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Prohormone convertase 2 enzymatic activity and its regulation in neuro-endocrine cells and tissues

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Abstract

We used the fluorometric substrate, pGlu-Arg-Thr-Lys-Arg-MCA and the C-terminal peptide of human $7B2_{155-185}$, a specific inhibitor of prohormone convertase 2 (PC2), to specifically measure the enzymatic activity of the prohormone convertases, PC2. Using lysates from the pancreatic α cell line, αTC1-6 cells, which contain moderate levels of PC2 enzymatic activity, we determined that the PC2 assay was linear with respect to time of incubation and protein added and had a pH optimum of 5.5 and a calcium optimum of 2.5 mM. Rat pituitary contained high levels of PC2 enzymatic activity, while the hypothalamus and other brain regions contained moderate levels. This enzyme assay was used to document that both mice null for PC2 as well as mice null for the PC2 cofactor, 7B2, had only trace levels of PC2 activity in various brain regions, while mice heterozygous for these alleles had approximately half of the PC2 activity in most brain regions. PC2 enzymatic activity and PC2 mRNA levels were somewhat discordant suggesting that PC2 mRNA levels do not always reflect PC2 enzymatic activity.

Keywords: Prohormone; Fluorometric; SAAS; Post-translational processing; Processing enzyme; Pituitary

1. Introduction

The majority of prohormones are cleaved at paired basic residues to generate bioactive hormones in a cell-specific manner by prohormone convertases (PCs), members of the mammalian family of the subtilisin-like endoproteases [1,2]. Seven members of the PC family have been cloned: furin [3,4], PC1 [5–8] (also referred to as PC3 [7]), PC2 [5,9], PACE4 [10], PC4 [11,12], PC5/6 [13,14] and PC7/PC8 [15,16]. Both PC1 and PC2 are found exclusively in neural and endocrine cells equipped with a regulatory secretory pathway [5,6], and process a variety of prohormones including POMC, prosomatostatin, provasopressin, proneurotensin, pro-TRH, pro-enkephalin and pro-CRH [17–22].

Various endocrine cell lines have been found to contain PC1 and PC2 mRNA and protein [19,23]. Protein and mRNA levels of these enzymes are affected by different agents including glucose [24,25], fatty acids [26], glucocorticoids [19,27], cytokines [28] and agents which stimulate adenylate cyclase and protein kinase A [29,30]. In all these studies, it was assumed that changes in mRNA levels reflect changes in enzymatic activity. Enzymatic activity is likely to represent the critical parameter, since enzymatic activity determines prohormone processing. mRNA levels and enzymatic activity may differ for the following reasons: (i) some mRNA may not be translated; (ii) some of the protein may be bioinactive, either because it is an inactive zymogen or a degraded form; (iii) intracellular activators and inhibitors may influence enzymatic activity [31]; and/or (iv) enzyme may be secreted from the cell and changes in mRNA may not reflect cellular content of the enzyme [30,32]. Even quantitation of PC protein level by Western blotting or RIA does not necessarily represent a measure of bioactive enzyme. The establishment of an assay to directly measure PC1 and PC2 therefore represents an important goal.

The 31 amino acid carboxyl-terminal peptide of 7B2 (7B2-CT) functions as an endogenous inhibitor of PC2 and

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functions to control the activity of activated PC2 in early secretory pathway compartments [33–35]. The 7B2-CT also acts as a potent inhibitor of purified PC2 in vitro [36]. Similarly, various synthetic peptides have been found to specifically inhibit PC1 enzymatic activity of which the peptide Ac-Leu-Leu-Arg-Val-Lys-Arg-NH_2 was the most potent with a K_i of 3.2 nM [37]. A fluorometric assay, which nonspecifically measured paired basic cleavage in purified insulinoma secretory granules, has previously been described [38]. However, this assay did not distinguish between activity derived from PC1 and PC2, and also detected activity by other members of the PC family and other paired basic cleaving enzymes. Our efforts have been directed towards developing convertase-specific assays capable of measuring PCs in crude tissue and cell line extracts, using specific convertase inhibitors to identify activity due to each convertase. While this manuscript was in preparation, Berman et al. [39,40] used a similar fluorimetric assay with 7B2-CT to measure PC2 activity in various mouse brain regions. This assay was not fully characterized (i.e. pH optimum, calcium optimum, time-course and dose response) and comparing corresponding brain regions, PC2 enzymatic activity was quite discordant between the two papers, suggesting the need for further assay characterization. Therefore, one of our goals was to describe and characterize a PC2 enzymatic assay using 7B2-CT to convey specificity. We wanted to establish an assay that would be rapid (i.e. not requiring partial purification of the enzyme or immunoprecipitation), reproducible and specific for each convertase. This assay could then be used to monitor the regulation of enzymatic activity by various agents in endocrine cell lysates and in neuroendocrine tissues. Here, we present a robust assay for the specific measurement of PC2 activity and demonstrate that this enzyme activity is regulated under different manipulations.

2. Materials and methods

2.1. Cell culture

GH3 cells, COS-7 and AtT-20 cells were obtained from American Type Culture Collection (Rockville, MD), InRI-G9 cells were obtained from Dr. Patricia Brubaker (University of Toronto), αTC1-6 cells were obtained from Dr. Douglas Hanahan (UCSF) and βTC3 cells were obtained from Dr. Bruce Verchere (British Columbia Research Institute for Children's and Women's Health). All cells were maintained at 37 °C and 5% CO_2 in Dulbecco's Minimum Essential Medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD) supplemented with 0.075% sodium bicarbonate, 50 IU/ml penicillin–streptomycin and 0.125 mg/ml Fungizone (amphotericin B) (Gibco BRL). To induce secretory granule formation [41,42], GH3 cells were incubated for 24 h with 1 μM β-estradiol and 300 nM human insulin as previously described [43]. In other regulation studies, GH3 cells were preincubated overnight in serum-free medium with 4.5 mM glucose prior to incubation for 6 h in serum-free medium with and without 50 μM *N*-(2-[*p*-Bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H-89). Similarly, αTC1-6 cells were preincubated overnight in serum-free medium with 4.5 mM glucose prior to incubation for 6 h with 0 and 10 mM glucose in serum-free medium.

2.2. Northern blot analysis of PC2 mRNA in GH3 cells

Total RNA (20 μg), extracted from different cell lines using TRIzol reagent (Gibco BRL), was loaded onto a 1.2% formaldehyde denaturing agarose gel. After horizontal electrophoresis, the gel was transferred to a GeneScreen Plus hybridization transfer membrane (Dupont NEN, Boston, MA). The pBluescript-rPC2 construct, kindly provided by Dr. Richard Mains (Johns Hopkins University), was used for generating the PC2 riboprobe. A 531 bp ^{32}P-UTP-labeled rPC2 antisense riboprobe was synthesized using T7 RNA polymerase (Gibco BRL). The membrane was hybridized with the probe at 60 °C in 50% formamide, 5× saline-sodium citrate (SSC), 1% sodium dodecyl sulfate (SDS), 5× Denhardt's, 5% Dextran sulfate, 100 μg/ml salmon sperm DNA and washed at room temperature with 2× SSC, 0.1% SDS followed by two washes at 70 °C for 1 h with 0.1× SSC, 0.1% SDS. The blot was developed by exposure to X-ray film with an intensifying screen for 16–18 h. The blot was then stripped with Probe Degradation Buffer (Ambion) and Blot Reconstitution Buffer (Ambion) and was further probed with a 111 bp rat cyclophilin riboprobe [44] using the same conditions described above. The mRNA for PC2 was quantitated on an AlphaImager 2000 densitometry (Alpha Innotech, San Leandro, CA) using cyclophilin as an internal control.

2.3. Preparation of cell and tissue lysates for measurement of PC2 activity

For all cells, the media was removed, the cells washed with phosphate-buffered saline (PBS), harvested in PBS and centrifuged at 1000 × *g* for 5 min. After placing on ice, 500 μl of cold cell lysate buffer [0.1 M sodium acetate, pH 5.5, 1% triton-X, 1 μM *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64), 1 μg/ml pepstatin and 1 mM phenylmethanesulfonyl fluoride (PMSF)] was added and the cells were sonicated. After centrifugation at 10,000 × *g* for 10 min, the supernatant was saved for the enzyme assay. Similarly, the pituitary and various brain regions were removed from adult Sprague–Dawley rats, rinsed with PBS and sonicated in five volumes (i.e. 500 μl/100 mg of tissue) of lysate buffer, with the exception of the pituitary which was sonicated in 10 volumes of lysate buffer. PC2 homozygous and heterozygous knockout mice and wildtype litter mates were identified by PCR as described [45]. 7B2 homozygous and heterozygous knockout mice and wildtype

litter mates were also identified by PCR in a similar manner [46]. Brain regions were dissected from these mice and lysates prepared as above. All tissues, cells and buffers were kept on ice. Freezing of the lysates did not impair subsequently measured activity.

2.4. PC2 enzymatic assay

Each assay (final volume 200 μl) contained 0.1 M sodium acetate (pH 5.5) buffer (except for pH studies), 2.5 mM calcium chloride (except for calcium studies), the enzyme source (usually in a volume of 50 μl) and the inhibitors, 1 μM E64, 1 μg/ml pepstatin and 1 mM PMSF, which inhibit cathepsin D and B and other nonspecific enzymes, but not PC2 [47]. To measure optimum pH, 100 mM Tris/100 mM acetate buffer with pH adjusted between 4.5 and 8.5 with acetic acid was used. Each assay was performed in duplicate and was done in parallel tubes with and without the synthetic peptide corresponding to the C-terminal region of human $7B2_{155-185}$ (custom-synthesized at the Louisiana State University Medical Center peptide core facility) (final concentration 3×10^{-6} M, except for studies on inhibitor-dose dependence). The reaction was preincubated for 15 min at 37 °C prior to addition of the substrate, pGlu-Arg-Thr-Lys-Arg-MCA [final concentration 0.2 mM, stock solution dissolved in dimethyl sulfoxide (DMSO)] (Peptides International, Louisville, KY). The final concentration of DMSO in the reaction mixture was 2%. The reaction continued for 17 h (except for the time-course experiment). The reaction mixture was briefly centrifuged to remove insoluble material and the liberated aminomethylcoumarin (AMC) was measured using a Sequoia Turner fluorometer (excitation 380 nm; emission 460 nm). Controls without enzyme and without substrate were included in each assay. Activity specifically attributable to PC2 was calculated as PC2 activity in the absence of 7B2-CT minus PC2 activity in the presence of 7B2-CT. The amount of AMC was quantitated using a standard curve of known amounts of AMC and the protein concentration of the lysates determined using the method of Bradford [48].

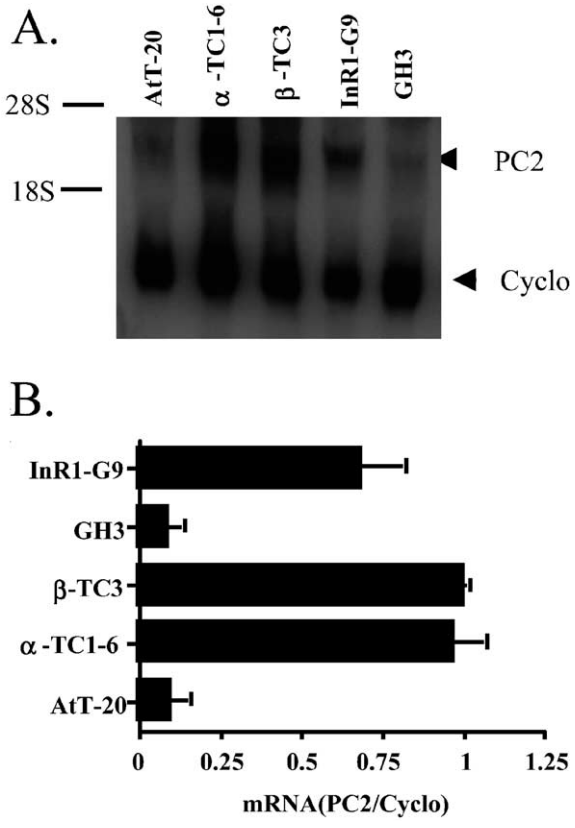

Fig. 1. (A) Representative Northern blot analysis of PC2 mRNA in various endocrine cell lines and (B) quantitation of PC2 mRNA in various endocrine cell lines. PC2 mRNA levels (corrected for cyclophilin levels) are measured as described in the Materials and methods. mRNA levels for each cell line represents mean ± S.E.M. of three independent experiments. Cyclo = cyclophilin.

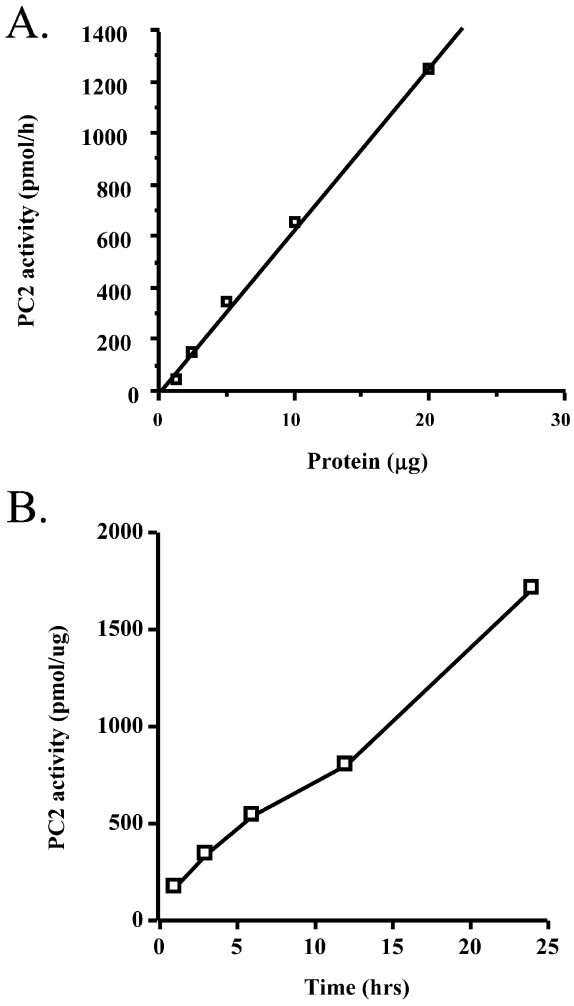

Fig. 2. Protein (A) and time (B) dependence of PC2 enzymatic activity in αTC1-6 cell lysates. The effect of different concentrations of αTC1-6 cell lysate on PC2 enzymatic activity [calculated as PC2 activity in the absence of 7B2-CT minus PC2 activity in the presence of 7B2-CT (3×10^{-6} M)] in reactions incubated for 17 h (A). Time course of PC2 enzymatic activity using αTC1-6 cell lysates (B).

2.5. Statistical analyses

Statistical analysis comparing two groups was performed using the Student's *t*-test. Statistical analysis comparing multiple groups was performed with the InStat 2.03 program using one-way ANOVA for multiple groups and post hoc Student's *t*-test (corrected using the Dunnett correction factor) for comparing treatment with control.

Correlations between mRNA levels and enzyme assay levels in different cell lines were performed on three separate occasions for each set of cell lines. The weighted mean of correlation (r_w) of the three individual correlations was determined according to standard statistical methods [49].

3. Results and discussion

3.1. PC2 enzymatic activity in endocrine cells

We initially analyzed different endocrine cell lines for PC2 mRNA levels by Northern blot (Fig. 1A) in order to identify a cell line with moderate PC2 expression. Quantitation of three independent Northern blots showed that βTC3 and αTC1-6 cells had the highest level of PC2 mRNA, with lower levels in other endocrine lines (Fig. 1B). As αTC1-6 cells had moderate PC2 mRNA but no PC1 mRNA [23], we used lysates from these cells to characterize the PC2 assay.

Fig. 2 demonstrates that PC2 enzymatic activity in αTC1-6 cell lysates was linear with respect to protein concentration and time of incubation. Although we typically incubated the αTC1-6 cell lysates for 17 h, much shorter incubation times (1–3 h) could be used in cell lines containing high levels of PC2. The assay was quite sensitive as one 10-cm dish of αTC1-6 cells provided enough enzymes to easily measure PC2 enzymatic activity for 10–20 assays. The assay is also convenient as cells can be harvested and the cell pellet or lysate stored at −80 °C, without appreciable loss of activity.

Fig. 3A demonstrates that the optimal calcium concentration for PC2 enzymatic activity using αTC1-6 cell lysates was 2.5 mM. In contrast to recombinant PC2 [47], PC2 enzymatic activity from αTC1-6 cell lysates was inhibited at

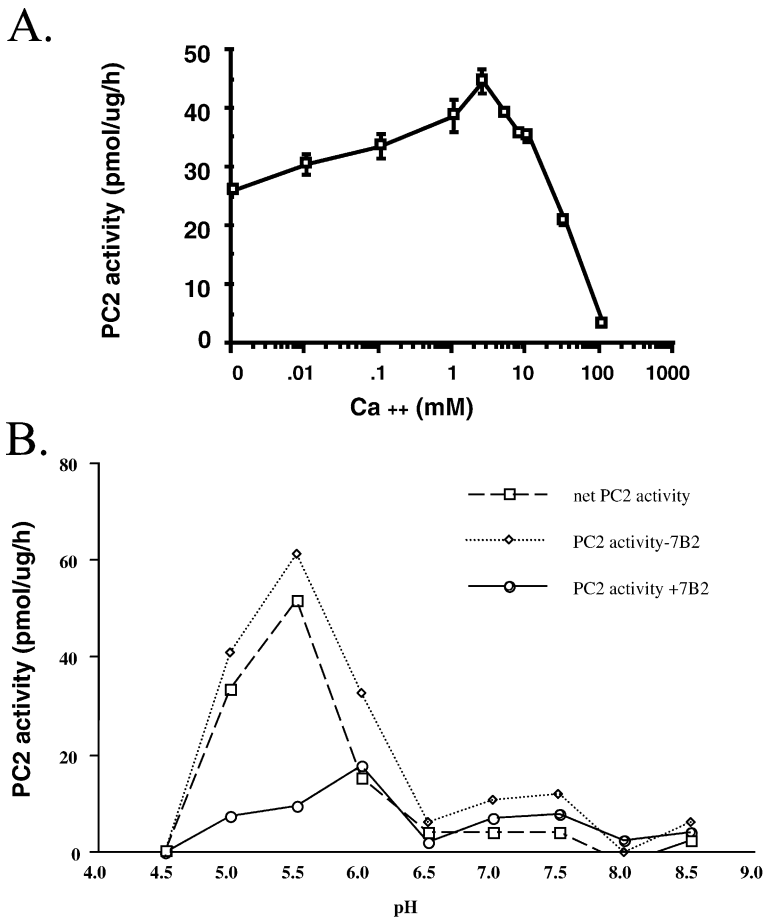

Fig. 3. Calcium (A) and pH (B) profile of PC2 enzymatic activity in αTC1-6 cell lysates. The effect of different calcium concentrations (A) on PC2 enzymatic activity [calculated as PC2 activity in the absence of 7B2-CT minus PC2 activity in the presence of 7B2-CT (3×10^{-6} M)] using αTC1-6 cell lysates. The effect of pH (B) on PC2 enzymatic activity. For the representative pH profile (B), enzymatic activity in the presence and absence of 7B2-CT is shown with the net activity [(activity − 7B2-CT) − (activity + 7B2-CT)] indicating activity specifically attributable to PC2.

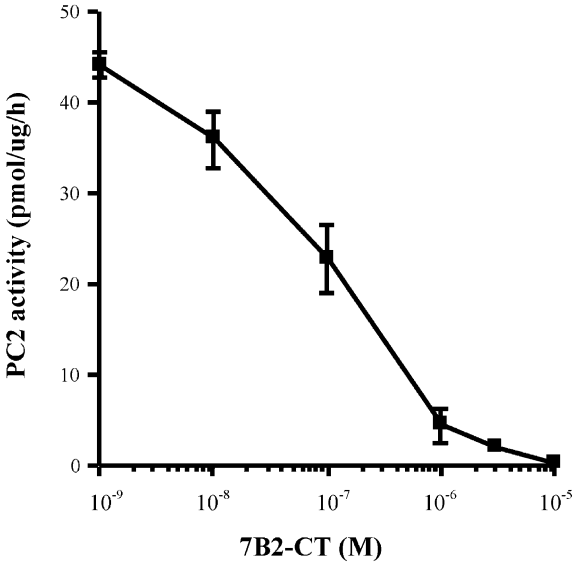

Fig. 4. Effect of different concentrations of 7B2-CT on PC2 enzymatic activity in αTC1-6 cell lysates.

high calcium concentrations. Fig. 3B demonstrates that the pH optimum for PC2 enzymatic activity using αTC1-6 cell lysates was 5.5, which was similar to the pH optimum of 5.0 found using purified recombinant PC2 [47], but differs from the pH optimum of 7.0 of PC2 purified from bovine adrenal medullary chromaffin granules [50]. In Fig. 3B, the enzymatic activity in the presence and absence of 7B2-CT, is shown, with the net activity [(activity – 7B2-CT) – (activity + 7B2-CT)] indicating activity specifically attributable to PC2. A pH of 5.5 and a calcium concentration of 2.5 mM were used for all further experiments on PC2 enzymatic activity.

Fig. 4 shows the effect of different concentrations of 7B2-CT on PC2 enzymatic activity. We used a concentration

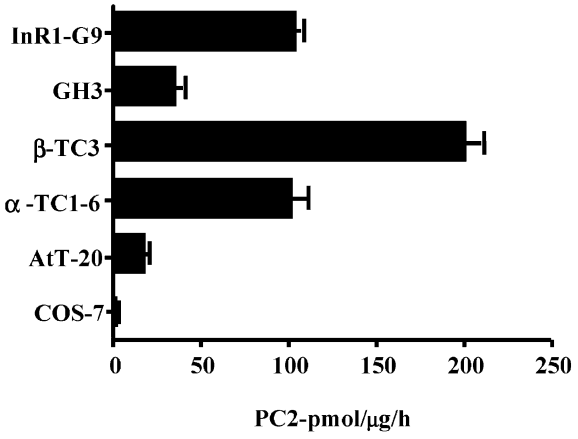

Fig. 5. PC2 enzymatic activity in various endocrine cell lines. PC2 enzymatic activity [calculated as PC2 activity in the absence of 7B2-CT minus PC2 activity in the presence of 7B2-CT (3×10^{-6} M)] and PC2 mRNA levels (corrected for cyclophilin levels) was measured as described in the Materials and methods. Enzymatic activity for each cell line represents mean ± S.E.M. of three independent experiments.

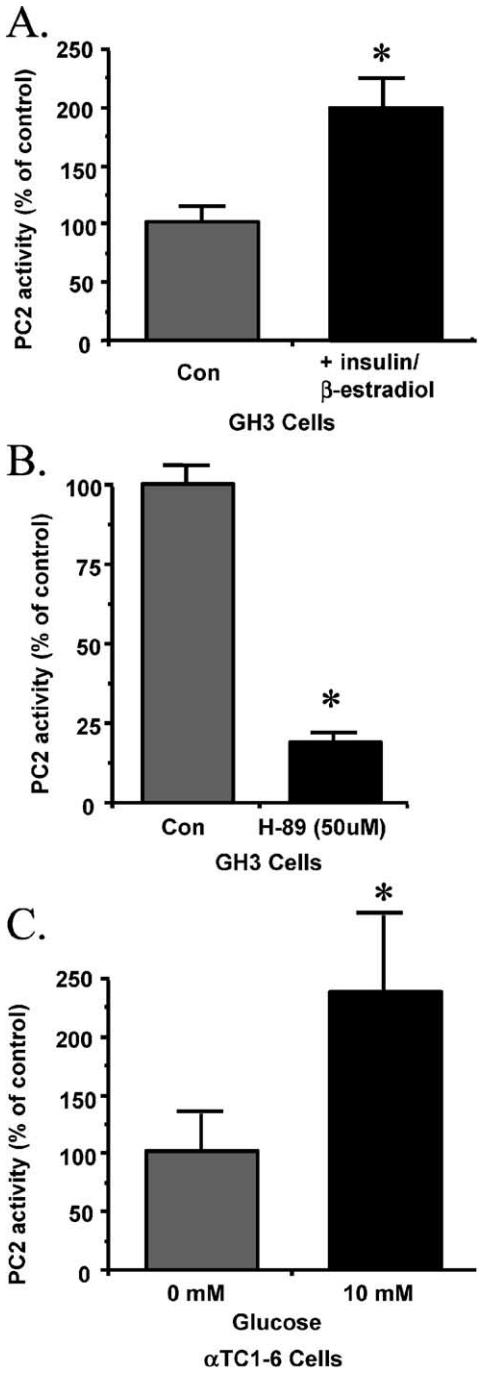

Fig. 6. Regulation of PC2 enzymatic activity in endocrine cell lines. GH3 cells were incubated for 24 h with 1 μM β-estradiol and 300 nM human insulin (A). GH3 cells were preincubated overnight in serum-free medium with 4.5 mM glucose prior to incubation for 6 h in serum-free medium with and without H-89 (B). αTC1-6 cells were preincubated overnight in serum-free medium with 4.5 mM glucose prior to incubation for 6 h with 0 and 10 mM glucose in serum-free medium (C). For all studies, PC2 enzymatic activity [calculated as PC2 activity in the absence of 7B2 minus PC2-CT activity in the presence of 7B2-CT (3×10^{-6} M)] was measured as described in the Materials and methods. Enzymatic activity represents mean ± S.E.M. of two to three independent experiments. *$p < 0.01$.

Table 1
Distribution of PC2 enzymatic activity in rat pituitary, brain regions and peripheral tissue

Tissue	PC2 activity (pmol/μg/h)
Anterior pituitary	32 ± 8.7
Neurointermediate pituitary	111 ± 10
Hypothalamus	8.7 ± 2.4
Frontal cortex	3.1 ± 1.8
Mid-brain	5.1 ± 3.0
Cerebellum	2.9 ± 1.7
Muscle	0
Liver	0

Mean ± S.E.M., $n = 3-4$ animals.

of 3×10^{-6} M that inhibited about 95% of the enzymatic activity for all experiments.

We next measured PC2 enzymatic activity in various cell lines. As shown in Fig. 5, we found the highest levels of PC2 enzymatic activity in extracts prepared from βTC3 cells, a beta cell tumor line [51]. αTC1-6 cells, which are derived from a glucagonoma [52] and InRI-G9 cells (derived from an insulinoma [53]), had moderate levels of PC2 enzymatic activity. GH3 cells (a sommatotroph cell line) and AtT-20 cells (a corticotroph cell line) had lower levels of PC2 enzymatic activity. The non-endocrine cell line, COS-7 cells, had no detectable PC2 activity. Enzymatic activity for PC2 was correlated to a moderate degree ($r_w = 0.89$, $r_w^2 = 0.79$; $p < 0.05$) with levels of PC2 mRNA. Certain cell lines, such as αTC1-6 and InRI-G9 cells, had higher levels of PC2 mRNA compared to enzymatic activity, while GH3 cells had higher levels of PC2 enzymatic activity compared to mRNA. We interpret this discordance as a demonstration that mRNA levels do not completely reflect enzymatic activity.

We postulated that agents that induce secretory granule formation [41,42] would increase PC2 activity. Addition of 1 μM β-estradiol and 300 nM human insulin to GH3 cells for 24 h increased ($p < 0.01$) PC2 activity 1.7-fold (Fig. 6A), demonstrating that agents that increase secretory granule formation increase PC2 activity.

One of the goals of developing this assay was to study the in vivo regulation of PC2 enzymatic activity. GH3 cells exposed to the protein kinase A inhibitor, H-89 [54], had a 5-fold reduction in PC2 enzymatic activity (Fig. 6B). Although this concentration of H-89 inhibits other kinases, this result, along with the findings by Jansen et al. [55] that the cAMP response elements of the proximal PC1 promoter confer basal and hormonal regulated promoter activity, provides evidence that PC1/PC2 enzymatic activity (probably by regulation of gene expression) in neuroendocrine cells likely involves the cyclic AMP–cAMP response element binding protein (CREB) pathway.

αTC1-6 cells exposed to 10 mM glucose had a 2.5-fold increase in PC2 enzymatic activity compared to cells exposed to 0 mM glucose (Fig. 6C). These results also demonstrate that αTC1-6 cells are glucose-responsive, as InR1-G9 cells have recently been found to be [56].

Thus, our studies using agents that increase secretory granule formation and our studies using glucose and H-89 demonstrate that the PC2 enzymatic assay can be used to study the regulation of enzymatic activity of this enzyme in a cell culture system.

3.2. PC2 enzymatic activity in neuro-endocrine tissues

We also measured PC2 enzymatic activity in the pituitary and various brain regions (Table 1) of the rat. The rat was

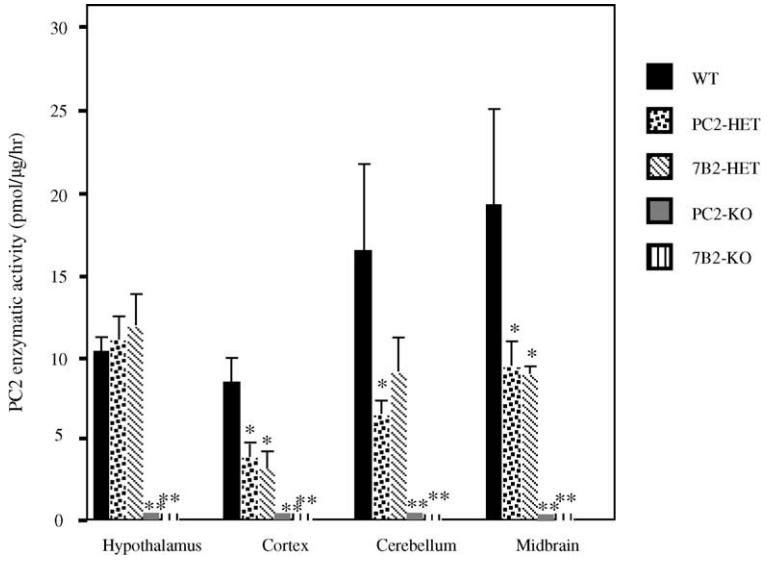

Fig. 7. Levels of PC2 in different brain regions of wildtype, PC2 heterozygous, 7B2 heterozygous, PC2 knockout and 7B2 knockout mice. PC2 enzymatic activity [calculated as PC2 activity in the absence of 7B2-CT minus PC2 activity in the presence of 7B2-CT (3×10^{-6} M)] for different brain regions is measured as described in the Materials and methods. Enzymatic activity represents mean ± S.E.M. from four to six mice. *$p < 0.05$ compared to the wildtype mice, **$p < 0.01$ compared to the wildtype mice.

chosen in order to have adequate tissue after dissection of the anterior and neurointermediate lobes of the pituitary. Rat neurointermediate lobe pituitary contained the highest levels of PC2 enzymatic activity, followed by anterior lobe. The frontal cortex, hypothalamus and other brain regions contained lower levels. The peripheral tissues, liver and muscle, had no PC2 enzymatic activity. The distribution of PC2 enzymatic activity in the brain was similar to the distribution of PC2 mRNA [57,58].

Fig. 7 demonstrates the use of the enzyme assay in various brain regions of wildtype, PC2 heterozygotes, 7B2 heterozygotes, PC2 knockout and 7B2 knockout mice. In all brain regions examined, only trace PC2 activity was present in PC2 knockout and 7B2 knockout mice. Heterozygous PC2 and 7B2 mice had approximately half of the PC2 activity of wildtype mice in the frontal cortex, cerebellum and midbrain. Interestingly, the activity of PC2 in the hypothalamus of heterozygous PC2 and 7B2 mice was similar to that of wildtype mice suggesting the importance of PC2 in the processing of proneuropeptides in that brain region. We speculate that compensatory upregulation of PC2 enzymatic activity occurs in the hypothalamus of heterozygous PC2 and 7B2 in order to maintain levels similar to that of wildtype mice. Brain PC2 enzymatic activity was higher in the mouse than the rat. Pituitaries from PC2 and 7B2 knockout mice were not available for these studies.

Another important advantage of our PC2 enzyme assay is illustrated by examining the previously published Western blots of PC2 [45] and 7B2 [59]. PC2 wildtype mice islets expressed 75 and 64 kDa PC2 immunoreactive proteins. The PC2 null mice islets expressed only a 72-kDa protein, reflecting the deletion of a segment of exon 3 of PC2 during the generation of the PC2 null mice and the resultant impaired conversion from the larger to the smaller, bioactive form. Differentiating between the 75, 72 and 64 kDa forms by Western blot would be difficult, while the PC2 assay reflects only bioactive (64 kDa) PC2. Similarly, brains from the 7B2 null mice [59] express a higher proportion of a larger PC2 immunoreactive protein (75 kDa form, Lindberg et al., unpublished observations) compared to the smaller form (presumably the 64 kDA form) than the wildtype mice, reflecting the impaired maturation of pro-PC2. Interestingly, in the 7B2 null mice, the lower molecular weight band of PC2 is still present, but is biologically inactive and reflects unproductive maturation of pro-PC2 [60]. Thus, the difference between bioactive and biologically inactive PC2, impossible to appreciate by Western blot analysis, is readily assessed by our PC2 enzyme assay.

4. Conclusion

We describe a rapid and reproducible enzyme assay to measure PC2 activity in endocrine cell lysates and pituitary and brain tissues. In cells and tissues with high levels of PC2, this assay can be performed rapidly and requires only a small amount of cells or tissues that express high levels of PC2. As this assay actually measures enzymatic activity, it is more informative, quantitative and rapid than measuring mRNA levels by Northern blot or protein levels by Western blotting.

Acknowledgements

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