Prosomatostatin Processing in Permeabilized Cells

CA I L U M I S I S R E Q U I R E D F O R P R O H O R M O N E C L E A V A G E B U T N O T F O R M A T I O N O F N A S C E N T S E C R E T O R Y V E S I C L E S*

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Our laboratory has been using a permeabilized cell system derived from rat anterior pituitary GH3 cells expressing prosomatostatin (pro-SRIF) to study prohormone processing and nascent secretory vesicle formation in vitro. Because calcium is necessary for prohormone processing enzyme activity, secretory granule fusion with the plasma membrane, and possibly sorting to the regulated pathway, we treated permeabilized cells with the calcium ionophore A23187 to determine the role of calcium in pro-SRIF cleavage and nascent vesicle formation from the trans-Golgi network (TGN). Here we demonstrate that pro-SRIF cleavage was markedly inhibited when luminal free calcium was chelated with EGTA in the presence of A23187. Surprisingly, submillimolar free calcium (−15 μM) was sufficient to maintain prohormone cleavage efficiency, a value far lower than that estimated for total calcium levels in the TGN and secretory granules. Experiments using both A23187 and the nonproionophore CCCP revealed that free calcium is absolutely required for efficient pro-SRIF cleavage, even at the optimal pH of 6.1. Secretory vesicle formation by contrast was not inhibited by calcium chelation but rather by millimolar extraluminal free calcium. Together, these observations demonstrate that pro-SRIF processing and budding of nascent secretory vesicles from the TGN can be uncoupled and therefore have distinct biochemical requirements. Interestingly, our data using intact GH3 cells demonstrate that basal secretion of SRIF-related material is largely calcium-dependent and therefore cannot be equated with constitutive pathway secretion. These results underscore the importance of determining calcium requirements before assigning a secretion event to either the constitutive or regulated secretory pathway.

The secretary pathway in eukaryotes consists of a series of topologically distinct intracellular membrane-enclosed compartments that are specialized in composition and function. Vesicular transport permits sorting without compromising compartmental identity. In polypeptide hormone-producing cells, the trans-Golgi network (TGN) has been implicated in specific protein trafficking and endoproteolytic maturation events in the distal secretory pathway (for review see Shields and Danoff, 1993). These cells can store and concentrate hormones in dense core secretory granules that deliver their contents to the cell surface by calcium-dependent fusion with the plasma membrane after an appropriate extracellular stimulus. In addition, neuroendocrine cells possess the ubiquitous constitutive pathway, whereby secretory proteins are continually delivered to the cell surface in a calcium-independent manner (Turner et al., 1992; Miller and Moore, 1991). Morphological evidence indicates that sorting of nascent secretory proteins into the regulated versus constitutive pathway occurs in the TGN (Sossin et al., 1990; Orci et al., 1987), although recent studies suggest that in pancreatic cells sorting may also occur after TGN exit in immature secretory granules through a pathway called “constitutive-like secretion” (Kuliawat and Arvan, 1994; Arvan and Castle, 1992). Biochemical evidence supports a model whereby the low pH and high calcium in the TGN lumen triggers selective aggregation and hence sorting of proteins destined for secretory granules (Song and Fricker, 1995; Colomer et al., 1994; Chaten and Huttner, 1991), whereas constitutively secreted proteins are excluded from such aggregates.

Luminal ionic conditions also appear to play an important role in hormone activation. Many peptide hormones are initially synthesized as larger inactive polyprotein precursors or prohormones that undergo endoproteolytic maturation during intracellular transport. Prohormone cleavage occurs within the TGN and secretory granules at a defined set of basic residues by the recently discovered prohormone convertase enzymes (PCs) (for reviews see Seidah et al. (1993) and Steiner et al. (1992)). PCs1 and PC2, which are the predominant PCs expressed in neuroendocrine tissue, are calcium-dependent endoproteases that require an acidic pH for maximal activity (Shennan et al., 1995; Rhodes et al., 1993; Davidson et al., 1998).

To investigate prohormone sorting, endoproteolytic maturation, and secretory vesicle formation in vitro, we have developed a permeabilized cell system (Xu and Shields, 1994, 1993) derived from growth hormone and prolactin secreting rat anterior pituitary GH3 cells stably expressing human or anglerfish prosomatostatin (pro-SRIF) (Elgort and Shields, 1994; Stoller and Shields, 1988). These cells possess significant levels of PC2 and cleave pro-SRIF with ~70% efficiency to generate the amino-terminal 75-80 amino acid propeptide and the carboxy-terminal 14-amino acid mature SRIF (Elgort and Shields, 1994; Stoller and Shields, 1988). Pro-SRIF cleavage in permeabilized GH3 cells requires ATP to generate an acidic

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The abbreviations used are: TGN, trans-Golgi network; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N",N"-tetraacetic acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; SRIF, somatostatin; PC, prohormone convertase enzyme; MES, 4-morpholineethanesulfonic acid.

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Calcium Requirements for Prosomatostatin Processing

TGN luminal pH (Xu and Shields, 1994). For concomitant formation of nascent secretory vesicles, both ATP and GTP are required (Xu and Shields, 1993).

We have employed the permeabilized cell system in conjunction with the calcium ionophore A23187 to examine the role of free calcium in prohormone processing and vesicle formation from the TGN. Here, we demonstrate that luminal free calcium is absolutely required for efficient pro-SRIF cleavage but not for secretory vesicle formation. We also show that luminal ionic conditions permissive for pro-SRIF cleavage in the TGN are nonpermissive extraluminally for vesicle formation.

EXPERIMENTAL PROCEDURES

Materials—[35S]Methionine was purchased from Amersham Corp. EGTA was purchased from J. T. Baker, Inc., A23187, CCCP, and BAPTA were purchased from Calbiochem. Rabbit anti-growth hormone serum (Xu and Shields, 1993), anti-SRIF-propeptide serum, which recognizes prepro-SRIF, pro-SRIF, and the free propeptide (Elgort and Shields, 1994), and anti-SRIF serum (RSS1), which recognizes prepro-SRIF, pro-SRIF, and the carboxyl-terminal mature SRIF-14 (Warren and Shields, 1984), were described previously. Rabbit antiprolactin serum (Stoller and Shields, 1984), were described previously. Rabbit anti-prolactin serum raised against a peptide corresponding to residues 200–213 of rat prolactin (CLRRDSHKVDNYLK), which was cross-linked to maleimide-activated hemocyanin (Pierce), was raised against a peptide corresponding to residues 200–213 of rat prolactin (CLRRDSHKVDNYLK), which was cross-linked to maleimide-activated hemocyanin (Pierce). Rat anterior pituitary GH3 cells stably expressing human prepro-SRIF (GH3.Hu.S) were described previously (Elgort and Shields, 1994). Cell Culture and Pulse Labeling—GH3.Hu.S cells were grown and pulsed with 500 μCi/mg [35S]methionine, which labels the amino-terminal propeptide of pro-SRIF as described previously (Stoller and Shields, 1988), and chased in the presence of complete growth medium for 2 h at 19°C. These conditions result in retention of ∼95% of the radiolabeled hormones in the TGN (Xu and Shields, 1993). Cells were then placed on ice prior to subsequent treatment.

Permeabilized Cell Preparation and in Vitro Incubations—Preparation of mechanically permeabilized cells (~95% breakage as assessed by trypan blue staining) was described previously (Xu and Shields, 1993). The standard incubation condition for these experiments contained 5 × 10^6 permeabilized cells, 25 mM Hepes-KOH, pH 7.3, 125 mM KCl, 2.5 mM MgCl2, 1 mM ATP, 200 μM GTP, 10 mM creatine phosphate, 160 μg/ml creatine phosphate kinase (ATP regenerating system), 0.5 mM phenylmethylsulfon fluoride, and 5 μg/ml trasyol in 300 μl. Background free calcium was determined to be 10–20 μM by measurement with a calcium-selective electrode (see below). Incubation for 2 h at 37°C under these conditions is sufficient for both pro-SRIF maturation and nascent secretory vesicle formation (Xu and Shields, 1993). Because our previous studies demonstrated that pro-SRIF processing can be effectively monitored by measuring the consumption of pro-SRIF concomitantly with the accumulation of either the free propeptide by SDS-polyacrylamide gel electrophoresis or mature SRIF from HPLC (Elgort and Shields, 1994), we used the former protocol for these experiments. For incubations in the presence of A23187 (dissolved in dimethyl sulfoxide) or CCCP (dissolved in ethanol), final concentrations of solvent in the reactions did not exceed 0.5%. For incubations at pH 6.1, Hepes in the assay buffer was replaced by 25 mM MES, pH 6.1.

Intact Cell Incubations—After the 19°C chase, cells were incubated for 10 min at 4°C followed by 2 h at 37°C in buffer containing 25 mM Hepes, pH 7.3, 130 mM NaCl, 3.2 mM KCl, 0.6 mM MgSO4, 1.3 mM KH2PO4, 6 mM glucose, 0.1% bovine serum albumin, and either 10 μM EGTA, 10 μM CaCl2, or background levels of free calcium determined to be 10–50 μM by measurement with a calcium-selective electrode (see below). Incubation for 30 min in the absence of calcium resulted in 30% processing.

Free Calcium Measurements—Background free calcium concentrations were determined with a calcium-selective electrode (Philips IS 561) equipped with a fresh poly(vinyl chloride) membrane. The potential difference between the calcium electrode and a reference electrode (double-junction Orion) was monitored with an Orion Expandable ion-Analyzer. The calibration curve of this electrode was linear between 10^-6 and 10^-3 M in CaCl2.

Immunoprecipitation and Densitometry—Sedimented intact or permeabilized cell pellets were lysed in 100 μl of phosphate-buffered saline containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and a mixture of protease inhibitors as described previously (Stoller and Shields, 1988). Lysates and supernatants containing secreted or budding material were treated sequentially with each antiserum in a buffer containing 2.5% Triton X-100 and precipitated with protein A-Sepharose as described previously (Xu and Shields, 1993). Immunoreactive material was resolved by SDS-polyacrylamide gel electrophoresis and detected by fluorography. Band intensities were quantitated using a Molecular Dynamics Model 300A computing densitometer and Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Luminal Free Calcium Is Required for Endoproteolytic Maturation of pro-SRIF.—To determine the free calcium requirement for pro-SRIF processing in permeabilized cells, the calcium concentration of the assay buffer was manipulated in the presence or the absence of the calcium ionophore A23187. As shown in Fig. 1, pro-SRIF cleavage efficiency was not significantly affected by perturbations of extraluminal calcium in the absence of A23187. Our standard in vitro incubation, which permits ∼35–50% pro-SRIF cleavage (Fig. 1B; Xu and Shields (1994)), includes only trace levels (∼15 μM) of free calcium. Chelation of this extraluminal free calcium with EGTA did not significantly alter cleavage efficiency (Fig. 1, A and B, hatched bars). Similarly, addition of up to 10 mM free calcium, a concentration similar to estimates for total (free plus bound) calcium in the TGN lumen (Chanut and Huttnner, 1991), had little effect on pro-SRIF processing. However, chelation of free calcium with EGTA markedly inhibited pro-SRIF cleavage when A23187 was included to access the luminal free calcium pools (Fig. 1, A and B, black bars). In contrast, cleavage efficiency was maintained in the presence of A23187 when free calcium was included in the incubations. Surprisingly, the trace free calcium of our standard incubation maintained efficient pro-SRIF cleavage in the presence of A23187. To eliminate the unlikely possibility that EGTA was inhibiting pro-SRIF cleavage by a mechanism other than chelation of free calcium, both EGTA and calcium were included simultaneously in the presence of A23187 (data not shown). Pro-SRIF cleavage was not inhibited by EGTA in these experiments, provided that the amount of added calcium was equal to or greater than that of EGTA (i.e., ∼50 μM free calcium or greater, as determined with a calcium-selective electrode). These results demonstrate that luminal free calcium in the low micromolar range was sufficient for efficient endoproteolytic maturation of pro-SRIF in permeabilized cells. We cannot exclude the possibility, however, that a lower free calcium concentration would also support processing.

To confirm the luminal calcium requirement for pro-SRIF cleavage in vivo, intact cells were incubated at 37°C with or without free calcium in the presence of A23187 following a 2 h chase at 19°C. Consistent with the in vitro results, chelation of cellular free calcium with EGTA in the presence of A23187 inhibited pro-SRIF cleavage (Fig. 2). Pro-SRIF cleavage efficiency in the presence of A23187 was significantly higher in samples containing trace (10–50 μM) or 10 mM added calcium, although for unknown reasons, processing was not completely restored to control levels. Thus luminal free calcium in the low micromolar range was also sufficient for endoproteolytic maturation of pro-SRIF in intact cells.

Our earlier studies demonstrated that a pH of 6.0–6.2 is necessary for pro-SRIF cleavage in the TGN (Xu and Shields, 1994), a value that corresponds to estimates of the endogenous luminal pH in the TGN (Sebek et al., 1995; Yilla et al., 1993; Anderson and Orci, 1988). To determine if the luminal free calcium requirement for pro-SRIF cleavage is independent of the pH requirement, permeabilized cells were incubated under varied calcium and pH conditions in the presence of both A23187 and the protonophore CCCP simultaneously (Fig. 3, black bars). When luminal calcium was chelated with EGTA at the suboptimal pH of 7.3 (double nonpermissive condition), cleavage was inhibited. In contrast, when millimolar free calcium was present at the optimal pH of 6.1 (double permissive condition), pro-SRIF cleavage was 45–50% efficient. Chelation
Calcium Requirements for Prosomatostatin Processing

**Fig. 1.** Calcium requirement for pro-SRIF cleavage in vitro. A, cells were pulse-labeled with [35S]methionine for 12 min at 37°C, chased for 2 h at 19°C, and permeabilized. Permeabilized cells were then preincubated with or without 1 μM calcium ionophore A23187 in the presence of 2 mM EGTA or ~0.015 (standard condition), 0.5, 2.0, 5.0, or 10.0 mM additional calcium chloride for 10 min at 4°C, followed by a further incubation with ATP and GTP for 2 h at 37°C. Samples were then separated into pellet (P) and supernatant (S) fractions to monitor vesicle formation (see Fig. 4) by centrifugation at 15,000 × g for 10 s and immunoprecipitated with rabbit anti-SRIF-propeptide or anti-SRIF (RSS1) serum (see “Experimental Procedures”). Immunoreactive peptides were resolved by SDS-polyacrylamide gel electrophoresis (20% acrylamide) and detected by fluorography. **B,** cleavage of pro-SRIF in permeabilized cells requires submillimolar luminal free calcium. Quantitation by densitometry of fluorograms was as in A. Pro-SRIF cleavage efficiency was determined by subtracting the sum of the propeptide band intensities from the pellet and supernatant multiplied by 100. The values represent means ± S.E. from at least three experiments. Incubations with up to 10 mM EGTA resulted in similar cleavage efficiencies to those with 2 mM EGTA (not shown).

Of luminal free calcium with EGTA at the optimal pH of 6.1 (single nonpermissive condition) only partially inhibited pro-SRIF cleavage because the affinity of EGTA for calcium is strongly pH-dependent and greatly diminished at low pH (Bers et al., 1994). The calcium chelator BAPTA exhibits a much lower pH sensitivity and consequently is more effective at pH 6.1 (Bers et al., 1994). When permeabilized cells were treated with BAPTA at the optimal pH of 6.1, pro-SRIF maturation was completely inhibited (~100% cleavage). As observed for the single ionophore experiments, trace micromolar free calcium maintained efficient pro-SRIF cleavage at pH 6.1 in the presence of A23187 and CCCP (data not shown). Thus even at the optimal pH of 6.1 luminal free calcium (no more than ~15 μM) is absolutely required for efficient endoproteolytic maturation of pro-SRIF. In contrast to our previous single ionophore experiments with CCCP at pH 7.3 (Xu and Shields, 1994), inhibition of cleavage at this pH was not complete in the presence of both ionophores and 10 mM calcium (data not shown) for reasons that are unclear at present. Nevertheless, our data here demonstrate that although acidic luminal pH is important, luminal free calcium is absolutely required for efficient pro-SRIF cleavage. Cleavage efficiency was much less sensitive to calcium and pH manipulations when ionophores were omitted from the incubations (Fig. 3, hatched bars), consistent with the ability of the TGN and nascent secretory vesicles of the permeabilized cells to maintain a relatively stable luminal environment in the presence of variations in the extraluminal environment.

Free Calcium Is Not Required for Nascent Secretory Vesicle Formation—To determine the free calcium requirement for nascent vesicle formation, we monitored budding efficiency from the TGN in vitro (Fig. 4). Vesicle budding from the TGN was very efficient (35–50% for growth hormone, prolactin, and pro-SRIF) when extraluminal calcium was chelated with EGTA in the absence of A23187 (Figs. 1A and 4). Identical results were obtained when incubations were performed in the presence of A23187 (Fig. 1A; data not shown). Our assay for vesicle budding is based on quantitating the release of radiolabeled hormones into a 15,000 × g supernatant following an in vitro incubation. To confirm that the appearance of radiolabeled hormones in the supernatant after incubation with A23187 and EGTA resulted from intact vesicle release rather than TGN lysis, sedimentation and protease protection assays were performed (Fig. 5). Most (66%) of the radiolabeled hormones appearing in the 15,000 × g supernatant after a standard incubation with EGTA (lane 2) could be sedimented at 200,000 × g (lane 4) with less (34%) remaining in the supernatant (lane 5). Furthermore, the radiolabeled hormones appearing in the 15,000 × g supernatant were protease-resistant (lane 7), whereas addition of detergent rendered them protease-sensitive (lanes 8 and 9). Thus the released radiolabeled hormones were membrane-enclosed and did not result from membrane lysis. We conclude that formation of nascent vesicles from the TGN of permeabilized cells does not require extraluminal nor luminal free calcium.

Free Calcium Inhibits Nascent Secretory Vesicle Formation in Vitro but Is Required for Efficient Basal Secretion of pro-SRIF in Vivo—Formation of nascent secretory vesicles from the TGN of permeabilized cells was strongly inhibited by millimolar concentrations of extraluminal free calcium (Figs. 1 and 4).

2 A requirement for low nanomolar free calcium (Ronnin and Martin, 1986), however, cannot be excluded.
Calcium Requirements for Prosomatostatin Processing

This inhibitory effect of millimolar calcium was not due to nonspecific vesicle aggregation or adherence to the permeabilized cells. Inhibition was half-maximal at ~2–5 μM free calcium independent of the presence of A23187 (Figs. 1A and 4, and data not shown). To determine if calcium was also inhibitory in vivo, we monitored basal secretion from intact cells (Fig. 6). In contrast to the observations in vitro, basal hormone secretion from intact cells in the presence of A23187 was not inhibited by millimolar free calcium (Fig. 6). Thus the calcium-sensitivity of TGN exit in vitro could not be recapitulated in vivo. Interestingly, basal secretion of pro-SRIF and the SRIF propeptide was dramatically inhibited in the presence of A23187 when free calcium was chelated with EGTA (68 ± 7% inhibition, Fig. 6, hatched bars). Inhibition was less pronounced for prolactin (40 ± 13% inhibition, Fig. 6, shaded bars) and growth hormone (~50% inhibition, data not shown). Trace free calcium (10–50 μM) was sufficient to maintain efficient secretion in the presence of A23187. Thus free calcium is required for efficient basal secretion, particularly for pro-SRIF and the SRIF propeptide.

DISCUSSION

An advantage of using a permeabilized cell system to study organelle function is that cytosolic conditions can be directly manipulated without necessarily changing conditions within the lumen of the organelle. Our permeabilized cell system allows us to examine an important event in the lumen of the TGN and immature secretory granules: the endoproteolytic maturation of a prohormone. In addition, this system permits examination of an isolated step in the transport of hormones from the TGN to the cell surface: the formation of nascent secretory vesicles. In this study, we exploited the permeabilized cell system to (a) determine the luminal free calcium requirement for endoproteolytic maturation of pro-SRIF, (b) compare the requirements for low pH versus calcium for pro-SRIF processing, and (c) determine the effect of perturbed calcium concentration and pH on the formation of vesicles from the TGN. Our data demonstrate that low levels (~15 μM) of luminal free calcium are sufficient for efficient endoproteolytic maturation of pro-SRIF in permeabilized GH3 cells (Fig. 1A and B). This is independent of the requirement for low pH, because inefficient cleavage occurred at the optimal pH of 6.1 when luminal free calcium was chelated with BAPTA (Fig. 3). The requirement for submillimolar free calcium could also be demonstrated in intact cells (Fig. 2).

In light of the high levels of intraluminal calcium (estimated to be 10 mM or higher) in the TGN and secretory granules (Chanat and Huttner, 1991), it was surprising that such low concentrations of free calcium were sufficient to support pro-SRIF cleavage. However, much of this calcium may in fact be bound to high capacity, low affinity calcium-binding proteins such as the chromogranins (Rosa et al., 1992; Huttner et al., 1991; Reiffen and Gratzi, 1986). Indeed, studies using a calcium-selective electrode suggest that in chromaffin granules, only ~24 μM calcium exists in the free unbound state (Bulenda and Gratzi, 1985). Thus our present observations showing a low micromolar free calcium requirement for pro-SRIF cleavage is consistent with earlier measurements of endogenously available luminal free calcium in regulated secretory granules. We do not know if the exchange between free and protein-bound pools of calcium is sufficiently rapid to permit depletion of the protein-bound pool when free calcium is chelated during...
Calcium Requirements for Prosomatostatin Processing

Fig. 4. Nascent secretory vesicle formation does not require free calcium and is inhibited by millimolar extraluminal free calcium. The pellet and supernatant (nascent vesicle) fractions from Fig. 1 were treated sequentially with anti-SRIF-propeptide, antiprolactin, and growth hormone antisera and the immunoprecipitated material quantitated by densitometry of fluorograms. Budding efficiency = ([sum of the hormone band intensities from the supernatant] + [sum of the hormone band intensities from the pellet and supernatant]) × 100. The values represent the means ± S.E. from at least three experiments. Budding efficiency in the absence of A23187 is shown and was not significantly affected by inclusion of 1 μM A23187 (Fig. 1A and data not shown).

| Treatment: | CON | SED | PKI | PKI/Det |
|------------|-----|-----|-----|---------|
| % Budding: | 46  | 30  | 56  |         |
| kDa        | P   | P   | S   |         |
| Prolactin  | 16.4| 18.4|      |         |

Treatment (no ionophore)

| Concentration (mM) | Budding Efficiency (%) |
|--------------------|------------------------|
| 2 mM EGTA          | 50                     |
| 0.015 mM Ca²⁺      | 40                     |
| 0.5 mM Ca²⁺        | 30                     |
| 2 mM Ca²⁺          | 20                     |
| 5 mM Ca²⁺          | 10                     |
| 10 mM Ca²⁺         | 5                      |

Fig. 5. Prolactin is packaged into sedimentable and membrane-enclosed secretory vesicles. Permeabilized cells were incubated as in Fig. 1 with 1 μM A23187 in the presence of 2 mM EGTA. One sample (CON) was separated into 15,000 × g pellet (lane 1) and supernatant (lane 2) fractions; a second sample (SED) separated into 15,000 × g pellet (lane 3) and supernatant fractions. This supernatant was then centrifuged at 200,000 × g for 20 min to generate a second pellet (lane 4) (nascent vesicle) and supernatant (lane 5) fractions. Samples PKI and PKI/Det were incubated with 25 μg/ml proteinase K in the absence or the presence of 1% Triton X-100, respectively, for 15 min at 4°C, quenched with 1 mM phenylmethylsulfonyl fluoride, and separated into 15,000 × g pellet (lanes 6 and 8) and supernatant (lanes 7 and 9) fractions. Budding efficiency was determined by immunoprecipitation using rabbit anti-prolactin serum. For the SED sample, budding efficiency is defined as (prolactin band intensity from the SP fraction) + (sum of the prolactin band intensities from the P, SP, and SS fractions). Similar results were obtained for budding of growth hormone and SRIF propeptide + pro-SRIF (not shown).

Our data also demonstrate that free calcium is not required for vesicle formation from the TGN of permeabilized cells (Figs. 1A and 4). A similar observation was made previously using permeabilized MDCK cells (Bennett et al., 1988). Interestingly, a recent study investigating the sorting of secretogranin II from the TGN of permeabilized PC12 cells (Carnell and Moore, 1994) suggests that luminal free calcium may not be required for sorting. In our system, formation of vesicles from the TGN was strongly inhibited by extraluminal free calcium roughly 5 orders of magnitude greater than physiological cytoplasmic conditions (Figs. 1A and 4). We were unable to demonstrate a calcium block on TGN exit in intact cells (Fig. 6) and presume that in permeabilized cells, millimolar calcium nonspecifically disrupts the TGN budding machinery. Interestingly, elevated cytosolic calcium levels (~100 μM) in permeabilized cells can cause mistargeting of plasma membrane clathrin-coated vesicle adaptor complexes (Seaman et al., 1993). We also found that an acidic extraluminal pH of 6.1 strongly inhibits both budding in permeabilized cells and secretion in intact cells (not shown).

Inhibition of clathrin-mediated transport events from the TGN and plasma membrane by cytosol acidification is well documented (Hansen et al., 1993; Cosson et al., 1989; Heuser and Anderson, 1989; Heuser, 1989; Sandvig et al., 1987) and appears to involve reversible structural alterations in the clathrin cages.

Significantly, our results reveal that pro-SRIF endoproteolytic maturation and entry into nascent secretory vesicles are consistent with the observed in vitro properties of PC2, the enzyme likely responsible for pro-SRIF cleavage in our system (Brachch et al., 1995; Siedah et al., 1994; Xu and Shields, 1994; Mackin et al., 1991). Using PC2 purified from rat insulinoma granules, Hutton and colleagues observed low micromolar calcium optima for cleavage of purified chromogranin A (K_m = 11 μM) (Arden et al., 1994), proopiomelanocortin (K_m = 5–80 μM) (Rhodes et al., 1993), and proinsulin (K_m = 100 μM) (Bennett et al., 1992). The calcium optimum for cleavage by PC2 synthesized in vitro using Xenopus egg extracts, however, was higher (K_m = 1–4 mM) (Shennan et al., 1995). In contrast to PC2 purified from granules, PC1 displayed a much higher calcium optimum in vitro (K_m = 5 μM for proinsulin cleavage; Davidson et al., 1988 and Zhou and Lindberg, 1993).
secretory vesicles with the plasma membrane has been well regulated pathway by contrast, calcium-dependent fusion of secretion. Distal steps in constitutive secretion do not require be made between basal secretion and constitutive pathway and growth hormone, reveals a calcium-dependent transport Overall, our data demonstrate that by using the permeabilized calcium and lower pH requirements than the latter (Zhou and activities of PC1 and PC2, the former having significantly higher is noteworthy that proinsulin processing requires both the ac-
substrate-specific differences or methodological differences. It endoproteolytic maturation cannot proceed if TGN exit is blocked. It is not clear if this discrepancy is due to tissue- or finding that pro-SRIF endoproteolytic maturation can proceed in the TGN if exit is prevented (Xu and Shields, 1993). These observations contrast with recent studies using pancreatic is-
in the TGN if exit is prevented (Xu and Shields, 1993). These observations contrast with recent studies using pancreatic is-
not obligatorily coupled events (compare Figs. 1B and 4). Pro-SRIF cleavage was inhibited by chelation of luminal free calcium, whereas formation of nascent vesicles was unimpaired. Conversely, vesicle formation but not pro-SRIF cleavage was inhibited by raising extraluminal free calcium to millimolar levels or by lowering the pH, which reinforces our previous finding that pro-SRIF endoproteolytic maturation can proceed in the TGN if exit is prevented (Xu and Shields, 1993). These observations contrast with recent studies using pancreatic islets (Huang and Arvan, 1994), which suggested that proinsulin endoproteolytic maturation cannot proceed if TGN exit is blocked. It is not clear if this discrepancy is due to tissue- or substrate-specific differences or methodological differences. It is noteworthy that proinsulin processing requires both the activities of PC1 and PC2, the former having significantly higher calcium and lower pH requirements than the latter (Zhou and Lindberg, 1993; Rhodes et al., 1993; Davidson et al., 1988). Overall, our data demonstrate that by using the permeabilized cell system, prohormone endoproteolytic maturation can be dissociated from entry into secretory vesicles.

Inhibition of pro-SRIF basal secretion from intact cells by chelation of free calcium, and to a lesser extent that of prolactin and growth hormone, reveals a calcium-dependent transport step distal to TGN exit (Fig. 6). An important distinction must be made between basal secretion and constitutive pathway secretion. Distal steps in constitutive secretion do not require calcium (Turner et al., 1992; Miller and Moore, 1991). In the regulated pathway by contrast, calcium-dependent fusion of secretory vesicles with the plasma membrane has been well documented (Turner et al. (1992) and Martin and Walent

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