Molecular Biology of the Cell
Vol. 6, 1271–1285, October 1995

Ionic Milieu Controls the Compartment-specific Activation of Pro-Opiomelanocortin Processing in AtT-20 Cells

Walter K. Schmidt* and Hsiao-Ping H. Moore†

University of California at Berkeley, Department of Molecular and Cell Biology, Berkeley, California 94720-3200

Submitted June 22, 1995; Accepted August 8, 1995
Monitoring Editor: Suzanne R. Pfeffer

Newly synthesized prohormones and their processing enzymes transit through the same compartments before being packaged into regulated secretory granules. Despite this coordinated intracellular transport, prohormone processing does not occur until late in the secretory pathway. In the mouse pituitary AtT-20 cell line, conversion of pro-opiomelanocortin (POMC) to mature adrenocorticotropic hormone involves the prohormone convertase PC1. The mechanism by which this proteolytic processing is restricted to late secretory compartments is unknown; PC1 activity could be regulated by compartment-specific activators/inhibitors, or through changes in the ionic milieu that influence its activity. By arresting transport in a semi-intact cell system, we have addressed whether metabolically labeled POMC trapped in early secretory compartments can be induced to undergo conversion if the ionic milieu in these compartments is experimentally manipulated. Prolonged incubation of labeled POMC trapped in the endoplasmic reticulum or Golgi/*trans*-Golgi network did not result in processing, thereby supporting the theory that processing is normally a post-Golgi/*trans*-Golgi network event. However, acidification of these compartments allowed effective processing of POMC to the intermediate and mature forms. The observed processing increased sharply at a pH below 6.0 and required millimolar calcium, regardless of the compartment in which labeled POMC resided. These conditions also resulted in the coordinate conversion of PC1 from the 84/87 kDa into the 74-kDa and 66-kDa forms. We propose that POMC processing is predominantly restricted to acidifying secretory granules, and that a change in pH within these granules is both necessary and sufficient to activate POMC processing.

INTRODUCTION

Peptide hormones that are targeted to dense core granules often traverse early parts of the regulated secretory pathway as inactive precursors. Bioactive peptides are generated from prohormones by proteolytic cleavage at paired basic residues (reviewed in Hutton, 1990; Lindberg, 1991; Steiner and James, 1992; Seidah *et al.*, 1993). In corticotrophs of the anterior pituitary, pro-opiomelanocortin (POMC)[1] is processed to adrenocorticotropic hormone (ACTH) and β-lipotropin (Mains *et al.*, 1977; Mains and Eipper, 1978; Roberts and Herbert, 1977; Roberts *et al.*, 1978). In melanotrophs of the intermediate lobe, the same precursor is cleaved to produce a different set of peptides (α-melanocyte–stimulating hormone, γ-lipo-

* Present address: Johns Hopkins University School of Medicine, Department of Cell Biology and Anatomy, Baltimore, MD 21205.
† Corresponding author.

[1] Abbreviations used: ACTH, adrenocorticotropic hormone; BFA, brefeldin A; EGTA, ethylene glycol-*O,O'*-bis[β-aminoethyl]-*N,N,N',N'*-tetraacetic acid; ER, endoplasmic reticulum; NEM, *N*-ethylmaleimide; PC1, prohormone convertase 1; POMC, pro-opiomelanocortin; SLO, streptolysin-O; TGN, *trans*-Golgi network.

© 1995 by The American Society for Cell Biology

tropin, and β-endorphin) (Gianoulakis *et al.*, 1979; Mains and Eipper, 1979).

Prohormone convertases 1 and 2 (PC1 and PC2) are members of the subtilisin family of mammalian endoproteases involved in prohormone processing (Seidah *et al.*, 1990, 1991; Smeekens and Steiner, 1990; Smeekens *et al.*, 1991). Coexpression studies have shown that PC1 alone can generate the POMC processing pattern of the anterior lobe, whereas PC1 and PC2 are both required to generate the peptide pattern of the intermediate lobe (Benjannet *et al.*, 1991; Thomas *et al.*, 1991; Day *et al.*, 1993; Rhodes *et al.*, 1993; Zhou *et al.*, 1993). PC1 is expressed in both the anterior and intermediate pituitary, whereas PC2 is highly expressed only in the intermediate lobe (Seidah *et al.*, 1991; Day *et al.*, 1992). Thus, the differential expression of PCs may be responsible for generating different peptides from POMC in different tissues.

The prohormone convertases themselves are also synthesized as precursors. ProPC1 has been shown to undergo rapid NH_2-terminal processing within the endoplasmic reticulum (ER), resulting in the removal of signal peptide and prosegment (Vindrola and Lindberg, 1992; Benjannet *et al.*, 1993; Lindberg, 1994; Milgram and Mains, 1994; Vindrola, 1994; Zhou and Mains, 1994). This cleavage occurs by an autocatalytic mechanism and is optimal at pH 7.0–8.0 in vitro (Shennan *et al.*, 1995), the pH range of the ER. The protein then undergoes glycosylation to generate an 84-kDa form and a fully glycosylated 87-kDa form (Benjannet *et al.*, 1993). Later in the pathway, further processing of the COOH-terminal segment converts the enzyme to a 74-kDa form and a 66-kDa form. The 66-kDa enzyme is stored in dense core granules and can undergo regulated secretion along with ACTH (Vindrola and Lindberg, 1992).

Even though both POMC and PC1 are transported through the same secretory pathway, processing of POMC appears to be restricted to late secretory compartments (Gumbiner and Kelly, 1981, 1982; Mains *et al.*, 1987; Tooze *et al.*, 1987; Schnabel *et al.*, 1989; Milgram and Mains, 1994). This raises an important question, i.e., why is POMC processing not observed in the ER or the Golgi? The possibility that PC1 precursors in the ER and the Golgi are enzymatically inactive but are activated upon further processing can be ruled out because both the early forms (84/87 kDa) and late forms (74/66 kDa) of PC1 exhibit enzymatic activities in vitro (Jean *et al.*, 1993; Rufaut *et al.*, 1993; Zhou and Lindberg, 1993; Friedman *et al.*, 1994; Zhou and Lindberg, 1994). Two alternative mechanisms, not mutually exclusive, can also explain restricted processing. In one mechanism, processing is regulated by inhibitors or activators that are functional only in a subset of secretory compartments. Recent evidence suggests that this may in fact be the mechanism involved in PC2 activation; the 7B2 protein appears to inhibit newly synthesized PC2 by transient association (Braks and Martens, 1994; Martens *et al.*, 1994). However, 7B2 does not interact with PC1 (Martens *et al.*, 1994), and to our knowledge, protein factors that influence PC1 activity have not been found. In an alternate "ionic milieu" mechanism, activation of processing is effected through changes in the lumenal ionic environment along the secretory path; the converting enzymes would first transit through compartments with lumenal environments that are nonpermissive for processing (ER and Golgi), and then through distal secretory compartments where the ionic conditions are permissive (*trans*-Golgi network [TGN] or immature secretory granules). This mechanism is based on the finding that PC1 purified from transfected cells requires acidic conditions and millimolar calcium for activity (Jean *et al.*, 1993; Rufaut *et al.*, 1993; Zhou and Lindberg, 1993). These conditions are also those thought to exist in distal secretory compartments (Anderson and Pathak, 1985; Mellman *et al.*, 1986; Orci *et al.*, 1986, 1994; Roos, 1988; Chandra *et al.*, 1991).

In this study, we have addressed the regulation of POMC processing in the anterior pituitary corticotroph-derived AtT-20 cell line. The ionic milieu model predicts that processing should take place in the ER or the Golgi if the ionic conditions within these compartments are manipulated to mimic those in distal compartments of the secretory pathway. Using secretion inhibitors or cell permeabilization, metabolically labeled POMC was trapped in various pre-processing secretory compartments, and the ionic conditions within these compartments were manipulated. This system has allowed us to address whether an ionic component plays a key role in the activation of POMC processing.

MATERIALS AND METHODS

Antisera and Reagents

Affinity-purified rabbit anti-porcine ACTH was prepared as previously described (Moore and Kelly, 1985). Anti-PC1 antiserum was a generous gift of Dr. Lindberg (Louisiana State University Medical Center, New Orleans, LA). Streptolysin-O (SLO) was purchased from Burroughs-Wellcome (Research Triangle Park, NC) or the Institute for Medical Microbiology and Hygiene, University of Mainz, Germany (Bhakdi *et al.*, 1993).

Cell Culture and Radiolabeling

AtT-20 cells were routinely maintained in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum under 15% CO_2 at 37°C. For radiolabeling experiments, cells were seeded at a density of 2×10^5 cells/well onto a 12-well dish (Corning, Oneonta, NY) and grown for 36–48 h. The cells were preincubated for 30 min at 37°C in buffer A (110 mM NaCl, 5.4 mM KCl, 0.9 mM Na_2HPO_4, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1 g/l glucose, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.2) or methionine-free DMEM for sulfate or methionine labeling, respectively. The cells were labeled with 2.5 mCi/ml $^{35}SO_4$ (ICN, Costa Mesa, CA) in buffer A or 0.5 mCi/ml tran^{35}S label (ICN) in

methionine-free DMEM at 37°C for the indicated times. For *N*-ethylmaleimide (NEM) or bafilomycin A_1 treatment, labeled cells were chilled to 0°C and pretreated for 10–15 min on ice with either 1 mM NEM (Sigma, St. Louis, MO) or 20 μM bafilomycin A_1 (LC Laboratories, Woburn, MA), respectively; the NEM-treated cells were incubated on ice for an additional 15 min with 2 mM dithiothreitol (DTT; Sigma). The cells were then chased for the appropriate periods in DMEM before collection of media when required (Figure 2). Cells were extracted with NDET (1% NP40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris, pH 7.4). When $CaCl_2$ manipulation was required (Figure 4, A and B), a 30-min preincubation at 37°C with calcium-free DMEM containing 5 μg/ml brefeldin A (BFA; Boehringer Mannheim, Indianapolis, IN), 10 μM A23187 (Boehringer Mannheim), and 5 μM nigericin (Sigma) preceded the chases to arrest transport and deplete internal calcium stores.

Preparation of Buffered Media and $CaCl_2$/EGTA Buffers

Buffered DMEM (Figure 3) was prepared with the addition of 25 mM HEPES (pH 7.0) or 25 mM MES (pH 5.0–6.4) to $NaHCO_3$-free DMEM (University of California, San Francisco, Cell Culture Facility, San Francisco, CA). Buffered DMEM with or without calcium was prepared by the addition of 10 mM $CaCl_2$ or 5 mM EGTA (Fluka, Ronkonkoma, NY), respectively, to $NaHCO_3$/calcium-free DMEM that had been buffered with HEPES or MES to the appropriate pH (Figure 4A). When calcium buffering was required (Figure 4B), the solutions were prepared by the addition of the appropriate amount of a Ca^{2+}/EGTA buffering system (K_2CaEGTA, magnesium acetate, and EGTA) to buffer B (25 mM glucose, 130 mM NaCl, 5 mM KCl, 25 mM MES, or HEPES, pH 5.0–7.0) to a final concentration of 5 mM free Mg^{2+} and 5 mM total EGTA. The amounts of K_2CaEGTA, Mg^{2+}, and EGTA needed to yield the desired free concentrations of Ca^{2+} were calculated using algorithms derived by Martell and Smith (1974) after correcting the stability constants of the EGTA complexes for temperature and pH.

Streptolysin-O Permeabilization

Cells were cultured and radiolabeled as described above, chilled to 0°C, and washed twice with 0.5 ml of cold buffer C (125 mM sodium glutamate, 15 mM NaCl, 5 mM magnesium acetate, 5 mM EGTA, 20 mM HEPES-NaOH, pH 7.2). The cells were incubated with 200 μl of cold buffer C containing 10 mM DTT and 2 IU/ml SLO (Burroughs-Wellcome) or 1,000 U/ml SLO (Bhakdi *et al.* 1985) for 5–10 min on ice. The permeabilization buffer was removed, and the cells were incubated in 250 μl warmed buffer C at 37°C for 2–3 min to induce pore formation. Permeabilized cells were incubated in 0.5 ml of buffer D (125 mM K glutamate, 15 mM KCl, 6 mM NaCl, 5 mM magnesium acetate, 25 mM MES-KOH, or HEPES buffered to the desired pH) containing an ATP-depleting system (30 IU/ml hexokinase and 2 mM glucose) to arrest transport. To manipulate the intra-lumenal pH and calcium, 5 μM nigericin and 10 μM A23187 were included. In some experiments, $CaCl_2$ was added to a final concentration of 10 mM. When calcium chelation was required (Figure 7), 5 mM EGTA was substituted for the $CaCl_2$. In the case of the pulse control for Figure 7, the radiolabeled cells were extracted immediately after a mock permeabilization. Otherwise, the cells were extracted with NDET buffer at the end of the chase periods. All samples were processed for immunoprecipitation.

Immunoprecipitation and Immunoblotting

Radiolabeled samples were immunoprecipitated using affinity-purified anti-ACTH (Moore and Kelly, 1985) or anti-PC1 antiserum (Vindrola and Lindberg, 1992) as previously described. The resultant immunoprecipitates were subjected to reducing SDS-PAGE and exposed for 48–72 h onto a PhosphorImager cassette (Molecular Dynamics, Sunnyvale, CA) for analysis. To determine the effect of secretagogue on PC1 release, a 10-cm dish of cells was incubated in DMEM for 3 h to collect basal unstimulated secretion. The same cells were then incubated for 3 h in DMEM containing 5 mM 8-Br-cAMP (Sigma) to recover a secretagogue-stimulated releasate. The media were cleared of cell debris by centrifugation (300 × *g*, 5 min) and the secreted proteins were precipitated with trichloroacetic acid. The precipitates were resuspended in sample buffer (8 M urea, 5% β-ME, 0.1% SDS, and 125 mM Tris, pH 6.8), subjected to 8.8% SDS-PAGE, and then transferred onto nitrocellulose (Schleicher & Schuell, Keene, NH) for 2 h at 350 mA in 10 mM sodium tetraborate (Sigma). The nitrocellulose was probed for PC1 immunoreactivity as previously described (Vindrola and Lindberg, 1992); immunoreactive PC1 was detected by the chemiluminescence method (ECL, Amersham, Arlington Heights, IL).

RESULTS

POMC Processing Occurs in a Post-Sulfation Compartment

In AtT-20 cells, POMC is sulfated on both N-linked oligosaccharides and tyrosine residues (Moore *et al.*, 1983b; Bateman *et al.*, 1990); these sulfation events occur predominantly in the *trans*-Golgi or TGN (Spiro *et al.*, 1991; Niehrs *et al.*, 1994). As shown in Figure 1,

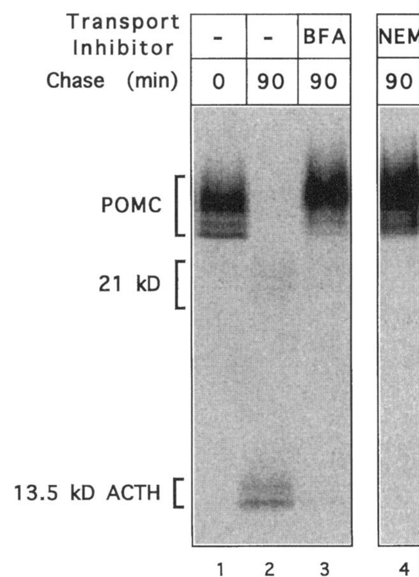

Figure 1. Proteolytic processing of sulfated POMC is blocked by transport inhibitors. AtT-20 cells were radiolabeled for 5 min with 2.5 mCi/ml $^{35}SO_4^{2-}$ in buffer A. The radiolabel was removed, and a sample well was extracted with NDET detergent buffer (lane 1, 0 min time point). The cells were then chased for 90 min in DMEM with or without BFA or NEM treatment. For BFA treatment, the cells were chased in DMEM containing 5 μg/ml BFA. For NEM treatment, the cells were first chilled to 0°C then incubated successively in DMEM containing 1 mM NEM and then 2 mM DTT. The cells were then chased in DMEM at 37°C. At the end of the chase periods, the cells were extracted and then immunoprecipitated with an anti-ACTH antibody. Immunoprecipitates were analyzed by 18% SDS-PAGE and a PhosphorImager. Proteolytic conversion of POMC into the mature 13.5-kDa ACTH is evident after chase without inhibitors (lane 2). BFA (lane 3) or NEM (lane 4) blocks this conversion.

POMC can be pulse labeled during a 5-min incubation with $^{35}SO_4^{2-}$. Only unprocessed POMC was recovered after the label (Figure 1, lane 1). When the cells were chased for 90 min, sulfate-labeled POMC was converted into several labeled fragments, two of which–the 21-kDa and 13.5-kDa forms–can be immunoprecipitated with an anti-ACTH antiserum (Figure 1, lane 2); the 21-kDa intermediate results from an initial cleavage at the C-terminal side of ACTH, and the 13.5-kDa form represents the glycosylated mature ACTH after further cleavage of the 21-kDa intermediate. The 21-kDa form does not accumulate to a large extent during the chase, indicating that the second cleavage occurs soon after the first cleavage. The reduced band intensity of mature glycosylated ACTH as compared with POMC is partly due to inefficient storage of POMC-derived peptides in AtT-20 cells (Moore *et al.*, 1983b); a significant fraction of labeled POMC-derived peptides was released during the chase (see Figure 2). In addition, processing results in the generation of multiple sulfated fragments of which only the ACTH-containing fragments are recognized by the immunoprecipitating antibody. Thus the immunoprecipitated materials represent only a portion of the total radiolabel in POMC.

Because sulfation of POMC precedes proteolytic processing, we next addressed whether these two events occur in different compartments by testing if they could be uncoupled by transport inhibitors. BFA has been shown to block transport between *trans*-Golgi and TGN and also between the TGN and secretory granules (Miller *et al.*, 1992; Rosa *et al.*, 1992; Carnell and Moore, 1994; Huang and Arvan, 1994). NEM is also a potent inhibitor of protein export from the Golgi and TGN (Salamero *et al.*, 1990; Miller and Moore, unpublished observation). If sulfation occurs in a compartment that is distinct and located before the processing compartment, then inhibition of transport with BFA or NEM should inhibit processing of sulfate-labeled POMC. This is indeed the case; the conversion of sulfate-labeled POMC into mature ACTH was inhibited by BFA (Figure 1, lane 3) or NEM (Figure 1, lane 4). The secretion of POMC and POMC-derived peptides was also completely blocked (our unpublished results). The effect of BFA is not due to direct inhibition of the processing enzymes, because no inhibition was observed if labeled POMC was first chased out of the sulfation compartment before drug treatment (Fernandez and Moore, unpublished observation). Thus, sulfated POMC can be effectively trapped in the *trans*-Golgi/TGN, and processing normally occurs after exit from this compartment. It should be noted that because POMC sulfation has not been directly localized to the TGN, it is as yet unclear whether sulfation and processing of POMC strictly take place in two consecutive compartments, or some intermediates are involved.

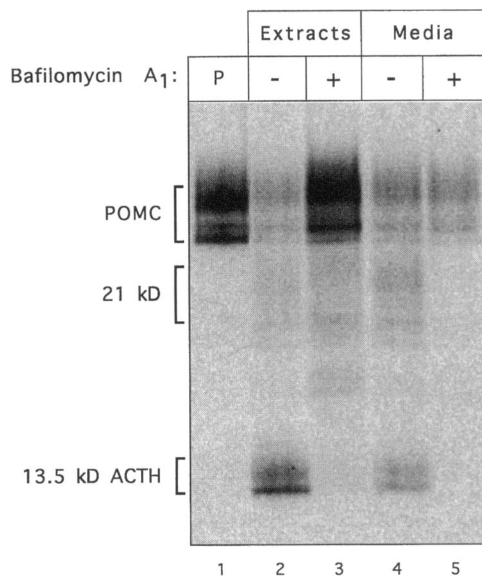

Figure 2. Treatment with bafilomycin A_1 inhibits the proteolytic processing of sulfated POMC. AtT-20 cells were radiolabeled for 5 min with 2.5 mCi/ml $^{35}SO_4^{2-}$ in buffer A. The radiolabel was removed, and a sample well was extracted with NDET buffer at the 0 min time point (P). The cells were then chased for 90 min in DMEM with (+) or without (−) 20 μM bafilomycin A_1. At the end of the chase period, the media and cell extracts were collected. Immunoprecipitates of the media and cell extracts with anti-ACTH were analyzed by 18% SDS-PAGE and a PhosphorImager.

POMC Processing Requires an Acidic Environment

Although PC1 and PC2 are both expressed in AtT-20 cells, POMC processing is likely to be PC1-mediated because PC1 expression is much higher than PC2 (Seidah *et al.*, 1990; Zhou and Mains, 1994), and the initial steps of POMC processing can be accelerated by overexpression of PC1 (Zhou and Mains, 1994) and inhibited by anti-sense PC1 (Bloomquist *et al.*, 1991). In vitro studies have shown that partially purified PC1 requires acidic conditions for optimal activity (Jean *et al.*, 1993; Rufaut *et al.*, 1993; Zhou and Lindberg, 1993). We therefore sought to determine whether POMC processing in vivo also required acidic lumenal conditions. Bafilomycin A_1, a specific inhibitor of the vacuolar H^+-ATPase, has been shown to inhibit PC2-mediated processing (Xu and Shields, 1994). This agent was used to examine effects on POMC processing (Figure 2). As before, $^{35}SO_4^{2-}$ pulse-labeled POMC (Figure 2, lane 1) underwent processing events during the 90-min chase resulting in the production of 21-kDa and 13.5-kDa forms (Figure 2, lane 2). Some of the precursor and processed forms were released into the medium (Figure 2, lane 4), indicating that storage by the regulated pathway was not efficient in these cells. In the cell extracts, mature ACTH represented the

major recovered form (Figure 2, lane 2) whereas POMC, the 21-kDa intermediate, and ACTH were recovered from the media (Figure 2, lane 4). When bafilomycin A_1 was added during the chase period, the extent of processing was drastically reduced and the majority of ACTH immunoreactivities in the cells was unprocessed POMC (Figure 2, lane 3). Unlike BFA or NEM, which block TGN export and abolish POMC secretion, the effect of bafilomycin cannot be simply explained by a general inhibition of protein transport. Under bafilomycin treatment, some labeled POMC continued to be exported from the TGN and secreted into the medium (Figure 2, lane 5), although the amount appeared to be reduced as compared with the control. These results are summarized in Table 1. They support the hypothesis that acidification is necessary for efficient POMC processing, and that bafilomycin inhibits this acidification leading to reduced conversion and secretion of only unprocessed precursors.

Acidification of the Sulfation Compartment Can Induce POMC Processing

POMC is not processed in the sulfation compartment even after prolonged periods (Figure 1). This may reflect the absence of processing enzymes, the presence of endoprotease inhibitors, or an inappropriate ionic environment (e.g., pH or calcium) within this compartment. Because PC1 is secreted along with ACTH, it must therefore transit through both the *trans*-Golgi and TGN. It also acquires terminal carbohydrate modifications characteristic of the late Golgi (Benjannet *et al.*, 1993). The lack of processing is therefore not likely to be due to the absence of PC1 in the sulfation compartment. To address whether the ionic environment within this compartment is inappropriate for processing, we have therefore addressed what effect lowering lumenal pH would have on the processing of POMC trapped in the *trans*-Golgi/TGN.

Because cellular membranes are leaky to protons, the cytoplasmic pH within the cell can be manipulated by simply lowering the extracellular pH (Aubert and Motais, 1974; L'Allemain *et al.*, 1984; Davoust *et al.*, 1987; Heuser, 1989). We infer that internal compartments such as the sulfation compartment may also be acidified by a similar mechanism. We therefore tested the effects of lowering the pH of extracellular medium on POMC trapped in the sulfation compartment (Figure 3). Cells were labeled with $^{35}SO_4^{2-}$ for 5 min and treated with BFA to prevent further transport. When labeled cells were chased in medium buffered at pH 7.0 (Figure 3, lane 2) or pH 6.0 (Figure 3, lane 3) for 90 min, processed forms of POMC were not observed. Conversion of POMC into the 13.5-kDa form, however, was observed when the extracellular pH was adjusted to pH 5.0 (Figure 3, lane 4). Similar results were obtained when using NEM to arrest protein transport (Figure 3, lane 5). Compared with untreated control chased in normal medium (Figure 3, lane 1), the conversion induced by low pH in BFA or NEM-treated cells (Figure 3, lanes 4 and 5) was not as efficient. Although direct lumenal pH measurements were not performed in this study, we surmise that the lower efficiency may be attributable to intraorganelle acidity failing to equilibrate with the extracellular pH in the absence of ionophores and plasma membrane permeabilization (see below, Figure 5A). Nonetheless, these results suggest that a POMC endoprotease is present and activatable by acidic conditions within the sulfation compartment.

Calcium Is Also Required for the Activation of POMC Processing

A possible interpretation of the above results is that the observed processing at acidic pH may have been due to the action of lysosomal proteases that also transit through the secretory pathway and are activated at acidic pH (Kornfeld and Mellman, 1989; Kornfeld, 1992). Lysosomal-type activities can be distinguished from prohormone convertase activities by different calcium requirements; convertases require calcium for their activity whereas lysosomal acid hydrolases, such as the cathepsins, are calcium independent (Kress *et al.*, 1966; Barrett, 1967–1972). Therefore, we addressed how manipulations of lumenal calcium affected the observed processing of POMC in the sulfation compartment.

Table 1. Quantitation of ACTH-containing proteins in bafilomycin A_1-treated cells

	Control extract		Bafilomycin extract	Control	Bafilomycin
	pulse only	with chase	with chase	medium	medium
POMC	549,546	103,574	608,271	95,707	83,305
21 kDa intermediate	0	34,819	60,316	52,405	7,383
13.5 kDa mature	0	130,378	5,522	33,491	0

The bands corresponding to POMC, 21-kDa intermediate, and 13.5-kDa mature ACTH in Figure 2 were quantitated with the ImageQuant Program (Molecular Dynamics). The counts for each band represent arbitrary scanning units that have been corrected for background.

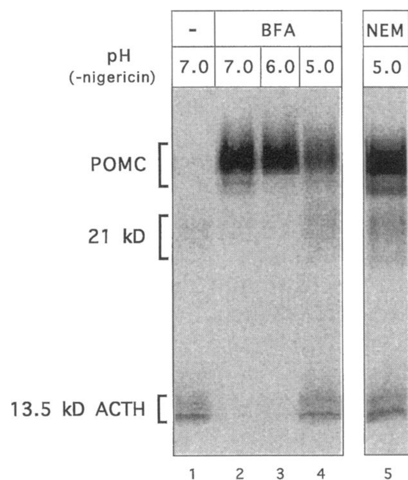

Figure 3. Induction of POMC processing in the sulfation compartment by acidification. AtT-20 cells were radiolabeled with sulfate for 5 min as described in Figure 1. The cells were then incubated for 90 min in media containing BFA to trap labeled POMC in the sulfation compartment. The chase media were adjusted to pH 7.0, 6.0, or 5.0 to acidify the cytosolic and membrane compartments. In some cases, transport was blocked by treatment with NEM instead of BFA as described in the legend to Figure 1. At the end of the chase periods, the cells from each condition were extracted, immunoprecipitated with anti-ACTH, and analyzed as described in Figure 1.

The calcium levels were manipulated using a calcium/EGTA buffering system in conjunction with the ionophores A23187 and nigericin (Martell and Smith, 1974). A23187 has previously been shown to effectively deplete lumenal stores of calcium, so cells were pretreated with this ionophore to remove excess calcium before the addition of the appropriate incubation buffer (West, 1981; Booth and Koch, 1989; Lodish and Kong, 1990; Chandra *et al.*, 1991; Fasolato *et al.*, 1991; Oda, 1992; Carnell and Moore, 1994). Because A23187 is a Ca^{2+}/H^{+} exchanger, the ionophore nigericin was also included to allow the free movement of H^{+} across membranes. In the presence of nigericin alone, the processing of sulfated POMC in BFA-treated cells was efficient at pH 5.5 (our unpublished results); therefore this pH was selected for the incubation buffers. The calcium concentration was fixed at 10 mM because PC1 processing activity in vitro is most efficient at this concentration (Benjannet *et al.*, 1993; Zhou and Lindberg, 1993, 1994). As shown in Figure 4A, POMC processing was not observed in the absence (0 mM, Figure 4A, lane 1) or presence (10 mM, Figure 4A, lane 2) of calcium under neutral pH. The same was true when the cells were incubated at pH 6.5 (our unpublished results). Under more acidic incubation conditions (pH 5.5), processing occurred in the presence of calcium (Figure 4A, lane 4) but not in its absence (Figure 4A, lane 3). Note that two bands just below POMC were recovered under the latter condition; the nature of these bands is unknown but they may represent differently modified forms of POMC. Thus, the observed POMC processing is calcium dependent and is unlikely to be due to the activation of lysosomal acid proteases.

We next determined the range of calcium concentrations required to sustain processing activity. Sulfate-labeled cells were first depleted of lumenal calcium stores by pretreatment with A23187 and EGTA. The cells were then incubated at pH 5.5 in the presence of ionophores and a calcium-buffering system (Miller and Moore, 1991; Carnell and Moore, 1994). POMC processing was not evident when the calcium was buffered at 10 μM or below (Figure 4B, lanes 1 and 2). A small amount of intermediate, however, was formed at 10 μM levels of calcium (Figure 4B, lane 2). Limited processing, as measured by the formation of 13.5-kDa mature ACTH, was restored when the calcium was buffered at 100 μM (Figure 4B, lane 3). Even more processing was evident at 1 mM free calcium levels (Figure 4B, lane 4). These results suggest that POMC processing induced by acidic conditions requires at least 100 μM free calcium, but is optimal at millimolar free calcium. The optimal calcium concentration for PC1 activity in vitro is also in the millimolar range (Jean *et al.*, 1993; Rufaut *et al.*, 1993; Zhou and Lindberg, 1993).

Induction of POMC Processing in the Sulfation Compartment in Semi-intact Cells

One potential problem with using transport inhibitors such as BFA is that these drugs may induce unwanted membrane fusion events (Wood *et al.*, 1991). To circumvent this problem, we turned to a permeabilized cell system employing the cytolysin SLO (Miller and Moore, 1991; Carnell and Moore, 1994). Radiolabeled cells are incubated with SLO to produce 15- to 20-nm plasma membrane pores. These pores were sufficiently large to allow exchange of macromolecules up to 200 kDa but were small enough to avoid the diffusion of transport vesicles (80–200 nm in diameter) (Bhakdi *et al.*, 1985; Ahnert-Hilger *et al.*, 1989). The permeabilized cells can then be washed to remove cytosol and incubated with an ATP-depleting system. Under these conditions, vesicular transport is effectively inhibited but can be restored upon the addition of ATP and cytosol (Miller and Moore, 1991). Permeabilized cells thus provide a well-defined system to arrest vesicular transport; this system also enhances the accessibility of ionophores and buffers for the manipulation of intraorganelle ionic conditions. In these experiments, we included nigericin and A23187 in the incubation buffers to equilibrate the lumenal pH and Ca^{2+}.

This permeabilized system was used to study the pH dependence for the activation of POMC processing within the sulfation compartment (Figure 5A). The

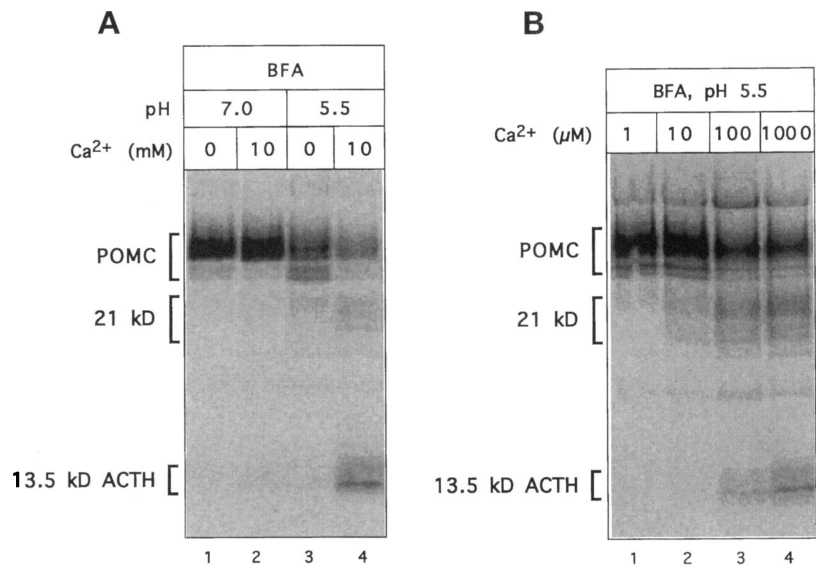

Figure 4. Calcium is required but is not sufficient for the activation of POMC processing in the *trans*-Golgi/TGN. (A) Low pH-induced POMC processing occurs only in the presence of calcium. AtT-20 cells were radiolabeled with sulfate as described in Figure 1. The cells were then incubated for 90 min in the presence of BFA, 10 μM A23187, and 5 μM nigericin in pH 7.0 or pH 5.5 DMEM with or without $CaCl_2$. At the end of the incubation period, the cells from each condition were extracted, immunoprecipitated, and analyzed as described in Figure 1. (B) Titration of the calcium requirement for efficient processing of POMC. Cells were radiolabeled as above and then depleted of internal calcium by incubating the cells for 30 min at 37°C in calcium free DMEM (pH 7.0) containing BFA, A23187, nigericin, and 10 mM EGTA. The calcium-depleted cells were washed once with PBS lacking calcium, then incubated in calcium-buffered media containing BFA, A23187, and nigericin at pH 5.5. The free calcium was buffered to the indicated values with a Ca^{2+}/EGTA buffering system (Martell and Smith, 1974). After a 90-min incubation, the cells from each condition were extracted, immunoprecipitated, and analyzed.

cells were pulse-labeled for 5 min with $^{35}SO_4^{2-}$ before their permeabilization with SLO. The permeabilized cells were then incubated in the absence of cytosol and in the presence of an ATP-depleting system to arrest transport. The labeled POMC was trapped in the sulfation compartment and no processing was observed when chased for 90 min at neutral pH (Figure 5A, lane 1). The effects of incubation with ionophores at various pH and 10 mM $CaCl_2$ were then examined. No processing was evident after a 90-min incubation under mildly acidic (pH 6.4 and pH 6.0, Figure 5A, lanes 2 and 3) conditions. POMC processing was triggered at pH 5.5 and 5.0 (Figure 5A, lanes 4 and 5), consistent with experiments using intact cells and transport inhibitors (see Figure 3). The published in vitro pH optimum of the 74/66-kDa PC1 is pH 5.5 (Zhou and Lindberg, 1994).

Comparison of the pH Dependence of POMC Processing in the Sulfation Compartment and the Immature Secretory Granules

As was shown in Figure 1, the majority of POMC processing normally occurs after its export from the sulfation compartment. Presumably by lowering the pH within the sulfation compartment, we activated PC1 prematurely. This induced activity should therefore have resembled the PC1 activity in the processing compartment, unless other factors were involved. We therefore compared the pH dependence of POMC processing in the sulfation compartment to that within maturing secretory granules. We took advantage of the fact that sulfate pulse-labeled POMC can be chased within 15 min into immature secretory granules, a compartment in which processing is no longer sensitive to BFA (Fernandez and Moore, unpublished observation). For the experiment presented in Figure 5B, sulfate-labeled cells were chased for 15 min to allow entry of POMC into maturing secretory granules (Tooze and Huttner, 1990; Tooze *et al.*, 1991; Grimes and Kelly, 1992; Xu and Shields, 1994). The cells were then permeabilized with SLO and incubated under the ionic conditions described in Figure 5A. Upon incubation at pH 6.4 (Figure 5B, lane 2) or pH 6.0 (Figure 5B, lane 3), the extent of POMC processing increased slightly from that observed at neutral pH (Figure 5B, lane 1). There was considerable processing under neutral conditions; this processing occurred primarily during the 15-min chase and permeabilization procedure and should be considered as the background processing level. Processing over background levels, however, was most evident at pH 5.5 (Figure 5B, lane 4) and pH 5.0 (Figure 5B, lane 5); the generation of the 13.5-kDa ACTH peptide was also accompanied by a corresponding decrease in the amount of the POMC precursor. Quantitation of the pH dependence of processing in the *trans*-Golgi/TGN (Figure 5A) and in immature granules (Figure 5B) is shown in Figure 5C. The amount of mature 13.5-kDa ACTH increased sharply between pH 6.0 and 5.5, and this occurred regardless of whether POMC was compartmentalized in either the sulfation compartment (*trans*-Golgi/TGN) (solid line) or immature secretory granules (dotted line). We did notice, however, that the relative activity at pH 6.0–6.5 was higher in the immature granules than in the sulfation compartment. The significance of this difference is unknown, but it may be due to a higher concentration of POMC and/or PC1 in the secretory granules.

Acidic Lumenal Conditions Can Also Activate POMC Processing in the ER

To determine whether low pH can also activate POMC processing in an early secretory compartment, we examined POMC in the ER after radiolabeling cells for 10 min with [^{35}S]methionine. The radiolabeled cells were permeabilized with SLO, further transport was prevented by depleting ATP and cytosol, and the cells were then incubated under increasingly acidic conditions in the presence of ionophores and excess (10 mM) calcium as described in Figure 5. As shown in Figure 6A, little if any POMC processing was observed under neutral (pH 7.0, Figure 6A, lane 1) or mildly acidic (pH 6.4 and 6.0, Figure 6A, lanes 2 and 3) conditions. Processing was clearly evident at pH 5.5 (Figure 6A, lane 4) and pH 5.0 (Figure 6A, lane 5). The processing pattern of [^{35}S]methionine-labeled POMC was different from that of sulfate-labeled POMC. The added complexity of the processed forms results from the labeling of a nonglycosylated form of ACTH (4.5 kDa), which was not detected by $^{35}SO_4^{2-}$ labeling. Thus, mature ACTH recovered from sulfate-labeled cells is the 13.5-kDa form, whereas in methionine-labeled cells the 4.5-kDa form predominates. A quantitation of the total 4.5-kDa ACTH recovered under these incubation conditions is shown in Figure 6B. Again, the recovery of this processed form of ACTH was optimal at pH ≤ 5.5, conditions that were also optimal for the recovery of mature ACTH from both the sulfation compartment and immature granules (compare with Figure 5C). This processing was also calcium dependent (our unpublished results), consistent with a PC1-mediated instead of a lysosomal hydrolase–mediated event. Thus, POMC processing can also be prematurely activated in the ER.

Processing of PC1 Is Correlated with Activation of POMC Processing

Previous studies have revealed that there are several forms of PC1 derived from a single precursor. By

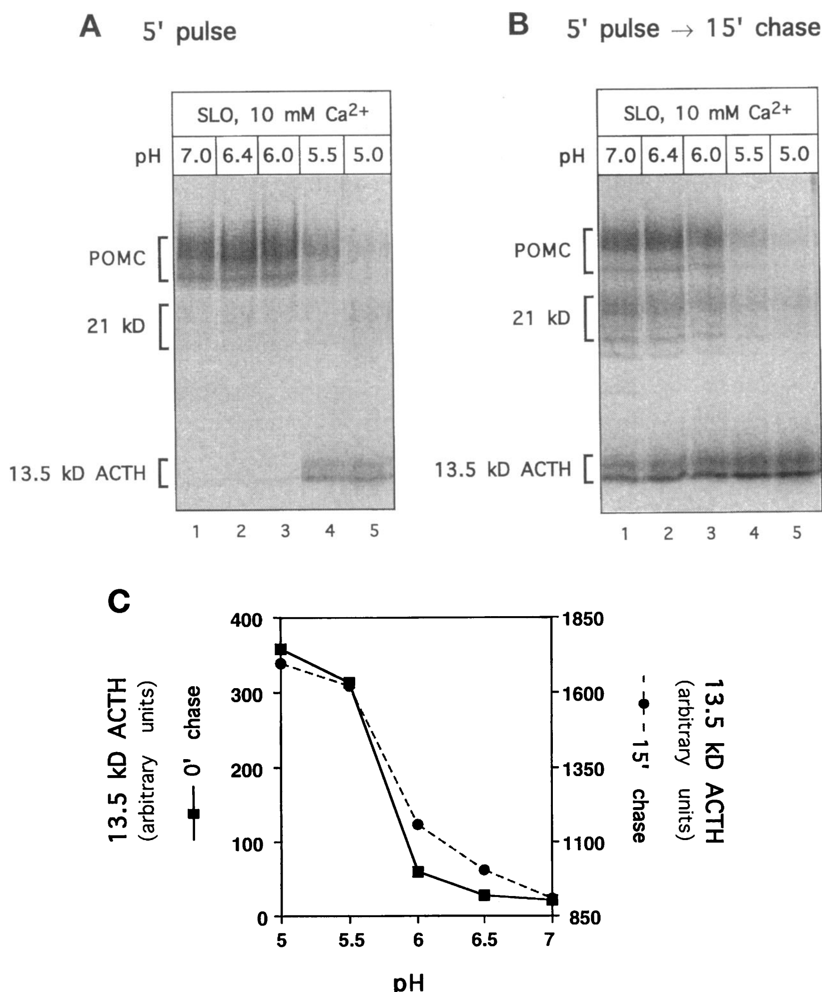

Figure 5. A comparison of the production of POMC in the *trans*-Golgi/TGN and maturing secretory granules of semi-intact AtT-20 cells. (A) pH dependence of processing within the sulfation compartment. AtT-20 cells were pulse labeled with sulfate for 5 min as described in Figure 1. The cells were then washed and permeabilized with SLO. Cells were then incubated in transport buffer D containing an ATP-depleting system in the absence of cytosol to arrest labeled POMC in the sulfation compartment. The buffer was then adjusted to the indicated pH and A23187, nigericin, and 10 mM $CaCl_2$ were added. After a 90-min incubation period at 37°C, the cell extracts were collected. All samples were then processed for immunoprecipitation, SDS-PAGE, and PhosphorImager analysis. (B) pH-dependent processing within maturing secretory granules. Cells were treated as above except that pulse-labeled cells were first chased in buffer A for 15 min at 37°C to allow packaging of labeled POMC into immature granules before permeabilization. (C) The total ACTH peptide (13.5 kDa) immunoprecipitated from each condition was quantitated and is expressed in arbitrary scanning units. The results from panel A are shown as closed squares and the results from panel B are shown as closed circles.

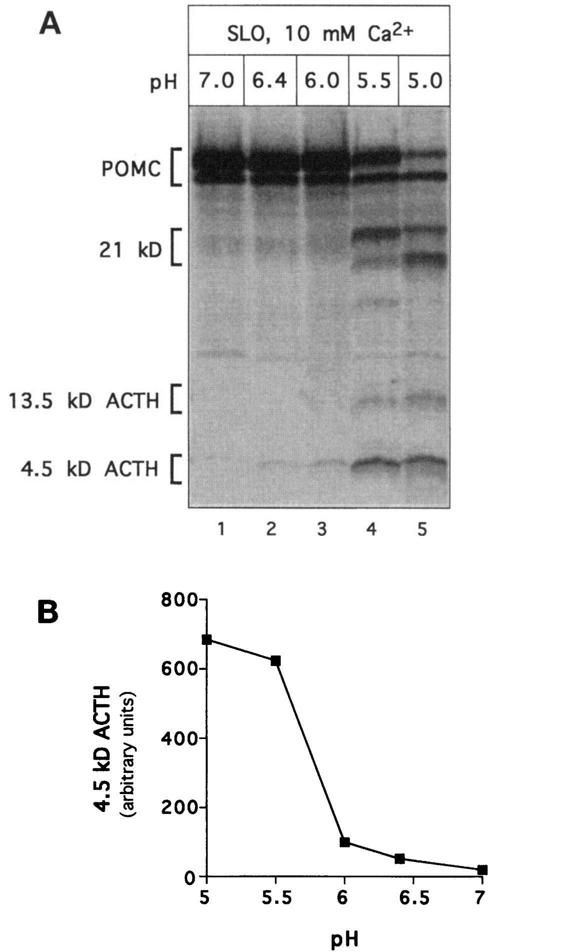

Figure 6. The pH dependence of POMC processing in the ER of semi-intact AtT-20 cells. (A) POMC processing can be activated by low pH in the ER. AtT-20 cells were radiolabeled for 10 min with 0.5 mCi/ml tran^{35}S-label. The cells were then permeabilized with SLO after which the cells representing the 0 min time point (P) were extracted. Transport buffer D containing an ATP-depleting system, A23187, and nigericin was added to the remaining cells. The pH of the buffers was adjusted to the indicated values and the calcium was kept at 10 mM. After a 90-min incubation period, the cell extracts were collected. At the end of the incubation period, the cell extracts were immunoprecipitated for ACTH-containing peptides, subjected to 18% SDS-PAGE, and analyzed by a PhosphorImager. (B) The total ACTH peptide (4.5 kDa) immunoprecipitated from each condition was quantitated and is expressed in arbitrary scanning units.

Western analysis, the major forms of PC1 secreted basally from unstimulated AtT-20 cells during a 3-h incubation were the 87-kDa and 66-kDa forms; no 74-kDa PC1 was detected under these conditions, possibly due to the low cellular level of this form at steady state (Figure 7A, lane 1). The antibody used in this analysis has been previously shown to recognize all forms of PC1 so the absence of the 74-kDa PC1 is not due to a lack of antibody reactivity (Vindrola and Lindberg, 1992). When AtT-20 cells were treated with the secretagogue 8-Br-cAMP, the release of 66-kDa PC1 was increased; we also observed the appearance of a minor band with a relative mobility consistent with that of 74-kDa PC1 (Figure 7A, lane 2). The release of 87-kDa PC1, however, was not stimulated by the secretagogue. This data, which is in agreement with previously published results (Vindrola and Lindberg, 1992), suggests that a portion of the PC1 precursor is secreted constitutively as the 87-kDa form while

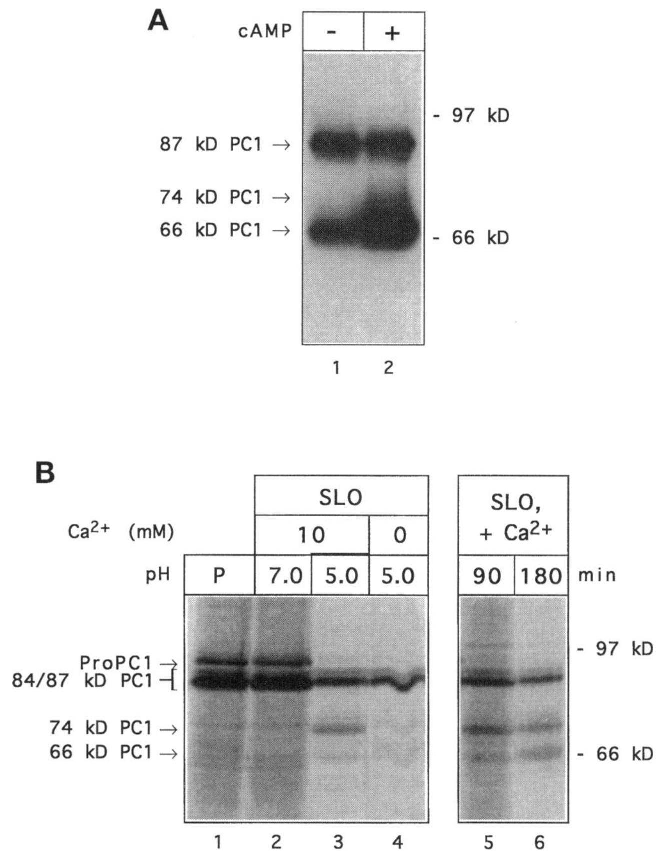

Figure 7. Biosynthesis and secretion studies of PC1. (A) The 74-kDa and 66-kDa forms of PC1 are released in a regulated manner from AtT-20 cells. A basal releasate was collected from a semi-confluent 10-cm dish of AtT-20 cells by incubating the cells for 3 h in serum-free media. The same cells were then treated with 5 mM 8-Br-cAMP for the same duration to recover secretagogue-stimulated secretion. These samples were then trichloroacetic acid precipitated before analysis by SDS-PAGE and Western blot to detect PC1 immunoreactivity. (B) The formation of the 74-kDa and 66-kDa forms of PC1 occurs under the ionic conditions that activate POMC processing in the ER. AtT-20 cells were radiolabeled for 20 min with [^{35}S]Met and then permeabilized with SLO as in Figure 5. One sample well was then extracted (P, lane 1). The remaining sample wells were incubated in transport buffer D containing an ATP-depleting system, A23187, nigericin, and either 10 mM $CaCl_2$ (lanes 2–3 and 5–6) or 5 mM EGTA (lane 4). The pH of the transport buffer was adjusted to 7.0 (lane 2) or pH 5.0 (lanes 3–6). After a 90-min (lanes 2–5) or 180-min (lane 6) incubation period, the cell extracts were collected. All samples were then processed for PC1 immunoprecipitation, SDS-PAGE, and PhosphorImager analysis.

the remainder is diverted into the regulated secretory pathway where it is converted to the 66-kDa and 74-kDa forms and subsequently secreted in a regulated fashion.

Given that there are various active forms of PC1, we were interested in determining which form(s) of PC1 was present in the ER of AtT-20 cells under conditions that activate POMC processing. AtT-20 cells were incubated with [^{35}S]methionine for 20 min to label PC1 in the ER (Lindberg, 1994), then permeabilized, depleted of cytosol and ATP, and incubated under various ionic conditions in the presence of ionophores. As shown in Figure 7B, after the pulse the 84/87-kDa form of PC1 represented the major labeled form although some ProPC1 was recovered (Figure 7B, lane 1). This is consistent with the efficient conversion of ProPC1 (95-kDa) to the 84/87-kDa form within the ER (Benjannet *et al.*, 1993; Lindberg, 1994; Shennan *et al.*, 1995). When the cells were permeabilized immediately after the pulse and incubated under neutral conditions in the presence of calcium, no conversion of 84/87 kDa PC1 to the 74-kDa or 66-kDa forms was observed during the subsequent 90-min chase (Figure 7B, lane 2). The same was true for permeabilized cells incubated at pH 6.0 (our unpublished results). Incubation at pH 5.0 (Figure 7B, lane 3), however, resulted in recovery of an additional band with a mobility expected for that of 74-kDa PC1; a faint band of 66 kDa was also observed. The generation of these forms required calcium (Figure 7B, compare lanes 3 and 4). Increasing the incubation time at pH 5.0 and in the presence of calcium from 90 min (Figure 7B, lane 5) to 180 min (Figure 7B, lane 6) resulted in a decrease in the recovery of both 87-kDa and 74-kDa PC1 and an increase in the recovery of 66-kDa PC1. These effects are quantitated in Table 2. Thus, like POMC processing, the conversion of PC1 from 84/87-kDa forms to the 74-kDa and 66-kDa forms could also be activated in an early compartment under acidic incubation conditions.

Table 2. Quantitation of recovered PC1 forms

	90 min		180 min	
	counts	% total	counts	% total
84/87 kDa	145,968	69.9	43,395	46.5
74 kDa	49,476	23.7	26,546	28.4
66 kDa	13,469	6.4	23,447	25.1
Total	208,913	100	93,388	100

The bands corresponding to 84/87 kDa, 74-kDa, and 66-kDa PC1 in Figure 7B (lanes 5 and 6) were quantitated with the ImageQuant Program (Molecular Dynamics). The counts for each band represent arbitrary scanning units that have been corrected for background and normalized for methionine contents. The % total represents the relative amount of each individual band with respect to the total recovery of all bands under each condition.

We have noted that under acidic incubation conditions, the production of the 66-kDa and 74-kDa PC1 forms did not quantitatively account for the reduced recoveries of ProPC1 and 84/87-kDa PC1. This is in part due to the instability of newly synthesized PC1 under acidic conditions; in a separate experiment, we have determined that ProPC1 and 84/87-kDa PC1 are rapidly degraded if exposed to acidic conditions within 5–10 min after synthesis (our unpublished results).

In summary, we have presented evidence that POMC processing can be induced in the proximal secretory pathway as a result of lumenal acidification. Furthermore, these maturation events occur concomitantly with the production of processed forms of PC1. We propose that normal activation of these processing events in the secretory pathway is tightly coupled to the regulation of lumenal pH.

DISCUSSION

Although both prohormones and prohormone endoproteases are known to transit the secretory pathway, the site of processing and the exact mechanisms for the regulation of prohormone processing remain unclear. The production of mature insulin is dependent upon an initial PC2-mediated cleavage (C-A junction) and a subsequent PC1-mediated cleavage (B-C junction), which serve to remove the C-peptide from proinsulin (Davidson *et al.*, 1988; Bailyes *et al.*, 1992; Bennett *et al.*, 1992; Orci *et al.*, 1994). Earlier studies have suggested that C-A cleavage occurs in the Golgi whereas B-C cleavage occurs in maturing granules (Davidson *et al.*, 1988). However, recent morphological and biochemical studies have argued that both events occur in maturing secretory granules (Huang and Arvan, 1994; Orci *et al.*, 1994). Other studies of prohormone maturation have yielded slightly different conclusions. The processing of prosomatostatin in GH_3 cells appears to occur in the TGN (Xu and Shields, 1993). Temperature block and immunolocalization studies also suggest that POMC processing is initiated in the TGN (Schnabel *et al.*, 1989; Milgram and Mains, 1994). Taken together, these results suggest that prohormone processing does not occur until relatively late in the secretory pathway, in the TGN or maturing secretory granules.

In this study, we provide evidence that POMC processing takes place in a post-sulfation compartment because the processing of sulfated POMC was blocked by transport inhibitors (Figure 1, BFA, NEM) or energy depletion (Figure 5A, SLO). As sulfation is known to take place in late Golgi compartments, these observations are consistent with the idea that processing occurs either in the TGN or maturing granules. However, the pH dependence of POMC processing that we observed in this study is more consistent with POMC processing in maturing granules rather than in

the TGN. Significant processing occurred only when the pH of the compartment was below 6.0 (Figures 5C and 6B). It has been argued that the pH of the TGN is higher than 6.0, otherwise the fusion activities of viral glycoproteins would be irreversibly inactivated during transit through the secretory pathway (Griffiths and Simons, 1986). This would place POMC processing in a compartment that is more acidic than the TGN, i.e., in maturing secretory granules. This conclusion is consistent with recent data that shows that PC1-dependent proinsulin processing occurs in newly formed granules after exit from the TGN (Orci *et al.*, 1994).

We have shown that POMC processing is inhibited by treatment with bafilomycin A_1, a specific inhibitor of the vacuolar H^+-ATPase (Figure 2). This result corroborates our previous finding that POMC processing was inhibited by treatment with weak bases that neutralize acidic compartments (Moore *et al.*, 1983a). Thus, an acidic environment is necessary for prohormone processing to occur. We have further shown that POMC processing may be induced prematurely in the *trans*-Golgi/TGN (Figures 3 and 5) or even in the ER (Figure 6). Taken together, the results suggest that a change in the lumenal pH is both necessary and sufficient to trigger POMC processing in a specific compartment.

A major concern is whether the POMC processing induced in early compartments by low pH was mediated by the physiologically relevant enzyme(s). Because the proximal secretory pathway (ER to TGN) is also involved in the transport of lysosomal enzymes, it was necessary to verify that the low pH induced processing of POMC was not due to lysosomal proteases. An important distinction is that lysosomal acid hydrolases such as the cathepsins do not require calcium for activity (Kress *et al.*, 1966; Barrett, 1967–1972), whereas PC1 is highly calcium dependent (Davidson *et al.*, 1988; Bailyes *et al.*, 1992; Jean *et al.*, 1993; Rufaut *et al.*, 1993; Zhou and Lindberg, 1993). The low pH-induced processing events that we observed were dependent on millimolar calcium (Figure 4), which is consistent with a calcium requirement for PC1 activity (Bailyes *et al.*, 1992; Jean *et al.*, 1993; Rufaut *et al.*, 1993; Zhou and Lindberg, 1993), and the expected calcium level of TGN and secretory granules (Reiffen and Gratzl, 1986; Roos, 1988; Chandra *et al.*, 1991). Lysosomal proteases should also yield distinct processing patterns from those generated by the specific endoprotease, as has been shown for chromogranin A (Hutton *et al.*, 1987). The processing patterns that we observed, however, were indistinguishable from controls (Figures 3, 5, and 6). Moreover, the POMC processing activated in proximal secretory compartments showed a sharp increase between pH 5.5 and 6.0, similar to that seen in maturing granules (Figure 5). Because the pH optimum for the purified 74/66 kDa PC1 is 5.5 (Zhou and Lindberg, 1994), these results are consistent with a PC1-mediated mechanism.

A model for the activation of POMC processing is presented in Figure 8. First, the prohormone POMC and the 84/87-kDa forms of PC1 are co-transported through the proximal secretory pathway. Because we did not observe significant POMC processing above pH 5.5 (Figures 3, 5, and 6), we propose that the lumen of the ER, Golgi, and the bulk of TGN is not sufficiently acidic to activate PC1 and thus no POMC processing occurs. Upon exit from the TGN, the prohormone and endoprotease are sorted into immature secretory granules. Acidification of this compartment to pH 5.5 activates PC1, which processes POMC to the 21-kDa intermediate and then the 4.5/13.5-kDa mature forms of ACTH. Concomitant with POMC processing, PC1 is also processed into the 74-kDa and 66-kDa forms. A portion of unprocessed POMC and 87-kDa PC1 are mis-sorted and secreted by constitutive vesicles. According to this model, acidification of the lumenal environment in maturing granules plays a key role in restricting processing to this post-TGN compartment. We have also shown that calcium is required for POMC processing as well as the conversion of 87-kDa PC1 into the 66-kDa and 74-kDa forms (Figures 4 and 7). In principle, an increasing gradient

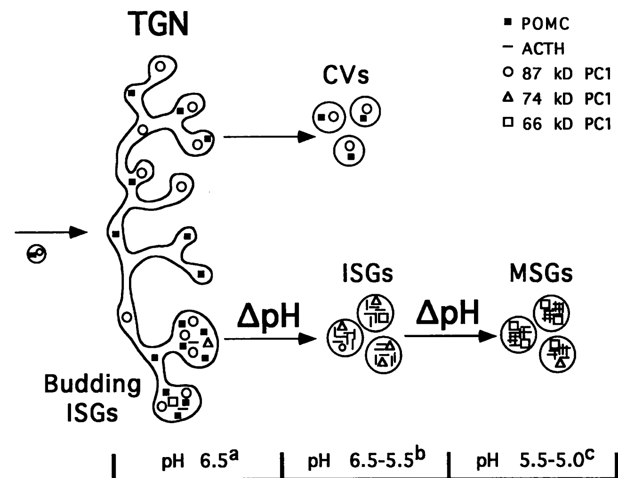

Figure 8. A prohormone processing model. The prohormone POMC (closed squares) and the 87-kDa PC1 (open circles) are co-transported through the proximal secretory pathway and into the TGN. Efficient processing of POMC is not observed in the TGN because this compartment is not sufficiently acidified (pH 6.5–6.0). Upon budding of immature secretory granules (ISGs), acidification occurs within these compartments (pH 5.5) leading to the activation of 87-kDa PC1. As a result, the processing of POMC into ACTH (thin lines) is observed within the granules. This acidification also initiates the conversion of 87-kDa PC1 into the 74-kDa (open triangles) and 66-kDa forms (open squares). Continued acidification of the maturing granules (pH 5.5–5.0) eventually leads to complete processing of PC1 into the 66-kDa form. [a] Boulay *et al.*, 1987; [b], [c] Orci *et al.*, 1994.

of free calcium concentrations from the ER and Golgi/TGN to the secretory granules could also result in the activation of PC1 activity in late compartments. However, lumenal calcium does not appear to be a determining factor for the spatial restriction of PC1 activity; increasing the *trans*-Golgi/TGN calcium concentrations with A23187 by itself did not result in POMC processing (our unpublished results). In contrast, lowering pH alone was sufficient to activate POMC processing (Figure 3).

The above model is in agreement with a recent study of pancreatic islets, which showed a close correlation of the pH of secretory granules with PC1 processing activities (Orci *et al.*, 1994); a sharp increase in proinsulin B-C cleavage was observed at a pH around 5.6, which is similar to the activation of POMC processing observed in our study. This model is also consistent with the observation that the conversion of 84/87-kDa PC1 into 74-kDa and 66-kDa PC1 in semi-intact cells is highly pH dependent (Figure 7B), indicating that this conversion occurs in acidifying secretory granules after exit from the TGN. This explains why only the 74-kDa and 66-kDa PC1 underwent stimulated release (Figure 7A), because the conversion of 84/87 kDa-PC1 into processed forms requires previous sorting into the regulated secretory pathway. The lower recovery of 74-kDa PC1 from the stimulated releasate as compared with 66-kDa PC1 suggests that it may be an intermediate for the production of the 66-kDa form; indeed, upon longer incubation at low pH in vitro, the amount of 74-kDa decreased slightly while the amount of 66-kDa increased (Figure 7B). These results suggest that the 74-kDa PC1 may be first generated within immature granules while 66-kDa PC1 may appear at a later stage during granule maturation. According to this model, one would also expect that PC1-mediated POMC processing should not occur when both PC1 and POMC are co-transfected into cells lacking acidic secretory granules. However, Thomas *et al.* (1991) have found that overexpression of PC1 and POMC in a constitutive secretory cell line (BSC-40) results in the conversion of mouse POMC into ACTH. A possible explanation is that this study employed a vaccinia virus expression system, which produced extremely high levels of PC1. This level of PC1 may have produced some enzymatic activities in the TGN even though its ionic environment is suboptimal.

Several studies have reported that purified PC1 in vitro exhibits significant activity at neutral pH (Jean *et al.*, 1993; Rufaut *et al.*, 1993; Zhou and Lindberg, 1993). Zhou and Lindberg (1994) have purified the different forms of PC1 and found that 84/87 kDa PC1 exhibits a wider pH and calcium range for activity than does 74/66 kDa-PC1; the 84/87-kDa PC1 is 30–90% active between pH 6.5 and pH 7.0, while the 74/66-kDa PC1 is much less active in this pH range (≤ 10%) (see below, Figure 9). These in vitro studies thus imply that PC1 should be somewhat active in the neutral environment of the ER. It is therefore difficult to explain why POMC processing was not observed in the proximal secretory pathway even after prolonged incubations (Figures 3 and 6; see also Milgram and Mains, 1994). Furthermore, one would also expect some 84/87-kDa PC1 to be converted into the 74/66-kDa forms in the ER or Golgi, because this processing event is thought to be autocatalytic (Zhou and Mains, 1994; Zhou and Lindberg, 1994). Again, conversion of PC1 was not observed in the ER (Figure 7; see also Vindrola and Lindberg, 1992; Milgram and Mains, 1994). The fact that there is no processing of POMC or 84/87-kDa PC1 in proximal secretory compartments suggests that 84/87-kDa PC1 in vivo behaves differently from the purified enzyme in vitro. This conclusion is supported by our data. A comparison of the pH profiles of POMC processing in our permeabilized system and the published in vitro PC1 activities (Zhou and Lindberg, 1994) are shown in Figure 9. The POMC processing observed in the ER of permeabilized cells exhibits a pH dependence that closely resembles the activities of purified 74/66-kDa PC1 and not of 84/87-kDa PC1. The pH-dependent processing profiles within the environments of the *trans*-Golgi/TGN and maturing secretory granules are also similar to the in vitro activity profile of 74/66-kDa PC1. This is especially true for the POMC processing observed in maturing secretory granules where PC1 is expected to be

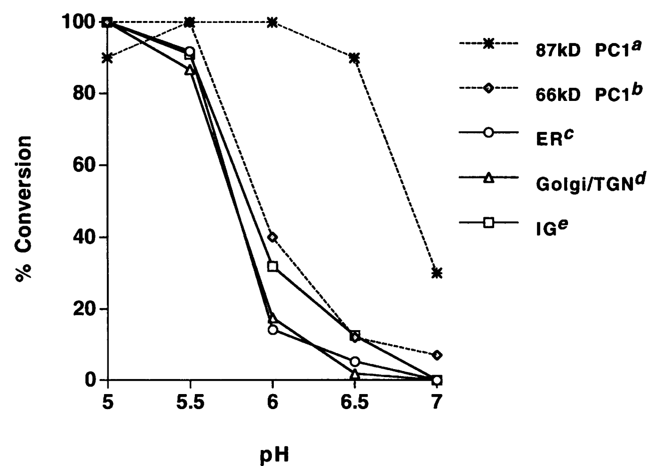

Figure 9. Comparison of the pH dependence of POMC processing observed in this study with the enzymatic properties of purified PC1 forms (Zhou and Lindberg, 1994). ([a] and [b]) The relative activities of the purified 87/84 kDa PC1 (*) and 74/66 kDa PC1 (◆) toward fluorogenic peptide substrates in vitro were taken from Zhou and Lindberg, 1994. ([c], [d], and [e]) The relative extent of POMC conversion in the ER (○, Figure 6B), Golgi/TGN (△, Figure 5C), or immature granules (□, Figure 5C) of semi-intact AtT-20 cells were plotted as a function of pH. The extent of conversion was calculated as a percentage of the maximal conversion for each experiment.

present as the 74/66-kDa form. Our data thus suggest that, unlike the activity of purified 84/87-kDa PC1 in vitro, POMC processing enzyme(s) within the environments of the ER and Golgi have strict low pH requirements for activity.

Why do in vitro PC1 activities differ from those expected from the processing we have observed in AtT-20 cells? There are several possible explanations. First, the in vitro studies employ small fluorogenic peptides as substrates, which may be cleaved more readily at neutral pH than are protein substrates. For instance, the activity of the 84/87-kDa PC1 toward itself or POMC could show a more acidic requirement than toward peptide substrates. If this is true, autocatalytic conversion of PC1 to the 74/66-kDa forms and POMC processing would be confined to secretory granules simply because this is the first secretory compartment that is sufficiently acidic. Second, protein factors may be present in proximal secretory compartments that modulate 84/87-kDa PC1 activity so that it is only active under acidic conditions. Such a protein factor could be a pH-dependent inhibitor that transiently associates with 84/87-kDa; dissociation of the inhibitor would occur under acidic conditions. Our experiments do not allow a distinction between these possibilities. The mechanism by which 84/87-kDa PC1 remains inactive in proximal secretory compartments deserves future investigation.

ACKNOWLEDGMENTS

The authors thank the members of the Moore lab for critical reading of the manuscript. The authors also thank Dr. Iris Lindberg for generous gifts of antibodies. This work was supported by grants from the Public Health Service (GM-35239) and the American Cancer Society (CB-89C). W.K.S. was supported by a Howard Hughes Predoctoral Fellowship.

REFERENCES

Ahnert-Hilger, G., Bader, M.-F., Bhakdi, S., and Gratzl, M. (1989). Introduction of macromolecules into bovine adrenal medullary chromaffin cells and rat pheochromocytoma cells (PC12) by permeabilization with streptolysin O: inhibitory effect of tetanus toxin on catecholamine secretion. J. Neurochem. *52*, 1751–1758.

Anderson, R.G.W., and Pathak, R.K. (1985). Vesicles and cisternae in the *trans* Golgi apparatus of human fibroblasts are acidic compartments. Cell *40*, 635–643.

Aubert, L., and Motais, R. (1974). Molecular features of organic anion permeability in ox red blood cell. J. Physiol. *246*, 159–179.

Bailyes, E.M., Shennan, K.I.J., Seal, A.J., Smeekens, S.P., Steiner, D.F., Hutton, J.C., and Docherty, K. (1992). A member of the eukaryotic subtilisn family (PC3) has the enzymic properties of the proinsulin-converting endopeptidase. Biochem. J. *285*, 391–394.

Barrett, A.J. (1967). Lysosomal acid proteinase of rabbit liver. Biochem. J. *104*, 601–608.

Barrett, A.J. (1969). Properties of lysosomal enzymes. In: Lysosomes in Biology and Pathology, London, United Kingdom: North-Holland Publishing Company, 245–312.

Barrett, A.J. (1972). Lysosomal enzymes. In: Lysosomes: A Laboratory Handbook, London, United Kingdom: North-Holland Publishing Company, 46–135.

Bateman, A., Solomon, S., and Bennett, H.P. (1990). Post-translational modification of bovine pro-opiomelanocortin: tyrosine sulfation and pyroglutamate formation, a mass spectrometric study. J. Biol. Chem. *265*, 22130–22136.

Benjannet, S., Rondeau, N., Day, R., Chretien, M., and Seidah, N. (1991). PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. Proc. Natl. Acad. Sci. USA *88*, 3564–3568.

Benjannet, S., Rondeau, N., Paquet, L., Boudreault, A., Lazure, C., Chretien, M., and Seidah, N. (1993). Comparative biosynthesis, covalent post-translational modifications and efficiency of prosegment cleavage of the prohormone convertases PC1 and PC2, glycosylation, sulphation and identification of the intracellular site of prosegment cleavage of PC1 and PC2. Biochem. J. *294*, 735–743.

Bennett, D.L., Bailyes, E.M., Nielsen, E., Guest, P.C., Rutherford, N.G., Arden, S.D., and Hutton, J.C. (1992). Identification of the type 2 proinsulin processing endopeptidase as PC2, a member of the eukaryote subtilisin family. J. Biol. Chem. *267*, 15229–15236.

Bhakdi, S., Tranum-Jensen, J., and Sziegoleit, A. (1985). Mechanism of membrane damage by streptolysin-O. Infect. Immun. *47*, 52–60.

Bhakdi, S., Weller, U., Walev, I., Martin, E., Jonas, D., and Palmer, M. (1993). A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes. Med. Microbiol. Immunol. *182*, 167–175.

Bloomquist, B., Eipper, B., and Mains, R. (1991). Prohormone-converting enzymes, regulation and evaluation of function using antisense RNA. Mol. Endocrinol. *5*, 2014–2024.

Booth, C., and Koch, G.L.E. (1989). Perturbation of cellular calcium induces secretion of luminal ER proteins. Cell *59*, 729–737.

Boulay, F., Doms, R., Wilson, I., and Helenius, A. (1987). The influenza hemagglutinin precursor as an acid-sensitive probe of the biosynthetic pathway. EMBO J. *6*, 2643–2650.

Braks, J., and Martens, G. (1994). 7B2 is a neuroendocrine chaperone that transiently interacts with prohormone convertase PC2 in the secretory pathway. Cell *78*, 263–273.

Carnell, L., and Moore, H.-P.H. (1994). Transport via the regulated secretory pathway in semi-intact PC12 cells, role of intra-cisternal calcium and pH in the transport and sorting of Secretogranin II. J. Cell Biol. *127*, 693–705.

Chandra, S., Kable, E.P.W., Morrison, G.H., and Webb, W.W. (1991). Calcium sequestration in the Golgi apparatus of cultured mammalian cells revealed by laser scanning confocal microscopy and ion microscopy. J. Cell Sci. *100*, 747–752.

Davidson, H.W., Rhodes, C.J., and Hutton, J.C. (1988). Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic beta cell via two distinct site-specific endopeptidases. Nature *333*, 93–96.

Davoust, J., Gruenberg, J., and Howell, K. (1987). Two threshold values of low pH block endocytosis at different stages. EMBO J. *6*, 3601–3609.

Day, N., Lin, H., Ueda, Y., Meador-Woodruff, J., and Akil, H. (1993). Characterization of pro-opiomelanocortin processing in heterologous neuronal cells that express PC2 mRNA. Neuropeptides *24*, 253–262.

Day, R., Schafe, M., Watson, S., Chretien, M., and Seidah, N. (1992). Distribution and regulation of the prohormone convertases PC1 and PC2 in the rat pituitary. Mol. Endocrinol. *6*, 485–497.

Fasolato, C., Zottini, M., Clementis, E., Zacchettis, D., Meldolesis, J., and Pozzan, T. (1991). Intracellular Ca^{2+} pools in PC12 cells. J. Biol. Chem. *266*, 20159–20167.

Friedman, T., Loh, Y., and Birch, N. (1994). In vitro processing of proopiomelanocortin by recombinant PC1 (SPC3). Endocrinology *135*, 854–862.

Gianoulakis, C., Seidah, N.G., Routhier, R., and Chretien, M. (1979). Biosynthesis and characterization of adrenocorticotropic hormone, alpha-melanocyte-stimulating hormone, and an NH2-terminal fragment of the adrenocorticotropic hormone/beta-lipotropin precursor from rat pars intermedia. J. Biol. Chem. *254*, 11903–11906.

Griffiths, G., and Simons, K. (1986). The *trans* Golgi network, sorting at the exit site of the Golgi complex. Science *234*, 438–443.

Grimes, M., and Kelly, R.B. (1992). Intermediates in the constitutive and regulated secretory pathways released in vitro from semi-intact cells. J. Cell Biol. *117*, 539–549.

Gumbiner, B., and Kelly, R.B. (1982). Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. Cell *28*, 51–59.

Gumbiner, B., and Kelly, R.B. (1981). Secretory granules of an anterior pituitary cell line, AtT-20, contain only mature forms of corticotropin and beta-lipotropin. Proc. Natl. Acad. Sci. USA *78*, 318–322.

Heuser, J. (1989). Effects of cytoplasmic acidification on clathrin lattice morphology. J. Cell Biol. *108*, 401–411.

Huang, X.F., and Arvan, P. (1994). Formation of the insulin-containing secretory granule core occurs within immature beta-granules. J. Biol. Chem. *269*, 20838–20844.

Hutton, J. (1990). Subtilisin-like proteinases involved in the activation of proproteins of the eukaryotic secretory pathway. Curr. Opin. Cell Biol. *2*, 1131–1142.

Hutton, J., Davidson, H., and Peshavaria, M. (1987). Proteolytic processing of chromogranin A in purified insulin granules: formation of a 20 kDa N-terminal fragment (betagranin) by the concerted action of a Ca^{2+}-dependent endopeptidase and carboxypeptidase H (EC 3.4.17.10). Biochem. J. *244*, 457–464.

Jean, F., Basak, A., Rondeau, N., Benjannet, S., Hendy, G., Seidah, N., Chretien, M., and Lazure, C. (1993). Enzymic characterization of murine and human prohormone convertase-1 (mPC1 and hPC1) expressed in mammalian GH4C1 cells. Biochem. J. *292*, 891–900.

Kornfeld, S. (1992). Structure and function of the mannose 6-phosphate/insulin like growth factor II receptors. Annu. Rev. Biochem. *61*, 307–330.

Kornfeld, S., and Mellman, I. (1989). The biogenesis of lysosomes. Annu. Rev. Cell Biol. *5*, 483–525.

Kress, L.F., Peanasky, R.J., and Klitgaard, H.M. (1966). Purification, properties, and specificity of hog thyroid proteinase. Biochim. Biophys. Acta *113*, 375–389.

L'Allemain, G., Paris, S., and Pouyssegur, J. (1984). Growth factor action and intracellular pH regulation in fibroblasts: evidence for a major role of the Na^+/H^+ antiport. J. Biol. Chem. *259*, 5809–5815.

Lindberg, I. (1991). The new eukaryotic precursor processing proteinases. Mol. Endocrinol. *5*, 1361–1365.

Lindberg, I. (1994). Evidence for cleavage of the PC1/PC3 prosegment in the endoplasmic reticulum. Mol. Cell. Neurosci. *5*, 263–268.

Lodish, H.F., and Kong, N. (1990). Perturbation of cellular calcium blocks exit of secretory proteins from the rough endoplasmic reticulum. J. Biol. Chem. *19*, 10893–10899.

Mains, R., and Eipper, B. (1979). Synthesis and secretion of corticotropins, melanotropins, and endorphins by rat intermediate pituitary cells. J. Biol. Chem. *254*, 7885–7894.

Mains, R.E., Cullen, E.I., May, V., and Eipper, B.A. (1987). The role of secretory granules in peptide biosynthesis. Ann. NY Acad. Sci. *493*, 278–291.

Mains, R.E., and Eipper, B.A. (1978). Coordinate synthesis of corticotropins and endorphins by mouse pituitary tumor cells. J. Biol. Chem. *253*, 651–655.

Mains, R.E., Eipper, B.A., and Ling, N. (1977). Common precursor to corticotropins and endorphins. Proc. Natl. Acad. Sci. USA *74*, 3014–3018.

Martell, A.E., and Smith, R.M. (1974). Critical Stability Constants, New York: Plenum Publishing Corporation.

Martens, G.J., Braks, J.A., Eib, D.W., Zhou, Y., and Lindberg, I. (1994). The neuroendocrine polypeptide 7B2 is an endogenous inhibitor of prohormone convertase PC2. Proc. Natl. Acad. Sci. USA *91*, 5784–5787.

Mellman, I., Fuchs, R., and Helenius, A. (1986). Acidification of the endocytic and exocytic pathways. Annu. Rev. Biochem. *55*, 663–700.

Milgram, S., and Mains, R. (1994). Differential effects of temperature blockade on the proteolytic processing of three secretory granule-associated proteins. J. Cell Sci. *107*, 737–745.

Miller, S.G., Carnell, L., and Moore, H.-P.H. (1992). Post-Golgi membrane traffic, brefeldin A inhibits export from the distal Golgi compartment to the cell surface but not recycling. J. Cell Biol. *118*, 267–283.

Miller, S.G., and Moore, H.-P.H. (1991). Reconstitution of constitutive secretion using semi-intact cells, regulation by GTP but not calcium. J. Cell Biol. *112*, 39–54.

Moore, H.-P.H., Gumbiner, B., and Kelly, R.B. (1983a). Chloroquine diverts ACTH from a regulated to a constitutive secretory pathway in AtT-20 cells. Nature *302*, 434–436.

Moore, H.-P.H., Gumbiner, B., and Kelly, R.B. (1983b). A subclass of proteins and sulfated macromolecules secreted by AtT-20 (mouse pituitary tumor) cells is sorted with adrenocorticotropin into dense secretory granules. J. Cell Biol. *97*, 810–817.

Moore, H.-P.H., and Kelly, R.B. (1985). Secretory protein targeting in a pituitary cell line, differential transport of foreign secretory proteins to distinct secretory pathways. J. Cell Biol. *101*, 1773–1781.

Niehrs, C., Beisswanger, R., and Huttner, W.B. (1994). Protein tyrosine sulfation, 1993: an update. Chemico-Biol. International *92*, 257–271.

Oda, K. (1992). Calcium depletion blocks proteolytic cleavages of plasma protein precursors which occur at the Golgi and/or *trans*-Golgi network. J. Biol. Chem. *267*, 17465–17471.

Orci, L., Halban, P., Perrelet, A., Amherdt, M., Ravazzola, M., and Anderson, R. (1994). pH-independent and -dependent cleavage of proinsulin in the same secretory vesicle. J. Cell Biol. *126*, 1149–1156.

Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Perrelet, A., Vassalli, J., and Anderson, R. (1986). Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory vesicles. J. Cell Biol. *103*, 2273–2281.

Reiffen, F.U., and Gratzl, M. (1986). Ca^{2+} binding to chromaffin vesicle matrix proteins, effect of pH, Mg^{2+}, and ionic strength. Biochemistry *25*, 4402–4406.

Rhodes, C.J., Thorne, B.A., Lincoln, B., Nielsen, E., Hutton, J.C., and Thomas, G. (1993). Processing of proopiomelanocortin by insulin secretory granule proinsulin processing endopeptidases. J. Biol. Chem. *268*, 4267–4275.

Roberts, J.L., and Herbert, E. (1977). Characterization of a common precursor to corticotropin and beta-lipotropin, identification of beta-lipotropin peptides and their arrangement relative to corticotropin in the precursor synthesized in a cell-free system. Proc. Natl. Acad. Sci. USA *74*, 5300–5304.

Roberts, J.L., Phillips, M., Rosa, P.A., and Herbert, E. (1978). Steps involved in the processing of common precursor forms of adrenocorticotropin and endorphin in cultures of mouse pituitary cells. Biochemistry *17*, 3609–3618.

Roos, N. (1988). A possible site of calcium regulation in rat exocrine pancreas cells, an X-ray microanalytical study. Scanning Microsc. *2*, 323–329.

Rosa, P., Barr, F.A., Stinchcombe, J.C., Binacchi, C., and Huttner, W.B. (1992). Brefeldin A inhibits the formation of constitutive secretory vesicles and immature secretory granules for the trans-Golgi network. Eur. J. Cell Biol. *59*, 265–274.

Rufaut, N.W., Brennan, S.O., Hakes, D.J., Dixon, J.E., and Birch, N.P. (1993). Purification and characterization of the candidate prohormone-processing enzyme SPC3 produced in a mouse L cell line. J. Biol. Chem. *268*, 20291–20298.

Salamero, J., Sztul, E.S., and Howell, K.E. (1990). Exocytic transport vesicles generated in vitro from the *trans*-Golgi network carry secretory and plasma membrane proteins. Proc. Natl. Acad. Sci. USA *87*, 7717–7721.

Schnabel, E., Mains, R.E., and Farquhar, M.G. (1989). Proteolytic processing of pro-ACTH endorphin begins in the Golgi complex of pituitary corticotropes and AtT-20 cells. Mol. Endocrinol. *3*, 1223–1235.

Seidah, N., Day, R., and Chretien, M. (1993). The family of prohormone and pro-protein convertases. Biochem. Soc. Trans. *21*, 685–691.

Seidah, N., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M., and Chretien, M. (1990). cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products, tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. DNA Cell Biol. *9*, 415–424.

Seidah, N., Marcinkiewicz, M., Benjannet, S., Gaspa, R.L., Beaubien, G., Mattei, M., Lazure, C., Mbikay, M., and Chretien, M. (1991). Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, Furin, and Kex2, distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2. Mol. Endocrinol. *5*, 111–122.

Shennan, K.I.J., Taylor, N.A., Jermany, J.L., Matthews, G., and Docherty, K. (1995). Differences in pH optima and calcium requirements for maturation of the prohormone convertases PC2 and PC3 indicates different intracellular locations for these events. J. Biol. Chem. *270*, 1402–1407.

Smeekens, S., Avruch, A., Lamendola, J., Chan, S., and Steiner, D. (1991). Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of langerhans. Proc. Natl. Acad. Sci. USA *88*, 340–344.

Smeekens, S., and Steiner, D. (1990). Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast diabasic processing protease Kex2. J. Biol. Chem. *265*, 2997–3000.

Spiro, R.C., Freeze, H.H., Sampath, D., and Garcia, J.A. (1991). Uncoupling of chondroitin sulfate glycosaminoglycan synthesis by brefeldin A. J. Cell Biol. *115*, 1463–1473.

Steiner, D., and James, D. (1992). Cellular and molecular biology of the beta cell. Diabetologia *35*(suppl 2), S41–S48.

Thomas, L., Leduc, R., Smeekens, S.P., Steiner, D.F., and Thomas, G. (1991). Kex2-like endoproteases PC2 and PC3 accurately cleave a model prohormone in mammalian cells, evidence for a common core of neuroendocrine processing enzymes. Proc. Natl. Acad. Sci. USA *88*, 5297–5301.

Tooze, J., Hollinshead, M., Frank, R., and Burke, B. (1987). An antibody specific for an endoproteolytic cleavage site provides evidence that pro-opiomelanocortin is packaged into secretory granules in AtT20 cells before its cleavage. J. Cell Biol. *105*, 155–162.

Tooze, S.A., Flatmark, R., Tooze, J., and Huttner, W.B. (1991). Characterization of the immature secretory granule, an intermediate in granule biogenesis. J. Cell Biol. *115*, 1491–1503.

Tooze, S.A., and Huttner, W.B. (1990). Cell-free protein sorting to the regulated and constitutive secretory pathways. Cell *60*, 837–847.

Vindrola, O. (1994). Rapid cleavage of the endogenous PC3 prosegment and slow conversion to 74 kDa and 66 kDa proteins in AtT-20 cells. Neuropeptides *27*, 109–120.

Vindrola, O., and Lindberg, I. (1992). Biosynthesis of the prohormone convertase mPC1 in AtT-20 cells. Mol. Endocrinol. *6*, 1088–1094.

West, D.W. (1981). Energy-dependent calcium sequestration activity in a Golgi apparatus fraction derived from lactating rat mammary glands. Biochim. Biophys. Acta *673*, 374–386.

Wood, S., Park, J., and Brown, W. (1991). Brefeldin A causes a microtubule-mediated fusion of the *trans*-Golgi network and early endosomes. Cell *67*, 591–600.

Xu, H., and Shields, D. (1993). Prohormone processing in the *trans*-Golgi network, endoproteolytic cleavage of prosomatostatin and formation of nascent secretory vesicles in permeabilized cells. J. Cell Biol. *122*, 1169–1184.

Xu, H., and Shields, D. (1994). Prosomatostatin processing in permeabilized cells: endoproteolytic cleavage is mediated by a vacuolar ATPase that generates an acidic pH in the *trans*-Golgi network. J. Biol. Chem. *269*, 22875–22881.

Zhou, A., Bloomquist, B., and Mains, R. (1993). The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in a strict temporal order during proopiomelanocortin biosynthetic processing. J. Biol. Chem. *268*, 1763–1769.

Zhou, A., and Mains, R. (1994). Endoproteolytic processing of proopiomelanocortin and prohormone convertases 1 and 2 in neuroendocrine cells overexpressing prohormone convertases 1 or 2. J. Biol. Chem. *269*, 17440–17447.

Zhou, Y., and Lindberg, I. (1993). Purification and characterization of the prohormone convertase PC1 (PC3). J. Biol. Chem. *268*, 5615–5623.

Zhou, Y., and Lindberg, I. (1994). Enzymatic properties of carboxyl terminally truncated prohormone convertase 1 (PC1/SPC3) and evidence for autocatalytic conversion. J. Biol. Chem. *269*, 18408–18413.