The constitutive and regulated secretory pathways represent the classical routes for secretion of proteins from neuroendocrine cells. Selective aggregation of secretory granule constituents in an acidic, bivalent cation-rich environment is considered to be a prerequisite for sorting to the regulated secretory pathway. The effect of selective vacuolar H\(^+\)-ATPase (V-ATPase) inhibitor bafilomycin A1 on the pH gradient along the secretory pathway was used here to study the role of acidification on the trafficking of the regulated secretory protein chromogranin A (CgA) in PC12 cells. Sorting of CgA was assessed by three-dimensional deconvolution microscopy, subcellular fractionation, and secretagogue-stimulated release, examining a series of full-length or truncated domains of human CgA (CgA-(1–115), CgA-(233–439)) fused to either green fluorescent protein or to a novel form of secreted embryonic alkaline phosphatase (EAP). We show that a full-length CgA/EAP chimera is sorted to chromaffin granules for exocytosis. Inhibition of V-ATPase by bafilomycin A1 markedly reduced the secretagogue-stimulated release of CgA-EAP by perturbing sorting of the chimera (at the trans-Golgi network or immature secretory granule) rather than the late steps of exocytosis. The effect of bafilomycin A1 on CgA secretion depends on a sorting determinant located within the amino terminus (CgA-(1–115)) but not the C-terminal region of the granin. Moreover, examination of chromaffin granule abundance in PC12 cells exposed to bafilomycin A1 reveals a substantial decrease in the number of dense-core vesicles. We propose that a V-ATPase-mediated pH gradient in the secretory pathway is an important factor for the formation of dense-core granules by regulating the ability of CgA to form aggregates, a crucial step that may underlie the granulogenic function of the protein.

The constitutive and regulated secretory pathways represent the classical routes for secretion of proteins from neuroendocrine cells (1). The constitutive pathway allows rapid transport of proteins in small vesicles originating from the outermost layer of the Golgi complex (the trans-Golgi network, or TGN\(^1\)) and destined for rapid fusion with the plasma membrane. The regulated secretory pathway is characterized by the concentration of a selected (segmented) pool of secretory proteins into granules with a typical electron-dense appearance on transmission electron microscopy. These granules may remain in the cell for an extended period of time after their formation until prompted to undergo exocytotic fusion with the plasma membrane by a secretagogue characteristic for a particular cell type.

Selective aggregation or condensation of secretory granule constituents in a mildly acidic environment and in the presence of millimolar concentrations of bivalent cations like Ca\(^{2+}\) is considered to be an important prerequisite for two proposed models of sorting within the regulated secretory pathway, namely sorting-for-entry and sorting-by-retention (2–4). In the sorting-for-entry model, the lumen of the TGN serves as a unique trafficking station where selective aggregation of the secretory protein may take place followed by subsequent binding of the protein aggregate to the membrane of the budding secretory granule or to a sorting receptor therein. In the sorting-by-retention hypothesis sorting takes place in a secretory organelle distal to the TGN, the short-lived immature secretory granule, wherein selective aggregation/condensation of regulated secretory proteins occurs; non-retained proteins are then removed from maturing granules, possibly into a constitutive-like secretory pathway.

Chromogranin A (CgA) belongs to the chromogranins/secreto-granins (or “granins”) family of regulated secretory proteins, which are ubiquitously distributed in secretory granules of endocrine, neuroendocrine, and neuronal cells. Because of their widespread occurrence, granins and particularly CgA have often been used as model proteins to understand mechanisms of protein targeting into dense-core secretory granules (4–8). A key determinant for secretory granule storage of CgA may be its propensity to form aggregates in a mildly acidic pH environment in the presence of Ca\(^{2+}\) (9–14), conditions that are fulfilled in the lumen of the TGN.

The pH of organelles along the secretory pathway decreases progressively from the endoplasmic reticulum to the secretory granule. For instance, determination of organelle pH in live

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\(^1\) The abbreviations used are: TGN, trans-Golgi network; CgA, chromogranin A; CgB, chromogranin B; CFP, cyan fluorescent protein; CMV, cytomegalovirus (immediate-early gene promoter); EAP, embryonic alkaline phosphatase devoid of the SEAP 17 amino acid signal peptide; GFP, green fluorescent protein; EGFP, enhanced GFP; GalT, 1,4-galactosyltransferase cyan fluorescent protein (Golgi marker); PBS, phosphate-buffered saline; PLAP, human placental alkaline phosphatase; SepP, 18-amino acid chromogranin A signal peptide; SEAP, secreted embryonic alkaline phosphatase; V-ATPase, vacuolar-type ATPase proton pump; \(\lambda_{\text{ex}}\), excitation maximum wavelength; \(\lambda_{\text{em}}\), emission maximum wavelength; ANF, atrial natriuretic factor.
cells showed a pH value of ~7.2–7.4 in the endoplasmic reticulum, to ~6.2 in the Golgi, and finally ~5.5 in secretory granules (15–18). A large number of studies have shown that the proton gradient that exists within the cellular compartments that form the secretory pathway is established and maintained essentially by electrogenic vacuolar-type ATPase proton pumps (V-ATPase). Although several studies point to a contribution of TGN acidification to some aspects of secretory granule biogenesis in several neuroendocrine cell types, the ultimate role of V-ATPase-mediated acidification on the sorting and release of regulated secretory proteins in sympathoadrenal chromaffin cells is incompletely understood. Also, significant discrepancies have been reported on the effect of pH perturbation on the intracellular trafficking of regulated secretory proteins in neuroendocrine cells. For example, inhibition of V-ATPase in pituitary cells provoked intracellular accumulation of proopiomelanocortin and prolactin in large electron-dense vacuolar structures (19, 20), whereas other results obtained in the same cell type reported re-routing of prohormone convertase or pro-opiomelanocortin to the constitutive pathway of secretion (21).

We previously reported that a CgA-green fluorescent protein (GFP) fusion protein expressed in PC12 cells is trafficked to dense-core secretory granules (22, 23), providing a tool to investigate which determinants of the secretory apparatus, for instance lumenal pH in secretory organelles, influence CgA sorting into the regulated pathway and its subsequent storage in and exocytotic release from the core of the catecholamine storage granule. Using a series of full-length or truncated domains of CgA fused to GFP or to a newly engineered form of embryonic alkaline phosphatase (EAP), the present work sought to examine the effect of selective perturbation of the vacuolar V-ATPase proton pump by the highly specific inhibitor bafilomycin A1 (24, 25) on the sorting and trafficking of the regulated secretory protein CgA in sympathoadrenal PC12 cells. Our results indicate that a functional pH- V-ATPase along the regulated secretory pathway is essential for the sorting of the granin to dense-core granules for exocytosis. We propose that acidification of late compartments of the secretory pathway mediates the routing of CgA by a mechanism recruiting a sorting determinant located in the amino-terminal domain of the mature protein (CgA-1–115) but not its carboxy-terminal region. Moreover, our data reveal that a pH gradient over the secretory pathway is an important factor for the formation of dense-core chromaffin secretory granules in PC12 cells, perhaps by modulating the granulogenic function of CgA.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transient Transfections**—PC12 rat pheochromocytoma cells were cultured in Ham’s F-12K medium supplemented with 15% heat-inactivated horse serum and 2.5% heat-inactivated bovine serum (Gemini Bioproducts), streptomycin (100 μg/ml), and penicillin (100 units/ml) (Invitrogen). Superoiled plasmid DNA for transfection was grown in Escherichia coli strain DH5α (Invitrogen) and purified on columns (Qiagen). Two days before transfection, PC12 cells were split onto either poly-L-lysine (Sigma) plus collagen (Upstate)–purified on columns (Qiagen). Two days before transfection, PC12 cells grown on collagen- and poly-L-lysine-coated 100-mm tissue culture dishes were transfected with pcDNA6-CgA-EAP, which carries the resistance gene for the eukaryotic protein synthesis inhibitor blastidicin S. For 48 h of transfection, transiently transfected cells were exposed to 10 μg/ml blastidicin S (Invitrogen) for 10 days, with the antibiotic-containing medium being changed every 3 days. Blastidicin S sensitivity of the parent PC12 cells was determined in a preliminary experiment. Twenty hours after transfection, cells were trypsinized, plated on collagen-coated cloning rings and further grown on collagen- and poly-L-lysine-coated 24-well tissue culture plates in a culture medium supplemented with 1 μg/ml blastidicin S. Blastidicin S-resistant cells were screened for regulated secretion of SgP-CgA-EAP by chemiluminescence (PhospaLight, Applied Biosystems).

**Three-dimensional Imaging by Deconvolution Microscopy**—Images were collected on a DeltaVision deconvolution microscopy system (Applied Precision) operated by SoftWoRx software (Applied Precision) on a Silicon Graphics O2 work station using with 60× (NA 1.4) or 100× (NA 1.4) oil immersion objectives. The system included a Photometrics CoolSnap HQ CCD camera mounted on a Nikon inverted fluorescence/differential interference contrast microscope and a mercury arc lamp light source. Pixel intensities were kept in the linear response range of the camera. Optical sections along the z axis were acquired with increments of 0.2 μm. The fluorescent data sets were deconvoluted and analyzed by Delta Vision SoftWoRx programs (Applied Precision) on a Silicon Graphics Octane workstation to generate optical sections or three-dimensional images of the data sets. The following excitation and emission wavelengths were used for imaging: GFP, λex 490 ± 10/λem 526 ± 36 nm; cyan fluorescent protein (CFP), λex 485 ± 35/λem 473 ± 35 nm; Alexa Fluor 555, λex 546–580 nm; Alexa Fluor 594, λex 580–610 nm; Hoechst 33342 (nuclear DNA stain), λex 350/λem 461 nm.

**Chimeric Photoprotein Fluorescence and Immunocytochemistry**—Transfected PC12 cells cultured on poly-L-lysine- and collagen-coated glass coverslips were fixed for 1 h at room temperature with 2% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, permeabilized with 0.1% Triton X-100 in PBS (10 min) and exposed to 1 μg/ml nuclear acid stain Hoechst 33342 (Molecular Probes) for nuclei visualization. Coverslips were subsequently washed with PBS, mounted in buffered Gelvatol, and processed for three-dimensional imaging by deconvolution microscopy. For immunocytochemistry, fixed PC12 cells were incubated for 5 min in PBS, glycine (0.1 M) buffer and subsequently exposed for 30 min to PBS containing 5% fetal calf serum to reduce nonspecific antibody labeling.

Cells were then incubated for 1 h at room temperature with a rabbit polyclonal anti-human plasmatic alkaline phosphatase (anti-PLAP, 1:50; Biomedia) in buffer containing 1% bovine serum albumin in PBS. Cells were then washed and incubated for 30 min with a Alexa Fluor 594-conjugated (red) goat anti-mouse IgG, F(ab’)2, (1:250; Molecular Probes) together with 1 μg/ml nucleic acid stain Hoechst 33342 (Molecular Probes). Coverslips were subsequently washed with PBS, mounted in buffered Gelvatol or Cellv, and processed for three-dimensional imaging by deconvolution microscopy.

**Electron Microscopy**—Cells were fixed in modified Karnovsky’s fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) overnight at 4 °C followed by 1% OsO4 in 0.1 M sodium cacodylate buffer, pH 7.4 and subsequently dehydrated using a series of ethanol solutions followed by propylene oxide and infiltration with epoxy resin. After polymerization at 65 °C overnight, thin sections were cut and stained with uranyl acetate (4% uranyl acetate in 50% ethanol) followed by bismuth subnitrate. Sections were examined at an accelerating voltage of 60 kV using a Zeiss EM10B electron microscope.

**Sucrose Gradient Fractionation**—Transiently transfected PC12 cells were labeled for 2 h with 1 μCi/ml l-[3H]norepinephrine (71.7

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**H⁺-ATPase-mediated Acidification and Chromogranin A Sorting**

Whereas the SEAP 17 amino acid signal peptide (MLLLLILGLR-LQLSLG)) pSEAP2 basic plasmid was used as a template (Clontech). Full-length human CgA and two human CgA domains chimeric proteins were designed by ligating the EAP gene into the KpnI and NotI cloning sites of EGFP gene in pCMV-CgA-EGFP, pCMV-CgA481-EGFP, or pCMV-panCgA-EGFP (23) to generate pCMV-CgA-EAP, pCMV-CgA481-EAP, or pCMV-panCgA-EAP. Each plasmid encodes, respectively, for the SgP-CgA-EAP, SgP-CgA-(1–115)-EAP, or SgP-CgA-(233–439)-EAP recombiant proteins (including the CgA 18 amino acid signal peptide (MRSAAVLALLCAGQVTA), or SgP.)

A CgA-EAP cDNA domain incorporating a HindIII and a Pmel signal site was obtained by PCR using primers incorporating an KpnI restriction site at the 5′/H11032 NCBI_U89937) gene was obtained by PCR using specific oligonucleotide primers (devoid of the SEAP 17 amino acid signal peptide (MLLLLILGLR-LQLSLG)) pSEAP2 basic plasmid was used as a template (Clontech). Full-length human CgA and two human CgA domains chimeric proteins were designed by ligating the EAP gene into the KpnI and NotI cloning sites of EGFP gene in pCMV-CgA-EGFP, pCMV-CgA481-EGFP, or pCMV-panCgA-EGFP (23) to generate pCMV-CgA-EAP, pCMV-CgA481-EAP, or pCMV-panCgA-EAP. Each plasmid encodes, respectively, for the SgP-CgA-EAP, SgP-CgA-(1–115)-EAP, or SgP-CgA-(233–439)-EAP recombiant proteins (including the CgA 18 amino acid signal peptide (MRSAAVLALLCAGQVTA), or SgP.)

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Fig. 1. Schematic representation of the organization of the EAP and GFP reporter proteins designed to study CgA trafficking to the dense core secretory granules of PC12 cells. Numbers along the diagram of the chimera indicate amino acid residue positions.

**RESULTS**

A CgA Human EAP Chimeric Protein Can Be Transiently or Stably Expressed in Sympathoadrenal PC12 Cells Where It Is Sorted to the Catecholamine Storage Vesicle and Routed to the Regulated Pathway of Secretion—The SEAP reporter gene encodes a heat-stable, truncated form of the placent enzyme that lacks the membrane anchoring domain, thereby allowing constitutive secretion of the protein in transfected cells (6, 27). We further engineered the full-length SEAP gene by PCR to generate an EAP devoid of the SEAP 17 amino hydrophobic acid signal peptide (MLLLLLLGLRLQLSLG). A SgP-CgA-EAP chimeric protein was designed by fusing the EAP amino terminus to the carboxyl terminus of full-length human CgA (including the CgA 18 amino acid signal peptide (MRSAAVLALLLCAGQVTA), or SgP), (Fig. 1).

**Three-dimensional Immunofluorescence Deconvolution Microscopy**—The subcellular distribution of expressed SgP-CgA-EAP was first assessed by immunofluorescence microscopy in paraformaldehyde-fixed PC12 cells (Fig. 2A). Three-dimensional reconstruction of SgP-CgA-EAP intracellular localization revealed a non-uniform punctate distribution of the fluorescence signal throughout much of the cytoplasm but completely excluded from the nucleus. A 0.2-μm optical section acquired in the middle region of SgP-CgA-EAP-expressing PC12 cells revealed discrete peripheral/subplasmalemmal punctate fluorescence signals characteristic of the granular distribution previously reported for the photoprotein SgP-CgA-GFP (see Fig. 10 and Ref. 23). These findings, therefore, suggest storage of the EAP-tagged chimera in dense-core secretory granules.

**Subcellular Fractionation**—To further define the subcellular localization of SgP-CgA-EAP, we performed equilibrium sucrose density gradient fractionations on post-nuclear superna-
Fig. 2. Subcellular distribution of a human CgA-EAP chimeric protein in PC12 cells. A, three-dimensional (3D) reconstruction of SgP-CgA-EAP subcellular distribution. 48 h after transfection with pCMV-CgA-EAP, cells were aldehyde-fixed and processed for immuno-cytochemistry. Cells were incubated with an anti-human PLAP followed by an Alexa Fluor 594 secondary antibody. A series of optical sections along the z axis was acquired with increments of 0.2 μm using a 100× (NA 1.4) oil immersion objective. The data set was deconvolved and analyzed to generate three-dimensional/volume or section views. Alexa Fluor 594 conjugate was imaged at λex 555 nm/λem 580 nm. Nuclei were visualized with Hoechst 33342 (100 μM) using a 100× objectivem. B, detection of SgP-CgA-EAP in sucrose density gradient fractions. Post-nuclear supernatants prepared from l-[3H]norepinephrine-labeled PC12 cells transiently expressing SgP-CgA-EAP were centrifuged to equilibrium on sucrose density gradients. Fractions were collected from the bottom of the gradient and assayed for sucrose concentration, alkaline phosphatase activity, and scintillation counting.

Correct targeting of SgP-CgA-EAP to chromaffin secre-

tory granules would predict that the chimera undergo

regulated exocytosis. Ba2+ (2 mM, 15 min), a potent secre-
tagogue for chromaffin cells (23, 28), triggered the release of SgP-CgA-EAP in the same sucrose region as obtained from PC12 cells transiently expressing SgP-CgA-(1–115)-EAP (Fig. 4B), characteristic of the vesicular localization observed for SgP-CgA-EAP (Fig. 2) or SgP-CgA-GFP chimeras expressing the carboxyl-terminal-half region of CgA fused to EAP (23). This suggests either that some degree of non-selective entry of the chimera from the constitutive into the regulated pathway of secretion or co-purification of heterogeneous organelles into the same fraction. Sucrose gradient fractionations may be limited by the potential for co-sedimentation of heterogeneous organelles of the same buoyant density to the same density fraction (29, 30). We, therefore, localized the cellular distribution of the EAP chimeras in other ways, both optical and biochemical.

We assessed by three-dimensional deconvolution microscopy the subcellular colocalization of the EAP chimeras with proANF-GFP, a well described fluorescent marker of neuroendocrine secretory granules (23, 31, 32). When expressed in PC12 cells, SgP-CgA-(1–115)-EAP exhibited a punctate distribution (Fig. 4B) characteristic of the vesicular localization observed for SgP-CgA-EAP (Fig. 2) or SgP-CgA-GFP chimeras (see Fig. 10 and Ref. 23). The degree of colocalization between stably expressing SgP-CgA-EAP. Exposure of such PC12 cells to Ba2+ (2 mM, 15 min) triggered SgP-CgA-EAP secretion by ~10.7-fold, and such release was blocked after Ca2+ channel blockade by 100 μM ZnCl2 (Fig. 3A).

The intracellular localization of SgP-CgA-EAP was finally investigated by immunodetection of the release of the chimeric protein into the extracellular medium. As shown in Fig. 3B, immunoblotting analysis of the extracellular medium in the absence of stimulation showed a major product band with electrophoretic mobility of ~140–150 kDa, which is in agreement with the mobility predicted for SgP-CgA-EAP (~75 kDa for CgA plus ~60 kDa for SEAP). Exposure of cells to Ba2+ substantially increased the release of SgP-CgA-EAP (Fig. 3B), further documenting storage of the fusion protein in regulated secretory vesicles. Taken together these results indicate that human CgA fused to the chemiluminescent reporter EAP and transiently or stably expressed in sympathoadrenal PC12 cells undergoes routing to chromaffin granules and, hence, sorting to the regulated pathway for exocytosis.

Alkalization of Cellular Compartment(s) of the Secretory Pathway Perturbs the Regulated Secretion of a CgA/EAP Fusion Protein in PC12 Cells—The acidic milieu within the lumen of intracellular compartments, including the Golgi, the TGN, and secretory granules, is generated and maintained by a V-ATPase. The acidic microenvironment of the TGN and the secretory granule is generally considered to be critical for sorting of regulated secretory proteins. We, therefore, questioned whether pH perturbation by the selective V-ATPase inhibitor baflomycin A1 (24, 25) might alter the regulated trafficking of CgA in chromaffin cells.

Trafficking of Truncated CgA-EAP Fusion Proteins—Using a series of full-length, point-mutant, or truncated CgA-GFP chimeras, we previously reported that information necessary for the regulated trafficking of human CgA in PC12 cells is contained within the amino-terminal region of the mature protein (CgA(1–115)), although not the carboxyl terminus (23). We wondered whether this sorting domain could also steer the ordinarily constitutively secreted EAP into the regulated secretory pathway of PC12 cells.

Equilibrium sucrose density gradient fractionation of cells expressing SgP-CgA-(1–115)-EAP revealed that chemiluminescent intensity was high in the ~1.4 M sucrose region containing l-[3H]norepinephrine-labeled chromaffin granules (Fig. 4A). In contrast, EAP activity was reduced in the same sucrose region obtained from PC12 cells transiently expressing the carboxyl-terminal-half region of CgA fused to EAP (SgP-CgA-(233–439)-EAP). Although reduced as compared with SgP-CgA-(1–115)-EAP, SgP-CgA-(233–439)-EAP was still present in the region of the gradient inhabited by chromaffin granules, which might suggest either some degree of non-selective entry of the chimera from the constitutive into the regulated pathway of secretion or co-purification of heterogeneous organelles into the same fraction.

H+ -ATPase-mediated Acidification and Chromogranin A Sorting
SgP-CgA-(1–115)-EAP and ANF-GFP was high, as shown by the “merged” image (yellow) of the green (ANF-GFP) plus red (SgP-CgA-(1–115)-EAP) fluorescence signals (Fig. 4B). In contrast, SgP-CgA-(233–439)-EAP accumulated mainly in a perinuclear region, with a distribution pattern virtually identical to the Golgi accumulation previously reported for the constitutively trafficked SgP-CgA-(233–439)-GFP photoprotein (23) and displayed low colocalization with the secretory granule marker ANF-GFP (Fig. 4B).

Further insight into the routing of SgP-CgA-(1–115)-EAP and SgP-CgA-(233–439)-EAP was achieved by examining secretagogue-evoked release of the chimeras. Ba2+/H11001 (2 mM, 15 min) triggered the release of transiently expressed SgP-CgA-EAP by approximately 48-fold (Fig. 4C) and the secretion of SgP-CgA-(1–115)-EAP by about 22-fold. In contrast, secretagogue-evoked release of SgP-CgA-(233–439)-EAP was far lower (only about 3.1-fold over basal; Fig. 4C) and of the same magnitude as unfused SEAP (−2.4-fold over basal, Fig. 4C), a marker of the constitutive pathway of secretion (6, 27).

FIG. 3. Chemiluminescence or immunochemical detection of unfused SEAP and chimeric SgP-CgA-EAP secretion in PC12 cells. Cells transiently transfected with either pCMV-CgA-EAP or pSEAP2 (A, left panels) or stably expressing SgP-CgA-EAP (A, right panel) were exposed for 15 min to secretion medium alone (mock), 2 mM BaCl2, or to 2 mM BaCl2 plus 100 μM ZnCl2. SEAP/EAP expression and release was evaluated by chemiluminescent assay of enzymatic activity (A) or by immunoblotting (B). SEAP/EAP secretion was calculated relative to total enzymatic activity present in the cell before stimulation. Total enzymatic activity is the sum of the amount released plus the amount remaining in the cell. In the left panels, release of EAP is expressed either as % EAP activity secretion or relative to EAP activity secretion in the absence of secretagogue. For the immunobchemical detection (B), extracellular media were collected and concentrated using C-18 SepPak cartridge and processed for immunoblot using an anti-human PLAP antibody (B). Values are given as the means of triplicate determinations ± S.E.

Effect of Bafilomycin A1 on Trafficking of Full-length or Truncated CgA-EAP Fusion Proteins—The effect of bafilomycin A1 was tested on trafficking of SgP-CgA-EAP transiently expressed in PC12 cells. 22 h of exposure of SgP-CgA-EAP-expressing PC12 cells to nanomolar amounts of bafilomycin A1 inhibited Ba2+/H11001-induced SgP-CgA-SEAP secretion by up to 80% (Fig. 5A). In contrast, unstimulated release of the fusion protein was increased by about 3.2-fold at 10 nM bafilomycin A1, indicating that inhibition of V-ATPase may prevent retention of SgP-CgA-EAP in the regulated secretory pathway, hence, diverting the chimera into the constitutive pathway of secretion (Fig. 5A).

Acidification of the lumen of the secretory vesicle by V-ATPases is critical for the transport of small transmitter molecules and biogenic amines such as catecholamines (33–35). To access whether V-ATPase activity is effectively reduced at low concentrations of bafilomycin A1, we evaluated the effect of the inhibitor on vesicular norepinephrine uptake in PC12 cells. As shown in Fig. 6, equilibrium sucrose density gradient fractionation of L-[3H]norepinephrine-loaded cells revealed an 83% reduction of L-[3H]norepinephrine uptake after 10 nM bafilomycin A1 for 22 h. This result indicates that carrier-mediated catecholamine uptake can be inhibited by the effects of even nanomolar amounts of bafilomycin A1 on the V-ATPase in chromaffin granules.
In PC12 cells transiently expressing unfused SEAP, SgP-CgA-(1–115)-EAP or SgP-CgA-(233–439)-EAP exposure to 10 nM bafilomycin A1 for 22 h inhibited Ba\textsuperscript{2+}/H\textsubscript{11001}-induced SgP-CgA-(1–115)-EAP secretion by 83% (p \textsuperscript{0.02}; Fig. 7), whereas the constitutive release of SEAP or SgP-CgA-(233–439)-EAP was not affected by bafilomycin A1 (Fig. 7). Consistent with the secretagogue-mediated release results, subcellular fractionation of SgP-CgA-(1–115)-EAP-expressing PC12 cells labeled with L-\textsuperscript{[3H]}norepinephrine indicated that bafilomycin A1 dramatically reduced the chemiluminescence signal found in the 1.4 M sucrose fraction containing chromaffin granules (Fig. 7). Consistent with the results of Fig. 6, reduction of V-ATPase activity was effectively achieved at 10 nM bafilomycin A1, as shown by the inhibition of vesicular L-\textsuperscript{[3H]}norepinephrine uptake (Fig. 7). These results suggest the effect of V-ATPase blockade on regulated secretion of CgA in PC12 cells is dependent on a sorting determinant located within the amino-terminal (CgA-(1–115)) region of CgA.

Effect of the Protonophores Nigericin and Monensin—Perturbation of intracellular pH using a combination of the proton-
selective ionophores nigericin plus monensin perturb the stimulated release of SgP-CgA-EAP transiently expressed in PC12 cells. As shown in Fig. 5B, after a 22-h exposure of cells to nigericin plus monensin, Ba\(^{2+}\)-induced SgP-CgA-EAP release was decreased in a dose-dependent manner by the ionophores, reaching ~60% inhibition at 10 nM dose. Concomitantly, exposure of cells to this combination of ionophores enhanced the unstimulated release of SgP-CgA-EAP, suggesting rerouting of the chimera to the constitutive secretory pathway.

Alkalization by Bafilomycin A1 Diminishes Release of SgP-CgA-EAP by Altering Its Routing into the Regulated Pathway of Secretion Rather than Inhibition of the Final Stages of Exocytosis—Granular acidification driven by V-ATPases located in the vesicle membrane may be a crucial step in the docking/priming of secretory vesicles before exocytotic fusion with the plasma membrane (36–38). To test whether acidic vesicle pH is required for the final stages of exocytosis, we examined the effect of V-ATPase inhibition on Ba\(^{2+}\)-stimulated release of either catecholamines (Fig. 8, A and B) or SgP-CgA-EAP (Fig. 8B). Increasing concentrations of bafilomycin A1 (0–10 nM, 22 h) did not affect Ba\(^{2+}\)-evoked L-[\(^{3}H\)]norepinephrine secretion (Fig. 8, A and B; \(p > 0.05\)) but slightly increased unstimulated catecholamine release (Fig. 8A, \(p = 0.013\)), consistent with non-exocytotic release after alkalinization of the secretory granule core (39).

In contrast, 10 nM bafilomycin A1 (22 h) strongly inhibited Ba\(^{2+}\)-evoked regulated secretion of SgP-CgA-EAP (~80% inhibition, \(p < 0.01\)) but did not affect stimulated catecholamine release (Fig. 8B). These results suggest that alkalization by bafilomycin A1 reduces stimulated release of SgP-CgA-EAP primarily by perturbing the granular targeting of the chimera rather than inhibiting the late stages (i.e. docking/priming or fusion steps) of exocytosis.

Perturbation of Biogenic Amine Vesicular Uptake Does Not Alter Regulated Release of the SgP-CgA-EAP Fusion Protein—In addition to peptide hormone and neurotransmitter cargos, dense-core vesicles of chromaffin cells contain high concentrations of catecholamines, nucleotides, and calcium (40). In vitro studies have shown that catecholamines may bind to CgA and aggregate the granin, perhaps promoting condensation of the catecholamine secretory granule core, thereby reducing intragranular osmotic pressure (14, 41). We wondered whether depletion of endogenous granular catecholamine storage may affect the regulated trafficking of CgA. As shown in Fig. 9, the vesicular monoamine transporter inhibitor reserpine did not affect stimulated release of SgP-CgA-EAP from transiently transfected PC12 cells. In contrast, L-[\(^{3}H\)]norepinephrine vesicular uptake was blocked by reserpine, reaching ~90% inhibition at 100 nM dose (Fig. 9). These data, therefore, indicate that functional vesicular monoamine uptake (and high vesicular concentrations of catecholamines) are not essential for correct sorting of CgA into chromaffin granules. Hence, bafilomycin A1 inhibition of CgA trafficking (Fig. 5, 7, and 8) is independent of any effect the drug might have on vesicular catecholamine stores.

Effect of V-ATPase Blockade on Golgi Apparatus Morphology and Dense-core Secretory Granule Biogenesis—To further explore how the V-ATPase might contribute to proper routing and

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**Fig. 5. Intracellular alkalization by V-ATPase inhibitor bafilomycin A1 or by the protonophores nigericin and monensin perturbs the regulated secretion of SgP-CgA-EAP from sympatheticadrenal cells.** PC12 cells were transiently transfected with pCMV-CgA-EAP and exposed for 22 h to the indicated concentrations of bafilomycin A1 (A) or monensin plus nigericin (B). Cells were washed with secretion medium and subsequently exposed for 15 min to secretion medium alone (mock) or to 2 mM BaCl\(_2\). SgP-CgA-EAP activity present in the extracellular medium and in the cell lysate was assayed by chemiluminescence. The chimera secretion rate was calculated relative to total enzymatic activity present in the cell before stimulation. Total enzymatic activity is the sum of the amount released plus the amount remaining in the cell. Values are given as the means of triplicate determinations ± S.E.

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**Fig. 6. Effect of low amounts of bafilomycin A1 on vesicular norepinephrine uptake.** PC12 cells were exposed for 22 h to 10 nM bafilomycin A1 were labeled for 2 h with L-[\(^{3}H\)]norepinephrine. Post-nuclear supernatants prepared from L-[\(^{3}H\)]norepinephrine-labeled PC12 cells were centrifuged to equilibrium on sucrose density gradients. Fractions were collected and assayed for sucrose concentration and scintillation counting.
release of regulated secretory proteins, we studied the effect of bafilomycin A1 on secretory granule and Golgi morphology. In particular, we evaluated the intracellular localizations of the dense-core secretory granule marker SgP-CgA-GFP as well as the classic trans-Golgi/TGN marker β1,4-galactosyltransferase fused to cyan fluorescent protein (GalT-CFP). As expected, SgP-CgA-GFP displayed a punctate distribution pattern consistent with a granular targeting of the photoprotein (Fig. 10A). GalT-CFP distribution was clustered in the perinuclear region, characteristic of the Golgi complex (Fig. 10A).

Bafilomycin A1 (10 nm, 22 h) reduced the number of secretory granules positive for SgP-CgA-GFP (Fig. 10B); this effect on regulated granule abundance might result from prevention of appropriate trafficking of this granulogenic protein (7, 42, 43). In addition, bafilomycin A1 provoked extensive relocation of GalT-CFP from Golgi stacks (Fig. 10A) to dispersed peripheral granular structures, perhaps of endosomal nature (Fig. 10B), suggesting a contribution of the low pH of the trans-Golgi/TGN to appropriate localization and/or steady-state retention of the glycosyltransferase to that organelle.

Could Golgi morphology also be affected by alkalinization? The effect of V-ATPase blockade was, therefore, studied at the ultrastructural level. Electron microscopy of control PC12 cells showed typical Golgi complexes that consisted of flattened stacks of several cisternae. Abundant numbers of dense-core secretory granules were present in untreated cells, most of them being observed in close proximity to the plasma membrane (Figs. 11 and 12). Although a modest dilatation of the Golgi cisternae could be observed in a subset of bafilomycin A1-treated cells (Fig. 11, E and F), the overall morphological appearance of the Golgi apparatus was largely similar to that in control cells. Axelsson et al. (44) also found that neutralization of pH in the Golgi apparatus by bafilomycin A1 caused relocalization of GalT and other glycosyltransferases in the absence of structural effects on the Golgi complex (44).

What happens to chromaffin granule morphology after alkalinization? Examination of chromaffin granule abundance revealed a marked decrease in the number of dense-core vesicles (Fig. 12A). Two such parameters, granules/μm² and granules/cell plane, were used to quantify the number of dense-core granules present (Fig. 12B). Number of granules/μm² was defined as the number of granules divided by the area of the cell body minus the surface of the nucleus, whereas granules/cell plane indicated the number of granules found in an xy section of the mid-cell body. Bafilomycin A1 reduced the number of chromaffin granules per μm² from 0.79 ± 0.07 to 0.24 ± 0.05 (p < 0.0001) and the number of granules per cell plane from 62.24 ± 5.22 to 16.52 ± 2.01 (p < 0.0001). Thus, V-ATPase-mediated acidification of subcompartments of the secretory pathway seems to be required for the formation of secretory granules. Consistent with this morphologic effect of bafilomycin A1 on granules is the diminution of L-[3H]norepinephrine in the peak granule fraction of the sucrose gradients (Fig. 6).

**DISCUSSION**

As noted earlier, the processes by which secretory proteins such as CgA are sorted into the regulated pathway of secretion is unsettled. Regardless of the level of sorting ("for-entry" or
the sum of the amount released plus the amount remaining in the cell. Values are given as the means of triplicate determinations calculated relative to total enzymatic activity or radioactivity present in the cell before stimulation. Total enzymatic activity (or radioactivity) is the sum of the amount released plus the amount remaining in the cell. Values are given as the means of triplicate determinations calculated relative to total enzymatic activity present in the cell before stimulation. Total enzymatic activity is the sum of the amount released plus the amount remaining in the cell.

Effect of reserpine on SgP-CgA-EAP exocytotic release and norepinephrine uptake. Effect of reserpine on Ba\(^{2+}\)-triggered SgP-CgA-EAP secretion (solid triangle). PC12 cells transiently transfected with pCMV-CgA-EAP were exposed for 22 h to the indicated concentrations of reserpine. Cells were subsequently exposed for 15 min to secretion medium alone or to 2 mM BaCl\(_2\). Extracellular media and cell lysates were assayed for SgP-CgA-EAP activity and/or l-\([^{3}\text{H}]\)-norepinephrine radioactivity. Secretion rate of either the chemiluminescent chimera or norepinephrine was calculated relative to total enzymatic activity or radioactivity present in the cell before stimulation. Total enzymatic activity (or radioactivity) is the sum of the amount released plus the amount remaining in the cell. Values are given as the means of triplicate determinations ± S.E.

"by-retention"), a key feature of secretory granule storage of CgA may be its dependence on low pH and high Ca\(^{2+}\) for aggregation/condensation (9–14). Such multimerization has been documented in vitro for CgA and chromogranin B (CgB), another member of the granin family (9–14). For instance, CgA exists in a monomer-dimer equilibrium at pH 7.5 and in a monomer-tetramer equilibrium at pH 5.5 (13), suggesting that the state of oligomerization of the granin may transition from mostly dimeric in the endoplasmic reticulum to a mostly tetrameric in the mildly acidic TGN and secretory granules. High Ca\(^{2+}\)/low pH-mediated aggregation of CgA has long been suspected to constitute a key step in the formation of secretory granules (6, 9–11). Indeed, recent studies carried out in living cells provide compelling evidence that CgA is an essential factor driving the biogenesis of dense-core secretory granules in chromaffin cells and even in non-neuroendocrine cells after adventitious expression (7, 42, 43).

In this study, we used a series of full-length or truncated CgA-GFP or -EAP chimeras to explore how selective perturbation of secretory organelles pH affects the regulated traffic of CgA in sympatoadrenal cells. Our results indicate that a functional H\(^{+}\)-V-ATPase along the regulated secretory pathway is required for proper granular sorting of CgA as well as for the biogenesis of chromaffin secretory granules.

Intracellular Trafficking of a Human CgA Embryonic Alkaline Phosphatase Chimeric Chemiluminescent Protein—We previously reported that a human Sgp-CgA-GFP fusion protein is trafficked to dense core secretory granules and thereby sorted to the regulated pathway for exocytosis (23). Although the use of the CgA-GFP photoprotein provided visualization by fluorescence microscopy of the chimera transport and storage along the secretory pathway, quantitative analysis of GFP chimera release in the extracellular milieu by fluorometry has been problematic. Indeed, substantial cellular autofluorescence (principally due to FAD and FMN coenzymes (15) occurring in the GFP excitation/emission spectrum (15) may prevent accurate determination of subtle changes in fluorescence and, therefore, in the secretory profile of the GFP photoprotein.

We, therefore, considered an alternative strategy and engineered an EAP tag, a truncated domain of human secreted embryonic alkaline phosphatase SEAP devoid of its hydrophobic signal peptide (Fig. 1). The advantages of using EAP over GFP to measure the steady-state release of a chimeric protein are multiple. For instance, chemiluminescence detection of SEAP/EAP is typically ~100 times more sensitive than GFP.
GFP and the Golgi-targeted
tion of the dense-core granule marker photoprotein SgP-CgA-
or 10 nM bafilomycin A1 (GalT-CFP) were exposed to mock medium (panel A) or 10 nM bafilomycin A1 (panel B) for 22 h. Aldehyde-fixed cells were examined by deconvolution microscopy. GFP was excited at \( \lambda_{em} 490 \pm 10 \) nm and imaged at \( \lambda_{ex} 528 \pm 38 \) nm; CFP was excited at \( \lambda_{ex} 436 \pm 10 \) nm and imaged at \( \lambda_{em} 465 \pm 30 \) nm. Optical sections along the z axis were acquired with increments of 0.2 \( \mu \)m using a 100× oil immersion objective (1.4 NA). Data were processed to generate combined three-dimensional views of the GFP and CFP chimera distribution. Shown in panels A or B are composite images of four replicate cells. Scale bars, 5 \( \mu \)m.

**FIG. 10.** Effect of bafilomycin A1 on the subcellular distribution of the dense-core granule marker photoprotein SgP-CgA-GFP and the Golgi-targeted \( \beta_{1,4}\)-galactosyltransferase cyan fluorescent protein (GalT-CFP) chimera. PC12 cells co-transfected with pCMV-CgA-EGFP together with the expression plasmid encoding for the targeting sequence of \( \beta_{1,4}\)-galactosyltransferase fused to CFP (GalT-CFP) were exposed to mock medium (panel A) or 10 nM bafilomycin A1 (panel B) for 22 h. Aldehyde-fixed cells were examined by deconvolution microscopy. GFP was excited at \( \lambda_{em} 490 \pm 10 \) nm and imaged at \( \lambda_{ex} 528 \pm 38 \) nm; CFP was excited at \( \lambda_{ex} 436 \pm 10 \) nm and imaged at \( \lambda_{em} 465 \pm 30 \) nm. Optical sections along the z axis were acquired with increments of 0.2 \( \mu \)m using a 100× oil immersion objective (1.4 NA). Data were processed to generate combined three-dimensional views of the GFP and CFP chimera distribution. Shown in panels A or B are composite images of four replicate cells. Scale bars, 5 \( \mu \)m.

fluorescence detection. Also, background from endogenous alkaline phosphatase can be virtually eliminated by heat treatment. Based on our earlier trafficking studies of a series of CgA domain/GFP fusion proteins (23) and other investigators’ use of a CgA/full-length SEAP chimeric protein (6), we reasoned that a CgA fused to a truncated form of SEAP might be trafficked to chromaffin granules, providing a sensitive way to report and quantify regulated secretion (Fig. 1).

Here we establish that a SgP-CgA-EAP chimeric protein correctly localizes to chromaffin secretory granules. Three-dimensional immunofluorescence microscopy of PC12 cells expressing SgP-CgA-EAP revealed a subplasmalemmal, punctate fluorescence characteristic of chromaffin granules (Fig. 2). Sucrose gradient studies colocalized SgP-CgA-EAP and cathecolamines to the same subcellular fraction at \( \sim 1.4 \) M sucrose (Fig. 2); this peak is consistent with the buoyant density reported previously for chromaffin granules from PC12 cells (23, 29, 30). Sucrose gradient fractionation results may be limited by the potential for co-purification of heterogeneous organelles to the same fraction (29, 30); hence, we also localized the chimera in a biochemical way. Barium chloride-evoked exocytosis (Fig. 3) demonstrated a regulated secretory profile for SgP-CgA-EAP, further demonstrating that the fusion protein is effectively sorted into dense-core chromaffin granules. Finally, analyses of the subcellular distribution and release profiles of truncated CgA domains fused to EAP indicate that a sorting signal contained within the CgA-(1–115) amino-terminal domain (but not the carboxyl-terminal CgA-(233–439) region) is sufficient to re-route the ordinarily constitutively secreted EAP tag into the regulated secretory pathway (Figs. 4 and 7). This finding is in line with previous work from our laboratory, localizing a sorting determinant for the regulated pathway within the CgA-(77–115) domain of the mature protein (23).

A Functional \( H^+\)-V-ATPase Is Required for the Regulated Trafficking of CgA—We found that alkalinalization of the secretory pathway by either the V-ATPase inhibitor bafilomycin A1 or the protonophores monensin and nigericin decreased Ba\(^{2+}\)-evoked SgP-CgA-EAP secretion. Earlier studies have shown that perturbation of the pH of subcellular compartments with weak bases or with V-ATPases inhibitors may prevent entry of secretory proteins into the regulated pathway (19–21, 45). However, it remains unclear whether alkalinalization of the luminal pH of secretory pathway organelles diverts secretory proteins to a constitutive (or perhaps to a constitutive-like (2)) secretory pathway or, alternatively, promotes intracellular retention of the secretory protein.

We show here that reduction of SgP-CgA-EAP regulated release by alkalinalization was associated with enhanced release of the chimera through an unstimulated, constitutive pathway of secretion, indicating re-routing rather than intracellular retention of the protein (Fig. 5). Consistent with this finding, examination of the intracellular distribution of the photoprotein SgP-CgA-GFP (Fig. 10) and ultrastructural analysis of bafilomycin A1-treated cells (Figs. 11 and 12) did not suggest accumulation of the granin in the trans-Golgi area or in Golgi-derived vesicles. At substantially higher dosage of bafilomycin A1 (1 \( \mu \)M), accumulation of dense-core material may occur in vacuolar structures near the TGN of pituitary cells (20). Depending on the model of sorting (entry versus retention) within the regulated secretory pathway (2–4), increased constitutive release of SgP-CgA-EAP induced by bafilomycin A1 and concurrently decreased regulated secretion (Fig. 5A) may be interpreted as the result of perturbed sorting of CgA at either the TGN stage (sorting-for-entry) or the immature secretory granule stage (sorting-by-retention).

Regardless of the sorting level, a key feature for the regulated sorting of CgA is a low pH/high Ca\(^{2+}\)-dependent aggregation/condensation of the protein. Hence, in the sorting-for-entry model, bafilomycin A1 would perturb selective aggregation of the secretory protein within the lumen of the TGN. Thus, enhanced release of SgP-CgA-EAP under unstimu-
lated conditions would reflect redirection of the chimera from the regulated into the constitutive pathway, acting as a default route for secretion. In the sorting-by-retention model, bafilomycin A1 may perturb the aggregation/condensation of CgA within the immature secretory granule and would then increase the rate of removal of CgA from maturing granules by a constitutive-like secretory pathway.

As noted above, Ca\(^{2+}\)/H\(^{-}\)-and pH-dependent homodimerization/homotetramerization processes may initiate aggregation-mediated sorting of CgA into the regulated secretory pathway. Discrete regions within not only the amino-terminal (46, 47) but also the carboxyl-terminal domains of CgA (6, 11, 48) mediate multimerization, perhaps acting as aggregative signals for sorting into the regulated pathway. Subcellular fractionation and Ba\(^{2+}\)-evoked secretion results for the chimeric proteins SgP-CgA-(1–115)-EAP and SgP-CgA-(233–439)-EAP (Fig. 7) suggest that V-ATPase blockade may alter the granular trafficking of CgA by disrupting the sorting process of the granin at a site in cis dependent upon sequences located between amino acid residues 1 and 115, consistent with a sorting determinant for the regulated pathway (23).

Controversy exists as to whether the H\(^{+}\)/H\(^{-}\)gradient generated by the V-ATPase is required for secretory granule exocytosis from neuroendocrine cells, with some studies excluding (49, 50) and others supporting (32, 36, 51, 52) such a role. For instance, granule acidification by V-ATPase is a decisive step in the ATP-dependent priming of insulin granules for exocytosis in pancreatic \(\beta\) cells (36). In contrast, other studies reported that Ca\(^{2+}\)-dependent alkalization of PC12 dense-core secretory granules may facilitate decondensation and exocytotic release of the protein cargo (32). According to the granule acidification hypothesis (36), one may speculate that bafilomycin A1-mediated inhibition of SgP-CgA-EAP exocytosis (Figs. 5 and 8) is a consequence of perturbation of the priming of chromaffin granules before secretion rather than perturbation of sorting into the granules. Conversely, the alkalization hypothesis (32) would predict that V-ATPase inhibition may increase the rate or extent of exocytosis.

We found that V-ATPase blockade reduced Ba\(^{2+}\)-induced SgP-CgA-EAP secretion but not catecholamine release from SgP-CgA-EAP-expressing PC12 cells, in which the pool of secretory granules was labeled with \(^{3}\text{H}\)norepinephrine (Fig. 8). This result clearly indicates that the final stages of exocytosis are unaffected by vesicular alkalization; thus, bafilomycin A1 is likely to decrease stimulus-induced exocytosis of CgA by impairing its trafficking into the regulated pathway at an intracellular locus that is proximal to the priming/docking stages of exocytosis.

Alteration of the chromaffin granule matrix may also be achieved by interfering with the vesicular monoamine transporter vesicular monoamine transporter (34) and could also affect the granular sorting of CgA. Because bafilomycin disruption of the vesicular pH gradient may result in non-exocytotic catecholamine discharge (Fig. 8), we wondered whether catecholamine depletion per se might alter exocytosis. However, lowering catecholamine levels in chromaffin granules with the
vesicular monoamine transporter amine-proton exchange inhibitor reserpine did not alter secretagogue-stimulated release of SgP-CgA-EAP (Fig. 9). Thus, sorting of the CgA chimera into dense-core granules does not depend on biogenic amines loading into these same granules. This finding is in line with previous studies in chromaffin cells showing that catecholamine-free granules may be competent for fusion to the plasma membrane (53).

Although the present data demonstrate the requirement of a functional V-ATPase for proper trafficking of CgA, the precise site perturbed by bafilomycin A1 exposure (and hence, the CgA actual site of selective sorting) is unclear. Recent studies in HeLa and anterior pituitary AtT-20 cells (17, 18) found a steady-state pH in the endoplasmic reticulum similar to cytosolic pH, whereas only the Golgi apparatus and secretory granules require active, bafilomycin-sensitive V-ATPases for acidification. Hence, in our experiments, bafilomycin A1 may perturb a sorting step of CgA involving low pH/high Ca\(^{2+}\)-dependent aggregation/condensation of the protein either at the TGN stage (sorting-for-entry) or at the immature secretory granule stage (sorting-by-retention). Consequently, such perturbation would then lead to increased constitutive or constitutive-like release of CgA (Fig. 5).

A Functional H\(^+-\)V-ATPase Is Required for Dense-core Granule Biogenesis—Our study points to an important effect of V-ATPase blockade not solely on the sorting and trafficking of CgA but also on the formation of chromaffin granules themselves. The ability of CgA and other granins to undergo low pH/high Ca\(^{2+}\)-induced aggregation in vitro and to interact with other components of the matrix of the secretory granule has long suggested that CgA may contribute fundamentally to the biogenesis of secretory granules (8, 10–14, 54). Indeed, recent studies provide evidence that CgA and perhaps other granins play a crucial role in the initiation and regulation of dense-core secretory granule biogenesis and hormone sequestration in living neuroendocrine cells, including sympathoadrenal PC12 cells (7, 42, 43). If CgA aggregation does not simply contribute to the sorting mechanism of the protein at the TGN lumen but is also an important factor driving formation of secretory granules, then perturbing the ability of CgA to form aggregates by neutralizing the pH gradient in the TGN or within the immature secretory granules may interfere with the assembly of the secretory organelle into mature dense-core granules. Consistent with this hypothesis, we found that exposure of PC12 cells to bafilomycin A1 reduced the number of secretory granules expressing the regulated secretory photoprotein SgP-CgA-GFP (Fig. 10). Moreover, ultrastructural examination of bafilomycin A1-treated cells revealed a decreased number of secretory granules per μm\(^2\) of cytosol and of granules per cell plane; both parameters were reduced by ~70–73% as compared with naive cells (Fig. 12B). Evaluation of the morphology of Golgi stacks by electron microscopy revealed an essentially preserved Golgi

![Image](image-url)
architecture (Fig. 11), suggesting that the effect of V-ATPase blockade on secretory granule formation is not simply a reflection of disruption of the Golgi complex.

Our data show that bafilomycin A1 inhibits regulated secretion of Sgp-CgA-SEAP while increasing the constitutive (unstimulated) release of the fusion protein (Fig. 4A). Increased constitutive release suggests that in the presence of bafilomycin A1, CgA may transit through the Golgi complex to the TGN and be routed to the constitutive secretory pathway as a default route for release. We propose that inhibition of the V-ATPase impairs retention of Sgp-CgA-EAP within the regulated secretory pathway, likely by perturbing selective aggregation of CgA at either the TGN stage or the immature secretory granule stage, decisive steps that may underlie the granulogenic role of this secretory protein.

In summary, we have found that selective disruption of the pH gradient along the secretory pathway by the vacuolar V-ATPase proton pump inhibitor bafilomycin A1 interferes with the sorting of CgA into chromaffin granules and reroutes the granin to a constitutive pathway of secretion. We propose that neutralization of the TGN (and/or the immature secretory granule) perturbs a pH/Ca\(^{2+}\)-dependent sorting mechanism of CgA that mobilizes a trafficking determinant within the amionic terminal but not the carboxyl-terminal region of the protein. Finally, these studies suggest V-ATPase as an important factor for the formation of dense-core chromaffin secretory granules in PC12 cells, perhaps by modulating the ability of CgA to form aggregates, a crucial step that may underlie the granulogenic function of CgA.

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Role of H^+-ATPase-mediated Acidification in Sorting and Release of the Regulated Secretory Protein Chromogranin A: EVIDENCE FOR A VESICULOGENIC FUNCTION
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