Evidence That PC2 Is the Endogenous Pro-neurotensin Convertase in rMTC 6-23 Cells and That PC1- and PC2-transfected PC12 Cells Differentially Process Pro-neurotensin*

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The neuropeptide precursor proneurotensin/neuromedin N (pro-NT/NN) is mainly expressed and differentially processed in the brain and in the small intestine. We showed previously that rMTC 6-23 cells process pro-NT/NN with a pattern similar to brain tissue and increase pro-NT/NN expression in response to dexamethasone, and that PC12 cells also produce pro-NT/NN but are virtually unable to process it. In addition, PC12 cells were reported to be devoid of the prohormone convertases PC1 and PC2. The present study was designed to identify the proprotein convertase(s) (PC) involved in pro-NT/NN processing in rMTC 6-23 cells and to compare PC1- and PC2-transfected PC12 cells for their ability to process pro-NT/NN. rMTC 6-23 cells were devoid of PC1, PC4, and PC5 but expressed furin and PC2. Stable expression of antisense PC2 RNA in rMTC 6-23 cells led to a 90% decrease in PC2 protein levels that correlated with a >80% reduction of pro-NT/NN processing. PC2 expression was stimulated by dexamethasone in a time- and concentration-dependent manner. Stable PC12/PC2 transfectants processed pro-NT/NN with a pattern similar to that observed in the brain and in rMTC 6-23 cells. In contrast, stable PC12/PC1 transfectants reproduced the pro-NT/NN processing pattern seen in the gut. We conclude that (i) PC2 is the major pro-NT/NN convertase in rMTC 6-23 cells; (ii) its expression is coregulated with that of pro-NT/NN in this cell line; and (iii) PC2 and PC1 differentially process pro-NT/NN with brain and intestinal phenotype, respectively.

Neuropeptides and peptide hormones are synthesized as part of larger inactive polypeptide precursors from which they are produced by cleavage at specific sites, usually pairs of basic residues, by proprotein convertases (PCs)1 (reviewed in Refs. 1–3). The mammalian PCs belong to a recently identified family of subtilisin-like proteases that were identified by their homology with the yeast Kex2 protease involved in the processing of pro-α-mating factor (4, 5). These Kex2-related enzymes exhibit different tissue and cellular distributions (reviewed in Refs. 2 and 3). Thus, furin (6) and PACE 4 (7) are expressed in most neuroendocrine and endocrine tissues in the body. PC1 (also designated PC3) and PC2 (8–11) are restricted to endocrine and neuronal cells. PC4 (12, 13) is exclusively expressed in germ cells of testes and ovaries. PC5 (also designated PC6) (14, 15) is widely distributed in neural, endocrine, and nonendocrine tissues, being abundant in the periphery, especially in the gut and adrenal. At the cellular level, furin appears to be confined to the Golgi apparatus while PC1 and PC2 are found in the various compartments of the regulated secretory pathway including the secretory granules. Consistent with such tissue and cellular distributions, furin has been shown to efficiently process protein precursors that are destined to the constitutive secretory pathway such as pro-β-nerve growth factor, proalbumin, or pro-von Willebrand factor (2, 16), while PC1 and PC2 have been reported to cleave peptide hormone and neuropeptide precursors that are routed to the regulated secretory pathway, like proinsulin (17–19), proglucagon (20, 21), or POMC (22, 23). Furthermore, a tissue-specific action of PC1 and PC2 has been shown to be responsible for the differential processing of POMC in the anterior and intermediate pituitary lobes (22, 24). The roles of PACE4, PC4, and PC5 in proprotein processing are as yet unknown. Even as regards furin, PC1, and PC2, only a few of the potential physiological substrates for these enzymes have been identified. The number of proprotein and hormone/neuropeptide precursors undergoing post-translational cleavage at basic sequences is considerable, and a major task in the future will be to identify the enzyme(s) involved in the maturation, often tissue-specific, of each of these precursors.

Neurotensin (NT) and neuromedin N (NN) are two structurally related brain and gut regulatory peptides which are encoded in the same precursor (25, 26). Rat pro-NT/NN is depicted in Fig. 1. The four Lys-Arg sequences in the precursor represent putative processing sites, the cleavage of which could generate various sets of peptides in addition to NT and NN. Recent evidence indicates that pro-NT/NN is differentially processed in brain versus intestinal tissues. Thus, in all rat brain regions examined pro-NT/NN is primarily cleaved at the three most C-terminal dibasic sequences to generate similar amounts of NT, NN, and a large N-terminal precursor fragment ending with the residue that precedes Lys140 (27, 28). In the gut, the precursor is preferentially cleaved at the two most C-terminal pairs of basic residues, giving rise to comparable amounts of NT and a large biologically active peptide starting after the signal peptide and ending with the NN sequence.

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1 The abbreviations used are: PC, proprotein convertase; POMC, proopiomelanocortin; NT, neurotensin; NN, neuromedin N; FBS, fetal bovine serum; HS, horse serum; PCR, polymerase chain reaction; INT, immunoreactive NT; INN, immunoreactive NN; CTNN, cysteaylated, trypsin-digested INN; ACTH, adrenocorticotropic hormone; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high performance liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; r, rat; h, human; m, mouse.
Fig. 1. Schematic representation of rat pro-NT/NN and the various products detected in the present study. Rat prepro-NT/NN is 169 amino acids long and starts with a 22-residue signal peptide not represented here. The positions of the four Lys-Arg (KR) dibasic sequences are shown.

(large NN, Fig. 1) (29–31). The first dibasic sequence (Lys85–50
and concomitantly increases the cellular content of precursor-derived peptides in tissues.

Neuroendocrine cell lines have proven to be useful models to identify the PCs involved in the maturation of neuropeptide/hormone precursors such as proinsulin (19), proglucagon (20), and POMC (22, 24), and to explain the processing patterns observed for these precursors in the tissues that normally express them. Recently, we reported that the rat medullothyroid carcinoma rMTC 6-23 cell line (32) expresses and processes the NT/NN precursor to yield NT, NN, and other precursor-derived peptides.

rMTC 6-23 Cell Transfection with PC1 and PC2 cDNAs—To construct a PC2 antisense cDNA-containing vector, the full-length human protein was excised by XbaI from Bluescript (SK-) and cloned into Bluescript (SK-) as an XbaI fragment and subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen) previously digested with the same restriction enzymes. The antisense orientation of the insert was verified with restriction enzymes. The cells were transfected with pcDNA3 alone or hPC2 antisense-pcDNA3 by the electroporation method described above for PC12 cells. Transfected cells produced colonies after approximately 3–4 weeks. The colonies were subcloned in 24-well plates, grown until they reached confluency, and passed 2–3 times in 100-mm Petri dishes. Six PC12/PC2 clones were selected for the studies reported here by Western blotting with anti-PC1 2B6 and anti-PC2 7BF antisera. These clones are denoted E/L, 12/E, E standing for electroporation, L for lipofection, 1 for PC1, 2 for PC2, and n being an identification number. Wild-type and transfected PC12 cells at 60–80% confluency were stimulated with optimal concentrations of nerve growth factor (200 ng/ml), dexamethasone (1 μM), forskolin (1 μM), and LiCl (20 mM) for 48 h. The cells were extracted and analyzed for PC expression and pro-NT/NN processing as described below.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 and rMTC 6-23 cells were grown and propagated as described previously (33, 38). GH3 and Att-20 cells were cultured in DMEM containing 7.5% (GH3) or 10% (Att-20) heat-inactivated horse serum (HS); 2.5% (GH3); or 10% (Att-20) fetal bovine serum (FBS) and 50 μg/ml gentamycin.

PC12 Cell Transfection with PC1 and PC2 cDNAs—The mPC1 and hPC2 cDNAs (gifts from Donald Steiner, University of Chicago, Chicago) were subcloned in the eukaryotic expression vector pcDNA3 (Invitrogen, Leek, The Netherlands). PC12 cells were grown and propagated as described previously (38). For transfection experiments, PC12 cells were grown in DMEM containing 5% FBS and 10% HS. The cells were transfected either by electroporation or lipofection. For electroporation, approximately 40 million cells were suspended in 0.5 ml of DMEM containing 20% FBS to which were added 25 μg of mPC1- or hPC2-pcDNA3. After 10 min the cells were electroporated with a Eurogentec apparatus (setting: 400 V, 500 microfarads, infinite resistance). The cells were then kept for 15 min at room temperature, plated in DMEM containing 5% FBS and 10% HS, and allowed to attach for 48 h. For lipofection, the cells plated in 60-mm dishes were grown to 70–80% confluency. The dishes were rinsed twice with pre-warmed serum-free DMEM medium (Opti-MEM, Life Technologies, Eragny, France). A 10-μl aliquot of Lipofectamin (Life Technologies) was placed into 100 μl of serum-free medium, vigorously mixed with 100 μl of serum-free medium containing 2 μg of DNA, and kept for 20 min at room temperature. This solution was added to the plated cells which were incubated for 2 h at 37°C. The medium was then replaced with regular medium (DMEM, 5% FBS, 10% HS) and after 48 h the cells were grown for 15 days in the same medium containing 0.5 mg/ml G418, after which time the G418 concentration was reduced to 0.25 mg/ml. Transfected cells produced colonies after approximately 3–4 weeks. The colonies were subcloned in 24-well plates, grown until they reached confluency, and passed 2–3 times in 100-mm Petri dishes. The colonies were screened for PC expression and pro-NT/NN processing as described below.

rMTC 6-23 Cell Transfection with PC2 Antisense cDNA—To construct a PC2 antisense cDNA-containing vector, the full-length human protein was excised by XbaI from Bluescript (SK-) as an XbaI fragment and subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen) previously digested with the same restriction enzymes. The antisense orientation of the insert was verified with restriction enzymes. The cells were transfected with pcDNA3 alone or hPC2 antisense-pcDNA3 by the electroporation method described above for PC12 cells. Transfected cells produced colonies after approximately 3–4 weeks. The colonies were subcloned in 24-well plates, grown until they reached confluency, and passed 2–3 times in 100-mm Petri dishes. The clones were selected by Western blotting with anti-PC2 7BF antisera. Fifty μg of protein were subjected to electrophoresis on a 10% polyacrylamide minigel and transferred to nitrocellulose. Protein loading uniformity and blotting efficiency were monitored by staining the nitrocellulose sheet with Ponceau Red and the transferred gel with Coomassie Blue R-250. The blots were incubated overnight at 4°C in phosphate-buffered saline, pH 7.4, containing 1 μg/ml EDTA, 0.3 μg/ml phenylmethylsulfonyl fluoride, 0.1% Triton X-100, 0.5% Nonidet P-40, and 0.1% Na3VO4. The blots were centrifuged and the protein content of the supernatants was determined using the Bio-Rad protein assay reagent, following the procedure recommended by the manufacturer. Fifty μg of protein were subjected to electrophoresis on a 9% polyacrylamide minigel and transferred to nitrocellulose. Protein loading uniformity and blotting efficiency were monitored by staining the nitrocellulose sheet with Ponceau Red and the transferred gel with Coomassie Blue R-250. The blots were incubated overnight at 4°C in phosphate-buffered saline, pH 7.4, containing 5% non-fat dry milk and then incubated with primary antibodies at the appropriate dilution for 4 h at room temperature in phosphate-buffered saline containing 5% non-fat dry milk. After three 5 min washes in Tris buffered saline (10 min Tris buffered saline [pH 7.4] containing 0.5% Nonidet P-40, and 0.1% Na3VO4), the blots were incubated at room temperature for 45 min in Tris buffered saline containing 5% non-fat dry milk with a 1:10,000 dilution of peroxidase-coupled goat anti-rabbit F(ab')2 fragment antibody (ImmunoNote S.A., Marseille, France). After washing in Tris buffered saline buffer, the blots were revealed by enhanced chemiluminescence (ECL kit, Boehringer, Meylan, France) according to the procedure recommended by the manufacturer. In some experiments, the blots were scanned and the protein bands were quantified by densitometry. PCs antisera were kindly provided by Iris Lindberg (Louisiana State University, New Orleans) and were used at a 1:10,000 dilution. The anti-PC1 antisera 2B6 directed against the N-terminal portion of mPC1 (42) recognizes both the 78- and 66-kDa active forms of the enzyme. The C-terminally directed anti-PC2 antisera 48F (39) detects equally well the 75- and 66-kDa proPC2 and the mature 66-kDa PC2 forms. The N-terminally directed antisera 7BF was made against a synthetic peptide that corresponds...
to the 13 amino acid sequence that follows the N-terminal tetrabasic cleavage site of mouse proPC2. It detects preferentially the mature 66-kDa PC2 and more faintly the 75-kDa proPC2.2. Antiserum 7BF was used to detect hPC2 in transfected PC12 cells because we observed that antiserum 4BF reacted poorly with hPC2, most likely because the C-terminal amino acid sequence of mPC2 recognized by this antiserum is not well conserved in hPC2. On the other hand, the mPC2 sequence recognized by 7BF is almost identical to the corresponding sequence in hPC2. The anti-furin antiserum (kindly provided by Nabil G. Seidah, Clinical Research Institute of Montreal, Montreal) was raised against a 17-amino acid sequence that follows the pro-region of furin and corresponds to the N-terminal sequence of the enzyme catalytic domain (43). It was used at a 1:200 dilution.

RNA Isolation and Analysis—Cells were washed with phosphate-buffered saline. Total RNA from the various cell lines and from rat small intestine and testis was extracted by the guanidine-phenol-chloroform method (44). Ten μg of total RNA was electrophoresed on 1% formaldehyde-agarose gels and transferred onto nylon membranes. RNA was UV cross-linked to filters and prefibrydized in 5 × SSPE (1 × SSPE is 0.15 M NaCl, 0.015 M sodium citrate, 1 mM EDTA, pH 7.0), 0.5% SDS, 5 × Denhardt's (1 × Denhardt's is 0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% bovine serum albumin), 50% formamide, 0.2 mg/ml denatured salmon sperm DNA 6 h at 42°C. Full-length hfurin, rpC4, mPC5 (gifts from Nabil G. Seidah), rpro-NT/NN (gift from Paul R. Doherty, University of Massachusetts, Worcester, MA), mPC1 and hPC2 cDNAs were radiolabeled by random priming with 5'-[32P]dCTP (>3000 Ci/mmol; ICN). The labeled probes were added to the hybridization mixture at the concentration of 1–3 × 106 cpm/ml and hybridized overnight at 42°C. The filters were then washed successively in 5 × SSPE, 0.1% SDS, twice at 50°C (15 min each). Filters were wrapped in Saran Wrap and exposed to Kodak X-Omat AR films at −70°C. In some experiments, the filters were washed out and rehybridized with a control labeled probe encoding GAPDH (gift from Françoise Presse, IPMC-CNRS, Valbonne). The films were scanned and mRNA bands were quantified by densitometry relative to GAPDH mRNA.

Reverse Transcription-Polymerase Chain Reaction (PCR) Analysis of PC2 Antisense RNA in Transfected rMTC 6-23 Cells—One μg of RNA from each transfected rMTC 6-23 clone was denatured at 65°C for 10 min and incubated for 1 h at 37°C in 20 μl of RT buffer (50 mM Tris-HCl, 10 mM MgCl2, 50 mM KCl, 10 mM dithiothreitol, 0.5 mM spermidine, pH 8.3) containing 2 μM of each deoxynucleotide phosphate, 25 units of RNasin (Promega, Charbonnieres, France), 0.1 μg of oligo(dT)15, and 15 units of avian myeloblastosis virus reverse transcriptase (Promega). The reverse transcriptase reaction mixture was then treated at 95°C for 3 min and kept on ice. Five μl of reverse transcriptase mixture was added to 50 μl of PCR reaction buffer (10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 0.2 mM Mg2+ bovine serum albumin, pH 9) containing 0.25 μM of each deoxynucleotide phosphate, 1 μM of each 5' and 3'-primers, and 0.5 unit of Taq polymerase (Appligene, Illkirch, France). Two sets of oligonucleotide primers (Eurogentec, Seraing, Belgium) were designed to hybridize with nonidentical hPC2 and rPC2 coding regions and to amplify DNA fragments of different sizes: hPC2, 5'-GGAGGAGTTACAGGAGAGATCC-3' (sense) and 5'-TCGCAGAGTCGGAGCAGCTCC-3' (antisense); and rPC2, 5'-GCTGACATTGGATGTCCTGAGT-3' (sense) and 5'-GGTCGACCCTGCGATGTGTT-3' (antisense) (underlined bases indicate points of homology between hPC2 and rPC2 sequences). The reaction mixture was overlaid with 50 μl of mineral oil (Sigma, St. Quentin Fallavier, France), treated at 94°C for 3 min, and submitted to 30 PCR cycles composed of successive 45-s denaturation periods at 94°C followed by 1-min annealing periods at 55°C and 1-min elongation periods at 72°C. The amplified products were separated by electrophoresis on a 1% agarose gel and bands with expected sizes of 635 and 525 base pairs for hPC2 and rPC2 cDNAs, respectively, were revealed by ethidium bromide staining.

Analysis of Pro-NT/NN Processing Products—The specificities of the NT (29G), NN (NN-Ah), and K6L (K6L-Af) antisera used here as well as the radioimmunoassay procedures employing these antisera have been previously described in detail (33, 38). Briefly, the NT antisera reacts with the free C terminus of NT, the NN antisera recognizes the free N terminus of NN, and the K6L antisera detects the free N terminus of the sequence that follows the Lys163-Arg164, Lys148-Arg149, Lys140-Arg141, and Lys84-Arg85 sequences were calculated by dividing post-HPLC iNT, C-terminal iNN, and iK6L, respectively, by CTiNN. Portions of acid extracts from PC12 and rMTC 6-23 cells were coomassie-stained, submitted to Arg-directed tryptic digestion (33, 38), and assayed for NT and large NT, respectively. 2 Fig. 2. Analysis of PCs content in rMTC 6-23 cells. rMTC 6-23 cells were unstimulated (−) or stimulated (+) by 1 μM dexamethasone for 48 h. Act-20 cells, GH3 cells, rat testis, and rat ileum were used as positive controls for PC1, PC2, PC4, and PC5 expression, respectively. PC12 cells served as a negative control for PC1 and PC2 expression and as a positive control for furin expression. Total RNA (10 μg) and proteins (50 μg) were analyzed by Northern and Western blotting as described under “Experimental Procedures.” A, Northern blot analysis of PC1, PC2, furin, PC4, and PC5 mRNAs. B, Western blot analysis of PC1, PC2, and furin proteins. The C-terminally directed PC2 antisense 4BF was used for immunoblotting. University of Massachusetts, Worcester) was used. This antiserum detects the free C terminus of NN and cross-reacts poorly (<3%) with NT or with C-terminally extended NN sequences (29). The tracer used in radioimmunoassays with antisemum NN was 125I-Baton-Hunter-NN prepared as described in Ref. 45.

Results

PCs Expression in rMTC 6-23 Cell Line—PC1, PC4, and PC5 mRNAs could not be detected in untreated rMTC 6-23 cells and in cells that had been exposed for 48 h to 1 μM dexamethasone (Fig. 2A). PC2 mRNA was present in control rMTC 6-23 cells.
Exposure of cells to dexamethasone in rMTC 6-23 cells was investigated. Cells—
The concentration and time dependence of PC2 induction by dexamethasone (Fig. 2) resulted in a graded increase in the 2.8-kilobase PC2 transcript and was increased by dexamethasone treatment. Furin mRNA was expressed in rMTC 6-23 cells and was not affected by dexamethasone (Fig. 2A). The expression of PC2 and furin and the lack of expression of PC1 in rMTC 6-23 cells were confirmed at the protein level by Western blot analysis (Fig. 2B). Att-20 cells, GH3 cells, rat testis, and rat ileum were used as positive controls for PC1, PC2, PC4, and PC5 expression, respectively (Fig. 2, A and B). PC12 cells served as a negative control for PC1 and PC2 expression and as a positive control for furin expression (Fig. 2, A and B).

Effect of Dexamethasone on PC2 Expression in rMTC 6-23 Cells—The concentration and time dependence of PC2 induction by dexamethasone in rMTC 6-23 cells was investigated. Exposure of cells to 10⁻⁹ to 10⁻⁵ M dexamethasone for 48 h resulted in a graded increase in the 2.8-kilobase PC2 transcript with maximal expression for 10⁻⁵ M dexamethasone (Fig. 3, A and C). The pattern of PC2 protein expression as determined by immunoblotting was similar (Fig. 3, B and C). PC2 mRNA levels increased as a function of time and were maximal after 72-96 h of treatment with 1 μM dexamethasone (Fig. 4, A and C). A similar time course was observed for PC2 protein synthesis (Fig. 4, B and C).

Expression of Antisense PC2 mRNA in rMTC 6-23 Cells—To investigate the role of PC2 in pro-NT/NN processing, human antisense PC2 RNA was stably expressed in rMTC 6-23 cells in an attempt to decrease PC2 levels. The presence of human antisense and rat sense PC2 mRNAs in transfected cells was detected by reverse transcriptase-PCR experiments as described under “Experimental Procedures.” Fig. 5A shows the results obtained with control cells (clone 10 transfected with expression vector alone) and PC2 antisense cDNA-transfected cells (clones E7, E5, and E14). All clones showed the 525-base pair band corresponding to rPC2 mRNA. The 635-base pair band corresponding to antisense hPC2 RNA was only detected in clones E5 and E14. PC2 protein expression was analyzed by immunoblotting (Fig. 5, B and C). Clone E7 expressed slightly lower levels of PC2 than clone 10. In contrast, clones E5 and E14 showed a marked reduction in PC2 levels (>80% and >90%, respectively). Examination of pro-NT/NN processing (Fig. 5C) shows that, in agreement with previous data (33, 34), control rMTC 6-23 cells (clone 10) quite efficiently (90%) converted pro-NT/NN. Conversion efficiency was slightly lower in clone E7. In contrast, processing was decreased by >50% and >80% in clones E5 and E14, respectively. Thus, the reduction in pro-NT/NN processing paralleled that observed for PC2 protein levels in PC2 antisense RNA-expressing rMTC 6-23 cells.

Pro-NT/NN Processing in PC1- and PC2-transfected PC12 Cells—Northern blot analysis of PC1 mRNA in stimulated wild type and PC1-expressing PC12 cells is shown in Fig. 6A. Wild type PC12 cells were devoid of PC1 mRNA whereas the transfected clones all expressed varying amounts of the 3.0-kilobase PC1 mRNA. These results were confirmed at the protein level by Western blot analysis with the N-terminally directed PC1 antiserum that detects the 87- and 66-kDa PC1 forms (Fig. 6B). Stimulated wild type and PC1-transfected PC12 cells expressed varying levels of the 1.1- and 1.5-kilobase pro-NT/NN mRNAs (Fig. 6A). These two mRNA species have been shown to...
were largely unable to generate pro-NT/NN-derived products. Further processing analysis showed that wild type PC12 cells (unprocessed) stored during the 48-h induction period (Table I). In contrast, the PC1 transfectants produced similar amounts of NT and large NN. They also produced NN in concentrations that were, however, 4 times lower on the average than those of large NN and NT. It should be pointed out that iK6L was undetectable in untransfected and PC1-transfected PC12 cells (not shown). This indicates that the Lys85-Arg86 dibasic in pro-NT/NN was not cleaved by PC1 in our transfectants. From these data, the percentages of cleavage at the three C-terminal Lys-Arg doublets in pro-NT/NN were calculated for each PC12/PC1 clone (Table I) and averaged in Fig. 8. PC1 cleaved pro-NT/NN with an order of preference for the dibasic sites that was Lys148-Arg149 > Lys163-Arg164 > Lys140-Arg141.

PC2-transfected PC12 cells expressed varying levels of the 2.8-kilobase PC2 mRNA whereas wild type PC12 cells were devoid of PC2 mRNA (Fig. 7A). Western blot analysis with a N-terminally directed PC2 antiserum which preferentially detects the 66-kDa PC2 protein confirmed these data (Fig. 7B). The levels of pro-NT/NN mRNA expression were variable in the selected PC12/PC2 transfectants (Fig. 7A). They were mirrored by the amounts of CTiNN measured in these clones (Table I). Pro-NT/NN processing analysis showed that the PC2 transfectants produced principally NN and NT (Table I). Large NN was in general not detectable except in the two clones (E2.11 and L2.2) that had the highest concentrations of CTiNN. In these clones, large NN was 5–10 times less abundant than NN. Large NT was detected only in the highest CTiNN-producing clone (L2.2). As with the PC12/PC1 cells, no iK6L could be detected in the PC2 transfectants (not shown), indicating that PC2 did not cleave the Lys85-Arg86 dibasic site in pro-NT/NN. The percentages of cleavage of the Lys-Arg sequences in pro-NT/NN (Table II, Fig. 8) revealed an order of preference for PC2 that was Lys140-Arg141 = Lys146-Arg147 > Lys163-Arg164. Note that this order markedly differs from that found for PC1.

**DISCUSSION**

One of the goals of the present study was to identify the PC(s) responsible for pro-NT/NN processing in the rMTC 6-23 cell line. Among the convertases whose presence was tested in rMTC 6-23 cells, only furin and PC2 were detected. This suggests that furin or PC2 could be involved in pro-NT/NN processing. Two pieces of evidence stand against furin as being a pro-NT/NN convertase. First, as recalled in the Introduction, furin, a ubiquitous enzyme, appears to be principally involved in the processing of precursor proteins that, unlike pro-NT/NN, are routed to the constitutive secretory pathway. Second, PC12 cells which do express furin (present study and Refs. 2 and 3) are virtually unable to process pro-NT/NN into mature products (37–39). This leaves the neuroendocrine cell-specific convertase, PC2, as the most likely pro-NT/NN convertase candidate in rMTC 6-23 cells. This hypothesis was directly tested by stably transfecting antisense PC2 mRNA in rMTC 6-23 cells and assessing the consequences of antisense expression on PC2 protein levels and pro-NT/NN processing. PC antisense strategies have been successfully used by others for demonstrating the role of PC2 in proglucagon processing in αTC1-6 cells (20) and of PC1 in POMC processing in AtT-20 cells (23). Our data show that rMTC 6-23 clones which expressed PC2 antisense mRNA exhibited a massive reduction of PC2 protein levels (up to 90%) which was paralleled by a marked inhibition of pro-NT/NN processing (>80%). These observations strongly argue in favor of PC2 being the major endogenous pro-NT/NN convertase in rMTC 6-23 cells.

Previous studies have shown that pro-NT/NN mRNA and protein expression was stimulated by dexamethasone in rMTC 6-23 cells (34). We show here that the glucocorticoid also increased PC2 mRNA and protein levels in these cells. The concentration-response and time dependence for the effect of dex-
With PC2, the major products observed were NT and NN, large different patterns of peptide production. Thus, the major cells were both able to process the NT/NN precursor but with PC2-mediated pro-NT/NN processing patterns. A major finding was that inhibitory (48).

Cells, although in this case the glucocorticoid effect was dexamethasone coregulated POMC and PC2 levels in AtT-20 nomenon (23, 48). In particular, it has been reported that and precursor substrate(s) expression may be a general phenomenon that produces pro-NT/NN. Hormonal coregulation of PC(s) a dexamethasone sensitive manner in the hypothalamic neurons both to up-regulate pro-NT/NN mRNA and NT levels in hypothalamic cells showing that PC1 colocalizes with pro-NT/NN in the endocrine N cells of the rat ileum.3 Thus, the coexpression profile of PC1 and PC2 with pro-NT/NN in systems that produce pro-NT/NN-derived products is consistent with the pattern of pro-NT/NN processing by these enzymes as determined here in transfected PC12 cells.

Differential processing by PC1 and PC2 has been reported for other prohormone precursors such as POMC and pro-insulin. In the case of POMC, PC1 appears to be responsible for the formation of large products including ACTH and β-lipotropin in the corticotrophs of the anterior pituitary, whereas PC2 processes these products further to generate smaller peptides such as the melanotropins, corticotropin-like intermediate lobe peptide, and β-endorphin in the intermediate lobe of the pituitary (22–24). In proinsulin, the insulin B and A chains are separated by a connecting peptide (C peptide) which is linked to both chains by dibasic sequences. Several lines of evidence indicate that in the insulin secretory β-granules PC1 preferentially cleaves the B chain-C peptide junction, while PC2 cleaves the C peptide-A chain junction (17, 18, 49, 50). Most recently, proglucagon was also shown to be differentially processed by PC1 and PC2, PC1 reproducing the processing pattern observed in the endocrine L cell of the gut and PC2 generating the pattern found in the pancreatic α cell (51). In general, it appears that PC1 cleaves multipepptide-producing precursors at a limited number of dibasic sites to generate large biologically active peptides, whereas PC2 processes additional dibasic sites to liberate the smaller active peptides. Such a pattern of action for PC1 and PC2 is consistent with the present observation that PC1 cleaves the Lys-Arg sequence that precedes NN much less efficiently than the two Lys-Arg sequences that flank NT in contrast to PC2 which processes the three Lys-Arg dibasic sites with a similar efficiency.

The analysis of a number of PC12 transfecteds that expressed varying amounts of PCs allows us to evaluate the incidence of PC1 and PC2 expression level on pro-NT/NN processing pattern and efficiency. Although there was some variability in the extent of dibasic site cleavages among the PC1- and PC2-transfected cells, the order of site preference for each endoprotease was similar within either series of transfectants. Thus, the level of convertase expression does not seem to greatly influence the qualitative pattern of pro-NT/NN processing by either PC1 or PC2. Similar conclusions were reached 3 P. Barbero, unpublished results.
PC12 cells were stimulated with NGF, dexamethasone, forskolin, and Li+ for 48 h. The amounts of CT1N and pro-NT/NN-derived peptides (see Fig. 1) and the percentages of cleavage at the dibasic sites were determined as described under "Experimental Procedures." The values are the means from duplicate determinations in a single experiment. Similar results were obtained in a separate experiment.

| Cell line | CT1N | Large NT | Large NN | NN | DT |
|-----------|------|----------|----------|----|----|
|           | pmol/mg | % cleavage |          |    |    |
| WT        | 19   | ND*      | ND       | 0.1 | 0.6 |
| E2.4      | 1.5  | ND       | ND       | 0.45 | 31 |
| E2.8      | 2.6  | ND       | ND       | 0.38 | 15 |
| E2.11     | 43   | ND       | 0.3      | 3   | 17 |
| E2.15     | 8    | ND       | 1.7      | 0.8 | 21.2 |
| E2.16     | 1.8  | ND       | 0.8      | 0.8 | 44 |
| L2.2      | 55   | 2        | 1.5      | 8  | 14.6 |

* ND, not detectable.

FIG. 8. Percentages of cleavage of the dibasic sequences in pro-NT/NN by PC1- and PC2-transfected PC12 cells. The values represent the mean ± S.E. from the determinations obtained in the six individual PC1/PC1 and PC1/PC2 transfectants (see Tables I and II).

regarding the processing of POMC in AtT-20 cells that overexpress PC1 or PC2 (24, 52). With respect to processing efficiency, somewhat higher pro-NT/NN conversion ratios were obtained in PC1/PC1 compared to PC1/PC2 transfectants. This was also the case in another study (39). It is, however, difficult to compare the efficiency of PC1 and PC2 in the present work because their concentration relative to one another is unknown. Our data with rMTC 6-23 cells show that PC2 can quite efficiently process pro-NT/NN (>95%). Recent studies have revealed that PC2 activation and enzymatic activity in the cell is under control of another neuroendocrine tissue-specific cellular protein designated 7B2 (53–55). It is therefore possible that the efficiency of PC2 may vary from one cell line to another depending on the cellular levels of 7B2 expression.

It would be interesting to know if the dibasic sites in pro-NT/NN are cleaved by PC1 and PC2 in a precise temporal order and if this order differs for both enzymes. This could provide some clues as to the pro-NT/NN sequence elements that direct PC1 and PC2 substrate specificity. The rMTC 6-23 cell line and the stable PC1/PC1 and PC1/PC2 transfectants characterized here should provide useful models to address this issue.

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