pH-dependent processing of secretogranin II by the endopeptidase PC2 in isolated immature secretory granules

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We have previously characterized the processing of secretogranin II (SgII) in PC12 cells that were stably transfected with the endopeptidase PC2. Here we show that processing of SgII can be observed in isolated immature secretory granules (ISGs) derived from this cell line in a temperature- and ATP-dependent manner. The stimulatory effect of ATP on processing can be attributed to the activation of the vacuolar H^+-ATPase and a concomitant decrease in intragranular pH. The immature secretory granule therefore provides an adequate environment for correct processing of SgII by PC2. The rate of SgII processing was strongly dependent on the intragranular pH, suggesting that processing of SgII can be used as a pH indicator for the granule interior. A standard curve was prepared using SgII processing in ISGs equilibrated at a range of pH values. The extent of processing in ISGs incubated in the presence of ATP at physiological pH was compared with the standard curve, and the intragranular pH was determined. From these observations, we propose an intragranular pH of 6.3 ± 0.1 for ISGs in a physiological buffer in the presence of ATP. Hence, the pH of ISGs seems to be similar to the pH of the *trans*-Golgi network (TGN) and is clearly higher than the pH of mature secretory granules (pH 5.0–5.5). Interestingly, no processing of SgII could be observed in a membrane fraction that is highly enriched in TGN under conditions for which processing was readily obtained in isolated ISGs.

INTRODUCTION

An unique feature of the eukaryotic cell is the compartmentalization of cellular functions involved in the uptake and secretion of macromolecules. These membrane-bound compartments connect with each other via controlled membrane-fusion events. The luminal milieu of these compartments dictates, to some extent, which reactions will take place. A crucial determinant for the activities within both endocytic and exocytic pathways appears to be the relative acidity of this milieu [1,2]. The activities of many enzymes within these compartments, and the transport of proteins along both pathways, have been shown to be dependent on the maintenance of this acidic pH [3–9]. The proton concentration inside these compartments is controlled by the characteristic ion permeabilities of their membrane and by the activity of an ATP-dependent proton pump of the vacuolar type [10,11]. The fundamental importance of the maintenance of an acidic pH is most apparent in the biogenesis of the secretory granules, the storage organelles of endocrine and neuroendocrine cells [12]. Sorting of regulated secretory proteins from constitutively secreted molecules begins in the TGN and involves their selective aggregation in response to the acidic pH of this compartment [13]. This aggregate is packaged into a vesicle that is referred to as an immature secretory granule (ISG) [14]. The ISG undergoes a series of morphological and biochemical changes [15], including: (1) increase in size and loss of the partial clathrin coat [16]; (2) accumulation of neurotransmitters; and (3) processing of peptide hormones to the mature forms. Both the uptake of neurotransmitters [3,17] and the endoproteolytic cleavage of prohormones [18,19] are regulated by and are dependent on the progressive acidification of the secretory granule interior.

We have studied the processing of a secretory granule protein in isolated ISGs in order to gain further information on the activities and intraluminal milieu of this intermediate. The model secretory protein used in this study, secretogranin II (SgII), is a member of the granin family of regulated secretory proteins that are a major component of the cores of secretory granules [20]. We have recently shown that SgII can be proteolytically cleaved to low-molecular-mass products in PC12 cells that were stably transfected with the endopeptidase PC2 (PC12/PC2 cells) [21]. PC2 belongs to the family of prohormone convertases (PC enzymes) that process prohormones at dibasic amino acid cleavage sites [22]. PC2 itself is synthesized as an inactive proform, and its transport along the secretory pathway [23] as well as its autocatalytic activation [24] are regulated by its association with another endocrine protein, 7B2 [25]. The site of prohormone processing has remained controversial: processing is thought to commence either in the TGN [26,27] or only after packaging of secretory proteins into ISGs [19,28]. In the present study we compare the pH-dependent processing of SgII by PC2 in ISGs and in the TGN isolated from PC12/PC2 cells and provide additional evidence that the ISG is indeed a functionally distinct organelle from the TGN.

EXPERIMENTAL

Materials

ATP, creatine phosphate and creatine phosphokinase were from Boehringer Mannheim (Mannheim, Germany). Concanamycin A was a gift from Ciba-Geigy (Basel, Switzerland). Nigericin and valinomycin were purchased from Sigma (Poole, Dorset, U.K.). All cell-culture materials were obtained from Gibco (Paisley, U.K.), and unless specified all other chemicals were obtained from Sigma or BDH (Poole, Dorset, U.K.). Radiochemicals

Abbreviations used: ISG, immature secretory granule; MSG, mature secretory granule; SgII, secretogranin II; TGN, *trans*-Golgi network.
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were obtained from Amersham (Slough, U.K.). The PC2 antiserum (4BF), used at a dilution of 1:2000, was provided by Dr. Iris Lindberg, LSU Medical Center, New Orleans, LA, U.S.A.

Cell culture

PC12/PC2 cells, clone 1.2 [21], were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) horse serum and 5% (v/v) fetal calf serum (growth medium), as previously described for PC12 cells [29].

[^{35}S]Sulphate labelling and subcellular fractionation of PC12/PC2 cells

PC12/PC2 cells in six 150 cm² dishes were grown to 80% confluency and were pulse-labelled for 5 min with [^{35}S]sulphate or were pulse-labelled for 5 min and chased for 15 min as described previously [29]. In some experiments the cells were depleted and were pulse-labelled in BSS [125 mM NaCl, 4.8 mM KCl, 1.4 mM $MgCl_2$, 10 mM glucose, 25 mM Hepes, pH 7.4, 0.1% (v/v) horse serum and 0.05% (v/v) fetal calf serum] instead of sulphate-free Dulbecco's modified Eagle's medium. The cells were harvested at 4 °C in TBS (Tris-buffered saline: 25 mM Tris/HCl, pH 7.4, 137 mM NaCl, 4.5 mM KCl and 0.7 mM Na_2HPO_4), resuspended in homogenization buffer [HB: 250 mM sucrose in 10 mM Hepes, pH 7.2, 1 mM EDTA and 1 mM $Mg(OAc)_2$] and homogenized as detailed previously [30]. A post-nuclear supernatant was prepared exactly as described for PC12 cells [30]. ISGs were separated from the TGN by velocity and equilibrium sucrose-gradient centrifugation as previously described [29,30], except that a step gradient (0.8–1.6 M sucrose) was used instead of a continuous gradient for the equilibrium gradient centrifugation. Fractions 2–4 of the velocity gradient, containing the bulk of the ISGs, were subjected to equilibrium sucrose-gradient centrifugation. Fractions 7–9 of the equilibrium gradient were pooled and used as 'isolated ISGs' in all experiments. Fractions 9–11 of the velocity gradient, containing the TGN [29], were subjected to equilibrium sucrose-gradient centrifugation. Fractions 6–8 of the equilibrium gradient were pooled and used as 'isolated TGN' (see Figures 5 and 6). For experiments on intact cells, PC12/PC2 cells (in one 35 cm² dish) were pulse-labelled for 5 min with [^{35}S]sulphate and were chased as indicated in the Figure legends. The cells were washed with TBS and extracted with TNTE [20 mM Tris, pH 7.5, 150 mM NaCl, 0.3% (w/v) Triton X-100, 5 mM EDTA, 0.5 mM PMSF and 1 μM leupeptin], and a heat-stable fraction was prepared by boiling the samples for 5 min. The heat-stable proteins were acetone precipitated, resuspended in Laemmli sample buffer and analysed by SDS/12% PAGE.

Processing in isolated ISGs and TGN

Aliquots of 125 μl of 'isolated ISGs' (approx. 75 μg) or 'isolated TGN' (approx. 50 μg) were adjusted to 50 mM KOAc/1 mM $Mg(OAc)_2$/20 mM (Hepes, Tris or Mes) buffered at a variable pH (as indicated in the Figures) in a final volume of 375 μl. In some experiments the KOAc and the $Mg(OAc)_2$ were replaced by KCl and $MgCl_2$. ISGs or TGN were supplemented with an ATP-regenerating system [29] where indicated and were incubated at 4 °C or 37 °C for the indicated periods of time. Nigericin, valinomycin, concanamycin (each stock solutions in ethanol) and NH_4Cl were added at 4 °C, 15 min before incubation at 37 °C. The samples were cooled at 4 °C, and the membranes were recovered by ultracentrifugation at 100000 ***g*** for 1 h at 4 °C. The pellets were resuspended in Laemmli sample buffer and analysed by SDS/12% PAGE, followed by fluorography and autoradiography [31]. The [^{35}S]sulphate radioactivity was quantified with a PhosphorImager and the ImageQuant Software package (Molecular Dynamics, Chesham, U.K.). The efficiency of processing was assessed by individual quantification of the ^{35}S-labelled processing products that could be resolved by SDS/12% PAGE was expressed as a percentage of the sum of the values obtained for SgII and each SgII-derived band.

Processing in semi-intact cells

PC12/PC2 cells (in one 150 cm² dish) were pulse-labelled for 5 min with [^{35}S]sulphate as described above. Then they either were incubated for 2 h at 20 °C in chase medium before permeabilization or were washed with cold KGlu buffer (20 mM Hepes, pH 7.2, 120 mM potassium glutamate, 20 mM KOAc and 2 mM EGTA) containing 0.1% (w/v) BSA and were permeabilized using six passages through a 22 G needle followed by two passages through a cell-cracker (EMBL workshop, Heidelberg, Germany) with an 18 μm clearance. The cells were washed several times with KGlu buffer, were distributed into five aliquots and then were finally resuspended in 120 μl of buffer (50 mM KOAc, 1 mM MgOAc and 20 mM Hepes, pH 7.2, or 20 mM Mes, pH 6.2 or 5.5). An ATP-regenerating system and nigericin (100 nM final concentration) were added where indicated. After a 10 min preincubation on ice, the cells were incubated for 45 min at 37 °C, were cooled for 5 min on ice and were pelleted for 30 min at 3000 ***g*** (4 °C) in a Heraeus Megafuge 1.0R. The pellets were resuspended in TNTE, and a heat-stable fraction was prepared by boiling the samples for 5 min. The heat-stable proteins were acetone precipitated, resuspended in Laemmli sample buffer and analysed by SDS/12% PAGE.

Immunoblotting

'Isolated ISGs' (100 μg) and 'isolated TGN' (100 μg) were incubated under processing conditions and subsequently were precipitated with trichloroacetic acid. The precipitated proteins were solubilized, separated by SDS/7.5% PAGE and transferred to nitrocellulose (S&S, Dossel, Germany). Immunoblotting was performed using standard techniques: the membrane was blocked and washed with 5% (w/v) low-fat milk in PBS containing 0.2% (w/v) Triton X-100, and the antiserum 4BF was diluted 1:2000 in the same buffer. Bound antibody was detected by ^{125}I-labelled Protein A and was quantified with a PhosphorImager system (Molecular Dynamics).

RESULTS

Processing of SgII in isolated ISGs requires activation of the vacuolar H^+-ATPase

ISGs were isolated by subcellular fractionation from PC12/PC2 cells that had been labelled by a 5 min [^{35}S]sulphate pulse followed by a 15 min chase. This protocol allows selective labelling of the regulated secretory proteins in ISGs and serves throughout this study as an operational definition of the ISG. SgII is sulphated on a single tyrosine residue (Tyr 126) [31], and this sulphation site is preserved in all five N-terminal PC2 processing products (Figure 1) that have been previously characterized [21].

Isolated ISGs were incubated in a neutral pH buffer [20 mM Tris or Hepes, pH 7.2, 50 mM KOAc and 1 mM $Mg(OAc)_2$] in the presence or absence of an ATP-regenerating system ($\pm$ATP) at 4 °C or 37 °C for 45 min. After incubation at 4 °C, analysis of the sulphated proteins by SDS/12% PAGE and autoradiography revealed bands for SgII (p86) and p38 (previously identified as a

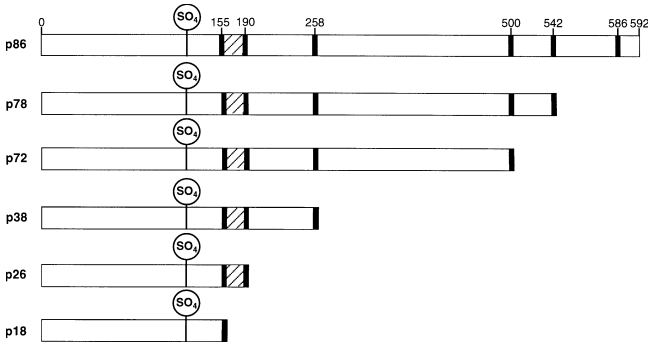
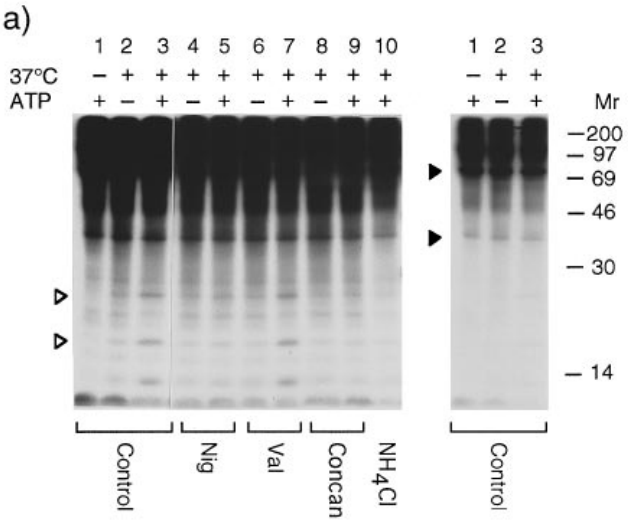


Figure 1 Schematic representation of SgII and sulphated processing products

p86 refers to full-length SgII ($M_r \sim 86000$). p78, p72, p38, p26 and p18 refer to SgII-derived processing products of $M_r \sim 78000$, 72000, 38000, 26000, and 18000 respectively. Black bars indicate dibasic cleavage sites (Arg–Lys) utilized by PC2. The hatched area between two cleavage sites corresponds to the position of secretoneurin. SgII is sulphated on Tyr-126 (SO_4).



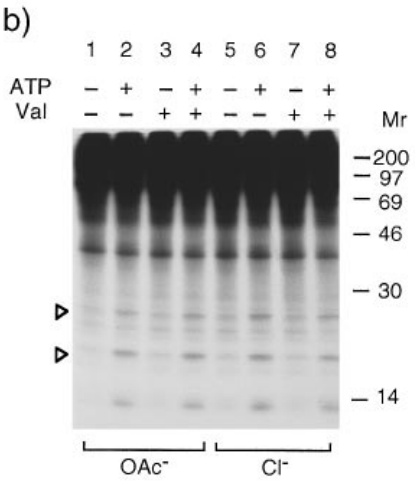


Figure 2 Processing of SgII in isolated ISGs requires activation of the vacuolar H^+-ATPase

(**a**) [^{35}S]Sulphate-labelled ISGs were isolated from PC12/PC2 cells and were incubated in 20 mM Hepes (pH 7.2)/50 mM KOAc/1 mM $Mg(OAc)_2$ for 45 min at 4 °C or 37 °C in the presence or absence of an ATP-regenerating system. Lanes 1–3 are shown twice: in the right panel a lighter exposure is shown. Nigericin (Nig; 100 nM), valinomycin (Val; 250 nM), concanamycin (Concan; 100 nM) and NH_4Cl (10 mM) (all final concentrations) were added where indicated. ISGs were recovered by ultracentrifugation and analysed by SDS/12% PAGE and autoradiography. Open arrowheads indicate the position of the SgII-processing products p26 and p18. Filled arrowheads indicate the full-length SgII (p86) and the SgII-processing product p38. (**b**) [^{35}S]Sulphate-labelled ISGs were isolated from PC12/PC2 cells and were incubated in 20 mM Hepes (pH 7.2)/50 mM KOAc/1 mM $Mg(OAc)_2$ (lanes 1–4) or in 20 mM Hepes (pH 7.2)/50 mM KCl/1 mM $MgCl_2$ (lanes 5–8) for 45 min at 37 °C. An ATP-regenerating system and/or valinomycin (1 μM final concentration) were added where indicated. ISGs were recovered by ultracentrifugation and were analysed by SDS/12% PAGE and autoradiography. Open arrowheads indicate the position of the SgII-processing products p26 and p18. The positions of molecular-mass markers (expressed as thousands) are indicated on the right-hand side of both panels.

SgII-derived processing fragment) [21] (Figure 2a, lanes 1). Further characteristic SgII processing fragments, p26 and p18, appeared only upon incubation at 37 °C in the presence of ATP (compare lanes 2 and 3 in Figure 2a). The sulphated band of approximately M_r 12000 is not derived from SgII and has not yet been identified [21]. Full-length SgII and the high-molecular-mass processing products p78 and p72 were largely masked by a sulphated proteoglycan. Unfortunately, this proteoglycan smear interfered with an accurate quantitative analysis of the processing efficiency. We chose therefore to assess the relative efficiency of processing under different conditions by quantification of the sulphated end product p18 (see below).

Processing of various substrates by the endopeptidase PC2 has been studied *in vitro* in several different cell and granule lysates, and has been shown to have a characteristic acidic pH optimum (pH 5.0–5.5) [32–35]. Chromaffin and other secretory granules in a variety of tissues contain an H^+-ATPase of the vacuolar type [36,37]. This enzyme plays a central role in the acidification of the dense-core secretory granules [38–40] (for a review see [36]). Therefore, it seemed likely that ATP might stimulate processing in isolated ISGs by activating the vacuolar H^+-ATPase of the ISG, thereby leading to a decrease in the intragranular pH. Consistent with this hypothesis, inhibition of the vacuolar H^+-ATPase by the fungal metabolite concanamycin A (a specific inhibitor of v-type H^+-ATPases [41]) abolished the ATP-dependent processing (Figure 2a, lanes 8 and 9). Furthermore, inclusion of nigericin was sufficient to eliminate the appearance of SgII fragments, in accordance with its function of facilitating proton release in exchange for potassium uptake (Figure 2a, lanes 4 and 5). In contrast, the K^+ ionophore valinomycin, which uncouples the membrane potential from the proton-pumping activity, had no effect on processing (Figure 2a, lanes 6 and 7). This suggests that the observed effect of ATP on processing results from the establishment of an acidic pH in the lumen of the granule and is neither dependent on, nor limited by, the generation of a membrane potential. As expected, addition of NH_4Cl, a weak base that accumulates in acidic membrane compartments, where it is protonated, leading to alkalinization of the intragranular milieu, also prevented processing (Figure 2a, lane 10).

Previous work by others on the bioenergetics of chromaffin granules has shown a requirement for chloride anions in the generation of a pH gradient across the granule membrane [17,37,38]. Interestingly, replacement of the acetate anions in the incubation buffer by chloride anions did not further increase the extent of processing, suggesting that the conductance of the

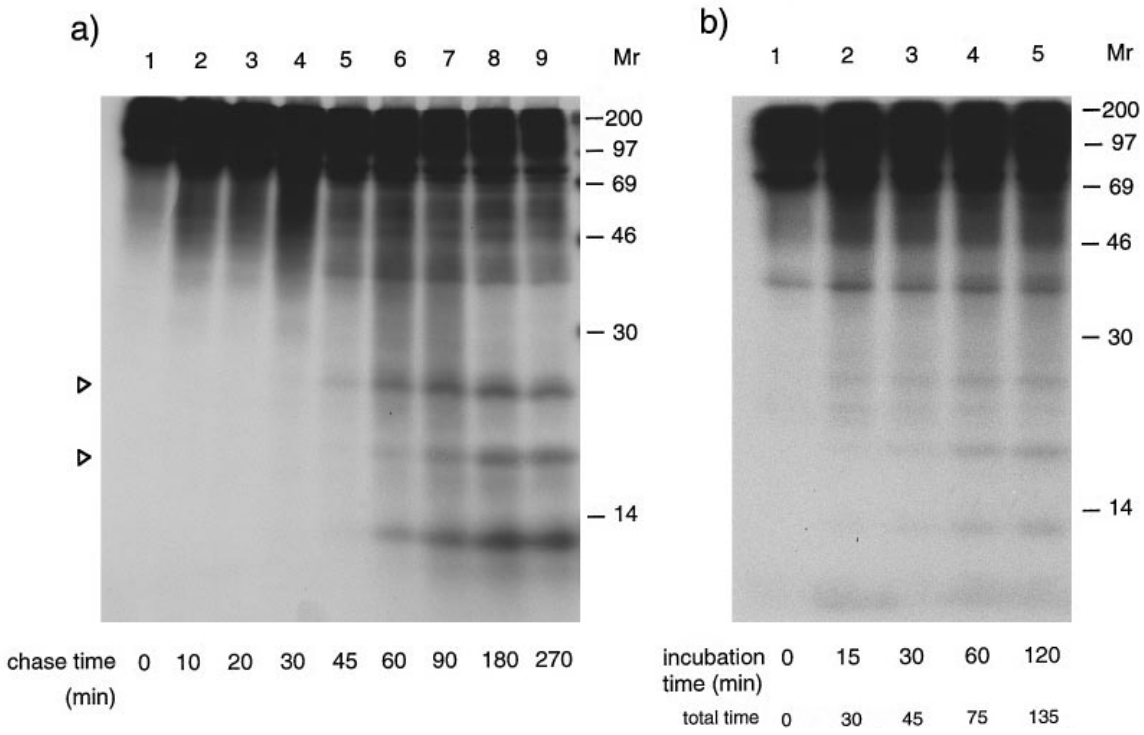


Figure 3 Processing in intact cells and in isolated ISGs

(**a**) PC12/PC2 cells were pulse-labelled for 5 min with [^{35}S]sulphate and were chased for the indicated length of time. The cells were lysed, a heat-stable fraction was prepared, and the heat-stable proteins were subjected to SDS/12% PAGE followed by autoradiography. (**b**) PC12/PC2 cells were labelled with a 5 min [^{35}S]sulphate pulse followed by a 15 min chase. The [^{35}S]sulphate-labelled ISGs were isolated and were incubated in 20 mM Hepes (pH 7.2)/50 mM KOAc/1 mM $Mg(OAc)_2$ at 37 °C in the presence of an ATP-regenerating system for various lengths of time (incubation time). The equivalent chase time (15 min chase *in vivo* + incubation time *in vitro* = total time) is indicated to facilitate the comparison of the *in vivo* and *in vitro* time courses. ISGs were recovered by ultracentrifugation and analysed by SDS/12% PAGE and autoradiography. Open arrowheads indicate the position of the SgII-processing products p26 and p18. The positions of molecular-mass markers (expressed as thousands) are indicated on the right-hand side of both panels.

PC12/PC2 ISG membrane for acetate is sufficient to dissipate any membrane potential that might limit maximal acidification (Figure 2b; see also [42]).

Comparison of the time course of processing in intact cells and in isolated ISGs

The profile of the processing products obtained by incubation of isolated ISGs was reminiscent of that observed *in vivo* [21]. Figure 3 shows a direct comparison of a time course of processing in intact PC12/PC2 cells pulse-labelled with [^{35}S]sulphate for 5 min and chased for various lengths of time and the sequential appearance of processing products in isolated ISGs incubated at pH 7.2 in the presence of ATP. The kinetics of both p26 and p18 appearance as well as p18 accumulation in isolated ISGs under these conditions appears to be very similar to the time course in intact cells. At early time points ($t = 30$ min of chase in intact cells, which corresponds to $t = 15$ min in isolated ISGs) only very little p18 was generated. It has previously been suggested that the half-time of maturation for secretory granules in PC12 cells is approx. 45 min [16]; therefore up to 90 min of chase time the labelled granules can be considered as ISGs at various stages of maturation. The appearance of p26 and p18 *in vitro* within this time window nicely parallels the time course *in vivo* and suggests that processing can indeed be reconstituted in isolated ISGs. At later time points ($t = 90$–180 min) the accumulation of p18 reaches a plateau in intact cells and processing seems to come to an end [as observed in isolated ISGs incubated at pH 5.5 (see below)]. Given the limitations imposed by our inability to resolve p86, p78, p72 and the proteoglycan, we estimate that approx. 44% of the radioactivity remains within the p86 band and 14% is found in p18 after 270 min of chase time in intact cells.

Acidification of the ISG lumen is sufficient for SgII processing by PC2

The data shown in Figure 2 suggest that processing in isolated ISGs solely depends on the establishment of an acidic intragranular pH. As all manipulations leading to the isolation of ISGs are carried out at 4 °C in the absence of ATP, conditions under which the H^+-ATPase would be expected to be inactive, this might result in the neutralization of the intragranular pH through equilibration with the sucrose-gradient buffer (10 mM Hepes, pH 7.2). If ATP is only required to re-acidify the ISG lumen after isolation, then it should be possible to observe ATP-independent processing after preincubating the ISGs in a low pH buffer. Figure 4 shows that processing can indeed be observed at 37 °C in the absence of ATP in ISGs that have been pre-equilibrated at pH 6.2 for 1 h on ice (lane 6). Addition of ATP to these granules still enhanced processing by approximately 2-fold [Figure 4a (compare lanes 6 and 7) and Figure 4b] as compared with the nearly 4-fold stimulation by ATP observed at pH 7.2 [Figure 4a (compare lanes 2 and 3) and Figure 4b]. Similar amounts of processing were observed after pre-equilibration at pH 5.5 compared with pH 6.2 in the presence of ATP

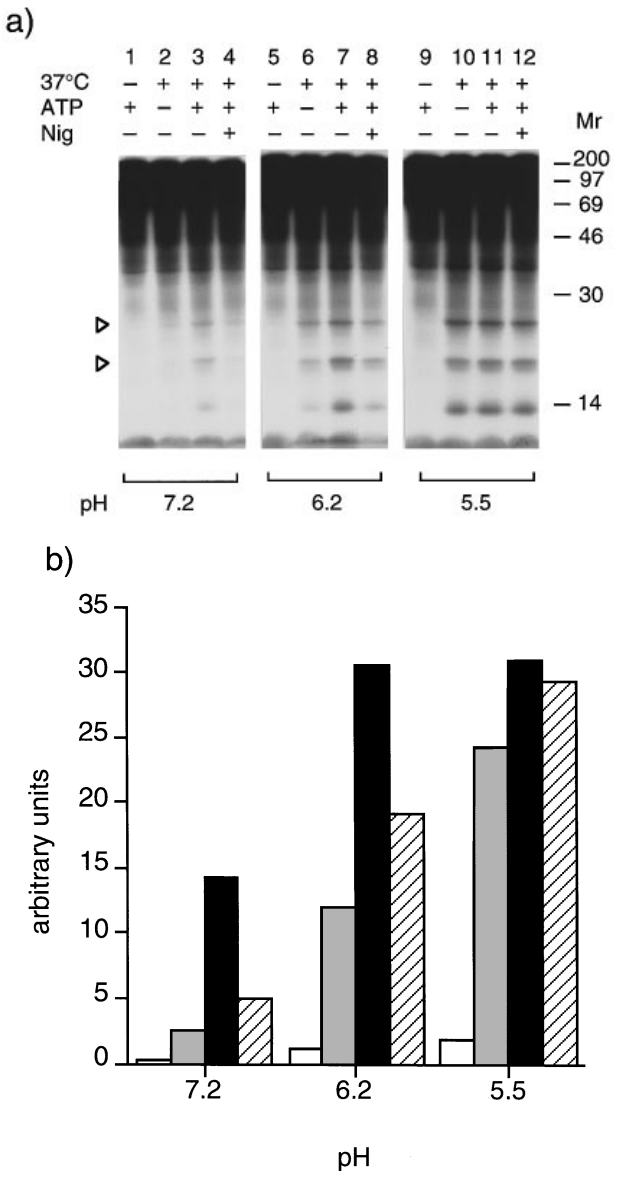


Figure 4 Acidification of the ISG lumen is sufficient for SgII processing by PC2

PC12/PC2 cells were labelled with a 5 min [^{35}S]sulphate pulse followed by a 15 min chase. (**a**) ISGs were prepared from the labelled cells and were preincubated in 20 mM Hepes (pH 7.2)/50 mM KOAc/1 mM $Mg(OAc)_2$ (lanes 1–4), 20 mM Mes (pH 6.2)/50 mM KOAc/1 mM $Mg(OAc)_2$ (lanes 5–8) or 20 mM Mes (pH 5.5)/50 mM KAc/1 mM $Mg(OAc)_2$ (lanes 9–12) for 1 h at 4 °C. Nigericin (Nig) was added to a final concentration of 100 nM, and an ATP-regenerating system was added where indicated. The samples were incubated for 45 min at 4 °C or 37 °C. ISGs were recovered by ultracentrifugation, and the samples were analysed by SDS/12% PAGE followed by autoradiography. Open arrowheads indicate the position of the SgII-processing products p26 and p18. The positions of molecular-mass markers (expressed as thousands) are indicated on the right-hand side. (**b**) The M_r 18000 band (p18) shown in (**a**) was quantified by PhosphorImager, and the extent of processing for each condition (open columns: 4 °C, +ATP; grey columns: 37 °C, −ATP; black columns: 37 °C, +ATP; hatched columns; 37 °C, +ATP, +nigericin) is shown in arbitrary units.

(Figure 4a, compare lanes 7 and 11); however, the ATP-dependent stimulation of processing was much reduced [Figure 4a (lanes 10 and 11) and Figure 4b]. Inclusion of nigericin during the incubation at 37 °C resulted in a signal that was comparable with the processing in the absence of ATP, suggesting that the intragranular pH was largely equilibrated with the external pH after a 1 h incubation at 4 °C [Figure 4a (compare lanes 2 and 4, 6 and 8, and 10 and 12) and Figure 4b].

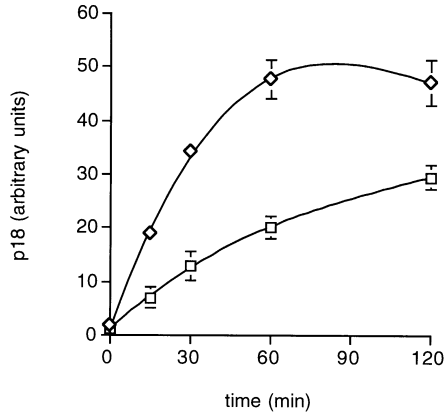


Figure 5 Time course of SgII processing by PC2 in isolated ISGs

[^{35}S]Sulphate-labelled ISGs were incubated in 20 mM Hepes (pH 7.2)/50 mM KOAc/1 mM $Mg(OAc)_2$ (□) or in 20 mM Mes (pH 5.5)/50 mM KOAc/1 mM $Mg(OAc)_2$ (◇) for 1 h at 4 °C. An ATP-regenerating system was added to those samples buffered at pH 7.2 (□), and all samples were incubated at 37 °C for the indicated periods of times. ISGs were recovered by ultracentrifugation, and the samples were analysed by SDS/12% PAGE. The appearance of the sulphated band corresponding to the processing product p18 was quantified with a PhosphorImager. Error bars represent the S.D.; pH 7.2: $n = 4$; pH 5.5: $n = 5$.

Time course of SgII processing by PC2 in isolated ISGs

Interestingly, the extent of processing obtained after 45 min incubation in the presence of ATP in a pH 7.2 buffer (Figure 4a, lane 3) was significantly less than the extent observed in pH 5.5 buffer in the presence or absence of ATP (Figure 4a, lanes 10 and 11) but was comparable with the processing obtained in pH 6.2 buffer in the absence of ATP (Figure 4a, lane 6). In order to see whether this result reflects different kinetics of processing at different pH values, we compared a time course of processing in ISGs at pH 7.2 in the presence of ATP with that of the processing in ISGs that had been pre-equilibrated in pH 5.5 buffer. Figure 5 shows that processing in granules that have been acidified by the activity of the vacuolar H^+-ATPase was indeed characterized by a much slower rate than that for processing in granules pre-equilibrated in pH 5.5 buffer.

The data presented in Figure 5 suggest a half-time of p18 accumulation of approx. 20 min at pH 5.5 compared with a much slower rate at pH 7.2 in the presence of ATP. As p18 appears to be an end product of processing (both *in vivo* and *in vitro*) we suggest that the half-time of SgII processing by PC2 in isolated ISGs at pH 5.5 is approx. 20 min. Given that acidification of a small vesicle like the ISG can be expected to reach a steady-state equilibrium within minutes [10], the slow kinetics of SgII processing at pH 7.2 in the presence of ATP indicates that the intragranular pH in ISGs generated by the vacuolar H^+-ATPase does not reach the optimal value for PC2 processing, and reflects the slower processing rate of PC2 at pH values above its pH optimum (pH 5.0–5.5).

From the data used to generate the curve in Figure 5, we could estimate that after 120 min of incubation at pH 5.5 approx. 40% of the radioactivity remains in p86 while the p18 band now accounts for about 16% of the total radioactivity (results not shown). This result is comparable with the extent of conversion

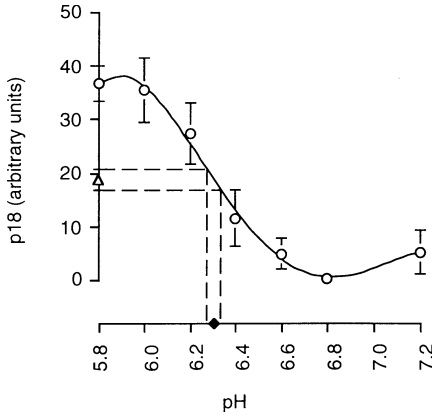


Figure 6 pH in isolated immature secretory granules

[^{35}S]Sulphate-labelled ISGs were incubated in buffers of different pH values (5.8–7.2) for 45 min at 37 °C in the presence of 100 nM nigericin, and the sulphated product p18 was quantified with a PhosphorImager to generate a standard curve. The extent of SgII processing in ISGs incubated at pH 7.2 in the presence of ATP was determined by quantifying p18, and the result (△; 18.82 ± 1.90 arbitrary units) was fitted on the standard curve (error bars indicate S.D.; $n = 6$). ◆ indicates the extent of processing in ISGs buffered at pH 6.3 ± 0.1.

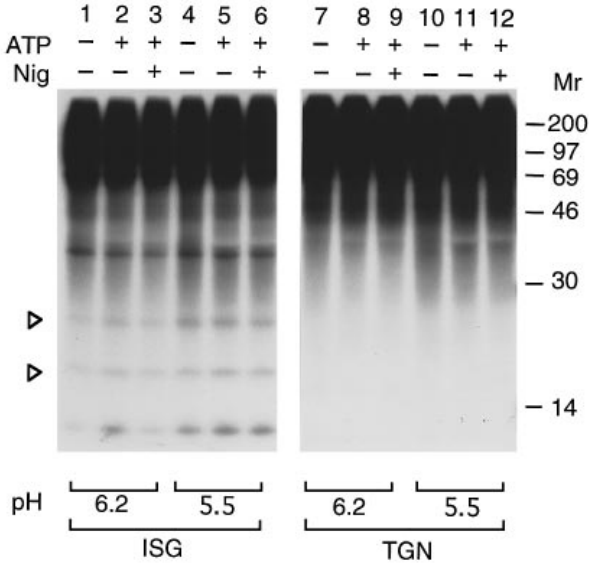


Figure 7 pH-dependent processing of SgII cannot be observed in isolated TGN

[^{35}S]Sulphate-labelled ISGs (lanes 1–6) and [^{35}S]sulphate-labelled TGN (lanes 7–12) were isolated in parallel and were preincubated in 20 mM Mes (pH 6.2)/50 mM KOAc/1 mM $Mg(OAc)_2$ (lanes 1–3 and 7–9) or 20 mM Mes (pH 5.5)/50 mM KOAc/1 mM $Mg(OAc)_2$ (lanes 4–6 and 10–12) for 1 h at 4 °C. Nigericin (Nig; final concentration of 100 nM) and an ATP-regenerating system were added where indicated. All samples were incubated for 45 min at 37 °C. ISGs were recovered by ultracentrifugation, and the samples were analysed by SDS/12% PAGE followed by autoradiography. Open arrowheads indicate the position of the SgII-processing products p26 and p18. The positions of molecular-mass markers (expressed as thousands) are indicated on the right-hand side.

observed in intact cells pulse-labelled for 5 min with [^{35}S]sulphate and chased for 270 min (see Figure 3).

SgII processing can be used as an indicator for intragranular pH

From these observations it seemed possible to use the processing of SgII by PC2 as an intrinsic pH indicator for ISGs. This type of 'functional measurement of vesicle acidification' [2] has the advantage that the acidification event takes place in an unequivocally defined organelle and therefore does not rely on the purity of the preparation. In other words, the observed processing of SgII labelled with a 5 min pulse of [^{35}S]sulphate followed by a 15 min chase by definition reflects an ISG-specific event, as ISGs are defined by this labelling procedure. Figure 6 shows the result of the pH calibration of SgII processing (solid line). Isolated ISGs were incubated in buffers of different pH values for 45 min at 37 °C, and the sulphated product p18 was quantified to generate a standard curve. To ensure complete equilibration, nigericin was added to these incubations. The rate of processing was linear for up to 45 min at 37 °C within the range of pH values used for this calibration (results not shown).

In the same experiment, the extent of SgII processing in the presence of ATP at pH 7.2 was determined, and the result of the p18 quantification from this sample was fitted on the standard curve. The extent of SgII processing observed in isolated ISGs under these conditions corresponds to the extent of processing in ISGs buffered at pH 6.3 ± 0.1 [see Figure 6 (◆)]. We would therefore propose a pH gradient close to 1.0 and an intragranular pH for ISGs of 6.3 ± 0.1 in a buffer with a physiological pH of 7.2. Interestingly, this value is comparable with the pH of the TGN as recently determined in an elegant study by Seksek et al. (pH 6.17 ± 0.02) [43]. In contrast, mature secretory granules isolated from different neuroendocrine cell types, including chromaffin cells, have been reported to be far more acidic (pH 5.0–5.5) [44,45].

pH-dependent processing of SgII cannot be observed in isolated TGN

Given the similarity between the pH values reported for the TGN [43] and the ISGs (Figure 6), we wondered whether processing of SgII by PC2 could actually take place in the TGN. Pulse–chase experiments in PC12/PC2 cells have shown that [^{35}S]sulphate-labelled SgII is not processed before its exit from the TGN [21]. However, this result could be simply a consequence of the short residence time of the labelled substrate in the TGN: it was previously shown that vesicles form at the TGN with a half-time of approx. 5 min [29]. We have shown here that processing of SgII by PC2 at a luminal pH of 6.3 is, in comparison, a relatively slow process ($t_{1/2} > 30$ min; Figure 5 and results not shown). We therefore isolated TGN from PC12/PC2 cells that contained SgII labelled by a 5 min [^{35}S]sulphate pulse [30] and analysed whether processing of SgII could occur in this isolated TGN in the absence of vesicle formation. ISGs were isolated from a parallel culture labelled for 5 min with [^{35}S]sulphate and were chased for 15 min.

First, ISGs and TGN were incubated under identical conditions at pH 7.2 in the absence or presence of ATP (in the absence of cytosol and GTP, that is under conditions that are unlikely to sustain the budding of vesicles [27]). As before (see Figure 2b), processing was observed in an ATP-dependent manner in ISGs but no genuine processing fragments could be detected in the TGN (results not shown). To ensure that acidification of the TGN was not limited by the generation of a membrane potential [46] we also assayed for processing both in the presence of chloride anions and in the presence of valinomycin and still did not observe any processing activity in the TGN (results not shown). Although the $^{+}$H-ATPase of the TGN is likely to share at least several subunits with the ISG H^+ pump [47], it is possible that the $^{+}$H-ATPase is inactive after isolation of the TGN. We

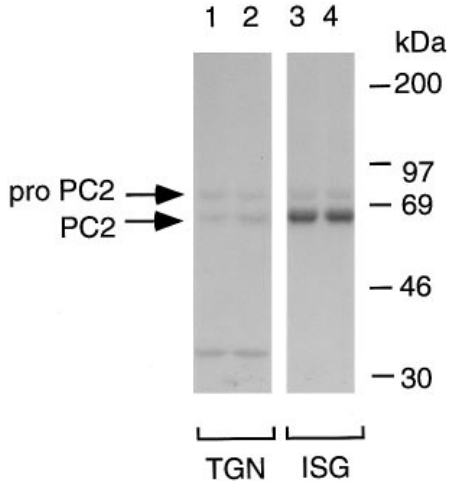


Figure 8 Relative abundance of pro-PC2 and mature PC2 in the TGN and in the ISG

Isolated ISGs (100 μg of total protein) and isolated TGN (100 μg of total protein) were incubated as for Figure 5, either in 20 mM Mes (pH 6.2)/50 mM KOAc/1 mM $Mg(OAc)_2$ (lanes 1 and 3) or 20 mM Mes (pH 5.5)/50 mM KOAc/1 mM $Mg(OAc)_2$ (lanes 2 and 4) for 1 h at 4 °C. After addition of 100 nM nigericin and an ATP-regenerating system, the samples were transferred to 37 °C for 45 min. The proteins were recovered by trichloroacetic acid precipitation, separated by SDS/7.5% PAGE and subjected to immunoblotting with antiserum 4BF. Bound antibody was detected with ^{125}I-labelled Protein A and was analysed by autoradiography. The positions of molecular-mass markers (expressed as thousands) are indicated on the right-hand side.

Table 1 Quantification of pro-PC2 and PC2 in TGN- and ISG-enriched fractions and determination of the ratio of PC2 to pro-PC2

The amounts of pro-PC2 and PC2 detected in TGN and ISG fractions for the representative experiment shown in Figure 6 were quantified and are shown in arbitrary units. Three independent experiments were carried out in duplicates, and the mean ratio of PC2 to pro-PC2 was determined.

	Pro-PC2 (arbitrary units)		PC2 (arbitrary units)		PC2/pro-PC2 (±S.E.M.)	
Fraction	pH 6.2	pH 5.5	pH 6.2	pH 5.5	pH 6.2	pH 5.5
TGN	11678	9835	10735	16988	1.22±0.09	2.28±0.42
ISG	13947	13450	107356	110174	8.78±1.65	8.89±1.33

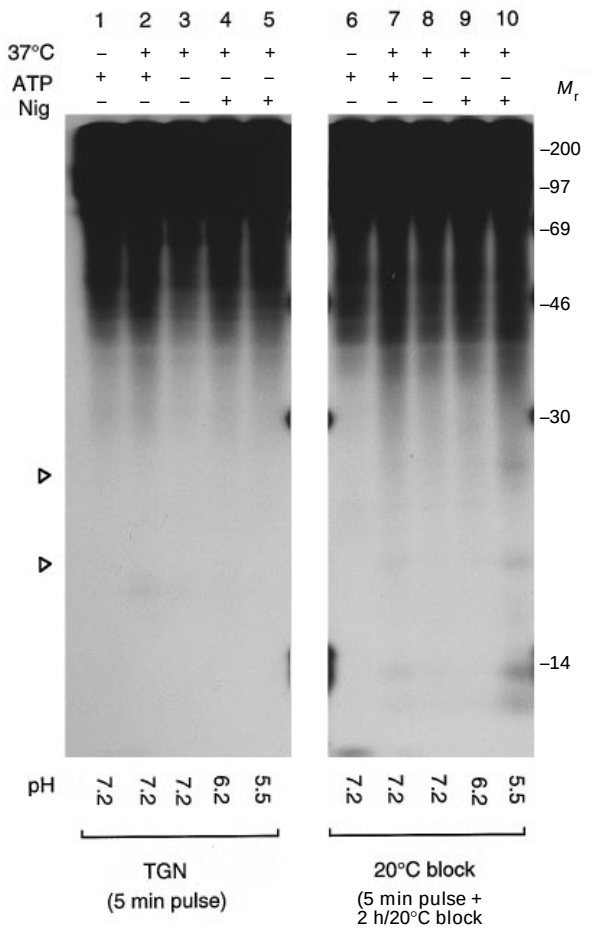


Figure 9 Processing of SgII in semi-permeabilized cells after a 20 °C block

PC12/PC2 cells were labelled with a 5 min [^{35}S]sulphate pulse and were either permeabilized immediately (lanes 1–5) or chased for 2 h at 20 °C before permeabilization (lanes 6–10). The semi-permeabilized cells were washed to remove the cytosol and were incubated for 45 min in buffers of pH 7.2, 6.2 or 5.5 (see Figure 3). An ATP-regenerating system (ATP) and nigericin (Nig; final concentration 100 nM) were added where indicated 10 min before the incubation at 37 °C. After incubation the cells were lysed, a heat-stable fraction was prepared, and the heat-stable proteins were analysed by SDS/12% PAGE and autoradiography. Open arrowheads indicate the position of the SgII-processing products p26 and p18. The positions of molecular-mass markers (expressed as thousands) are indicated on the right-hand side.

therefore preincubated TGN in pH 6.2 or pH 5.5 buffer in the presence of nigericin in order to acidify the TGN lumen. Even under these conditions we were not able to obtain any evidence for processing of SgII in this compartment (Figure 7).

Activation of PC2 requires cleavage of the pro-form to the mature form. The lack of PC2 activity in the TGN might thus reflect the state of PC2 maturation in this compartment. We therefore analysed the relative ratios of the pro- and mature PC2 forms in TGN and ISGs after incubation at pH 6.2 or pH 5.5 for 45 min (identical conditions as in Figure 7, lanes 3, 6, 9 and 12). Immunoblotting of TGN and ISGs (equal amounts of protein were loaded) with an antibody directed against the C-terminus of PC2 suggested that pro-PC2 and PC2 are found at a 1:1 ratio in the TGN, whereas in the ISGs the mature form of PC2 is enriched approx. 9 times greater than pro-PC2 (Figure 8 and Table 1). Although a larger proportion of PC2 is mature in the ISGs, the lack of SgII processing in the TGN cannot be explained by the absence of mature PC2 in this compartment. It should, however, be noted that PC2 is enriched by 3–6-fold in ISGs as compared with the TGN: the increased concentration of PC2 upon packaging into ISGs might enhance PC2 conversion and be crucial for PC2 activity towards SgII.

Processing of SgII by PC2 can be observed in semi-permeabilized cells after a 20 °C block

Recently Xu and Shields [27] showed that prosomatostatin processing by PC2 in permeabilized GH3 cells can be uncoupled from budding of secretory vesicles from the TGN using a 20 °C block. Processing in the TGN was solely ATP dependent, whereas formation of secretory vesicles needed the addition of both ATP and GTP [27]. The requirement of ATP for prosomatostatin processing in the TGN was circumvented by incubation of the permeabilized cells in acidic buffers in the presence of a protono-

phore [9]. We have shown above that after similar treatments we do not observe processing of SgII by PC2 in isolated TGN (Figure 7). This discrepancy led us to wonder whether the TGN, defined by a 5 min pulse of [^{35}S]sulphate, is functionally equivalent to the TGN in cells blocked for 2 h at 20 °C. We therefore compared processing in semi-intact cells that had been previously pulse-labelled for 5 min and chased at 20 °C for 2 h with cells that had been permeabilized immediately after the 5 min [^{35}S]-sulphate pulse. The semi-intact cells were washed extensively to remove the cytosol and were incubated under conditions equivalent to the experiments in Figures 2 and 4. At pH 5.5 in the presence of nigericin the processing products p18 and p26 are clearly visible only in cells that have been blocked at 20 °C (Figure 9, compare lanes 5 and 10). At pH 7.2 in the presence of ATP, the signals for p18 and p26 are much weaker but still recognizable (lane 7). In contrast, in cells that have only been pulse-labelled a band of approx. M_r 16000 appears at pH 7.2 in the presence of ATP, but no p26 or p18 was detected even after prolonged exposure (Figure 9, lane 2). The M_r 16000 protein has also been seen in experiments with isolated TGN-membranes (results not shown) but has not been observed in intact cells and might be generated by a different endopeptidase with a neutral pH optimum.

DISCUSSION

We have previously established a PC12 cell line that is stably transfected with the endopeptidase PC2. Pulse–chase experiments using radioactive sulphate in combination with antibody mapping were used to show that in these PC12/PC2 cells SgII was converted by PC2 to distinct lower-molecular-mass products [21], including secretoneurin ([48]; H. Winkler and S. A. Tooze, unpublished work). We have now demonstrated that processing of SgII to an identical set of products can be observed in isolated ISGs derived from PC12/PC2 cells. The sequential appearance of the different processing products correlated well with the results obtained from the time course in intact cells (Figure 3; [21]).

Addition of ATP at pH 7.2 to isolated ISGs stimulated processing by approx. 4-fold. This ATP-induced processing could be completely abolished by either inhibition of the vacuolar H^+-ATPase with concanamycin A or by neutralizing its activity through inclusion of a K^+/H^+ ionophore or a weak base. These results are in good agreement with the previous finding by others that proinsulin conversion can be observed in immature β-granules isolated from rat pancreatic islets [49]. Both PC2 and PC1 (also known as PC3, another member of the eukaryote subtilisin family) are required for conversion of proinsulin to insulin [35,50,51]. Rhodes et al. [49] also observed a protonophore-sensitive stimulation of processing by ATP and concluded that the ATP effect reflected a requirement for an acidic intragranular pH. The use of the specific inhibitor concanamycin A allows us to show unequivocally that stimulation of processing by ATP is indeed due to activation of the vacuolar H^+-ATPase.

Processing of SgII by PC2 is not complete either in isolated ISGs or in intact PC12/PC2 cells (Figure 3; [21]). The final extent of processing obtained in isolated ISGs at pH 5.5 is, however, similar to the extent of processing observed in intact cells. A possible explanation for the incomplete processing of SgII both *in vitro* and *in vivo* in PC12/PC2 cells might be that a population of full-length SgII, and processing intermediates, remains or becomes inaccessible to PC2 as a result of the progressive condensation of the granule core. This could explain why SgII processing does not reach completion even after prolonged storage in the secretory granules.

Variations in the extent of SgII processing in other cell types [48] have been observed. PC12 cells are derived from a phaeochromocytoma, and the processing efficiency in our PC12/PC2 cells is therefore best compared with chromaffin cells that express both SgII and PC2 endogenously. Interestingly, the processing efficiency observed in our PC12/PC2 cells is very comparable with that observed in bovine chromaffin cells, where up to 50 % of the proprotein remains unprocessed [52], attesting to the validity of our cellular model. However, it cannot be excluded that the expression level or the activity of the PC2 chaperone 7B2 is a limiting factor for SgII processing in PC12/PC2 cells. 7B2 has been shown to play a principal role in the regulation of PC2 [25,53] and is present in PC12 cells [54] and in PC12/PC2 cells (I. Lindberg and S. A. Tooze, unpublished work). However, the level of 7B2 in PC12/PC2 cells is significantly less than in rat chromaffin cells (results not shown). Overexpression of 7B2 has been shown to faciliate the conversion of proPC2 into mature PC2 [53], and the processed form of 7B2 was able to enhance the *in vitro* cleavage of POMC in lysates from *Xenopus* intermediate pituitary cells [55]. The majority of PC2 that is stored in secretory granules in PC12/PC2 cells appears to be converted into the mature form (Figure 8; results not shown); hence PC2 activation is not impaired in PC12/PC2 cells. Further work on the activity of 7B2 on substrate processing in general, and the role of 7B2 in PC2-dependent SgII-processing in PC12/PC2 cells in particular, will be required to address this question fully.

The intriguing observation that processing cannot be observed in the TGN, defined operationally as the sulphation compartment, but can be observed in the 20 °C block compartment might point to a fundamental difference in the functional characteristics of the TGN in a cell at steady state and in a cell that has been subjected to a temperature block. Secretory proteins accumulate at 20 °C, and it is conceivable that microdomains are formed within the TGN in cells incubated at 20 °C that mimic the environment of the secretory granule. In these microdomains, the local concentration of PC2 and SgII (or any other granule enzyme and its substrate) might be greater than in the TGN of a cell at steady state. Co-aggregation of enzyme and substrate might be crucial for efficient proteolytic activity. This could explain why PC2 does not appear to be active in the TGN of PC12/PC2 cells even though approx. 50 % of PC2 is in the mature form.

Alternatively, the passage from the TGN to the 20 °C block environment or ISG environment could result in the removal of inhibitory factors which may limit PC2 activity. The PC2 chaperone 7B2 has been shown to inhibit PC2 activity *in vitro* [56]. The full-length protein (intact 7B2) was effective as an inhibitor, whereas the cleaved protein was not. It has been proposed that cleavage of 7B2 precedes PC2 conversion [25,53]. However, it is conceivable that the C-terminal fragment of 7B2, to which the inhibitory properties of the protein are confined [56,57], does not dissociate from the mature form of PC2 in the milieu of the TGN. Alternatively, another, as yet unidentified, factor might influence PC2 activity in the TGN of PC12/PC2 cells. This point could be addressed in the future by further analysing the activation of PC2 in the secretory pathway of PC12/PC2 cells.

Although the internal pH of mature secretory granules (MSGs) in a variety of tissues has been studied in detail by others [44,45,58], very few data are so far available on the luminal pH of ISGs. Conventional methods for pH measurements, such as accumulation of radioactively labelled weak bases in acidic compartments, are amenable only when large quantities of easily purified vesicles (e.g. chromaffin granules from adrenal medulla) are available [44,45,58,59]. The following two reasons in par-

ticular contribute to the difficulty in obtaining data on the internal pH of ISGs. (1) ISGs in any cell type constitute a short kinetic intermediate in the biogenesis of secretory granules (half-time of maturation: 45 min) and therefore represent a very small proportion of the total secretory granule population. (2) It is difficult to obtain a pure preparation of ISGs that is absolutely free of any contamination from the large pool of MSGs.

In this study we have undertaken the measurement of the pH of isolated ISGs *in vitro*. The use of an endogenous pH-dependent reaction in the ISG, i.e. processing of a granule-specific protein (SgII) by a granule-specific enzyme (PC2), as an indicator for internal pH eliminates the possibility that the results might be attributed to a contaminating membrane population. In addition, the ISG is operationally defined by a pulse–chase labelling protocol, thereby strictly limiting all observations to the population of secretory granules that contain SgII labelled by a 5 min [^{35}S]sulphate pulse followed by a 15 min chase. From our observations, we have obtained a pH value of 6.3 ± 0.1 for isolated ISGs derived from PC12/PC2 cells.

In a recent study, Orci and co-workers correlated the pH in β-cell granules with processing of proinsulin [60]. This study is very informative regarding the pH dependence of the endoproteolytic cleavages that underlie proinsulin processing at steady state. The pH of the granules was measured by immunolocalization of DAMP [3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine], a weak base that accumulates in acidic compartments [1]. Processing of proinsulin in these granules was followed by double-labelling with an antibody that specifically recognizes proinsulin. Immature β-cell granules were identified by their high proinsulin content. The mean pH of these proinsulin-rich granules was found to be 6.3 and can be taken to support the data presented here.

The similarity of the pH value obtained both for the immature β-cell granules and ISGs isolated from PC12/PC2 cells to the pH determined by others for the TGN [43] suggests that acidification below pH 6.3 is not a direct consequence of budding from the TGN but requires additional factors and/or events to reach the final pH of 5.5 that is characteristic for MSGs. Previous studies have suggested that maturation in PC12 cells involves fusion of several ISGs to form one MSG that is characterized by both a larger size and a greater density [15]. It was suggested that the partial clathrin coat seen on ISGs in a variety of cell types [61,62] functions to remove excess membrane (a result of the fusion of more than four spheres) via clathrin-coated vesicle formation [16]. It is tempting to speculate that acidification to a value below that of both the TGN and the ISG might require membrane fusion and retrieval of excess membrane. These membrane remodelling events could allow both a concentration of the vacuolar H^+-pumping ATPase (via an increase in linear density) and removal of proteins with a potentially adverse activity on acidification (e.g. Na^+/K^+-ATPase [63,64]).

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REFERENCES

1 Anderson, R. G. and Orci, L. (1988) J. Cell Biol. **106**, 539–543
2 Mellman, I., Fuchs, R. and Helenius, A. (1986) Annu. Rev. Biochem. **55**, 663–700
3 Johnson, R. G. (1988) Physiol. Rev. **68**, 232–307
4 Clague, M. J., Urbé, S., Aniento, F. and Gruenberg, J. (1994) J. Biol. Chem. **269**, 21–24
5 Johnson, L. S., Dunn, K. W., Pytowski, B. and McGraw, T. E. (1993) Mol. Biol. Cell **4**, 1251–1266
6 Henomatsu, N., Yoshimori, T., Yamamoto, A., Moriyama, Y. and Tashiro, Y. (1993) Eur. J. Cell Biol. **62**, 127–139
7 Yilla, M., Tan, A., Ito, K., Miwa, K. and Ploegh, H. L. (1993) J. Biol. Chem. **268**, 19092–19100
8 Reaves, B. and Banting, G. (1994) FEBS Lett. **345**, 61–66
9 Xu, H. and Shields, D. (1994) J. Biol. Chem. **269**, 22875–22881
10 Al-Awqati, Q. (1986) Annu. Rev. Cell Biol. **2**, 179–199
11 Forgac, M. (1989) Physiol. Rev. **69**, 765–796
12 Njus, D., Kelly, P. M. and Harnadek, G. J. (1986) Biochim. Biophys. Acta **853**, 237–265
13 Chanat, E. and Huttner, W. B. (1991) J. Cell Biol. **115**, 1505–1519
14 Halban, P. A. and Irminger, J. C. (1994) Biochem. J. **299**, 1–18
15 Tooze, S. A. and Stinchcombe, J. C. (1992) Semin. Cell Biol. **3**, 357–366
16 Tooze, S. A., Flatmark, T., Tooze, J. and Huttner, W. B. (1991) J. Cell Biol. **115**, 1491–1503
17 Johnson, R. G., Carty, S. E. and Scarpa, A. (1985) Ann. N.Y. Acad. Sci. **456**, 254–267
18 Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Perrelet, A., Vassalli, J.-D. and Anderson, R. G. W. (1986) J. Cell Biol. **103**, 2273–2281
19 Orci, L., Ravazzola, M., Storch, M. J., Anderson, R. G., Vassalli, J. D. and Perrelet, A. (1987) Cell **49**, 865–868
20 Huttner, W. B., Gerdes, H.-H. and Rosa, P. (1991) Trends Biochem. Sci. **16**, 27–30
21 Dittié, A. S. and Tooze, S. A. (1995) Biochem. J. **310**, 777–787
22 Seidah, N. G., Chrétien, M. and Day, R. (1994) Biochimie **76**, 197–209
23 Benjannet, S., Rondeau, N., Paquet, L., Boudreault, A., Lazure, C., Chrétien, M. and Seidah, N. G. (1993) Biochem. J. **294**, 735–743
24 Matthews, G., Shennan, K. I. J., Seal, A. J., Taylor, N. A., Colman, A. and Docherty, K. (1994) J. Biol. Chem. **269**, 588–592
25 Braks, J. A. M. and Martens, G. J. M. (1994) Cell **78**, 263–273
26 Schnabel, E., Mains, R. E. and Farquhar, M. G. (1989) Mol. Endocrinol. **3**, 1223–1235
27 Xu, H. and Shields, D. (1993) J. Cell Biol. **122**, 1169–1184
28 Tooze, J., Hollinshead, M., Frank, R. and Burke, B. (1987) J. Cell Biol. **105**, 155–162
29 Tooze, S. A. and Huttner, W. B. (1990) Cell **60**, 837–847
30 Tooze, S. A. and Huttner, W. B. (1992) Methods Enzymol. **219**, 81–93
31 Lee, R. W. H. and Huttner, W. B. (1983) J. Biol. Chem. **258**, 11326–11334
32 Shennan, K. I. J., Taylor, N. A., Jermany, J. L., Matthews, G. and Docherty, K. (1995) J. Biol. Chem. **270**, 1402–1407
33 Shennan, K. I. J., Smeekens, S. P., Steiner, D. F. and Docherty, K. (1991) FEBS Lett. **284**, 277–280
34 Arden, S. D., Rutherford, N. G., Guest, P. C., Curry, W. J., Bailyes, E. M., Johnston, C. F. and Hutton, J. C. (1994) Biochem. J. **298**, 521–528
35 Davidson, H. W., Rhodes, C. J. and Hutton, J. C. (1988) Nature **333**, 93–96
36 Nelson, N., Cidon, S. and Moriyama, Y. (1988) Methods Enzymol. **157**, 619–633
37 Al-Awqati, Q., Barasch, J. and Landry, D. W. (1989) Soc. Gen. Physiol. Ser. **44**, 283–294
38 Cidon, S. and Nelson, N. (1983) J. Biol. Chem. **258**, 2892–2898
39 Cidon, S. and Nelson, N. (1986) J. Biol. Chem. **261**, 9222–9227
40 Moriyama, Y. and Nelson, N. (1987) J. Biol. Chem. **262**, 9175–9180
41 Droese, S., Bindseil, K. U., Bowman, E. J., Siebers, A., Zeeck, A. and Altendorf, K. (1993) Biochemistry **32**, 3902–3906
42 Johnson, R. G. and Scarpa, A. (1976) J. Gen. Physiol. **68**, 601–631
43 Seksek, O., Biwersi, J. and Verkman, A. S. (1995) J. Biol. Chem. **270**, 4967–4970
44 Johnson, R. G. and Scarpa, A. (1975) J. Biol. Chem. **251**, 2189–2191
45 Hutton, J. C. (1982) Biochem. J. **204**, 171–178
46 Glickman, J., Croen, K., Kelly, S. and Al-Awqati, Q. (1983) J. Cell Biol. **97**, 1303–1308
47 Moriyama, Y. and Nelson, N. (1989) J. Biol. Chem. **264**, 18445–18450
48 Kirchmair, R., Hogue Angeletti, R., Gutierrez, J., Fischer-Colbrie, R. and Winkler, H. (1993) Neuroscience **53**, 359–365
49 Rhodes, C. J., Lucas, C. A., Mutkoski, R. L., Orci, L. and Halban, P. (1987) J. Biol. Chem. **262**, 10712–10717
50 Bennett, D. L., Bailyes, E. M., Nielsen, E., Guest, P. C., Rutherford, N. G., Arden, S. D. and Hutton, J. C. (1992) J. Biol. Chem. **267**, 15229–15236
51 Bailyes, E. M., Shennan, K. I., Seal, A. J., Smeekens, S. P., Steiner, D. F., Hutton, J. C. and Docherty, K. (1992) Biochem. J. **285**, 391–394
52 Fischer-Colbrie, R., Hagn, C. and Schober, M. (1987) Ann. N.Y. Acad. Sci. **493**, 120–134
53 Zhu, X. and Lindberg, I. (1995) J. Cell Biol. **129**, 1641–1650
54 Seidah, N. G., Chrétien, M. and Day, D. (1994) Biochimie **76**, 197–209
55 Braks, J. A. M. and Martens, G. J. M. (1995) FEBS Lett. **371**, 154–158
56 Martens, G. J., Braks, J. A. M., Eib, D. W., Zhou, Y. and Lindberg, I. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 5784–5787
57 Van Horssen, A. M., Van den Hurk, W. H., Bailyes, E. M., Hutton, J. C., Martens, G. J. M. and Lindberg, I. (1995) J. Biol. Chem. **270**, 14292–14296

58 Johnson, R. G., Carty, S. E., Fingerhood, B. J. and Scarpa, A. (1980) FEBS Lett. **120**, 75–79
59 Russell, J. T. and Holz, R. W. (1981) J. Biol. Chem. **256**, 5950–5953
60 Orci, L., Halban, P., Perrelet, A., Amherdt, M., Ravazzola, M. and Anderson, R. G. W. (1994) J. Cell Biol. **126**, 1149–1156
61 Tooze, J. and Tooze, S. A. (1986) J. Cell Biol. **103**, 839–850
62 Orci, L., Ravazzola, M., Amherdt, M., Louvard, D. and Perrelet, A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 5385–5389
63 Cain, C. C., Sipe, D. M. and Murphy, R. F. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 544–548
64 Fuchs, R., Schmid, S. and Mellman, I. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 539–543

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