Retention and stimulus-dependent recycling of dense core vesicle content in neuroendocrine cells

Roslyn A. Bauer, Ruth L. Overlease, Janet L. Lieber and Joseph K. Angleson*

Department of Biological Sciences, University of Denver, Denver, CO 80208, USA

*Author for correspondence (e-mail: jangleso@du.edu)

Accepted 9 January 2004
Journal of Cell Science 117, 2193-2202 Published by The Company of Biologists 2004
doi:10.1242/jcs.01093

Summary

We have used fluorescence imaging of individual exocytic events in combination with immunogold electron microscopy and FM1-43 photoconversion to study the stimulus-dependent recycling of dense core vesicle content in isolated rat pituitary lactotrophs. Secretory stimulation with high external [K^+] resulted in 100 exocytic sites per cell that were labeled by extracellular antibodies against the peptide hormone prolactin. Morphological analysis demonstrated that the prolactin was retained and internalized in intact dense cores. Vesicles containing non-secreted, internalized prolactin did not colocalize with DiI-LDL that had been chased into lysosomes but did transiently colocalize with internalized transferrin. The recycling vesicles also trafficked through a syntaxin 6-positive compartment but not the TGN38-positive trans-Golgi. Recycling vesicles, which returned to the cell surface in a slow basal manner, could also be stimulated to undergo exocytosis with a high release probability during subsequent exocytic stimulation with external K^+. These studies suggest a functional role for recycling vesicles that retain prolactin.

Key words: Exocytosis, Transferrin, Prolactin, FM1-43, TGN38, Syntaxin 6

Introduction

Exocytic secretion of peptide hormones from endocrine cells is often viewed to be a process in which the peptide contents in dense core vesicles fully dissolve upon exposure to the extracellular solution. However there is evidence that complete release of peptide hormones does not always occur. Previous studies have demonstrated retention of insulin in dense cores at the surface of rat and human pancreatic β-cells following exocytic stimulation (Larsson et al., 1989). Similar observations have been made for a number of hormones in anterior pituitary cells (St. John et al., 1986; Angleson et al., 1999). Retained dense core content was internalized in pituitary lactotrophs (Angleson et al., 1999), however the consequence of partial secretion from individual granules and the fate of the retained hormone has not been resolved.

Lactotrophs of the anterior pituitary produce and secrete the peptide hormone prolactin (PRL). Like other peptide hormones, newly synthesized PRL enters the TGN where specific aggregation of the hormone initiates the processes of protein sorting and packaging for regulated secretion (Arvan and Castle, 1998; Dannies, 1999). PRL aggregates bud off the TGN into immature secretory granules which then go through a series of intracellular fusions yielding several types of morphologically distinct immature vesicles (Farquhar et al., 1978). Ultimately this processing gives rise to mature secretory granules that store PRL until a signal triggers the exocytic release of PRL. Secretion is controlled by a number of stimulatory and inhibitory signaling pathways within the lactotroph that are initiated by hormonal and neuronal input (Lamberts and MacCleod, 1990). Ultimately PRL secretion is due to Ca^{2+}-dependent fusion of dense core vesicles with the plasma membrane (Zorec et al., 1991).

The dense core vesicle membrane is retrieved for potential recycling or degradation following exocytic fusion. The relatively long half-life of some protein constituents of the dense core vesicle membrane (Wasmeier and Hutton, 1996) suggests that vesicles are then recycled and used for more than one round of regulated secretion. A great deal has been learned regarding the kinetics and regulation of the initial endocytic events from patch clamp capacitance measurements in endocrine cells including chromaffin cells (e.g. Smith and Neher, 1997), pancreatic β-cells (Eliasson et. al., 1996), melanotrophs (Thomas et al., 1994), corticotrophs (Lee and Tse, 2001) and somatotrophs (Kilic et. al., 2001). Much of the information about trafficking pathways that follow endocytosis has been derived from studies using fairly non-specific electron dense traces such as horse radish peroxidase (HRP) or cationized ferritin (Farquhar, 1978; Orci et al., 1986; Bäck et al., 1993). While such studies have provided insight into potential destinations for material taken up during coupled exocytosis and endocytosis, many questions remain regarding the fate of specific protein components of the dense core vesicle.

Recently we have found that under conditions where the dense cores were not fully solublized they were subsequently internalized (Angleson et al., 1999; Cochilla et al., 2000). In the present study, we have used a combination of fluorescence imaging and electron microscopy to investigate the fate of retained PRL. We found that the non-secreted PRL was internalized slowly as intact dense cores in vesicles that then underwent intracellular processing. The retained PRL entered a pool of vesicles that were used with high release probability during subsequent stimulated exocytosis. Recycling granules transiently colocalized with internalized transferrin and

syntaxin 6 but not the TGN38-positive trans-Golgi or lysosomes loaded with fluorescent LDL. Since retention of peptide hormones appears to be a feature shared by many endocrine cells, the recycling pathway and preferential use of vesicles containing retrieved hormone revealed in this study may be of relevance to a wide range of cell types.

Materials and Methods

Cell preparation and solutions

Anterior pituitary cells were obtained from male Sprague-Dawley rats (250-350 g) by enzymatic dispersion and isolated as described previously (Ben-Tabou et al., 1994; Angleson et al., 1999). Cells were cultured in 8-well Permanox chamber slides (Nunc) for 2-5 days in DMEM (Gibco BRL) with 10% FBS. Media was changed every 24 hours.

The standard external solution contained 140 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM D-glucose and 10 mM Hepes, pH 7.2. High [K^+] external solution was identical except it contained 100 mM KCl and 42 mM NaCl.

Immuno-detection of non-secreted PRL

Antibodies against PRL were used to detect prolactin in dense cores exposed to the extracellular solution as a result of exocytosis (Angleson et al., 1999). Cells were stimulated at room temperature (~22°C) for 5 minutes in high [K^+] external solution containing 1% BSA and anti-PRL antibodies (1:1000 rabbit anti-ratPRL-IC-5 or 1:1000 guinea pig anti-rat PRL both from Ed Parlow (UCLA Medical School, Torrance, CA). At the end of stimulation, cells were either fixed (t=0) or incubated for 10 or 20 minutes at 37°C in standard external solution containing anti-PRL antibodies and 1% BSA before fixation at the indicated time (i.e. t=10 or 20 minutes). For time points exceeding 20 minutes, cells were then transferred to culture media at 37°C without anti-PRL and incubated for the indicated additional time before fixation. Cells were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature and then permeablized with 0.3% Triton X-100 for 10 minutes at room temperature. Cy3-labeled donkey anti-rabbit secondary antibodies (1:400) were then added in PBS containing 1% BSA and incubated for 30 minutes at 37°C. Vectashield (Vector Laboratories) with DAPI was used to mount slides. This procedure would detect both anti-PRL that had been internalized and anti-PRL that was still exposed to the extracellular solution. In control experiments, secondary antibody (1:100) was used instead of primary anti-PRL antibody during high [K^+] stimulation and subsequent incubation with standard external solution. Experiments testing the amount of internalization of anti-PRL were conducted as above except the cells were not permeablized with Triton following fixation. Under these conditions, only anti-PRL that had not been internalized by the time of fixation would be detected.

Dense core recycling assay

An assay was developed to characterize both basal and stimulus-dependent recycling of internalized dense cores. Cells that had internalized anti-PRL antibodies in response to the first exocytic stimulation (as described above for the 20 minute time point) were incubated in antibody-free media for an additional 30 or 60 minutes as indicated. Cells were then incubated in standard external solution containing Cy2-labeled donkey anti-rabbit antibodies for an additional 30 minutes, rinsed, and stimulated a second time for 5 minutes with high [K^+] external solution containing Cy3-labeled donkey anti-rabbit antibodies. This was followed by fixation and processing for microscopy. The second stimulation times were designated as either t=80 or t=110, which reflects the total number of minutes between the end of the first high [K^+] stimulation and the beginning of the second high [K^+] stimulation. This protocol allows for the distinction between granules that recycled without stimulation from those that recycled in response to the second exocytic stimulus. Cy2-labeling reflects anti-PRL that had recycled back to the cell surface in the absence of KCl stimulation (i.e. basal recycling). Cy3 labeling reflects anti-PRL that had recycled back to the cell surface in response to the second KCl-stimulation. All steps of the recycling assays occurred at 37°C.

Colocalization of internalized anti-PRL with TGN38 or syntaxin 6

Anti-PRL uptake experiments were performed as described above. Following fixation and permeablization, cells were incubated with either mouse anti-TGN38 (1:200 Clone 2F7.1) (Horn and Banting, 1994) or mouse anti-syntaxin 6 for one hour at 37°C, washed and then stained with Cy3 donkey anti-rabbit (to detect internalized anti-PRL) and Cy2 donkey anti-mouse secondary antibodies (1:400) and processed as described above. Three different clones of mouse monoclonal anti-syntaxin 6 antibodies were used for these experiments and gave indistinguishable results (mouse anti-syntaxin 6, BD Transduction Labs; mouse anti-syntaxin 6 clones Y3D6 and 8F4) (Bock et al., 1997). Cy2 and Cy3 labeled secondary antibodies used for all experiments were from Jackson ImmunoResearch Labs.

Colocalization of internalized Alexa488-anti-PRL with Alexa546 transferrin or DiI-LDL

For comparison of internalized anti-PRL with either internalized transferrin or internalized LDL, the anti-PRL antibody was directly labeled with Alexa 488 using the protein labeling kit from Molecular Probes (Eugene, OR) following the manufacturer's protocol. Anti-PRL uptake was then carried out as described above except cells were not permeablized following fixation. DiI-LDL was used for experiments testing for colocalization of the internalized anti-PRL with degradative compartments. Cells were incubated for 30 min at 37°C in standard external solution containing 50 μg/ml fluorescent DiI-LDL (Molecular Probes, Eugene, OR) then rinsed and incubated at 37 ^{0}C in culture media for 5 hours before the beginning of the Alexa 488-anti-PRL uptake experiments. Experiments testing the colocalization of internalized Alexa 488 labeled anti-PRL with transferrin were conducted in the standard manner except that 100 μg/ml Alexa546-transferrin (Molecular Probes) was included for the last 45 minutes before fixation.

Fluorescence imaging

Image acquisition and analysis was performed with a system running Slidebook software (Intelligent Imaging Innovations, Denver, CO) comprised of a Zeiss Axiovert S100 inverted microscope equipped with a z-stepper motor, Sutter filter wheels, Cooke Sensicam CCD camera, and G4 Apple Macintosh computer. The pixel size of the SVGA chip is 6.8×6.8 μm giving 108 nm per image pixel with a 63× oil immersion objective (1.4 NA, PlanApochromat, Zeiss). Excitation and emission wavelengths were controlled with a Sedat qaud-pass mirror (Chroma Technology) and the following excitation and emission bandpass filters: Cy2 or Alexa 488(490/20; 528/38); Cy3, Alexa 546, or DiI-LDL (555/28; 617/73). Z-sections were acquired at 300 nm spacing.

Image processing and data analysis

Images were digitally deconvolved using the constrained iterative algorithm in Slidebook. The number of anti-PRL positive puncta per cell and the brightness of individual puncta were determined from analysis of z-series of images that completely spanned the thickness of cells. Images were thresholded to background fluorescence intensity. Individual puncta were defined using the object counting

routine in Slidebook. This routine groups all pixels with an intensity value above threshold that were continuous in 3D space as one object (puncta). After the puncta were defined and counted, the integrated fluorescence intensity of each puncta was determined.

The statistical comparisons were performed with SPSS v11.1 software. Histograms of the fluorescence intensity of individual puncta were generated and analyzed with Igor Pro (Wavemetrics). Quantitative analysis of the extent of colocalization between internalized anti-PRL immunoreactivity and the various markers (i.e. TGN38 immunoreactivity, syntaxin 6 immunoreactivity, internalized Alexa546-transferrin or internalized DiI-LDL) was performed on z-series of images. PRL-positive and marker-positive pixels were defined blindly and independently of each other using the thresholding segmentation routine in Slidebook. Regions of overlap between anti-PRL-positive and marker-positive pixels were determined using the image math 'AND' function in Slidebook. The extent to which internalized anti-PRL was colocalized with the respective marker in each cell is expressed as the percent of pixels that were PRL- and marker-positive over the number of pixels that were PRL-positive.

Electron microscopy and pre-embedding immunogold labeling of internalized anti-PRL

The stimulation-dependent anti-PRL uptake procedure was the same as that used for the t=20 minute time point described above for the fluorescence experiments. Cells were washed in PBS and fixed in 100 mM PBS containing 3% paraformaldehyde and 0.15% glutaraldehyde for 15 minutes, then incubated in 0.1% sodium borohydride in 100 mM phosphate buffer for 15 minutes followed by a 30 minute incubation in 0.05% Triton X-100, all at room temperature. Cells were blocked with Goat Blocking Solution (Aurion, Electron Microscopy Sciences) for 60 minutes, rinsed with phosphate buffer containing BSA-c (Aurion, EMS) and incubated for 22 hours with goat anti-rabbit ultrasmall gold conjugate (Aurion, EMS) in 0.2% BSA at 4°C. Cells were rinsed with phosphate buffer containing BSA-c and re-fixed in 2.5% glutaraldehyde for 1 hour before silver enhancement with R-Gent SE-EM (Aurion, EMS) following manufacturer's protocol. Cells were post-fixed with 0.5% OsO_4 for 15 minutes before ethanol dehydration and embedding in Epon. Thin sections (100 nm) were post-stained with 5% uranyl acetate and Reynolds lead citrate for 4 minutes each and viewed with a Hitatchi 7000 transmission electron microscope. Control experiments were performed in an identical manner except that the anti-PRL antibody was not included during the treatment with high [K^+] solution.

To view the surface-exposed anti-PRL at the end of the exocytic stimulation, cells were stained with rabbit anti-PRL for the t=0 time point as above for the fluorescence experiments, then fixed in 2% glutaraldehyde and 2% paraformaldehyde in phosphate buffer for 10 minutes. Cells were then incubated with for 20 min with 6 nm gold labeled donkey anti-rabbit antibody (Aurion, EMS) without permeabilization of the plasma membrane. Cells were then processed as above.

Photoconversion of FM1-43

Cells were stimulated for 5 minutes with high [K^+] external solution containing 4 μM FM1-43 (Molecular Probes) and then switched to standard external solution with FM1-43 for an additional 20 minutes at room temperature (~22°C). Cells were then rinsed three times with PBS, fixed with ice cold 2% glutaraldehyde 2% paraformaldehyde in 100 mM phosphate buffer for 20 minutes and then incubated in 100 mM NH_4Cl in 100 mM phosphate buffer for 10 minutes. Cells were switched to 100 mM phosphate buffer containing 1.5 mg/ml diaminobezidine (DAB) for photoconversion. To achieve photoconversion of FM1-43 (Henkel at al., 1996), the sample was illuminated for 45 min with light from a 175 watt Xenon lamp passed through a 485/30 HQ emission filter (Chroma Technology Corp) and focussed on the cells with a 20× objective (0.5 NA Plan-neofluar, Zeiss). Cells were then rinsed in phosphate buffer and postfixed for 1 hour in 1.0% OsO_4 prior to ethanol dehydration and embedding in Epon. Thin sections were viewed without post-staining to clearly distinguish granules containing electron dense FM1-43-DAB reaction product from unlabeled granules.

Results

Retention and internalization of dense cores containing non-secreted PRL

Previous studies have demonstrated that the dense core granule contents, including PRL itself, are not fully released from primary lactotrophs during exocytic fusion of secretory granules with the plasma membrane (Angleson et al., 1999; Cochilla et al., 2000). We used anti-PRL antibodies to monitor the stimulation-dependent surface labeling and fate of non-secreted PRL. Anterior pituitary cells fixed at the end of a 5 minute stimulation with high [K^+] external solution containing anti-PRL antibodies displayed discrete surface staining (Fig. 1A, t=0). Such staining was observed in 43% of cells, consistent with the proportion of cells in this preparation that are PRL-positive lactotrophs (Angleson et al., 1999). Staining was stimulation dependent; staining was not observed in the absence of high [K^+] treatment. Staining was specific in that substitution of a fivefold higher concentration of labeled secondary-antibody for the anti-PRL antibody during stimulation did not result in staining (data not shown). Quantitative analysis of complete z-series through cells revealed an average of 100 stained exocytic structures per cell (Fig. 2A).

To determine whether the anti-PRL had been internalized during the 5 minute stimulation, we compared the staining between samples in which the membranes were permeablized prior to application of the secondary antibody (Fig. 1A) to samples in which the membrane permeablization step was omitted (Fig. 1E). The same type of experiments were conducted at the t=10 minute time point. (Fig. 1B vs 1F). The staining was indistinguishable between the permeablized and nonpermeablized samples at both the t=0 and t=10 time points. The anti-PRL staining was thus restricted to surface-exposed structures up to 10 minutes after the end of stimulation, which indicates a significant delay before endocytosis of the anti-PRL. These results are consistent with our previous report of slow (~20 minute at room temperature) internalization of FM1-43 stained dense cores in this preparation (Angleson et al., 1999).

When a delay of 20 or 80 minutes was added between the end of high [K^+] stimulation and fixation and permeablization, the anti-PRL stained structures were internalized and remained visible as discrete puncta (Fig. 1C,D). When secondary antibody was added without permeabilization 20 minutes after the end of stimulation there was no detectable staining, which indicates that internalization occurred between the 10- and 20-minute time points (data not shown). The number of anti-PRL stained puncta per cell was reduced by 60% from the t=0 time point to both the 20 or 80 minute time points (Fig. 2A). The integrated fluorescence of each puncta was determined to provide an estimate of relative size. The number of bright structures was increased at the 20 and 80 minute time points relative to the t=0 time (Fig. 2B). These analyses suggest that

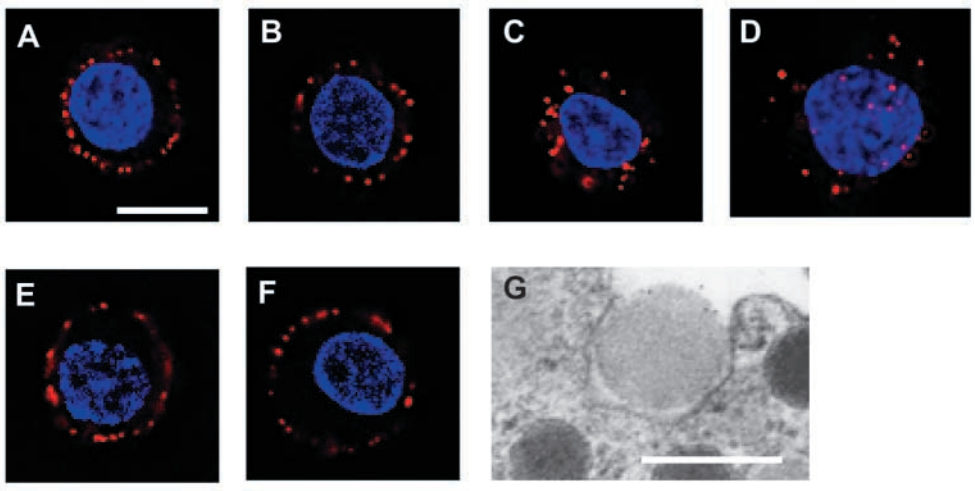

Fig. 1. Stimulation-dependent surface labeling and internalization of non-secreted PRL. (A-D) Lactotrophs were stimulated for 5 min at room temperature in high [K^+] external solution containing anti-PRL antibodies, then rinsed, and fixed at the indicated time point, permeablized and processed for indirect immunofluorescence. (A) t=0; (B) t=10; (C) t=20; (D) t=80 minutes. (E,F) Surface labeling of non-internalized anti-PRL. Cells were labeled with anti-PRL as above and then fixed and processed for indirect immunofluorescence without permeablization of the plasma membrane. (E) t=0; (F) t=10 minutes. Deconvolved images from the middle of a z-series are shown. DAPI staining shown in blue. Scale bar is 10 μm for all fluorescence images. (G) Gold-labeled secondary antibody was applied to non-permeablized cells fixed at t=0 to reveal anti-PRL in surface-exposed dense cores. Bar, 400 nm.

some of the structures containing the non-secreted PRL had either fused or clustered together after internalization.

Immunogold electron microscopy was used to determine the morphological characteristics of the structures containing the non-secreted PRL. To determine the ultrastructural characteristics of the surface-bound anti-PRL, 6 nm gold-labeled secondary antibodies were applied to cells fixed at the t=0 time point without membrane permeablization. The gold label was found associated with dense cores exposed to the extracellular space (Fig. 1G). To visualize the internalized anti-PRL, cells were fixed at the 20 minute time point, permeablized and stained with gold-labeled secondary antibodies. These cells displayed gold particles associated with structures that had an appearance similar to the core of a dense core vesicle except that the gold-labeled cores were often in clusters (Fig. 3). The gold-labeled clusters were found throughout the cell (Fig. 3A). Control experiments in which the cells were not exposed to the anti-PRL antibody during high [K^+] treatment did not show gold labeling but did still possess clusters of small cores similar in appearance to the labeled structures in the experimental condition (Fig. 3D,E). This indicates that the retention and clustering of the cores was not an artifact due to uptake of the anti-PRL antibodies. Such clustered small cores are not observed in resting lactotrophs in our preparation.

While some gold-labeled clusters of granules appeared to be contained within one structure (Fig. 3C) the lack of good membrane preservation obtained with the protocol used for the immunogold labeling prevented clear determination of whether granule clusters were contained within a single membrane compartment (Fig. 3B). To further address this question we used photoconversion of FM1-43 (Henkel et al., 1996) to determine whether endocytosed cores were clustered within a

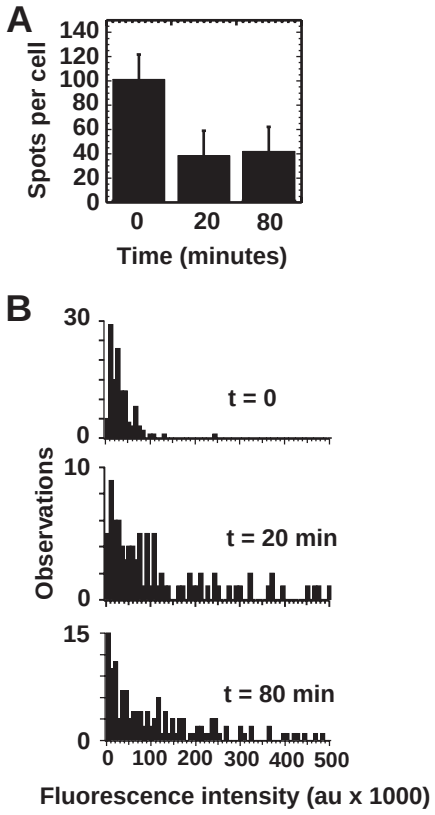

Fig. 2. Intracellular fusion of vesicles containing internalized anti-PRL. Quantitative fluorescence analysis of data from experiments described in Fig. 1 suggest fusion of the structures containing the internalized anti-PRL. Analysis was performed on complete z-series of images as described in Materials and Methods. (A) The number of structures containing internalized anti-PRL decreased over time. The graph shows the mean (±s.e.m.) number of stained puncta per cell at each time point. The difference between the means at t=0 (100.6±7.0) and t=20 (38.6±4.3) and between t=0 and t=80 (42.0±5.0) were significant (P<0.005; Student's t-test). (B). Relative size of the structures containing internalized anti-PRL over time was estimated from the fluorescence intensity of individual puncta. Histograms of the integrated fluorescence of individual puncta in cells at each time point demonstrate a clear increase in the proportion of brightly stained (large) structures at the 20 and 80 minute time points compared to the 0 time point (means were significantly different; P<0.001; Mann-Whitney U-test). Data are pooled from 10-12 randomly selected cells per time point.

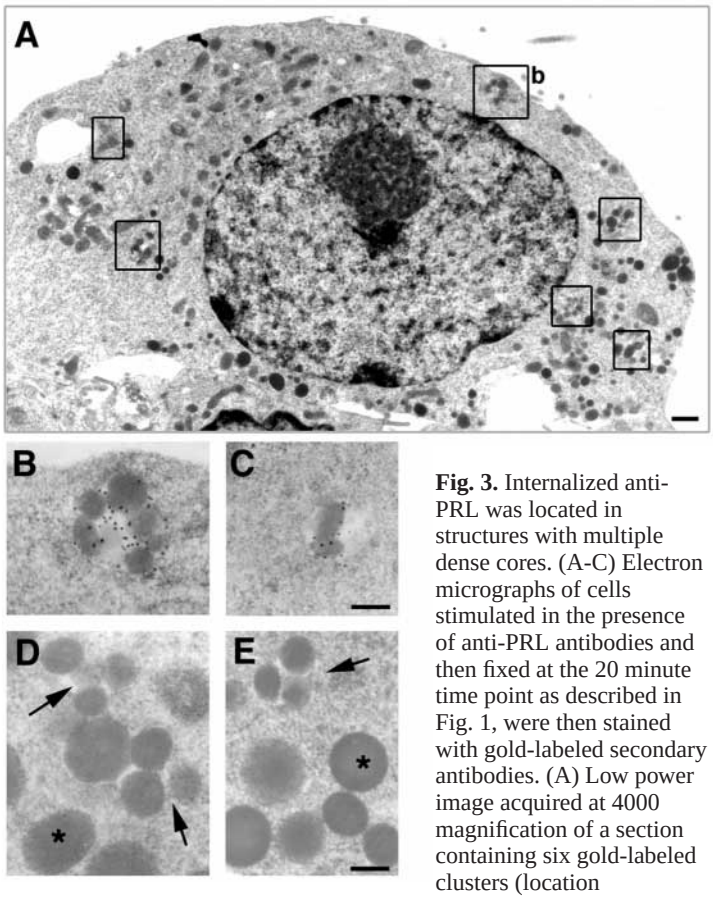

Fig. 3. Internalized anti-PRL was located in structures with multiple dense cores. (A-C) Electron micrographs of cells stimulated in the presence of anti-PRL antibodies and then fixed at the 20 minute time point as described in Fig. 1, were then stained with gold-labeled secondary antibodies. (A) Low power image acquired at 4000 magnification of a section containing six gold-labeled clusters (location highlighted with boxes, box marked 'b' is displayed at high magnification in B) demonstrates that the internalized anti-PRL was throughout the cell. (B) The gold-labeled dense cores often clustered together or (C) appeared to have begun to coalesce. (D,E) Control cells processed as for above except that anti-PRL primary antibody was omitted from the stimulation solution. Note the presence of multi-granule clusters containing small cores (arrows). For B-E micrographs were acquired at 30,000 magnification. Bars, 300 nm (A); 200 nm (B-E).

common membrane. FM1-43 labels the dense cores of lactotrophs and co-stains with anti-PRL (Angleson et al., 1999; Cochilla et al., 2000). Cores were labeled with FM1-43 under conditions identical to the 20 minute time point used for anti-PRL internalization except that the cells were maintained at room temperature throughout the experiment. Under these conditions internalized FM1-43 is almost exclusively due to stained dense cores and internalized dye from other endocytic mechanisms is negligible (Angleson et al., 1999). The electron-dense FM1-43 photoconversion reaction product was found in granule matrices (not membrane) that were clustered together within a single membrane (Fig. 4). Furthermore, this result provides additional evidence that the retention of cores is not due to anti-PRL binding.

Basal and stimulus-dependent recycling of granules

Cells that had internalized anti-PRL primary antibodies as a result of a first round of coupled exocytosis-endocytosis (as described above) were tested for recycling of anti-PRL back to the cell surface (Fig. 5, Table 1). To test for stimulus-dependent recycling, a second exocytic stimulation with high [K^+] external solution containing Cy3-labeled secondary antibody was applied either 80 or 110 minutes after the end of the first stimulation. To test for basal recycling, Cy2-donkey anti-rabbit antibodies in standard external solution were added 30 minutes prior to the second exocytic stimulation (i.e. from t=50 to t=80 for cells stimulated at t=80, or from t=80 to t=110 for cells stimulated at t=110). Cy2-labeled structures represent basal recycling of anti-PRL (i.e. without stimulation) whereas structures labeled with Cy3 alone represent granules that had recycled to the cell surface in response to the second KCl-induced exocytosis (Fig. 5). The number of Cy2- and Cy3-positive puncta were quantified for each cell (Table 1). The results demonstrate both a low rate of basal recycling and a significant stimulus-dependent recycling of granules containing anti-PRL.

Recycling of non-secreted PRL through syntaxin 6-positive and TGN38-negative compartments

We tested whether the recycling granules returned to the trans-Golgi by co-staining cells that had internalized anti-PRL following a first exocytic stimulation with the TGN markers anti-TGN38 or anti-syntaxin 6. There was little, if any, colocalization of anti-PRL and anti-TGN38 at any time point (Fig. 6A,E), which indicates a low level of traffic to the TGN. The results with syntaxin 6 colocalization were strikingly different. More than 25% of the internalized anti-PRL colocalized with anti-syntaxin 6 at the 20 minute time point, while at the 80 minute time point the extent of colocalization was reduced to ~7% (Fig. 6B,E). There was no colocalization of anti-syntaxin 6 and surface anti-PRL at t=0 or t=10 time points indicating that syntaxin 6 is not a component of the exocytic structure and thus is unlikely to be a constituent of mature granules. Together these results indicate that the recycling granules transiently interact with syntaxin-6-positive membranes while bypassing the TGN38-positive trans-Golgi.

We also compared the anti-PRL recycling pathway to the more well characterized pathways taken by recycling transferrin or internalized LDL. For these experiments, the anti-PRL uptake was performed as previously except that the anti-PRL antibody was directly conjugated to Alexa 488. This

Table 1. Basal and stimulus-dependent recycling of anti-PRL

Assay time	Basal		Stimulus-dependent	
Basal (stimulus)	Total/cell	Rate (event min^{-1})	Total/cell	Rate (min^{-1})
50-80 (80)	6.1 (±0.8)	0.20	11.7 (±1.1)	2.34
80-110 (110)	13.6 (±3.2)	0.45	27.6 (±5.3)	5.52

Basal and stimulus-dependent recycling assayed as described in Materials and Methods. Data are from experiments described in Fig. 5. Assay time refers to the 30 minute period in which basal recycling was assayed prior to the second exocytic stimulation, the time of second exocytic stimulation is in parentheses. Time is in minutes after the end of the first stimulation. The total number corresponds to the number of Cy2-puncta per cell for basal and Cy3-puncta per cell for stimulated. The rates are the total number/30 minutes for basal and total number/5 minutes for stimulated. Values are mean±s.e.m. from 10-20 cells per time point.

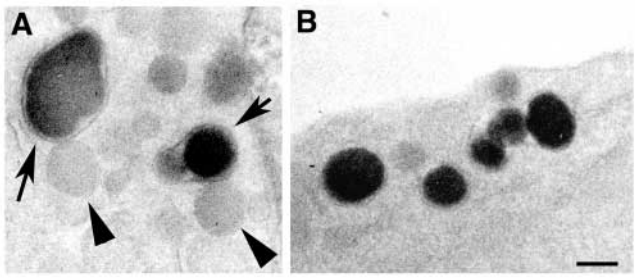

Fig. 4. Ultrastructural identification of internalized FM1-43 stained dense cores. Photoconversion revealed that internalized FM1-43 was located in dense cores that were clustered within a membrane. Dark FM1-43-DAB reaction product (arrows) is clearly distinguished from unlabeled dense core vesicles (arrowheads). FM1-43 was found in vesicles containing cores that appeared to coalesce (A) and in vesicles with multiple distinct cores (B). Bar, 200 nm for both images.

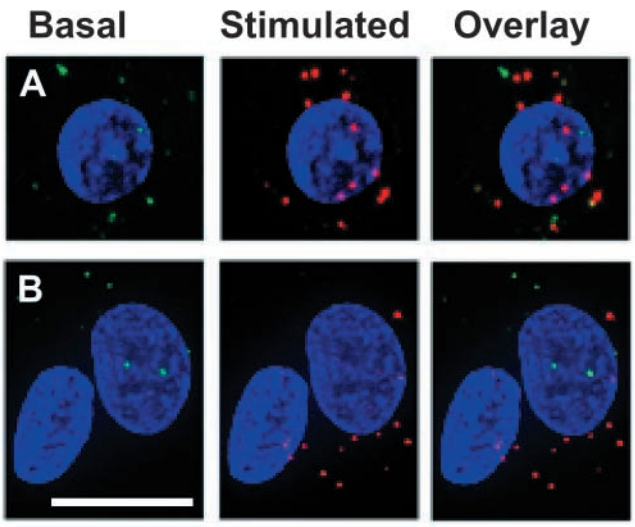

Fig. 5. Vesicles containing internalized anti-PRL recycle in a stimulus-dependent manner. To measure recycling, cells that had internalized anti-PRL in response to a first stimulation were sequentially incubated with Cy2-labeled secondary antibodies in standard external solution for 30 minutes and then Cy3-labeled secondary antibodies in high [K^+] external solution for 5 minutes. Anti-PRL that recycled to the cell surface in a basal manner was labeled with Cy2 (green). Granules that recycled back to the cell surface in a stimulus-dependent manner were labeled with Cy3 only (red). (A) Basal recycling (green) measured from t=50 to 80 minutes; stimulus-dependent recycling measured at t=80. (B) Basal recycling (green) measured from t=80 to 110 minutes; stimulus-dependent recycling measured at t=110. (See Materials and Methods for details of the assay and Table 1 for quantitation.) DAPI (blue) was included in the mounting media. The images shown are projection images from the entire z-series. Bar, 10 μm.

enabled internalized antibody to be detected without membrane permeablization, which would result in reduction or loss of signal from of internalized Alexa546 transferrin or DiI-LDL. DiI-LDL was loaded and then chased for 5 hours prior to beginning the anti-PRL uptake experiments so that the DiI-LDL had trafficked to lysosomes. There was essentially no colocalization of the internalized anti-PRL with the DiI-LDL, indicating that little if any of the internalized cores had accumulated in the lysosomes on the time scale of these experiments (Fig. 6C,E). In contrast, there was a high level of colocalization between the internalized anti-PRL and recycling transferrin, suggesting that the recycling PRL trafficked through early endosomes and the endosomal recycling compartment used by transferrin (Fig. 6D,E).

Discussion

We have found that dense core vesicles that had undergone exocytic fusion with the plasma membrane retained some non-secreted PRL in a dense core that was slowly internalized and then used during subsequent rounds of exocytosis. In addition to providing evidence for partial secretion from individual granules, these studies provide several significant findings concerning the recycling of dense core vesicle components and suggest possible roles for recycling retained hormone in neuroendocrine secretion.

Retention of dense core vesicle cargo

Our observations of partial release and recycling of a peptide hormone from individual dense core vesicles in primary lactotrophs may represent a process shared by other endocrine cells. While it is commonly believed that the entire contents of dense core vesicles are released upon exocytosis, evidence exists supporting the notion that vesicles may actually release only a portion of their contents (Artalejo et al., 1998). For example, stimulated bovine chromaffin cells did not show ultrastructural evidence for loss of granule content regardless of measurable secretion of catecholamines (Fox, 1996; Plattner et al., 1997). Amperometric measurements of catecholamine release from chromaffin cells have indicated that release of catecholamines can occur in a partial manner (Zhou et al., 1996). Stimulated PC12 cells were shown to retain a portion of chromogranin B (Piplikar and Huttner, 1992), which indicates that the peptide content of catecholamine-containing granules can also undergo partial release. Finally, endogenous insulin in human and rat pancreatic β-cells (Larsson et al., 1989) and a number of hormones (GH, ACTH, LH and TSH) in rat anterior pituitary cells (St John et al., 1986) (R.A.B. and J.K.A., unpublished) are retained at the cell surface following exocytic stimulation. Thus partial secretion and retention of peptide cargo in dense core vesicles appears to occur in a wide range of endocrine and neuroendocrine cell types.

The majority of studies addressing the fate of retained granule content have focused on cells containing chromaffin granules. It is possible that the dense cores in vesicles of these catecholamine-secreting cells are retrieved intact for local recycling involving the import from the cytosol of catecholamine precursors (Artalejo et al., 1998). If so, dense core vesicles of this type could possess a local recycling pathway similar to that of synaptic vesicles (Betz and Angleson, 1998). The well documented finding that antibodies against dopamine-beta-hydroxylase, an enzyme involved in catecholamine synthesis in the lumen of dense core vesicles, can be taken up and recycled in a stimulus-dependent fashion in chromaffin cells (Dowd et. al., 1983; Patzak and Winkler, 1986; Pender and Burgoyne, 1992; Hurtley, 1993; Wick et. al.,

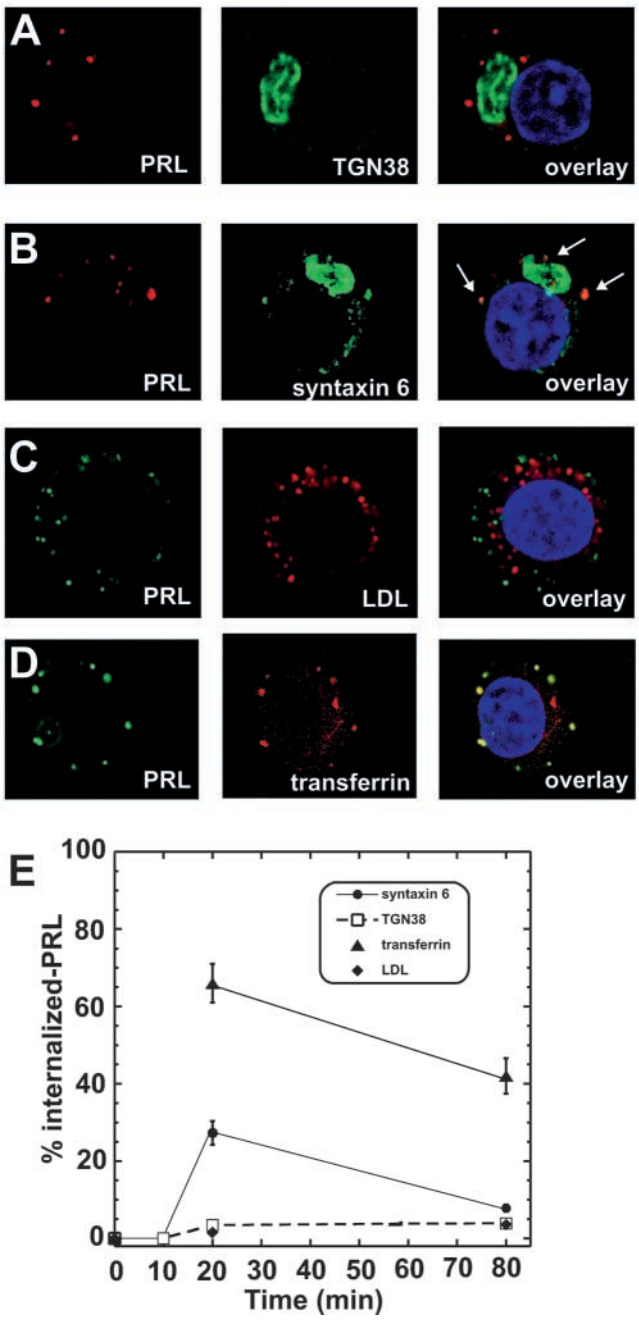

Fig. 6. Characterization of the anti-PRL recycling pathway. Cells were stained for internalized anti-PRL at the 0, 10, 20 and 80 min time points as described for Fig. 1. All images are from the t=20 min time point. (A,B) Following fixation and permeabilization, cells were also stained with anti-TGN38 or anti-syntaxin 6. (C) Cell fixed at the t=20 min time point, without permeablization, showing internalized Alexa 488 labeled anti-PRL (green) and DiI-LDL (red) that had been chased into the lysosomes. (D). Cell fixed at the t=20 minute time point that had been internalized Alexa 488 anti-PRL (green) and Alexa 546 transferrin (red). Bar, 10 μm. DAPI (blue) was included in the mounting media. (E) The extent of colocalization between internalized anti-PRL and the respective markers was determined from complete z-series of cells and is expressed as the percent of pixels that were positive for anti-PRL and positive for marker out of the number of pixels that were positive for anti-PRL. Data given are means (±s.e.m.) from 12 to 20 cells per condition and 30-50 z-sections per cell.

1997) is consistent with local recycling of catecholamine-containing dense core vesicles.

Our study addressed partial secretion of peptide hormones, as opposed to the contents of catecholamine-containing chromaffin granules. In the case of non-secreted insulin, it has been reported that fully processed insulin, but not proinsulin, was retained in cores at the β-cell surface (Larsson et al., 1989), which suggests that partial secretion of insulin did not simply reflect retention of inappropriately processed hormone. The non-secreted PRL in our studies did not traffic to lysosomes and instead formed a pool of granules that were available for subsequent rounds of exocytosis.

Preferential use of recycling granules

Dense core vesicles can be described in terms of functionally or morphologically defined pools of granules (Plattner et al., 1997; Neher, 1998, Olofsson et. al., 2002). In our study, some of the internalized anti-PRL could recycle to the surface in the absence of a second exocytic stimulation. Such recycling could contribute to basal secretion from lactotrophs. However a substantial portion of the recycling-PRL entered or formed a pool of vesicles that appeared to be preferentially used during the second exocytic stimulation. Since there were an average of 12 recycling vesicles detected per cell during the second stimulation (at t=80) and ~40 anti-PRL positive puncta from the first stimulation present at this time (Fig. 2), it can be concluded that these recycling vesicles had a release probability of 12/40 or ~30%. The number of dense core vesicles in primary cultures of endocrine cells, including lactotrophs, is in the order of 10,000 to 20,000 per cell (e.g. Ingram et al., 1988; Plattner et al., 1997; Olofsson et al., 2002). The first exocytic stimulation resulted in ~100 exocytic events with retained PRL per cell (Fig. 2). Our previous studies that employed patch clamp capacitance measurements of membrane fusion revealed that the amount of secretory membrane added to the plasma during exocytosis matched the amount predicted by counting granules with retained cores in the same cells (Angleson et al., 1999). Thus our estimate of 100 exocytic events measured by PRL-retention is a reasonable measure of the total number of exocytic events during the first stimulation. From this we can estimate the release probability during the first exocytic stimulation to be between 0.5 and 1% (100/20,000 to 100/10,000). If the vesicles containing recycling PRL had this low release probability then we predict on average 0.2 to 0.4 recycling granules per cell during the second stimulation and we would not have reliably detected recycling. There was an even higher number of stimulus-dependent recycling events at later time points (t=110 min; Table 1). Clearly a population of recycling granules is somehow made available for preferential use during stimulated exocytosis.

Recycling of non-secreted PRL

The extent to which the recycling pathway taken by the non-secreted PRL is shared by the dense core vesicle membrane remains to be determined. If a dense core vesicle were to completely release its peptide cargo upon exocytosis, the vesicle membrane would presumably have to traffic back to a compartment of the biosynthetic pathway for functional

recycling. Several studies addressing the fate of post-exocytic dense core vesicle membrane relied on uptake of tracers, such as HRP or cationized ferritin that could be visualized by electron microscopy. Such studies yielded a variety of results in different endocrine cells. For example, HRP was trafficked back to a cis compartment of the Golgi in pancreatic β-cells (Orci et al., 1986) but HRP was not found in the Golgi in pituitary melanotrophs (Bäck et al., 1993). In primary lactotrophs cationized-ferritin was found to traffic to a variety of structures including immature granules (Farquhar, 1978). The apparently contradictory results of such studies may reflect differential sorting and trafficking of the non-specific fluid phase and membrane markers that can occur during intracellular fusion and fission events. Indeed, cationized ferritin and HRP trafficked to distinct compartments in the same pituitary cells (Bäck et al., 1993). Other studies have relied on the endocytic-retrieval of antibodies against the lumenal domains of dense core vesicle membrane proteins. Antibodies against membrane-bound peptidylglycine α-amidating monooxygenase (PAM) accumulated in the TGN in the corticotrophic AtT-20 cell line (Milgram et al., 1993) but did not appear to traffic to such a compartment in primary cultures of anterior pituitary cells (El Meskini et. al., 2000). Antibodies against the lumenal domain of the dense core vesicle membrane protein ICA512 accumulated in perinucular structures in insulinoma cells (Solimena et al., 1996). Thus the endocytic traffic of dense core vesicle membrane is not yet fully resolved.

To characterize the recycling of retained secretory cargo in endocrine cells we compared the recycling pathway taken by retained PRL to other more well described recycling pathways such as those taken by transferrin or LDL (Gruenberg and Maxfield, 1995). We did not detect significant colocalization of the internalized anti-PRL with DiI-LDL that had been loaded into late degradative compartments by a 5 hour chase. The DiI-LDL should be exclusively present in lysosomes with this long chase. Thus the retained PRL did not traffic to lysosomes on the time scale of the recycling assays.

The majority of internalized anti-PRL did colocalize with internalized transferrin at the t=20 minute time point. It is important to keep in mind that internalization of the dense cores containing retained PRL was a slow process that occurred entirely between the t=10 and 20 minute time points at 37°C. Therefore structures co-stained with internalized anti-PRL and transferrin at the 20 minute time point could have been either early endosomes or the endosomal recycling compartment. At 80 minutes the amount of colocalization with transferrin was reduced but still significant. The internalized PRL that remained with the transferrin could have given rise to basal recycling of PRL, which could play a role in sustained secretion.

The recycling pathway in which some of the internalized PRL diverged from the transferrin recycling pathway and some of the PRL underwent stimulus-dependent recycling is reminiscent of the recycling of GLUT4 glucose transporter in adipocytes and muscle. Stimulated recycling of GLUT4 involves insulin-regulated sorting from transferrin in the endosomal recycling compartment (Lampson et al., 2001). GLUT4 recycling is also regulated by syntaxin 6 (Perera et al., 2003) and involves traffic through syntaxin 6-positive, TGN38-negative compartments (Shewan et al., 2003). These similarities raise the possibility that the stimulus-dependent recycling of retained PRL may share components of the GLUT4 recycling pathway. In this regard it is interesting to note that recycling GLUT4 is found in dense core vesicles in atrial cardiomyocytes, a specialized cell with both regulated peptide secretion and GLUT4 recycling (Slot et al., 1997).

We noted that the recycling PRL transiently colocalized with syntaxin 6 but not the TGN 38-positive trans-Golgi. Known recycling pathways from the cell surface to the TGN are typified by internalized furin and TGN38 (Gosh et al., 1998; Mallet and Maxfield, 1999). Internalized furin bypasses the endosomal recycling compartment and goes to late endosomes and then to the TGN (Mallet and Maxfield, 1999). P-selectin, a component of secretory granules in platelets and endothelial cells, traffics to the TGN via this late endosome pathway (Straley and Green, 2000).

In contrast, internalized TGN38 traffics to the endosomal recycling compartment, not the late endosome, before returning to the TGN (Gosh et al., 1998). The SNARE protein syntaxin 6 is likely to be involved in the traffic between the endosomal recycling compartment and the TGN. Syntaxin 6, a component of both the TGN and a subset of endosomes (Bock et al., 1997), has been found to interact with SNAREs of the early/recycling endosomes as well as the early endosome protein EEA1 (Mills et al., 2001; Mallard et al., 2002). Furthermore syntaxin 6 has been shown to be functionally involved in traffic between the recycling endosome and the TGN (Mallard et. al., 2002).

The extensive colocalization between the internalized PRL and transferrin in our studies indicates involvement of the endosomal recycling compartment, which suggests that the colocalization of the recycling PRL with syntaxin 6 could be in this compartment. This could provide a means of traffic between the recycling granules and the TGN. While there was no detectable transport of the recycling PRL to the TGN, it remains possible that traffic from the TGN to the recycling granule could occur via a syntaxin-6-containing pathway involving the endosomal recycling compartment.

Possible role for retention and recycling of prl

In addition to the TGN and endosomes, syntaxin 6 is also present in immature, but not mature, granules in endocrine cells (Klumperman et. al., 1998; Wendler et al., 2001). It has been shown that syntaxin 6 is involved in the homotypic fusion of immature secretory granules in PC12 cells (Wendler et al., 2001). TGN38 is absent from immature secretory granules (Dittié et al., 1997). Thus our findings of transient recycling through a syntaxin 6-positive compartment while bypassing the TGN38-positive TGN are also consistent with the recycling PRL trafficking to immature granules.

Our observation of preferential use of recycling granules during subsequent exocytic stimulation is also consistent with the possibility that the internalized anti-PRL trafficked back to a compartment that contains newly synthesized PRL. Newly synthesized hormones in endocrine cells, including PRL in pituitary cells, can be preferentially released (Walker and Farquhar, 1980; Gold et al., 1982; Rhodes and Halban, 1987; Chen at al., 1989). It may be possible that newly synthesized hormone merged with the recycling granules in an intermediate compartment such as immature granules or the endosomal recycling compartment..

Finally, the ultastructural characteristics of the granules containing recycling PRL in our study are very similar to the immature granules in lactotrophs undergoing sustained secretion. A series of studies by Farhquar and colleagues (Farquhar et al., 1978; Farquhar, 1978; Rosenzweig and Farquhar, 1980) combined autoradiography and electron microscopy to unravel the ultrastructural pathway taken by newly synthesized PRL in lactotrophs from lactating female rats. These studies defined four morphologically distinct types of maturing vesicles. After trafficking through the ER and Golgi, the newly synthesized PRL budded from the trans-Golgi in structures containing relatively small cores that were termed type I granules. These then fused to form type II granules that contained several small cores that coalesced into type III and eventually type IV, or mature, granules. Our ultrastructural studies (Figs 3, 4) revealed that internalized anti-PRL and FM1-43 were present in structures that are similar to the immature type II and III granules described previously. This is consistent with the finding that cationized ferritin taken up during secretion trafficked to granules containing newly synthesized PRL (Farquhar, 1978).

Delivery of PRL in a preformed aggregate to a site containing newly synthesized PRL may be advantageous. Aggregation of hormones is involved in maturation of dense core vesicles (Dannies, 1999; Arvan and Castle, 1998). The specific aggregation of soluble hormones, including PRL, is aided by a variety of factors including protein 'chaperones' that may help trigger or seed aggregation (Thiele et al., 1997; Jain et al., 2000). For example, it has been observed that as much as 20% of chromogranin B in PC12 cells is membrane bound and it was suggested that this membrane-bound population may aid the aggregation/sorting of more chromogranin or other regulated peptides into forming granules (Pimplikar and Huttner, 1992; Glombik et al., 1999). While purified PRL has been found to aggregate in vitro without membrane (Colomer et al., 1996), a means of attaching the aggregating PRL to the membrane may be necessary for sorting to, or retention in, forming granules. It is possible that the recycling of the non-secreted, membrane-bound, population of PRL to forming granules may act as a seed to enhance the efficiency of aggregation and/or help retain the newly synthesized PRL in the granule by providing a membrane attachment point.

Whether the recycling-PRL has such a role in granule formation remains to be determined. Recycling PRL could also contribute to the generation of multiple secretory pathways within lactotrophs. Exocrine cells, such as parotid acinar cells, possess several distinct pathways for release of secretory products. These include distinct major and minor regulated pathways, the latter of which preferentially releases newly synthesized secretory proteins, as well as stimulus-independent pathways (Huang et al., 2001). The major and minor regulated pathways also have distinct secretagogue dependence (Castle and Castle, 1996). The potential for multiple secretory pathways within endocrine cells has not been as extensively explored; however, it is possible that the basal and stimulus-dependent recycling of PRL that we observed along with secretion from de novo granules could represent distinct secretory pathways. In this regard, it will be interesting to test the many known PRL secretagogues for affects on each of the potential secretory pathways in lactotrophs.

We thank Judith Snyder (University of Denver) for use of the ultramicrotome, Rytis Prekeris (University of Colorado Health Sciences Center) for syntaxin 6 antibodies, Ed Parlow and the National Hormone Distribution Program of the NIDDK for anti-PRL antibodies, and Adam Zweifach and John Hutton (University of Colorado Health Sciences Center) for helpful discussions. Supported by NIH grant RO1 DK57999 to J.K.A.

References

Angleson, J. K., Cochilla, A. J., Kilic, G., Nussinovitch, I. and Betz, W. J. (1999). Regulation of dense core release from neuroendocrine cells revealed by imaging single exocytic events. *Nat. Neurosci*. **2**, 440-446.

Artalejo, C. R., Elhamdani, A. and Palfrey, C. C. (1998). Dense-core vesicles can kiss-and-run too. *Curr. Biol*. **8**, R62-R65.

Arvan, P. and Castle, D. (1998). Sorting and storage during secretory granule biogenesis: looking backward and looking forward. *Biochem. J*. **332**, 593-610.

Bäck, N., Soinila, S. and Virtanen, I. (1993). Endocytic pathways in the melanotroph of the rat pituitary. *Histochem. J*. **25**, 133-139.

Ben-Tabou, S., Keller, E. and Nussinovitch, I. (1994). Mechanosensitivity of voltage-gated calcium currents in rat anterior pituitary cells. *J. Physiol.* **476**, 29-39.

Betz, W. J. and Angleson, J. K. (1998). The synaptic vesicle cycle. *Ann. Rev. Physiol*. **60**, 347-363.

Bock, J. B., Klumperman, J., Davanger, S. and Scheller, R. H. (1997). Syntaxin 6 functions in *trans* Golgi network vesicle trafficking. *Mol. Biol. Cell* **8**, 1261-1271.

Castle, J. D. and Castle, A. M. (1996). Two regulated secretory pathways for newly synthesized parotid salivary proteins are distinguished by doses of secretgogues. *J. Cell Sci.* **109**, 2591-2599.

Chen, T. T., Kinemane, R. D., Betts, G., Hill, J. B. and Frawley, L. S. (1989). Relative importance of newly synthesized and stored hormone to basal secretion by growth hormone and prolactin cell. *Endocrinology* **125**, 1904-1909.

Cochilla, A. J., Angleson, J. K. and Betz, W. J., (2000). Differential regulation of granule-to-granule and granule-to-plasma membrane fusion during secretion from rat pituitary lactotrophs. *J. Cell Biol*. **150**, 839-848.

Colomer, V., Kicska, G. A. and Rinder, M. J. (1996). Secretory granule content proteins and the lumenal domains of granule membrane proteins aggregate *in vitro* at mildly acidic pH. *J. Biol. Chem.* **271**, 48-55.

Dannies, P. S. (1999). Protein hormone storage in secretory granules: Mechanisms for concentration and sorting. *Endocrine Rev*. **20**, 3-21.

Dittié, A. S., Thomas, L., Thomas, G. and Tooze, S. A. (1997). Interaction of furin in immature secretory granules from neuroendocrine cells with the AP-1 adaptor complex is modulatd by casein kinase II phosphorylation. *EMBO J*. **16**, 4859-4870.

Dowd, D. J., Edwards, C., Englert, D., Mazurkiewicz, J. E. and Ye, H. Z. (1983). Immunofluorescent evidence for exocytosis and internalization of secretory granule membrane in isolated chromaffin cells. *Neuroscience* **10**, 1025-1033.

Eliasson, L., Proks, P., Ämmälä, C., Ashcroft, F. M., Bokvist, K., Renström, E., Rorsman, P. and Smith, P. A. (1996). Endocytosis of secretory granules in mouse pancreatic β-cells evoked by transient elevation of cytosolic calcium. *J. Physiol.* **493**, 755-767.

El Meskini, R., Mains, R. E. and Eipper, B. A. (2000). Cell type-specific metabolism of peptidylglycine α-amidating monooxygenase in anterior pituitary. *Endocrinology* **141**, 3020-3034.

Farquhar, M. G., Reid, J. J. and Daniell, L. W. (1978). Intracellular transport and packaging of prolactin: A quantitative electron microscope autoradiographic study of mammotrophs dissociated from rat pituitaries. *Endocrinology* **102**, 296-311.

Farquhar, M. G. (1978). Recovery of surface membrane in anterior pituitary cells. Variations in traffic detected with anionic and cationic ferritin. *J. Cell Biol*. **77**, R35-R42.

Fox, G. Q. (1996). A morphometric analysis of exocytosis in KCl-stimulated bovine chromaffin cells. *Cell Tissue Re*s. **284**, 303-316.

Glombik, M. M., Krömer, A., Salm, T., Huttner, W. B. and Gerdes, H.-H. The disulfide-bonded loop of chromogranin B mediates membrane binding and directs sorting from the *trans*-Golgi network to secretory granules. *EMBO J.* **18**, 1059-1070.

Gold, G., Gishizky, M. L. and Grodsky. G. M. (1982). Evidence that glucose 'marks' beta cells resulting in preferential release of newly synthesized insulin. *Science* **218**, 56-58.

Gosh, R. N., Mallet, W. G., Soe, T. T., McGraw, T. E. and Maxfield, F. R. (1998). An endocytosed TGN38 chimeric protein is delivered to the TGN after trafficking through the endocytic recycling compartment in CHO cells. *J. Cell Biol*. **142**, 923-936.

Gruenberg, J. and Maxfield, F. R. (1995). Membrane transport in the endocytic pathway. *Curr. Opin. Cell Biol* **7**, 552-563.

Henkel, A. W., Lubke, J. and Betz, W. J. (1996). FM1-43 dye ultrastructural localization and release from frog motor nerve terminals. *Proc. Natl. Acad. Sci. USA* **93**, 1918-1923.

Horn, M. and Banting, G. (1994). Okadaic acid treatment leads to a fragmentation of the *trans* Golgi network and an increase in expression of TGN38 at the cells surface. *Biochem. J.* **301**, 69-73.

Huang, A. Y., Castle, A. M., Hinton, B. T. and Castle, J. D. (2001). Resting (basal) secretion of proteins is provided by the minor regulated and constitutive-like pathways and not granule exocytosis in parotid acinar cells. *J. Biol. Chem*. **276**, 22296-22306.

Hurtley, S. M. (1993). Recycling of a secretory granule membrane protein after stimulated secretion. *J. Cell Sci.* **106**, 649-656.

Ingram, D., Keefe, P. D., Wooding, F. B. P. and Bicknell, R. J. (1988). Morphological characteristics of lactotrophs separated from the bovine pituitary by a rapid enrichment technique. *Cell Tissue Res.* **252**, 655-659.

Jain, R. K., Joyce, P. B. M. and Gorr, S.-U. (2000). Aggregation chaperones enhance aggregation and storage of secretory proteins in endocrine cells. *J. Biol. Chem*. **275**, 27032-27036.

Kilic, G., Angleson, J. K., Cochilla, A. J., Nussinovitch, I. and Betz, W. J. (2001). Sustained stimulation of exocytosis triggers continuous membrane retrieval in rat pituitary somatotrophs. *J. Physiol*. **522**, 771-783.

Klumperman, J., Kuliawat, R., Griffith, J. M., Geuze, H. J. and Arvan, P. (1998). Mannose 6-phosphate receptors are sorted from immature secretory granules via adaptor protein AP-1, clathrin, and syntaxin 6-positive vesicles. *J. Cell Biol*. **141**, 359-371.

Lamberts, S. W. and Macleod. R. M. (1990). Regulation of prolactin secretion at the level of the lactotroph. *Physiol. Rev.* **70**, 279-318.

Lampson, M. A., Schmoranzer, J., Zeigerer, A., Simon, S. M. and McGraw, T. E. (2001). Insulin-regulated release from the endosomal recycling compartment is regulated by budding of specialized vesicles. *Mol. Biol. Cell* **12**, 3489-3501.

Larsson, L.-I., Nielsen, J. H., Hutton, J. C. and Madsen. O. D. (1989). Pancreatic hormones are expressed on the surface of human and rat islet cells through exocytic sites. *Euro. J. Cell Biol*. **48**, 45-51.

Lee, A. K. and Tse, A. (2001). Endocytosis in identified corticotrophs. *J. Physiol*. **533**, 389-405.

Mallard, F., Tang, B. L., Galli, T., Tenza, D., Saint-Pol, A., Yue, X., Antony, C., Hong, W., Goud, B., and Johannes, L. (2002). Early/recycling endosome-to-tgn transport involves two snare complexes and a rab6 isoform. *J. Cell Biol*. **156**, 653-664.

Mallet, W. G. and Maxfield, F. R. (1999). Chimeric forms of furin and TGN38 are transported from the plasma membrane to the trans-Golgi network via distinct endosomal pathways. *J. Cell Biol.* **146**, 345-359.

Milgram, S. L., Mains, R. E. and Eipper. B. A. (1993). COOH-terminal signals mediate the trafficking of a peptide processing enzyme in endocrine cells. *J. Cell Biol*. **121**, 23-36.

Mills, I. G., Urbe, S. and Clague, M. J. (2001). Relationship between EEA1 binding partners and their role in endosome fusion. *J. Cell Sci*. **114**, 1959-1965.

Neher, E. (1998). Vesicle pools and Ca^{2+} microdomains: new tools for understanding their roles in neurotransmitter release. *Neuron* **20**, 389-399.

Olofsson, C. S., Gopel, S. O., Barg, S., Galvanovskis, J., Ma, X., Salehi, A., Rorsman, P. and Eliasson, L. (2002). Fast insulin secretion reflects exocytosis of docked granules in mouse pancreatic β-cells. *Pflugers Arch*. **444**, 43-51.

Orci, L., Ravazzola, M., Amherdt, M., Brown, D. and Perrelet, A. (1986). Transport of horseradish peroxidase from the cell surface to the Golgi in insulin-secreting cells: preferential labeling of cisternae located in an intermediate position in the stack. *EMBO J.* **5**, 2097-2101.

Patzak, A. and Winkler, H. (1986). Exocytic exposure and recycling of membrane antigens of chromaffin granules: Ultrastructural evaluation after immunolabeling. *J. Cell Biol*. **102**, 510-515.

Pender, N. and Burgoyne, R. D. (1992). Histamine stimulates exocytosis in a sub-population of bovine adrenal chromaffin cells. *Neurosci. Lett*. **144**, 207-210.

Perera, H. K. I., Clarke, M., Morris, N. J., Hong, W., Chamberlain, L. H. and Gould, G. W. (2003). Syntaxin 6 regulates Glut4 trafficking in 3T3-L1 adipocytes. *Mol. Biol. Cell* **14**, 2946-2958.

Pimplikar, S. W. and Huttner, W. B. (1992). Chromogranin B (Secretogranin I), a secretory protein of the regulated pathway, is also present in a tightly membrane-associated form in PC12 cells. *J. Biol. Chem.* **267**, 4110-4118.

Plattner, H., Artalejo, A. R. and Neher. E. (1997). Ultrastructural organization of bovine chromaffin cell cortex- analysis by cryofixation and morphometry of aspects pertinent to exocytosis. *J. Cell Biol.* **139**, 1709-1717.

Rhodes, C. J. and Halban. P. A. (1987). Newly synthesized proinsulin-insulin and stored insulin are released from pancreatic β-cells predominantly via a regulated, rather than constitutive, pathway. *J. Cell Biol.* **105**, 145-153.

Rosenzweig, L. J. and Farquhar, M. G. (1980). Sites of sulfate incorporation into mammatotrophs and somatotrophs of the rat pituitary as determined by quantitative electron microscopic autoradiography. *Endocrinology* **107**, 422-431.

Shewan, A. M., van Dam, E. M., Martin, S., Bor Luen, T., Hong, W., Bryant, N. J. and James, D. E. (2003). GLUT4 recycles via a *trans* Golgi Network (TGN) subdomain enriched in syntaxins 6 and 16 but not TGN38: Involvement of an acidic targeting motif. *Mol. Biol. Cell* **14**, 973-986.

Solimena, M., Dirkx, R., Hermel, J.-M., Pleasic-Williams, S., Shapiro, J. A., Caron, L. and Rabin, D. U. (1996). ICA512, an autoantigen of type I diabetes, is an intrinsic membrane protein of neurosecretory granules. *EMBO J*. **15**, 2102-2114.

Slot, J. W., Garruti, G., Martin, S., Oorschot, V., Posthuma, G., Kraegen, E. W., Laybutt, R., Thibault, G. and James, D. E. (1997). Glucose transporter (GLUT-4) is targeted to secretory granules in rat atrial cardiomyocytes. *J. Cell Biol*. **137**, 1243-1254.

Smith, C. and Neher, E. (1997). Multiple forms of endocytosis in bovine adrenal chromaffin cells. *J. Cell Biol*. **139**, 885-894.

Straley, K. S. and Green, S. A. (2000). Rapid transport of internalized P-selectin to the late endosomes and the TGN: roles in regulating cell surface expression and recycling to secretory granules. *J. Cell Biol.* **151**, 107-116.

St. John, P. A., Dufy-Barbe, L. and Barker, J. L. (1986). Anti-prolactin cell surface immunoreactivity identifies subpopulations of lactotrophs from the rat anterior pituitary. *Endocrinology* **119**, 2783-2795.

Thiele, C., Gerdes, H.-H. and Huttner, W. B. (1997). Protein secretion: puzzling receptors. *Curr. Biol*. **7**, R496-R500.

Thomas, P., Lee, A. K., Wong, J. G. and Almers, W. (1994). A triggered mechanism retrieves membrane in seconds after Ca^{2+}-stimulated exocytosis in single pituitary cells. *J. Cell Biol*. **124**, 667-675.

Walker, A. M, and Farquhar, M. G. (1980). Preferential release of newly synthesized prolactin granules is the result of functional heterogeneity among mammatotrophs. *Endocrinology* **107**, 1095-1104.

Wasmeier, C. and Hutton, J. C. (1996). Molecular cloning of phogrin, a protein-tyrosine phosphatase homolog localized to insulin secretory granule membrane. *J. Biol. Chem*. **271**, 18161-18170.

Wendler, F., Page, L., Urbé, S. and Tooze, S. A. (2001). Homotypic fusion of immature secretory granules during maturation requires syntaxin 6. *Mol. Biol. Cell* **12**, 1699-1709.

Wick, P. F., Trenkle, J. M. and Holz., R. W. (1997). Punctate appearance of dopamine-beta-hydroxylase in the chromaffin cell surface reflects the fusion of individual chromaffin granules upon exocytosis. *Neuroscience* **80**, 847-860.

Zhou, Z., Misler, S. and Chow, R. H. (1996). Rapid fluctuations in transmitter release from single vesicles in bovine adrenal chomaffin cells. *Biophys. J*. **70**, 1543-1552.

Zorec, R., Sikdar, S. K. and Mason, W. T. (1991). Increased cytosolic calcium stimulates exocytosis in bovine lactotrophs. *J. Gen. Physiol*. **97**, 473-497.