The pituitary contains professional secretory cells, devoting a large fraction of their energy to the synthesis of hormones that are stored for secretion in response to a complex mixture of inputs. Ba^{2+}, a substitute for Ca^{2+}, and phorbol ester, a mimic for diacylglycerol, have a synergistic effect on exocytosis. By using these secretagogues, we developed a paradigm in which phorbol ester potentiates of Ba^{2+}-evoked exocytosis produces a robust secretory response in multiple pituitary cell types. Because cells subjected to this stimulatory paradigm remain healthy despite their greatly reduced hormone content, we used this paradigm to study the fate of granule membrane proteins. We examined the turnover of peptidylglycine α-amidating monoxygenase (PAM), a membrane enzyme involved in the final maturation of many peptides, and VAMP2, a vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE). The stability of recently synthesized PAM was increased by sustained exocytosis. Biotinylation studies established that the appearance of integral membrane PAM at the plasma membrane was stimulated along with hormone secretion. PAM biotinylated on the cell surface undergoes cleavage to yield soluble peptidylglycine-α-hydroxylating monoxygenase that can then be secreted in a regulated fashion. Consistent with a kiss-and-run or cavicapture mode of secretion (Taraska, J. W., Perrais, D., Ohara-Imaizumi, M., Nagamatsu, S., and Almers, W. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2070–2075), biotinylated prolactin was also retained by the cells and later released in response to secretagogues. Thus, pituitary cells can retrieve and reuse components of the machinery involved in the final stages of exocytosis (the SNAREs) as well as soluble and membrane granule proteins.

In neurons, synaptic vesicle membrane components are rapidly retrieved at the nerve terminal after secretion. The vesicles thus formed are replenished with locally synthesized neurotransmitter and are again able to undergo stimulated release (1). In contrast, neuroendocrine secretory granule membrane components must have a different fate following exocytosis. The peptides stored in secretory granules are often generated from propeptides as they enter immature secretory granules, which form in the trans-Golgi network region of the cell. If the membrane proteins of secretory granules are retrieved and reused, a connection between granule biogenesis and granule membrane recycling must occur. Sporadic evidence of functional recycling (retrieval and reuse) of secretory granule membrane proteins has been collected over the last 2 decades. In chromaffin cells, stimulation of secretion causes internalization of glycoprotein III/clusterin and dopamine β-hydroxylase from the plasma membrane and their subsequent re-entry into secretory granules (2–4). In insulinoma cells, stimulated secretion resulted in the endocytosis of two secretory granule membrane proteins, phogrin and ICA512, and their re-entry into newly forming insulin granules (5, 6). Both endogenous and heterologously overexpressed P-selectin, a protein involved in the first phase of the inflammatory response, can be retrieved from the plasma membrane and re-inserted in newly formed granules (7).

Peptidylglycine α-amidating monoxygenase (PAM), a type I membrane protein, is a processing enzyme present in secretory granules of neuroendocrine cells and neurons that is responsible for one of the final steps in the biosynthesis of many peptide hormones and neuropeptides (8). Studies on the biosynthesis and trafficking of exogenous membrane PAM in a mouse corticosterone tumor cell line, AtT-20, identified residues in the cytosolic domain of PAM that were essential for granule entry, internalization from the plasma membrane, and transit through the endosomal pathway (9). These studies revealed a role for the phosphorylation and dephosphorylation of specific residues in the cytosolic domain of PAM in determining whether PAM internalized from the plasma membrane entered lysosomes (10). Because overexpression of membrane PAM in AtT-20 cells causes striking differences in the cellular localization of secretory granules, the cytoskeleton, and the basal and evoked release of mature hormone compared with cells in which only endogenous PAM is present (11), we wanted to examine this process in primary pituitary cells, where secretory granules are much more prevalent.

We used cultured rat anterior pituitary cells to establish a stimulatory paradigm based on the synergistic actions of Ba^{2+}, a substitute for Ca^{2+}, and phorbol myristate acetate (PMA) to mimic diacylglycerol, which causes massive secretion of hormone from multiple cell types. Massive secretion in turn creates the necessity for sustained retrieval of secretory granule membrane proteins, including PAM, from the plasma membrane. The response to the stimulation paradigm of granule membrane proteins in primary pituitary cells was monitored using subcellular fractionation, immunocytochemistry, surface biotinylation and enzyme assays. The sustained exocytosis

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1 The abbreviations used are: PAM, peptidylglycine α-amidating monoxygenase; VAMP2, vesicle-associated membrane protein 2 (synaptobrevin 2); SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; PHM, peptidylglycine-α-hydroxylating monoxygenase; PAlm, membrane-bound peptidyl-α-hydroxylase-α-amidating lyase; PRL, prolactin; TGN, trans-Golgi network; PMA, phorbol 12-myristate 13-acetate; sPHM, soluble PHM; ACTH, adrenocorticotropic hormone; TES, N-Tris(hydroxymethyl)methyl-2-aminoethansulfonic acid; GH, growth hormone; BSA, bovine serum albumin.
yielded healthy cells with less than 20% of their normal content of hormone, causing depletion or redistribution of secretory granule membrane proteins. Combining biosynthetic labeling with the stimulation paradigm, we found that the rate of turn-over of recently synthesized PAM in primary pituitary cells was decreased by sustained exocytotic activity. By using biotinylation to label PAM on the plasma membrane, we found that a fraction of the PAM tagged on the cell surface was subsequently endoproteolytically processed to yield soluble PHM that was secreted in a regulated fashion. The ability of secretory granule components to be reused, if not released following exocytosis, is not limited to membrane proteins. Prolactin, a soluble granule protein, was also internalized following stimulation and was delivered to a cellular compartment from which it could be released again into the medium. Together, these data suggest that secretory granule proteins, if not consumed during one round of exocytosis, can be functionally recycled for subsequent use.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Antiseras to growth hormone (JH89) and ACTH (Kathy) were described previously (12), as was antiserum to exon A of PAM (JH629) (13). Polyclonal antiserum to prolactin (IC-5) was from the National Hormone and Peptide Program (NIDDK, National Institutes of Health), and monoclonal antibody to actin (JLA20) was from the Developmental Hybridoma Bank (University of Iowa). Monoclonal antibody to VAMP2 (clone 69.1) was obtained commercially from Synaptic Systems GmbH (Germany). Sulfo-NHS-LC-biotin and sulfo-NHS-SB-biotin were from Pierce (catalog numbers 21335 and 21331, respectively); all other chemicals were from Sigma unless otherwise stated. Antiseras to growth hormone (JH89), ACTH (Kathy), prolactin (IC-5), and actin (JLA20) were prepared as described previously (14) with only minor modifications. Briefly, anterior pituitaries from adult male and female Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were incubated for 2 h at 37 °C in pituitary homogenization buffer (150 mM NaCl, 4 mM KCl, 5 mM CaCl2, 15 mM HEPES-KOH, pH 7.5) containing protease inhibitors and then passed 5 times through a 26-gauge needle. The resulting pellets were washed twice with homogenization buffer, resuspended in ice-cold homogenization buffer, and collected by centrifugation at 1,100 × g. The resulting pellets were incubated for 20 min in ice-cold 1% Triton X-100, 5 mM sucrose, 60 mM KCl, 2.5 mM MgCl2, 0.3 mM phenylmethylsulfonyl fluoride, and 50 μM 4-5′-bis(N-methylamino)-phenacyl fluorescein. The resulting supernatants were separated by centrifugation at 140,000 × g for 15 min, removing cell debris and nuclei (P1). The resulting pellets were then centrifuged at 435,000 × g for 15 min to separate a light membrane P3 pellet from the soluble, cytosolic supernatant fraction (SN). SN pellets were resuspended in equal volumes of homogenization buffer, and the soluble fractions were adjusted to the same volume of resuspended pellets with homogenization buffer.

**Western Blot**—After fractionation by SDS-PAGE and electroblotting to polyvinylidene difluoride membranes (Schleicher & Schuell), membranes were probed with the indicated primary antibodies. The antigen-antibody complexes were detected using secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase-conjugated secondary antibody and Super Signal West Pico chemiluminescence substrate (Pierce). When quantification was performed, nonsaturated signals were desensitized using Scion Image software (National Institutes of Health) or acquired with a GeneGnome work station through the GeneSnap software (Syngene) and analyzed with the GeneTools software (Syngene).

**Labeling**—Functional recycling of granule proteins was investigated using biotinylated transport proteins. Cells were first fed with secretion medium alone for 2 min. The labeling was performed either before or after challenging the cells with the Ba2+/PMA paradigm. In the former case, the cells were chased for 2 h and extracted or further chased in medium devoid of hormone and growth factors (huTBTM) before challenging the cells with biotinylated transport proteins (see below with the exception that the BSA concentration was 2 mg/ml). For the latter case, the control and Ba2+/PMA-challenged cells were labeled and extracted with TMT buffer. Aliquots of the extracts were precipitated with trichloroacetic acid, solubilized, and either counted or loaded on gel, fractionated by SDS-PAGE, and exposed for fluorography. Other aliquots of the extracts were immunoprecipitated with the indicated antibodies, and the immunoprecipitates were fractionated by SDS-PAGE and exposed for fluorography.

**PAM Enzymatic Assays**—Pepidylviglycine α-hydroxylating monoxygenase assays were performed as described (15) using Ac-254-I-Tyr-Val-Gly as substrate.

**Surface Biotinylation**—Cultured rat anterior pituitary cells or AT-20 cells stably expressing PAM-1 were fed with CSFM air supplemented with 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, insulin/transferrin/selenium (from Invitrogen or Mediatech), and 1 mg/ml fatty acid-free bovine serum albumin. Cells were fed daily with the same medium until used.

**Fluorescence Microscopy**—After secretagogue treatment, cells were rinsed with warm HEPES saline buffer (120 mM NaCl, 4 mM KCl, 5 mM CaCl2, 15 mM HEPES-KOH, pH 7.5) and fixed for 30 min with 4% formaldehyde in the same pre-warmed buffer. After extensive rinsing, cells were permeabilized with 0.075% Triton X-100 in blocking buffer (2 mg/ml BSA in phosphate-buffered saline) for 20 min at room temperature and then further incubated in blocking buffer for 20 min at room temperature. Fixed cells were incubated with primary antibodies diluted in blocking buffer for 2 h at room temperature or overnight at 4°C. Following extensive rinsing with phosphate-buffered saline, cells were then incubated in the appropriate Cy3-conjugated secondary antibody for 1 h at room temperature in the dark. Slides were mounted with glass coverslips using Permaflour mounting medium (Immuno-tech, France). Image stacks (Δz = 0.2 μm) were created using Openlab 3.4 software (Improvision, UK) and a Nikon TE300 fluorescence microscope with a Hamamatsu digital CCD camera C4742-95-12ERG connected to an Orca-ER camera controller. Images were acquired using an oil immersion 60× objective (NA 1.4) and were deconvolved (iterative mode) using Velocity 3.0 software (Improvision, UK) and an appropri-
the biotinylation reactions performed at 0 °C, after being fed with CSFM air, cells were transferred to a bath containing melting ice. All the steps were as for the biotinylations at 37 °C except that all the media and buffers used were pre-chilled at 0 °C. In some experiments, surface biotinylation was performed with 5 mM sulfo-NHS-SS-biotin, a derivative whose biotin moiety can be removed by incubation of cells with buffer (50 mM Tris-HCl, pH 7.6, 100 mM NaCl) containing reduced glutathione (50 mM; GSH), a membrane-impermeable reducing agent (as described in Ref. 17 with modifications). Cells were extracted in TMT supplemented with protease inhibitors. The protein concentration of the lysates was determined by the bicinchoninic acid assay (Pierce), TMT supplemented with protease inhibitors. The protein concentration of the lysates was determined by the bicinchoninic acid assay (Pierce), and equal amounts of lysates from each period of stimulation for control (C) and stimulated samples (S) were analyzed for GH and PRL; signals were desensitized, and the hormone content of stimulated cells is plotted as a percentage of the hormone content of control cells. Data represent the range for determinations from two independent sets of samples; two additional experiments produced similar results. E, rat primary anterior pituitary cells were subjected to the paradigm described in B, fixed, and stained with antibodies specific for ACTH, GH, or PRL. The cortical region of corticotropes and somatotropes is indicated by arrows; peripheral puncta in lactotropes are indicated by arrows; isolated puncta in Ba²⁺/PMA stimulated corticotropes and somatotropes are indicated by asterisks, as is the perinuclear region of stimulated lactotropes. Bar, 10 μm. Similar results were obtained in two additional experiments.

**RESULTS**

Repetitive Stimulation Causes Massive Exocytosis and Depletion of Hormone Content in Pituitary Endocrine Cells—Our laboratory has long been interested in the synthesis, storage, and secretion of proteins targeted to the secretory pathway (18). Exocytosis is coupled to compensatory endocytosis (19); therefore, to study secretory granule membrane protein recycling, we developed a stimulation paradigm that causes massive secretion of hormone. Anterior pituitary endocrine cells typically respond to multiple secretagogues, and we used BaCl₂ and a phorbol ester (phorbol 12-myristate 13-acetate (PMA)) to mimic the physiological second messengers Ca²⁺ and diacylglycerol, respectively (20–22). Phorbol ester and barium are known to have a synergistic effect on the evoked exocytosis of luteinizing hormone in chicken pituitary cells (23). We observed a similar response in cultured rat primary pituitary cells (Fig. 1A). Basal secretion of growth hormone was compared with that elicited by PMA alone, barium alone, or their combination (Fig. 1A). Barium and PMA alone elicited a similar exocytotic response, with a 2–3-fold increase of GH secretion over basal levels. However, when the two secretagogues were administered together, the evoked secretion of GH was 8–9 times higher than basal levels (Fig. 1A, bottom).

Next, we tested a paradigm involving sequential stimulation with these secretagogues (Fig. 1B). The PMA exposure time was selected to maximize the secretory response and minimize protein kinase C down-regulation. When cultured anterior pituitary cells were subjected to this paradigm, a large secretory response was produced by somatotropes, lactotropes (Fig. 1C), and corticotropes (not shown); we refer to cells exposed to this stimulation paradigm as Ba²⁺/PMA-challenged. At the end of the stimulation paradigm, the GH and PRL cells had undergone a massive reduction of hormone content (Fig. 1D); Ba²⁺/PMA-challenged cells contained less than 20% as much hormone as control cells. These biochemical data were qualitatively confirmed...
Single granules was prevalent throughout the cytoplasm (Fig. 1, left panel). In stimulated cells, staining in the cortical region was less pronounced, and punctate staining likely corresponding to immunoreactivity, mainly in the plasma membrane region (Fig. 1, arrows). In contrast, in the perinuclear region (Fig. 1, asterisks), the cytoplasmic punctate staining almost completely lost.

The 5-fold drop in hormone content measured by biochemical analysis (Fig. 1D) was more dramatic than the decrease observed by immunocytochemistry. 

The Ba\textsuperscript{2+}/PMA stimulation paradigm causes depletion of soluble secretory granule markers and redistribution of secretory granule membrane markers. A, at the end of the secretion paradigm, control and Ba\textsuperscript{2+}/PMA-stimulated cells were labeled for 30 min with \textsuperscript{[35}S\textsuperscript{]methionine and extracted. Total protein synthesis was analyzed by precipitation with trichloroacetic acid (TCA, left panel), and GH synthesis was analyzed by immunoprecipitation (right panel). B, control cells (C) and cells challenged with Ba\textsuperscript{2+}/PMA (S) were homogenized and subjected to differential centrifugation. Equal proportions of each fraction were analyzed by Western blot using antibodies specific for actin, growth hormone, PAM (antiserum JHR69), and VAMP2. C, control and Ba\textsuperscript{2+}/PMA-challenged anterior pituitary cells were fixed and stained with antibody against VAMP2, arrows, cortical staining in control cells; asterisks, perinuclear staining; and arrowheads, isolated puncta, in Ba\textsuperscript{2+}/PMA-challenged cells. Bar, 10 \(\mu\text{m}\). A similar response was observed in most cells, and the entire experiment was replicated with similar results.

The Ba\textsuperscript{2+}/PMA-stimulated and control cells were fixed and probed for ACTH, GH, and PRL (Fig. 1E). Corticotropes and somatotropes under control conditions showed strong immunoreactivity, mainly in the plasma membrane region (Fig. 1E, arrows). In stimulated cells, staining in the cortical region was less pronounced, and punctate staining likely corresponding to single granules was prevalent throughout the cytoplasm (Fig. 1E, asterisks). Control lactotropes showed a characteristic strong staining in the Golgi/TGN region along with punctate staining located eccentrically to the nucleus (Fig. 1E, arrows). In contrast, in the stimulated lactotropes immunoreactivity was present only in the perinuclear region (Fig. 1E, asterisks), with the cytoplasmic punctate staining almost completely lost.

The 5-fold drop in hormone content measured by biochemical analysis (Fig. 1D) was more dramatic than the decrease observed by immunocytochemistry. This discrepancy suggested that some endocrine cells might be so depleted that they became difficult to detect based on their remaining hormone content. Each type of anterior pituitary endocrine cell contains VAMP2, an integral membrane granule protein that participates in exocytosis through formation of the SNARE complex (24). VAMP2 is not found at high levels in non-endocrine cells (25). When endocrine cells were identified using VAMP2 and co-stained with hormone-specific antibodies, it became clear that responses ranged from almost complete depletion of the hormone content (very few granules stained) to little discernible effect (not shown). The stimulated cells shown in Fig. 1E retained enough hormone following the Ba\textsuperscript{2+}/PMA challenge to allow easy identification based on visualization of hormone.

The Ba\textsuperscript{2+}/PMA Stimulation Paradigm Is Not Toxic and Primarily Affects Secretory Granule Proteins—The Ba\textsuperscript{2+}/PMA paradigm involves long stimulation times, raising the possibility that cells could be damaged. By phase contrast microscopy, the Ba\textsuperscript{2+}/PMA-challenged cells did not show any evident signs of stress (not shown). To obtain a physiological parameter of cellular health, protein synthesis was analyzed. At the end of the Ba\textsuperscript{2+}/PMA stimulation paradigm, control and Ba\textsuperscript{2+}/PMA-challenged cells were pulse-labeled using \textsuperscript{[35}S\textsuperscript{]methionine, chased briefly in nonradioactive medium, and extracted. Aliquots of the extracts were subjected to trichloroacetic acid precipitation to measure total protein synthesis or to GH immunoprecipitation to look specifically at synthesis of a secretory product. The total radioactivity incorporated was not significantly different (not shown), and fluorography of equivalent samples from control and Ba\textsuperscript{2+}/PMA-challenged cells revealed indistinguishable patterns of total protein synthesis and similar rates of GH synthesis (Fig. 2A). Therefore, this stimulation paradigm, while causing massive depletion of hormone, does not compromise the viability of the cells.

We next analyzed the effect of the Ba\textsuperscript{2+}/PMA challenge on a variety of cellular markers. Stimulated and control cells were homogenized in isotonic buffer and subjected to differential centrifugation. A crude nuclear pellet (P1), a secretory granule-enriched fraction (P2), a fraction corresponding to lighter membrane compartments (P3), and soluble cytosolic proteins (SN) were subjected to Western blot analysis. The subcellular fractions were probed for a general marker, actin, and for three secretory granule proteins, GH, PAM, and VAMP2 (Fig. 2B). In control cells, actin localizes to the particulate fractions containing nuclei and secretory granules and to cytosol, whereas the microsomal fraction (P3) is largely devoid of actin. In the stimulated cells, the actin distribution was unchanged. Analyzed in this way, the Ba\textsuperscript{2+}/PMA paradigm affects neither the actin distribution nor its total amount. As expected, the GH signal was reduced by immunocytochemistry.
Stimulation of Exocytosis Reduces the Turnover of Recently Synthesized PAM—We used metabolic labeling to explore the effect of sustained exocytosis on PAM turnover (Fig. 4). Quadruplicate wells of cultured rat primary pituitary cells were labeled for 30 min with [35S]methionine and chased for 2 h to allow newly synthesized PAM to move out of the endoplasmic
The turnover rate of newly synthesized PAM is reduced by massive stimulation of exocytosis. A, cells labeled for 30 min with [35S]methionine (L) were chased for 2 h to allow the newly synthesized PAM to reach the distal part of the secretory pathway. Cells were either extracted (initial, I) or further chased in the absence of secretagogues (basal, B) or challenged with the Ba2+/PMA paradigm in the absence (stimulated, S) or presence of 20 μM chloroquine (chloroquine, C). The media collected from each period of each treatment were pooled and the cells lysed. B, equal proportions of cell lysates and pooled media were immunoprecipitated (IP) with a polyclonal antibody to PHM (JH1781; directed to residues 37–382 of rat PAM-1); membrane PAM (mPAM), soluble bifunctional PAM (sPAM), soluble PHM (sPHM) are indicated. mPAM includes equal proportions of cell lysates and pooled media were immunoprecipitated (IP) with a polyclonal antibody to PHM (JH1761; directed to residues 37–382 of rat PAM-1); membrane PAM, soluble PHM and PAM secreted (Fig. 4, B and C). Chloroquine treatment during stimulation had a slight effect on the turnover of recently synthesized PAM, with total amounts of PAM and its derived products similar to those of the samples stimulated in the absence of this drug (Fig. 4C). The fact that levels of cellular PAM protein, as assessed by Western blot (Fig. 2B), were decreased in the presence of Ba2+/PMA reflects the occurrence of exocytosis, which was not quantified in that experiment.

The turnover of recently synthesized VAMP2 was examined in the same cell extracts (Fig. 4D). Under basal conditions, the amount of the recently synthesized VAMP2 remained unchanged during the 5.5-h chase. Ba2+/PMA challenge, regardless of the presence of chloroquine, had a slight effect on VAMP2 turnover during the 5.5-h chase. In both cases, an ~15% reduction in the amount of recently synthesized VAMP2 was observed. Although strong exocytotic stimulation altered the subcellular distribution of VAMP2 (Fig. 2, B and C), it had a similarly minor effect on the total cellular content of VAMP2. These observations indicate that VAMP2 is very stable and is effectively recycled, regardless of the level of stimulation.

Biotinylated PAM-1 Can Undergo Processing and Yield Mature Products in AtT-20 PAM-1 Cells—Antibodies directed against the ectodomains of PAM are internalized by primary pituitary endocrine cells (26, 28). Determinants governing the routing of endocytosed PAM were identified using corticotrope tumor cell lines expressing cytosolic domain mutants of PAM-1.
In this study, we set out to establish the fate of PAM molecules that visit the cell surface using surface biotinylation. Methods were first optimized using a stable AtT-20 cell line overexpressing PAM-1 (31). To establish which forms of PAM-1 could be biotinylated at the cell surface, trafficking was suppressed by chilling the cells to 0 °C; proteins biotinylated at 0 °C were isolated using avidin-agarose beads. PAM proteins in total cell lysates and avidin eluates were compared by Western blot (Fig. 5A). Without biotinylation, no signal was detected in the avidin eluates. Approximately 5% of the total PAM-1 and 2.5% of the PALm was on the plasma membrane under basal conditions (Fig. 5B, white bars). These estimates agree well with data obtained using antibodies to assess the amount of PAM on the plasma membrane (32). More importantly, no soluble PHM becomes biotinylated at 0 °C, indicating that no detectable PHM is on the cell surface.

Because the levels of surface PAM are lower in primary pituitary endocrine cells, we wanted to determine whether we could label more PAM by carrying out the biotinylation reaction at 37 °C without compromising cell viability. During 30 min at 37 °C, 20–25% of the total cellular content of PAM-1 and PALm was biotinylated (Fig. 5B, black bars). This is consistent with the large flux of intact PAM-1 and PALm onto and off of the plasma membrane observed in AtT-20 PAM-1 cells (32). After biotinylation for 30 min at 37 °C, but not at 0 °C, ~5% of the total cellular pool of sPHM is biotinylated. The biotinylated sPHM observed at 37 °C must be generated from PAM-1 biotinylated at 0 °C. Soluble PHM from biotinylated PAM-1 was stored in a cellular compartment from which it could be secreted in a regulated manner (Fig. 6A).

We next tested the effect of secretagogues on the access of PAM to the plasma membrane. Anterior pituitary cells were exposed to biotin for 30 min at 37 °C in the absence or presence of secretagogues (Fig. 6B, s− or +). Cells were either extracted immediately (Fig. 6B, label) or chased in medium for 2 h; secretagogues were not present during the chase. Under resting conditions, ~0.7% of the PAM-1 and 1.0% of the PALm was biotinylated (Fig. 6, C and D); 0.5% of the total sPHM was biotinylated. These values are consistent with prior estimates that pituitary endocrine cells secrete ~1% of their cell content of PHM activity per h under basal conditions (26). When secretagogues were present during the labeling period, biotinylation of PAM-1 and PALm increased between 2- and 3-fold (Fig. 6, C and D); this is consistent with the presence of PAM-1 and PALm in cellular compartments that are responsive to stimulation. Addition of secretagogue also increased the amount of biotinylated sPHM in the cells. Because biotinylated sPHM is likely produced by processing of biotinylated PAM-1, this increase suggests that secretagogues affect the processing of biotinylated PAM-1. We next examined the stability of biotinylated PAM proteins during the chase period (Fig. 6E). Whether biotinylation was carried out in the presence or absence of secretagogue, the total amount of biotinylated PAM was remarkably stable throughout the 2-h chase.

**Soluble PHM Derived from Biotinylated PAM-1 Is Secreted upon Stimulation of Exocytosis**—Surface biotin labeling experiments demonstrated that PAM-1, after biotinylation at the plasma membrane, was processed to yield mature products in both AtT-20 PAM-1 cells and primary pituitary cells (Figs. 5 and 6). We wanted to determine whether the processing of biotinylated PAM-1 was performed intracellularly and whether the biotinylated sPHM produced was stored in a cellular compartment from which it could be secreted in a regulated manner. We first examined AtT-20 PAM-1 cells. Biotinylation was carried out for 30 min at 37 °C in resting conditions; secretagogues were added only after the cells had been chased for 30 min (Fig. 7A, top). Biotinylated soluble PHM was secreted during the first 30-min chase period (Fig. 7A, bottom, 0–30 min B and S); at 30–60 min after biotinylation, basal release of
FIG. 6. PAM-1 that traverses the plasma membrane of pituitary endocrine cells is stable. A, primary rat anterior pituitary cells were exposed (+) or not (–) to 5 mM sulfo-NHS-LC-biotin for 30 min at 37 °C and lysed. Equal amounts of lysate were incubated with avidin beads, and eluates were analyzed by immunoblotting using PAM antibody JH629. 10% of the respective lysates were analyzed in parallel. The asterisk indicates a nonspecific doublet. B, schematic for biotinylation of primary rat anterior pituitary cells at 37 °C. Cells were labeled with 5 mM sulfo-NHS-LC-biotin for 30 min in the absence or presence of secretagogues (2 mM Ba2+ plus 1 μM PMA, indicated as Sec in the figure) and extracted (label) or chased for 2 h and then extracted (chase). C, biotinylated proteins were analyzed as in A; asterisk, nonspecific doublet. For calculations, proportional aliquots of cell lysates (10% of the amount incubated with avidin beads) were probed at the same time with the same antibody (one lysate only, input, is shown on the left). Duplicates of each treatment are shown. D, the amounts of biotinylated PAM-1, PALm, and PHM were quantified and expressed as a percentage of the total corresponding cellular form in the lysate; error bars, range for duplicate determinations. E, signals for biotinylated PAM-1, PALm, and sPHM were summed and expressed as a percentage of the PAM biotinylated during 30 min in the absence of secretagogues (white bar); bars, range of duplicates.

FIG. 7. PHM derived from processing of biotinylated PAM-1 is routed to a regulated secretory compartment. A, top, duplicate wells of AtT-20 PAM-1 cells (B, basal, and S, stimulated) were biotinylated at 37 °C for 30 min, chased for 30 min in the absence of secretagogues, and then either challenged (S) or not (B) with secretagogues (2 mM Ba2+ plus 1 μM PMA) for 30 min. The media from each period were collected and incubated with avidin beads; the eluates were probed for PHM by immunoblotting using PAM antibody JH629 (A, bottom). B, a similar experiment was repeated with primary rat pituitary cells. To increase the fraction of PAM-1 at the plasma membrane, cells in duplicate wells (B, basal, and S, stimulated) were first “primed” by incubation in medium containing 1 μM PMA and then biotinylated for two consecutive 30-min periods in buffer containing 2 mM Ba2+. The cells were then chased for two consecutive 30-min periods in medium devoid of secretagogues and a final 30-min period in the absence (B) or presence (S) of secretagogues (top). The media from every period were analyzed; equal proportions of avidin eluates were probed for the presence of PHM. Desensitized signals are plotted (bottom); bars are ranges for duplicate experiments.
biotinylated sPHM declined, but its secretion could be stimulated by secretagogues (Fig. 7A, bottom, 30–60 min, compare B and S).

We carried out a similar experiment using cultured primary pituitary cells but modified the protocol based on the Ba$^{2+}$/PMA challenge paradigm to increase the amount of PAM-1 at the plasma membrane during incubation with the activated biotin derivative. This involved priming the pituitary cells using a 30-min treatment with PMA and then stimulating them for two periods of 30 min with barium in the presence of biotinylation reagent (Fig. 8B). Again, we found that biotinylated sPHM was secreted at a basal rate. More importantly, the secretion rate for PHM was increased 3-fold following stimulation with secretagogues (Fig. 8B). These data provide clear evidence that PAM-1 that has reached the cell surface can undergo endoproteolytic processing to yield mature soluble PHM, some of which is stored in a cellular compartment from which its secretion can be stimulated.

A Subset of Post-exocytic PRL Undergoes Internalization and Subsequent Secretion—We also analyzed the behavior of prolactin (PRL), a soluble granule protein (Fig. 8). Primary pituitary cells were biotinylated for 30 min in the absence or presence of secretagogue (Fig. 8, sec – or +) and harvested immediately or chased in the absence of secretagogue (as described in Fig. 6B). In culture, the tonic dopaminergic inhibition of lactotropes (33) is removed, and basal release of PRL is high. In a recent report (34), upon depolarization with high K$^+$ (in physiological saline and at 22 °C), prolactin cores were detected at the plasma membrane, subsequently internalized, and reappeared at the cell surface after a second high potassium stimulation. These observations suggest that PRL packed in secretory granules can be reused if not spent during one round of exocytosis. In our experimental conditions, 10% of the cellular PRL was biotinylated at rest (Fig. 8A); the percentage of cellular PRL that was biotinylated increased 2-fold upon secretagogue stimulation. These observations suggest that PRL packed in secretory granules can be reused if not spent during one round of exocytosis. In our experimental conditions, 10% of the cellular PRL was biotinylated at rest (Fig. 8A); the percentage of cellular PRL that was biotinylated increased 2-fold upon secretagogue stimulation. The biotinylated PRL was quite stable; following a 2-h chase in the absence of secretagogue there was little change in the cellular content of biotinylated PRL (Fig. 8A, right).

This experiment shows that, in basal and stimulated conditions, a significant fraction of the PRL that reaches the cell surface in a manner that allows access to the activated biotin reagent does not immediately diffuse away and remains associated with the cell lysate. These data do not provide any insight into the topology of the biotinylated PRL molecules. To determine whether the PRL exposed to the extracellular space and retained by the cells was actually internalized, we made use of a cleavable biotin derivative, sulfo-NHS-SS-biotin. If biotinylated PRL were internalized, it would be identified by its resistance to removal by glutathione. Indeed this was what we observed (Fig. 8). Duplicate wells of cells were exposed to sulfo-NHS-SS-biotin or to uncleavable sulfo-NHS-LC-biotin for 15 min in the presence of secretagogues (2 mM Ba$^{2+}$ plus 1 µM PMA). Under these conditions, ~13% of the cellular PRL was

FIG. 8. After exocytosis, a fraction of prolactin is internalized and routed to a secretory compartment. A, primary pituitary cells were subjected to the paradigm described in Fig. 6B. Avidin eluates and total cell lysates were probed for prolactin (left panel). The amount of biotinylated PRL was expressed as a percentage of total cell content (right panel). B, to establish whether the biotinylated PRL remains on the cell surface or is internalized, primary pituitary cells were subjected to surface biotinylation following the paradigm described (top panel). Cells were exposed to 5 mM sulfo-NHS-SS-biotin for 15 min at 37 °C in the presence of secretagogues (2 mM Ba$^{2+}$ plus 1 µM PMA) and either extracted (labeled) or chased for 1 h at 37 °C; the chased samples were then chilled on ice, and the surface biotin was stripped (using glutathione; see “Experimental Procedures”); a set of samples was then extracted while another was warmed at 37 °C for 1 h (chased samples) and the surface biotin was stripped (using glutathione; see “Experimental Procedures”). Avidin eluates and total cell lysates were probed (as described in Fig. 6) for prolactin together with an internal control (Fig. 8B).

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biotinylated, irrespective of the biotin derivative used (Fig. 8B, bottom left, labeled, white and black bars). When the cells exposed to the cleavable biotin were chased for 1 h at 37 °C, chilled at 0 °C to block membrane traffic, and then stripped of any surface biotin, ~9% of the cellular PRL was still biotinylated, an indication that a large fraction of the PRL labeled at the cell surface and retained by the cells had been internalized (Fig. 8B, bottom left, chased).

To determine whether the internalized PRLs were sorted into a cellular compartment that could undergo regulated secretion, cells subjected to the same label/chase/stripping paradigm were further chased at 37 °C in medium containing secretagogues (2 mM Ba2+ plus 1 μM PMA) for 15 min. The collected media were probed for biotin conjugates by incubation with avidin beads (Fig. 8B, bottom right). About 7% of the PRL present in the medium was biotinylated, evidence that after internalization from the surface PRL can be sorted to a regulated secretory organelle.

**DISCUSSION**

The fate of secretory granule membrane proteins is poorly understood. Because granule exocytosis must be accompanied by compensatory endocytosis, we set out to establish a paradigm capable of reliably inducing a massive secretory response in anterior pituitary cells, professional secretory cells that contain many secretory granules. In order to stimulate secretion by the majority of the cell types in the anterior pituitary, we used generic secretagogues, barium and phorbol ester. The paradigm developed was then combined with metabolic labeling and surface biotinylation to study the response of both soluble and membrane secretory granule proteins.

**Secretory Granule Proteins Respond Differently to Sustained Exocytosis**—As observed for chicken gonadotropes (23), simultaneous challenge with barium and PMA produces a synergistic secretory response from rat anterior pituitary cells. Moreover, sequential and repeated stimulation with barium and PMA caused sustained secretion, depleting somatotropes and lactotropes of about 80% of their hormone content, while maintaining cell viability. Corticotropes responded in a similar manner. When we compared the content and subcellular distribution of several granule proteins before and after stimulation, clear differences were apparent. Both biochemical and morphological approaches indicated that soluble proteins (GH and sPHM) were largely depleted from secretory granules. Granule membrane proteins (VAMP2, PAM-1, and PALm) exhibited a variety of responses. Based on subcellular fractionation and immunocytochemistry, a significant fraction of the VAMP2 moved from secretory granules into lighter membranes, consistent with its recycling (24). Although the secretory granule-enriched fraction was depleted of PAM-1 and PALm, neither protein accumulated in lighter membrane fractions. Subsequent experiments demonstrated that the observed loss of total PAM reflected both its conversion into secreted products and its degradation, with secretagogues affecting both processes.

**PMA Potentiation of Ba2+ -evoked Secretion Explains Their Synergistic Effect**—In a series of pilot experiments, we found that barium alone produced sustained secretion of GH, prolactin, ACTH, and PHM; however, the secretory response declined with time, and exposure to barium alone did not deplete cells of these soluble granule proteins. Although PMA alone produced only a modest secretory response, prior exposure to PMA greatly enhanced the ability of pituitary cells to respond to subsequent application of barium. Because both sequential and simultaneous challenges with PMA and barium cause a strong exocytotic response, the effects of PMA on exocytosis are immediate (on the time scale of our experiments) and last after its removal. Therefore, in addition to acting as a secretagogue, PMA is a potent enhancer of Ba2+ -evoked secretion.

The anterior pituitary is a complex tissue, composed of several different cell types integrating a variety of signals from the hypothalamus and from target tissues. In addition, pituitary cells respond to a variety of paracrine factors secreted by the different cell types that compose this gland (35). To exclude the possibility that the PMA-mediated potentiation of secretion required communication between different endocrine cell types or a contribution from the folliculostellate cells, we demonstrated that AtT-20 corticotrope tumor cells, a homogeneous ACTH-producing cell line, also exhibited PMA-induced enhancement of Ba2+ -evoked exocytosis (not shown). The potentiation appears to be a cell-autonomous phenomenon, not requiring cross-talk between different cell types.

**Sustained Secretory Activity Decreases Degradation of Recently Synthesized PAM**—In order to observe the effects of secretagogues on the metabolism of recently synthesized granule proteins, we incubated pituitary cells with [35S]methionine and allowed the newly synthesized proteins to mature before exposing the cells to the Ba2+/PMA challenge. During the chase, the PAM proteins synthesized during the pulse process through the secretory pathway, as signaled by the appearance of sPAM and sPHM. Under basal conditions the processing of membrane PAM continued during the following 5.5 h, with little release of radiolabeled PAM into the medium. The products identified after the 5.5-h chase accounted for less than half of the PAM present at the start of the chase, indicating that recently synthesized PAM was being degraded. The 5.5-h Ba2+/PMA challenge both increased the processing and decreased the degradation of PAM. Together, these findings may indicate that, in resting conditions, pituitary cells synthesize PAM at a rate that exceeds the needs of the cell, and therefore, its steady state is regulated by degradation. A study from our laboratory established that long term stimulation of exocytosis results in up-regulation of mRNA and protein the levels of pro-hormone and processing enzymes (36). Our present results suggest the interesting possibility that sustained exocytotic stimulation (like the one produced by the Ba2+/PMA paradigm) might regulate PAM protein levels post-translationally diverting newly synthesized enzyme molecules from a degradative pathway. An appealing hypothesis is that the fraction of newly synthesized PAM rescued from degradation is routed to secretory granule membranes as it is retrieved by compensatory endocytosis. Chloroquine, which discharges transmembrane pH gradients, had little effect on the turnover of PAM during sustained stimulation, suggesting that the lysosomal pathway contributes little to the degradation of newly synthesized PAM in primary pituitary cells. An alternative explanation is that massive stimulation decreases PAM turnover by diverting the targeting of newly synthesized PAM from lysosomes to secretory granules. In this case, sustained stimulation would mask any chloroquine effect.

The effect of the Ba2+/PMA stimulation on PAM metabolism was revealed because biosynthetic labeling allowed us to focus on the recently synthesized PAM that was moving through the trans-Golgi network and immature secretory granules. Consistent with the response observed, PAM-1 mutated to mimic cytosolic domain phosphorylation by protein kinase C (PAM-1/Ser937→Asp) is both more efficiently delivered to the regulated secretory pathway and turns over more slowly than PAM-1 when expressed in AtT-20 cells (10). Our present observations indicate that the same two messengers that stimulate secretion also regulate the delivery of PAM to secretory granules.

The Ba2+/PMA challenge had little effect on the turnover of newly synthesized or total cellular VAMP2. These data, along with the morphological evidence about its cellular redistribu-
tion, point to the stability of VAMP2 under resting conditions and to its very effective recycling during sustained exocytosis.

**PAM-1 Is Subjected to Functional Recycling**—The biogenesis of secretory granules requires packaging of newly synthesized peptides emerging from the Golgi complex. If the membrane components of secretory granules are retrieved after exocytosis, their functional recycling requires a route from the endocytic to the biosynthetic pathway. In chromaffin cells, antibody internalization and surface biotinylation have produced experimental evidence for the retrieval and delivery of glycoprotein III/clusterin and dopamine β-hydroxylase to newly formed secretory granules through a post-Golgi compartment (2–4). Studies in primary endothelial cells, insulinoma, and AtT-20 cell lines produced evidence in support of the functional recycling of granule membrane proteins such as P-selectin, phogrin, and ICA512 (5–7). In PC12 cells, the majority of the secretory granules that undergo exocytosis are thought to maintain their membrane identity without collapsing into the plasma membrane; they reseal in few seconds, retrieving their membrane intact by a mode defined as “kiss-and-stay” or “cavicapture” (37).

Antibody internalization studies using AtT-20 cells demonstrated that PAM-1 localized on the plasma membrane undergoes rapid retrieval followed by delivery to the TGN region, whereas PAM-1/A99, a truncation mutant lacking most of the cytosolic domain, accumulates on the cell surface (32). Besides regulating the efficiency of PAM delivery to secretory granules, phosphorylation of the protein kinase C site, Ser<sup>937</sup> (38), also plays a role in its endocytotic trafficking. PAM cannot be phosphorylated at this site (PAM/Ser<sup>937</sup> → Asp) is internalized and delivered to lysosomes (38), whereas PAM with a phosphomimetic mutation at this site (PAM/Ser<sup>937</sup> → Asp) accumulates at a perinuclear site, unable to return to the TGN area like wild type PAM-1 (10). Both phosphorylation and dephosphorylation at this site are important for late steps in endocytosis/recycling.

Primary anterior pituitary cells internalize antibodies to the luminal domains of PAM, suggesting post-exocytotic retrieval and the possibility of functional recycling to secretory granules (26, 28). In the present study, we used biotinylation to specifically label and follow the fate of the subpopulation of PAM that traverses the plasma membrane. Making use of AtT-20 cells overexpressing PAM-1, we were able to establish that, when membrane traffic was blocked, the membrane forms of PAM (PAM-1 and PAlm) were biotinylated, whereas soluble PHM was not. When membrane traffic was allowed, a much larger fraction of the total membrane PAM was biotinylated, along with a small fraction of the cell-associated soluble PHM.

Under similar basal conditions (30 min at 37 °C), in primary anterior pituitary cells only about 1% of the membrane PAM was biotinylated. Consistent with this, when examining the steady state localization of PAM in anterior pituitary cells, we had previously established that only ~4% of the total PAM, mainly membrane forms, is associated with cellular compartments that contained plasma membrane or endosomal markers (27). The percentage of PAM-1 on the plasma membrane was increased by secretagogue challenge, indicating that some PAM-1 is present in a regulated compartment.

The biotinylated soluble PHM detected in both AtT-20 cells and primary pituitary cells following a 30-min biotinylation at 37 °C could be derived from the cleavage of internalized PAM-1 or processing of PAM-1 on the plasma membrane. The first hypothesis is in agreement with our previous observations on AtT-20 cells and primary pituitary cells (26, 28, 29). In primary pituitary cells, there is almost full recovery of biotinylated PAM after a 2-h chase; this stability suggests that the biotinylated PAM is internalized because proteolytic cleavage of biotinylated PAM-1 on the cell surface should lead to the loss of soluble products. In both AtT-20 cells and anterior pituitary cells, we observed the regulated release of biotinylated soluble PHM derived from PAM-1 that had been biotinylated on the cell surface. Taken together, our results demonstrate that unprocessed PAM-1 that traverses the plasma membrane can be functionally recycled, generating cleavage products stored in a regulated compartment from which secretion can be stimulated by secretagogues.

**Prolactin Can Be Recycled and Reused**—We also evaluated the behavior of a soluble secretory granule protein, prolactin. It has been reported recently that, upon stimulation with high K<sup>+</sup> at 22 °C, prolactin cores remain associated with the plasma membrane, undergo internalization, and following restimulation, reappear at the cell surface (34). We found that in resting conditions 10% of the total prolactin was biotinylated in 30 min at 37 °C, signaling its appearance at the cell surface. The tonic dopaminergic inhibition that controls prolactin secretion in vivo is absent in culture, leading to high basal levels of release (33). The fraction of PRL biotinylated was doubled by secretagogue challenge, consistent with the stimulation of exocytosis. After a 2-h chase, a consistent fraction of biotinylated prolactin was still associated with cells, regardless of whether secretagogue challenge had been administered or not. These data clearly indicate that a fraction of the prolactin molecules that are exposed to the extracellular space remain associated with the cells and do not diffuse away into the medium. In the case of PAM-1, the production of biotinylated soluble PHM and its ability to be released into medium in resting and stimulated conditions are clear indications of the internalization of PAM-1 and sorting to a secretory compartment. By comparison, in the case of prolactin, the lack of a similar processing step prevented us from establishing the cellular topology of the molecules tagged at the cell surface in these experiments. In order to establish whether prolactin biotinylated at the cell surface could be internalized and later released, we used another biotin-coupling reagent, sulfo-NHS-SS-biotin, bearing a disulfide bond that can be reduced, thus detaching the biotin moiety. This approach allowed us to determine that 1 h after labeling ~75% of the prolactin molecules tagged were resistant to biotin stripping, indicating their disappearance from the cell surface and internalization. When this labeling protocol was repeated and the cells were challenged with secretagogue 1 h after labeling, we were able to identify an appreciable fraction of secreted prolactin as biotinylated. Together, our results indicate that after secretion the prolactin that remains associated with the cells is internalized and sorted to a secretory compartment, processes that imply its functional recycling. Although these findings are in agreement with the observations by others (34), it remains to be established whether functional recycling of unspent post-exocytotic hormone is peculiar to prolactin or a phenomenon common to other regulated secretory peptides.

In summary, we report the design and validation of a paradigm that produces sustained exocytosis, allowing the study of granule membrane protein recycling in secretory cells. Although the mechanistic features of phorbol ester potentiation of barium-evoked secretion need further investigation, this paradigm might represent a useful tool for studying the kinetics of hormone replenishment following depletion. Our data support the following hypotheses. First, exo- and endocytotic trafficking of secretory granule membrane proteins is regulated by secretagogues. Second, soluble and membrane proteins of secretory granules are subjected to functional recycling and reuse.
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