Role of Prohormone Convertases in Pro-Neuropeptide Y Processing: Coexpression and in Vitro Kinetic Investigations†

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ABSTRACT: Proneuropeptide Y (ProNPY) undergoes cleavage at a single dibasic site Lys38-Arg39 resulting in the formation of 1−39 amino acid NPY which is further processed successively by carboxypeptidase-like and peptidylglycine α-amidating monooxygenase enzymes. To investigate whether prohormone convertases are involved in ProNPY processing, a vaccinia virus derived expression system was used to coexpress recombinant ProNPY with each of the prohormone convertases PC1/3, PC2, furin, and PACE4 in Neuro2A and NIH 3T3 cell lines as regulated neuroendocrine and constitutive prototype cell lines, respectively. The analysis of processed products shows that only PC1/3 generates NPY in NIH 3T3 cells while both PC1/3 and PC2 are able to generate NPY in Neuro2A cells. The convertases furin and PACE4 are unable to process ProNPY in either cell line. Moreover, comparative in vitro cleavage of recombinant NPY precursor by the enzymes PC1/3, PC2 and furin shows that only PC1/3 and PC2 are involved in specific cleavage of the dibasic site. Kinetic studies demonstrate that PC1/3 cleaves ProNPY more efficiently than PC2. The main difference between the cleavage efficiency is observed in the V_{max} values whereas no major difference is observed in K_m values. In addition the cleavage by PC1/3 and PC2 of two peptides reproducing the dibasic cleavage site with different amino acid sequence lengths namely (20−49)-ProNPY and (28−43)-ProNPY was studied. These shortened ProNPY substrates, when recognized by the enzymes, are more efficiently cleaved than ProNPY itself. The shortest peptide is not cleaved by PC2 while it is by PC1/3. On the basis of these observations it is proposed, first, that the constitutive secreted NPY does not result from the cleavage carried out by ubiquitously expressed enzymes furin and PACE4; second, that PC1/3 and PC2 are not equipotent in the cleavage of ProNPY; and third, substrate peptide length might discriminate PC1/3 and PC2 processing activity.

Peptide hormones, neuropeptide neurotransmitters, and protein, synthesized as parts of larger biologically inactive precursor proteins, are rendered biologically functional following a series of postranslational modifications. Such modification invariably involves endoproteolytic activity which often occurs at either single or pairs of amino acids. Genetic evidence suggests that pairs of basic amino acids are necessary for prohormone processing, since mutations in one of the basic residues present in the cleavage site of proinsulin, for example, result in secretion of the precursor rather than the mature protein (*1*, *2*). There is now evidence that the presence of basic amino acids, although necessary, is not sufficient for recognition by endoproteases and that secondary structure determinants favor the cleavage of a physiologically relevant site within a precursor (*3*, *4*).

A family of mammalian proteases has been identified and shown to cleave prohormones at basic amino acid pairs to produce bioactive peptides. The known members of the prohormone convertase family are PC1/3, PC2, furin, PACE4, PC4, PC5/6 (*5*−*13*), and the recently discovered PC7 (*14*, *15*). Northern blot analysis and in situ hybridization have demonstrated highly specific localization of PC1/3 and PC2 to endocrine and neuroendocrine tissues and of PC4 to the testis whereas the other PCs have a widespread distribution (*16*).

Proneuropeptide Y undergoes cleavage at single dibasic site Lys-Arg resulting in the formation of neuropeptide Y (NPY).[1] NPY is a 36-amino acid peptide which is widely distributed in the central and peripheral nervous system (*17*) and serves as mediator in several physiological responses. In the cardiovascular system, NPY exerts a number of important regulatory actions; it acts as a potent vasoconstritor on certain blood vessels (*18*), potentiates the action of vasoactive substances such as norepinephrine and angiotensin

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[1] Abbreviations: ACN, acetonitrile; FCS, fetal calf serum; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; RIA, radioimmunoassay; NPY neuropeptide Y; ProNPY proneuropeptide Y; FBS, fetal bovine serum; DMEM, Dulbecco's modified eagle medium; DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium methylsulfate; KH, Krebs−Hepes buffer; VVProNPY, recombinant ProNPY vaccinia virus; IRNPY, neuropeptide Y immunoreactive-like material; IRProNPY, proneuropeptide Y immunoreactive-like material; PC(s), proconvertase(s); NPY-GKR, neuropeptide Y (1−39); CPON, C flanking peptide of NPY.

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II (*19*), and inhibits presynaptically the release of catecholamines (*20*). NPY also inhibits glucose-induced insulin secretion and when administered centrally, causes hyperphagia and obesity (*21*).

Therefore, NPY represents a target for the development of therapeutic drugs. One of the possible targets is the inhibition of the proteolytic processing of ProNPY into NPY. To intervene in this pathway a complete knowledge of processing of proNPY is necessary.

The roles of PC1/3 and PC2 have been elucidated in the processing of proinsulin (*22*−*25*), propancreatic polypeptide (*26*), while PC1/3, PC2, furin, and PACE4 have all been implicated in the processing of prosomatostatin (*27, 28*).

So far, heterologous expression of ProNPY in cells expressing endogenous PC1/3 or PC2 resulted in NPY (*29*−*31*). However, the presence of NPY and of ProNPY mRNA (*32*) in peripheral tissues suggests that ProNPY could be processed as well by a widely distributed proconvertase, such as furin or PACE4. Specific affinities of PC1/3 compared to PC2 in ProNPY processing and the possible involvement of PACE4 or furin have not been assessed.

To evaluate the role of PC1/3, PC2, furin, and PACE4 in the maturation process of NPY precursor we used a vaccinia virus system to coexpress each of these convertases with ProNPY. In addition we performed an in vitro ProNPY cleavage assay. Our results demonstrate that enzymes reported to be active in constitutive secretory pathaway (i.e., furin and PACE4) are unable to cleave ProNPY and that PC1/3 is a more efficient NPY converting enzyme than PC2 in both cultured secretory cells and in the in vitro assay.

MATERIALS AND METHODS

DNA Manipulations and Vector Construction

DNA manipulations were performed according to standard procedures (*33*). The recombinant M13 (M13-ProNPY) containing the cDNA encoding rat ProNPY was constructed as follows: a 0.4 kb fragment excised from pGme3-ProNPY by *Eco*RI digestion (*34*) was subcloned into a single *Eco*RI restriction site of M13 (mp18). In order to delete the 5′ noncoding region a new *Eco*RI restriction site was created by site-directed mutagenesis (Amersham Site-Directed Mutagenesis Kit). A 0.3 kb *Eco*RI fragment from M13-ProNPY was then subcloned into the single *Eco*RI site of the vaccinia insertion plasmid pHGS1 (*35*). In the resulting insertion plasmid pHGS1-ProNPY, the initiator codon from the ProNPY cDNA was fused immediately downstream of the vaccinia 11K promotor transcription initiation site. The preparation and screening of recombinant vaccinia viruses were performed essentially as described by Bertholet et al. (*35*).

Recombinant p11K (mouse) mPC1/3, (human) hPC2, (mouse) mfurin, and (rat) rPACE4 plasmids were constructed by inserting mPC1/3, hPC2, mfurin, and rPACE4 coding regions, respectively, into p11K, a pHGS1-derived vector, using *Bam*HI and/or *Eco*RI as single restriction sites.

Cell Cultures

Neuro2A and NIH 3T3 cells were grown in DMEM (Gibco, Life Technologie) containing 10% FBS (*36*). For preparation and screening of recombinant vaccinia virus ProNPY CV1 and TK$^-$ cells were cultured in DMEM containing 8% FCS.

ProNPY and Prohormone Convertase (PC) Coexpression

Infection, Transfection, and Peptide Extraction. To identify the enzyme(s) able to process ProNPY we used a vaccinia virus modified expression system leading a coexpression of ProNPY and each of the PCs. This involved the infection of cells with recombinant rat vaccinia viruses. One hour postinfection, the cells were transfected with expression vectors in which the vaccinia 11K late promoter was linked to a cDNA fragment encoding PC1/3, PC2, furin, or PACE4. The recombinant rat NPY vaccinia virus provides the transcription factors necessary for transactivation of the 11K promotor used to drive the expression of its own linked cDNA and the 11K promotor of cDNA of transfected vector. For coexpression of ProNPY and prohormone convertases, Neuro2A or NIH 3T3 cells were grown to confluency in six-well Costar plates (5×10^5 cell per well) using DMEM supplemented with 10% FBS. Infection with ProNPY recombinant vaccinia virus at an infection multiplicity of 5 was carried out. One hour postinfection, the cells were transfected with 10 μg (per six well) of prohormone convertase constructs using DOTAP (Boehringer Mannheim) under the conditions described by the supplier. Figure 1 illustrates the design of the transient expression experiments.

After transfection the cells were incubated for 16−18 h at 37 °C. Subsequently cells were washed with 2 mL of PBS containing 0.001% Tween-20 (Pierce) and incubated with 1 mL of buffer A [0.1% BSA, 0.001% Tween-20 in KH buffer, pH 7.4 at 37 °C (KH buffer: 100 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM $MgSO_4^{2-}$, 1 mM K_2HPO_4, 25 mM $NaHCO_3$, 20 mM HepesNa, and 10 mM glucose) for 4 h. The medium collected was analyzed for PCs activities presence and ProNPY processing. Cells were washed with PBS containing 0.001% Tween-20 three times and harvested with a rubber policeman in EDTA 50 mM, 0.05% Tween-20 in 50 mM Tris buffer at pH 7.5 (1 mL per well dish). These extraction conditions show a 80% of recovery yield, no degradation of ProNPY and NPY was observed (NPY and ProNPY were used as externe references). The cell suspension was subjected to sonication for three cycles of 2 min. The samples were stored at −20 °C until fractionation by HPLC. Each experiment was repeated three times.

Western Blot

SDS−*Polyacrylamide Electrophoresis and Western Blotting*. Cell extracts were electrophorized on 1.5-mm minigels (Bio-Rad) containing 7.5% polyacrylamide in a Tris-glycine buffer system (*38*). Western blotting of samples transferred to nitrocellulose was achieved using anti-PC1/3 (2B6) and anti-PC2 (C-term) rabbit polyclonal antisera 1/200 final dilution (PC antisera were a generously gift from Dr. I. Lindberg, New Orleans). Western blots were carried out using alkaline phosphatase-coupled horse anti-rabbit second antibody (Amersham).

Protein Assay. Protein contents were assayed using the Bradford method (Bio-Rad protein assay). Bovine serum albumin was used as a standard.

A

^{1}YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRYGKR-SSPETLISDLLMRESTENVPRTRLEDPAMW^{69}

^{20}YSALRHYINLITRQRYGKR-SSPETLISDL^{49}

^{28}NLITRQRYGKR-SSPE^{43}

B

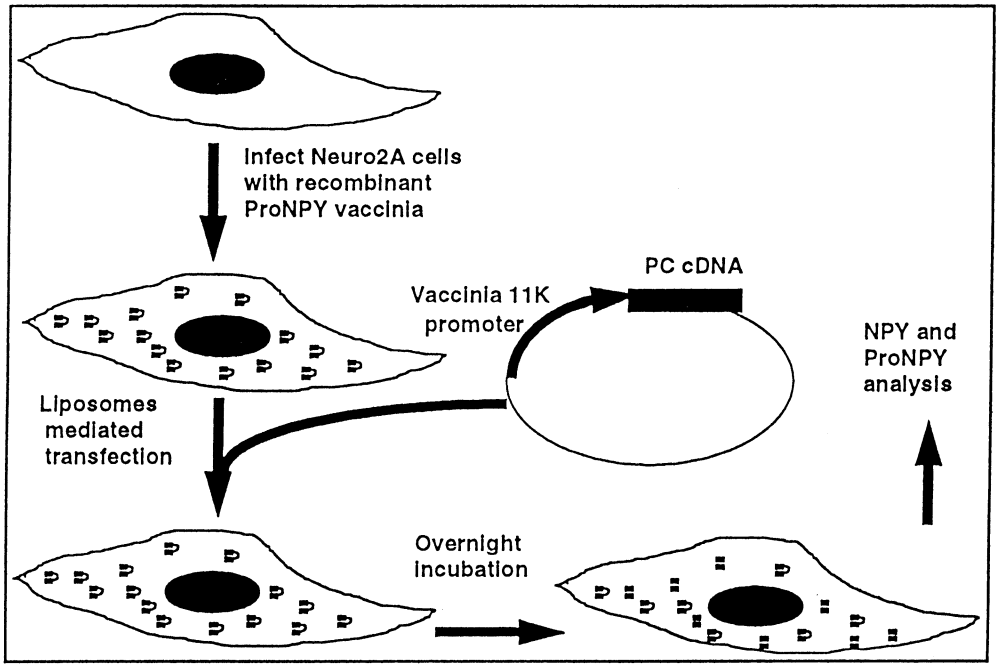

FIGURE 1: (A) Amino acid sequences of ProNPY, (20−49)-ProNPY, (28−43)-ProNPY. (B) Schematic representation of the protocol of vaccinia virus derived transient coexpression system for ProNPY and the prohormone convertases.

Expression and Purification of ProNPY

The recombinant MS2-polymerase−ProNPY cDNA was constructed as follows: for ProNPY all three Met amino acids were replaced by Leu to use a Met residue for BrCN cleaving of the fusion protein. MS2-ProNPY was cloned into *Escherichia coli* strain 537 and expressed under the control of the P_L promotor, which is regulated by cl 857, a temperature sensitive repressor.

The transformed *E. coli* 537 cells were grown over night at 32 °C in 1-L batches under vigorous shaking. Protein expression was induced by increasing temperature to 42 °C, where it was kept for 2 h. Fermentation was stopped, and cells were harvested and lyzed by ultrasonication. The fusion protein was purified by gel permeation chromatography, precipitated with trifluoroacetate acid, dried, and redissolved in 1% formic acid. BrCN was added, and ProNPY was purified by gel permeation chromatography and preparative HPLC. In total, 10−15 mg of ProNPY (purity >95%) was obtained from a 1-L fermentation batch. ProNPY was characterized by SDS−PAGE and Edman degradation of the N-terminal 21 amino acids.

HPLC

The HPLC elution of immunoreactive NPY and ProNPY (Figures 4 and 5) was carried out with a linear gradient of ACN in 0.05% trifluroacetic acid at a flow rate of 1 mL/min using reverse phase phenyl column (Machery-Nagel AG). A gradient of 10−32% ACN was run over 10 min followed by 32−35% ACN over the next 10 min, and finally 35% ACN was maintained for another 10 min. Fractions of 1 mL were collected into 50 μL of 0.1% Tween-20, lyophilized, and assayed for immunoreactive NPY and ProNPY.

Radioimmunoassay for NPY

NPY immunoreactivity was measured using NPY02, a specific anti-NPY monoclonal antibody, as previously described (*37*). The capacity of the antibody to bind to NPY was tested by a RIA. A constant amount of tracer (3000 cpm of [^{125}I]NPY) was incubated with a constant amount of antibody (1 ng) and with increasing amounts of unlabled NPY (10^{-11}−10^{-8} M) to establish a standard curve. The limit of detection of the assay is 10 fmol/tube.

Peptide Synthesis and Analysis

Fmoc-protected amino acids were obtained from Alexis (Läufelfingen, Switzerland), 2-chlorotrityl chloride resin from NovaBiochem (Läufelfingen, Switzerland), diisopropylcarbodiimide and thiocresol from Aldrich, 1-hydroxybenzotriazole (HOBt), trifluoroacetic acid, thioanisole, piperidine,

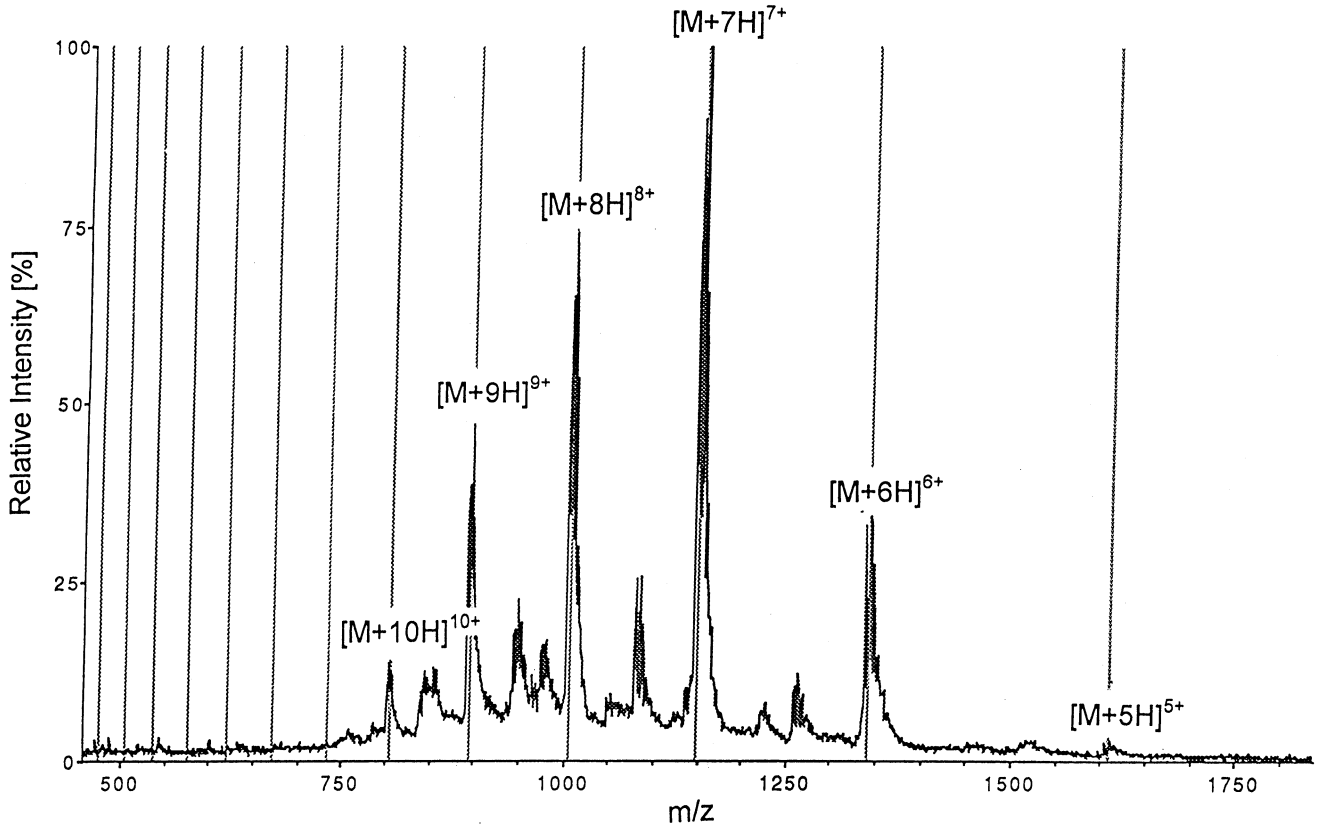

FIGURE 2: Electrospray mass spectrum of *E. coli* expressed and purified ProNPY (see Materials and Methods). Hypermass calculation revealed 8042 ± 2.9 as the main peak, which corresponds to M + Na^+ (M_{calcd} 8040). Further peaks correlated with *M* (hypermass 8015.5 ± 0.6, M_{calcd} 8017) and M + $2K^+$ (hypermass 8098.3 ± 2.1, M_{calcd} 8098).

and dimethylformamide (p.a. grade) from Fluka, and diethyl ether, acetonitrile, and *tert*-butyl alcohol from Merck.

The peptides were synthesized by automated, multiple solid phase peptide syntheses using a robot system (Syro, MultiSynTech, Bochum) as described previously (*39*). To obtain peptide acids 2-chlorotrityl chloride resin was used for anchoring to polystyrene−1%-divinylbenzene (30 mg, 15 μM/peptide). For side chain protection Tyr(*tert*-butyl), Asp(*tert*-butyl) Glu(*tert*-butyl), Ser(*tert*-butyl), Thr(*tert*-butyl), Arg(2,2,3,5,5-pentamethylchromanesulfonyl), His(trityl), Gln(trityl), Asn(trityl), and Lys(*tert*-butyloxycarbonyl) were used. The C-terminal 15 residues were obtained using single couplings with diisopropylcarbodiimide/1-hydroxybenzotriazole activation, 8-fold excess, and a coupling time of 1 h. To prolong the peptides double coupling cycles of two times 40 min were chosen.

The peptide acids were cleaved with trifluoroacetic acid/thioanisole/thiocresol within 3 h, collected by centrifugation, and lyophilized from water/*tert*-butyl alcohol.

The peptides were analyzed by reverse phase HPLC (Merck-Hitachi, Darmstadt) on a Nucleosil 5-μm, C-18 column (125 × 3 mm). Furthermore, they were analyzed by electrospray mass spectrometry (Figure 2) (API III, Sciex, Toronto). Purity was >95% for both peptides according to MS and HPLC.

(28−43)-ProNPY. Retention time, 10.5 min (gradient 15% acetonitrile to 60% acetonitrile within 30 min); mass spectromety, M(theor) 1918.1, *M*(exp) 1918.

(20−49)-ProNPY. Retention time, 14.4 min (gradient 25% acetonitrile to 75% acetonitrile within 30 min); mass spectrometry, *M*(theor) 3615.1, *M*(exp) 3614.

Enzymatic Assays: Kinetic Studies and ProNPY Substrate Specificty

Incubations with ProNPY or synthetic related substrates (20−49)-ProNPY and (28−43)-ProNPY were conducted for various time intervals with 10 μL of purified PC1/3, PC2, or furin (average activity of cleavage of 10 nmol/h of pGlu-Arg-Thr-Lys-Arg-MCA) at 37 °C in a total volume of 25 or 50 μL (*52*). The protein content of the enzymes PC1/3, PC2, and furin used correspond to 0.6, 1, and 0.5 μg, respectively. The purified PC1/3, furin, and PC2 were gifts of Dr. N. Seidah (IRCM Montréal) and Dr. I. Lindberg (New Orleans), respectively (*41*, *52*). The final incubation mixture contained 50 mM sodium acetate and 5 mM $CaCl_2$, and the pH was adjusted to 6.0, 5.0, and 7.0 for PC1/3, PC2, and furin, respectively. The separation of processed substrate was carried out on a C18 reverse phase HPLC column (peps, Pharmacia) with a linear gradient of ACN in 0.5% trifluoroacetic acid at a flow rate of 1 mL/min. Different mobile phase concentrations were used depending on the substrate: ProNPY, two successive gradients of 30−32% ACN over 40 min followed by 32−60% over 10 min; (20−48)-ProNPY, 5−30% ACN over 40 min; (28−43)-ProNPY, 0−15% ACN over 40 min. The endproducts generated by the cleavage were identified by mass spectrometry (Dr. P. Dumy, Institut de Chimie Organique, Lausanne).

Kinetic parameters (K_m and V_{max}) were determined under the following conditions: Substrates were incubated at a final concentration ranging from 40 to 500 μM to identify the K_m range and subsequently in the range of K_m (from 40 to 200 μM for ProNPY cleavage). Incubations were stopped when less than 20% of the substrate was digested. The substrates

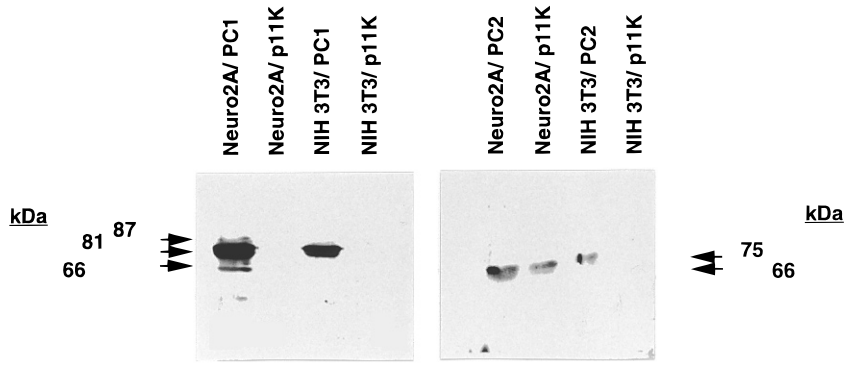

FIGURE 3: Cell extracts of transfected and non-transfected NIH 3T3 and Neuro2A cells with PC1/3 and PC2 were subjected to gel electrophoresis (see Materials and Methods). PC1/3 and PC2 were detected by western blotting using amino terminal polyclonal rabit antiserum (2B6) and a C-terminal polyclonal rabit antiserum respectively. p11K vector (without any convertase cDNA).

Table 1: Effect of Various Coexpression on ProNPY Processing in NIH 3T3 Cells[a]

		NIH 3T3 cell extracts			
fmol/100 μL	CTL	PC1/3	PC2	furin	PACE 4
NPY	73 ± 9 (18%)	240 ± 10 (50%)	84 ± 15 (17%)	71 ± 10 (13%)	80 ± 11 (17%)
ProNPY	328 ± 25 (82%)	234 ± 20 (50%)	400 ± 40 (83%)	491 ± 40 (87%)	401± 20 (83%)

[a] The amounts of ProNPY and NPY were evaluated by a radioimmunoassay using NPY02 antibody against N-terminal NPY epitope. Each value indicates the mean ± SD obtained from three experiments. Numbers in parentheses represent the fraction of respective forms as the percentage of total immunoreactivity.

Table 2: Effect of Various Coexpression on ProNPY Processing in Neuro2A Cells[a]

		Neuro2A cell extracts			
fmol/100 μL	CTL	PC1/3	PC2	furin	PACE 4
NPY	637 ± 32 (13%)	1584 ± 99 (32%)	781 ± 52 (16%)	559 ± 35 (12%)	562 ± 40 (12%)
ProNPY	4265 ± 300 (87%)	3257 ± 200 (68%)	4149 ± 210 (84%)	4278 ± 350 (88%)	4250 ± 400 (88%)

[a] The detection of peptides was as described in legend of Table 1.

and products were separated by HPLC as described above. K_m and V_{max} were determined from initial velocity measurements plotted versus various substrate concentrations using the Lineweaver−Burk or Eadie−Hofstee representation (Figures 7 and 8).

RESULTS

Coexpression of Rat ProNPY with Each of Prohormone Convertases PC1/3, PC2, Furin, and PACE4

The expression level of transient expression of prohormone convertases in Neuro2A cells was monitored in the media collected after 4 h of incubation, by incubating an aliquot of conditioned culture medium in the presence of pGlu-Arg-Thr-Lys-Arg-MCA, which represent an average of cleavage activity about 50 pmol/h above background per aliquot of 20 μL in total volume of 50 μL. For each specific enzyme expression a control was carried out using the same transfection vector with no coding region for PC(s). PC1/3 and furin exhibited higher enzymatic activity than PC2 and PACE4 above the background seen in the control experiment. The enzymatic activities monitored in the conditioned cultured medium of NIH 3T3 cells, after expression of different convertases are similar to those in Neuro2A cells. However, this is not the case for PC2 which is not active. The levels of activated endogenous compared to expressed PC1/3 and PC2 have been estimated in both cell lines by western blot (Figure 3).

PC1/3 Is Detectable in Neither NIH 3T3 nor Neuro2A. The expressed PC1/3 is completely processed in Neuro2A cells when compared to NIH 3T3 cells. In Neuro2A cells we observe the "three form" described for PC1/3 in neuroendocrine tissues; ProPC1/3 = 87 kDa, PC1/3 = 81 kDa, and the C-terminal processed PC1/3 = 66 kDa. In NIH 3T3 cells the C-terminal processed PC1/3 (66 kDa) is absent.

PC2 Enzyme Is Endogenously Produced by Neuro2A Cells. As expected PC2 is absent in NIH 3T3. The overexpressed PC2 shows only the ProPC2 (74 kDa) which is different from Neuro2A where the (66 kDa) PC2 represents a major form. These data show that PC1/3 and PC2 are well expressed in NIH 3T3 and that Neuro2A cells and are more processed in the latter cell line.

ProNPY is Processed by PC1/3 and PC2. To point out the enzyme(s) involved in the generation of NPY, a RIA of NPY was performed on fractions obtained from reverse phase phenyl HPLC two kinds of cells, were used, i.e. NIH 3T3 cells which express a small amount of furin and Neuro2A neuroendocrine cells which possess both regulated and constitutive secretory pathways and express small amounts of PC1/3, PC2, and more PACE4 (*16*). NIH 3T3 and Neuro2A cells infected with recombinant ProNPY and transfected with the control plasmid lacking the PCs coding region produced a limited intracellular cleavage of ProNPY into NPY, 18% and 13%, respectively (see Tables 1 and 2). This cleavage may be attributed to endogenous convertase activities. NIH 3T3 cells contained less intracellular NPY and ProNPY immunoreactive material than Neuro2A cells in accordance with the constitutive and regulated character of each kind of cells, respectively. Above these endogenous activities, transfection with expression vectors carrying

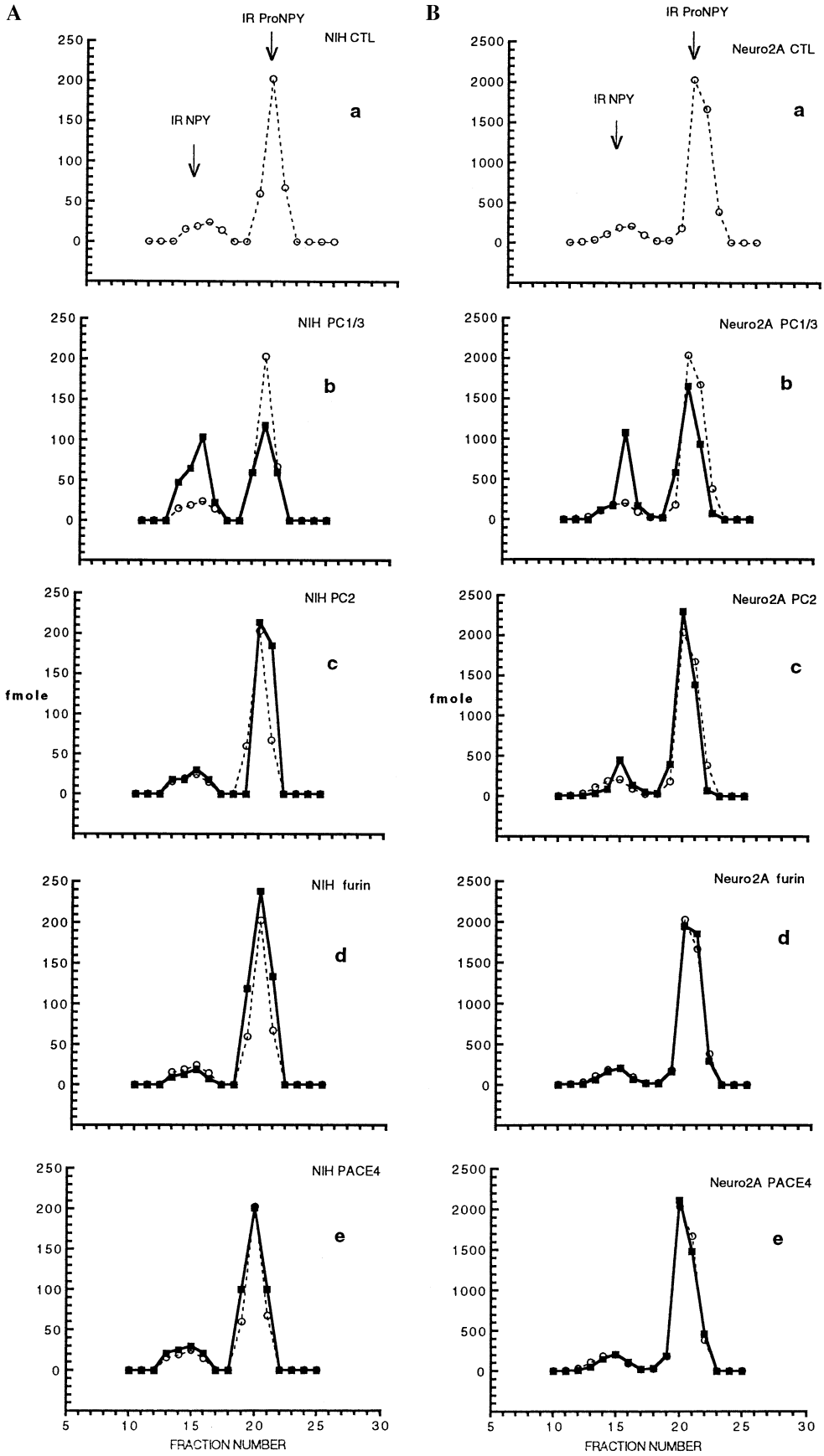

FIGURE 4: HPLC analysis of ProNPY processing. (A) ProNPY coexpressed in NIH 3T3 and (B) ProNPY coexpressed in Neuro2A with (a) p11K vector (without any convertase cDNA), (b) p11K PC1/3, (c) p11K PC2, (d) p11k furin, and (e) p11k PACE4.

cDNA for different PCs is expected to enhance the extent of processing of ProNPY.

From the results presented in Figure 4 and the calculation of the amount of each product with respect to the total NPY-like immunoreactivity (IRNPY), we were able to deduce the convertases which are likely to be responsible for the

Table 3: ProNPY and NPY Analysis of Cultutre Media of NIH 3T3 Cells with Various Coexpression[a]

		NIH 3T3 cell medium			
fmol/100 μL	CTL	PC1/3	PC2	furin	PACE 4
NPY	150 ± 10 (6%)	123 ± 10 (5%)	141 ± 20 (6%)	89 ± 9 (4%)	100 ± 15 (4%)
ProNPY	2246 ± 80 (94%)	2277 ± 100 (95%)	2258 ± 150 (94%)	2250 ± 200 (96%)	2357 ± 180 (96%)

[a] The detection of peptides was as described in legend of Table 1.

Table 4: ProNPY and NPY Analysis of Cultutre Media of Neuro2A Cells with Various Coexpression[a]

		Neuro2A cell medium			
fmol/100 μL	CTL	PC1/3	PC2	furin	PACE 4
NPY	597 ± 27 (71%)	817 ± 50 (100%)	800 ± 60 (100%)	608 ± 40 (73%)	680 ± 35 (72%)
ProNPY	242 ± 16 (29%)	not detected	not detected	205 ± 15 (27%)	200 ± 20 (28%)

[a] The detection of peptides was as described in legend of Table 1.

Table 5: Kinetic Constant for the Cleavage of ProNPY, (20−49)-ProNPY, and (28−43)-ProNPY by PC1/3 and PC2[a]

	PC/3			PC2		
	K_m (μM)	V_{max} (pmol/min)	V_{max}/K_m (10^{-9} L/min)	K_m (μM)	V_{max} (pmol/min)	V_{max}/K_m (10^{-9} L/min)
ProNPY	96 ± 18	47 ± 9	494	69 ± 10	3 ± 1	46
(20−48)-ProNPY	415 ± 50	297 ± 60	715	204 ± 30	42.5 ± 7	208
(28−43)-ProNPY	175 ± 25	255 ± 70	1457	not cleaved	not cleaved	not cleaved

[a] V_{max}/K_m represent the cleavage efficacy.

cleavage of ProNPY into NPY in either NIH 3T3 (Figure 4A) or Neuro2A (Figure 4B). Only PC1/3 (50% of total IR NPY (Figure 4Ab) was able to cleave ProNPY in NIH 3T3 cells (see Table 1). In Neuro2A cells the major ProNPY processing activity can also be attributed to PC1/3 (32%) (Figure 4Bb, Table 2). Furin and PACE4 were not able to produce significant amounts of NPY in either cells. In addition, we consistently observed weak PC2 mediated processing of ProNPY in latter cells when compared to the control, 16 ± 1% vs 13 ± 0.7%, respectively (Figure 4Bc, Table 2). Since the cleavage difference occasioned by PC2 above the control (16%−13%) was statistically not significant ($n = 3$, $P > 0.05$), PC1/3 is suggested as a prime candidate for the processing of ProNPY. The secreted product from NIH 3T3 cells seems not to be influenced by the expression of the different convertases, no major difference of ProNPY processing was observed with the presence of the different convertases (Table 3). This is in contrast to the secreted products from Neuro2A cells, where the expression of both PC1/3 and PC2 shows no detectable amount of ProNPY while furin and PACE4 had no enhancement on the NPY processing (Table 4).

ProNPY Production and Purification

Protein expression of ProNPY in transformed *E. coli* was induced by increasing temperature for 2 h. The fusion protein obtained from the cell extracts was purified by gel permeation and subjected to BrCN cleavage. The cleavage product was purified on HPLC. A 1-L fermentation yielded 10−15 mg of pure ProNPY (purity >95%). ProNPY was characterized by SDS−PAGE, Edman degradation of the N-terminal 21 amino acids and finally with Electrospray mass spectroscopy. Electrospray mass spectrum reveals 8042 ± 2.9 as a main peak, which corresponds to M + Na^+ (M_{calcd} 8040). Further peaks correlated with *M* (hypermass 8015.5 ± 0.6, M_{calcd} 8017) and M + $2K^+$ (hypermass 8098.3 ± 2.1, M_{calcd} 8098) (Figure 2).

In Vitro Digestion of ProNPY by PC1/3, PC2, and Furin

To further characterize prohormone convertase activities on ProNPY, in vitro digestion of ProNPY was performed by coincubation of 0.5 nmol of ProNPY with either PC1/3, PC2, or furin. Enzyme activities were controlled using the fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-MCA. Following ProNPY digestion, reaction mixtures were separated by C18 HPLC and a sample of each fraction collected was assayed for NPY by RIA. Figure 5 shows that, under these conditions, ProNPY was completely cleaved by PC1/3, partially by PC2 and not at all by furin. Chelating calcium with EDTA abolished the activities of both PC1/3 and PC2. These data are in agreement with our coexpression cell experiments that showed considerably higher ProNPY cleavage activity for PC1/3 than for PC2. Furthermore this difference in activity is rather due to the substrate recognition than to the difference of PC1/3 and PC2 behavior depending on the cell type or the expression system.

Kinetic Study of the Cleavage of ProNPY by PC1/3 and PC2

To understand the significance of the difference in ProNPY recognition between PC1/3 and PC2, kinetic studies were performed (Figures 7 and 8). We were able to separate NPY-GKR, CPON, and ProNPY by HPLC as shown in Figure 6A. The data presented in Table 5 demonstrate that PC1/3 cleaves ProNPY more efficiently than PC2. This is in agreement with the results obtained with the comparative enzyme assays (Figure 5). Interestingly the main difference between the cleavage efficiency can be attributed to a change in V_{max} (47 pmol/min for PC1/3 and 3 pmol/min for PC2), while the observed K_m values are within a narrow range (95 μM for PC1/3 and 69 μM for PC2). The kinetics of PC1/3 and PC2 cleavages were plotted first using the hyperbolic regression plot and secondly the Eadie−Hofstee plot to deduce the kinetic constants V_{max} and K_m (Figures 7 and 8).

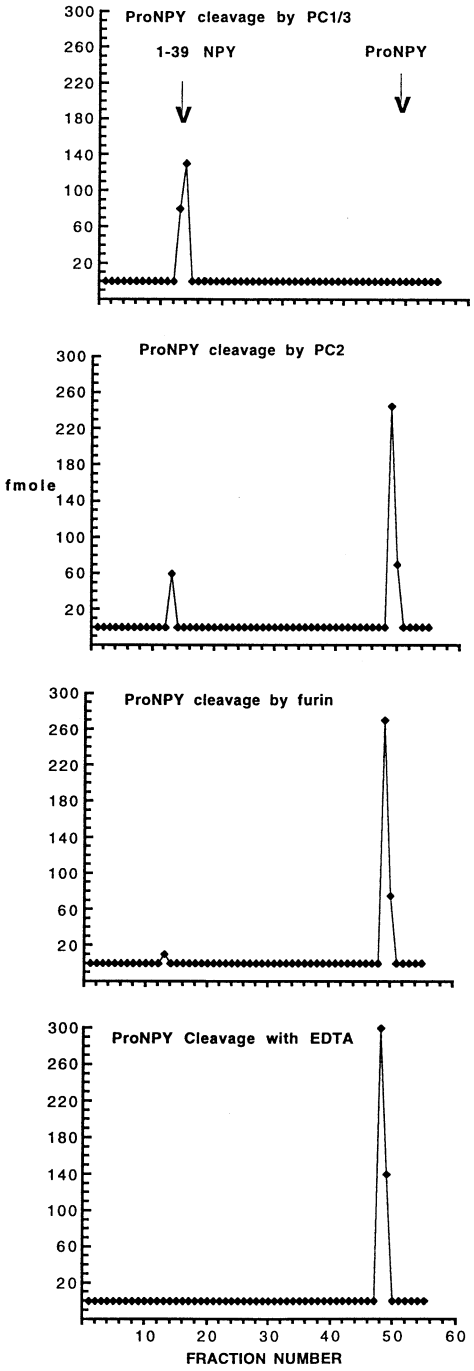

FIGURE 5: HPLC analysis of ProNPY and its processing fragment generated by PC1/3, PC2, and furin. Immunochemical evaluation of ProNPY and (1−39)-NPY after cleavage and reverse phase HPLC separation of reaction mixture.

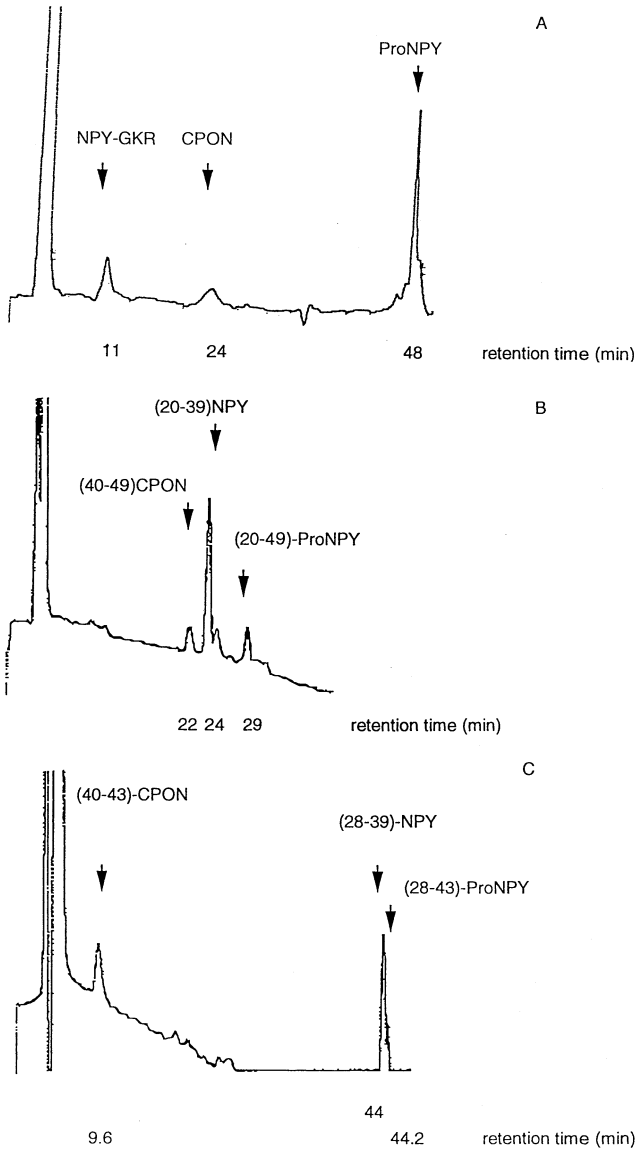

FIGURE 6: HPLC analysis of enzyme assays. (A) Elution profile of ProNPY after enzymatic incubation with PC1/3 shows the peaks corresponding to ProNPY and resulting N-terminal (1−39)-NPY and C-terminal fragment CPON. (B, C) Elution profile of (20−49)-ProNPY and (28−43)-ProNPY after incubation with PC1; the resulting C- and N-terminal fragments are indicated.

Digestion of ProNPY Synthetic Peptides by PC1/3 and PC2

To determine if structural conformation located in the NH_2- and COOH-terminal parts of the precursor are involved in enzymatic recognition, kinetic studies have been extended to two partial ProNPY peptides with different amino acid sequence lengths, namely (20−49)-ProNPY and (28−43)-ProNPY.

Figure 6B,C shows the HPLC separation of ProNPY, (20−49)-ProNPY, and (28−43)-ProNPY, respectively, digested with PC1/3. The shortened sequences of proNPY are cleaved more efficiently in the order (28−43)-ProNPY > (20−49)-ProNPY > ProNPY for PC1/3 and (20−49)-ProNPY > ProNPY for PC2 (see Table 5). This is reflected by the increase of V_{max}. The K_m values are also higher for short peptides than for ProNPY suggesting that the interactions of short peptides with the enzymes are less favorable compared to the whole precursor. In addition, the shortest peptide (28−43)-ProNPY is not cleaved by PC2 at all, while it is still cleaved by PC1/3. This confirms that PC1/3 and PC2 exhibit different requirements for the cleavage of ProNPY which is indicated by the cleavage efficacy as defined by V_{max}/K_m.

DISCUSSION

Within the last few years, the discovery and the molecular characterization of the enzymes belonging of the new subtilisin−kexin family of endoproteases brought a better understanding to the synthesis and release of biologically active peptides and proteins. There are so far seven described members within this family, all possessing particular features with regard to their subcellular localization

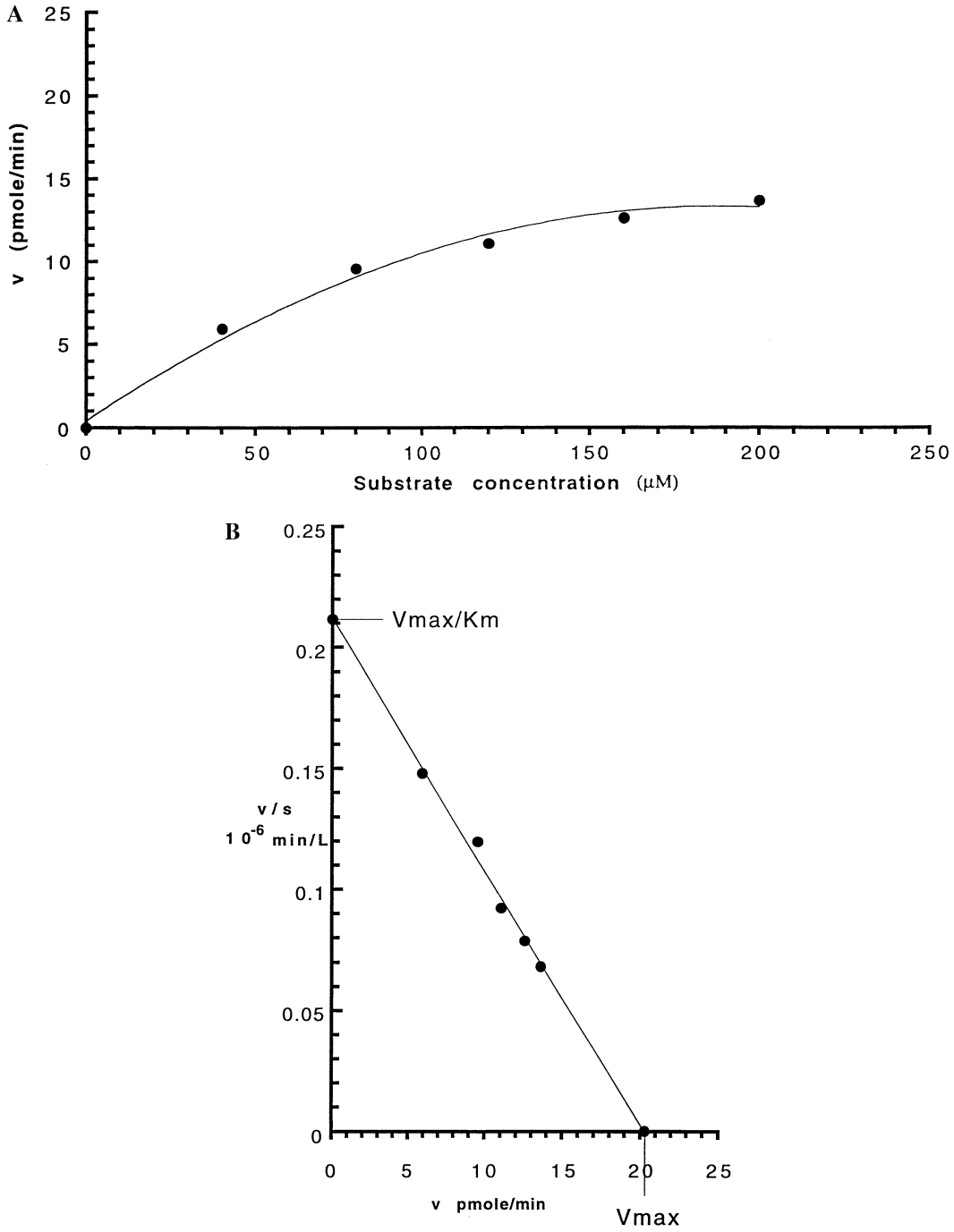

FIGURE 7: (A) Hyperbolic and (B) Eadie−Hofstee plots of kinetic cleavage of ProNPY by PC1/3, V_{max} = 47 pmol/min, K_m = 96 μM.

and tissue distribution. The furin, PACE4, PC5/6 and PC7 enzymes are more ubiquitously distributed while the PC1/3 and, PC2 are localized to specialized endocrine or neuroendocrine tissues and PC4 to spermatids. It appears that PC1/3 and PC2 are generally associated with the secretory pathway regulated by extracellular signals, while constitutively secreted proteins are processed by either one of the other convertases (*16*).

NPY is derived from a 69-amino acid precursor which possesses a single Lys-Arg cleavage site. Based on the observations that NPY and ProNPY mRNA (*18, 32*) are present in different tissues (*18, 32*), one can hypothesize that this precursor might be processed in either a constitutive or a regulated secretory pathway.

Heterologous cellular expression of ProNPY has shown that PC1/3 and or PC2 are involved in the processing of propeptide (*29*−*31*). Recently, in sympathetic neurones, PC2 was demonstrated to be the major ProNPY processing enzyme (*42*). The results of this study clarify the role of PC1/3 and PC2 in the cleavage of ProNPY and exlude PACE4 and furin as candidates in this process. A NIH 3T3 cell line was used as a prototype of constitutive cells, and only furin was shown to be present, despite the absence of detectable expression of prohormone convertases other than furin, NIH 3T3 cells infected with the ProNPY virus were able to convert 18% of the substrate. This result suggests that endogenous furin might be linked to the synthesis of mature NPY. However, when these cells were in addition transfected with cDNAs encoding either PC1/3, PC2, PACE4, or furin, only the ones expressing exogenous PC1/3 contained increased levels of immunodetectable NPY (32% over control). This observation makes it unlikely that endogenous

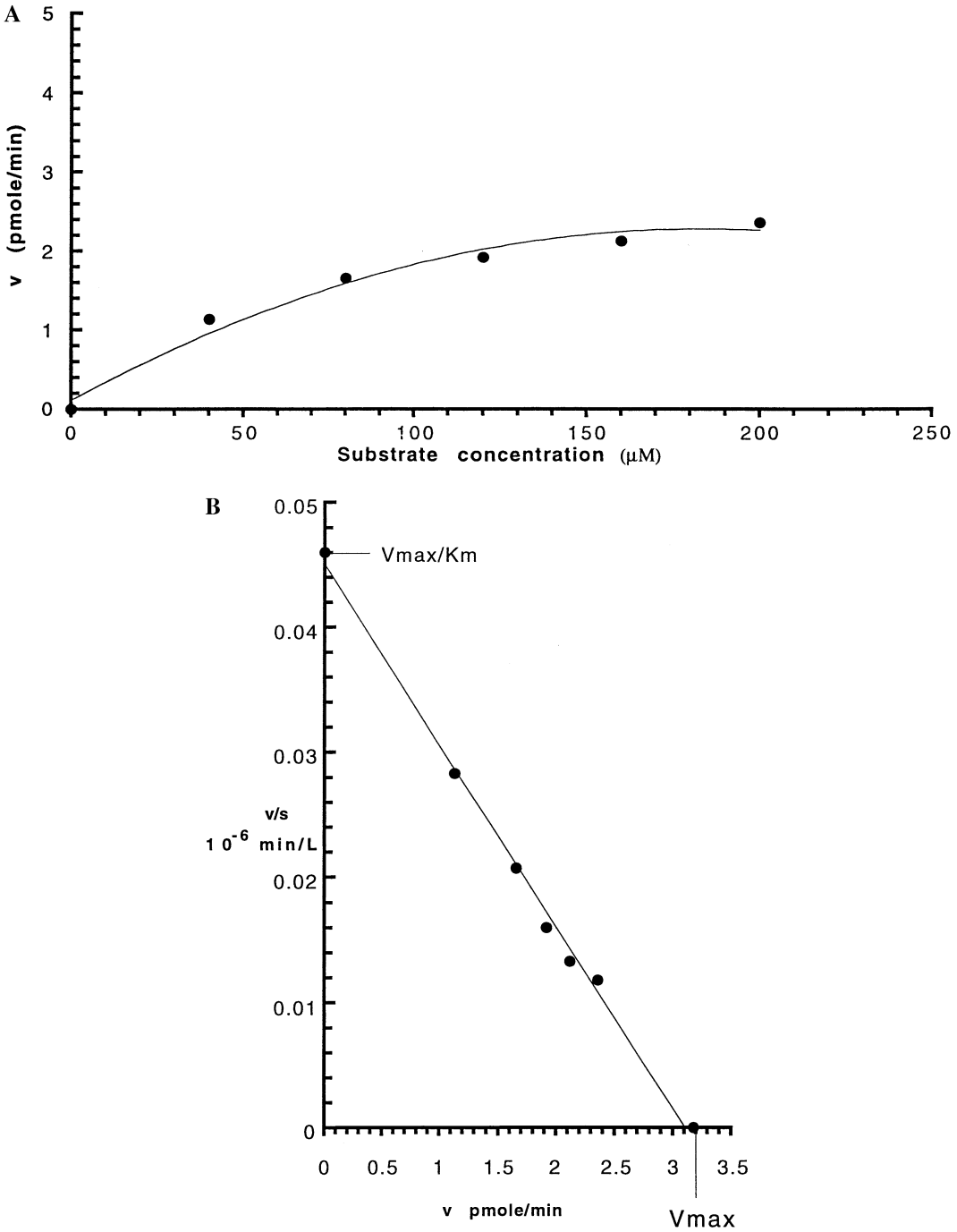

FIGURE 8: (A) Hyperbolic and (B) Eadie−Hofstee plots of kinetic cleavage of ProNPY by PC2, V_{max} = 3 pmol/min, K_m = 46 μM.

furin is responsible for the background level of ProNPY processing in NIH 3T3 cells. The expression level of furin in the medium of transfected cells was monitored and was shown to be high. Since furin is a transmembrane enzyme, the furin detected in the medium corresponds to shed furin or the C-terminal autoprocessed furin. Wise et al. (*43*) have shown that Cos cells transfected with an expression plasmid for furin with a transmembrane domain secrete 75 kDa furin protein, whereas higher molecular mass forms, associated with membranes, remain in side cells. The secretion of the truncated form of furin could be a common feature of subtilisin−kexin family of endoproteases. Germain et al. (*44*) reported that, when expressed in insect Sf9 cells, the yeast Kex2 protease remains inside cells as a high molecular mass form (120 kDa), whereas a C-terminally processed form (70 kDa) is secreted into the medium. Moreover ProNPY has Arg residue at position −5 and −7 from the dibasic site. These basic residues are not localized at position −4 and/or −6 found in furin and PACE4 substrate. This show that the position of the Arg upstream of the dibasic site is precise, and could not be substituted even by two Arg at −5 and −7. This fits well with the Nakayama's rules (*45*), which shows that furin requires a basic residue at position −4 or −6 beside the pairs (Arg-X-Lys/Arg-Arg or Arg-X-X-X-Lys/Arg-Arg).

Neuro2A cell line was used as a prototype of regulated cells which express PC2 (Figure 3) and a considerable amount of PACE4 (*16*). Neuro2A cells were described to contain the neuroendocrine peptide 7B2, a key cofactor peptide in the synthesis of active PC2 and a hallmark of regulated secretory pathway (*40*, *41*, *48*−*51*). As in NIH 3T3 cells, substantial background activity (13% of the

substrate converted) was detected in Neuro2A cells. So far we do not know which metabolic process is involved in this endogenous ProNPY conversion. In contrast to NIH 3T3 cells, PC1/3 (19% over control) was not the only convertase active in ProNPY processing. Cells transfected with the vector carrying the coding sequence for PC2 where consistently found to contain marginally increased mature NPY (3% over control). This PC2 activity could be attributed either to the weak 7B2 activity or to a difference in substrate recognition by PC2 in Neuro2A compared to the NIH 3T3 cell line. The coexpression of PC1/3 with ProNPY in NIH 3T3 cells process a high ratio of ProNPY (50%). This seems to be high, but in fact it represents only a small amount (240 fmol/100 μL) due to the fact that NIH 3T3 cells do not store ProNPY. In contrast, Neuro2A cells do store ProNPY and show a lower processing ratio which represents only 37%. This corresponds to a large amount (1580 fmol/100 μL). In the medium of NIH 3T3 cells we observe the presence of almost only ProNPY (95%). This reflects the constitutive secretion feature of NIH 3T3 cells, ProNPY is not stored but is secreted constitutively. In contrast with Neuro2A cells we observe an amount of NPY in the medium which is probably secreted via a constitutive secretory pathway and might be cleaved by constitutive enzymes. Interestingly, this "constitutive processing" is observed only in Neuro2A cells and not in NIH 3T3 cells. This "constitutive processing" could be carried out by other enzymes other than PCs used in our present work, such as prohormone thiol protease, the 70 kDa aspartic protease (*46*), PC5/6 or PC7, or other unknown enzymes. The favored constitutive processing observed when PC1/3 and PC2 are expressed is unclear. We could speculate that this apparent constitutive processing could be due to a default in vaccinia virus expression system which is known to be less adequate to study secretory events.

One can suppose that the cleavage efficiency observed by PC1/3 in Neuro2A cells corresponds only to an active autoprocessed PC1/3, and that the low efficacy of PC2 corresponds to a non-autoprocessed PC2. The observation made in our study should suggest this is not the case. Our data from Figure 3, reveal that the local environment in which PC1/3 and PC2 are placed is important. The autoprocessing of ProPC1/3 is complete in Neuro2A cells, we can observe ProPC1/3 (87 kDa), PC1/3 (81 kDa) and the C-terminal processed form (66 kDa). In contrast to these cells in NIH 3T3 cells PC1/3 is present only in two forms: ProPC1/3 (87 kDa) and PC1/3 (81 kDa). PC2 is not autoprocessed in NIH 3T3 cells. This is different from Neuro2A cells where the major part of PC2 is processed (Figure 3). In summary, we observe that in Neuro2A cells both PC1/3 and PC2 are processed and activated more than in NIH 3T3. This is in agreement with the study, suggesting that proprotein conversion is determined by a multiplicity of factors, including convertase processing, substrate specificity, and intracellular environment (*47*).

Recently, a study examined the effects of alteration in incubation time and temperature or in intracellular pH on the biosynthetic processing of ProNPY in transfected AtT20 and GH3 cells. It revealed a difference in the kinetics of PC1/3 and PC2 activity (*31*). The authors showed that cleavage with PC2 occurred at a slower rate than with PC1/3.

In performing in vitro processing assays we were able to show that PC1/3 is, indeed, more efficient than PC2 in the processing of ProNPY. In addition, such assays revealed no specific activity of furin, as expected from our coexpression experiments. To better understand the difference in substrate handling between PC1/3 and PC2, we determined the kinetic parameters K_m and V_{max} for these reactions.

Surprisingly, K_m values for the ProNPY cleavage by PC1/3 and PC2 are very similar and, moreover, fit well with those reported for the cleavage of fluorogenic and short synthetic peptides reproducing the cleavage site (*41*, *52*−*56*). These K_m values are unusually high (69 and 95 μM, respectively), considering the K_d values generally observed in ligand-receptor interactions (nM range). Hook et al. (*46*) have investigated whether different prohormones may be preferentially processed by certain processing enzyme(s): They have compared the relative processing of proenkephalin, ProNPY, POMC by different processing enzymes prohormone thiol protease (PTP), 70 kDa aspartic protease, and PC1/3 and PC2. They showed that ProNPY is a good substrate for the PTP enzyme compared to its cleavage by the 70 kDa aspartic proteinase or PC1/3 and PC2. We would therefore expect a nM range as a K_m value for conversion by PTP enzyme; however, this remains to be established.

In contrast to the enzyme−substrate affinity, the interaction velocity as defined by V_{max} differs by a factor 10 between the two enzymes, reflecting a much slower PC2 than PC1/3 activity. When a shortened synthetic peptide (20−49)-ProNPY was used as substrate, K_m and V_{max} increased for both enzymes. The rise in V_{max} might reflect a higher flexibility of short peptides compared to the full-length substrate. An even shorter precursor, (28−43)-ProNPY, was still efficiently cleaved by PC1/3 but was no longer processed by PC2. This difference suggests that PC2 might have more stringent requirements on substrate structure than PC1/3. This could be a starting point to build molecules that can serve as specific ProNPY processing inhibitors.

On the basis of these results, it is clear that ProNPY is not processed by the the proconvertases furin and PACE4, PC1/3, and PC2 are not equipotent in the cleavage of ProNPY with major difference in the rate of the cleavage and not in the enzyme substrate interaction and eventually the substrate peptide feature might discriminate between PC1/3 and PC2 enzymatic activities.

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