indicate the elution positions of the standards used: *PE* (proenkephalin); peptide B (*B*), Met-enk-Arg-Phe, peptide E (*E*), Leu-enk, peptide F (*F*), and Met-enk.

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 $>1 \times 10^4$ M^{-1} 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
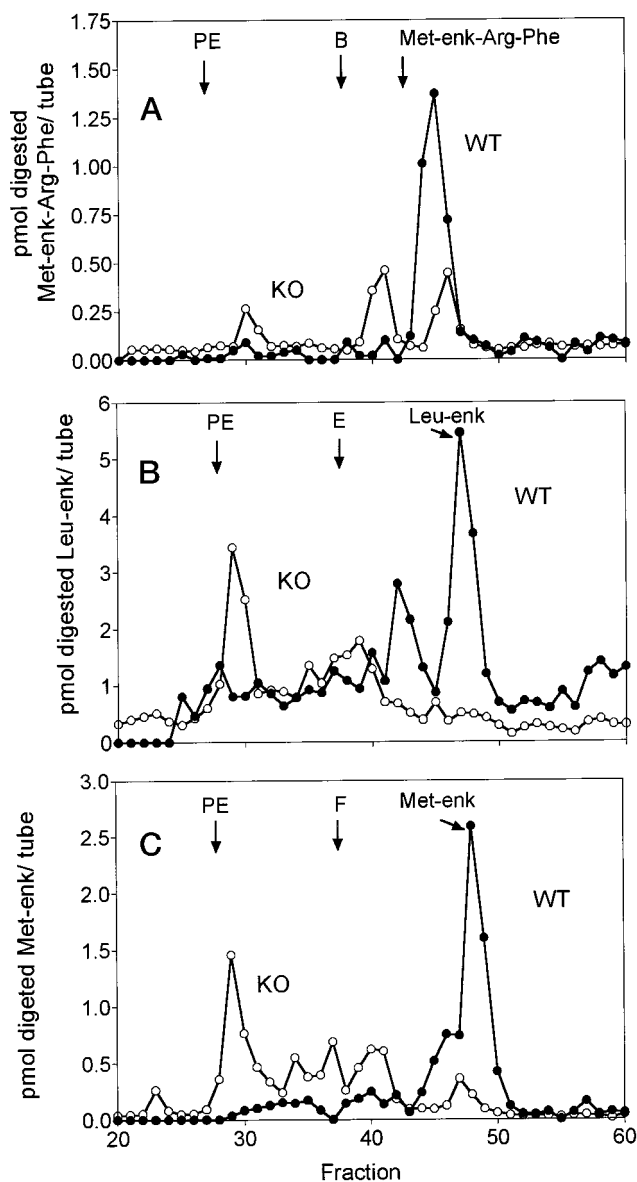

FIG. 6. **Analysis of enkephalins in PC2-knock-out and wild-type mouse brain.** Frozen brains from knock-out (*KO*) and wild-type (*WT*) control mice were homogenized and centrifuged, and an aliquot of the supernatant was subjected to HPGPC. Enkephalin RIAs were then performed on duplicate aliquots of fractions. *B* and *C*, aliquots were digested with trypsin and carboxypeptidase B prior to RIA to reveal immunoreactivity contained within larger fragments. *Closed circles*, wild-type brain; *open circles*, PC2-knock-out brain. *A*, Met-enk-Arg-Phe; *B*, Leu-enkephalin; *C*, Met-enkephalin. *Arrows* indicate the elution positions of the standards used: *PE* (proenkephalin), peptide B (*B*), Met-enk-Arg-Phe, peptide E (*E*), Leu-enk, peptide F (*F*), and Met-enk.

and PC1 (46), a P4 Arg-containing sequence (GSRKKR-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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[4] E. Apletalina, J. Appel, N. S. Lamango, R. A. Houghten, and I. Lindberg, submitted for publication.

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 subsites; 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 hydrolysis 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 kex2 showed an effect neither on K_m nor on k_{cat} 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).[5]

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 K_m 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 k_{cat}/K_m compared with Arg, attributable mainly to an effect on K_m) and an even greater effect on furin (280-fold reduction in k_{cat}/K_m, due mainly to k_{cat}) (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 k_{cat} 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 *in vitro* to form Met-enk-Arg-Phe,[2] 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*[2] compared with the known presence of this heptapeptide in AtT-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 AtT-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 in peptide-containing pathways.

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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[5] N. S. Lamango, E. Apletalina, J. Liu, and I. Lindberg, submitted for publication.

Specificity of PC2 on Proenkephalin

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