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

Lucinda Carnell and Hsiao-Ping H. Moore

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

Abstract. To gain insight into the mechanisms governing protein sorting, we have developed a system that reconstitutes both the formation of immature secretory granules and their fusion with the plasma membrane. Semi-intact PC12 cells were incubated with ATP and cytosol for 15 min to allow immature granules to form, and then in a buffer containing 30 μM $[Ca^{2+}]_{free}$ to induce exocytosis. Transport via the regulated pathway, as assayed by the release of secretogranin II (SgII) labeled in the TGN, was inhibited by depletion of ATP, or by the inclusion of 100 μM GTPγS, 50 μM AlF$_{3-5}$ or 5 μg/ml BFA. When added after immature granules had formed, GTPγS stimulated rather than inhibited exocytosis. Thus, exocytosis of immature granules in this system resembles the characteristics of fully matured granules. Transport of SgII via the regulated pathway occurred at a fourfold higher efficiency than glycosaminoglycan chains, indicating that SgII is sorted to some extent upon exit from the TGN. Addition of A23187 to release Ca^{2+} from the TGN had no significant effect on sorting of SgII into immature granules. In contrast, depletion of lumenal calcium inhibited the endoproteolytic cleavage of POMC and proinsulin. These results establish the importance of *intra*-cisternal Ca^{2+} in prohormone processing, but raise the question whether lumenal calcium is required for proper sorting of SgII into immature granules. Disruption of organelle pH gradients with an ionophore or a weak base resulted in the inhibition of transport via both the constitutive and the regulated pathways.

N eucaryotic cells, proteins directed to the plasma membrane enter the ER and travel via transport vesicles through the Golgi before delivery to the cell surface (Pfeffer and Rothman, 1987). In the TGN, proteins targeted for secretion are sorted into two distinct exocytic pathways: constitutive and regulated (for reviews, see Burgess and Kelly, 1987; Miller and Moore, 1990). The regulated pathway, characteristic of neuronal, exocrine, and endocrine cell types, packages proteins into granules where they are stored until an appropriate physiological signal triggers their release, whereas the constitutive pathway delivers proteins directly to the plasma membrane. One cell line that has been useful for studying regulated secretion is the PC12 cell line (Greene and Tischler, 1976), derived from a rat adrenal medulla tumor. PC12 cells have may characteristics of neuronal cells; they secrete the neurotransmitter norepinephrine in response to membrane depolarization (Green and Rein, 1977) and form neuronal processes upon treatment with nerve growth factor (Greene and Tischler, 1976). PC12 cells also have the ability to sort regulated proteins very efficiently (>95%) into the regulated pathway (Rosa et al., 1985; Gerdes et al., 1989; Miller et al., 1992). The sorting can be measured using an easily identifiable marker, secretogranin II (SgII),[1] which is tyrosine sulfated in TGN (Baeuerle and Huttner, 1987; Niehrs and Huttner, 1990; Rosa et al., 1992a) and therefore can be monitored by pulse labeling with [^{35}S]sulfate. Several previous studies have used PC12 cells to investigate individual steps of the regulated secretory pathway, such as the formation of regulated granules from the TGN (Tooze and Huttner, 1990), or the fusion of mature secretory granules with the plasma membrane (Ahnert-Hilger et al., 1985; Peppers and Holz, 1986; Ahnert-Hilger and Gratzl, 1987; Carroll et al., 1990; Hay and Martin, 1992). In this paper, we describe a semi-intact cell system using streptolysin-O (SL-O)–permeabilized cells to reconstitute the formation of immature regulated granules from the TGN and their targeting/fusion with the plasma membrane. Such a system provides us the opportunity to examine the processes of sorting,

1. *Abbreviations used in this paper:* ACTH, adrenocorticotropic hormone; GAG chains, glycosaminoglycan chains; NEM, N-ethylmaleimide; POMC, proopiomelanocortin; SgII, secretogranin II; SL-O, streptolysin-O.

budding, targeting, and fusion of regulated secretory granules within the same experimental system.

One advantage to using an in vitro system to study transport is that we are able to control cytoplasmic ionic conditions during the transport process. The ionic conditions in the cytoplasm and within the lumen of organelles are essential in a variety of vesicular transport events. In particular, it has been proposed that Golgi/TGN lumenal calcium and pH are critical in sorting proteins into the regulated secretory pathway (see below). The role of these ionic conditions in sorting has been difficult to assess in intact cells. The problem with intact cells is that one cannot properly control both cytoplasmic and lumenal ionic conditions by relying on ionophores to work effectively across both plasma and organelle membranes. Furthermore, moderate increases in cytoplasmic calcium (e.g., due to ineffective buffering) could cause release of proteins from regulated secretory granules thereby complicating sorting measurements. In SL-O–perforated cells, cytoplasmic ionic conditions can be effectively buffered and the lumenal ions can be manipulated with ionophores or other reagents which can gain access directly to the cytoplasm.

The mechanisms for protein sorting between the constitutive and the regulated pathway are not well understood. Entry of proteins from TGN into the constitutive pathway is thought to proceed by a bulk flow process, while transport of proteins into the regulated pathway requires a positive sorting signal (Burgess and Kelly, 1987; Miller and Moore, 1990). A postulated model for the sorting of regulated proteins is that selective aggregation of these proteins in the TGN causes them to be segregated away from nonaggregated, constitutive proteins (Burgess and Kelly, 1987; Pfeffer and Rothman, 1987; Huttner and Tooze, 1989; Chanat and Huttner, 1991). This model is based on morphological data (Orci et al., 1987) that proteins destined for the regulated pathway appear to aggregate near budding sites at the TGN. The model is also corroborated by biochemical evidence which suggests that the ionic milieu of the TGN may facilitate selective aggregation of certain regulated secretory proteins. In vitro studies have shown that under low pH (5.2 and 6.4) and high calcium (10 mM) conditions, secretogranins and other exocrine granule proteins aggregate while constitutive proteins do not (Gerdes et al., 1989; Gorr et al., 1989; Yoo and Albanesi, 1990; Chanat and Huttner, 1991; LeBlond et al., 1993). Aggregation under these conditions is consistent with the estimated pH of 6.4 within the TGN (Anderson and Pathak, 1985), and the estimated calcium concentration within the Golgi lumen, believed to be in the millimolar range (Roos, 1988; Chandra et al., 1991). However, the importance of these lumenal ionic conditions in sorting has not been directly tested in reconstituted transport systems where these conditions can be more easily manipulated. In this report, we have used our semi-intact cell transport system to examine the role of TGN lumenal calcium and pH in the transport and the sorting of regulated secretory proteins in PC12 cells.

Materials and Methods

Materials

SL-O was purchased from Burroughs-Wellcome (Research Triangle Park,

NC). It was reconstituted as 20 I.U./ml stock solution in distilled H_2O and frozen in aliquots at $-80°C$ until use. EGTA was purchased from Fluka AG (Buchs, Switzerland). ATP, creatine kinase, creatine phosphate, BFA, and A23187 were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). N-ethylmaleimide, nigericin, hexokinase, 4-methylumbelliferyl β-D-xyloside, and fetal calf serum were obtained from Sigma Chemical Co. (St. Louis, MO). [^{35}S]Na_2SO_4 was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). DME was purchased from Bio-Whittaker (Walkersville, MD). Enriched calf serum and horse serum were purchased from Gemini Bioproducts, Inc. (Calabasas, CA).

Cell Culture

PC12 cells were obtained from Dr. Stuart Feinstein (University of California, Santa Barbara, CA) and were maintained in DME containing 5% enriched calf serum and 5% horse serum under 10% CO_2 atmosphere, AtT-20-NIH cells (a subclone of the AtT-20 cell line) were maintained in DME containing 10% fetal calf serum under 15% CO_2 atmosphere.

Semi-intact Cell Transport Assay

Transport of Secretogranin from the TGN to the Cell Surface via the Regulated Secretory Pathway. PC12 cells were plated onto 12-well plates coated with poly-D-lysine (50 μg/ml) at a density of 5×10^5 per well and grown for 2–3 d. The cells were rinsed twice in buffer A (20 mM Na-Hepes, pH 7.2, 110 mM NaCl, 5.4 mM KCl, 0.9 mM Na_2HPO_4, 10 mM $MgCl_2$, 2 mM $CaCl_2$, and 1 g/l glucose) and then incubated in buffer A for 30 min at 37°C to deplete cells of unlabeled sulfate. The cells were pulse labeled with buffer A containing 1 mCi/ml [^{35}S]sulfate (250 μl/well) for 5 min at 37°C to label SgII in the TGN. The labeling buffer was aspirated and the cells were washed in buffer B (125 mM Na-glutamate, 15 mM NaCl, 20 mM Hepes, pH 7.2, 5 mM Mg-acetate, 5 mM EGTA, and 5 mM Na_2SO_4). Semi-intact cells were prepared as follows. The cells were permeabilized by the addition of 250 μl of buffer B containing 3 IU/ml SL-O for 8 min on ice. After prebinding, the excess SL-O was removed and replaced with ice-cold buffer B. The plate was then floated in a 37°C water bath for 3 min to allow for pore formation. The cells were then incubated on ice for 10 min in transport buffer C (125 mM K glutamate, 15 mM KCl, 5 mM EGTA, Mg-acetate to 5 mM free magnesium ions, 20 mM Hepes, pH 7.2) and an ATP-regenerating system (500 μM ATP, 40 IU/ml creatine kinase, and 2 mM creatinine phosphate). In some experiments, the concentration of free calcium in buffer C was adjusted with a Ca/EGTA buffering system, and the concentrations of free calcium and magnesium ions were determined using the program Mathematica (Wolfram Research Inc., Champaign, IL), with the stability constants of the EGTA complexes corrected for temperature and pH as described by Martell and Smith (1974). Where indicated, bovine brain cytosol (prepared as described in Miller and Moore, 1992) was added to each reaction mixture at 60–75 μg/ml. The plate was then floated in a water bath for 15 min at 37°C to allow for the budding of vesicles from the TGN. To induce release of labeled SgII from granules, the buffer was collected from the cells and replaced with buffer C containing an ATP-regenerating system and 30 μM buffered free calcium ions. The cells were then incubated for an additional 15 min at 37°C. Unstimulated release was determined during the same time period in a parallel culture using a calcium-free transport buffer (buffer C). For each experiment a control was included, in which parallel cultures were treated with an ATP-depleting system consisting of 30 IU/ml hexokinase and 2 mM glucose instead of an ATP-regenerating system. Release of SgII from intact PC12 cells was performed as previously described in (Miller et al., 1992). Media were collected into microfuge tubes and centrifuged to pellet any detached cells. The supernatants containing 5 μg BSA as a carrier were then precipitated with trichloroacetic acid (10% final concentration) on ice for 10 min, and centrifuged at 4°C for 30 min at 3,000 rpm in a GPR centrifuge (Beckman Instruments, Inc., Fullerton, CA). The pellets were rinsed with acetone ($-20°C$) and spun for 20 min at 3,000 rpm at 4°C. The supernatants were aspirated and the pellet air dried. Laemmli sample buffer (Laemmli, 1970) was added to the pellets and the samples were boiled for 5 min before analysis by SDS-PAGE (12.5% gel). The cells were extracted with 100 μl of NDET buffer (1% Nonidet P-40, 0.4% wt/vol, sodium deoxycholate, 66 mM EDTA, and 10 mM Tris, pH 7.4). 1/10 of the cell extract was added to an equal volume of 2× Laemmli sample buffer and boiled for 5 min before analysis by SDS-PAGE. Radiolabeled SgII bands were quantitated by exposing the gels to a PhosphorImager screen and analyzed with the Image-Quant program (Molecular Dynamics, Sunnyvale, CA).

Constitutive Transport of Glycosaminoglycan Chains from the TGN to the Cell Surface. PC12 cells were plated at 5×10^5 per well onto 12-

well plates and rinsed three times in buffer A. The cells were incubated in buffer A containing 1 mM 4-methylumbelliferyl xyloside (xyloside) for 30 min to induce synthesis of free glycosaminoglycan chains. Cells were then pulse labeled with 250 μl of buffer A containing 1 mM xyloside and [^{35}S]sulfate at 0.5 mCi/ml for 3 min at 37°C. The labeled cells were rinsed in buffer B, permeabilized as described above, and incubated for the indicated times in buffer C containing 60–75 μg/ml bovine brain cytosol and an ATP-regenerating system. Media were collected and cells were extracted with PBS containing 0.5% Triton X-100. The amount of labeled GAG chains was quantitated using the precipitation/filtration assay as previously described (Miller and Moore, 1992).

Processing of Proopiomelanocortin in AtT-20 Cells

AtT-20 cells were plated at a density of 5 × 10^5 cells per well onto a six-well plate and grown for 3–4 d. The cells were rinsed twice and starved for 30 min in buffer A. The cells were then labeled in 250 μl buffer A containing [^{35}S]sulfate (2.5 mCi/ml) for 5 min followed by a rinse with buffer D (127 mM NaCl, 5 mM KCl, 0.33 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 4.2 mM NaHCO$_3$, 20 mM Hepes, pH 7.2, and 5.6 mM glucose) containing 2.2 mM CaCl$_2$ and 5 mM Na$_2$SO$_4$. Next the cells were pretreated on ice for 3 min with buffer D containing 5 mM MgCl$_2$ plus 5 mM EGTA either in the presence or absence of 10 μM A23187. The buffer was replaced with fresh buffer D containing 5 mM MgCl$_2$ and 10 μM A23187 and preincubated on ice for 5 min. To initiate transport, the dish was floated in a 37°C water bath for 45 min. The media samples were collected and stored at −20°C. The cells were extracted with ice-cold 5 N acetic acid and subjected to three freeze–thaw cycles. The cell debris was removed by centrifugation in a microfuge at 14,000 rpm and the supernatant was lyophilized and resuspended in NDET. Both the cell extracts and media samples were double-immunoprecipitated using an antibody to ACTH as described in (Moore and Kelly, 1986). The samples were analyzed using SDS-PAGE (10–18% polyacrylamide gradient gel).

Transfection and Processing of Rat Proinsulin II in PC12 Cells

PC12 cells were plated at 1.5 × 10^5 per well in a poly-D-lysine–coated six-well plate for 12–24 h. The cells were transfected with rat proinsulin II genomic DNA using the lipofectAMINE (GIBCO BRL, Gaithersburg, MD) procedure. 32 μg of lipofectAMINE reagent, 2.5 μg pRSV-rat proinsulin (Orci et al., 1987), .6 μg of a plasmid carrying neomycin resistance (pSV2 neo) were added to 100 μL of DME (no serum). The mixture was added to .9 mL of DME and incubated at room temperature for 15–45 min before adding to the cells. After 6 h, 1 ml of DME supplemented with 10% enriched calf serum and 10% horse serum was added to the cells containing the lipofectinAMINE solution. 8–12 h later, the lipofectinAMINE solution was replaced with normal PC12 media for 30 h. Stable clones were selected with 0.325 mg/ml G418 (active concentration). For the pulse–chase processing experiments, stably transfected PC12 cells expressing proinsulin were plated at 1 × 10^6 per well into a six-well plate coated with poly-D-lysine and grown for 3 d. The cells were rinsed twice in cysteine-free DME and then incubated for 30 min in the same media. The cells were then pulse labeled with [^{35}S]cysteine (500 μCi/ml) for 45 min. Buffer D containing 5 mM MgCl$_2$, 5 mM EGTA, and 10 μM A23187 was added to the cells for 5 min at 0°C. Calcium-free DME containing 10 μM A23187 was then added to the cells and incubated on ice for an additional 10 min before incubation at 37°C for 2 h. The media was collected and cells were extracted with NDET. Both the media and cell extracts were double-immunoprecipitated with an antibody against porcine insulin. No A23187 controls were performed in DME containing normal levels of calcium during the entire chase period. The samples were analyzed by SDS-PAGE (10–18% polyacrylamide gradient gel) and visualized using a phosphoImager.

Results

Reconstitution of Transport via the Regulated Pathway of SgII in SL-O–permeabilized Cell

A system that reconstitutes the budding and sorting events accompanying the formation of regulated granules and targeting and fusion events leading to exocytosis would facilitate the analysis of these processes. Fig. 1 shows a schematic
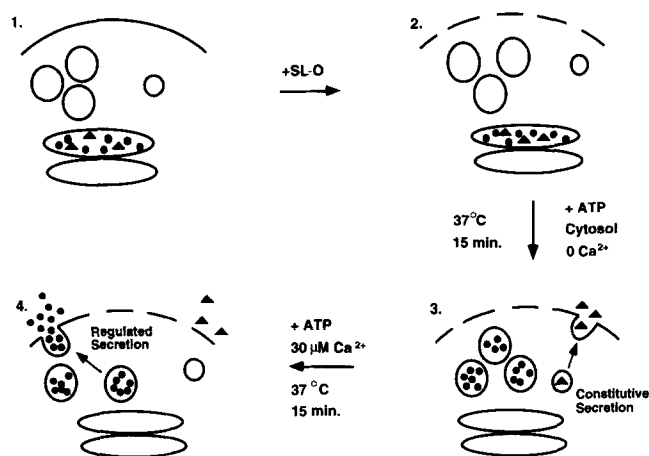

Figure 1. Scheme for in vitro transport from the TGN to the cell surface via the regulated secretory pathway in semi-intact PC12 cells. PC12 cells were pulse labeled with [^{35}S]sulfate for 5 min to label SgII (*circle*) and GAG chains (*triangle*) in the TGN, which are markers for the regulated and constitutive pathways, respectively. The plasma membrane was then selectively perforated using SL-O. Export from the TGN was initiated by the addition of a transport buffer containing an ATP-regenerating system and incubating at 37°C for 15 min (chase I). In some cases, bovine brain cytosol was added to the incubation mixture during the chase period. Free calcium was buffered to less than 10 nM to allow budding of both constitutive and regulated granules from the TGN which prevented regulated secretion of SgII. After the 15-min incubation, the cells were incubated for an additional 15 min in transport buffer with an ATP-regenerating system containing 30 μM buffered calcium to induce secretion of SgII from the regulated granules (chase II).

illustration of our in vitro reconstitution of transport of molecules through the regulated secretory pathway in PC12 cells. In the first step, the cells are pulse labeled for 5 min with [^{35}S]sulfate which labels both a soluble marker, free glycosaminoglycan (GAG) chains, as well as the regulated protein, SgII, in the TGN (Tooze and Huttner, 1990). The cells are then permeabilized using the cytolysin, SL-O, that forms pores 15–20 nm in diameter and is specific for the plasma membrane (step 2). These pores are large enough to allow passage of molecules up to about 200 kD (Bhakdi et al., 1985), but too small for TGN or transport vesicles (80–200 nm in diameter) (Orci et al., 1987) to leak out of the cell boundary. Transport of regulated proteins from the TGN to immature granules and secretion of constitutive proteins can be initiated by incubation at 37°C for 15 min in a buffer that resembles the ionic environment of the cytoplasm and an ATP-regenerating system (step 3). In most reactions, the transport buffer contained EGTA to chelate the calcium concentrations to <10 nM (herein referred to as "0 Ca^{2+}"). Where indicated, the free Ca^{2+} concentration was buffered to 100 nM to mimic physiological cytoplasmic conditions. During this chase period (chase I) the pulse-labeled SgII is expected to exit the TGN and enter immature secretory granules, since previous studies in intact PC12 cells have shown that sulfated SgII is chased into immature granules within 15 min (Tooze and Huttner, 1990). Some of the labeled GAG chains are also expected to be exported via the constitutive pathway during this period (the $t_{1/2}$ for this pathway is on the order of 7–10 min, Kelly, 1985). In intact cells, immature

granules formed during a 15-min chase period are fusion competent (Fig. 2 C; Tooze et al., 1991). Thus after chase I, we induced exocytosis of immature secretory granules by incubating the semi-intact cells in the complete transport buffer containing 30 μM buffered calcium for 15 min at 37°C (step 4, chase II).

Fig. 2 shows a typical result of the transport of labeled SgII in semi-intact PC12 cells. During chase I, very little labeled SgII was detected in the medium (Fig. 2 A, lanes 1 and 2), consistent with the previous finding that it is sorted efficiently to the regulated pathway in PC12 cells (Tooze and Huttner, 1990). After the 15 min chase, a portion of the labeled SgII had entered a compartment which could undergo

calcium-induced exocytosis, since secretion was stimulated by the addition of 30 μM buffered calcium during chase II (Fig. 2 A, lane 4, stimulated; lane 3, unstimulated). The stimulated release of SgII that we observed after 15 min of chase most probably originates from immature granules rather than from fully matured granules, since in intact PC12 cells maturation of secretory granules has been shown to take 90 min or longer after their formation from the TGN (Tooze et al. 1991; Grimes and Kelly, 1992). In these experiments, we noticed that the heparan sulfate proteoglycan (Fig. 2 A, the smear migrating just below the interface between stacking and separation gels) was also released upon calcium stimulation. This result is consistent with the finding of Sal-
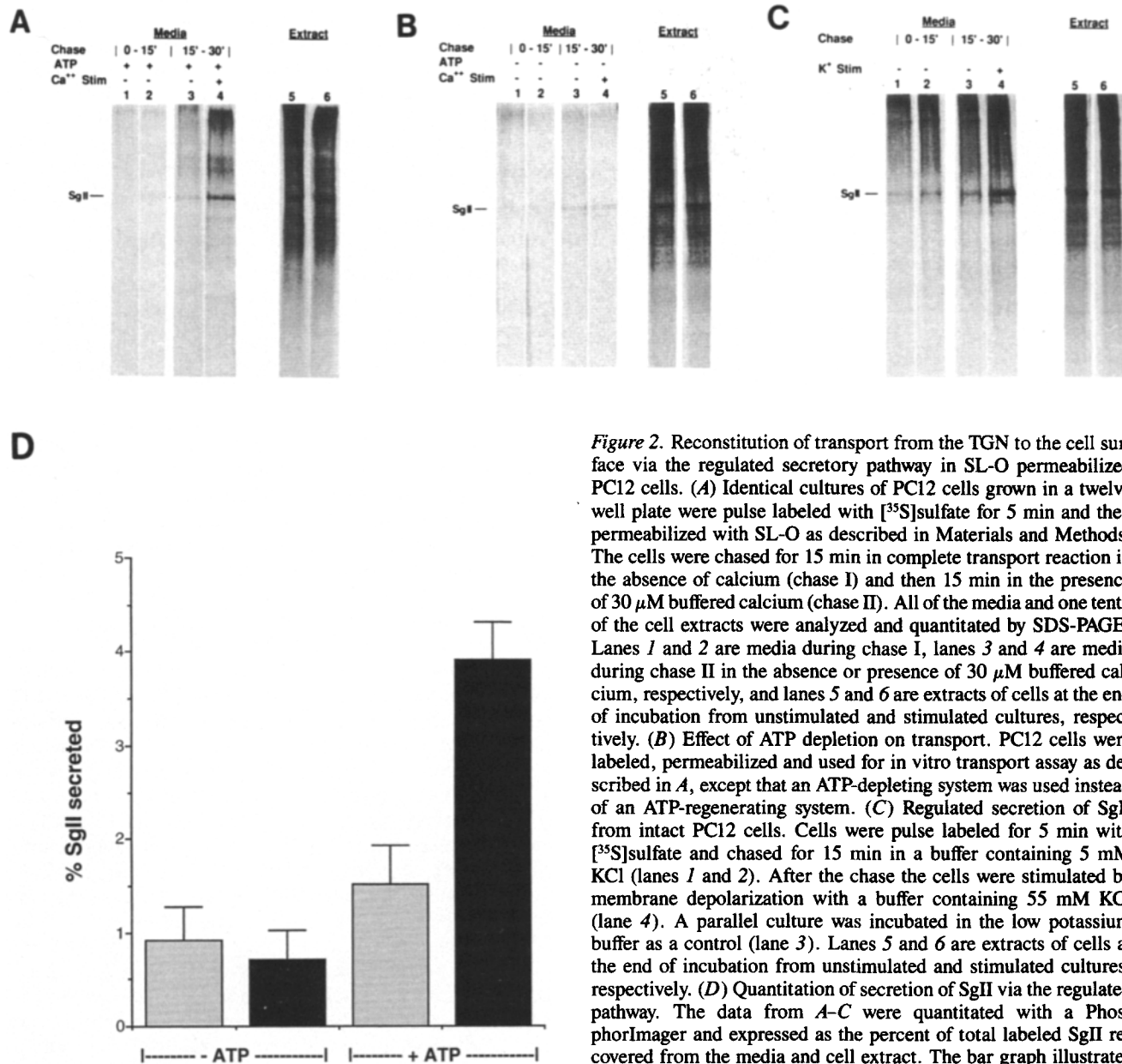

Figure 2. Reconstitution of transport from the TGN to the cell surface via the regulated secretory pathway in SL-O permeabilized PC12 cells. (A) Identical cultures of PC12 cells grown in a twelve well plate were pulse labeled with [^{35}S]sulfate for 5 min and then permeabilized with SL-O as described in Materials and Methods. The cells were chased for 15 min in complete transport reaction in the absence of calcium (chase I) and then 15 min in the presence of 30 μM buffered calcium (chase II). All of the media and one tenth of the cell extracts were analyzed and quantitated by SDS-PAGE. Lanes 1 and 2 are media during chase I, lanes 3 and 4 are media during chase II in the absence or presence of 30 μM buffered calcium, respectively, and lanes 5 and 6 are extracts of cells at the end of incubation from unstimulated and stimulated cultures, respectively. (B) Effect of ATP depletion on transport. PC12 cells were labeled, permeabilized and used for in vitro transport assay as described in A, except that an ATP-depleting system was used instead of an ATP-regenerating system. (C) Regulated secretion of SgII from intact PC12 cells. Cells were pulse labeled for 5 min with [^{35}S]sulfate and chased for 15 min in a buffer containing 5 mM KCl (lanes 1 and 2). After the chase the cells were stimulated by membrane depolarization with a buffer containing 55 mM KCl (lane 4). A parallel culture was incubated in the low potassium buffer as a control (lane 3). Lanes 5 and 6 are extracts of cells at the end of incubation from unstimulated and stimulated cultures, respectively. (D) Quantitation of secretion of SgII via the regulated pathway. The data from A-C were quantitated with a PhosphorImager and expressed as the percent of total labeled SgII recovered from the media and cell extract. The bar graph illustrates the percent of labeled SgII released into the medium during chase II under unstimulated (▨, 0 Ca^{2+}) or stimulated conditions (■, 30 μM Ca^{2+}) in transport reactions containing an ATP-regenerating system (+ATP) or an ATP-depleting system (−ATP). In comparison, intact cells secrete 1.94% ± 0.23% of labeled SgII in a buffer containing low K$^+$ and 7.10% ± 0.59% in buffer containing high K$^+$ during this period (quantitated from C). Values shown are the mean of triplicate points ± SEM.

ton et al. (1983) that proteoglycans are released from depolarized PC12 cells, and may be explained by the findings of Grimes and Kelly (1992) that some of this constitutively released proteoglycan is first packaged into immature granules that become subsequently sorted (see Discussion).

The observed release of labeled SgII is not due to cell lysis, since depletion of energy using an ATP depletion system completely abolished release during chases I and II (Fig. 2 B, lanes 1 and 2, chase I; lanes 3 and 4, chase II). Exocytosis of preformed mature granules from a variety of permeabilized cells is known to occur in the absence of ATP (Gomperts, 1990), whereas the formation of granules from the TGN is energy dependent (Tooze and Huttner, 1990). The requirement for ATP and the calcium-induced secretion in our system suggest that we have reconstituted both the formation of immature secretory granules from the TGN and their regulated exocytosis. Thus, similar to mature stored granules (Ahnert-Hilger et al., 1985; Peppers and Holz, 1986; Senyshyn et al., 1992), immature granules can also be stimulated to fuse with the plasma membrane by micromolar calcium.

The efficiency of transport in the semi-intact cell system was compared to that in intact cells (Fig. 2 C). Intact PC12 cells were labeled and chased for the same periods of time. During chase II, the cells were stimulated by membrane depolarization with high potassium (55 mM KCl) in the presence of millimolar calcium (Fig. 2 C). Secretion from intact cells follows a similar pattern as in semi-intact cells; relatively little is secreted during chase I, while high potassium (Fig. 2 C, lane 4) causes an enhanced secretion of SgII over the unstimulated control (Fig. 2 C, lane 3). Overall, secretion from intact cells is higher than that in semi-intact cells. Quantitation of the experiment shown in Fig. 2 (A–C) is summarized in Fig. 2 D. In SL-O–permeabilized cells, calcium stimulation caused on average 3.91% of the total-labeled SgII to be secreted into the medium during chase II. This is two- to threefold higher than the 1.51% release seen in the absence of calcium. In intact cells, high potassium caused 7.1% total labeled SgII to be released during the same chase period, or three- to fourfold higher than that released from unstimulated cells (1.94%). Thus, the calcium stimulated release of SgII attained in SL-O–permeabilized cells (3.91%) is ~50% of the level seen in intact cells (7.1%). This degree of reconstitution is similar to the level achieved for

constitutive secretion in SL-O–treated CHO cells (Miller and Moore, 1992).

To determine if sorting occurs in this reconstituted system, we compared the transport of SgII to a fluid phase tracer, sulfated GAG chains. GAG chains are synthesized and sulfated in the trans-Golgi/TGN when cells are incubated with a membrane permeant xyloside (Schwartz et al., 1974; Kimura et al., 1984; Farquhar, 1985; Velasco et al., 1988). In several systems they have been shown to behave as a soluble marker which enters transport vesicles exiting the TGN (Burgess and Kelly, 1984; Brion et al., 1992; Matsuuchi and Kelly, 1991). For each marker, we measured a "sorting index" in semi-intact cells (Moore and Kelly, 1985), which corresponds to the efficiency at which a given molecule enters the regulated pathway relative to the constitutive (or unregulated) pathway. Table I shows the sorting index measured under various conditions; the sorting index was calculated by taking the ratio of calcium-induced secretion over constituted secretion (see Table I). The sorting index of SgII measured when transport was carried out in physiological calcium (100 nM buffered calcium) was 1.43, which is similar to that found in intact cells (1.8). When the reaction was carried out in a transport buffer containing 5 mM EGTA to reduce the free calcium level to <10 nM (see Table I, 0 Ca^{2+} condition), the sorting index was 3.02. We found that labeled GAG chains also entered the regulated pathway to some extent in the semi-intact PC12 cells, consistent with earlier studies in intact AtT-20 cells (Matsuuchi and Kelly, 1991). The sorting index of GAG chains in 0 Ca^{2+} transport buffer was .81, which is about fourfold lower than the corresponding number for SgII (3.02). This indicates that a larger fraction of SgII compared to GAG chains is secreted via the regulated pathway. Thus, sorting of molecules has occurred in this in vitro system. Addition of bovine brain cytosol (60–75 μg/ml) to the transport buffer increased both the calcium-dependent and -independent release of SgII by about twofold (Table II), but did not increase the percentage of SgII entering the regulated pathway.

Inhibition of Transport by NEM, GTPγS, AlF$_{3-5}$, and BFA

To further characterize SgII transport in this system, we investigated the effects of several reagents that are known to

Table I. The Sorting Index of Secretogranin II and GAG Chains in Intact and Semi-intact PC12 Cells

Conditions	SgII	GAG chains
Intact	1.80 ± 0.24 (5)	
Semi-intact		
100 nM Ca^{2+}	1.43 ± 0.03 (5)	0.47 ± 0.10 (6)
0 Ca^{2+}	3.02 ± 0.27 (8)	0.81 ± 0.13 (6)
0 Ca^{2+}/10 μM A23187	3.86 ± 1.07 (8)	0.69 ± 0.06 (6)
(added after pulse − label)		
0 Ca^{2+}/10 μM A23187	3.06 ± 0.39 (4)	
(added prior to pulse − label)		

Transport in semi-intact and intact PC12 cells was carried out as in Fig. 2, A and C, respectively, except the free calcium concentration in the transport reaction was buffered to either the physiological level (100 nM), or <10 nM with EGTA (0 Ca^{2+}). In some experiments, 10 μM A23187 was added to the cells in 0 Ca^{2+} transport buffer either before the pulse label, or after pulse labeling before the start of the reaction. SgII in the media and the extract was analyzed by SDS-PAGE and a PhosphorImager as described in Fig. 2. Transport of GAG chains was carried out as in Fig. 5. The sorting index is defined as (stimulated release − basal release during chase II)/(constitutive release during chase II). Numbers in parenthesis indicate the number of data points averaged.

Table II. The Requirements for In Vitro Transport of SgII from the TGN to Cell Surface Via the Regulated Secretory Pathway

Conditions	Relative extent of transport
Complete	100.0
−ATP	18.2 ± 7.8
NEM	11.3 ± 2.0
No cytosol	54.3 ± 14.1
GTPγS	29.7 ± 2.7
AlF$_{3-5}$	2.0 ± 0.6
BFA	11.9 ± 2.5

Transport of SgII semi-intact PC12 cells was carried out as described in Fig. 2. The percentage of labeled SgII secreted in response to 30 μM Ca^{2+} during chase II was normalized to complete transport reaction containing cytosol and ATP.

affect vesicular transport (Balch et al., 1984; Melancon et al., 1987; Barr et al., 1991; Schwaninger et al., 1992). In each case, transport was normalized to the amount observed in control cells without the reagent. Fig. 3 *A* shows the relative amount of SgII secreted during chase II from stimulated (30 μM Ca^{2+}) or unstimulated cells (0 Ca^{2+}) compared to control cells. Transport of SgII in our assay was inhibited by the alkylating agent NEM, consistent with the notion that

vesicular fusion requires the NEM-sensitive factor (NSF) (Malhotra, 1988; Beckers and Balch, 1989; Carroll et al., 1990; Miller and Moore, 1991; Yang et al., 1992). NEM also inhibited SgII secretion when added after the first chase (data not shown), indicating it indeed exerts its effect at a late step during transport. Addition of 100 μM of the non-hydrolyzable analogue GTPγS at the start of the reaction inhibited both unstimulated and stimulated release of SgII: secretion in the absence of calcium was reduced from 46 to 25.2% (0 Ca^{2+}), and secretion in the presence of calcium was decreased from 100 to 29.7% (30 μM Ca^{2+}). Similarly, AlF$_{3-5}$, which activates trimeric G proteins but not low molecular weight GTP-binding proteins (Kahn, 1991), completely abolished transport when added immediately after permeabilization to a final concentration of 50 μM: 0.9% from unstimulated cells (−Ca^{2+}) and 1.9% from stimulated cells (+Ca^{2+}). Very little labeled SgII was recovered in the medium during chase I (data not shown), implying that the lack of secretion via the regulated pathway did not result from a diversion to the constitutive pathway. Consistent with earlier studies (Miller et al., 1992; Rosa et al., 1992a), transport of SgII from the TGN to the cell surface was inhibited by 5 μg/ml BFA when added early during the transport reaction (Fig. 3 *A*). These results are summarized in Table II.
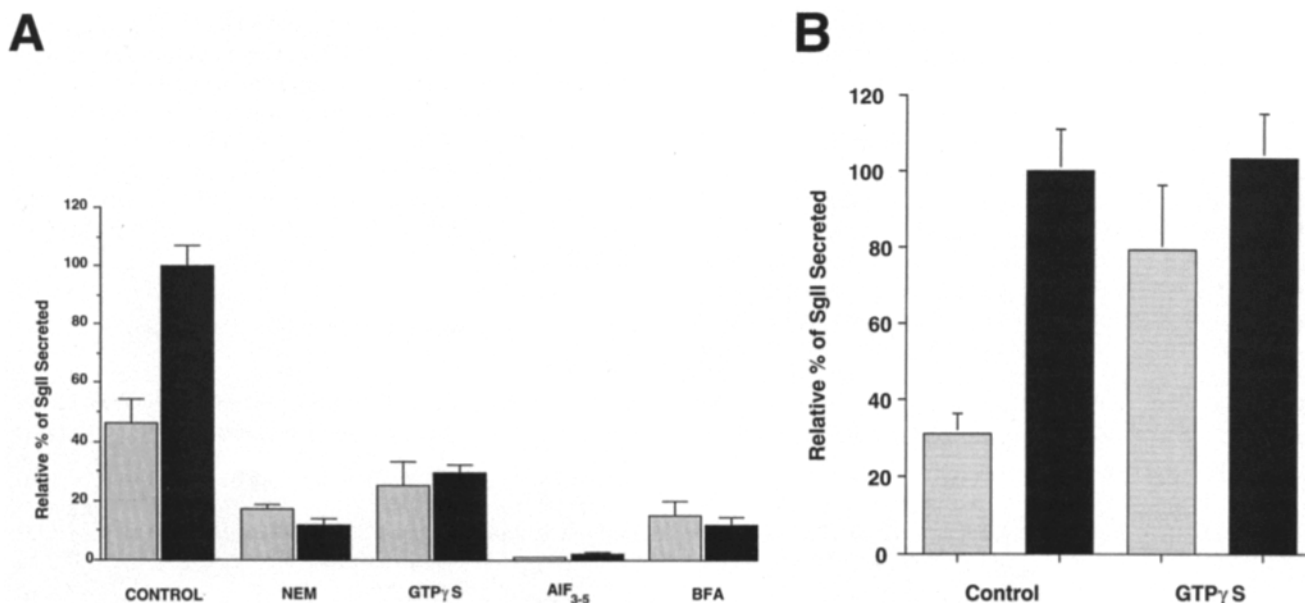
We found that GTPγS exerts distinct effects depending on

Figure 3. (*A*) In vitro transport of SgII from the TGN to the cell surface via the regulated pathway is inhibited by NEM, GTPγS, AlF$_{3-5}$, and BFA. Cells were pulse-labeled with [^{35}S]sulfate for 5 min to label SgII in the TGN. The cells were permeabilized with SL-O, and treated with 100 μM GTPγS, AlF$_{3-5}$ (50 μM AlCl$_3$ and 20 mM NaF), or 5 μg/ml BFA in buffer C on ice for 10 min. 500 μM NEM was added for 15 min on ice and then removed and replaced with a buffer containing 1 mM DTT to inactivate the NEM. To initiate the transport reaction, the cells were shifted to 37°C and chased for 15 min in transport buffer containing the various reagents to allow granule formation. The buffer was collected and the fresh transport buffer containing GTPγS, AlF$_{3-5}$, and BFA were added. The cells were then incubated in the absence or the presence of 30 μM buffered calcium for 15 min. The percent of total labeled SgII secreted during chase II was calculated for each condition, and normalized to control untreated cells. Hundred percent is defined as secretion from control stimulated cells. (*B*) Effect of GTPγS on exocytosis of immature granules. Cells were pulse labeled, permeabilized, and chased for 15 min as in *A* except without GTPγS. To examine the effects of GTPγS on immature granules that had formed during chase I, 100 μM GTPγS was added to the transport buffers during chase II for an additional 15 min. The percent of total labeled SgII secreted during chase II was calculated, and normalized to control untreated cells. 100% is defined as secretion from control stimulated cells. Values shown are the mean of triplicate points ±SEM. ▨, 0 Ca^{2+}; ■, 30 μm Ca^{2+}.

the time of addition. If GTPγS was added to the assay immediately after pulse, it inhibited both calcium and calcium-independent secretion of SgII from the TGN (see above). However, when introduced after chase I (i.e., after some immature granules had already formed) GTPγS did not inhibit SgII secretion; Instead, it enhanced calcium-independent release of SgII (from 30 to 79% of the calcium-dependent release) (Fig. 3 B); secretion in the presence of calcium was not affected significantly. This property of immature granules is reminiscent of that of mature granules: nonhydrolyzable GTP analogues are known to enhance release from stored secretory granules in a number of systems including PC12 cells (for a review see Carroll et al., 1990; Gomperts, 1990). This result suggests that immature granules are not only capable of fusion, but they have also acquired the biochemical components for proper modulation by both GTPase(s) and calcium that exist for mature granules. The distinct effects of GTP analogues also suggest that transport from the TGN to cell surface is likely to involve more than one GTPase (see Discussion).

Effect of Calcium Depletion on Prohormone Processing

Intra-cisternal calcium ions have been postulated to play a role in a variety of steps in TGN to cell surface transport, including sorting of proteins into the regulated secretory and proteolytic processing of secretory products. To test if cisternal calcium plays a role in the proteolytic processing of prohormones, we first examined the proteolytic cleavage of proopiomelanocortin (POMC) in intact mouse pituitary AtT-20 cells In vitro studies show that the endoproteases, PC1 and PC2, that cleave prohormones at dibasic residues are calcium dependent (Shennan et al., 1991; Zhou and Lindberg, 1993) but a requirement for calcium has not been directly tested in vivo. In AtT-20 cells and pituitary corticotrophs, POMC is processed into a variety of intermediate forms with the final product being the adrenocorticotropic hormone (ACTH). Processing of POMC can be conveniently monitored by pulse labeling with [^{35}S]SO$_4$ in the TGN (Moore et al., 1983b). Proteolytic processing begins at the TGN and continues in immature granules (Tooze et al., 1987; Schnabel et al., 1989), and thus can be followed by chasing the cells for 1–2 h after the label.

To disrupt intra-TGN calcium, we used the calcium ionophore, A23187, since it has been shown to release Golgi/TGN calcium stores in a variety of cultured cells within minutes (Chandra et al., 1991; Oda, 1992). It has also been shown to be effective in releasing ER calcium stores (Booth and Koch, 1989; Lodish and Kong, 1990). AtT-20 cells were pulse labeled with [^{35}S]sulfate for 5 min, and placed in a calcium-free chase medium containing 10 μM A23187 and 5 mM EGTA to chelate any remaining calcium. The cells were then chased for 45 min in the same medium. Fig. 4 A shows that depletion of lumenal calcium ions inhibited processing of POMC significantly; only a small fraction of the labeled POMC was cleaved to the intermediate forms, and no fully processed, mature ACTH was detected compared to the control. The large fraction of the POMC-related peptides secreted during the chase is due to high levels of constitutive-like secretion from AtT-20 cells (Moore et al., 1983a).

We also confirmed the above results in PC12 cells. PC12 cells do not express significant levels of PC1 or PC2 (Benjan-
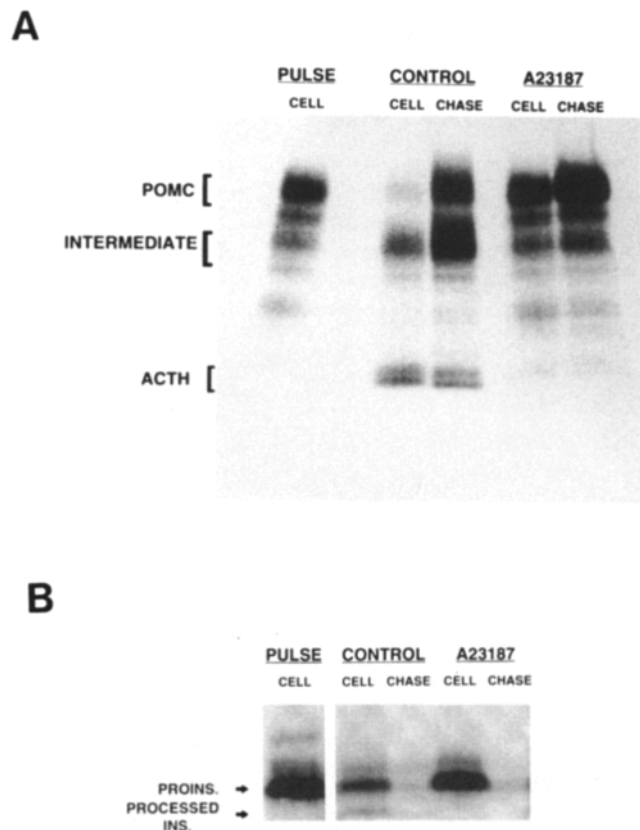

Figure 4. Lumenal calcium is required for prohormone processing and can be blocked by treatment with A23187. (A) Processing of POMC in AtT-20 cells. Cells grown in identical six-well dishes were pulse labeled for 5 min with [^{35}S]sulfate. Following the pulse the cells were either extracted immediately (PULSE lane), or pretreated on ice in a calcium free buffer containing 10 μM A23187 followed by incubation for 45 min at 37°C. Media (CHASE lane) and cell extracts (CELL lane) were collected, double immunoprecipitated with an antibody against ACTH, and analyzed using SDS-PAGE. Control cells were incubated in DME without A23187 throughout the chase period. The positions of the 29–31-kD POMC precursor, the 21–23-kD intermediate forms, and the 13-kD glycosylated mature ACTH are indicated in brackets. (B) Processing of rat proinsulin II in PC12 cells. Identical cultures of PC12 cells stably transfected with rat proinsulin II were pulse labeled for 45 min with [^{35}S]cysteine. The cells were either extracted immediately (PULSE lane), or pretreated on ice in a calcium-free buffer containing 10 μM A23187 followed by incubation for 2 h at 37°C. Media and cell extracts were collected, double immunoprecipitated with an antibody to porcine insulin, and analyzed using SDS-PAGE. Control cells were incubated in DME without A23187. Lane 1 shows insulin-related peptides recovered from the cell extracts immediately after the pulse. Top arrow on the left indicates the position of proinsulin (PROINS). Lanes 2 and 3 are cell extract (CELL) and media samples (CHASE), respectively, from control untreated cultures collected after the 2-h chase. Bottom arrow on the left indicates the appearance of a processed insulin (processed ins.) in the cell extract. Lanes 4 and 5 are cell extract (CELL) and media samples (CHASE), respectively, from cultures treated with A23187 after the chase.

net et al., 1991; Galanopoulou et al., 1993), but contain high levels of furin, a Kex2-like processing enzyme that functions in the constitutive secretory pathway in mammalian cells (Molloy et al., 1994). Rat proinsulins contain a furin con-

sensus site (RQKR) in the C-A peptide junction (discussed in Yanagita et al., 1993), which should be a substrate for furin in PC12 cells. We therefore made a stably transfected PC12 cell line expressing rat proinsulin II. Cells pulse labeled with [^{35}S]cysteine and immunoprecipitated with an anti–insulin antiserum showed the production of unprocessed proinsulin within the cells (Fig. 4 B, *pulse* lane). After a 2-h chase, some proinsulin was converted to a faster migrating, processed form that was found in the cell extract (Fig. 4 B, *control*, *cell* lane, *lower arrow*). Secretion of the insulin-related proteins is slower than in AtT-20 cells, since very little was secreted into the media during the 2 h chase (Fig. 4 B, *control*, *chase* lane). In cells treated with A23187 in calcium-free media, no processing was observed after the 2-h chase either in the cells (Fig. 4 B, *A23187*, *cell* lane) or in the media (Fig. 4 B, *A23187*, *chase* lane). These results suggest that lumenal calcium ions within the TGN or immature granule are required for proper processing of POMC and rat proinsulin. It also suggests that A23187 is a useful reagent to deplete intra-cisternal calcium store in compartments involved in the biogenesis of regulated secretory granules in both AtT-20 and PC12 cells.
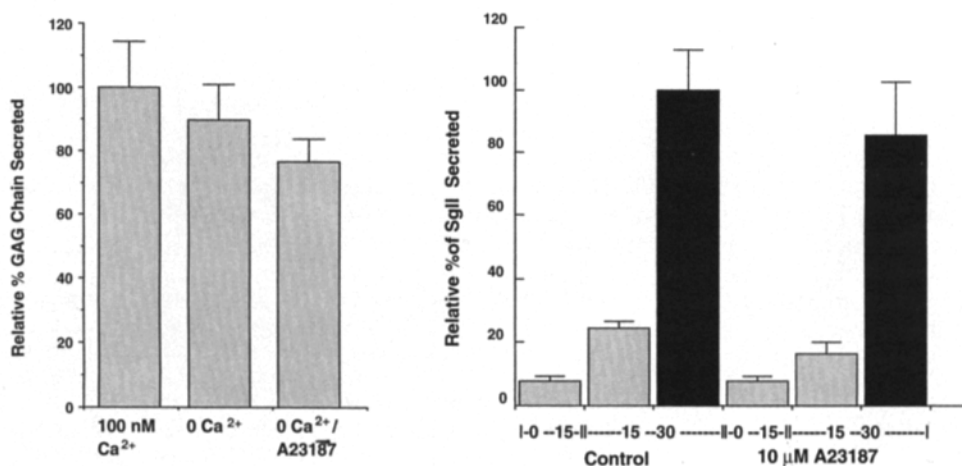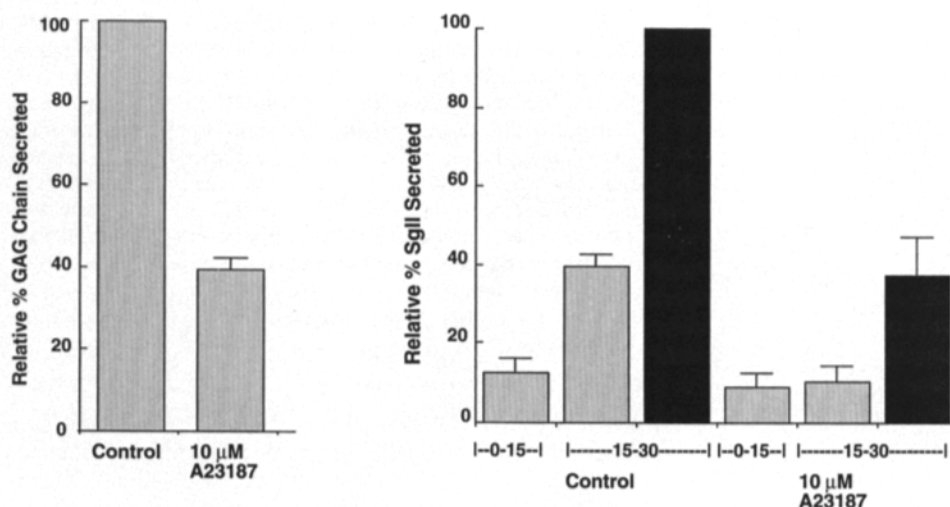

Figure 5. Effect of depletion of *intra*-cisternal calcium on sorting and transport of SgII and GAG chains from the TGN to the cell surface. (*A*) A23187 treatment after pulse-labeling. (*Left*) Constitutive secretion of GAG chains. Cells were pulse labeled for 3 min with [^{35}S]sulfate, permeabilized, and chased for 15 min in complete transport reaction containing 100 nM buffered Ca^{2+}, 0 Ca^{2+}, or 0 Ca^{2+} plus 10 μM A23187. The buffer was collected and the cells were incubated for a second 15 min with fresh buffer with the same composition. The chases were combined to determine amount of transport for a 30-min period. (*Right*) Transport of SgII via the regulated pathway. The cells were pulse labeled, permeabilized, and preincubated for 10 min at 0°C with 10 μM A23187 in a transport reaction containing 0 Ca^{2+}, and then chased for 15 min at 37°C (*0'–15'*). The cells were then stimulated in 30 μM buffered calcium containing A23187 for 15 min at 37°C (*15'–30'*). Control unstimulated release was measured in 0 Ca^{2+}. Labeled SgII in media and extracts were processed and quantitated as in Fig. 3. The numbers shown are relative secretion during chase I and chase II. (100% being secretion from control stimulated cells during chase II.) (*B*) Pretreatment with A23187 before pulse labeling. The experiments were performed as in *A*, except that A23187 was added to the cultures 5 min before pulse labeling and is present throughout the labeling and chase periods. ▨, 0 Ca^{2+}; ■, 30 μm Ca^{2+}.

Effect of Intra-Cisternal Golgi/TGN Calcium Depletion on the Sorting and Secretion of SgII via the Regulated Pathway

Next, we wished to determine the effect of depleting TGN calcium on the sorting and transport of proteins into the regulated secretory pathway. Intact cells are not suitable for these studies since treatment of intact cells with ionophores makes it difficult to control the levels of cytoplasmic calcium ions. This is particularly important in studies measuring the sorting between the constitutive and the regulated pathways; since high cytoplasmic calcium levels will cause release of regulated granules. Thus, we used the semi-intact PC12 system described above to study the role of lumenal calcium in sorting.

Before testing the role of intra-cisternal calcium on the sorting of SgII, we examined the effect of depletion of both cytoplasmic and intra-cisternal calcium ions on the rate of transport from the TGN to the cell surface. To test whether cytoplasmic calcium concentrations affect constitutive secretion in semi-intact PC12, transport of GAG chains was carried out in a transport buffer containing physiological levels of cytoplasmic calcium (buffered to 100 nM) and compared to transport in a buffer containing no calcium (Fig. 5 A). The amount of GAG chain secretion during a 30-min incubation period in 100 nM free Ca^{2+} was similar to that in 0 Ca^{2+} (89.7% of the 100 nM level). These data are consistent with previous studies reconstituting constitutive secretion in CHO cells (Miller and Moore, 1991), which showed that cytoplasmic calcium was not required for constitutive transport from the TGN to the cell surface. Addition of 10 μM A23187 at the start of transport in 0 Ca^{2+} caused only a slight decrease in the rate of GAG chain secretion (from 89.7% of control 100 nM level to 76.6%, Fig. 5 A). A23187 also caused a slight decrease in the amount of GAG chains secreted via the immature granules; therefore the sorting index of GAG chains is similar with or without A23187 treatment (.69 versus .81, see Table I).

To study SgII sorting, A23187 was added to a calcium-free transport buffer containing 5 mM EGTA to chelate any calcium released from the TGN in our in vitro transport system. Depleting lumenal calcium caused a slight decrease in the unstimulated and stimulated release of SgII (Fig. 5 A); secretion in 0 Ca^{2+} during chase II was 16.2% from cells treated with A23187 compared to 24.2% from control cells, and 85.3% in 30 μM Ca^{2+} compared to 100% of control. The slight decreases in transport rates of SgII were similar to GAG chains described above. Importantly, we did not observe a diversion of SgII to the constitutive pathway, since the amount of labeled SgII recovered in the media during chase I or II from unstimulated cells was not increased (Fig. 5 A, 0–15-min chase, control versus A23187). The sorting index of SgII in A23187 treated cells was 3.86, which is not significantly different from 3.02 in control cells (Table I). The observation that sorting is not disrupted by depletion of intra-cisternal calcium ions implies that a calcium-dependent aggregation of SgII in the TGN is not essential for its initial sorting into immature secretory granules.

In the above experiments, A23187 was added to the cultures after the pulse labeling. It is possible that an effect was not observed because labeled SgII had already passed beyond the site of aggregation when A23187 was added. There-

fore, we performed a pretreatment experiment in which cultures were treated with A23187 before the pulse. Fig. 5 B shows that preincubation of semi-intact PC12 cells in calcium-free buffer containing A23187 for 5 min reduced constitutive secretion of GAG chains to 39% of control untreated cultures (Fig. 5 B, left). Similarly, both basal and calcium-induced release of SgII was reduced to 30–40% of control (Fig. 5 B, right). As was seen with postlabel treatment, we did not observe missorting of SgII: the sorting index for the treated culture (3.06) is similar to control cultures (3.02). In PC12 cells, manipulations that result in missorting of SgII are known to increase constitutive release from unstimulated cells by at least 10-fold (Gerdes et al., 1989); this was not observed. Thus, lumenal TGN calcium per se does not appear to be critical for the initial sorting of SgII into immature granules in this system.

A potential problem in interpreting the above experiments is the necessity of adding 30 μM buffered calcium to triggered release from immature granules during chase II. Since A23187 is still present during the chase II, one could argue that the added 30 μM calcium might restore some of the TGN Ca^{2+} pool during this period, which could effect the sorting of SgII during this period. We consider this unlikely, since 30 μM calcium is much lower than the concentrations (mM) needed for aggregation in vitro (Gerdes et al., 1989). However, to further eliminate this possibility we made use of the observation that BFA specifically inhibits the budding of granules from the TGN without affecting granule exocytosis (Miller et al., 1992 and Rosa et al., 1992a). We repeated the A23187 experiment described in Fig. 5 A, but included 5 μg/ml BFA during chase II to inhibit further budding of new granules from the TGN. This allowed us to monitor secretion from immature secretory granules that had already formed under calcium-free conditions. BFA treatment alone during chase II produced a slight decrease in the amount of calcium-induced secretion of SgII as compared to control without BFA treatment (Fig. 6); this might be due to the fact that very few new granules were formed during the second 15-min chase. Again, we do not observe significant differences in sorting caused by A23187 using this protocol (Fig. 6); the level of regulated secretion of SgII from A23187-treated cells was similar to that from cells that had not been treated with A23187. Comparison of SgII sorting indices in A23187/BFA and BFA-treated cells (2.05 and 1.68, respectively) confirms the finding that release of TGN calcium does not lead to missorting of SgII into immature granules in this system.

Transport Via the Regulated Pathway Is Inhibited by Disruption of Organelle pH Gradients in SL-O–permeabilized Cells

In addition to calcium, the acidic pH (6.4) of the TGN has also been proposed to be essential for the aggregation and sorting of regulated proteins (Gerdes et al., 1989). We therefore also tested the effects of alternating intra-Golgi pH on protein sorting and transport into immature granules in our system. The intra-Golgi/TGN pH was perturbed with a protonophore (nigericin) or a weak base (NH$_4$Cl) before transport reaction was carried out in semi-intact PC12 cells (Fig. 7). Addition of 10 μM nigericin completely abolished export of SgII from the TGN; the amounts secreted from unstimu-
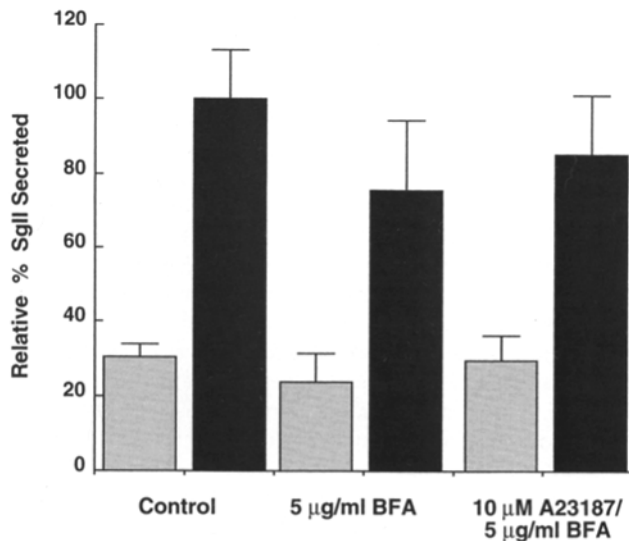
Figure 6. Control experiment to show that the lack of sorting defects in the presence of A23187 cannot be explained by reversal of internal stores due to calcium added during the stimulation period. Cells were pulse labeled, permeabilized, and treated with 10 μM A23187 as in Fig. 5 B, except that during chase II BFA (5 μg/ml) was added to the transport reaction to prevent further budding from the TGN during the stimulation period. Parallel cultures were treated identically, except that no A23187 was added. Relative secretion of SgII during chase II is shown. The pair of bars on the left shows unstimulated and stimulated secretion from control cells that had not been treated with either BFA or A23187. The pair in the middle shows secretion from cells that were treated with BFA during chase II. The pair on the right shows secretion from cells that were treated with A23187 during the entire transport reaction, and BFA during chase II. ▨, 0 Ca^{2+}; ■, 30 μm Ca^{2+}.
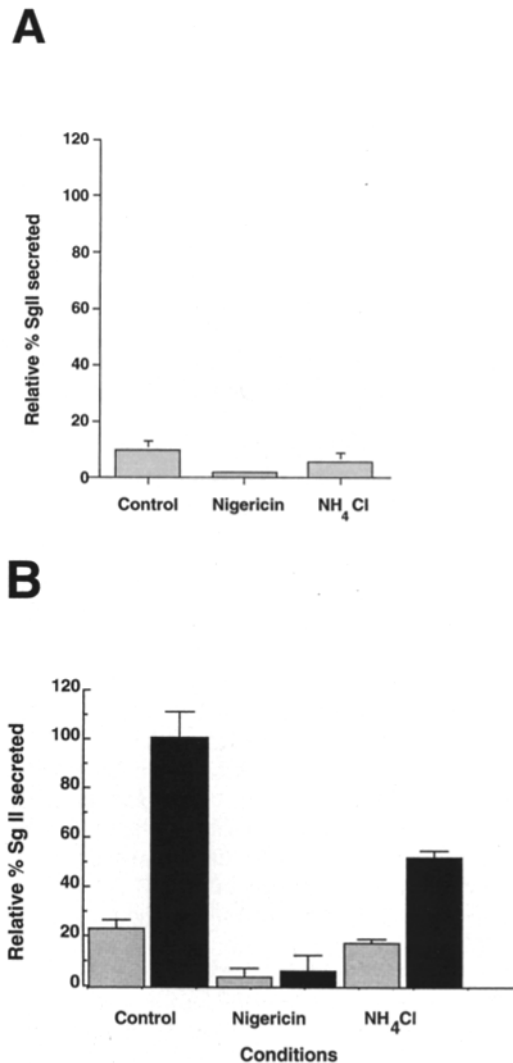

Figure 7. Regulated transport is inhibited by disruption of organelle pH gradients in SL-O–permeabilized cells. PC12 cells were pulsed for 5 min with ^{35}S-SO$_4$ and then permeabilized as described in Fig. 3. After permeabilization the cells were pretreated with either 10 μM nigericin or 50 mM NH$_4$Cl. The cells were then chased for 15 min to allow vesicle formation to proceed. After the chase the buffer was collected and the cells were stimulated to release SgII using a high calcium transport buffer in the continued presence of nigericin or NH$_4$Cl. (A) Relative secretion of SgII during chase I. (B) Relative secretion of SgII during chase II. 100% is defined as secretion from control stimulated cells during chase II. ▨, 0 Ca^{2+}; ■, 30 μm Ca^{2+}.

lated cells during chase I (Fig. 7 A) and from stimulated or unstimulated cells during chase II (Fig. 7 B) were less than 10% of control stimulated release. The weak base, NH$_4$Cl, at 50 mM also reduced the extent of transport, albeit less dramatically; calcium-induced secretion was decreased from 100 to 51.7% (Fig. 7 B). Interestingly, no missorting was observed in NH$_4$Cl treated cells in this system (see Discussion), since the amount released under nonstimulating conditions was not increased (see Fig. 7 A, chase I, and Fig. 7 B, chase II/0 Ca^{2+}). We have previously shown that the acidic lumenal TGN pH is critical for proper formation of constitutive vesicles (Miller, S., and H.-P. Moore, unpublished observations). Taken together, these results suggest that organelle pH gradient is required for the proper transport from TGN to the cell surface via both the constitutive and the regulated pathway.

Discussion

In this report, we developed a semi-intact cell system to test some aspects of current models for sorting and transport of regulated secretory proteins. Transport of immature granules is dependent on added ATP and cytosol, and is inhibited by GTPγS, AlF$_{3-5}$, and BFA when added at the start of the reaction (Fig. 3 A). These characteristics are consistent with previous studies demonstrating that the budding of exocytic vesicles from the TGN is inhibited by BFA, GTPγS, and

AlF$_{3-5}$ (Leyte et al., 1992; Miller et al., 1992; Rosa et al., 1992a; Tooze and Huttner, 1990). In addition to vesicle budding, our system also reconstitutes exocytosis of newly formed immature granules. This is supported by the finding that secretion of SgII from immature granules is dependent on micromolar calcium levels, and is enhanced by GTPγS added during chase II (Fig. 3 B). The differential effects of GTPγS added at different time points of incubation suggest that both the early steps (formation of granules) and the later steps (targeting and fusion) are reconstituted. Most likely, distinct GTP-binding proteins are involved in the early vs late steps of transport.

Using this system, we tested whether the lumenal ionic conditions within the TGN are required for the proper sorting and transport of specific proteins into the regulated pathway. Studies have shown that the lumen of TGN is mildly acidic (Anderson and Pathak, 1985), with a concentration of total calcium in the Golgi in the millimolar range (Roos, 1988; Chandra et al., 1991). In our reconstituted system, depleting lumenal calcium with A23187 at the start of the reaction had little effect on the sorting of SgII into immature granules (Fig. 5 A). A potential problem is that A23187 failed to deplete Golgi calcium. However, this appears unlikely for the following reasons: (a) direct measurements of calcium with ion microscopy and fluorescent dyes in isolated Golgi vesicles or intact cultured cells have shown that calcium sequestered in the Golgi can be released with A23187 or ionomycin (West, 1981; Chandra et al., 1991; Fasolato et al., 1991). (b) A23187 added to intact cultured cells perturbs ER sorting and export as well as proteolytic cleavage of pro-C3 and HA, indicating that the ionophore can deplete calcium from ER, Golgi, and TGN (Klenk et al., 1984; Booth and Koch, 1989; Lodish and Kong, 1990; Oda, 1992). (c) We show that proteolytic processing of POMC and rat proinsulin II is inhibited by A23287; thus under our experimental conditions the ionophore can effectively discharge lumenal calcium ions, even from acidic compartments such as immature granules (Orci et al., 1986). (d) Addition of A23187 before pulse labeling, while reducing the overall extent of export from the TGN, did not result in the missorting of SgII from the regulated pathway. Another concern is that SgII may be already aggregated in the TGN and thus addition of A23187 to the reaction has little effect on sorting of sulfated SgII. However, sulfated SgII can be released from permeabilized Golgi vesicles in a buffer lacking calcium (Chanat and Huttner, 1991), suggesting that aggregation would be reversible under such conditions. Taken together, these results raise the question of whether luminal calcium is required for the proper sorting of SgII into immature granules. A possible explanation is that aggregation of SgII may be achieved by several different mechanisms; thus perturbation of lumenal calcium by itself does not lead to significant missorting. Future experiments will be needed to resolve this issue.

Previous studies have suggested that disruption of intra-TGN pH by weak bases such as chloroquine and ammonium chloride causes regulated proteins to be secreted constitutively (Moore et al., 1983a; Rosa et al., 1985). In our semi-intact PC12 cell system, disruption of the pH gradient across organelle membranes with nigericin completely blocked transport via the regulated pathway. The lack of calcium-induced secretion of SgII was not accompanied by an increase in secretion at low calcium (Fig. 7 A), suggesting that labeled SgII was not diverted to the constitutive pathway. Using a similar semi-intact CHO cell system, we found that nigericin inhibited constitutive secretion of GAG chains from the TGN. Kinetic studies have shown that the inhibition resulting from pH disruption is at an early step, most likely at the level of budding of constitutive vesicles from the TGN (Miller, S. G., and H.-P. H. Moore, unpublished). These results are consistent with other studies that showed inhibitors of the vacuolar-type ATPase blocks transport at different stages in the Golgi and TGN (Miller, S. G., and H.-P. Moore, unpublished observation; Yilla et al., 1993; Muroi et al., 1993). In SL-O-perforated PC12 cells, secretion of GAG

chains via the constitutive pathway is also inhibited by nigericin. Thus, the most likely explanation for the inhibition of the regulated secretion of secretogranin in this system is that budding of both constitutive and regulated vesicles from the TGN is blocked when the pH gradient is abolished by nigericin. These results from our semi-intact cell system are thus different from those obtained with intact cells in which weak bases caused regulated secretory products to be secreted constitutively. The difference may be explained by two factors. First, weak bases may not have inhibited vesicle budding from the TGN in earlier experiments because of inefficient neutralization of intra-Golgi pH in intact cells. Second, the time courses of pulse–chase experiments in the previous experiments in which diversion of regulated proteins to the constitutive pathway was observed were longer than those used in the semi-intact cell system. Unregulated secretion of POMC-related products and SgII occurred during a long (2 h) chase period (Moore et al., 1983; Rosa et al., 1985). In contrast, the studies described here measured early events of transport during the first 30 min of chase. The long chases needed to observe constitutive release of these proteins suggest that missorting may occur after they have entered immature granules. This interpretation is supported by the finding that ammonium chloride causes increased basal release of zymogens from a post-TGN compartment (von Zastrow et al., 1989).

The system that we have developed differs from previous studies on exocytosis using permeabilized cells (see for a review Gomperts, 1990), in that all of the previous studies examined the secretion characteristics of granules that have fully matured. By choosing a time window (15–30 min after the pulse label) in which only immature but not mature granules were produced (Tooze et al., 1991; Grimes and Kelly, 1992; Xu and Shields, 1993), we could study the exocytic properties of immature secretory granules and compared them to those of mature granules reported in the literature. We found that immature granules formed in semi-intact PC12 cells exhibit properties very similar to those of mature granules. In most permeabilized systems examined previously, exocytosis of mature granules is activated by micromolar of cytoplasmic free calcium ions. We found that upon exit from the TGN or shortly thereafter, labeled SgII can be released by micromolar of free calcium ions (Fig. 2), indicating that immature granules can already undergo calcium-induced exocytosis. This result is consistent with the previous finding that SgII in immature granules can be released from intact PC12 cells by potassium depolarization (Tooze et al., 1991). Although the observed secretion during 15–30-min chase may result from a small portion of granules that have already matured, we consider this possibility unlikely. If immature granules were fusion incompetent and only mature granules could fuse, one would predict that the percentage of secretion of labeled SgII in response to stimulation would increase with increasing chase times; but this is not the case: the percentage of labeled SgII secreted from PC12 cells during a 15-min period does not increase with increasing chase times (15-min chase, 7%, Fig. 2 C; 1-h chase, 6.6%, Miller et al., 1992; overnight, 3%; Rosa et al., 1985; and our unpublished data). We have also found that GTPγS enhances release of immature granules at low calcium concentrations (Fig. 3 B) similar to mature granules (Carroll et al., 1990; Gomperts, 1990; Yang et al., 1992). A GTP-

binding protein, G_E, has been postulated to mediate this effect (Gomperts, 1990), although its identity is still controversial (Oberhauser et al., 1992; Lillie and Gomperts, 1993; Morgan and Burgoyne, 1993). The results imply that secretory granule membrane components, especially those involved in vesicular targeting (e.g., rab3A and binding protein, rabphilin 3A; Shirataki et al., 1993), in docking (e.g., VAMPs and syntaxins, Sollner et al., 1993), and in fusion (e.g., synaptotagmin; reviewed in Debello et al., 1993) may be recruited to nascent granules quickly upon their emergence from the TGN. Future experiments will be necessary to test this hypothesis directly.

Sorting of "cargos" appears to be achieved progressively during granule biogenesis. Although Tooze and Huttner (1990) reported that a constitutively secreted sulfated proteoglycan was sorted from SgII into different vesicles upon exit from the TGN, Grimes and Kelly (1992) found that a significant fraction of this proteoglycan entered the immature granules first and was subsequently sorted during granule maturation. Similarly, in pancreatic β cells insulin C-peptide was preferentially released from maturing granules (Kuliawat and Arvan, 1992; Neerman and Halban, 1993). In our system, some of the proteoglycans destined for constitutive release also appeared to enter the immature granules; they were secreted together with SgII upon stimulation with calcium (Fig. 2 A, material migrating just below the start of the running gel). Very little of this proteoglycan was secreted in transport buffer containing <10 nM calcium (Fig. 2 A, lane 3). However, since GAG chains entered the immature granules with a fourfold lower efficiency than SgII (see Table I), the contents are probably sorted to some extent upon or shortly after the formation of immature granules. Taken together, these data are consistent with the notion that granule contents are sorted progressively, starting from the formation of immature granules at the TGN and ending with the maturation of secretory granules.

In summary, our results suggest that lumenal calcium ions play an important role in prohormone processing in the distal part of the secretory pathway, but their presence may not be absolutely required for sorting of SgII from the TGN into immature granules. In contrast, the pH gradient of the TGN is essential for export of proteins from the TGN. Our results do not exclude the possible involvement of lumenal calcium ions during granule maturation.

References

Ahnert-Hilger, F., S. Bhakdi, and M. Gratzl. 1985. Minimal requirements for exocytosis. J. Biol. Chem. 260:12730-12734.

Ahnert-Hilger, G., and M. Gratzl. 1987. Further characterization of dopamine release by permeabilized PC12 cells. J. Neurochem. 49:764-770.

Anderson, R. G. W., and R. K. Pathak. 1985. Vesicles and cisternae in the trans golgi apparatus of human fibroblasts are acidic compartments. Cell. 40:635-643.

Baeuerle, P. A., and W. B. Huttner. 1987. Tyrosine sulfation is a trans-Golgi-specific protein modification. J. Cell Biol. 105:2655-2664.

Balch, W. E., W. G. Dunphy, W. A. Braell, and J. E. Rothman. 1984. Reconstitution of the transport of protein between successive compartments of the golgi measured by the coupled incorporation of N-acetylglucosamine. Cell. 39:405-406.

Barr, F. A., A. Leyte, S. Millner, T. Preuffer, S. A. Tooze, and W. B. Huttner. 1991. Trimeric G-proteins of the trans-Golgi network are involved in the formation of constitutive secretory vesicles and immature secretory granules. FEBS (Fed. Eur. Biochem. Soc.) Lett. 294:239-243.

Beckers, C. J. M., and W. E. Balch. 1989. Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. J. Cell Biol. 108:1245-1256.

Benjannet, S., N. Rondeau, R. Day, M. Chretien, and N. G. Seidah. 1991. PC1 and PC2 are protein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. Proc. Natl. Acad. Sci. USA. 88:3564-3568.

Bhakdi, S., J.Tranum-Jenson, and A. Sziegoleit. 1985. Mechanism of membrane damage by streptolysin O. Infect. Immunol. 47:52-60.

Booth, C., and G. L. E. Koch. 1989. Perturbation of cellular calcium induces secretion of luminal ER proteins. Cell. 59:729-737.

Brion, C., S. G. Miller, and H. P. H. Moore. 1992. Regulated and constitutive secretion. Differential effects of protein synthesis arrest on transport of glycosaminoglycan chains to the two secretory pathways. J. Biol. Chem. 267:1477-1483.

Burgess, T. L., and R. B. Kelly. 1984. Sorting and secretion of adrenocorticotropin in a pituitary tumor cell line after perturbation of the level of a secretory granule-specific proteoglycan. J. Cell Biol. 99:2223-2230.

Burgess, T. L., and R. B. Kelly. 1987. Constitutive and regulated secretion. Annu. Rev. Cell Biol. 3:243-293.

Carroll, A., A. R. Rhoads, and P. D. Wagner. 1990. Hydrolysis-resistant GTP analogs stimulate catecholamine release from digitonin-permeabilized PC12 cells. J. Neurochem. 55:930-936.

Chanat, E., and W. B. Huttner. 1991. Milieu-induced, selective aggregation of regulated secretory proteins in the trans-Golgi network. J. Cell Biol. 115:1505-1519.

Chandra, S., E. P. W. Kable, G. H. Morrison, and W. W. Webb. 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.

Debello, W. M., H. Betz, and G. J. Augustine. 1993. Synaptotagmin and neurotransmitter release. Cell. 74:947-950.

Farquhar, M. G. 1985. Progress in unraveling pathways of Golgi traffic. Annu. Rev. Cell Biol. 1:447-448.

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

Galanopoulou, A. S., G. Kent, S. N. Rabbani, N. G. Seidah, and Y. C. Patel. 1993. Heterologous processing of prosomatostatin in constitutive and regulated secretory pathways. J. Biol. Chem. 268:6041-6049.

Gerdes, H. H., P. Rosa, E. Phillips, P. A. Baeuerle, R. Frank, P. Argos, and W. B. Huttner. 1989. The primary structure of human secretogranin II, a widespread tyrosine-sulfated secretory granule protein that exhibits low pH- and calcium-induced aggregation. J. Biol. Chem. 264:12009-12015.

Gomperts, B. D. 1990. G_E: a GTP-binding protein mediating exocytosis. Annu. Rev. Physiol. 52:591-606.

Gorr, S.-U., J. Shioi, and D. V. Cohn. 1989. Interaction of calcium with porcine adrenal chromogranin A (secretory protein-I) and chromogranin B (secretogranin I). Am. J. Physiol. 257:E247-E254.

Green, L., and G. Rein. 1977. Release, storage and uptake of catecholamines by a clonal cell line of Nerve Growth Factor (NGF) responsive pheochromocytoma cells. Brain Res. 129:247-263.

Green, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of a rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA. 73:2424-2428.

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

Hay, J. C., and T. F. J. Martin. 1992. Resolution of regulated secretion into sequential MgATP-dependent and calcium-dependent stages mediated by distinct cytosolic proteins. J. Cell Biol. 119:139-151.

Huttner, W., and S. Tooze. 1989. Biosynthetic protein transport in the secretory pathway. Curr. Opin. Cell Biol. 1:648-654.

Kahn, R. A. 1991. Fluoride is not an activator of the smaller (20-25kDa) GTP-binding proteins. J. Biol. Chem. 266:15595-15597.

Kelly, R. B. 1985. Pathways of protein secretion in eukaryotes. Science (Wash. DC). 230:25-32.

Kimura, J. H., S. Lohmander, and V. C. Hascall. 1984. Studies on the biosynthesis of cartilage proteoglycan in a model system of cultured chondrocytes from the Swarm rat chondrosarcoma. J. Cell. Biochem. 26:261-278.

Klenk, H. D., W. Garten, and R. Rott. 1984. Inhibition of proteolytic cleavage of the hemaglutinin of influenza virus by the calcium-specific ionophore A23187. EMBO (Eur. Mol. Biol. Organ.) J. 3:2911-2915.

Kuliawat, R., and P. Arvan. 1992. Protein targeting via the constitutive-like secretory pathway in isolated pancreatic islets-passive sorting in the immature granule compartment. J. Cell Biol. 118:521-529.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of

the head of bacteriophage T4. *Nature (Lond.)*. 227:680–685.

LeBlond, F. A., G. Viau, J. Laine, and D. LeBel. 1993. Reconstitution in vitro of the pH-dependent aggregation of pancreatic zymogens en route to the secretory granule: implication of GP-2. *Biochem. J.* 291:289–296.

Leyte, A., F. A. Barr, R. H. Kehlenbach, and W. B. Huttner. 1992. Multiple trimeric G-proteins on the trans-Golgi network exert stimulatory and inhibitory effects on secretory vesicle formation. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4795–4804.

Lillie, T. H., and B. D. Gomperts. 1993. Kinetic characterization of guanine-nucleotide-induced exocytosis from permeabilized rat mast cells. *Biochem. J.* 389–394.

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

Malhotra, V., L. Orci, B. S. Glick, M. R. Block, and J. E. Rothman. 1988. Role of an N-ethylmaleimide-sensitive transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack. *Cell.* 54:221–227.

Martell, A. E., and R. M. Smith. 1974. Critical Stability Constants. Plenum Publishing Corp., New York. 469 pp.

Matsuuchi, L., and R. B. Kelly. 1991. Constitutive and basal secretion from the endocrine cell line, AtT-20. *J. Cell Biol.* 112:843–852.

Melancon, P., B. S. Glick, V. Malhotra, P. J. Weidman, T. Serafini, M. L. Gleason, L. Orci, and J. E. Rothman. 1987. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. *Cell.* 51:1053–1062.

Miller, S. G., and H.-P. H. Moore. 1990. Regulated secretion. *Curr. Opin. Cell Biol.* 2:642–647.

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

Miller, S. G., and H.-P. H. Moore. 1992. Movement from trans-Golgi network to cell surface in semiintact cells. *Methods Enzymol.* 219:234–247.

Miller, S. G., L. Carnell, and H.-P. H. Moore. 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.

Molloy, S. S., L. Thomas, J. K. VanSlyke, P. E. Stenberg, and G. Thomas. 1994. Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:18–33.

Moore, H.-P. H., and R. B. Kelly. 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.

Moore, H. P. H., and R. B. Kelly. 1986. Rerouting of a secretory protein by fusion with human growth hormone sequences. *Nature (Lond.).* 321:443–446.

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

Moore, H. P. H., B. Gumbiner, and R. B. Kelly. 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.

Morgan, A., and R. D. Burgoyne. 1993. A synthetic peptide of the N-terminus of ADP-ribosylation factor (ARF) inhibits regulated exocytosis in adrenal chromaffin cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 329:121–124.

Neerman, A. M., and P. A. Halban. 1993. Novel, non-crinophagic, degradation of connecting peptide in transformed pancreatic beta cells. *J. Biol. Chem.* 268:16248–16252.

Niehrs, C., and W. B. Huttner. 1990. Purification and characterization of tyrosylprotein sulfotransferase. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:35–42.

Oberhauser, A. F., J. R. Monck, W. E. Balch, and J. M. Fernandez. 1992. Exocytotic fusion is activated by Rab3a peptides. *Nature (Lond.).* 360:270–273.

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., M. Ravazzola, M. Amherdt, O. Madsen, A. Perrelet, J. Vassalli, and R. Anderson. 1986. Conversion of proinsulin occurs coordinately with acidification of maturing secretory vesicles. *J. Cell Biol.* 103:2273–2282.

Orci, L., M. Ravazzola, M. Amherdt, A. Perrelet, S. Powell, D. Quinn, and H.-P. Moore. 1987. The trans-most cisternae of the Golgi complex: a compartment for sorting of secretory and plasma membrane proteins. *Cell.* 51:1039–1051.

Peppers, S., and R. Holz. 1986. Catecholamine secretion from digitonin-treated PC12 cells. *J. Biol. Chem.* 261:14665–14669.

Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sort-

ing by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56:829–852.

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., A. Hille, R. W. H. Lee, A. Zanini, P. De Camilli, and W. B. Huttner. 1985. Secretogranins I and II: two tyrosine-sulfated secretory proteins common to a variety of cells secreting peptides by the regulated pathway. *J. Cell Biol.* 101:1999–2011.

Rosa, P., F. A. Barr, J. C. Stinchcombe, C. Binacchi, and W. B. Huttner. 1992a. 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.

Rosa, P., S. Mantovani, R. Rosboch, and W. B. Huttner. 1992b. Monensin and brefeldin a differently affect the phosphorylation and sulfation of secretory proteins. *J. Biol. Chem.* 267:12227–12232.

Salton, S. R. J., R. U. Margolis, and R. K. Margolis. 1983. Release of chromaffin granule glycoproteins and proteoglycans from potassium-stimulated PC12 pheochromocytoma cells. *J. Neurochem.* 41:1165–1170.

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

Schwaninger, R., H. Plutner, G. M. Bokoch, and W. E. Balch. 1992. Multiple GTP-binding proteins regulate vesicular transport from the ER to Golgi membranes. *J. Cell Biol.* 119:1077–1096.

Schwartz, N. B., L. Galligani, P.-L. Ho, and A. Dorfman. 1974. Stimulation of synthesis of free chondroiton sulfate chains by b-D-xylosides in cultured cells. *Proc. Natl. Acad. Sci. USA.* 71:4047–4051.

Senyshyn, J., W. E. Balch, and R. W. Holz. 1992. Synthetic peptides of the effector-binding domain of rab enhance secretion from digitonin-permeabilized chromaffin cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 309:41–46.

Shennan, K. I. J., S. P. Smeekens, D. F. Steiner, and K. Doherty. 1991. Characterization of PC2, a mammalian Kex2 homolog, following expression of the cDNA in microinjected Xenopus oocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 284:277–280.

Shirataki, H., K. Kaibuchi, T. Sakoda, S. Kishida, T. Yamaguchi, K. Wada, M. Miyazaki and Y. Takai. 1993. Rabphilin 3A, a putative target protein for smg p25A/rab3A p25 small GTP-binding protein related to synaptotagmin. *Mol. Cell Biol.* 13:2061–2068.

Sollner, T., M. K. Bennett, S. W. Whiteheart, R. H. Scheller, and J. E. Rothman. 1993. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell.* 75:409–418.

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

Tooze, J., M. Hollinshead, R. Frank, and B. Burke. 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., R. Flatmark, J. Tooze, and W. B. Huttner. 1991. Characterization of the immature secretory granule, an intermediate in granule biogenesis. *J. Cell Biol.* 115:1491–1503.

Velasco, A., J. Hidalgo, J. Perez-Vilar, G. Garcia-Herdugo, and P. Navas. 1988. Detection of glycosaminoglycans in the Golgi complex of chondrocytes. *Eur. J. Cell Biol.* 47:241–250.

von Zastrow, M., A. M. Castle, and J. D. Castle. 1989. Ammonium chloride alters secretory protein sorting within the maturing exocrine storage compartment. *J. Biol. Chem.* 264:6566–6571.

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

Xu, H., and D. Shields. 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.

Yanagita, M., H. Hoshino, K. Nakayama, and T. Takeuchi. 1993. Processing of mutated proinsulin with tetrabasic cleavage sites to mature insulin reflects the expression of furin in nonendocrine cell lines. *Endocrinology.* 133:639–644.

Yang, Y. C., N. C. Vu, and P. D. Wagner. 1992. Guanine nucleotide stimulation of norepinephrine secretion from permeabilized PC12 cells: effects of Mg++, other nucleotide triphosphates and N-ethylmaleimide. *Biochem. Biophys. Acta.* 1134:285–291.

Yoo, S. H., and J. P. Albanesi. 1990. Ca^{2+}-induced conformational change and aggregation of chromogranin A*. *J. Biol. Chem.* 265:14414–14421.

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