Differential Regulation of Granule-to-Granule and Granule-to-Plasma Membrane Fusion during Secretion from Rat Pituitary Lactotrophs

Amanda J. Cochilla,* Joseph K. Angleson,‡ and William J. Betz*

*Department of Physiology and Biophysics, University of Colorado Medical School, Denver, Colorado 80220; and ‡Department of Biological Sciences, University of Denver, Denver, Colorado 80208

Abstract. We used fluorescence imaging of individual exocytic events together with electron microscopy to study the regulation of dense core granule-to-plasma membrane fusion and granule-to-granule fusion events that occur during secretion from rat pituitary lactotrophs. Stimulating secretion with elevated extracellular potassium, with the calcium ionophore ionomycin, or with thyrotropin releasing hormone or vasoactive intestinal polypeptide resulted in abundant exocytic structures. Approximately 67% of these structures consisted of multiple granules fused together sharing a single exocytic opening with the plasma membrane, i.e., compound exocytosis. For all of these stimulation conditions there appeared to be a finite number of plasma membrane fusion sites, ~11 sites around each cellular equator. However, a granule could fuse directly with another granule that had already fused with the plasma membrane even before all plasma membrane sites were occupied. Granule-to-plasma membrane and granule-to-granule fusion events were subject to different regulations. Forskolin, which can elevate cAMP, increased the number of granule-to-granule fusion events without altering the number of granule-to-plasma membrane fusion events. In contrast, the phorbol ester PMA, which activates protein kinase C increased both granule-to-granule and granule-to-plasma membrane fusion events. These results provide a cellular mechanism that can account for the previously demonstrated potentiation of secretion from lactotrophs by cAMP- and PKC-dependent pathways.

Key words: FM1-43 • fluorescence • homotypic fusion • compound exocytosis • phorbol ester

Introduction

Both exocytic release of neurotransmitters from small clear vesicles and secretion of peptide hormones and neuropeptides from dense core granules are Ca²⁺-dependent processes that are subject to potentiation and inhibition from various cellular signaling pathways. Modulation of rapid neurotransmitter release from vesicles in neurons is transduced by increasing residual calcium within the terminal; the excess Ca²⁺ facilitates vesicle fusion with the plasma membrane (for review see Zucker, 1999). In the case of slower secretion from dense core granules, the size and rate of refilling of functionally releasable pools of granules is a target of second messenger modulation, which can alter the time course and the amount of exocytosis (Gillis et al., 1996; Smith et al., 1998).

Biochemical assays of catecholamine release from permeabilized chromaffin cells indicate that Ca²⁺-dependent secretion can be enhanced by protein kinase C (PKC; Pocotte et al., 1985). These findings were expanded by patch-clamp capacitance recordings that provide high resolution measurements of exocytosis and reveal the existence of different functional pools of granules (Neher, 1998). Studies with chromaffin cells indicated that both the size of the readily releasable pool (von Rueden and Neher, 1993; Gillis et al., 1996) and the rate of refilling of this pool (Smith et al., 1998) are subject to modulation by residual internal Ca²⁺ and by PKC. Refilling the releasable pool in pancreatic β-cells is enhanced by protein kinase A, Ca²⁺, and GTP (A mmala et al., 1993; Proks et al., 1996; Renstrom et al., 1997). Capacitance recordings also indicate that the size of exocytic events in melanotrophs is increased by cAMP (Sikdar et al., 1998) and that the amplitude of the exocytic response in lactotrophs is increased by cAMP during Ca²⁺-dialysis (Sikdar et al., 1990).

Abbreviations used in this paper: IBMX, 3-isobutyl-1-methylxanthine; PKC, protein kinase C; PRL, prolactin; TRH, thyrotropin releasing hormone; VIP, vasoactive intestinal polypeptide.

© The Rockefeller University Press, 0021-9525/2000/08/839/10 $5.00
The Journal of Cell Biology, Volume 150, Number 4, August 21, 2000 839–848
http://www.jcb.org
Prolactin (PRL) secretion from pituitary lactotrophs is a Ca\textsuperscript{2+}-dependent process that is subject to regulation from inhibitory and stimulatory signaling pathways arising from both hormonal and neuronal inputs (for review see Lamberts and Macleod, 1990). Dopaminergic input from the hypothalamus plays a major inhibitory role in PRL secretion. Dopamine has a number of effects on isolated lactotrophs including the following: G-protein-mediated reduction in cAMP (DeCamilli et al., 1979); inhibition of phospholipase C (Journot et al., 1987); activation of K\textsuperscript{+-} channels (Einhorn et al., 1991); assembly and disassembly of the cortical actin network (Carbajal and Vitale, 1997); and release of the dense cores of secretory granules (Anglestone et al., 1999). Hormones and signaling pathways that have a stimulatory effect on PRL secretion include TRH, which activates PLC (Martin, 1983); VIP, which activates adenylyl cyclase (Lamberts and Macleod, 1990); and the PRL-releasing peptide, which elevates arachidonic acid (H Inuma et al., 1998).

While numerous studies have begun to elucidate the complex signaling pathways initiated by the neurotransmitters and hormones that modulate prolactin secretion, little is known about how these pathways ultimately affect the exocytic release of PRL. In the present study, we combined electron microscopy with an optical assay that permits detection of exocytic events in lactotrophs isolated from the rat anterior pituitary (Anglestone et al., 1999). We found that various stimuli including thyrotropin releasing hormone (TRH) and vasoactive intestinal polypeptide (VIP) result in abundant compound exocytosis. Compound exocytosis occurs when multiple secretory granules fuse together via homotypic fusion and release their contents into the extracellular space through a single plasma membrane fusion pore. Pharmacological manipulations revealed that the relative amount and magnitude of compound exocytosis as well as the number of plasma membrane fusion sites are regulated independently. These regulations represent a point at which exocytic secretion can be potentiated by different second messenger pathways.

Materials and Methods

Cell Preparation

Anterior pituitary cells were obtained from male Sprague-Dawley rats (250–350 g) by enzymatic dispersion. Cells from the lactotroph-enriched band (Anglestone et al., 1999) were cultured for 2-4 d and transferred to polylysine-coated coverslips 10 min before experiments. A bout half of the cells in the lactotroph-enriched band displayed exocytotic spots in FM 1-43 when stimulated with high [K\textsuperscript{+}]. This fraction agreed with that of permeabilized cells staining positive with anti-PRL antibodies (Anglestone et al., 1999).

External solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 8 mM CaCl\textsubscript{2}, 10 mM d-glucose, and 10 mM Hepes, pH 7.2. High [K\textsuperscript{+}] solution contained either 100 mM KCl and 45 mM NaCl, 50 mM KCl and 90 mM NaCl, or 25 mM KCl and 120 mM NaCl. Drug treatments were as follows: PMA (400 nM for 10 min before stimulation and during KCl stimulation); forskolin (10 \mu M for 10 min before stimulation and during KCl stimulation); bombesin (2 \mu M as stimulation); TRH (200 nM as stimulation); and VIP (500 nM as stimulation). Compounds were purchased from RBI. Experiments were done at 22°C.

Electron Microscopy

Cells were cultured in Permanox culture chambers (Nunc) for 2 d. The medium was exchanged with high [K\textsuperscript{+}] for 2 min before a wash with 100 mM of ice-cold phosphate buffer. The first fixation comprised incubating the tissue in 2% paraformaldehyde/2% glutaraldehyde in 100 mM phosphate buffer for 1 h at 4°C. The tissue was washed and a second fixation was performed in 1% osmium in 100 mM phosphate buffer at 4°C for 1 h before being processed for electron microscopy (Henkel et al., 1996). The prevalent exocytic structures observed in stimulated lactotrophs are unlikely to be fixation artifacts, as identical treatment of bovine chromaffin cells did not produce such structures (data not shown). For ruthenium red experiments, 1 mM cacydolate buffer was used instead of phosphate buffer and 1% ruthenium red was added to both fixation solutions.

Imaging

Fluorescence imaging experiments were performed with a system comprising an Olympus IX 70 inverted microscope, a Photometrics Quantix camera with a K odak K A F1400 chip (6.7 × 6.7-µm physical pixels, giving 67 nm per image pixel with a 100× oil immersion objective [1:4 NA]) and a Silicon Graphics O2 computer with DeltaVision deconvolution software (Applied Precision). Fluorescence images of FM 1-43-stained cells were obtained with 490/20-nm excitation and 617/73-nm emission filters. Exposure lasted 0.4 s with 10% transmittance neutral density filters. Images were acquired at ~1 Hz. The chamber was perfused constantly by gravity feed; solution changes required ~10 s. Quantitative analyses were performed on raw images; deconvolution was used only to enhance images for presentation. Fluorescent images were analyzed using NIH Image and Igor Pro (WaveM etronis) on a Macintosh G3 computer. Fluorescence of individual spots was measured as the mean pixel value inside a circle centered around the spot (circle diam ~ 7 pixels ~470 nm). This analysis may underestimate the size of the largest exocytotic structures. Values were normalized to the mean surface membrane fluorescence inside the circle at the same location on the cell membrane before stimulation. A brightness of zero means the spot was the same brightness as the plasma membrane, and 1.0 means it was twice as bright. To measure the brightness of only compound spots in a given condition, the brightness of all spots whose amplitude was 1.0 times brighter than the membrane was averaged. The value 1.0 was chosen based on the width of the first peak in a histogram of spot amplitudes fitted with a Poisson distribution (see Fig. 2; A nglestone et al., 1999). However, the same significance levels were achieved if 0.8 or 1.2 was used as the cutoff value. Significance levels for the different stimulations indicated in the text and in Table I were compared with 100 mM KCl stimulation. Data were acquired from images of the cell equator. All data are reported as mean ± SEM.

The curves in Fig. 4 b were drawn as follows. The occupancy of surface membrane exocytic sites proceeds from empty to single to multiple as granules fuse. This is analogous to a consecutive chemical reaction of the form A → B → C. A assuming equal rate constants, the fractions of empty and singly occupied surface membrane fusion sites are given by $e^{-g x}$ and $(g/n) e^{-g x}$, respectively, where n is the number of exocytosed granules (see Fig. 4 b, x-axis), and m is the number of surface membrane fusion sites (assumed to be 11). The fraction of membrane sites with multiple (compound) events is simply $1 - e^{-g x} - (g/n) e^{-g x}$. The dotted lines in Fig. 4 b were drawn according to an iterative model in which probabilities of fusion to surface membrane and to already fused granules could be adjusted independently. To fit the observed data, the probability of a site progressing from single to multiple was slightly enhanced at early times, and significantly reduced at later times. This was accomplished by raising the granule-to-granule fusion probability to the power to 0.3 + 0.07g.

Results

Compound Exocytosis Occurs in Lactotrophs

Previous studies (Fujita et al., 1983; Anglestone et al., 1999) have shown ultrastructural evidence that compound exocytosis occurs in stimulated pituitary cells. Electron micrographs show multiple granules connected by a single membrane that is continuous with the plasma membrane (Fig. 1 a). We frequently observed compound exocytotic structures with up to four or five granule cores clearly contained within a single membrane that was connected to the plasma membrane (Fig. 1 a, left and middle). Yet, we also
observed structures that contained multiple granule cores with no clear evidence of a connection to the plasma membrane in single sections (Fig. 1a, lower right). This latter structure could reflect either intracellular fusion between granules without fusion with the plasma membrane, or it could reflect an exocytic structure where the connection to the plasma membrane is out of the plane of the 80-nm section. To determine whether granules in electron micrographs were contiguous with the extracellular space, the dye ruthenium red was applied during fixation. This dye becomes fixed to the extracellular surface of the cell and is electron dense so that any portion of the cellular membrane exposed to the extracellular surface during fixation contains an electron-dense coating visible in electron micrographs. Both of these micrographs show two cells directly opposed. The membranes delimiting the two cells are stained with ruthenium red (left, membranes are on the left side; right, membranes are at the top). Many granules that are located away from the surface membrane were stained with ruthenium red (arrowheads), indicating that they were contiguous with the extracellular surface at fixation. Presumably, the connection to the surface was via chains of connected granules that had fused together and were fused to the plasma membrane.

Compound Exocytosis Can Be Studied in Living Cells Using a Fluorescence Assay

Previously, we (Angleson et al., 1999) established that the contents of lactotroph secretory granules stain with the fluorescent dye FM1-43. During bath stimulation with high K⁺ in the presence of FM1-43, granules fuse with the plasma membrane and release prolactin (PRL); however, some PRL, as well as FM1-43, are retained in the dense cores that are exposed to the extracellular space allowing visualization of individual exocytic sites as bright fluorescent spots. The spots appear quickly, and sometimes show abrupt doublings or triplings in brightness during the time course of recording (Fig. 2b and c, closed circles). Histograms were generated from the final brightness of these spots that showed clear evenly spaced peaks, indicating that brightness varies in a quantal nature (Fig. 2d is reproduced from Figure 2b in Angleson et al., 1999). Considering this fluorescence data, together with ultrastructural evidence of compound exocytosis in these cells, we conclude that the brightness of a given fluorescent spot correlates with the number of granule cores undergoing exocytosis at that plasma membrane fusion site. By using this FM1-43 fluorescence assay, we can study compound exocytosis in living cells.

Compound Exocytosis Occurs in Response to Physiological Stimuli

Using the fluorescence assay described above to study compound exocytosis in lactotrophs, we found that lactotrophs undergo compound exocytosis in response to depolarization and in response to the physiological stimula-
tors TRH and VIP. For the following experiments, we stimulated lactotrophs in the presence of FM1-43 and measured all of the following: the number of fluorescent spots per cellular equator, which corresponds to the number of plasma membrane fusion sites; the brightness of each spot, which corresponds to the number of granules undergoing exocytosis at that site; and the percentage of spots whose brightness exceeded that of a single granule (estimated from the Poisson fit in Fig. 2 d; see Materials and Methods). Bath stimulation with high K\(^+\) (100 mM) in the presence of FM 1-43 evoked exocytosis from 10.7 ± 6.1.8 sites per cellular equator. Approximately 66.9 ± 6.10.7% of those events were compound, and the compound spots had a brightness that was 5.5 ± 6.0.7 times brighter than the membrane (Table I). Stimulating lactotrophs with physiological concentrations of TRH (200 nM) or VIP (500 nM) evoked compound exocytotic events in the same manner as depolarizing the cells with KCl (Fig. 3 and Table I). The number of exocytic fusion sites evoked by either TRH or VIP was not significantly different from the number evoked by 100 mM KCl. TRH stimulation produced 13.0 ± 4.0 spots/equator (P = 0.55; Fig. 3 a) and VIP stimulation produced 6.2 ± 1.2 spots/equator (P = 0.05; Fig. 3 b). Neither the percentage of compound events evoked by TRH nor VIP was significantly different from that evoked by KCl. 52.1 ± 16.7% of the exocytic events evoked by bath stimulation with TRH were compound (P = 0.18), as were 76.7 ± 8.7% of those evoked with VIP (P = 0.21, Table I). Finally, neither TRH- nor VIP-evoked spots were significantly dimmer or brighter than KCl-evoked spots (Fig. 3 c and Table I). The average brightness of spots evoked by TRH was 2.8 ± 0.2 times greater than the membrane brightness (P = 0.05, Table I), and the average brightness of spots evoked with VIP was 3.2 ± 0.2 times that of the membrane brightness (P = 0.12, Table I). These results demonstrate that both VIP and TRH evoke compound exocytosis from lactotrophs, and that this exocytosis is not significantly different from that evoked by KCl in the number of events per cell, the number of granules exocytosed at each site, or the percentage of exocytic events containing multiple granules.

**Compound Exocytosis Can Occur before all Plasma Membrane Fusion Sites Are Occupied**

Different stimulation strengths affected the number of
Cells stimulated with 25 mM KCl exocytosed about 1/4 as many granules as cells stimulated with 100 mM KCl, with fewer membrane fusion sites and less compound exocytosis. To explore quantitatively the relationship between granule-to-plasma membrane and granule-to-granule fusion, we fitted these data with a simple model (Fig. 4 b). The simplest model, based on an assumption of equal probability for all fusion events (solid lines) predicted the disappearance of empty membrane fusion sites (circles) well, but poorly estimated the fraction of sites with compound events (squares) and the fraction with single fusion events (triangles). This result suggests that some membrane fusion sites are less likely to proceed to compound events than others. The data could be fitted better (dotted lines) by a model in which the probability of granule-to-granule fusion was progressively reduced (see Materials and Methods).

### Granules Can Fuse to Prefused Granules

To determine whether granules could fuse to granules already fused with the plasma membrane, we performed sequential stimulations at increasing KCl concentrations. 25 mM KCl was washed into the preparation and images were acquired. Next, 100 mM KCl was washed into the preparation and images were acquired from the same cells (Fig. 5 a). During the second stimulation, the average fluorescence of spots that had appeared during the first stimulation increased from 1.94 ± 0.25 to 8.38 ± 1.2 times brighter than the membrane (Fig. 5 b). The percentage of those spots that was compound increased from 42.8 ± 11.1% to 98.9 ± 1.0%. This result indicates that spots that appeared in response to the first stimulation grew in size during the second stimulation. Occasionally, this type of sequential increase in brightness was observed during a single stimulation (Fig. 2, b and c). Taken together, these results suggest that compound exocytosis clearly can occur via fusion of one granule to a granule that is already fused with the plasma membrane. While we have no evidence for granule-to-granule fusion before fusion with the plasma membrane, we cannot, at present, rule out that this process also occurs.

### The Number of Plasma Membrane Fusion Sites and the Size of Compound Events Are Differentially Regulated

Manipulating various intracellular signaling pathways including PKC and cAMP affect the size and number of compound exocytic events. Both PMA, which activates PKC, and forskolin, which can elevate cAMP, increased the number of granules associated with each exocytic event, whereas only PMA altered the number of plasma membrane fusion sites. Forskolin did not affect either the number of plasma membrane fusion sites or the percentage of events that was compound during high K+ stimulation. Cells were incubated in forskolin and IBMX for 10 min before stimulation with 100 mM KCl, forskolin, and IBMX. This stimulation activated 12.5 ± 1.7 plasma membrane fusion sites per cellular equator (P = 0.13, Table I) and 87.4 ± 5.7% of events were compound (P = 0.46, Table I). However, the amplitude of compound events was increased by forskolin to nearly double that recorded in KCl alone (Table I, 10.1 ± 1.0 times membrane fluores-

Table I. Summary of Experimental Results

| Stimulation          | No. of spots per equator | Percentage of compound events | Compound event amplitude |
|----------------------|--------------------------|-------------------------------|--------------------------|
| 100 mM K+ (12/145)   | 10.7 ± 1.8               | 66.9 ± 1.07                   | 5.5 ± 0.7                |
| VIP (25/180)         | 6.2 ± 1.2                | 76.7 ± 8.7                    | 3.2 ± 0.2                |
| TRH (677)            | 13.0 ± 4.0               | 52.1 ± 16.7                   | 2.8 ± 0.2                |
| 25 mM K+ (21/192)    | 4.7 ± 0.8                | 42.8 ± 16.7                   | 3.4 ± 0.4                |
| Ionomycin (36/355)   | 12.1 ± 1.8               | 87.4 ± 3.9                    | 7.5 ± 0.6                |
| FSK and 100 mM K+ (19/237) | 12.5 ± 1.7          | 87.4 ± 5.7                    | 10.1 ± 1.0               |
| PMA and 100 mM K+ (17/287) | 18.1 ± 1.8           | 99.2 ± 0.7                    | 10.1 ± 0.7               |

*Data are shown for (left to right): the number of spots per cell measured at the cell’s equator, the percentage of events whose brightness indicated that they were compound events (see Materials and Methods), and the brightness of the compound events. The conditions summarized are (top to bottom): control stimulation with 100 mM KCl (in bold), VIP stimulation, TRH stimulation, weak stimulation with 25 mM KCl, ionomycin stimulation, stimulation with 100 mM KCl in the presence of forskolin and IBMX, and stimulation with 100 mM KCl in the presence of PMA. The number of cells and the number of spots recorded under each condition are indicated in parentheses under the stimulation paradigm. Asterisks indicate values that are significantly different from the standard stimulation with 100 mM KCl. Significance levels are as follows. Spots per equator between control and 25 mM KCl, P = 0.002; and between control and PMA, P = 0.009. Percent compound events between control and 25 mM KCl, P = 0.003; and between control and PMA, P = 0.007. Compound event amplitude between control and 25 mM KCl, P = 0.02; between control and forskolin, P = 0.01; and between control and PMA, P = 0.002.
cence, \( P < 0.05 \); Fig. 6). Incubating cells with forskolin alone caused a small number of exocytic events.

In contrast to the effect of forskolin on KCl-induced exocytosis, stimulation with KCl and PMA increased the number of plasma membrane fusion sites, the percentage of events that was compound, and the size of individual compound events. Cells were incubated in PMA for 10 min before stimulation with 100 mM KCl and PMA. There were many more compound events under these conditions than during 100 mM KCl stimulation alone; 99.2 \( \pm \) 0.7% of events recorded in PMA were compound (\( P < 0.01 \); Table I). The size of the compound events was enhanced by PMA as well. When stimulated in the presence of PMA, compound events’ fluorescence was 10.1 \( \pm \) 0.7 times greater than the baseline fluorescence (\( P < 0.002 \); Table I). PMA was the only compound tested that had an effect on the number of plasma membrane fusion sites. The number of fusion sites activated by PMA and KCl was almost double that activated by KCl alone (Table I, 18.1 \( \pm \) 1.8 spots/equator, \( P < 0.01 \)).

The calcium ionophore ionomycin did not mimic the potentiating effects of PMA or forskolin, indicating that these compounds did not simply allow a more sustained increase in intracellular calcium. When compared with stimulation with 100 mM KCl, stimulation of lactotrophs with ionomycin did not induce exocytosis through a significantly different number of plasma membrane fusion sites (Table I, 12.1 \( \pm \) 1.78 spots/equator, \( P = 0.6 \)). Approximately 87.4 \( \pm \) 3.9% of the events evoked by ionomycin was compound (\( P = 0.05 \); Table I). The average size of the compound events was not different from the average size of events evoked by KCl stimulation. The fluorescence was 7.47 \( \pm \) 0.6 times greater than the membrane (\( P = 0.08 \), Table I). From these data, we can conclude that compound exocytosis evoked by stimulation with ionomycin was not significantly different than that evoked by stimulation with KCl.

Discussion

Compound Exocytosis in Neuroendocrine Cells

Cellular secretions are typically thought to occur via exocytic fusion of individual secretory vesicles or granules with the plasma membrane. Compound exocytosis represents a less well characterized mode of secretion, where a
A single granule fuses with the plasma membrane and has additional granules fused to it. Compound exocytosis results in a chain or cluster of granules with a single plasma membrane fusion pore connecting the lumen of several granules to the extracellular space. Compound exocytosis is a relatively common mode of exocytosis in various lymphoid cells as revealed by ultrastructural studies of eosinophils and mast cells (McLaren et al., 1977; Lawson et al., 1978; Henderson et al., 1983), electrophysiological studies of eosinophils (Scepek and Lindau, 1993), and biochemical studies of mast cells (Gu et al., 1998). Compound exocytosis in these circulating lymphoid cells allows massive cytotoxic secretions to be precisely directed toward a target cell (Lawson et al., 1978).

Exocytic structures have been observed in many secretory cells (Palade, 1975) including pituitary lactotrophs (Farquhar, 1977). Compound exocytosis also has been observed in electron micrographs of several peptide-secreting cells, including pancreatic β-cells (Orci and Malaissè, 1980), acinar cells (Senda et al., 1989), pituitary somatotrophs (Nakagawa et al., 1995), lactotrophs, and possibly other anterior pituitary cells (Fujita et al., 1983). A recent study using patch-clamp capacitance recordings from melanoctrophs indicates that the size of individual exocytic events is increased by cAMP (Sikdar et al., 1998). However, this result could reflect either a cAMP-dependent increase in the occurrence of compound exocytosis or preferential exocytosis of larger granules in the presence of cAMP (as the authors suggest). While evidence for the existence of compound exocytosis in neuroendocrine cells is clear, a role for this mode of secretion in these cells has not been established.

In the present study, we found that a variety of stimulations including the physiological secretagogues TRH and VIP cause abundant compound exocytosis in lactotrophs. In addition, we found that the relative amount of compound exocytosis can be modulated by experimental manipulation of cellular signaling pathways known to alter the amount of PRL secretion from lactotrophs. These data

![Figure 4](image1.png)  
**Figure 4.** A simple model describes the relative numbers of single and compound fusion events. (a) Histogram of spot brightness after stimulation with 25 mM KCl. The smooth line represents the scaled unitary fluorescence values from Fig. 2 d. Approximately 43% of events are brighter than the curve representing single granule events, demonstrating that 25 mM KCl induced compound exocytosis. (b) The observed frequency of single (triangles) and compound (squares) fusion events and empty plasma membrane fusion sites (circles) for stimulation in 25 mM (open symbols) and 100 mM (closed symbols) KCl. The model assumes a total of 11 membrane fusion sites (equatorial optical section). Solid lines show predicted distributions assuming equal probabilities for all fusion events. To fit the observed data, it was necessary to progressively reduce the probability that a single site would progress to a compound site (dotted lines; see Materials and Methods).

![Figure 5](image2.png)  
**Figure 5.** Sequential stimulation indicates that spots can increase in brightness as granules fuse with prefused granules. (a) Sequential stimulations with low (25 mM) then high (100 mM) K⁺ were used to track the fate of spots. Cells were bathed in solution containing 4 μM FM 1-43 and stimulated with 25 mM KCl followed by 100 mM KCl (~5 min between stimulations). Low K⁺ caused fluorescent spots to appear on the surface of the cells (left). During the subsequent high K⁺ stimulation more spots appeared and some pre-existing spots increased in brightness (right). (b) This graph shows the brightness of 92 individual spots (open circles) during an initial 25-mM KCl stimulation and a subsequent 100-mM KCl stimulation. Data from individual spots were first normalized to membrane brightness, and all data were normalized to the initial brightness in 25 mM KCl to clearly show the increase in brightness that occurred during the subsequent stimulation with 100 mM KCl. A average ± 5D is shown as a filled diamond.
provide evidence for a likely physiological role for compound exocytosis in a neuroendocrine cell.

**Distinct Cellular Mechanisms for Potentiation of Exocytosis in Lactotrophs**

Biochemical secretion studies using various physiological or pharmacological manipulations reveal that a number of intracellular signaling pathways, including PKC and cAMP, increase PRL secretion from lactotrophs (Lambeerts and Macleod, 1990). We used the previously developed optical assay of exocytosis developed previously (Angleson et al., 1999) to test whether forskolin or PMA can alter either the relative amount of compound exocytosis or the number of plasma membrane fusion sites in lactotrophs. The effects of forskolin are likely due to activation of adenylyl cyclase and elevation of cAMP, whereas the effect of PMA could be due to the activation of PKC or enhancement of the phorbol ester binding protein Munc 13-1, which is known to enhance neurotransmitter release (Betz et al., 1998). We found that the number of granules undergoing exocytosis at each fusion site during elevated [K⁺]o stimulation (i.e., compound exocytosis) was increased significantly in both forskolin- and PMA-treated cells when compared with untreated control cells (Fig. 7). Similarly, we recently observed that the apparent frequency of compound exocytosis is reduced by treatment with dopamine analogues that are known to reduce cAMP (Angleson et al., 1999). These findings have two important implications: first, the molecular interactions responsible for the granule-to-granule fusion that occurs during compound exocytosis can be regulated; and second, regulation of compound exocytosis may serve to modulate the amount of cellular secretions from these cells.

While both PMA and forskolin increase homotypic fusion and allow more granules to undergo exocytosis at each plasma membrane fusion site, PMA has a second potentiating effect on exocytosis. Stimulation by elevated [K⁺]o caused an increase in the number of FM 1-43-stained exocytic structures in PMA, but not forskolin-treated cells, when compared with untreated control cells (Fig. 7). Thus, PMA treatment has two potentiating effects on exocytosis: PMA apparently activates otherwise inactive plasma membrane fusion sites, allowing more heterotypic fusion of granules to the plasma membrane; and, PMA also allows an increase in the amount of homotypic granule-to-granule fusion that occurs during compound exocytosis. Forskolin only leads to an increase in homotypic granule-to-granule fusion. Based on these findings, it appears that these distinct heterotypic and homotypic fusion reactions can be subject to different regulations. The fact that prolonged stimulation by ionomycin did not cause either an increase in compound exocytosis or the number of FM 1-43-stained structures when compared with elevated [K⁺]o-stimulated control cells suggests that neither forskolin nor PMA exert their potentiating effects simply by allowing for more sustained elevations of [Ca²⁺].

Based on the studies presented here, it appears that rat lactotrophs possess at least two distinct cellular mechanisms for potentiation of exocytosis: an increase in plasma membrane fusion sites and an increase in the occurrence of compound exocytosis. Compound exocytosis has been
observed in other neuroendocrine cells (see above), and several reagents, including PMA and forskolin, potentiate secretion in these cells. For example, compound exocytosis has been observed in pancreatic β-cells (Orci and M állasse, 1980), and cAMP potentiates exocytosis in these cells (A mmala et al., 1993). Potentiation of secretion is often attributed to an increase in the size of the readily releasable pool of granules (Néher, 1998) that could arise from an increase in granule mobility, an increase in plasma membrane fusion sites, an increase in the occurrence of compound exocytosis, or any combination of these processes. Since secretion studies measuring either released secretory products or changes in plasma membrane capacitance cannot readily distinguish between these mechanisms, the relative contribution of each mechanism in various cell types remains unresolved. The present study indicates that compound exocytosis is an abundant mechanism for secretion from lactotrophs, and that the homotypic granule-to-granule fusion employed in compound exocytosis can be increased by reagents known to potentiate secretion from these cells. Granule-to-granule fusion appears to be regulated by different mechanisms than heterotypic granule-to-plasma membrane fusion. This result is similar to observations in lymphoid cells (Scepek and Lindau, 1993; Guo et al., 1998). The findings in the present study reveal previously unrecognized mechanisms for modulating cellular secretion from neuroendocrine cells as well as raise intriguing questions regarding the molecules involved in the different types of fusion and their apparent distinct regulation.

We would like to thank Steve Fadul for invaluable assistance in many aspects of this project and Dot Dill (both from University of Colorado Health Sciences Center, Denver, CO) for expert assistance in electron microscopy. This work was funded by grants from the National Institutes of Health to A. J. Cochilla, J. K. A ngleson, and W. J. Betz.

Submitted: 5 May 2000
Revised: 19 June 2000
A ccepted: 21 June 2000

References

A mmala, C., F. M. A shcroft, and P. R orsman. 1993. Calcium-independent potentiation of insulin release by cyclic AMP in single beta-cells. Nature. 363: 356–358.
A ngleson, J. K., A. J. Cochilla, G. K ilic, I. Nussinovitch, and W. J. Betz. 1999. Regulation of dense core release from neuroendocrine cells revealed by imaging single exocytic events. Nat. Neurosci. 2:440–446.
B etz, A., U. A. shery, M. R. ckmann, I. A ugustin, E. Néher, T. C. Sudhoff, J. Ret etig, and N. B rose. 1998. Munc13-1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release. Neuron. 21:123–136.
C arbaajal, M. E., and M. L. V itale. 1997. The cortical actin cytoskeleton of lactotropes as an intracellular target for the control of prolactin secretion. Endocrinology. 138:5374–5384.
D e Camilli, P., D. M aconchi, and A. Soada. 1979. Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenomas. Nature. 278:252–254.
E inhorn, L. C., K. A. Gregerson, and G. S. Oxford. 1991. D 2 dopamine receptor activation of potassium channels in identified rat lactotrophs: whole-cell and single-channel recording. J. Neurosci. 11:3727–3737.
F arqahar, M. G., 1977. Secretion and cinchophagy in prolactin cells. Adv. Exp. Med. Biol. 80:37–94.
Fujita, H., H. K urihara, and J. Miyagawa. 1983. Ultrastructural aspects of the role of calcium ionophore A 23187 on incubated anterior pituitary cells of rats. Cell Tissue Res. 229:129–136.
G illis, K. D., R. M osner, and E. Néher. 1996. Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules. Neuron. 16:1209–1220.
G uo, Z., C. Turner, and D. Castle. 1996. Relocation of the t-SNARE SNA P-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. Cell. 94:537–548.
H enderson, W. R., E. Y. Chi., A. Jorg, and S. J. Klebanoff. 1983. Horse eosino phil degranulation induced by the ionophore A 23187. Ultrastructure and role of phospholipase A 2. Am. J. Pathol. 111:341–349.
H enkel, A. W., J. Lubke, and W. J. Betz. 1996. FM 1-43 dye ultrastructural localization in and release from frog motor nerve terminals. Proc. Nat. Acad. Sci. USA. 93:1918–1923.
H enley, J. R., E. W. K rueger, B. J. Osowal, and M. A. M c liven. 1998. D ynamin mediated internalization of caveolae. J. Cell Biol. 141:85–99.
H inuma, S., Y. H abata, R. Fujii, Y. K awamata, M. H osoya, S. Fukuusi, C. K i tadai, Y. Masuo, T. A sano, H. M atsumoto, et al. 1998. A prolactin-releasing peptide in the brain. Nature. 393:272–276.
J ollie, W. P., and T. J. Trich. 1971. R uttenham labeling of micropinocytotic activity in the rat visceral yolk-sac placenta. J. Ultrastruct. Res. 35:541–553.
J ournot, L. V., H omburger, C. Pantaloni, M. P riam, J. Bockaert, and A. E njal bert. 1987. A n id est activating protein-sensitive G protein is involved in dopamine inhibition of angiotensin and thyrotropin-releasing hormone-stimulated inositol phosphate production in anterior pituitary cells. J. Biol. Chem. 262:15106–15110.
L ambert s, S. W., and R. M. M acleod. 1990. Regulation of prolactin secretion at the level of the lactotroph. Physiol Rev. 70:279–318.
L awson, D. C., F ewtrell, and M. R aff. 1978. Localized mast cell degranulation induced by concanavalin A -Sepharose beads. J. Cell Biol. 79:394–400.
Martin, T.F. 1983. Thyrotropin-releasing hormone rapidly activates the phosphodiester hydrolysis of polyphosphoinositides in GH3 pituitary cells. Evidence for the role of a polyphosphoinositide-specific phospholipase C in hormone action. J. Biol. Chem. 258:14816–14822.

McLaren, D.J., C.D. MacKenzie, and F.J. Ramalho-Pinto. 1977. Ultrastructural observations on the in vitro interaction between rat eosinophils and some parasitic helminths (Schistosoma mansoni, Trichinella spiralis, and Nippostrongylus brasiliensis). Clin. Exp. Immunol. 30:105–118.

Nakagawa, J., H. Mori, T. Maeda, T. Matsuo, and Y. Okada. 1995. Dynamics of secretory granules in somatotrophs of rats after stimulation with growth hormone-releasing factor: a stereological analysis. Cell Tissue Res. 282:493–501.

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

Orci, L., and W. Malaisse. 1980. Hypothesis: single and chain release of insulin secretory granules is related to anionic transport at exocytic sites. Diabetes. 29:943–944.

Palade, G. 1975. Intracellular aspects of the process of protein synthesis. Science. 189:347–358.

Pocotte, S.L., R.A. Frye, R.A. Senter, D.R. TerBush, S.A. Lee, and R.W.Holz. 1985. Effects of phorbol ester on catecholamine secretion and protein phosphorylation in adrenal medullary cell cultures. Proc. Natl. Acad. Sci. USA. 82:930–934.

Proks, P., L. Eliasson, C. A mmala, P. Rorsman, and F.M. A shcroft. 1996. Ca\(^{2+}\)-and GTP-dependent exocytosis in mouse pancreatic beta-cells involves both common and distinct steps. J. Physiol. 496:255–264.

Rendstrom, E., L. Eliasson, and P. Rorsman. 1997. Protein kinase A-dependent and -independent stimulation of exocytosis by cAMP in mouse pancreatic B-cells. J. Physiol. 502:105–118.

Scepek, S., and M. Lindau. 1993. Focal exocytosis by eosinophils: compound exocytosis and cumulative fusion. EMBO (Eur. Mol. Biol. Organ.) J. 12: 1811–1817.

Senda, T., K. Yamashita, N. Sugimoto, K. Ozutsumi, M. Matsuda, and H. Fujita. 1989. Multigranular exocytosis induced by clostridium perfringens type A enterotoxin in incubated anterior pituitary cells and pancreatic acinar cells of rats. Biomed. Res. 10:385–393.

Sikdar, S.K., R. Zorec, and W.T. Mason. 1990. cAMP directly facilitates Ca-induced exocytosis in bovine lactotrophs. FEBS (Fed. Eur. Biochem. Soc.) Lett. 273:150–154.

Sikdar, S.K., M. Krefl, and R. Zorec. 1998. Modulation of the unitary exocytic event amplitude by cAMP in rat melanotrophs. J. Physiol. 511:851–859.

Smith, C., T. Moser, T. Xu, and E. Neher. 1998. Cytosolic Ca\(^{2+}\) acts by two separate pathways to modulate the supply of release-competent vesicles in chromaffin cells. Neuron. 20:1243–1253.

von Ruden, L., and E. Neher. 1993. A Ca-dependent early step in the release of catecholamines from adrenal chromaffin cells. Science. 262:1061–1065.

Zucker, R.S. 1999. Calcium- and activity-dependent synaptic plasticity. Curr Opin Neurobiol. 9:305–313.