THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 2, Issue of January 9, pp. 829–836, 1998
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Printed in U.S.A.

Prodynorphin Processing by Proprotein Convertase 2

CLEAVAGE AT SINGLE BASIC RESIDUES AND ENHANCED PROCESSING IN THE PRESENCE OF CARBOXYPEPTIDASE ACTIVITY*

(Received for publication, April 9, 1997, and in revised form, October 24, 1997)

Robert Day‡§, Claude Lazure¶, Ajoy Basak¶, Alain Boudreault¶, Paul Limperis‡, Weijia Dong‖, and Iris Lindberg**

*From the ‡Department of Pharmacology, University of Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada, the ¶Laboratory of Neuropeptide Structure and Metabolism, Clinical Research Institute of Montreal, Montreal, Quebec, Canada, the ‖Montreal Children's Hospital Research Institute, Montreal, Quebec H3H 1P3, Canada, and the **Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, Louisiana 70112*

Endoproteolytic processing of the 26-kDa protein precursor prodynorphin (proDyn) at paired and single basic residues is most likely carried out by the proprotein convertases (PCs); however, the role of PCs at single basic residues is unclear. In previous studies we showed that limited proDyn processing by PC1/PC3 at both paired and single basic residues resulted in the formation of 8- and 10-kDa intermediates. Because PC2 is colocalized with proDyn, we examined the potential role of this convertase in cleaving proDyn. PC2 cleaved proDyn to produce dynorphin (Dyn) A 1–17, Dyn B 1–13, and α-neo-endorphin, without a previous requirement for PC1/PC3. PC2 also cleaved at single basic residues, resulting in the formation of the C-peptide and Dyn A 1–8. Only PC2, but not furin or PC1/PC3, could cleave the Arg-Pro bond to yield Dyn 1–8. Structure-activity studies with Dyn A 1–17 showed that a P_4 Arg residue is important for single basic cleavage by PC2 and that the P_1' Pro residue impedes processing. Conversion of Dyn A 1–17 or Dyn B 1–13 into leucine-enkephalin (Leu-Enk) by PC2 was never observed; however, Dyn AB 1–32 cleavage yielded small amounts of Leu-Enk, suggesting that Leu-Enk can be generated from the proDyn precursor only through a specific pathway. Finally, PC2 cleavages at single and paired basic residues were enhanced when carried out in the presence of carboxypeptidase (CP) E. Enhancement was blocked by GEMSA, a specific inhibitor of CPE activity, and could be duplicated by other carboxypeptidases, including CPD, CPB, or CPM. Our data suggest that carboxypeptidase activity enhances PC2 processing by the elimination of product inhibition caused by basic residue-extended peptides.

Opioid peptides are produced by the endoproteolytic processing of three distinct polypeptide precursors: proopiomelanocortin, proenkephalin, and prodynorphin (proDyn).[1] ProDyn is the precursor of leucine-enkephalin (Leu-Enk) C-terminally extended sequences including dynorphin (Dyn) A 1–17, Dyn A 1–8, Dyn B 1–13, and α-neo-endorphin (αNE) (1). Flanking single and paired basic residue cleavage sites are observed within the proDyn precursor (see Fig. 1). Even though proDyn processing appears to be a complex model to study processing (2), it is common for neuropeptide precursors to have multiple cleavage sites. It is interesting to note that *in vivo* processing of this precursor results in defined sets of neuropeptide products such as Dyn A 1–17 and Dyn B 1–13, but little if any Leu-Enk is ever observed in the same neurons (2, 3). This suggests that not all apparent basic residue cleavage sites are used within the secretory pathway or that the intracellular processing enzymes responsible for proDyn processing have specific preferences for these processing sites.

As with other neuropeptide precursors, the paired basic residues within proDyn are most likely processed by the family of enzymes known as the proprotein convertases (PCs) (4). These include furin/paired amino acid cleaving enzyme (PACE) (5, 6), PC1/PC3 (7–9), PC2 (7, 10), PC4 (11, 12), PACE4 (13), PC5/PC6 (14, 15), and LPC/PC7/PC8 (16–18). The fact that PC1/PC3 and PC2 are principally expressed in neurons and endocrine cells (19–21) suggests that they are candidate enzymes for the processing of neuropeptide precursors such as proDyn (2). In previous studies we tested the cleavage specificity of PC1/PC3 for proDyn (22). PC1/PC3 cleaved proDyn at both single and paired basic residues to yield processing intermediates of 8 and 10 kDa (Fig. 1). This study suggested that further processing by another enzyme(s) was required to achieve biologically active opioid peptides. Based on the processing profiles of other protein precursors such as proopiomelanocortin (23), proenkephalin (24), and pro-insulin (25) and also based on colocalization studies in the central nervous system (20, 26, 27), we concluded that PC2 was the best candidate to produce the expected final proDyn products.

Processing of protein precursors also requires the action of carboxypeptidases to yield a fully mature peptide (28). Once endoproteolysis has occurred C-terminal to a single or paired basic residue site, a carboxypeptidase is required for C-terminal trimming of the basic residue(s). Although CPE was long thought to be the only carboxypeptidase to carry out this function within secretory pathways, novel carboxypeptidases have now been discovered that carry out this function, including

* This work was supported by Medical Research Council Grants MT-13196 and ME-13678 (to R. D.) and PG-11474 (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.

§ Scholar of the Fonds de la Recherche en Santé du Quebec. To whom correspondence should be addressed: Dept. of Pharmacology, Faculty of Medicine, University of Sherbrooke, 3001 12ième Ave. Nord, Sherbrooke, Quebec J1H 5N4, Canada. Tel.: 819-564-5428; Fax: 819-564-5400; E-mail: rday@courrier.usherb.ca.

[1] The abbreviations used are: proDyn, prodynorphin; Leu-Enk, leucine-enkephalin; Dyn, dynorphin; αNE, α-neo-endorphin; PC, proprotein convertase; HPLC, high pressure liquid chromatography; CP, carboxypeptidase; RIA, radioimmunoassay; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; GEMSA, guanidinoethylmercaptosuccinic acid; PACE, paired amino acid cleaving enzyme; CT, C-terminal; MCA, aminomethylcoumarin.

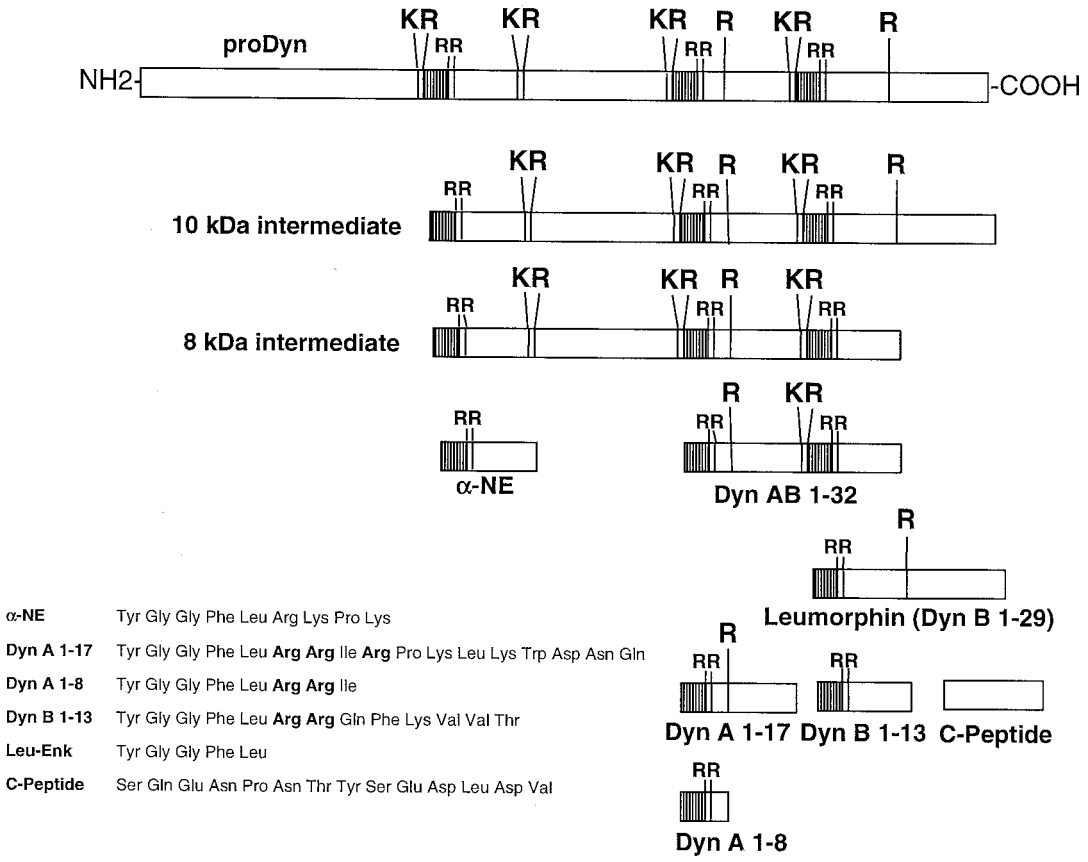


FIG. 1. **Representation of rat proDyn precursor showing possible paired and single basic cleavage sites.** Also shown are various known processing intermediates, such as the 8- and 10-kDa peptides, and final opioid peptide products. The *hatched portions* represent the opioid core of Leu-Enk.

CPD (29). This study focuses on proDyn processing by PC2 and examines the importance of carboxypeptidase activity on the efficiency of PC2 activity.

MATERIALS AND METHODS

cRNA Probes and in Situ Hybridization—The rat proDyn cRNA probe was transcribed from a *Bam*HI-*Hin*cII fragment of 733 nucleotides subcloned into pGEM4. The cRNA for rat PC2, achieved by reverse transcription-polymerase chain reaction from a rat striatum library (Stratagene, La Jolla, CA) represents a 425-base pair fragment covering the rat sequence from bases 1812 to 2236 (7). ^{35}S-UTP and ^{35}S-CTP (1250 Ci/mmol, Amersham) were used for radioactive label.

The *in situ* hybridization colocalization studies were carried out as described previously (30). Male Sprague-Dawley rats (250–300 g) were used for these studies. Sections were simultaneously hybridized with an ^{35}S-UTP/^{35}S-CTP-labeled PC2 cRNA probe (specific activity, 2.6×10^5 Ci/mmol) and a digoxigenin-UTP-labeled proDyn cRNA probe. ProDyn mRNA signal appeared as dark blue (or purple) staining, whereas PC2 mRNA appeared as silver autoradiographic grains, which could be revealed as white grains when viewed in dark field light microscope observations. Controls for specificity of hybridization were carried out by pretreatment of brain sections with RNase A or by the use of sense strand probes of the same size and specific activity. No specific labeling was observed in these experiments.

Recombinant PC2 and proDyn—Recombinant mouse proPC2 was purified from the conditioned medium of Chinese hamster ovary PC2/7B2 cells as described previously (31). Three different preparations were used during the course of the work with similar results. Rat proDyn was overexpressed in dhfr$^-$ Chinese hamster ovary cells and purified as described previously (22). No significant loss of PC2 activity is observed when the enzyme is incubated at temperatures below 42.5 °C (31). The specific activity of PC2 was 0.9 nmol/h/μg using the Cbz-Arg-Ser-Lys-Arg-aminomethylcoumarin substrate. Recombinant mouse PC1/PC3 and furin were purified from baculovirus (32). Each of these proteins was purified to apparent homogeneity. Analysis was carried out using SDS-polyacrylamide gel electrophoresis with Coomassie staining or Western blotting using specific antibodies.

Peptides and in Vitro Studies—The peptides Dyn A 1–17, Dyn A 1–8, Dyn A 1–9, porcine leumorphin, Dyn AB 1–32, and Dyn A 2–17 were purchased from Peninsula Laboratories (Belmont, CA). Dyn A 1–17 peptide analogs (see Fig. 5) were synthesized on an automated peptide synthesizer (Applied Biosystems) using Fast-Moc chemistry (33). The following side chain protecting groups were used: *tert*-butyloxy-carbonyl for Lys, *tert*-butyl for Tyr and Asp, trityl (triphenylmethyl) for Trp, and 2,2,5,7,8-pentamethylchroman-6-sulfonyl for Arg side chains, respectively. The crude peptides obtained upon cleavage from the resin and complete deprotection were purified by reverse phase HPLC performed on a semi-prep CSC-Excil C_{18} column (25 × 1.0 cm, Chromatography Sciences Co., St. Laurent, QC, Canada) using a linear gradient of 20–60% acetonitrile in 0.1% trifluoroacetic acid. The peptides were fully characterized by electron spray mass spectroscopy and amino acid analysis.

Between 5 and 20 μg of purified recombinant proDyn or various proDyn-derived synthetic peptides were incubated with PC2 (200 ng/reaction). The incubations were carried out in 50 mM sodium acetate, pH 5.0, containing 5 mM $CaCl_2$ and 0.1% Brij-30. Incubation times varied from 30 min to 24 h depending on the experiment. In some experiments different carboxypeptidases were coincubated in the reaction mixture. These included CPE, CPD, CPM (provided by Dr. Lloyd Fricker, Albert Einstein College of Medicine), and CPB (Sigma). The specific activity of CPE was determined using the substrate dansyl-Phe-Ala-Arg. 10 ng of CPE completely cleave 50 nmol of Ala-Arg bonds in 1 h. In the described experiments, where CPE was used, excess CPE conditions (>200 ng/24 h incubation) were always added to ensure that all C-terminal basic residues would be cleaved. Similar conditions (>200 ng/reaction) were also applied with the other carboxypeptidases, CPD, CPM, and CPB. To terminate the reaction, the samples were diluted 1:1 (vol:vol) with acidified methanol (0.1 N HCl). The samples were then submitted to gel filtration chromatography or reverse phase HPLC analysis.

Chromatography and RIAs—For HPLC, the samples were injected using an automated injector system (Hitachi L-7200) onto a Beckman 5-μm C_{18} column (25 × 0.46 cm). The samples were separated using a 15–35% acetonitrile gradient in 0.1% trifluoroacetic acid increasing at a rate of 0.5%/min. Peaks were detected by UV light at 210 nm and

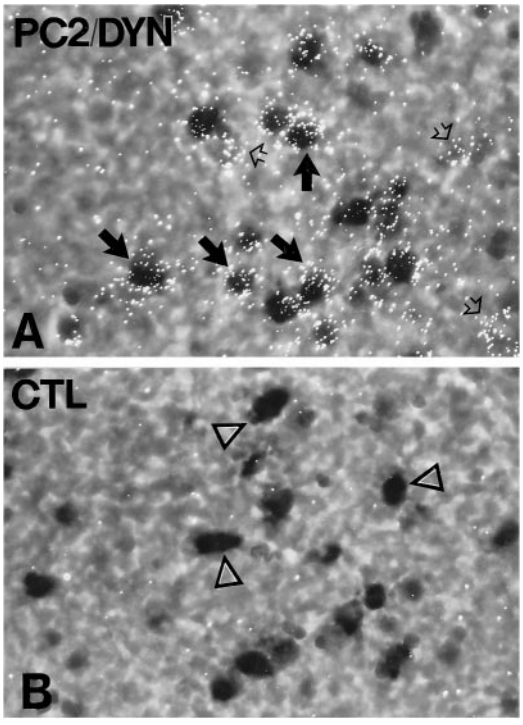


FIG. 2. *A*, *in situ* hybridization histochemistry showing colocalization of proDyn and PC2 mRNAs within the rat striatal neurons. ProDyn mRNA was detected using a nonradioactively labeled cRNA probe and appears as *dark cells*. PC2 was detected using a radioactively labeled cRNA probe and appears as *white grains* (autoradiographic grains observed in dark field). The *full arrows* show examples of colocalization, whereas the *small open arrows* show examples of PC2 expressing neurons that do not express proDyn. *B*, control brain section hybridized with sense strand PC2 probe. The *arrowheads* point to proDyn mRNA expressing neurons.

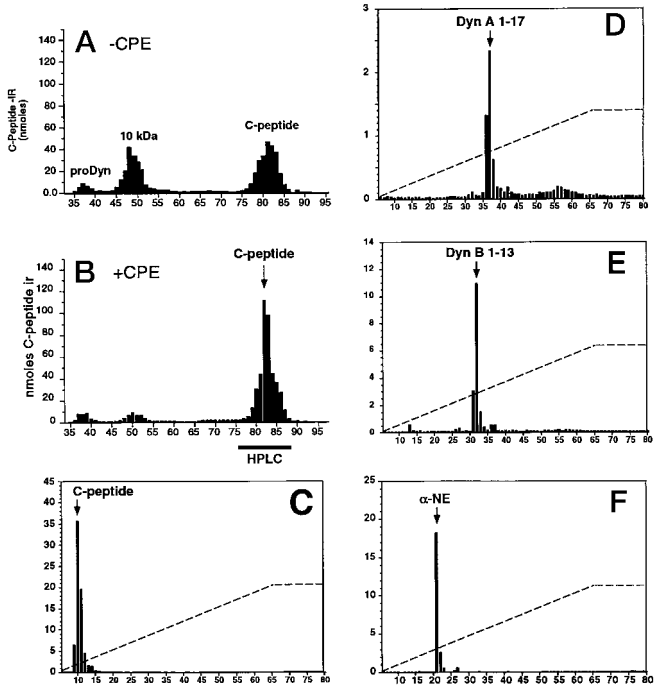


FIG. 3. **Seiving chromatography (*A* and *B*) and HPLC analysis (*C–F*) of proDyn incubated with PC2.** In *A* and *B* proDyn/PC2 were incubated for 24 h at 37 °C with or without CPE. The G-50 fractions obtained were analyzed using the C-peptide RIA. Note that in both cases the C-peptide is produced (a single basic cleavage site); however, PC2 processing is more efficient in the presence of CPE. The fractions indicated in *B* were pooled and then submitted to HPLC chromatography. Within this region of the chromatogram, peptides ranging in size from 10 to 30 amino acids should be found. In *C* the HPLC fractions were analyzed with the C-peptide RIA. A C-peptide immunoreactive peak was obtained eluting at the same retention time as the C-peptide standard. The same fractions were also analyzed for Dyn A 1–17 (*D*), Dyn B 1–13 (*E*), and αNE (*F*). Immunoreactive peaks were detected that correspond to the elution times of the respective peptide standards. For *C–F* the *y* axis represents immunoreactivity in nmol.

evaluated by peak height. All integrations were performed automatically with the Hitachi D-7000 HPLC System Manager program. HPLC fractions were dried down and submitted to amino acid analysis. Gel chromatography was performed using Sephadex G-50 using a running buffer of 0.1% formic acid with 0.1% bovine serum albumin (34). G-50 fractions were collected, dried, resuspended, and submitted to RIA. For both HPLC and G-50 chromatography peptide standards were run under the same conditions were used to determine the elution times of the peptide of interest. For the G-50 chromatography dextran blue and cobalt chloride were also used as markers of void and total volumes, respectively. The recovery yields for gel chromatography (≥85%) and HPLC analysis (95%) have been determined using radioactively labeled peptides as described previously (34).

The antibodies for Dyn A 1–17, Dyn A 1–8, Dyn B 1–13, C-peptide, and αNE were generously provided by Dr. Huda Akil (University of Michigan) and have been extensively characterized under RIA conditions (34). The Dyn B 1–13 and αNE antibodies are C terminus-directed, and under RIA conditions, neither antibody can recognize intermediates containing a hindered antigenic site at the C terminus. The C-peptide antibody recognizes any proDyn-derived intermediate with a C-peptide in its C terminus.

Fluorogenic Enzyme Assay—The effect of CPE on the hydrolysis of Cbz-Arg-Ser-Lys-Arg-aminomethylcoumarin (MCA) by PC2 was studied in a 50-μl reaction volume containing 6 ng of activated PC2, 200 mM substrate, in 0.1 M sodium acetate buffer, pH 5.0, containing 5 mM calcium chloride and 0.1% Brij-30. Reactions were carried out in duplicate, and the liberated aminomethylcoumarin was estimated by fluorometry at the time intervals stated. The experiment was repeated once with similar results.

RESULTS

In Situ Hybridization Colocalization Studies of PC2 and proDyn—In Fig. 2, we provide direct evidence of the colocalization of proDyn and PC2 in the central nervous system. Using a combined *in situ* hybridization technique, we observed PC2 mRNA within proDyn expressing neurons in the striatum. Other brain regions were also examined, including the paraventricular and supraoptic nuclei, the hippocampus, and the cortex. In each of these regions, proDyn and PC2 mRNA colocalization was observed. However, anterior lobe gonadotrophs, which are known to express proDyn (38), co-expressed PC1/PC3 but not PC2.[2]

Processing of Recombinant proDyn by PC2 in Vitro—To determine whether PC2 was important in the formation of biologically active opioid peptides, we tested the effect of PC2 on proDyn precursor processing *in vitro*. Purified recombinant proDyn and PC2 were incubated overnight and fractionated using size exclusion chromatography (Fig. 3, *A* and *B*) and HPLC chromatography (Fig. 3, *C*, *D*, *E*, and *F*). The same experiment was also carried out in the presence of CPE (Fig. 3*B*). The fractions were analyzed with RIAs using well characterized antibodies (34) to different regions of proDyn. The data demonstrated that PC2 could generate each of the biologically active opioid peptides, including Dyn A 1–17 (Fig. 3*D*), Dyn B (Fig. 3*E*), and αNE (Fig. 3*F*), from the proDyn precursor protein. Processing intermediates (10 kDa) were observed (Fig. 3, *A* and *B*), and smaller intermediates such as Dyn AB 1–32 were also identified (data not shown). Because processing of the proDyn precursor was not complete, the values of each of the obtained final opioid products examined vary as seen in Fig. 3 (*C*, *D*, *E*, and *F*). For example, in the case of Dyn A 1–17, the immunoreactive content varies from 1 to 3 nmol, whereas C-

[2] R. Day and W. Dong, unpublished observation.

peptide immunoreactivity is in the 20–40-nmol range. It should be remembered that the Dyn A 1–17 peptide was further processed to Dyn A 1–8 and that significant levels of Dyn AB 1–32 were also observed, both peptide species contributing to the lower Dyn A 1–17 immunoreactivity observed in the HPLC fractions collected.

In addition three other observations were made: 1) an extensive processing of the proDyn C-peptide was observed (Fig. 3, *A*, *B*, and *C*) providing evidence that PC2 cleaved at single basic residues; 2) Dyn A 1–8 immunoreactivity was detected in the chromatographed samples (data not shown). Processing of Dyn A 1–8 from proDyn requires cleavage at a single basic residue; and 3) coincubation of proDyn and PC2 in the presence of CPE resulted in an enhanced processing efficiency, as observed by the reduced quantities of proDyn high molecular weight intermediates and increased quantities of C-peptide produced (Fig. 3, *A* and *B*). The studies described below further explored each of these observations.

In Vitro Processing of Dyn A 1–17 by PC2—We first studied the formation of Dyn A 1–8 by PC2 using Dyn A 1–17 as the peptide substrate (Fig. 4). Dyn A 1–17 peptide was incubated with determined amounts of PC2 active enzyme in an overnight incubation. The amount of PC2 activity was measured using a fluorogenic assay with the substrate pGlu-Arg-Thr-Lys-Arg-MCA. Each sample was analyzed by reverse phase HPLC enabling the separation of each of the processed peptides. These experiments were carried out in the absence of CPE, and therefore if cleavage occurred in between the Arg ↓ Pro site of Dyn A 1–17, we would expect the formation of Dyn A 1–9 and Dyn A 10–17. The data demonstrated that Dyn A 1–17 was cleaved into two distinct peptides, which co-eluted with Dyn A 1–9 and Dyn A 10–17 peptide standards (Fig. 4*B*). To obtain unequivocal identification of these peptides, the fractions were collected and submitted to amino acid analysis. The two peaks obtained were identified as Dyn A 1–9 and Dyn A 10–17. There was no evidence for the production of Leu-Enk-Arg-Arg (Dyn A 1–7), demonstrating that PC2 had a cleavage preference for the single basic residue site rather than a paired basic residue site in the Dyn A 1–17 substrate. In Fig. 4*C*, we tested the specificity of the cleavage reaction using the C-terminal (CT) peptide of 7B2. The CT peptide was shown to be a highly potent (*i.e.* at nM concentrations) and specific inhibitor of PC2 activity (41). The CT peptide completely abolished the cleavage of Dyn A 1–17 by PC2.

In similar *in vitro* assays, we tested whether recombinant purified PC1/PC3 or furin could also cleave Dyn A 1–17. The enzymatic activities were matched to that of PC2, based on the fluorogenic assay using the substrate pGlu-Arg-Thr-Lys-Arg-MCA. Neither PC1/PC3 nor furin had any effect on the Dyn A 1–17 substrate, even using long incubation times (>24 h) or higher enzymatic concentrations (6-fold higher levels).

Processing of Dyn A 1–17 Analogs by PC2—To investigate the involvement of amino acids surrounding the single Arg cleavage site, we prepared a series of Dyn A 1–17 synthetic peptide analogs (Fig. 5). Cleavage of each peptide was compared with the processing of Dyn A 1–17. The conditions were set such that the measured amount of PC2 activity (based on fluorogenic assay) cleaved 75% of the Dyn A 1–17 peptide. Substitution of Arg^9 by Ala^9 resulted in the complete lack of cleavage, demonstrating the importance of this basic residue and further confirming the site of PC2 cleavage. It is worth noting that PC2 was still unable to cleave the paired basic amino acid residues to produce Leu-Enk-Arg-Arg from [Ala^9]-Dyn A 1–17. The [Lys^9]-Dyn A 1–17 analog was inefficiently processed (1.3%), suggesting that a single Lys residues at the P_1 cleavage position produces a poor substrate. The [Ala^6]-Dyn

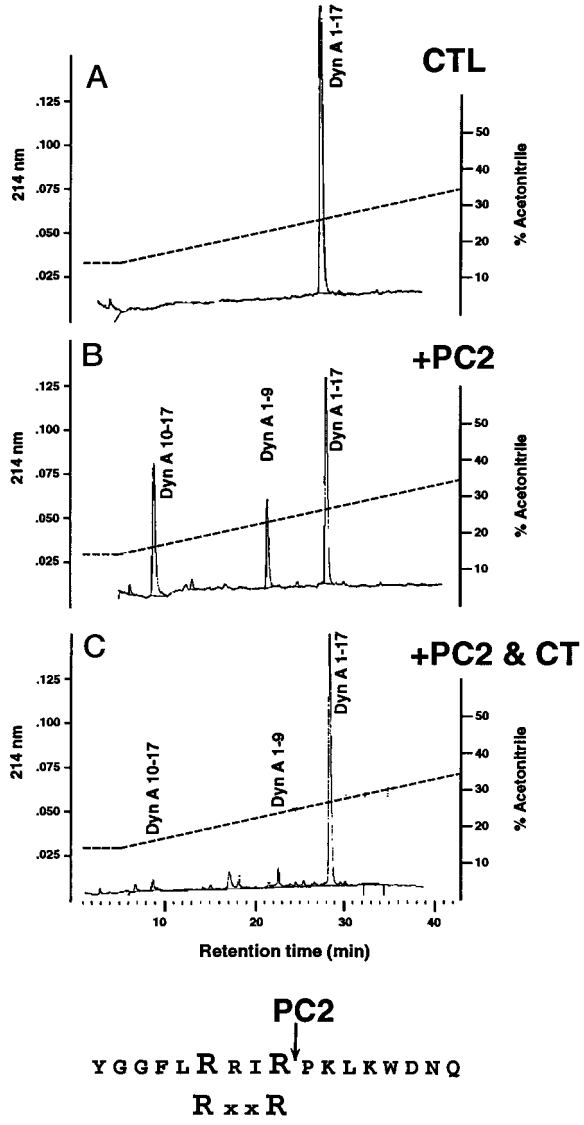


FIG. 4. **Representative HPLC chromatograms of Dyn A 1–17 incubated with PC2.** Incubation conditions were for 24 h at 37 °C. *A*, control showing retention time and amount of Dyn A 1–17 peptide used (10 μg). *CTL*, control. *B*, effect of PC2 on Dyn A 1–17 demonstrates cleavage into Dyn A 1–9 and Dyn A 10–17. Peptide peaks were collected, and amino acid analysis confirmed peak identities. *C*, specificity of cleavage is shown by coincubating with the 7B2 CT peptide, a specific inhibitor of PC2 activity. The processing of Dyn A 1–17 was completely abolished. This experiment was repeated three times with identical results.

A 1–17 analog was not cleaved by PC2, whereas efficient cleavage of the [Ala^7]-Dyn A 1–17 was observed, demonstrating that an Arg in P_4 is important for cleavage of Dyn A 1–17, whereas an Arg in P_3 is not. These data indicate that the motif R*XX*R is critical for the processing of Dyn A 1–17. Because a Pro residue at P_1' is predicted to impede processing, we tested an [Ala^{10}]-Dyn A 1–17 analog. This peptide was more efficiently cleaved by PC2 than Dyn A 1–17. Other substitutions, with related Pro analogs, where Pro^{10} was replaced with $^{D}Pro^{10}$ or hydroxy-Pro^{10} showed reduced cleavage. We also tested the influence of other basic residues located C-terminal to the cleavage site by synthesizing an [Ala^{11}]-Dyn A 1–17 analog. This peptide analog was cleaved more efficiently by PC2, suggesting that the basic residue (*i.e.* Lys) at P_2' interferes with PC2 cleavage. Finally, we examined the peptide des[Tyr^1]-Dyn A 1–17 (Dyn A 2–17). The Tyr residue is known to be conformationally important for interactions of Dyn A 1–17 with opioid receptors;

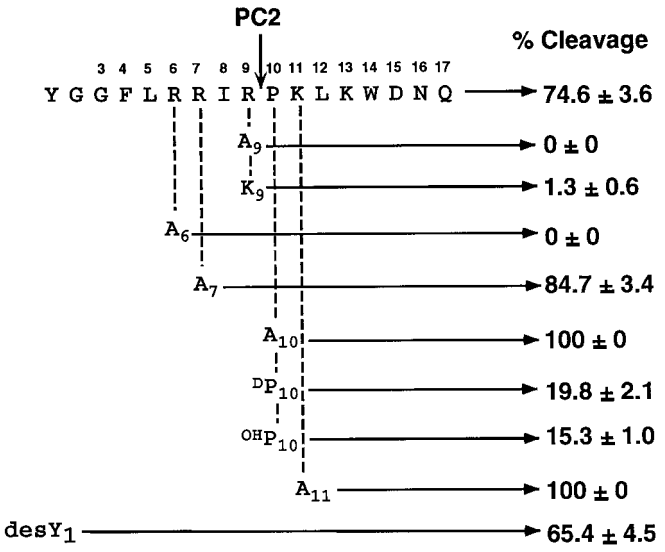
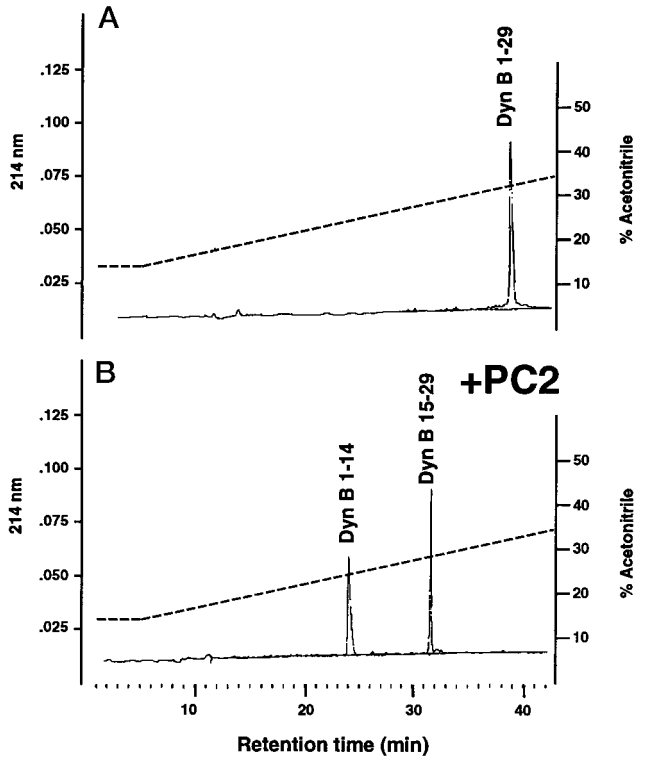


FIG. 5. **Amino acid substitutions of Dyn A 1–17 to examine the substrate specificity of PC2.** The assays were carried out simultaneously using equal concentrations of each peptide (100 μM) and PC2 enzyme activity. The samples were analyzed under the same HPLC conditions used in Fig. 4. The assay conditions were set such that approximately 75% of the control peptide, Dyn A 1–17, was cleaved. The percentage of cleavage was based on the peak height of the intact peptide used. See "Results" for a description. The values given represent the means ± S.E. (n = 3).



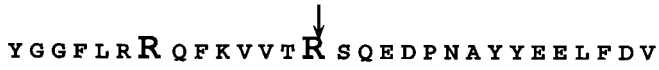


FIG. 6. **Representative HPLC analysis of porcine leumorphin incubated with PC2 demonstrating cleavage at single basic residue.** Incubation conditions were for 24 h at 37 °C. *A*, control shows retention time and amount of leumorphin (Dyn B 1–29) peptide used (10 μg). *B*, effect of PC2 on leumorphin demonstrates cleavage into Dyn B 1–14 and Dyn B 15–29. Peptide peaks were collected, and amino acid compositions confirmed peak identities. This experiment was repeated three times with identical results.

however, the effect of conformation on processing is unexplored. The data show that des[Tyr^1]-Dyn A 1–17 was well cleaved as compared with Dyn A 1–17 and that removal of the Tyr residue had only a minor effect on the ability of PC2 to cleave this peptide.

Single Basic Cleavage of PC2 in Leumorphin—As a comparative measure of PC2 processing at single basic residues, we investigated the single basic residue cleavage in leumorphin (Dyn B 1–29). In this peptide there is no Arg residue in the P_4 position; however, an Arg is found at P_8 (Fig. 6). The proDyn C-peptide was cleaved from recombinant proDyn by PC2, and thus we would also expect cleavage of leumorphin by PC2. Dyn B 1–29 was completely cleaved to yield Dyn B 1–14 (*i.e.* Arg-extended form of Dyn B 1–13) and Dyn B 15–29 (*i.e.* the proDyn C-peptide).

Enhanced Processing in the Presence of Carboxypeptidase—Addition of CPE appears to result in more efficient processing of proDyn by PC2 (Fig. 3, *A* and *B*). Further evidence of the enhancement of PC2 processing produced by CPE is provided using the fluorogenic substrate Cbz-Arg-Ser-Lys-Arg-MCA. This substrate was incubated with PC2 with or without the addition of CPE (Fig. 7). The addition of CPE resulted in a 25% increased PC2 processing efficiency.

It was unclear whether this enhancement was due to carboxypeptidase activity or the CPE protein itself. We therefore tested the reversiblity of this effect using the specific CPE inhibitor GEMSA (Fig. 8). This test was carried out using Dyn A 1–17 as the substrate. PC2 cleaved Dyn A 1–17 into Dyn A 1–9 and Dyn A 10–17 (Fig. 8*B*). The addition of CPE (Fig. 8*C*), resulted in the formation of Dyn A 1–8 instead of Dyn A 1–9, as verified by amino acid composition and a shift in retention time. Processing was more efficient in the presence of CPE (Fig. 8*C* compared with 8*B*), as determined by the reduced amount of remaining Dyn A 1–17 substrate and the concomitant increase in Dyn A 10–17 product. Based on repeated experiments, Dyn A 1–17 was cleaved with 24 ± 2% (n = 3) better efficiency in the presence of CPE. However, the addition of 100 mM GEMSA to the reaction mixture completely reversed the effect (Fig. 8*D*), because only Dyn A 1–9 was formed, and the levels of Dyn A 1–17 substrate remaining were identical to those in Fig. 8*B*. The same experiment was repeated using CPE that had been boiled for 5 min, with the result that no enhancement of PC2 processing was observed. Finally, we also tested the effects of other carboxypeptidases including CPD, CPM, and CPB. In all cases, we obtained an identical enhancement of PC2 processing (ranging from 21 to 26%).

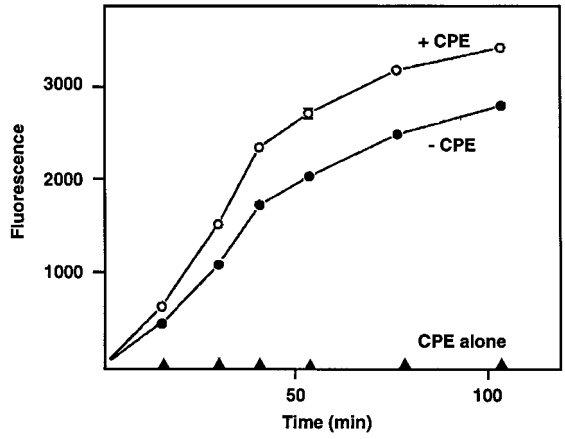


FIG. 7. **Effect of CPE on PC2 activity using fluorogenic assay.** CPE alone has no effect on this assay; however, it enhances the fluorescence produced by PC2. Other carboxypeptidases tested (CPD, CPM, and CPB) had similar effects.

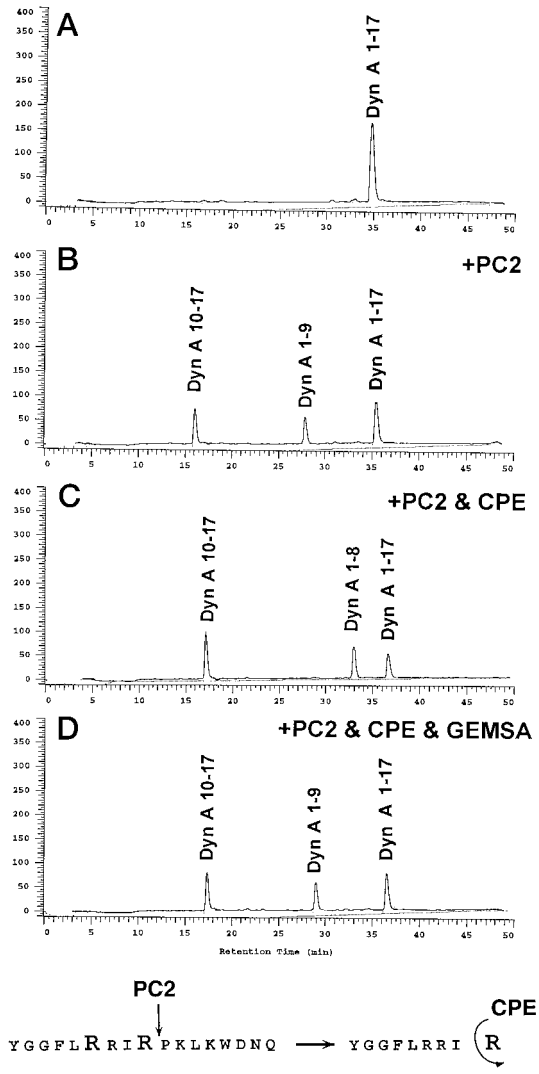


FIG. 8. **CPE enhancement of Dyn A 1–17 processing by PC2.** Incubation conditions were for 24 h at 37 °C. Representative HPLC chromatograms are shown: *A*, control Dyn A 1–17; *B*, Dyn A 1–17 with PC2; *C*, Dyn A 1–17 with PC2 and CPE; and *D*, Dyn A 1–17 with PC2, CPE, and GEMSA. Enhanced processing effect is best noted by comparing the reduction of the Dyn A 1–17 substrate in *C* as compared with *B*. In *D* the addition of GEMSA reversed this effect. Other carboxypeptidases tested (CPD, CPM, and CPB) had identical effects. In the presence of carboxypeptidase activity, Dyn A 1–9 is converted to Dyn A 1–8. This experiment was repeated three times with identical results.

The above data suggested that CPE was enhancing PC2 processing by its C-terminal trimming activity on the Dyn A 1–9 product. This would also suggest that product inhibition of PC2 was occurring. To examine this possibility, we incubated the substrate Dyn A 1–17 with PC2 with increasing concentrations of exogenous Dyn A 1–9 peptide. We then measured the levels of Dyn A 1–17 remaining, as well as the formation of the Dyn A 10–17 product (Fig. 9). Under the conditions used, initially almost 50% of the Dyn A 1–17 substrate was processed. However, with increasing concentrations of Dyn A 1–9 (50–400 mM), the levels of Dyn A 1–17 processing dropped (as measured by an increase in peak height). Concomitant decreased levels of the Dyn A 10–17 peptide product were also observed (as measured by a decrease in peak height).

CPE Enhancement of PC2 Processing on Dyn AB 1–32—We further investigated the enhancement effect of CPE on PC2 processing using a more complex peptide that has multiple cleavage sites. Dyn AB 1–32 is an intermediate in the formation of Dyn A 1–17 and Dyn B 1–13. Within this peptide, four

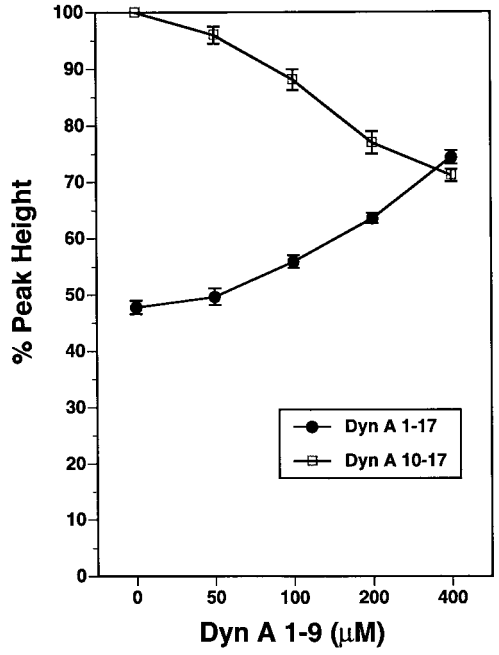


FIG. 9. **Effect of Dyn 1–9 as inhibitor of PC2 processing.** Increasing concentrations (50–400 μM) of Dyn A 1–9 (product) were added to the Dyn A 1–17/PC2 reaction mixture. The *line* with *solid circles* shows that Dyn A 1–17 processing is decreased (measured as an increase in peak height) as the exogenous concentration of Dyn A 1–9 is increased. Also shown (*open boxes*) is the decrease in Dyn A 10–17 product (also measured as a function of peak height). The values are the means ± S.E. (n = 3).

potential cleavage sites are found (*i.e.* two Arg-Arg sites, one Lys-Arg site, and a single Arg) (Fig. 10). The incubation of PC2 and Dyn AB 1–32 resulted in the formation of only Dyn A 1–19 (*i.e.* Lys-Arg extended) and Dyn B 1–13 (Fig. 10*B*). We could not detect the formation of either Dyn A 1–9 or Leu-Enk-Arg-Arg. However, when the same experiment was carried out in the presence of CPE (Fig. 10*C*), enhanced PC2 processing was revealed by the detection of Dyn A 10–17, Leu-Enk, Dyn B 8–13, and Dyn A 1–8.

Comparative Kinetic Measurements of PC2 Processing—We compared the K_m values of Dyn AB 1–32, Dyn A 1–17, and leumorphin (in the absence of CPE). These values were approximately the same for the three peptides (Dyn AB 1–32, 100 ± 17 μM; Dyn A 1–17, 79 ± 20 μM; and leumorphin 77 ± 19 μM). This suggests that the three peptides bind with equal affinity to PC2. However, we observed a major difference in turnover rates. Using the same cleavage conditions (*i.e.* enzyme and substrate concentrations) for the three peptides, we measured the time required for PC2 to cleave 50% of each substrate (*i.e.* $t_{1/2}$). The values obtained were as follows: leumorphin, $t_{1/2}$ = 1.25 h; Dyn A 1–17, $t_{1/2}$ = 13 h; and Dyn AB 1–32, $t_{1/2}$ = 19 h. Thus, leumorphin was cleaved at least 10 times faster than Dyn A 1–17 and 15 times faster than Dyn AB 1–32.

DISCUSSION

A major finding of the present study is that PC2 can process proDyn both at single and paired basic residues to produce biologically active opioid peptides. Taken together with data demonstrating the neuronal co-expression of PC2 and proDyn, a strong case can be made that PC2 is an important proDyn processing enzyme in the brain.

The distribution of proDyn mRNA, proDyn-derived peptides, and PC2 mRNA has been well studied (1, 3, 20, 34–37). ProDyn has been shown to be differentially processed in the brain and pituitary (34). Processing is more complete in the brain, resulting in final opioid peptide products, whereas only processing

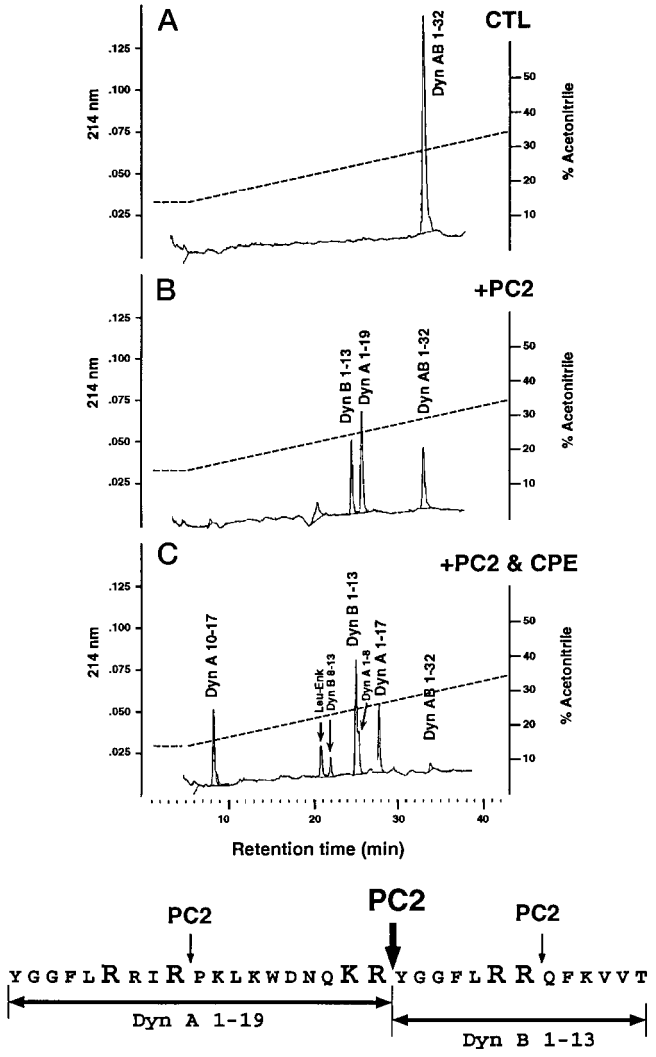


FIG. 10. **Representative HPLC chromatograms of Dyn AB 1–32 incubated with PC2.** Incubation conditions were for 24 h at 37 °C. *A*, control shows retention time and the amount of Dyn B 1–32 peptide used (10 μg). *B*, effect of PC2 on Dyn AB 1–32 demonstrates cleavage into Dyn A 1–19 (Dyn A 1–17 extended with Lys-Arg) and Dyn B 1–13. Peptide peaks were collected, and amino acid compositions confirmed peak identities. *C*, addition of CPE to the incubation mixture results in further processing and appearance of Dyn A 10–17, Leu-Enk, Dyn B 8–13, and Dyn A 1–8. Note that the Dyn AB 1–32 substrate is almost completely processed under these conditions. Identical results were obtained in duplicate experiments.

intermediates are observed in the anterior pituitary. A major difference between these two tissues are the low levels of PC2 in the anterior pituitary (21). We have previously proposed that PC2 would be critical for the processing of final proDyn opioid peptide products based on correlations between the *in vivo* cellular expression of PC2 and completeness of proDyn processing (26), where lack of PC2 expression correlated with lack of proDyn processing. The data of the present study (Fig. 3) provide direct evidence that PC2 is responsible for the formation of final opioid active peptide products (Dyn A 1–17, Dyn A 1–8, Dyn B 1–13, and αNE), which contrasts with our previous data showing that PC1/PC3 processed proDyn primarily into 8- and 10-kDa intermediates (22). To produce these final opioid active peptides, PC2 must cleave proDyn not only at paired basic residues but also at single basic residues. Two such sites are found in Dyn A 1–17 and leumorphin, resulting in the formation of Dyn A 1–8 and the C-peptide, respectively. The data of previous studies (22) with those presented here showed that PC1/PC3 could generate the C-peptide (22) but not Dyn A 1–8. Therefore, PC1/PC3 has a redundant function with PC2 to produce the C-peptide, but the formation of Dyn A 1–8 is exclusive to PC2.

Since PC1/PC3 can cleave at single basic residues, its inability to produce Dyn A 1–8 suggested that neighboring amino acids might be restricting catalysis. It is possible that the Pro residue at the P_1' position was causing an important steric hindrance. Conformational constraints can restrict proteolysis by trypsin and tryptic-like enzymes, especially when the Pro residue is found following the Arg cleavage site, as is the case with Dyn A 1–17. Indeed, trypsin cleaving C-terminal to single basic residues cannot cleave an Arg ↓ Pro bond. However, when this Arg-Pro motif is preceded by Gln (*i.e.* Gln-Arg ↓ Pro), the arginyl bond becomes susceptible again to trypsin action (47, 48). Similarly, based on the results presented using Dyn A 1–17 analogs, we believe that the presence of an Arg in the P_4 position permits PC2 to carry out the required catalysis. Thus, we suggest that the motif R*X*X*R* ↓ in Dyn A 1–17 is recognized by PC2 for cleavage, a recognition sequence that is largely preferred to the other potential cleavage site present in the same substrate, namely the Arg^6-Arg^7 pair. On the other hand, the presence of an Arg in the P_4 position does not appear to be an essential requirement for PC2 cleavage at single basic sites because we also observed the cleavage of leumorphin by PC2 (Fig. 6). In the case of leumorphin we propose that the Arg in position P_8 could be important. In this case PC2 cleavage would conform with the rules proposed by Devi (42) for single basic cleavage, which predicts that basic residues at position P_4, P_6, or P_8 are important (43).

Although Dyn A 1–17 is cleaved by PC2, the data obtained using peptide analogs (Fig. 5) provide evidence that both the Pro^9 and Lys^{11} residues (at the P_1' and P_2' positions, respectively) contribute restrictions to PC2 cleavage. Thus, structural constraints and charged residues at the P′ positions within the Dyn A 1–17 substrate are responsible for the incomplete processing of Dyn A 1–17 into Dyn A 1–8. These data may have some bearing on what is known about proDyn peptides *in vivo*. The varying ratios of Dyn A 1–17:Dyn A 1–8 that are found in different brain regions (49) could be a function of PC2 enzyme activity. There is growing evidence that both PC2 levels (21, 50) and activity (39) are regulated, and thus the Dyn A 1–17:Dyn A 1–8 ratio could be altered by changes in PC2. Although both Dyn A 1–17 and Dyn A 1–8 are selective for the κ opioid receptor, Dyn A 1–8 is more selective for the δ opioid receptor than Dyn A 1–17. Regarding behavior, κ and δ receptor are associated with aversive and rewarding behaviors. It is conceivable that modulation of PC2 activity within proDyn expressing neurons could have physiological consequences through Dyn A 1–17 processing.

Our data provide evidence for PC2 as the Dyn A 1–8 generating enzyme; however, an alternative possibility would be that a metalloendopeptidase cleaves Dyn A 1–17 N-terminal to the single ↓Arg site, directly producing Dyn A 1–8 without the need for a subsequent carboxypeptidase (44–46). Although the present data demonstrate the specificity of PC2 to cleave Dyn A 1–17 at the Arg ↓ Pro site (*i.e.* PC1/PC3 and furin had no effect), it does not exclude the above hypothesis. However, we have recently examined brain extracts of mice lacking active PC2 (51) and could not measure any Dyn A 1–8 immunoreactivity, whereas control mice showed normal levels of Dyn A 1–8. These recent observations suggest that no redundant function exists in the formation of Dyn A 1–8.

The data demonstrating enhanced PC2 processing by carboxypeptidase activity suggest an interesting relationship between the PC family of enzymes and the now growing family of

carboxypeptidases (29). The data indicate that the enhanced processing effects are not due to protein-protein interactions (between PC2 and CPE) but that carboxypeptidase activity is important. Evidence supporting this notion includes the fact that GEMSA, a specific inhibitor of carboxypeptidase activity, reversed the enhancement effect. Furthermore distinct proteins with carboxypeptidase activity (*i.e.* CPE, CPD, CPM, and CPB) produced identical enhancement effects. The enhancement of PC2 processing by carboxypeptidase was not substrate-dependent because it was observed using recombinant proDyn, Dyn A 1–17, Dyn AB 1–32, and a fluorogenic substrate. In regards to the mechanism of PC2 processing enhancement, we propose that C-terminal trimming of single or paired basic residues removes product inhibition. In support of this, our data also showed that the exogenous addition of the product could serve to inhibit the effectiveness of PC2 processing.

We believe our findings showing enhanced processing have a direct relevance to *fat*/*fat* mouse model, which has been shown to be deficient in CPE activity (52). While examining this animal model for basic residue extended peptides, several investigators noted that processing of protein precursors (*i.e.* proDyn and pro-insulin) was diminished in the *fat*/*fat* mouse (53, 54), an effect that implicates reduced processing by endoproteases such as the PCs. Based on the present data, it is likely that accumulation of basic residue extended peptides within the *fat*/*fat* mouse may cause a product inhibition of enzymes such as PC2. The data presented (Fig. 10*B*) show that products such as Dyn A 1–8, Dyn A 10–17, or even Leu-Enk cannot be generated from Dyn AB 1–32, even after long incubation periods, *unless* CPE is present (Fig. 10*C*). The presence of Dyn A 1–19 (*i.e.* Lys-Arg extended) is most likely restricting further processing of Dyn AB 1–32. Within the concentrated environment of the immature secretory granule, product inhibition could represent a significant factor in the overall processing of protein precursors.

Finally, the occurrence of the differential processing of protein precursors in distinct tissues is well established. Various mechanisms are possible, including the selective cellular expression of different enzymes responsible for the cleavage of the protein precursor substrate. The present data suggest that the activity levels of a single enzyme could also be important. In the case of proDyn, all the final opioid peptide products can be obtained with PC2. However, because there are structural constraints (for example in the Arg-Pro bond of Dyn A 1–17), changes in PC2 activity could be a mechanism used to select alternate processing pathways and ultimately leading to differential cellular biological output. Further studies examining precursor-product relationships are necessary to elucidate the mechanisms of processing by PC2, as well as further studies into the influence of carboxypeptidases on PC processing.

Acknowledgments—We thank Xue Wen Yuan for excellent technical assistance and Dany Gauthier for carrying out the amino acid compositions. We also thank Dr. Lloyd Fricker (Albert Einstein College of Medicine) for the purified carboxypeptidase enzymes (CPE, CPD, and CPM) and for helpful discussions. We thank Dr. Huda Akil (University of Michigan) for the proDyn peptide antisera against Dyn A 1–17, Dyn B 1–13, αNE, and C-peptide and Dr. Donald F. Steiner (Howard Hughes Medical Institute, Chicago, IL) for providing the brain tissues of PC2-null mice. The rat proDyn cDNA was obtained from Dr. Jim Douglass (Amgen, Thousand Oaks, CA).

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