PACE4: a subtilisin-like endoprotease with unique properties

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PACE4 is one of the neuroendocrine-specific mammalian subtilisin-related endoproteases believed to function in the secretory pathway. The biosynthesis and secretion of PACE4 have been studied using transfected neuroendocrine and fibroblast cell lines, as well as primary pituitary cultures. ProPACE4 (approx. 106 kDa) is cleaved intracellularly before secretion of PACE4 (approx. 97 kDa); the N-terminal propeptide cleavage is accelerated in a truncated form of PACE4 lacking the Cys-rich C-terminal region (PACE4s). Neither PACE4 nor PACE4s is stored in regulated neuroendocrine secretory granules, whereas pro-opiomelanocortin-derived peptides and prohormone convertase 1 enter the regulated secretory pathway efficiently.

The relatively slow cleavage of the proregion of proPACE4 in primary anterior pituitary cells, followed by rapid secretion of PACE4, is similar to the results for proPACE4 in transfected cell lines. The enzyme activity of PACE4 is distinct from furin and prohormone convertases, both in the marked sensitivity of PACE4 to inhibition by leupeptin and the relative insensitivity of PACE4 to inhibition by Ca^{2+} chelators and dithiothreitol; PACE4 is not inhibited by the α_1 -antitrypsin Portland variant that is very potent at inhibiting furin. The unique biosynthetic and enzymic patterns seen for PACE4 suggest a role for this neuroendocrine-specific subtilisin-like endoprotease outside the pathway for peptide biosynthesis.

INTRODUCTION

PACE4 is one of the newly discovered family of subtilisin-like endoproteases which are related to the yeast Kex2 enzyme [1–5]. Other members of this family include prohormone convertase (PC)1/3 and PC2, which are restricted to neurons and endocrine cells and clearly play major roles in the biosynthetic processing of propeptides into bioactive smaller peptides, furin, which is expressed in virtually all tissues and which plays an important role in processing of proteins in the constitutive pathway (such as the insulin receptor, proendothelin, viral glycoproteins and bacterial endotoxins), PC4, which is restricted to germ cells, PC5/6, which is found in brain and gastrointestinal tissue, and LPC or PC8, which is found in all tissues [1,5–12]. PC7 was originally used to refer to a fragment of rat PACE4 [13,14], but has recently also been used to refer to LPC/PC8 [10,12,15].

PACE4 is found at extremely high levels in most cells of the anterior pituitary and in cerebellar Purkinje cells; the pituitary level of PACE4 mRNA is strongly responsive to thyroid status [13,14,16,17]. PACE4 is also found in the β cells of the endocrine pancreas, along with PC1, but PACE4 is absent from the α , δ and PP cells [18]. PACE4 is also present at high levels in about a dozen selected subcortical nuclei [16]. PACE4 is not found at significant levels in a number of non-neuroendocrine tissues such as liver, kidney, spleen, lung, gastrointestinal tract, germ cells and submaxillary glands [13,16,17]. The *Aplysia* PACE4 homologue is localized to the secretory granules of bag cell neurons along with egg-laying hormone and the *Aplysia* PC1 homologue [19]. It has been proposed that PACE4 is crucial to β -cell-specific processing [18]. Thus the tissue and cellular localization of PACE4 suggests an important role in neuroendocrine function.

Since the tissue distribution and pattern of regulation of expression of PACE4 are indicative of important endocrine and neurobiological functions, we have examined the biosynthesis

and secretion of PACE4 in more detail. PACE4 was found to be secreted by stably transfected fibroblasts and neuroendocrine cells, and by primary anterior pituitary cells in culture; the recombinant PACE4 secreted by fibroblasts provides the protein needed for further enzymic characterization. Additional studies examined the role of the N-terminal proregion and the C-terminal region beyond the presumed catalytic domain. The enzymic activity of PACE4 was found to be distinct from the activities of furin and other known PCs. The results suggest unique biological roles for PACE4.

EXPERIMENTAL

Expression vectors

The nt 1–2848 fragment of the rat PACE4 cDNA was expressed using pCIS.2CXXNH as described [13]. The expression vector for rat PACE4s (truncated PACE4) was created in an identical manner but with a stop codon inserted to make the protein terminate after amino acid residue 650, producing a molecule the same size as PC2 [20]. The PACE4s protein lacks the entire Cys-rich region (residues 663–937) [13]. The PC1pro–PACE4 chimera was synthesized as described [21], attaching amino acids 1–110 of PC1 (signal, proregion and cleavage site) to residues 136–937 of PACE4 (catalytic region and Cys-rich region).

Transfection of cell lines to achieve expression of PACE4 and PACE4s

AtT-20 and hEK-293 cells stably expressing PACE4, PACE4s and PC1pro:PACE4 were created by transfection with lipofectin (Gibco–BRL, Gaithersburg, MD, U.S.A.) and selection in 0.5

Abbreviations used: PC, prohormone convertase; PMA, phorbol 12-myristate 13-acetate; POMC, pro-opiomelanocortin; PAM, peptidylglycine α -amidating mono-oxygenase; Mca, (7-methoxycoumarin-4-yl)acetyl; α_1 -AT, α_1 -antitrypsin; α_1 -AT-PDX, Portland variant of α_1 -AT; α_1 -AT-PITT, Pittsburgh variant of α_1 -AT.

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mg/ml G418 as described [21]. Transient expression in hLoVo cells was as described previously [21]; hLoVo cells were originally used in this work as a cell line lacking both furin and PACE4 [22], but other reports conclude that hLoVo cells indeed express PACE4 [2]. Chinese hamster ovary (CHO, clone DG44) cells were transfected in the same manner, but stable cell lines were selected by growth in alpha minimal essential medium containing 20% dialysed calf serum (Gibco-BRL) and maintained in the same medium with 10% dialysed calf serum.

Biosynthetic labelling

AtT-20, hEK-293 and hLoVo cells were incubated in medium containing [³⁵S]Met/Cys as described [21]; when indicated, secretion was stimulated using 250 nM phorbol myristate acetate (PMA) [23]. Cells were extracted with boiling SDS, and both cell extracts and medium were treated with a cocktail of protease inhibitors before immunoprecipitation and SDS/PAGE analysis [21]. Rabbit polyclonal antisera used included: pro-opiomelanocortin (POMC) antisera JH93 [for the N-terminal region of corticotropin (ACTH)] and JH2 (for β -endorphin) [21]; PC1 antiserum JH888 [24]; peptidylglycine α -amidating monooxygenase (PAM) antiserum JH475 (for the mono-oxygenase region) [25]; and PACE4 antisera JH1473 and JH1475, raised at Hazleton HRP (Denver, PA, U.S.A.) against a recombinant protein [PACE-(570–656)] expressed using the pET system and purified as described [25]. Primary cultures of adult rat anterior pituitary were prepared as described, using Sprague-Dawley rats from Harlan [26].

Immunocytochemistry

AtT-20 cells were fixed using cold methanol (for PACE4, PC1 and TGN38) or 4% paraformaldehyde (for POMC peptides), and primary antibody was visualized using goat anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate [21,27].

Enzyme preparation and protease assay

Complete serum-free medium [28] from monolayers of hEK-293 stable transfectants expressing and secreting rat PACE4 was diluted five times with 20 mM Tris/HCl, pH 7.5 (buffer A) and filtered through a 0.2 μ m filter. Truncated soluble furin (hFUR713) was secreted by BSC40 cells infected with a vaccinia virus recombinant expressing soluble furin [29,30]. The two preparations were purified by similar protocols. The filtered media were loaded (flow rate 0.75 ml/min) on to a Mem-Sep1000 DEAE ion-exchange column (Pharmacia). Proteins were eluted from the ion-exchange column with a gradient of 0–400 mM NaCl in buffer A. Fractions of 0.75 ml were collected and assayed for protease activity.

Fluorimetric assays were performed with the Boc-Arg-Val-Arg-Arg-Mca substrate [where Mca is (7-methoxycoumarin-4-yl)acetyl] in a 0.5 ml reaction volume as previously described [31]. Approximately the same amount of activity (1 unit, where a unit is defined as the quantity of enzyme capable of hydrolysing 1 pmol of fluorogenic substrate/min) were used for both furin and PACE4. Briefly, samples were assayed in 100 mM Hepes, pH 7.5, containing 0.5% Triton X-100, 1 mM CaCl₂, 1 mM β -mercaptoethanol and 25 μ M fluorogenic substrate. Appropriate substitutions or additions were made to the reaction mixture in order to analyse the effects of inhibitors. Assays were performed at 30 °C and terminated by dilution with 1 mM ZnCl₂ after a 1 h incubation. The 7-amino-4-methylcoumarin liberated by cleavage of the peptide was measured with a Hitachi F-2000 spectrofluorimeter (380 nm excitation, 460 nm emission).

RESULTS

Biosynthesis of PACE4 in fibroblasts and neuroendocrine cells

When hEK-293 fibroblasts were stably transfected with a vector encoding rat proPACE4, a protein of the expected size was synthesized and released into the medium [13]. However, no secretion of human PACE4 was detected from transfected COS-1 cells [32]. To investigate the synthesis and secretion of PACE4 more thoroughly, the hEK-293 line and additional stably transfected neuroendocrine and fibroblast cells were examined (Figure 1). In the hEK-293 cells, a protein the size of proPACE4 (106 kDa) was synthesized during the pulse (P) and converted efficiently during the chase (P/C) into a protein the size of mature PACE4 (97 kDa); this protein was secreted into the medium (M). About half of the protein synthesized in a 15 min pulse was secreted during the 2 h chase period, with the rest still in the cells. CHO and hLoVo cells also processed and secreted PACE4 efficiently.

By comparison, although a protein the size of proPACE4 was synthesized during the pulse in the AtT-20 neuroendocrine cells, the extent of conversion into the mature form of PACE4 was much lower and little PACE4 was secreted into the medium during the 2 h chase. The overall recovery of PACE4 during the chase was low, suggesting intracellular turnover of the newly synthesized protein. The inset shows, in a greatly overexposed autoradiogram, that a tiny fraction of the mature PACE4 was secreted by the transfected AtT-20 cells. Whereas PACE4 is efficiently secreted by fibroblasts, neuroendocrine cell lines as well as primary anterior pituitary cells (see below) degrade most of the PACE4 intracellularly.

Role of the Cys-rich C-terminal region in PACE4 biosynthesis

To investigate why AtT-20 cells do not process and secrete PACE4 efficiently whereas several mammalian PCs and even yeast Kex2 are efficiently processed and secreted in AtT-20 cells [4,21,29,33–41], we first investigated the effect of deleting the Cys-rich region from proPACE4 on its biosynthesis, processing, storage and secretion in AtT-20 cells. Similar Cys-rich regions

Figure 1 Biosynthesis of PACE4 in stably transfected cell lines

Stable hEK-293 and AtT-20 cells expressing proPACE4 were pulse-labelled for 15 min in medium containing [³⁵S]Met/Cys, extracted, immunoprecipitated and analysed by SDS/PAGE and fluorography (P). An identical well was labelled and then incubated in complete non-radioactive medium for an additional 2 h (cells, P/C; medium, M); the inset shows the P/C and M samples from an overexposed autoradiogram from AtT-20 cells. Similar results were obtained in three additional experiments with hEK-293 cells and in six additional experiments with AtT-20 cells.

Figure 2 Role of the C-terminus of proPACE4 in biosynthesis

AtT-20 cell lines stably expressing proPACE4 or proPACE4s were labelled and analysed as in Figure 1. Similar results were obtained in two additional experiments of this type, at chase times of 1, 2 and 3 h.

Figure 3 Immunocytochemistry of PACE4 in AtT-20 cells

The PACE4 and PACE4s stable cell lines were fixed and analysed. In the top row, non-transfected cells (Non-trans Cells) were stained using antisera against the endogenous subtilisin-like endoprotease PC1, TGN38 or PACE4 as shown. The nucleus (n), *trans*-Golgi network (tgn) and tips of cellular processes (arrows) are indicated. In the bottom row, the PACE4 antiserum was used to stain AtT-20 cells expressing PACE4 or PACE4s as shown. Staining of cells expressing PACE4 was blocked by addition of the purified bacterial fragment of PACE4 (PACE Cells; Block).

are found in furin and PC5/6 [2], and deletion of the Cys-rich domain of truncated soluble furin yields a secreted active enzyme [42]. ProPACE4s terminates at residue 650, making PACE4s equivalent in length to natural PC2 and to previously examined C-terminally truncated forms of PC1, furin and Kex2 [21,42–44]. By deleting the Cys-rich region, the rate of conversion from promolecule (72 kDa) into mature protein (66 kDa) was accelerated in AtT-20 cells (Figure 2). Similar acceleration of processing of PACE4, by deleting the C-terminal Cys-rich region, was seen in three fibroblast cell lines (hEK-293, CHO and hLoVo; not shown). Interestingly, the efficiency of secretion of

Figure 4 PACE4 and PACE4s do not alter intracellular POMC biosynthetic processing

Clonal AtT-20 cells stably expressing proPACE4 (■) or proPACE4s (▲) were compared with non-transfected (○) AtT-20 cells in a pulse–chase paradigm. Cells were labelled for 15 min in medium containing [³⁵S]Met/Cys and then chased in complete non-radioactive medium for 2 h. Cell extracts and medium were immunoprecipitated with antiserum JH93 directed against the N-terminus of corticotropin. No changes were seen in intracellular processing using β -endorphin antisera (not shown). Similar results were obtained in five experiments of this type with both PACE4 and PACE4s cell lines and with both corticotropin (ACTH) and endorphin antisera, for chase times of 30 min to 6 h.

the truncated PACE4s was also much higher than for PACE4, much of which was degraded in the cells and never released by the AtT-20 cells. The C-terminal non-enzymic portions of PC1 and Kex2 also retard transit through the cell and secretion into the medium, and the enzymic properties of PC1 are also changed by the presence or absence of the C-terminal non-enzymic region [21,43,44].

Immunocytochemistry of PACE4 in AtT-20 cells

The localization of PACE4 and PACE4s was investigated in AtT-20 cells using immunocytochemistry (Figure 3). For comparison, the pattern of staining for the endogenous convertase PC1 was also monitored; it was found in the tips of cell processes (arrow) and the *trans*-Golgi network region (tgn), as identified using a TGN38 antibody [27,45]. PACE4 showed some accumulation at the tips of processes (arrows), where secretory granules, POMC peptides and soluble forms of PAM are known

Figure 5 Secretagogues do not stimulate release of mature PACE4 or PACE4s

Paired wells of AtT-20 cells expressing proPACE4 or proPACE4s were labelled and chased as in Figure 1. After the 2 h chase incubation, cells were placed in fresh complete serum-free medium with or without PMA for an additional 30 min, and the medium was analysed using the PACE4, PC1 or β -endorphin (POMC) antisera. The top panel shows one set of results from cells expressing proPACE4 (PACE4 and PC1) or proPACE4s (PACE4s and POMC). The autoradiograms from five experiments of this type were density analysed [23], and the ratio of the amount of radiolabelled protein released in PMA-supplemented compared with basal medium was calculated. Data are means $\pm$ S.E.M. β LPH, β -lipotropin; β -endo, β -endorphin.

to be concentrated [23,46–50], but PACE4 did not accumulate in the tips to the same extent as PC1 or POMC. PACE4 immunostaining was excluded from the nucleus (n). PACE4s was also distributed throughout the cell, with only limited accumulation in the tips of processes. These data suggest that PACE4 and PACE4s do not reach secretory granules as well as the endogenous PC1 or POMC. By comparison, some transfected molecules (e.g. pro-neuropeptide Y) reach regulated granules far more efficiently than PC1 and POMC [45].

Expression of PACE4 and PACE4s does not alter POMC biosynthetic processing

The immunocytochemical data indicate that the majority of the PACE4 and PACE4s in transfected AtT-20 cells does not reach regulated secretory granules. The possibility remained that a small percentage could reach the POMC-containing granules and alter POMC biosynthetic processing. As seen in Figure 4, the pattern of intracellular POMC processing in clonal AtT-20 cells stably expressing proPACE4 and proPACE4s was identical with the pattern in non-transfected cells, whether the corticotropin pattern of peptides or the endorphin pattern of peptides (endorphin pattern not shown) was examined, for all chase times tested from 30 min to 6 h. From enzyme assays (see below), the PACE4 emerging into the culture medium is enzymically active.

Figure 6 Biosynthesis of PACE4 and PAM in primary anterior pituitary cultures

Primary cultures of adult rat anterior pituitary were labelled and chased as in Figure 1, except the chase period was lengthened to 4 h. Similar results were seen with an independent set of primary anterior pituitary cultures.

Secretagogues do not stimulate secretion of PACE4 or PACE4s

The immunocytochemical and metabolic labelling data suggested that stimulation of secretion from AtT-20 cells might be another useful way of determining the relative storage of PACE4 and PACE4s in secretory granules. AtT-20 cells were pulse-labelled, chased and then incubated with or without secretagogue for 30 min (Figure 5). There was no stimulation of release of mature PACE4 (97 kDa) or PACE4s (66 kDa), whereas the rate of secretion of β -endorphin, β -lipotropin and 63 kDa PC1 was markedly increased in the same samples. The pattern for PACE4 and PACE4s secretion matched the results for the 81 kDa form of PC1, the secretion of which was also not sensitive to PMA. Stimulation of release of the 63 kDa but not the 81 kDa form of PC1 was also seen in previous work [21,37,43,51]. This result indicates that PACE4 and PACE4s are not stored in regulated secretory granules along with PC1 and POMC-derived peptides.

Role of the N-terminal proregion in PACE4 biosynthesis

The proregion of PACE4 is slightly longer than the proregions of other PCs, and the signal sequence of PACE4 is much longer than the signal sequence of other members of this family of endoproteases [13], so it seemed possible that the N-terminal region of PACE4 was instrumental in the retarded processing of PACE4 in AtT-20 cells. Previous work established that the proregions of a number of convertases in the subtilisin-like family must be cleaved to activate the endoprotease and to allow transport out of the endoplasmic reticulum [21,30,44,52–56]. However, when expressed in AtT-20 or hLoVo cells, PC1-pro-PACE4 processing was indistinguishable from that of native PACE4, and the secretion of the resultant mature PACE4 was not improved (not shown).

Biosynthesis of PACE4 in primary pituitary cells

The inefficient processing and secretion of PACE4 in transfected pituitary cells might be caused by the use of a transfected stable cell line, so normal pituitary cells in primary culture were studied (Figure 6). The biosynthesis, storage and secretion of endogenous proPACE4 was compared with the results with PAM, another peptide-biosynthetic enzyme which is also expressed endogenously at high levels in pituitary as both integral membrane PAM-2 (104 kDa) and soluble bifunctional PAM-3 (92 kDa) [57]. The proPACE4 (108 kDa) made in the primary cultures was processed

Figure 7 Western-blot analysis of hEK-293 and CHO culture media

Non-transfected cells (nontrans) and cell lines stably expressing PACE4 or PACE4s were incubated in complete serum-free medium for 16 h and equal aliquots of medium were subjected to Western-blot analysis [23,39].

slowly during the incubation; some of the PACE4 was retained in the cells at the end of the 4 h chase as proPACE4, 108 kDa. However, the majority of the proPACE4 was cleaved in 4 h to the size of mature PACE4 (95 kDa) and was secreted during the chase incubation, mirroring the rapid secretion seen from the transfected fibroblast cell lines. By contrast, the two endogenous PAM proteins were well retained in the pituitary cells during the chase incubation; little secretion of membrane PAM-2 or soluble PAM-3 was observed during the 4 h chase. Both PAM and PACE4 are expressed in several of the pituitary cell types [13,57], so experiments of this type reflect results from a mixture of several cell types. Nonetheless, the comparison is very clear; PAM is stored whereas PACE4 is rapidly secreted during the chase period.

Production of PACE4 from stably transfected cells

Since the AtT-20 cells showed no changes in POMC processing or secretion when PACE4 or PACE4s were expressed (Figures 4 and 5), it was important to determine whether the cDNA constructs used could encode functional enzymes, and to determine their proteolytic properties. For this purpose, stably transfected hEK-293 lines were created, since this line has no endogenous PACE4 and works well for the production and purification of secreted soluble recombinant enzymes [4,28,58]. Conditioned medium from hEK-293-PACE4 cells was used to evaluate PACE4 activity. By Western blot we were unable to detect any PACE4 protein secreted by non-transfected hEK-293 cells whereas an intense band corresponding to PACE4, migrating at approx. 100 kDa, was detected in the media of hEK-293-PACE4 cells (Figure 7). After ion-exchange chromatography, enzymically active fractions were used to examine the inhibitory profile of the enzyme.

Inhibitor profiles for PACE4 and furin are distinct

The sensitivity of PACE4 activity to a variety of inhibitors was evaluated in parallel to the truncated soluble form of human

Table 1 Effects of inhibitors on furin and PACE4 activity

Activity was adjusted by subtracting the activity from similar samples from non-transfected hEK-293 cells, and is given as a percentage of the value without the additive; values are representative of three to seven experiments for each additive. P means PACE4 much more sensitive to inhibition than furin under the same conditions, and F means furin much more sensitive to inhibition than PACE4 under the same conditions. dec-RVKR-cmk, decanoyl-Arg-Val-Lys-Arg-chloromethylketone.

Additive	Concentration	Activity (%)	
		PACE4	Furin
No inhibitor		100	100
BSA	1 mg/ml	112	97
Soya-bean trypsin inhibitor	0.5 mg/ml	98	95
Bestatin	0.1 mg/ml	114	96
Leupeptin	0.1 mM	23	93
	1.0 mM	6	57
Pepstatin	1.0 mM	99	91
PMSF	3 mM	67	72
	10 mM	33	39
E-64	1 mM	84	81
Dithiothreitol	1 mM	61	23
	5 mM	55	1
<i>o</i> -Phenanthroline	1 mM	78	70
ZnCl ₂	1 mM	10	0
	2 mM	0	0
HgCl ₂	1 mM	1	0
CoCl ₂	1 mM	43	62
EDTA	1 mM	70	0
	2 mM	55	1
	10 mM	22	0
EGTA	1 mM	139	0
	2 mM	50	1
	10 mM	29	0
EGTA and MgCl ₂	2 mM each	63	0
EGTA and MnCl ₂	2 mM each	104	80
EGTA and CaCl ₂	2 mM each	124	98
MnCl ₂ (Ca ²⁺ -free)	1 mM	75	20
α_1 -AT wild-type	50 μ g/ml	112	97
α_1 -AT-PDX	2 μ g/ml	109	1
	20 μ g/ml	104	2
dec-RVKR-cmk	10 μ M	0	0

furin, hFUR713t [29,30,59], using the fluorogenic substrate Boc-Arg-Val-Arg-Arg-Mca. All of the data obtained with the soluble form of furin corresponded to the previously reported inhibitor profiles [29] and are included in Table 1. Similarly to furin, PACE4 proteolytic activity was unaffected by BSA, soya-bean trypsin inhibitor, α_1 -antitrypsin (α_1 -AT), bestatin (amino-peptidase inhibitor), E-64 (cysteine protease inhibitor) or pepstatin (aspartyl protease inhibitor). The serine protease inhibitor PMSF and the chelators *o*-phenanthroline and EDTA decreased both furin and PACE4 activity at relatively high concentrations. PACE4 and furin reacted very differently with specific inhibitors, however. Leupeptin (Ac-Leu-Leu-Argininal) produced a much more profound inhibition of PACE4 than of furin. By comparison, furin was strongly inhibited by 5 mM dithiothreitol, 2 mM EDTA and EGTA, and minute concentrations (1 μ g/ml) of the Portland variant of α_1 -AT (α_1 -AT-PDX) [60], whereas all of the PACE4 activity remained. As expected, the chloromethyl ketone derivative, decanoyl-Arg-Val-Lys-Arg-chloromethylketone, as well as the heavy metal ions Zn²⁺ and Hg²⁺, inhibited both PACE4 and furin, whereas Co²⁺ did not inhibit either enzymic activity.

Since furin activity was completely abolished by 2 mM levels of the chelators EDTA and EGTA whereas PACE4 activity was

relatively unaffected, we investigated the Ca^{2+} requirement in more detail. First, levels of these chelators up to 10 mM were used to remove the free Ca^{2+} presumably required for activation of the convertases. The results demonstrated that these high concentrations of EGTA/EDTA failed to abolish PACE4 activity completely. Increased Ca^{2+} reversed the partial inhibition of PACE4 and furin by low levels of EGTA. Unlike furin, MnCl_2 alone maintained most of the activity of PACE4. Reversal of the inhibition of PACE4 was also observed when concentrations of MnCl_2 , MgCl_2 or CaCl_2 equimolar to the chelator were used; this effect was not observed when MgCl_2 was used with furin.

We also examined the inhibitory properties of the furin serpin α_1 -AT-PDX on PACE4 activity in more detail. High concentrations of the native α_1 -AT did not inhibit furin or PACE4 activity, whereas α_1 -AT-PDX at very low levels completely blocked the ability of furin to cleave the substrate. Surprisingly, PACE4 activity remained unaffected when high levels of α_1 -AT-PDX were used.

DISCUSSION

Biosynthesis, intracellular routing and secretion of PACE4

Since PACE4 is encoded on the same chromosome as furin, and since some early reports suggested that PACE4 was not secreted and had a wide tissue distribution, the latter has been regarded as a soluble intracellular version of furin [32,61]. However, a further examination of PACE4 is important for several reasons. First, its tissue distribution indicates that it may have an important role in endocrine and neural function [13,16–18]. Second, it is very strongly regulated by endocrine status [13]. Third, the identity of furin as the only major endoprotease in the constitutive pathway has been questioned [60,62]. Fourth, endoprotease(s) in the constitutive pathway clearly participate in the processing of pro-nerve growth factor, *Aplysia* egg-laying hormone, pro-7B2 and parathyroid hormone [19,33,63–65].

The routing of foreign proteins after transfection into AtT-20 cells has come under intense study. Although many enzymes and prohormones that normally reside in regulated secretory granules arrive in such granules after transfection, including yeast Kex2 [21,23,34,35,37,39,45,66–68], the picture is more complex. Some exocrine enzymes (e.g. trypsin, expressed as trypsinogen or as active trypsin) are targeted efficiently to regulated granules [69], whereas other exocrine proteins (e.g. amylase, GP2) exit via the constitutive route [70]. Other proteins expressed after transfection can adopt an immunocytochemical pattern in the cell very similar to that of secretory granules, as in the case of immunoglobulins, yet not be subjected to regulated secretion [48]. Polarized cells also have unique trafficking patterns, with some soluble proteins being secreted only basolaterally or apically, and other proteins secreted equally at both surfaces [71]. It is not clear how PACE4 and PACE4s elude regulated secretory granules, since such granules are apparently the default pathway in some neuroendocrine cells [72], but the ability to aggregate in the lumen of the secretory pathway is thought to be crucial for secretory granule proteins [72,73].

The biosynthetic processing of proPACE4 to the size expected for mature PACE4 is much slower than the processing of endogenous and exogenous proPC1 in AtT-20 cells, and slower than endogenous proPC1 in primary pituitary cells. The rate of the proregion cleavage of proPACE4 is partially due to the C-terminal Cys-rich domain of PACE4, since PACE4s was processed and secreted more rapidly than full-sized proPACE4 (Figure 2). The low rate of proregion cleavage with proPACE4 was not alleviated by the replacement of the rapidly cleaved proregion of PC1.

Enzymic activity of PACE4

The poor cleavage of proPACE4 indicates that PACE4 is not efficiently activated in neuroendocrine cells, and correlates with the fact that no changes were detected in POMC processing as a result of expression of PACE4 or PACE4s. This is in marked contrast with the striking changes in POMC processing seen when PC1 or PC2 are over- or under-expressed in AtT-20 cells [20,21,35,36,74]. This raised the possibility that PACE4 might need activation by another factor such as 7B2 [75,76], that PACE4 is secreted as a zymogen (like the α -subunit of endopeptidase 24.18) [77,78] or that PACE4 may not have access to the POMC in the cell (as von Willebrand Factor is not a good intracellular substrate for PC1 and PC2, although furin activates von Willebrand Factor well) [79].

Thus we compared the inhibitor profile of furin, the closely related mammalian convertase, with that of PACE4. Using the same substrates and units of activity, the results show that PACE4 is a serine endoprotease like furin; however, PACE4 has a unique pattern of dependence on bivalent cations. Although EGTA/EDTA completely inhibited furin activity, a significant amount of PACE4 activity remained. Further analysis using Mn^{2+} demonstrated the regaining of activity in the presence of the chelators. Mn^{2+} has a higher affinity than Ca^{2+} for EGTA, so Mn^{2+} can liberate Ca^{2+} from EGTA [80]. Thus PACE4 may not require Ca^{2+} to express its full enzymic activity, or perhaps the Ca^{2+} ion is retained more strongly by PACE4 than it is by furin, so that the chelators are unable to displace it from the enzyme.

It has been established that subtilisin BPN', a bacterial counterpart to the mammalian convertases, naturally binds two Ca^{2+} ions [81]; one of the binding sites is the DXXDXDXXXQ motif, which is close to the histidine of the catalytic triad. Interestingly, PACE4 and furin exhibit similar sequences adjacent to the catalytic histidine. For instance, rat furin has the sequence D¹⁷⁴VNDQDPDPQ and rat PACE4 has the sequence D²⁰⁷VNGDYDPSPRYD. Other determinants influencing Ca^{2+} binding could also be involved in this process, since molecular modelling studies have shown close similarities of mammalian convertases to subtilisins and thermolysin [82].

The results with the serpin α_1 -AT-PDX are intriguing. Although α_1 -AT-PDX potently inhibits furin activity, no inhibitory effects were observed with PACE4, even at levels far above those required for inhibition of furin. Variants of α_1 -AT have been recently exploited to examine whether specific inhibition of a single convertase is feasible. For example, the inhibitory properties of the naturally occurring mutant α_1 -AT Pittsburgh (α_1 -AT-PITT), with a single Met $\rightarrow$ Arg alteration, indicated that it was unable to abolish activity of purified furin [29]. When tested in cells, co-expression of α_1 -AT-PITT was unable to abolish furin-mediated processing of pro- β -nerve growth factor or PACE4-mediated cleavage of the von Willebrand precursor [32,60], although α_1 -AT-PITT inhibited furin-mediated processing of pro-von Willebrand factor [32]. Whereas furin cleaves the α -subunit of endopeptidase-24.18, PACE4 cannot [77].

A second replacement within the α_1 -AT-binding site led to the α_1 -AT-PDX version of α_1 -AT and inhibition of furin activity [60]. We confirmed inhibition of furin activity by α_1 -AT-PDX, whereas PACE4 activity was unaffected (Table 1) [83]. However, co-expression of α_1 -AT-PDX with PACE4 was reported to block cleavage of gp160 of HIV-1, as well as cleavage of 7B2 [42]. Apparent disagreement between results for enzyme specificities from co-expression studies and test tube analyses has also been seen for other endoproteases [84]. Clearly, further experiments are needed to ascertain the specificity of inhibition of the α_1 -AT variants.

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