IMAGING EXOCYTOSIS AND RECAPTURE OF SECRETORY GRANULES IN CULTURED ENDOCRINE CELLS

by

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

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ABSTRACT

Classical models of exocytosis propose that vesicles collapse and disperse into the cell surface during fusion. Following exocytosis, a cell must retrieve by endocytosis any vesicle components it wishes to keep inside. We have tested whether this model applies to dense-core granules in PC12 cells. Granule components were labeled with fluorescent proteins or lipids in different colors, and two-color evanescent field microscopy was used to image single granules during and after exocytosis. Whereas the small luminal protein neuropeptide Y (NPY) was lost from granules in seconds, the larger protease tissue plasminogen activator (tPA) remained at the granule site for over a minute, marking post-exocytic granules. When tPA was imaged with cyan fluorescent protein (CFP) as a cytosolic marker, the volume occupied by the granule appeared as a dark spot where the granule excluded CFP. The dark spot remained even after tPA reported exocytosis, indicating that granules failed to flatten into the cell surface. In a majority of events, the granule membrane protein phogrin, labeled with either DsRed or mRFP on its cytosolic end, did not disperse into the plasma membrane during exocytosis but instead remained static at the granule site. Because phogrin does not leave the granule cavity, we labeled phogrin with green fluorescent protein (GFP) at its luminal end in order to sense pH changes in the granule's interior. When exocytosis caused the acidic granule interior to neutralize, GFP-phogrin at first brightened and later dimmed as the interior of the granule separated from the extracellular space and re-acidified. Re-acidification and dimming could be reversed by application of the membrane-permeant base

NH_4Cl. We conclude from these studies that granules selectively release certain proteins while retaining others. Additionally, we conclude that close to half of all granules re-seal after releasing cargo, and that these partially empty granules are recaptured otherwise intact. Work in neurons has suggested that synaptic vesicles do not exchange membrane components, lipid, or protein during transient exocytosis. To test whether dense-core granules behave similarly, we imaged the lipid probe FM4-64. During exocytosis, granules lost FM4-64 into the plasma membrane within milliseconds, regardless of whether or not they resealed later. From this work, we conclude that the lipid bilayers of the granule and the plasma membrane become continuous during exocytosis, allowing vesicle lipids to escape into the plasma membrane. Thus, the classical model of exocytosis does not apply to all exocytic events in cultured neuroendocrine cells. Indeed, a large number of granules do not collapse into the plasma membrane, but instead release some components such as small proteins and lipids, while retaining others. In addition, these granules can later reseal. We propose to call this mode of transient exocytosis "cavicapture," short for "cavity recapture," to distinguish it from both kiss-and-run exocytosis, where lipids and protein of synaptic vesicles are retained, and full-fusion exocytosis, where all components are lost.

CHAPTER 1

INTRODUCTION

Eukaryotic cells utilize an intricate network of membranous compartments to process and deliver material to the cell's exterior (Rothman and Wieland, 1996). In the final stage of this pathway, membrane-bound vesicles dock to and fuse with the plasma membrane through the process of exocytosis (Almers, 1990). During exocytosis, the lumen of a vesicle connects with the extracellular space, which allows components stored in the vesicle to escape. Exocytosis, found in all eukaryotic cells, can be classified into two basic types. In the first type, transport vesicles exit from the trans-Golgi Network (TGN) and approach to within a few nanometers of the plasma membrane. Once at the cell surface, the vesicles merge with the plasma membrane by exocytosis. This form of exocytosis, called "constitutive" or "unregulated" exocytosis, delivers receptors and other signaling molecules such as the EGF receptor or the transferrin receptor to the cell surface. In constitutive exocytosis, no specific signal has been described that regulates fusion. These vesicles generally fuse with the plasma membrane as soon as they reach the cell surface (Toomre et al., 1999). In the second form of exocytosis, secretory vesicles rest at the cell surface after approaching the plasma membrane and wait for a signal before undergoing exocytosis and releasing their contents. This form of exocytosis, known as "regulated" or "triggered" exocytosis, is essential for many cellular processes, including the precise release of

neurotransmitter during synaptic transmission and the release of hormones and peptides from endocrine cells.

There are five sequential stages of regulated exocytosis found in both neurons and endocrine cells. During the first stage, *vesicle biogenesis and transport*, vesicles bud from the TGN and are delivered to the cell surface by molecular motors. During the second stage, *docking*, and the third stage, *priming*, vesicles undergo a series of morphological and biochemical steps, which endows the vesicles with the ability to fuse. During the fourth stage, *membrane fusion*, the lumen of the vesicle connects with the extracellular space as the bilayer of the vesicle merges with that of the plasma membrane. In the final stage, *vesicle recapture*, the components of the vesicle are collected from the cell surface and internalized, which restores the molecular identity and surface area of the plasma membrane.

Biogenesis and Transport of Secretory Vesicles.

All proteins destined for export begin life in the membranous network of the endoplasmic reticulum (ER). Here, proteins are processed and their folding is monitored by quality control machinery (Hammond and Helenius, 1995; Rothman and Wieland, 1996). From the ER, proteins and lipids are then trafficked by vesicular carriers to the Golgi network for further processing and sorting. At the terminus of the Golgi stacks, the trans-Golgi Network (TGN), protein components of one type of exocytic organelle, the dense-core granule, aggregate and form the nascent core of the granule (Chanat and Huttner, 1991). The aggregation of

granule proteins, particularly a group of proteins known as "granins," is induced by the high calcium concentration and low pH of the TGN lumen (Chanat and Huttner, 1991; Huttner et al., 1991). Once formed, these dense aggregates bind lipid and drive the deformation of a membrane pocket, which later pinches off and forms an enveloped granule. In PC12 cells, newly budded hormone and neuropeptide-containing granules emerge from the TGN unfinished (Tooze, 1991). Such immature secretory granules (ISG) are rapidly transported along microtubules from the TGN to the cell's cortex by molecular motors (Rudolf et al., 2001). In insulinoma cells, the motor, ATP-dependent kinesin, has been shown to mediate this outward movement (Varadi et al., 2003). Once at the periphery, ISGs are captured by a thin actin cortex, where granules are further processed and transformed into mature granules within an hour (Lang et al., 2001; Rudolf et al., 2001; Tooze, 1991; Tooze et al., 1991). The maturation of ISGs includes homotyptic fusion, removal of missorted material by membrane budding, and the processing of granule components by granule-resident proteases (Tooze, 1991; Zhou et al., 1999). Mature granules are then dispersed under the surface of the plasma membrane along actin filaments by the molecular motor myosin Va (Rudolf et al., 2003). Disrupting the interaction of myosin Va with granules results in an accumulation of granule clusters at the cell's periphery and a strong reduction in the ability of granules to move lateral to the cell surface (Rudolf et al., 2003).

The biogenesis of synaptic vesicles (SV) proceeds via a different route than the biogenesis of dense-core granules. In neurons, synaptic vesicle precursors

emerge from the TGN as vesiculotubular structures that are similar to constitutive transport vesicles (Tsukita and Ishikawa, 1980). These containers are transported down the axon along microtubules by molecular motors. The transport vesicles then fuse with the presynaptic membrane, delivering the components of synaptic vesicles to the nerve terminal (Ahmari et al., 2000; Craig et al., 1995; Nakata et al., 1998). SV components are then internalized from the plasma membrane by clathrin-coated vesicles to form new synaptic vesicles (Cremona and De Camilli, 1997; Hannah et al., 1999). The biogenesis of synaptic vesicles at the nerve terminal is advantageous inasmuch as neurons require a constant supply of vesicles. The genesis of vesicles at their site of use ensures a steady supply during periods of high activity. If new synaptic vesicles were always made in the TGN, the pool of available vesicles would be limited by the slow transport of vesicles hundreds of microns down the axon to the synapse. High periods of activity would then leave the terminal depleted of vesicles. Once formed, SVs dock at sites of fusion known as "active zones." The active zone is defined by a dense matrix of protein adjoining the synaptic cleft (Landis et al., 1988; Pfenninger et al., 1972; Phillips et al., 2001). This proteinacous scaffold bundles a nest of vesicles close to the plasma membrane. Those vesicles resting closest to the membrane are thought to be released within milliseconds of stimulation and are thought to constitute a rapidly releasable pool (Dobrunz and Stevens, 1997; Murthy and De Camilli, 2003). Like dense-core granules, a mesh of actin surrounds the active zone, possibly organizing the cluster of vesicles and facilitating recycling (Shupliakov et al., 2002). Also similar to granules, the

molecular motor myosin Va has been shown to mediate the short range movements of synaptic vesicles at the nerve terminal along actin filaments (Langford, 2002).

Figure 1.1

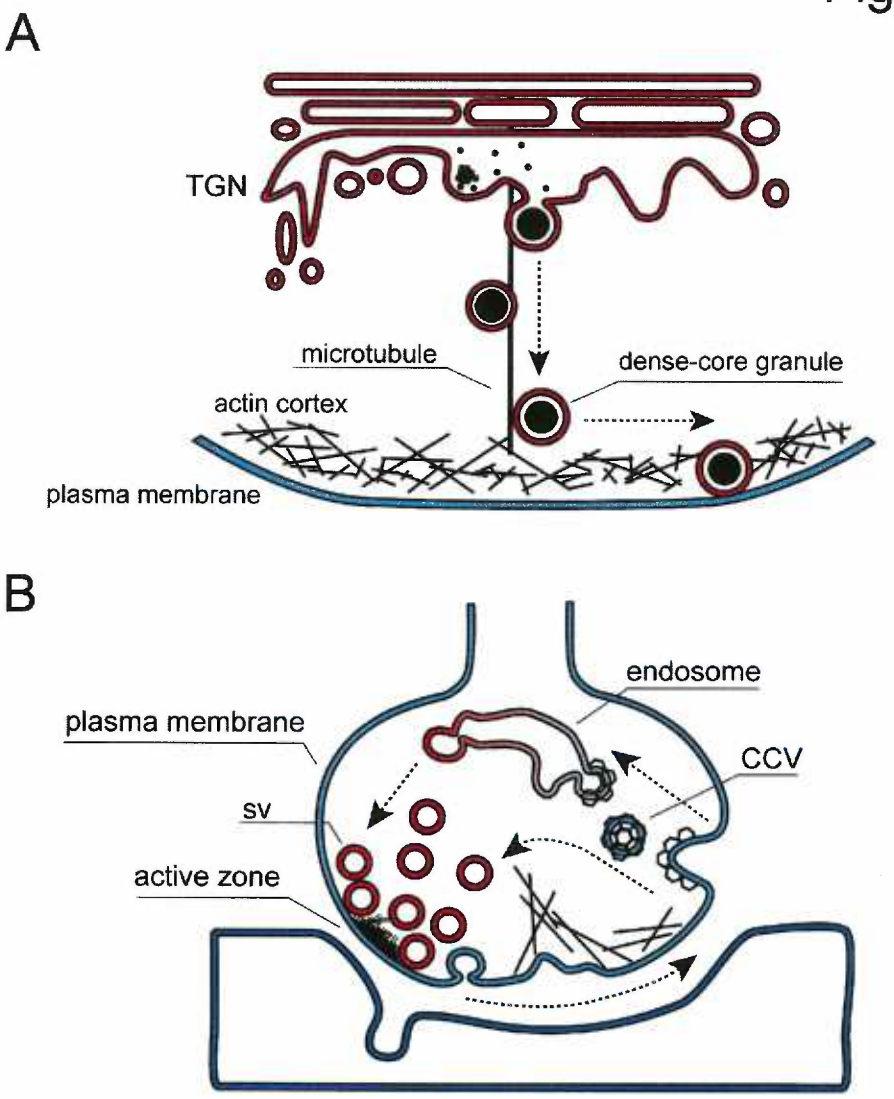

Fig. 1.1. Biogenesis and transport of vesicles in neurons and endocrine cells. A) In an endocrine cell, the selective aggregation of granule proteins in the trans-Golgi Network (TGN) leads to the budding of dense-core granules. Granules are transported by motors along microtubules to the actin cortex where some dock with the plasma membrane. B) In neurons, synaptic vesicles (SV) are generated at the synapse by local recycling of components. Clathrin-coated vesicles (CCVs) concentrate and retrieve synaptic vesicle components from the plasma membrane. These endocytic vesicles can uncoat, forming new vesicles directly, or fuse with sorting endosomes for further processing. New synaptic vesicles are then transported to the active zone where they dock with the plasma membrane.

Docking and priming.

After secretory vesicles are delivered to the cell's cortex, a subset of vesicles closely associates with the plasma membrane. This process, called "docking," is defined by the simple morphological criterion that a vesicle is physically close and likely tethered to the plasma membrane. Observations of chromaffin cells with electron microscopy have been used to estimate the docked granule pool (those granules within 10 nm of the plasma membrane) to be ~4 % (~1000 out of ~25,000) of the cell's granules (Plattner et al., 1997; Steyer et al., 1997). It is not yet clear, however, what molecules mediate this attachment. Synaptic terminals also contain a morphologically docked vesicle pool (Dobrunz and Stevens, 1997; Reist et al., 1998; Schikorski and Stevens, 1997). Again, this pool merely correlates to those vesicles that are closest to the presynaptic membrane. Docking, however, does not correlate directly to the aptitude for exocytosis. Indeed, some docked granules must undergo a biochemical transformation to become fusion-ready. This finding stems mainly from kinetic and morphological analysis of exocytosis, primarily in chromaffin cells. In chromaffin cells, it has been shown that there are distinct kinetic phases of release (Neher and Zucker, 1993; Thomas et al., 1993; Xu et al., 1998; Xu et al., 1999). There is a rapid exocytic burst that occurs within milliseconds of stimulation. The number of morphologically docked vesicles, however, far exceeds the number of vesicles released during the burst (Klenchin and Martin, 2000; Plattner et al., 1997; Steyer et al., 1997). This indicates that while docked granules could account for all vesicles released during

the burst, not all docked granules fuse rapidly. Following the burst, two slower phases of exocytosis occur within seconds and minutes, respectively. These phases likely represent exocytosis of vesicles that were less competent for fusion (Barg et al., 2002). Thus, it appears that there are several distinct classes of secretory vesicles occupying unique biochemical states. Those vesicles that are released first are in some way "primed" for rapid fusion while granules released later must undergo a biochemical or morphological transformation to become fusion-ready.

Priming has thus been defined as a series of biochemical steps that activate the machinery responsible for fusion (Sudhof, 1995). Like cocking a trigger, priming occurs in order to ready a vesicle to fuse as soon as the cellular signal stimulating exocytosis (in most cases a rise in intracellular Ca^{2+}) arrives below the plasma membrane. There are several molecular events that have been ascribed to the priming step. First, priming appears to require ATP because nonhydrolyzable ATP does not inhibit the rapid phase of exocytosis, the phase ascribed to the primed pool, but does inhibit the slow phase of release (Hay and Martin, 1993; Holz et al., 1989; Parsons et al., 1995). There are also several ATP-dependent enzymes that have been proposed to induce priming. N-ethylmaleimide-sensitive fusion (NSF) protein is the most well-studied candidate. NSF is proposed to interact with a set of proteins known as the "soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors," or "SNAREs," prior to exocytosis (Sollner et al., 1993). The SNARE hypothesis proposes that the formation of a complex composed of one light chain, the v-SNARE found on the vesicle, and

one heavy chain resident to the plasma membrane, the t-SNARE, mediates membrane targeting and vesicle fusion (Sudhof, 1995). The members of the complex, however, can assemble stable *cis*-complexes within their own membrane compartments, precluding the assembly of the *trans*-complex (Otto et al., 1997). NSF disassembles *cis*-SNARE complexes through hydrolysis of ATP and has been proposed to play a role in readying the SNAREs to interact in *trans* to mediate membrane fusion (Hayashi et al., 1994). Other ATP-dependent processes have been proposed to mediate some aspect of priming. For example, the phosphorylation of phosphatidylinositol (PI) to the phosphoinositide $PI(4,5)P_2$ has been shown to be essential for priming (Hay et al., 1995; Hay and Martin, 1993). Furthermore, protein kinases such as PKC and PKA have been shown to influence the number of primed vesicles. The treatment of cells with phorbol-ester, which activates PKC, stimulates exocytosis by increasing the number of fusion-ready, primed vesicles (Gillis et al., 1996). Other proteins not regulated by ATP also have been shown to play a role in priming, including the protein MUNC18, which binds to the t-SNARE syntaxin and might regulate its activity by holding syntaxin in a closed/inactive state (Gallwitz and Jahn, 2003). The protein MUNC13, which binds to MUNC18, also stimulates priming (Ashery et al., 2000). In conclusion, a series of biochemical steps occurs to ready a vesicle for membrane fusion. This primed pool of vesicles is then "cocked" for membrane fusion and most likely mediates the initial burst of exocytosis when the cell is stimulated.

Figure 1.2

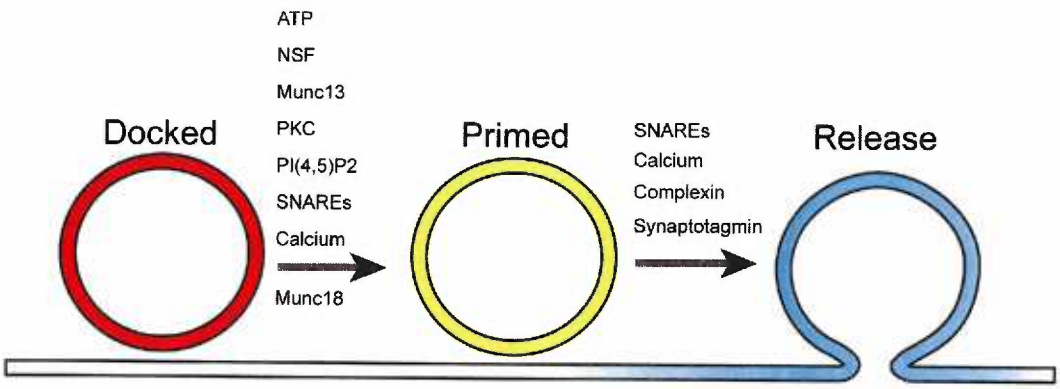

Fig. 1.2. Docking and priming steps of calcium-triggered exocytosis. Vesicles first approach and dock within nanometers of the plasma membrane. After docking, a series of enzymatic steps occurs to prime vesicles for rapid exocytosis. These steps might include the disassembly of cis-SNARE complexes and the formation of trans-SNARE complexes. Once priming has occured, membrane fusion requires SNAREs and is stimulated by an increase in intracellular calcium.

Membrane Fusion

The final stage of exocytosis is the fusion of the vesicle membrane and the plasma membrane. During fusion, the two bilayers are brought into close proximity, where a structural rearrangement occurs to form the initial aqueous connection linking the lumen of the vesicle to the extracellular space. This connection has been termed the "fusion pore" (Almers and Tse, 1990). The biophysical stages of fusion pore formation still are not well understood. Most of what we do know has been obtained through high-resolution physical measurements of single fusion events. For example, the capacitance across a membrane is proportional to its surface area. When a single vesicle adds to the plasma membrane during exocytosis, the additional membrane can be detected as a step increase in capacitance (Neher and Marty, 1982). Membrane capacitance has been used to monitor the exocytosis of individual vesicles in many cell types (Breckenridge and Almers, 1987b; Fernandez et al., 1984; Klyachko and Jackson, 2002; Neher and Marty, 1982; Nusse and Lindau, 1988). Single fusion events also can be detected electrically as vesicles discharge their membrane potential through the fusion pore (Breckenridge and Almers, 1987a). Conductance across the fusion pore is proportional to the pore's dimension. The initial conductance of vesicles during the first 10-20 ms of exocytosis has been shown to increase rapidly by several hundred picosiemens. This conductance is similar to that found in large ion channels such as the BK K^+(Ca) channels (130-240 pS) (Latorre and Miller, 1983) and single gap-junction (160 pS) (Veenstra and DeHaan, 1986). Evidently,

during the first few moments of exocytosis, the fusion pore is small, likely no larger than 1-2 nm in diameter (Unwin and Zampighi, 1980). Unlike ion channels, however, the variance of the initial conductance is large for fusion pores, suggesting that there is not a fixed diameter of the nascent pore (Breckenridge and Almers, 1987a). After formation, the pore expands slowly, sometimes reaching sizes close to 20 nm in diameter (Chandler and Heuser, 1980). Surprisingly, rapid drops in capacitance (flicker) have been seen after exocytosis (Albillos et al., 1997; Ales et al., 1999; Breckenridge and Almers, 1987b; Fernandez et al., 1984; Klyachko and Jackson, 2002). Because these drops have magnitudes similar to the initial increase during exocytosis, fusion pores are thought to be completely reversible.

Another technique, amperometery, has been used to probe the structure of fusion pores during exocytosis (Wightman et al., 1991). Amperometry detects a spike of current as catacholamines are released from individual vesicles and strike a carbon-fiber electrode placed close to the cell. The shape of this spike can be used to determine the ease with which catacholamines exit the vesicle, and hence the fusion pore's physical dimensions. Indeed, the overexpression and mutation of many proteins thought to be involved in exocytosis have been shown to change the shape of amperometric spikes (Fisher et al., 2001; Graham et al., 2002; Wang et al., 2001). One phase of the spike, the "foot signal," has been studied in detail to determine the structure of the fusion pore. Foot signals sometimes precede spikes and are thought to correspond to periods when catacholamines are released slowly through a narrow fusion pore (Chow et al., 1992). Occasionally, a "stand-

alone" foot signal without an accompanying spike has been seen and has been proposed to represent transient, reversible fusion events (Albillos et al., 1997; Alvarez de Toledo et al., 1993; Zhou et al., 1996)

How does the fusion of two hydrophobic, semi-crystalline structures (lipid bilayers) separated by a layer of water occur? Indeed, for exocytosis to happen, several energetically unfavorable processes must occur rapidly. There are two dominant models describing the physics of biological membrane fusion. Both models are based on studies of membrane fusion in living cells as well as viral-induced membrane fusion and the fusion of artificial lipid bilayers. The fundamental difference between the two models is whether fusion proceeds via a purely lipidic route, with proteins acting in a catalytic role, or whether proteins play a more central role, directly forming the fusion pore and associating with lipids at only a late step in the process. The lipidic model of membrane fusion is called the "stalk hypothesis" or "proximity model" (Chernomordik et al., 1987). The stalk hypothesis proposes that membrane fusion begins with the association of the two proximal monolayers (Chanturiya et al., 1997; Jahn et al., 2003; Melikyan et al., 1995). A small initial contact point, the nipple, forms between the two bilayers as membranes are pressed close and the water separating the two membranes is squeezed out. Out-of-plane thermal fluctuations of the lipids then induce the formation of a local non-bilayer intermediate, which connects the two proximal monolayers. This hemifusion intermediate, termed the "stalk," has a high negative curvature and would be stabilized by lipids that have intrinsic negative curvature (Chernomordik et al., 1995). After hemifusion, the inner

bilayers must rupture to connect the two compartments. The pore could then expand, allowing the vesicle to fully integrate into the target membrane. The stalk model suggests that the fundamental process of membrane fusion can function in the absence of proteins. Indeed, many behaviors observed in fusion pores in biological systems can be recreated in artificial lipid bilayers devoid of protein or in isolated secretory vesicles (Chanturiya et al., 1997; Oberhauser and Fernandez, 1993).

In the second model of membrane fusion, the initial contact between the bilayers is made by a proteinaceous complex that forms in *trans,* similar to a gap junction (Almers and Tse, 1990). After formation of the complex, conformational changes in the subunits would lead to the formation of an aqueous pore. The subunits may then break apart, exposing the amphipathic surfaces between them and allowing the migration of lipids into the pore. This model has gained interest as recent work has shown that the V0-ATPase proteolipid complex can form between and fuse vacuoles in yeast (Peters et al., 2001). Other proteins recently have been shown to affect the rate and extent of fusion pore opening, suggesting that a complex of several proteins likely facilitates membrane fusion. Proteins such as complexin (Archer et al., 2002), cysteine string protein (Graham and Burgoyne, 2000), Munc-18 (Fisher et al., 2001), synaptotagmin I & IV (Wang et al., 2001), and syntaxin (Han et al., 2004) have been shown to modulate fusion pore dynamics. Indeed, membrane fusion most likely proceeds by a mechanism that combines aspects of the stalk and proteinaceous pore model. In this hybrid model, a ringlike complex of several components could form between opposing

bilayers. This complex might undergo conformational changes induced by intracellular calcium and facilitate lipids associated with the complex to bend and merge. This semi-stable intermediate would then be able to flicker open and closed before the complex fully dilates, breaking the hemifusion intermediate and fusing the two compartments.

Figure 1.3

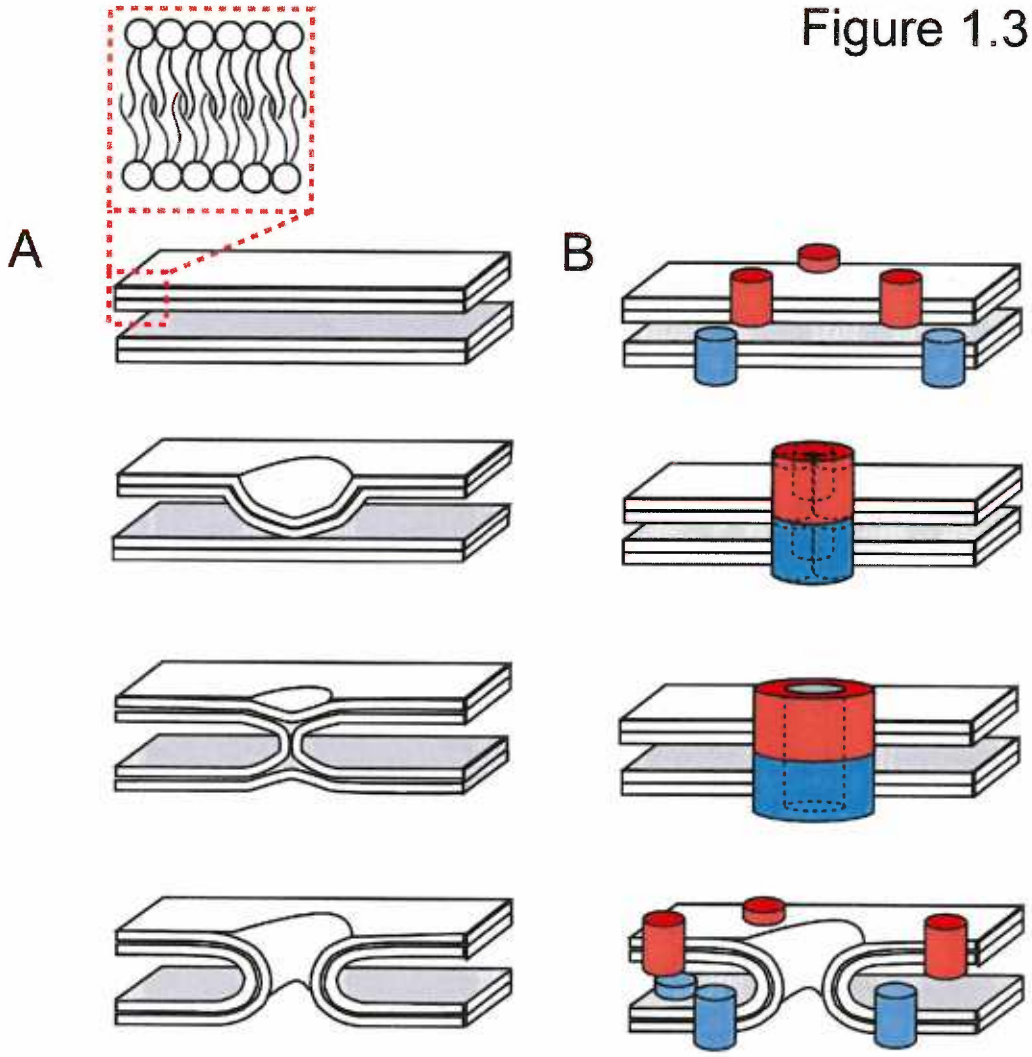

Fig. 1.3. Models of membrane fusion. A) The stalk/proximity model of exocytosis. First, a nipple forms in one bilayer, pressing the proximal leaflets together. Next, lipids in each of the proximal leaflets fuse, forming the stalk. The outer leaflets of the hemifusion intermediate then rupture and merge, forming the open fusion pore. B) The proteinaceous-pore model of fusion. First, protein subunits in opposing bilayers assemble in *trans*, forming a complex similar to a gap junction. Next, the complex dilates, opening a fusion pore made exclusively of the inner surface of the channel complex. Finally, the complex disassembles, allowing lipids to invade the pore and fuse the two bilayers.

The SNARE hypothesis and Ca^{2+}-triggered exocytosis.

While membrane fusion can occur in a pure lipid system, and several mechanisms may have evolved to fuse biological membranes, Ca^{2+}-triggered exocytosis *in vivo* is known to require the concerted action of several protein components. The core machinery proposed to regulate both the specificity of vesicle targeting and the process of membrane fusion itself is the SNAREs. SNAREs constitute a group of membrane proteins that share in common a central motif composed of eight heptad repeats that often precede a C-terminal transmembrane domain. In neurons and neuroendocrine cells, the secretory vesicle v-SNARE is a transmembrane protein VAMP2/synaptobrevin, and the two resident plasma membrane t-SNAREs are the transmembrane protein syntaxin and the palmitoylated protein SNAP-25. Alone, synaptobrevin and SNAP-25 are mostly unstructured, yet together with syntaxin they form an extremely stable alpha-helical coiled-coil bundle (Fasshauer et al., 1997a; Fasshauer et al., 1997b). Indeed, this complex is so energetically favorable that it is resistant to denaturation by SDS and is stable at temperatures upwards of 90°C (Hayashi et al., 1994). The proteins associate first through their membrane distal amino-terminal ends in a head-to-head fashion, suggesting that SNAREs "zipper" together, driving the two target membranes into close proximity (Hanson et al., 1997; Lin and Scheller, 1997). The crystal structure of the neuronal SNARE complex revealed a unique ionic layer in the otherwise hydrophobic core of the 4-helical bundle (Sutton et al., 1998). This "zero layer" is conserved among members of the SNARE family of

proteins (Fasshauer et al., 1998). During complex assembly, each helix contributes one charged glutamine or arginine to the zero layers in a 3Q:1R ratio. This generates a lock-and-key mechanism, which ensures that the correct ratio of SNAREs assembles between the membranes.

Much biochemical work has shown that SNAREs can mediate membrane fusion *in vitro*. Specifically, experiments have demonstrated that two populations of proteoliposomes, each carrying either the t-or v-SNAREs, will fuse and exchange lipid (Weber et al., 1998). These data suggest that SNAREs are the minimal machinery required for fusion. This idea also has been tested by evanescent-field microscopy, where proteoliposomes were seen to fuse with supported bilayers. In these experiments, when divalent cations were added to the solution, liposomes that contained synaptobrevin merged with supported bilayers that contained syntaxin and SNAP-25 (Fix et al., 2004). The speed of exocytosis in this system was rapid, suggesting that SNAREs alone could mediate fast exocytosis. Other studies have shown that cells expressing a v- "flipped" SNARE that is exposed to the extracellular space can fuse with cells expressing two flipped t-SNAREs (Hu et al., 2003).

In vivo, SNAREs have been shown to be essential factors in calcium-triggered exocytosis through a combination of molecular and genetic perturbations. For example, the clostridial neurotoxins, which poison neurotransmission, were shown to cleave SNAREs. Specifically, tetanus toxin cleaves synaptobrevin while members of the Botulinum toxin family were shown to act on syntaxin, SNAP-25 and synaptobrevin (Blasi et al., 1993a; Blasi et al.,

1993b; Schiavo et al., 1992). Because these toxins robustly poison synaptic transmission, SNAREs were argued to be necessary for calcium-triggered exocytosis (Niemann et al., 1994). This also was demonstrated in transgenic animals lacking SNAREs. Evoked neurotransmission was blocked by deleting the gene for synaptobrevin in *Mus musculus* and *Drosophila melanogaster* (Broadie et al., 1995; Schoch et al., 2001). Similarly, the deletion of syntaxin in *D. melanogaster* and *Caenorhabditis elegans* blocked exocytosis (Nonet et al., 1998; Schulze et al., 1995). Finally, deletion of SNAP-25 in *M. musculus* abolished calcium-triggered exocytosis (Washbourne et al., 2002). Surprisingly, in many of these animals, spontaneous exocytic events (minis) were still observed, indicating that SNAREs might not provide the final work in fusing bilayers and instead might act as cofactors or regulators of triggered exocytosis. SNAREs, nevertheless, are clearly important for exocytosis in neurons and neuroendocrine cells. From structural and functional studies, it appears that one job of SNAREs is to bring bilayers into close proximity. A fundamental question that remains is whether the SNARE core complex mediates the final mechanical work of calcium-triggered membrane fusion or whether another component associates with the core complex to mediate the fusion reaction (Almers, 2001; Tucker et al., 2004).

Along with members of the SNARE core complex several other proteins have been demonstrated to be necessary for calcium-triggered exocytosis. Specifically, the sec1/Munc18 proteins (SM), synaptotagmins, and complexins, have all been shown to participate in triggered exocytosis. SM proteins are 60-70

kD hydrophilic proteins that bind to syntaxin (Hata et al., 1993). Mutations in SM proteins block triggered exocytosis in *C. elegans*, *D. melanogaster*, and *M. musculus* (Verhage et al., 2000; Weimer et al., 2003; Wu et al., 1998). Munc18-1 binds to a closed conformation of syntaxin and holds syntaxin in an inactive conformation that inhibits core complex assembly (Betz et al., 1997). Another presynaptic protein that is essential for triggered exocytosis is Munc13. Munc13 binds to the amino terminus of syntaxin and displaces Munc18 (Betz et al., 1997; Varoqueaux et al., 2002). A second family of calcium-binding proteins, the synaptotagmins, has been shown to be necessary for calcium-triggered exocytosis (Brose et al., 1992). The structures of synaptotagmins share in common a transmembrane region and two C2 calcium-binding domains separated by a flexible linker. Synaptotagmin 1 binds a total of 5 calcium ions, interacts with the t-SNARE syntaxin, and associates with membranes in a calcium-dependent step (Chapman, 2002). All these qualities have suggested that synaptotagmin might be a calcium sensor that triggers exocytosis. In this model, synaptotagmin would bind calcium ions together with syntaxin. This interaction could perturb the SNARE's membrane anchors, driving the bilayers together. Synaptotagmin is clearly important for regulated exocytosis. Mice lacking synaptotagmin have greatly diminished fast synaptic transmission (Geppert et al., 1994). Deletion of synaptotagmin, however, increases the rate of spontaneous exocytosis, suggesting that synaptotagmin is not essential for membrane fusion but instead might regulate the timing and calcium-dependence of exocytosis (Littleton et al., 1994).

Finally, the small 15 kD complexins have been implicated in neuronal SNARE complex assembly (McMahon et al., 1995). Like Munc18, knockout of complexin blocked calcium-triggered exocytosis (Reim et al., 2001). Exocytosis stimulated by high osmolatriy, however, remained intact in these mice. These data suggest that complexin is involved in a late calcium-dependent step in exocytosis. Complexins contain a conserved alpha helical region that binds tightly to the assembled core complex, and has been proposed to hold the SNARE complex in a primed state, readying the SNAREs for rapid exocytosis (Chen et al., 2002; Hu et al., 2002). The numerous protein components required for calcium-triggered exocytosis allow cells to maintain tight control of exocytosis while providing for maximal speed. Indeed, exocytosis in neurons is thought to occur within fractions of a millisecond (Almers et al., 1991).

Endocytosis

Exocytosis must be followed by endocytosis in order to maintain the molecular composition and surface area of the plasma membrane. In a terminal with a limited number of vesicles, a cell must recycle and reuse the unique molecular components of the vesicle many times over to sustain high firing rates. Indeed, the synapse at the frog neuromuscular junction can fire at 2 Hz for 24 hours without a significant decrease in vesicle number or post-synaptic potential (Lynch, 1982). Clearly, a mechanism must exist to ensure the rapid retrieval and reuse of vesicle components. Two models of vesicle recycling were suggested over 30 years ago from morphological studies of the neuromuscular junction synapse (Ceccarelli et

al., 1972; Ceccarelli et al., 1973; Heuser and Reese, 1973). Using similar techniques (electron microscopy of quick-frozen tissue), the two groups arrived at very different models of vesicle recycling. The core difference between the two models is whether vesicles fully fuse and collapse into the plasma membrane (full fusion model), later being recaptured at a point distal to the release site by clathrin-mediated endocytosis, or whether vesicles never collapse into the plasma membrane but instead are rapidly recaptured without intermixing components with the plasma membrane (kiss-and-run model) (Valtorta et al., 2001; Wilkinson and Cole, 2001).

The full-fusion model has been the prevailing model when describing compensatory endocytosis in secretory cells. This model closely resembles other well-studied forms of receptor-mediated endocytosis in non-secretory cells that involve clathrin (Conner and Schmid, 2003). The full-fusion model of vesicle recapture originated with work of Heuser and Reese (Heuser and Reese, 1973). In their studies, nerves of isolated frog satorius muscle were stimulated at 10 Hz to induce massive exocytosis of synaptic vesicles. Vesicles were depleted in the terminal after several minutes of stimulation, and there was an increase in plasma membrane surface area and an appearance of membranous cisternae. Clathrin-coated vesicles were also observed to proliferate during nerve stimulation at the lateral edges of the terminal. Vesicles were observed to reappear in the terminal after a 15 minute rest, arising from coated structures that budded from the end of membrane-bound internal cisternae. The authors concluded that under such stimulation conditions, vesicles fully merged with the plasma membrane and their

components were then collected and re-internalized by clathrin-coated vesicles (CCV) outside the active zone. CCVs then fused with endosomes in the terminal, and new vesicles were reborn and transported to active zones for a second round of exocytosis. DeCamilli and colleagues proposed an amended version of this model in which synaptic vesicles also can be formed directly by budding from the plasma membrane in a clathrin/dynamin-dependent step, without needing to pass through a sorting endosome (Takei et al., 1996). Along with EM studies, the CCV model of vesicle recapture has been studied at the level of the light microscope. At the *D. melanogaster* neuromuscular junction, some molecular components of CCVs including dynamin, AP2, and DAP160 surround active zones (Roos and Kelly, 1999). This work supports the idea that CCVs retrieve components of synaptic vesicles at sites adjacent to active zones. Furthermore, data obtained from fluorescence microscopy of synaptic vesicles containing the vesicle protein VAMP2/synaptobrevin, which was fused to a pH-sensitive GFP (synapto-pHluorin) (Miesenbock et al., 1998), have shown that VAMP can escape vesicles during exocytosis and spread into neighboring regions of the axon. The VAMP is then retrieved with a half-time of 74 s (Sankaranarayanan and Ryan, 2000). This value is close to rates of endocytosis in hippocampal neurons measured by using the styryl dye FM1-43 (between 20 and 60s) (Murthy, 1999; Ryan et al., 1996). Furthermore, this time constant, while fast, could be consistent with the speed of clathrin-mediated endocytosis (Fernandez-Alfonso and Ryan, 2004; Merrifield et al., 2002). The accumulation of clathrin has been measured directly in stimulated hippocampal boutons (Mueller et al., 2004). In these studies, clathrin was

recruited to hippocampal boutons during stimulation and then was lost with a half time of 47 s, suggesting that clathrin can assemble and uncoat in less than a minute in nerve terminals.

Ceccarelli and colleagues have argued in a competing model that synaptic vesicles do not cycle through the CCV pathway but instead are recaptured from the active zone intact. In this model, called "kiss-and-run" exocytosis, vesicle components remain segregated from the plasma membrane, allowing for rapid retrieval (Valtorta et al., 2001). If frugality were preferred by nature, kiss-and-run would seem an attractive process. The cell could avoid the energetically costly process of collecting and concentrating vesicle components into coated pits (Bretscher et al., 1980). The cell also would avoid other processes such as vesicle transport, re-budding, and sorting. Indeed, Ceccarelli and colleagues found that under low frequency stimulation (2 Hz) there was no decrease in the number of synaptic vesicles at the nerve terminal, which supports the idea that there is a rapid and efficient recycling mechanism that could provide a steady supply of vesicles. The strongest arguments for direct vesicle recapture from these early studies were obtained when the extra-cellular marker horseradish peroxidase (HRP) was included in the external bathing media during stimulation. Under these conditions, many synaptic vesicles were stained with HRP, suggesting that vesicles resting close to the plasma membrane fused and then resealed directly, trapping HRP in their lumen (Ceccarelli and Hurlbut, 1980a; Ceccarelli et al., 1972; Ceccarelli et al., 1973). The appearance of HRP-labeled "Omega" structures also provided tantalizing evidence that vesicles could rest at the cell

surface with mouths gaping before they resealed. While the full-fusion model has dominated, the kiss-and-run mechanism has gained considerable attention over the last several years, mostly due to imaging studies using amphipathic styryl dyes such as FM1-43 (Betz and Bewick, 1992; Fesce et al., 1994; Valtorta et al., 2001). Styryl dyes are not fluorescent in solution, but acquire a higher quantum yield when they insert into one leaflet of the bilayer (Cochilla et al., 1999). Several studies have shown that synaptic vesicles stained with the yellow styryl dye FM1-43 lose dye much more slowly than would be expected if vesicles fully collapsed during exocytosis (Aravanis et al., 2003; Klingauf et al., 1998; Verstreken et al., 2002). If dye trapped in the vesicle's membrane left by lateral diffusion during exocytosis, then dye should leave the vesicle within milliseconds (Zenisek et al., 2002). It was found, however, that the rate of dye loss from vesicles is proportional to the dye's hydrophobicity (Klingauf et al., 1998). Because unbinding is rate limiting, dye must be prevented from leaving vesicles by lateral diffusion, supporting either the idea that vesicles open and close so rapidly that dye has little time to escape, or the idea that a mechanism exists to prevent the escape of vesicle components into the plasma membrane. Slow loss of dye recently has been detected even in single vesicles stained with FM1-43 (Aravanis et al., 2003). Imaging studies of single exocytic events using synapto-pHluorin have further supported a rapid recycling route for synaptic vesicles. VAMP was found to remain in vesicles and in some instances (44 %) was rapidly recaptured from the cell surface within milliseconds (Gandhi and Stevens, 2003).

The sequestering of secretory vesicle components also has been observed in endocrine cells. For example, when chromaffin cells were depolarized, immuno-reactive granule proteins appeared as patches on the cell surface (Patzak et al., 1987; Patzak et al., 1984; Patzak and Winkler, 1986; Phillips et al., 1983; Wick et al., 1997). These patches were then internalized. Clearly, not all of the components of secretory vesicles disperse into the plasma membrane during exocytosis. Patching of vesicle components also has been seen during the exocytosis of cortical granules in sea urchin eggs. When cortical granules underwent calcium-triggered exocytosis, patches of granule proteins remained separate from the plasma membrane and were recaptured intact following fusion (Smith et al., 2000; Whalley et al., 1995).

While many models have been based on the assumption that a cell uses either the full-fusion or kiss-and-run mechanism at the exclusion of the other pathway, several studies have shown that two distinct phases of endocytosis occur in neurons after triggered exocytosis. Capacitance measurements of the large terminals of retinal bipolar cells detected two phases of membrane recovery after exocytosis depending on the strength of the initial stimulus. With strong stimulation, the time constant was slow with a halftime of 20 s. With weaker stimulation, the recovery was much faster with a half time of 2 s (von Gersdorff and Matthews, 1994). The authors concluded from this study that the two observed rates of endocytosis might reflect two different endocytic mechanisms, one rapid and one slow. It is interesting to note that the slow phase, possibly corresponding to a clathrin-mediated process, occurred during strong stimulation

when the terminal was likely overwhelmed by added membrane. Other work has shown that the recovery of added membrane occurs at two distinct rates depending on internal calcium concentration in the cochlear inner hair cell. A slow (~15 s) phase was seen at lower intracellular calcium, and a rapid (~300 ms) phase was observed when calcium was higher (Beutner et al., 2001). Again, this work supports the view that there are two separate mechanisms for recovering membrane after exocytosis. In light of these studies it is possible that both Ceccarelli and Heuser were correct. Two modes of endocytosis could occur in the same terminal, yet they might be used for separate purposes depending on the activity of the cell.

Clearly, the numerous endocytic pathways provides the cell with many options for the recovery of vesicles components (Conner and Schmid, 2003; Jarousse and Kelly, 2001). Neurons and, to a lesser extent, neuroendocrine cells require a steady supply of vesicles to maintain high levels of secretion. This situation might have lead to parallel endocytic pathways in order to ensure the speedy recovery of vesicle components. Both kiss-and-run and full-fusion likely operate in even the same cell, possibly simultaneously (Gandhi and Stevens, 2003). The kiss-and-run pathway would offer cells the additional option of regulating the quantity of content released during fusion and even the speed with which molecules exit the vesicle. For example, dopamine neurons have been shown to release 25-30% of their vesicle dopamine content through flickering fusion pores (Staal et al., 2004). Thus, by utilizing the kiss-and-run pathway, the

cell might be able to regulate secretion by tightly controlling not only the number of vesicles that fuse but also the amount each releases.

Figure 1.4

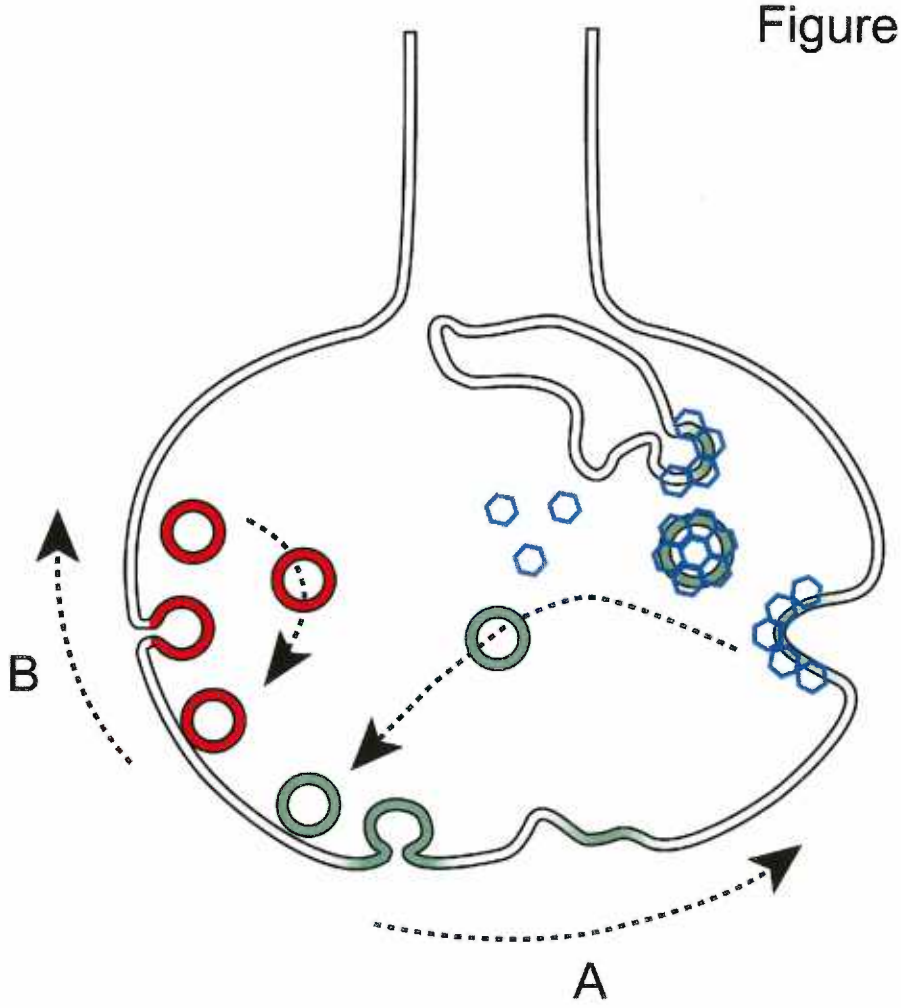

Fig. 1.4. Two modes of endocytosis. (A) In the full-fusion model, a vesicle (green) fuses and collapses into the plasma membrane. This added membrane is then collected into a clathrin-coated pit and internalized. The clathrin-coated vesicle then fuses with an endosome or uncoats to form a new synaptic vesicle. (B) In the kiss-and-run model of exocytosis, a vesicle (red) fuses with the plasma membrane but does not exchange membrane components. The fusion pore of this vesicle then reseals, recapturing the vesicle intact. The vesicle is then ready to undergo another round of exocytosis.

Experimental system and biological questions

In this thesis, we sought to answer several basic questions regarding the processes of regulated exocytosis and endocytosis in cultured endocrine cells using the imaging technique of evanescent field microscopy (Steyer and Almers, 2001). Evanescent field microscopy illuminates a thin region where a cell adheres closely to a glass coverslip (Axelrod, 2001). In this "footprint," individual secretory granules can be seen as they move within nanometers of the plasma membrane and undergo exocytosis (Steyer and Almers, 2001; Steyer et al., 1997). In the following work, we used the cultured neuroendocrine PC12 cell line to study exocytosis (Tischler and Greene, 1978). PC12 cells grow readily in culture, have numerous dense-core granules, and can be transfected easily with plasmid DNA. PC12 cells also can be stimulated to undergo exocytosis with a local superfusion of a high potassium solution (Zerby and Ewing, 1996). High potassium depolarizes these cells, which opens calcium channels in their plasma membranes and allows calcium to enter the cytoplasm, triggering granules to undergo exocytosis (Pozzan et al., 1984). These experimental conditions are non-invasive and closely mimic natural processes in neuroendocrine cells.

Using these methods, we first tested whether granules fuse and collapse into the plasma membrane during triggered exocytosis, releasing all their contents, or whether granules undergo transient fusion, do not collapse, and release only certain molecules during exocytosis. Second, we tested whether the fusion pore that forms between the granule and the plasma membrane during

triggered exocytosis is permeable to lipid. Lastly, we asked whether granules are capable of resealing from the plasma membrane following exocytosis.

CHAPTER 2

Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells.

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Abbreviations: NPY, neuropeptide Y; tPA, tissue plasminogen activator; EGFP, enhanced green fluorescent protein; CFP, cyan fluorescent protein; DsRed, *Discosoma* coral red fluorescent protein.

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ABSTRACT

Classical cell biology teaches that exocytosis causes the membrane of exocytic vesicles to disperse into the cell surface, and that a cell must later retrieve by molecular sorting whatever membrane components it wishes to keep inside. We have tested whether this view applies to secretory granules in intact PC12 cells. Three granule proteins were labeled with fluorescent proteins in different colors, and two-color evanescent field microscopy was used to view single granules during and after exocytosis. Whereas neuropeptide Y was lost from granules in seconds, tissue plasminogen activator (tPA) and the membrane protein phogrin remained at the granule site for over a minute, thus providing markers for post-exocytic granules. When tPA was imaged simultaneously with cyan fluorescent protein (CFP) as a cytosolic marker, the volume occupied by the granule appeared as a dark spot where it excluded CFP. The spot remained even after tPA reported exocytosis, indicating that granules failed to flatten into the cell surface. Phogrin was labeled with green fluorescent protein (GFP) at its luminal end and used to sense the pH in granules. When exocytosis caused the acidic granule interior to neutralize, GFP-phogrin at first brightened and later dimmed again as the interior separated from the extracellular space and re-acidified. Re-acidification and dimming could be reversed by application of NH_4Cl. We conclude that most granules reseal in less than ten seconds after releasing cargo, and that these empty or partially empty granules are recaptured otherwise intact.

INTRODUCTION

When vesicles undergo exocytosis, their membrane must be retrieved by endocytosis in order to maintain a constant cell surface area. Classical work holds that exocytic vesicles flatten into the cell surface and allow their components to mix with the plasma membrane. These membrane components are then retrieved by molecular sorting, as occurs during clathrin-mediated endocytosis. In contrast, for the stimulated exocytosis of secretory granules it has been proposed that granules release their contents through a fusion pore and then reseal again once cargo has been released (Henkel and Almers, 1996; Palfrey and Artalejo, 1998). Aside from the loss of cargo and some membrane components, the granule remains intact and may be re-used or degraded. This mechanism may be referred to as granule cavity recapture, "cavicapture" (Henkel and Almers, 1996; Palfrey and Artalejo, 1998) or granule recapture. A related mechanism was proposed decades ago for synaptic vesicles, where it is referred to as "kiss and run" exocytosis (Valtorta et al., 2001).

Three experimental approaches support granule recapture. In the first, exocytosis of single granules was detected as step increases in membrane capacitance, an assay of cell surface area. Such studies showed that exocytosis can be reversible in mast cells (Fernandez et al., 1984; Spruce et al., 1990). When the release of catecholamine from chromaffin cells was simultaneously detected as an amperometric spike, the spike occurred almost precisely while exocytosis increased the cell surface area in a step (Albillos et al., 1997; Ales et al., 1999). Sometimes, however, a normal spike was accompanied by only a transient

increase in membrane capacitance, indicating that the connection between granule lumen and the outside, or fusion pore, had opened and closed again (Albillos et al., 1997; Ales et al., 1999). The second approach explored how the kinetics of amperometric spikes changed in intact cells while stimulus patterns varied (Elhamdani et al., 2001), or in permeabilized cells while they over-expressed proteins involved in exo- and endocytosis (Fisher et al., 2001). In particular, treatments expected to interfere with the endocytic protein dynamin caused amperometric spikes to broaden and their charge to increase, suggesting that dynamin caused granules to reseal before they discharged all their catecholamine (Graham et al., 2002). The third approach was based on imaging single granules (Angleson et al., 1999; Whalley et al., 1995; Williams and Webb, 2000). Sea urchin eggs retrieved large vesicles after fertilization triggered exocytosis (Whalley et al., 1995). In lactotropes, exocytosis caused granule matrices to bind the externally applied dye FM1-43, and some granules retained the dye after it was washed from the cell (Angleson et al., 1999). In PC12 cell membrane patches, exocytosis caused some granules to take up and sequester an extracellular marker, indicating that the interior of the granule had transiently connected to the external space (Holroyd et al., 2002). In chromaffin cells, horseradish peroxidase appeared in organelles indistinguishable from granules soon after the stimulation. Once again this was interpreted as granules transiently connecting their lumen with the external space (Henkel et al., 2001).

The above results provide strong evidence for granule recapture but questions remain. In the first approach, cases of fusion pore closure were too rare

at normal external [Ca^{2+}] (Albillos et al., 1997; Ales et al., 1999) to support a significant role of this mechanism under physiologic conditions. This could have been the result of observing only spontaneous fusion events. In the second approach, the observed effects on catecholamine release were subtle, and it was not clear when changes in the amount released reflected changes in the degree of emptying rather than in the catecholamine content of the granule. Finally, in some imaging experiments it was not clear whether the large vesicles retrieved were empty granules or vesicles that newly formed through invagination of the plasma membrane (Whalley et al., 1995). Other imaging studies explored a time scale of 15 to 30 minutes and carried little information on how long granule cavities remained open, except that closure was unlikely to occur on the millisecond timescale implied by the duration of amperometric spikes (Graham et al., 2002).

Here we explore the post-exocytic fate of granules when intact and unperturbed PC12 cells were stimulated by membrane potential changes. Our results confirm three predictions of granule recapture in PC12 cells: that a granule membrane protein fails to disperse, that granules keep their shape, and that they reseal after exocytosis.

METHODS

Constructs and Cells.

NPY-EGFP and NPY-CFP were made by excising the open reading frame of human pro-neuropeptide Y (NPY) by restriction digestion from the HindIII and EcoRI sites in NPY-GFP (Lang et al., 1997) and by subcloning the fragment into the pEGFP-N1 and pECFP-N1 parent vectors (Clontech, BD Bioscience, Palo Alto, CA). To fuse rat tissue plasminogen activator (tPA) to the yellow fluorescent protein, Venus (tPA-Venus), we removed the coding sequence of EGFP from tPA-EGFP (Lochner et al., 1998) by digestion with Age1 and BsrG1, and replaced it with the open reading frame of Venus (Nagai et al., 2002). The predicted molecular mass of tPA-EGFP is 97 kD, 3 times that of processed NPY-EGFP (31 kD). Phogrin-DsRed was made by excising the phogrin open reading frame from the phogrin-EGFP plasmid (Pouli et al., 1998) and subcloning it into the EcoRI/Age1 site of the DsRed-N1 vector (Clontech). The open reading frame of rat syntaxin 1A was amplified with the Expand High Fidelity PCR system (Boehringer Mannheim, Mannheim, Germany) using the forward primer 5'-GAAGATCTCGAGGGAAGCTTGCCACCATGAAGGACCGAACCCAGGAG and the reverse primer 5'-CCATCGGGGGCATCTTTGGAGGGGTACCCGGGATCCGCG. The PCR product was subsequently ligated into the HindIII/Kpn1 site of pEGFP-N1 vector (Clontech). We obtained tPA-EGFP from B. Scalettar, Venus from A. Miyawaki, Phogrin-EGFP from G. Rutter, and syntaxin 1A from R. Scheller.

PC12-GR5 cell stocks (provided by R. Nishi) were maintained in T80 flasks (Nalgene Nunc, Rochester, NY) at 37°C, 10% CO_2 in DMEM high glucose (Invitrogen, Carlsbad, CA) supplemented with 5% New Calf Serum (NCS) and 5% Horse Serum (HS). For imaging, cells were replated onto Poly-L-Lysine (Sigma) coated high refractive index glass coverslips (n_{488}=1.80; Plan Optik, Elsoff, Germany) and allowed to adhere overnight. Cells were then transfected with 1 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

Experiments were performed 24 to 48 hours after transfection. Cells were placed in imaging buffer (in mM: 130 NaCl, 2.8 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4, 300 mOsm) and each coverslip was imaged for up to two hrs. To stimulate secretion, individual cells were locally perfused through a micropipette (4 μm tip diameter) with a solution of elevated $[K^+]$: (105 KCl, 50 NaCl, 2 $CaCl_2$, 0.7 $MgCl_2$, 1 NaH_2PO_4, 10 HEPES, as ph 7.4, 330 mOsm). In some experiments, cells were locally perfused with a solution where 50 mM NaCl was replaced by 50 mM NH_4Cl. All chemicals were obtained from Sigma. Experiments were carried out at room temperature (28 ^{o} C). Means are given $\pm$ SE.

Fluorescence microscopy.

To selectively illuminate the plasma membrane and its associated granules, we used evanescent field illumination (Axelrod, 2001; Steyer and Almers, 2001). Cells were grown on high refractive index glass coverslips and viewed with an

inverted microscope (IX-70; Olympus America, Melville, NY) using a 1.65 NA objective (Apo x 100 O HR, Olympus), as described (Merrifield et al., 2002). Fluorescent proteins of different colors were imaged simultaneously with an image splitter (Multispec MicroImager, Optical Insights, Santa Fe, NM) that separated the emission components of two fluorescent proteins into two channels that were then projected as side-by-side images on the back-illuminated chip of a CCD camera (Micromax, Roper Scientific, Trenton, NJ). Images were acquired at 2 Hz at 150 ms exposure using Metamorph software (Universal Imaging, Downingtown, PA). The two images were brought into focus in the same plane by adding weak lenses to one channel, and they were brought into register by careful adjustment of the mirrors in the image splitter. To correct for any residual misregistration or small differences in magnification, we took pictures in both colors before each experimental session of scattered 300 nm beads (2-FY-300, Interfacial dynamics, Portland, OR) fluorescing at both wavelengths. Beads in the two images were brought into superposition to within 1 pixel by shifting, stretching or shrinking one image using a program written in MATLAB. Optical magnification was to 119 nm/pixel except in Fig. 2.3 (67 nm/pixel).

EGFP and DsRed were both excited by the 488 nm laser line as described (Merrifield et al., 2002). Images were acquired for 3.3 min at 2 Hz. To excite CFP and Venus, an acousto-optic modulator (AOM) (Neos, Melbourne, FL) alternated the laser beam between 458 nm and 514 nm. The beam then passed through a custom dual wavelength filter passing the 450-464 nm and 504 -520 nm bands. Light was directed into the objective with a custom dichroic mirror that reflected

from 425 to 462 nm and from 507 to 537 nm, and transmitted from 463 nm to 506 nm and from 538 nm to 614nm. A 520 nm dichroic mirror (Chroma 520DCLP) in the image splitter separated cyan and yellow fluorescence. Cyan passed through a 25 nm band pass filter centered at 480 nm, and yellow through a 565 longpass filter. Since 458 nm and 516 nm excitation alternated, the resulting stack had to be de-interleaved off-line into 458 nm and 516 nm stacks. For analysis we used only the cyan image of the 458 nm stack and the yellow image of the 516 nm stack. Images were acquired at 1 image pair/s. Aside from being imaged with a camera, cells could also be observed by eye through dual emission filters placed into the eyepieces. Each transmitted from 470-496 nm and beyond 533. All filters and mirrors were from Chroma Technology Corp. (Brattleboro, VT).

Image Analysis

Evanescent field illumination selectively images the "footprint" of a cell where the cell adheres closely to the coverslip. Footprints were scanned by eye for individual exocytic events. The coordinates and time of occurrence of each event were marked and a 3.6 μm x 3.6 μm square region was centered on the brightest pixel of the first frame showing exocytosis. Onset of exocytosis was defined as the first frame showing a significant fluorescence increase of the granule. The 3.6 μm x 3.6 μm square was also placed on all other frames in the sequence and, in two-color experiments, were transferred to the corresponding point in the other-color image. The 3.6 μm x 3.6 μm squares thus defined were excised from the movies and stored as mini-stacks. A 1.2 μm diameter circle was centered on the fusing granules and the average fluorescence therein was measured. The circle

was also transferred into the corresponding location in the other-color image, and the fluorescence measured. The local background was determined as the average fluorescence in a concentric annulus with 3.6 μm outer diameter and subtracted. Since the 3.6 μm annulus often contained other granules, this method systematically subtracts too much background.

RESULTS

Imaging exocytosis in PC12 cells by evanescent field fluorescence microscopy. Since we wished to track the fate of single granules after exocytosis, first experiments were carried out to establish that we could reliably stimulate exocytosis in PC12 cells and detect it by imaging. Fig. 2.1A shows a PC12 cell expressing a 4 kD granule protein, neuropeptide Y, fused to green fluorescent protein (NPY-EGFP) (Lang et al., 1997). The evanescent field (EF) selectively illuminated fluorophores within about <100 nm of the plasma membrane (Steyer and Almers, 2001) and thus imaged only the "footprint" where the cell adhered tightly to the coverslip. Single NPY-EGFP -containing granules were visible as small fluorescent dots that were stationary for tens of seconds to minutes. Footprints as in Fig. 2.1A were outlined and their areas were measured. The average area was 310 μm^2 (n=5) and contained 0.358 ± 0.05 labeled granules/μm^2.

To stimulate exocytosis, external [K^+] was elevated by superfusing single cells through a micropipette. The elevated [K^+] made the plasma membrane potential less negative and thereby opened voltage-gated Ca channels. Fig. 2.1B shows a video clip of a granule undergoing exocytosis; the fluorescence at the granule site is plotted beneath. Upon fusion, the granule briefly lit up and then dimmed. The transient brightening is not well resolved at the image acquisition frequency used here, but faster recordings (data not shown) show NPY-EGFP emerging from a single granule in a cloud and rapidly diffusing away, as in bovine chromaffin cells (Steyer and Almers, 2001) and with acridine orange in INS-1 cells (Tsuboi et al., 2000). Brightening occurs both because the EGFP in the acidic granule encounters the neutral pH of the external medium, and because release against the coverslip moves NPY-EGFP closer into the evanescent field. After rising briefly in Fig. 2.1B, the fluorescence fell as NPY-EGFP was released. Sometimes measurable punctate fluorescence remained at the granule site even after exocytosis, indicating some granules failed to release all their NPY-EGFP.

Fig. 2.1C shows averaged results. Fluorescence rose for less than 1 s and then fell as NPY-EGFP was released. In differentiated PC12 cells, stimulation was reported to raise the pH in granules and cause EGFP to brighten already before exocytosis (Han et al., 1999) but any such effect in Fig. 2.1C must have been either too small to detect, or must have preceded the release of NPY-EGFP by less than 1 s. Fig. 2.1D shows a latency histogram of exocytic events. Elevated [K^+] caused vigorous exocytosis whose rate rose to a peak and then fell to lower

levels. However, fusion events occurred rarely or never before depolarization, hence the exocytosis observed here is a triggered event.

Other proteins leave granules more slowly. Although NPY-EGFP is well suited to report exocytosis, it leaves granules too fast to be useful as a marker for post-exocytic granule components. Two proteins were found to leave granules much more slowly, tissue plasminogen activator (tPA) and phogrin. tPA is a 70 kD serine protease normally found in PC12 cell and chromaffin granules (Parmer et al., 1997). To verify that tPA is targeted to granules, we fused tPA to the yellow fluorescent protein Venus (Nagai et al., 2002), and co-expressed tPA-Venus it with NPY-CFP. Fig. 2.2A shows a fluorescence image separated into its cyan and yellow components. Small circles were drawn around the tPA-Venus labeled structures in the yellow image and transferred into the cyan image. Nearly all tPA-Venus positive spots (86 ±2 % of 126 granules in 2 cells) co-localized with NPY-CFP positive granules. At least near the cell surface, therefore, most or all tPA-Venus labeled structures were granules. Next, exocytosis was stimulated in a cell expressing tPA-EGFP. A representative tPA-EGFP fusion event (Fig. 2.2B) is shown together with the time course of fluorescence at the granule site (Fig. 2.2C). After brightening the granule dimmed slowly, either because the tPA is released, and/or because the granule resealed and re-acidified (see later). Clearly tPA-EGFP was retained in granules much longer than NPY-EGFP (for similar results with tPA-Venus see Fig. 2.3B).

The granule membrane protein phogrin is made as a 112 kD precursor that is later shortened by intra-luminal cleavage to 60 kD (Wasmeier and Hutton,

1996). Phogrin was fused at its cytoplasmic end to the red fluorescent protein DsRed. To verify that phogrin was targeted to granules, cells were co-transfected with phogrin-DsRed and NPY-EGFP. Phogrin-DsRed fluorescence was punctate, and 84% ±6% of the red phogrin spots co-localized with green NPY-EGFP spots (137 granules in 3 cells from experiments as in Fig. 2.2A). Like tPA-Venus, therefore, phogrin-DsRed selectively labeled granules. Cells were stimulated with elevated [K^+] as in Figs. 2.1 and 2.2B. Fig. 2.2D shows a granule labeled with both NPY-EGFP and phogrin-DsRed undergoing exocytosis. Whereas the NPY-EGFP signal vanished after exocytosis, the phogrin-DsRed signal diminhished only slightly, and a fluorescent spot remained throughout. Related results have been obtained in INS-1 cells (Tsuboi et al., 2000). Fig. 2.2E shows averaged fluorescence measurements from experiments as in Fig. 2.2 D. Most NPY-EGFP was lost rapidly from the granule as in Fig. 2.1, but phogrin-DsRed fluorescence remained nearly constant; what changes did occurr are attributable at least in part to bleaching or minor movement. Evidently, most phogrin remained in the granule and failed to migrate into the plasma membrane for more than 1 min after exocytosis. The persistence of phogrin and, to a lesser extent, of tPA at exocytic sites makes both proteins useful probes for monitoring the fate of granules after exocytosis.

Most granules do not collapse into the plasma membrane after exocytosis. In chromaffin cells (Wick et al., 1997) and sea urchin eggs (Smith et al., 2000), membrane-associated granule components remain as patches on the cell surface after the granules have performed exocytosis (Wick et al., 1997), and have

flattened into the plasma membrane (Wick et al., 1997). The following experiment shows that PC12 cell granules do not readily flatten into the plasma membrane. Cells were co-transfected with tPA-Venus as a granule marker and with CFP as a cytoplasmic marker. Where the volume of a granule excludes cytosolic CFP, we expect diminished CFP fluorescence to provide a negative image of the granule. Exocytosis was stimulated as in Fig. 2.1 and movies were recorded as alternating images of tPA-Venus and CFP. The CFP channel showed a mottled fluorescence where tPA-Venus-labeled and unlabeled structures excluded CFP, and some cells showed extended dark regions possibly resulting where the cell membrane had lifted itself out of the evanescent field (not shown). Where such regions did not interfere, however, the CFP image showed a dark spot at the site of 21 of 31 granules (5 cells) that later performed exocytosis. In each of the 21 granules, the spot persisted after exocytosis. Fig. 2.3A shows images from a clip showing the average of movies temporally aligned to the moment of fusion. For 50 s before fusion, a dark spot in the center of the CFP image superimposed on the spot of tPA-Venus fluorescence, and the spot remained after exocytosis for the duration of the recording. While tPA-Venus fluorescence increased 12 fold and then declined, CFP fluorescence at the granule site showed a deficit that failed to diminish after fusion (Figs. 2.3B,C). GFP diffuses freely in cytoplasm with a diffusion coefficient one-third that in aqueous solution (Swaminathan et al., 1997), or about 30 μm^2 /s. The same may be assumed for CFP. Thus the failure for CFP to fill the dark spot at the granule site indicates that CFP continued to be excluded. Evidently granules do not, or do not fully, collapse into

the plasma membrane and instead retain their volume for >1 minute after exocytosis.

Most granule cavities reseal after exocytosis and re-acidify. We next asked whether the granules resealed or whether their lumen remained continuous with the external space during the 100 s post-exocytic interval explored in Fig. 2.3. Again phogrin was used as a granule marker, but this time it was conjugated with EGFP near the end of phogrin's luminal domain (EGFP-phogrin). A mutation blocked cleavage of phogrin at its intra-luminal cleavage site, and ensured that the EGFP remained on the protein. In addition, a C-terminal 18 kD fragment was removed to limit the size of the construct (Ohara-Imaizumi et al., 2002). Like tPA-EGFP, the construct served as a pH sensitive probe of the granule lumen. EGFP-phogrin labeled granules brightened as exocytosis connected their lumens with the external space and neutralized the acidic pH (Fig. 2.4A). Later the granules dimmed again, approximately to their pre-fusion values. This result is analyzed in Fig. 2.4B,D, and is in striking contrast to the result with phogrin-DsRed. Since the main difference between the constructs was the location and pH sensitivity of the chromophore, we conclude that the dimming of EGFP-phogrin reports the re-acidification of the granule after it had resealed and disconnected from the extracellular space (fission). To gain insight into the timing of fission, we plotted the time integral of Fig. 2.4B to remove high-frequency noise (Fig. 2.4C). The trace reached half its final value within 8.0 $\pm$ 1.4 s after fusion (14 events in 3 cells), indicating that half the fluorescence gained during exocytosis was quenched within 8 s. Clearly fission occurred in less than 8 s after the average

fusion event. It may have occurred in much less than 8 s, as much of this time may be taken up by acidification of already fissioned granules.

To confirm that dimming reports re-acidification of a sequestered compartment, we stimulated cells and waited for EGFP-phogrin labeled granules to brighten during exocytosis and then to dim. We then applied external ammonium chloride to supply NH_3, a membrane permeant base that neutralizes the acidic pH of organelles (Klempner and Styrt, 1983; Sankaranarayanan and Ryan, 2000). Fig. 2.4E, F shows fluorescence traces from two exocytic events. As in Fig. 2.4 A,B,D the granules first brightened and then dimmed. NH_4Cl caused the granules to brighten again (Fig. 2.4 E,F), and caused brightening even in granules that had not undergone exocytosis (Fig. 2.4G). As a control, cells were transfected with syntaxin-EGFP, a construct carrying EGFP on the extracellular side of the plasma membrane where NH_4Cl is not expected to cause pH changes. Whereas external acidification caused the strong and reversible drop in fluorescence expected from the pH dependence of EGFP (Llopis et al., 1998; Patterson et al., 1997), NH_4Cl caused little or no fluorescence change (Fig. 2.4H). We conclude that NH_4Cl caused granules to brighten in Figs. 2.4 E-G by collapsing proton gradients and not through a direct effect on EGFP.

We analyzed 21 granules that fused <100 seconds before the NH_4Cl perfusion. Thirteen were seen to brighten in response to NH_4Cl; these granules must have resealed and re-established a proton gradient. Five became invisible before NH_4Cl was applied and could not be scored. Three granules failed to

brighten and presumably lost most of their phogrin into the plasma membrane. In the majority of granules, therefore (between 62% and 86% in this dataset), the dimming reported resealing.

DISCUSSION

We have explored the release and retention of three proteins after the stimulated exocytosis of secretory granules in intact PC12 cells. The release of NPY-EGFP was so rapid that we could not resolve it well. However, tPA-EGFP and tPA-Venus were released more slowly, and the release of the membrane protein phogrin was altogether too slow to be detected reliably during our observation period. Phogrin and tPA were used as markers of post-exocytic granules or granule remnants. By imaging the volume excluded by a granule, we found that most granules failed to collapse into the cell surface and instead retained their volume for >100 s, thus offering refuge for proteins that were reluctant to leave. Within the 100 s most granules re-acidified their lumen and must therefore have disconnected from the cell surface. Hence most granules in PC12 cells reseal after exocytosis.

The advantages to a cell in adopting this type of exocytosis have been discussed (Henkel and Almers, 1996; Meldolesi and Ceccarelli, 1981; Palfrey and Artalejo, 1998). When secretory vesicles avoid mixing their membrane components with the cell surface in the first place, cells save themselves the effort of re-assembling these components during endocytosis. Some of this benefit would accrue even when granules membranes flatten into the cell surface as long

as their components stay together in patches, as seen after prolonged stimulation in bovine chromaffin cells (Wick et al., 1997). Retrieving intact secretory vesicle cavities as in "kiss and run" exocytosis would strongly benefit small synaptic terminals that must re-cycle a limited reservoir of synaptic vesicle membrane in repeated rounds of exo- and endocytosis (Harata et al., 2001). However, it is less clear how often recaptured granules are used again for exocytic hormone release. They lack the molecular machinery to re-fill with proteins, but may well re-accumulate small neurotransmitters such as catecholamines. Indeed, granule components and perhaps entire recaptured granules can participate in a second round of catecholamine release when chromaffin cells are re-stimulated after 5 minutes of rest (von Grafenstein and Knight, 1992). Exocytosis of recaptured granules was also observed in PC12 cell membrane patches (Holroyd et al., 2002).

We cannot tell exactly how rapidly granules resealed after exocytosis. However, half the exocytic brightening of EGFP-phogrin is reversed in less than 8 s. Granules in other cells reseal more slowly. In MIN6 pancreatic beta cells, EGFP-phogrin labeled granules remained bright for tens of seconds before they began to dim (Ohara-Imaizumi et al., 2002) and in chromaffin cells, granules occasionally stay open for minutes (D. Perrais, unpublished). In PC12 cells, 8 s is too short for a granule to completely release a protein such as tPA; and significant amounts of tPA remain in granules long after exocytosis. We do not know why tPA-EGFP is released so slowly, since its molecular mass is only about 3 times larger than that of NPY-EGFP. It may be bound to components of the granule

membrane. Nonetheless, the results show that resealing of granules results in the differential release of cargo.

How granules reseal is unknown. In principle, the resealing process could represent the molecular reverse of the opening of the fusion pore. If fusion pore opening results directly from the assembly of SNARE *cis*-complexes, then its molecular reversal requires the partial disassembly of *cis*-complexes. This may pose energetic puzzles (Holroyd et al., 2002); nonetheless molecular reversal has been considered for synaptic vesicles in hippocampal terminals where fusion pores are thought to stay open for < 6 ms (Stevens and Williams, 2000). Alternatively, the resealing process may require the recruitment of a dedicated fission machinery, either before or after exocytosis. The endocytic protein dynamin has been proposed to serve this function (Artalejo et al., 1995; Graham et al., 2002; Holroyd et al., 2002). Two-color evanescent field microscopy seems well suited to test this idea, and to test for the transient recruitment of other proteins to spatially defined sites in the plasma membrane (Merrifield et al., 2002).

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Figure 2.1

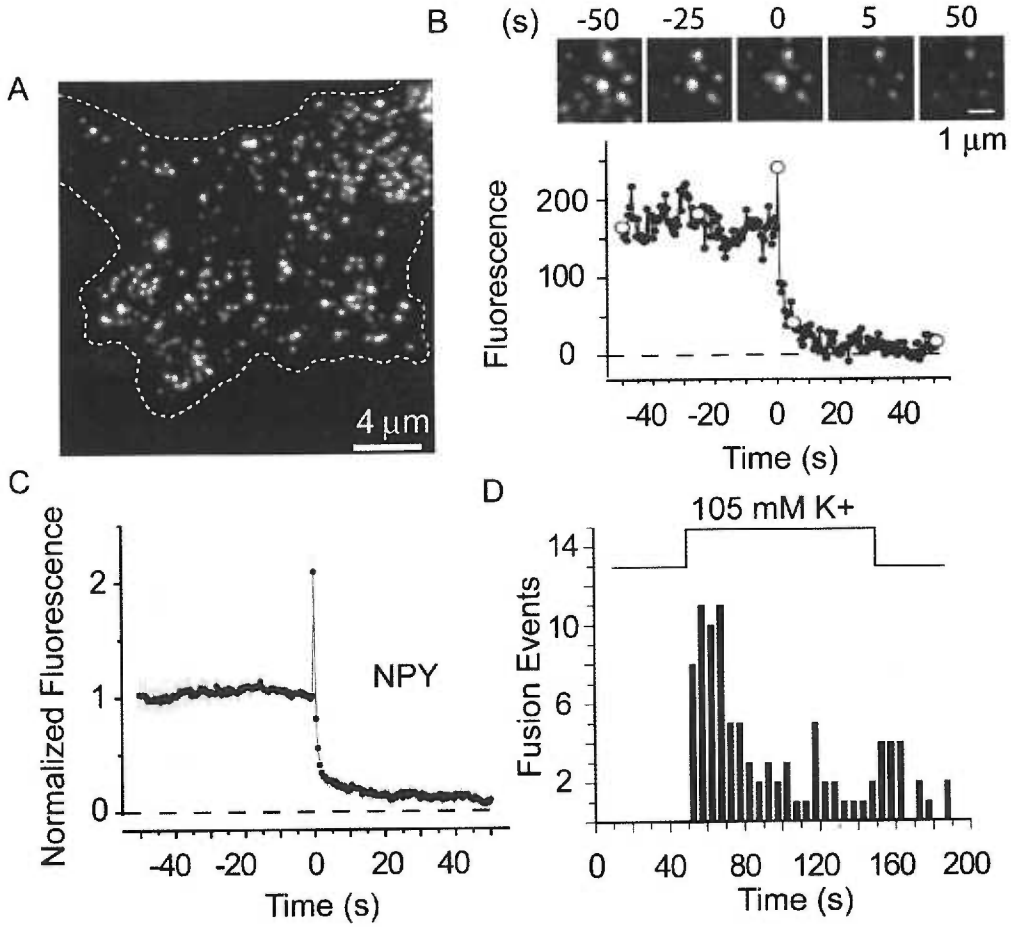

Fig. 2.1. Exocytosis of single NPY-EGFP labeled granules. (A) Evanescent field micrograph of the footprint of a live PC12 cell (dashed line) expressing NPY-EGFP. (B) A single NPY-EGFP labeled granule undergoing exocytosis (top) and the fluorescence at the granule site (bottom). Open circles refer to images shown. There is a strong fluorescence increase lasting for a single frame; this transient increase defines the moment of fusion and the time origin. Traces as in (B) were aligned to the moment of fusion and normalized to the intensity at the granule site during the last 2 seconds before fusion. The results were then averaged (C) (43 events 4 cells). (D) Number of fusion events in 5 s intervals is plotted against time after raising [K+] (8 cells, 96 events).

Figure 2.2

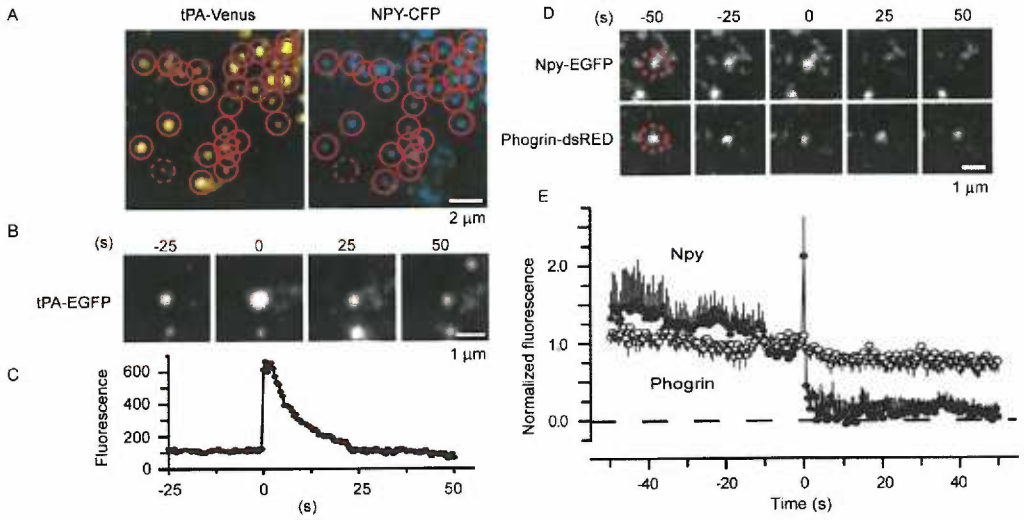

Fig. 2.2. Other granule proteins disperse more slowly after exocytosis. (A) Co-localization of tPA-Venus and NPY-CFP. Circles were drawn around fluorescent dots in the tPA-Venus image and transferred into the NPY-CFP image. The dotted circle shows an example where no NPY-CFP was detected at the site of a tPA-Venus granule, and where colocalization was scored negative. (B) tPA-EGFP labeled granule undergoing exocytosis. Here and in other figures, the apparent widening of the fluorescent spot at 0 s artifactally resulted from printing all four panels at the same contrast, the effect is not seen if the panel is displayed so that saturation of grey levels is avoided (not shown). (C) Fluorescence at the granule site. (D) Two-color imaging of a single NPY-EGFP and phogrin-DsRed labeled granule undergoing exocytosis. Granule position outlined by dashed circle. (E) Average fluorescence trace of granules containing both NPY-EGFP and phogrin-DsRed. Fluorescence traces were temporally aligned to the moment of fusion as reported by NPY, then normalized to the last 2 s before fusion and finally averaged as in Fig. 2.1B. Throughout, time is relative to the moment of fusion.

Figure 2.3

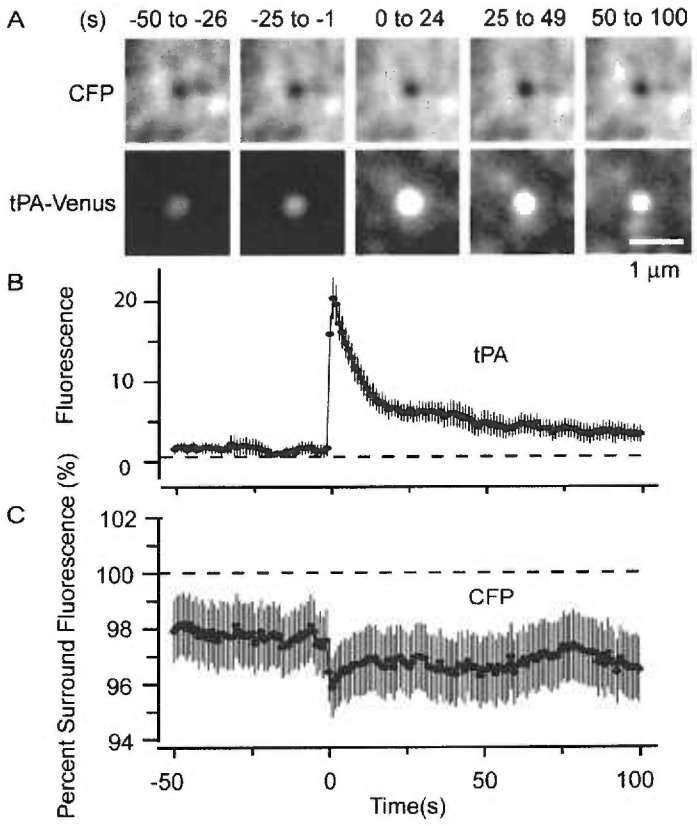

Fig. 2.3. Failure of granules to collapse into the plasma membrane. (A) Two-color imaging of single granules from cells co-transfected with CFP and tPA-Venus. Cyan (top) and yellow images (bottom) are averages of 18 fusion events in three cells lacking extended dark regions in the CFP channel that may have resulted from failure of cells to adhere uniformly. Movies showing the small regions were aligned to the moment of fusion as reported by tPA-Venus, and then averaged. The panels show averages of 25 successive frames each. (B) Fluorescence signals at the sites of a tPA-Venus labeled granules (including those in A) were plotted against time, aligned to the moment of fusion and averaged (5 cells, 31 events). (C) CFP fluorescence at granule sites in B. No background was subtracted; instead, the CFP fluorescence at the granule site was calculated as a percentage of the intensity in a concentric annulus of 3.5 μm outer diameter. The downward spike at the time of fusion and the apparent increase in the fluorescence deficit were not consistently observed and their origins are not understood.

Figure 2.4

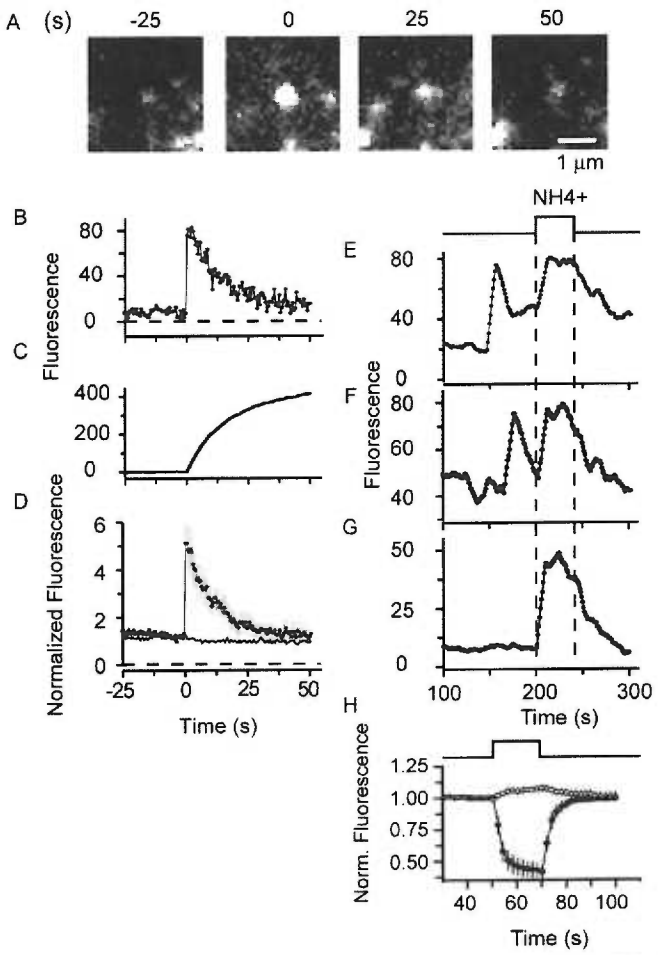

Fig. 2.4. Granules re-seal after exocytosis and re-acidify. (A) EGFP-phogrin labeled granules brighten transiently on exocytosis. (B) Plot of the fluorescence intensity at the granule site, along with its time integral (C). (D) Average of 14 events in 3 cells, obtained as in Fig. 2.1C. Continuous line re-plots data with phogrin-DsRed from fig. 2E. (E)-(G), brightening of three granules during application of 50 mM NH4Cl. The granules in (E), (F) had previously undergone exocytosis while the granule in (G) had not. (E)-(G) are plotted as rolling averages of 10 successive measurements to reduce noise. Background was measured as the average of intensities in seven 1.2 mm circles in the image of each cell placed where granules were lacking. (H) Fluorescence changes in cells expressing syntaxin-EGFP on the cell surface. The fluorescence drops markedly when the external medium is acidified (35 µm2 membrane area, average of 6 cells) but changed only slightly when the NH4Cl containing solution is applied (6 cells) This change may be due to acidic docked granules that containing small amounts of syntaxin.Since EGFP-phogrin was poorly expressed in our cells, we sought to increase the Ca influx and thus raise the frequency of exocytic events in experiments as in E, F. This was done by maintaining 50 mM CaCl2 externally, both before (62.5 NaCl, 3 KCl, 50 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose, pH 7.4, 300 mOsm) and during stimulation (105 KCl, 50 mM CaCl2, 0.7 mM MgCl2, 1 mM NaH2PO4, 10 mM HEPES, 379 mOsm; all concentrations in mM). The 50 mM [Ca2+] did not significantly change the fluorescence response during exocytosis in EGFP-phogrin expressing cells. Fluorescence rose abruptly by a factor 5.5 + 0.8 and declined with a half-time of 7.9 + 0.9 s (16 events in 5 cells).

CHAPTER 3

Bilayers merge even when exocytosis is transient.

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Abbreviations: EGFP, enhanced GFP; mRFP, monomeric red fluorescent protein; NPY, neuropeptide Y; ΔF/F, fractional fluorescence change; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor.

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ABSTRACT

During exocytosis, the lumen of secretory vesicles connects with the extracellular space. In some vesicles, this connection closes again, causing the vesicle to be recaptured mostly intact. The degree to which the bilayers of such vesicles mix with the plasma membrane is unknown. Work supporting the kiss-and-run model of transient exocytosis implies that synaptic vesicles allow neither lipid nor protein to escape into the plasma membrane, suggesting that the two bilayers never merge. Here we test whether neuroendocrine granules behave similarly. Using two-color evanescent field microscopy, we imaged the lipid probe FM4-64 and fluorescent proteins in single dense-core granules. During exocytosis, granules lost FM4-64 into the plasma membrane in small fractions of a second. While FM4-64 was lost, granules retained the membrane protein, GFP-phogrin. Using GFP-phogrin as a probe for resealing, it was found that even granules that reseal lose FM4-64. We conclude that the lipid bilayers of the granule and the plasma membrane become continuous even when exocytosis is transient.

INTRODUCTION

In many types of cells, exocytosis causes the membrane of a secretory vesicle to flatten into the cell surface, and to release its components into the plasma membrane. It is now clear, however, that the membranous cavity of some vesicles reseal after exocytosis, thereby disconnecting the vesicle from the plasma membrane. The most direct evidence was obtained where exocytosis and resealing could be studied at the level of single vesicles, as was done in mast cell granules (Fernandez et al., 1984; Spruce et al., 1990), in endocrine granules (Albillos et al., 1997; Ales et al., 1999; Angleson et al., 1999; Henkel and Almers, 1996; Holroyd et al., 2002; Taraska et al., 2003) and in synaptic-like microvesicles of an endocrine gland (Klyachko and Jackson, 2002). The resealing of secretory vesicles is thought to be advantageous to cells because it saves them the effort of retrieving vesicle membrane components by molecular sorting.

How far does fusion progress before a vesicle reseals? We consider two possibilities. In the first, fusion is complete and the bilayers of the fusing compartments merge. Now the vesicle's membrane components may be free to leave, and their dispersal can be avoided only partially, and only if the vesicle reseals quickly. In the second, the lipid bilayers of the fusing compartments remain separate. Such fusion was observed when a viral envelope protein mediated cell-cell fusion, and when electric currents could pass between the fusing cells for minutes before fluorescent lipids could do so (Chernomordik et al., 1998; Tse et al., 1993; Zimmerberg et al., 1994). During such incomplete fusion, it is thought that fusion-mediating proteins such as viral envelope proteins

or SNAREs may remain at the fusion site. There, they form a fusion pore that allows the exchange of water-soluble molecules, yet also constitute a fence (Chernomordik et al., 1998) that prevents vesicle membrane components from leaving the vesicle.

Optical measurements of FM dye loss from synaptic vesicles in hippocampal neurons of mammals (Aravanis et al., 2003; Gandhi and Stevens, 2003; Klingauf et al., 1998; Pyle et al., 2000; Stevens and Williams, 2000; Zakharenko et al., 2002) and in motor neurons of flies (Verstreken et al., 2002) support a model in which vesicles can reseal. Indeed, the concept of resealing was first suggested for synaptic vesicles (Ceccarelli et al., 1973) where it has become known under the name "kiss-and-run" (Valtorta et al., 2001). If the retention of membrane components is the goal, then incomplete fusion seems advantageous, especially in synaptic vesicles where diffusion distances are smallest and membrane components would escape the fastest. Synaptic vesicles undergoing kiss-and-run were found to retain their entire store of the membrane protein VAMP/synaptobrevin (Gandhi and Stevens, 2003; Sankaranarayanan and Ryan, 2000). Most importantly, they were also found to retain half or more of the fluorescent lipid probe FM1-43 (Aravanis et al., 2003) even when they remained open to the external space for an entire second (Pyle et al., 2000). In kiss-and-run, synaptic vesicles evidently reseal before their bilayers have merged with the plasma membrane (Aravanis et al., 2003; Klingauf et al., 1998). After more conventional fusion, bilayers become continuous in milliseconds. This was shown

by using FM1-43 in giant synaptic terminals from goldfish retinal bipolar cells (Zenisek et al., 2002).

Must one assume that exocytosis is reversible only as long as the lipid bilayers of vesicle and cell remain separate? Here we determined in single vesicles how rapidly a fluorescent lipid escaped after exocytosis, and whether that same vesicle later resealed. Single granules of PC12 cells were found to lose the lipid FM4-64 rapidly, regardless of whether or not they later resealed.

METHODS

Plasmids, Solutions, Cells. The NPY-EGFP and EGFP-phogrin plasmids were described previously (Ohara-Imaizumi et al., 2002; Taraska et al., 2003). Phogrin-mRFP was made by removing the ORF of EGFP by restriction digestion from the Kpn1/Not1 sites in phogrin-EGFP (Tsuboi et al., 2000) and replacing it with the ORF of mRFP (Campbell et al., 2002). mRFP was a gift of R.Y. Tsien (UCSD, San Diego, CA). PC12-GR5 cell stocks were cultured as described (Taraska et al., 2003). Briefly, cells were plated onto poly (L-lysine)-coated high refractive index glass coverslips (n_{488}=1.80, Plan Optik, Elsoff, Germany). Cells were transfected with 1 μg of plasmid DNA using lipofectamine 2000 (Invitrogen) according to the manufactures instructions and incubated for no less than 24 hours. In some experiments, cells were then incubated for an additional 12 to 24 hours in culture media containing 6.4 μM FM4-64. They were then thoroughly washed for 0.5 - 1 hr in dye free media. For imaging, cells were maintained in imaging buffer (in

mM: 130 NaCl, 2.8 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4). To stimulate exocytosis, individual cells were depolarized by a 20 - 100 s long, local superfusion with elevated [K^+] from a micropipette (105 KCl, 50 NaCl, 2 $CaCl_2$, 0.7 $MgCl_2$, 1 NaH_2PO_4, 10 HEPES, pH 7.4). The exocytic events analyzed herein occurred between 0.5 and 60 s after the start of perfusion. In some experiments, the perfusion with elevated [K^+] was immediately followed by a second perfusion with an imaging buffer wherein 50 mM NaCl was replaced by 50 mM NH_4Cl. In others, cells were ruptured by a strong jet of imaging solution through a pipette placed within a few microns of a cell. Experiments were carried out at room temperature (28°C).

Fluorescence Microscopy. Cells were imaged with an inverted microscope (IX-70; Olympus America, Melville, NY) configured for evanescent field excitation as described (Merrifield et al., 2002; Steyer and Almers, 2001; Taraska et al., 2003) and equipped with a 1.65 NA objective (Apo x 100 O HR, Olympus) and an image splitter (Multispec MicroImager, Optical Insights, Santa Fe, NM) for the simultaneous imaging of red and green fluorescent probes. Fluorescence was excited by an argon/krypton laser (Coherent, Santa Clara, CA), usually at 488 nm and in some experiments (mRFP) simultaneously at 488 nm and 568 nm. Optical filters and dichroic mirrors were as in Merrifield *et al.* (Merrifield et al., 2002). The resulting green and red images were then projected side-by-side onto a back-illuminated CCD camera (Quantix 57, and Cascade 512 B for mRFP-phogrin, both from Roper Scientific, Trenton, NJ). Images were acquired using Metamorph

software (Universal Imaging, Dowington, PA). For precise alignment of the red and green images, we took pictures of 200 nm fluorescent beads (Molecular Probes, Eugene, OR.) fluorescing both red and green, and then proceeded as described (Taraska et al., 2003). Pixel size was 61 nm. Frames were acquired as streams at 33 Hz, or in time-lapse recordings with 150 ms exposures given at 2 Hz. To adjust the polarization of the incident laser beam, a quarter wave plate was placed in the laser path and turned 45° to rotate the polarization of the laser from vertical to horizontal. Polarization direction was confirmed with an analyzer.

Image Analysis. For colocalization analysis, overlap was determined by drawing small circles (610 nm) around NPY-EGFP granules in the green image and transferring the circles to the red image. Colocalization was scored positive when a red dot was found within the circle. The percent colocalization between NPY-EGFP and FM4-64 was determined in single cells and these values were then averaged.

To locate exocytic events, movies of the entire cell were scanned by eye for brightening events. The time and location of each fusion event in a cell were cataloged and small regions (3.11 μm x 3.11 μm) containing each granule were excised as a ministack. As described previously (Taraska et al., 2003), onset of exocytosis was defined as the first frame showing a visible increase in EGFP fluorescence over background. Regions of interest were centered on the brightest pixel of the granule during the first frame of exocytosis. In two-color experiments, regions located in the green image were transferred to the corresponding

coordinates in the red image, and the red channel ministacks were excised for analysis.

Two methods were used to measure granule fluorescence. In the first, fluorescence from EGFP or mRFP was measured in a 610 nm-diameter circle centered on the granule, and the fluorescence in a concentric annulus (1.2 μm outer diameter) was subtracted as local background. FM4-64 fluorescence was determined similarly except that background was measured in the entire surrounding area (3.11 x 3.11 μm square) in order to average out disturbances from abundant non-granule organelles. The second method was used to determine EGFP-phogrin fluorescence in granules where NH_4Cl application caused large fluorescence changes in neighboring granules, some located in the 1.2 μm diameter annulus. To isolate better the fluorescence of a granule from that of its neighbors, we used a program written in MATLAB and kindly provided by Dr. David Perrais. The program fitted images in ministacks with the sum of an inclined plane, and two circularly symmetric Gaussian functions, one for the granule of interest and another for its nearest neighbor. The fit had nine free parameters, three each for the inclined plane (the fluorescence in the central pixel and the slopes in the x and y directions) and the two Gaussians (the amplitudes, widths and center positions). The procedure isolated the granule of interest from the nearest surrounding granule. As the granule of interest moved within the region, its position was updated because it was a free parameter. The fluorescence of a granule was taken to be the amplitude of the Gaussian function fitted to that granule.

RESULTS

Secretory granules accumulate the styryl dye FM4-64. PC12 cells expressing the green fluorescent granule marker neuropeptide Y- EGFP (NPY-EGFP) (Lang et al., 1997; Taraska et al., 2003) were cultured overnight in media containing the red lipidic styryl dye, FM4-64 and then washed. They were then imaged with evanescent field fluorescence microscopy (Axelrod, 2001; Steyer and Almers, 2001), a technique that selectively images the plasma membrane and adjacent cytoplasm in the "footprint" where a cell adheres closely to the coverslip. The images in Fig. 3.1A show numerous green dots, each a secretory granule, as well as red dots representing organelles containing FM. There were more red dots than green dots (shown in the merged image), indicating that FM4-64 does not label granules specifically. Nonetheless, some FM4-64 labeled structures clearly contained also NPY-EGFP, suggesting that the dye also enters granules. This is seen better at higher magnification (Fig. 3.1B).

Because of the high density of FM4-64-labeled structures, we tested whether the colocalization of NPY-EGFP and FM4-64 was due to random overlap. Images of 30 NPY-EGFP granules were excised and added together, and the same was done with the corresponding region from the red channel (Fig. 3.1C). Both panels show a dot in the center, demonstrating that FM4-64 was strongly enriched at granule sites and that colocalization was not an artifact of probe density. The higher background in the FM4-64 image arises from the staining of other organelles.

To test whether FM4-64 stained the outer or inner leaflet of the granule membrane, cells were ruptured with a strong jet of buffer from a perfusion pipette. Plasma membrane patches that remained stuck to the coverslip contained green granules (Avery et al., 2000), and even minutes after cell rupture, most or all of the granules still contained FM4-64 (Fig. 3.1D). Since styryl dyes desorb from membranes into a surrounding solution within seconds (Ryan et al., 1996), FM4-64 must have been trapped within the lumen of the granules.

To determine the rate of FM uptake by granules, we incubated NPY-EGFP-transfected cells with FM4-64 and then counted the number of green granules containing FM4-64 over a period of hours. FM4-64 labeled 80% of the green granules within 3 hours (Fig. 3.1E left). FM4-64 was subsequently lost from most granules after a 6 h-wash in dye-free medium (Fig. 3.1E right). Evidently, FM4-64 enters granules reversibly.

Granules lose FM4-64 during exocytosis. To test whether FM4-64 escapes from granules during exocytosis we imaged individual granules labeled with NPY-EGFP and FM4-64. Fig. 3.2A shows video stills of a single granule containing both NPY-EGFP and FM4-64. As before (Taraska et al., 2003), exocytosis caused NPY-EGFP first to brighten and then to dim. The granule brightened as it connected with the external space and its internal pH rose, and as NPY-EGFP was secreted against the coverslip. Granules then dimmed in small fractions of a second as some or all of their NPY-EGFP diffused away. FM4-64 fluorescence dimmed just as rapidly, indicating rapid loss of the dye from the granule. The

experiment is analyzed in Figs. 3.2B,C where the fluorescence from both fluorophores is plotted against time, with average results shown in Figs. 3.2D,E. As soon as NPY-EGFP brightened, FM4-64 fluorescence declined. Thus, on the time scale of our measurements (30 ms per frame), FM4-64 started to leave as soon as the lumen of the granule established continuity with the extra-cellular space. As a control, neighboring granules that did not show a change in NPY-EGFP fluorescence and did not undergo exocytosis, did not exhibit a change in the FM4-64 signal (Fig. 3.2E).

FM 4-64 spreads into the plasma membrane. In hippocampal synaptic terminals, FM1-43, a yellow homologue of FM4-64, was found to leave synaptic vesicles by de-sorption from the vesicle membrane and loss into the external fluid, a process that takes of the order of a second (Aravanis et al., 2003; Klingauf et al., 1998). In retinal bipolar neurons, by contrast, dye leaves synaptic vesicles in < 10 ms by lateral diffusion into the plasma membrane (Zenisek et al., 2002). In terms of speed, the escape of FM4-64 from granules is more reminiscent of bipolar than of hippocampal neurons, as it is complete in less than 100 ms (Fig. 3.2E). When an exponential function was fitted to 3.2E, the time constant was 45 ms. For two reasons, even this small value is substantially overestimated. First, Fig. 3.2E is an average obtained from sequences temporally aligned to the moment of fusion, and such alignment is accurate only to within the exposure time for a single image. Second, the moment of exocytosis is expected to occur randomly at any time during the 30 ms acquisition time of a single image. Even if

alignment were perfect and FM4-64 left instantly, the resulting change in fluorescence would appear to take two frames, not much less than observed in Fig. 3.2E. Most probably the discharge of FM4-64 was complete within 30 ms, the exposure time of a single image.

We wondered whether the dimming of FM4-64 in exocytic granules occurred by loss of the dye into the imaging buffer, or by spread into the plasma membrane. This was tested by measuring the fluorescence of a large region centered on a granule (Fig. 3.3A). The larger region did in fact dim over the course of the recording, but this was likely due to photobleaching, as it occurred continuously and was temporally uncorrelated with exocytosis. At the moment of exocytosis, there was instead an increase in fluorescence (open circles in Fig. 3.3A). The increase was only small when our laser beam was s- polarized (Sund et al., 1999) and generated an evanescent field oscillating in parallel to the plasma membrane. It was much larger with a p-polarized beam (filled circles) whereby the evanescent field contains a component oscillating in a direction perpendicular to the plasma membrane. This direction is especially favorable for exciting styryl dyes in the plasma membrane (Zenisek et al., 2002). Fig. 3.3A indicates that exocytosis causes styryl dye to spread into the plasma membrane rather than being lost. First, the total amount of fluorescence increased. Second, the increase required p-polarized light, which excites FM1-43 more effectively when the dye is located in the planar plasma membrane than in a spherical secretory vesicles (Zenisek et al., 2002). Spread into the plasma membrane could be observed directly when multiple exocytic events are averaged (Fig. 3.3B).

Recapture of granules after exocytosis. To test whether granules resealed, we used the granule membrane protein phogrin with EGFP attached near its luminal domain. EGFP-phogrin serves as a probe of the intra-granular pH (Miesenbock et al., 1998). Like NPY-EGFP, EGFP-phogrin brightened on exocytosis as granules lost acid, and then dimmed again, as in the first three panels of Fig. 3.4A. Dimming may indicate re-acidification of the granule lumen. This was tested by superfusing the cell with imaging buffer containing NH_4Cl. NH_4Cl collapses proton gradients across internal membranes (Miesenbock et al., 1998), and will cause all EGFP-phogrin in acidic compartments to brighten (Miesenbock et al., 1998). Four granules lit up. Three were virgin granules, and the fourth was the one that had undergone exocytosis. Evidently that granule had resealed, and most or all its post-exocytic dimming was due to re-acidification. The experiment is analyzed in Fig. 3.4B. Fig. 3.4B1 plots the fluorescence of virgin granules as NH_4Cl was applied and withdrawn; and marks the time course of NH_4Cl action. Fig. 3.4B2 plots the fluorescence of the granule that underwent exocytosis and then resealed, and Fig. 3.4B3 that of another granule that remained open. In similar experiments, 32 of 78 granules brightened visibly when NH_4Cl was applied, while 32 others failed to do so. No verdict could be reached on the remaining 14 granules. Most (13) became invisible before application of NH_4Cl. These granules either lost their phogrin before NH_4Cl was applied, or had resealed and retracted into the cytosol. In one case the granule's image merged

with that of another. The median time between fusion and NH_4Cl application was 17 s (5-47 for granules that resealed, and 6 - 43s for granules that did not).

We wished to quantify the effect of NH_4Cl on the fluorescence of post-exocytic granules. The main difficulty was that post-exocytic granules were often close to virgin granules that had not undergone exocytosis but also brightened strongly. To minimize the disturbance from virgin granules, we fitted Gaussian functions both to the post-exocytic granule and to its nearest virgin neighbor (see Methods). The amplitude of the Gaussian fitted to the post-exocytic granule was taken to represent that granule's fluorescence. Measurements were made just before and just after the application of NH_4Cl and again while NH_4Cl was present. The difference between the two values, $\Delta F/F$, varied strongly among post-exocytic granules (Fig. 3.4C). Many such granules brightened, presumably because they had restored their pH gradient after they had resealed. Others did not strongly change their fluorescence, and still others apparently dimmed further.

As a control, the analysis was carried out also on granules that had undergone no exocytosis, and were mock-perfused with imaging buffer that lacked NH_4Cl. The fractional fluorescence change, $\Delta F/F$, varied much less (Fig. 3.4D). Most such granules dimmed, presumably because of photobleaching or because they moved some distance into the cytosol. $\Delta F/F$ was rarely positive; the median was - 5% and greater than 22% in none of 100. We next tested how the brightening of virgin neighbors influenced our analysis. This was done by adding to the images of the cells analyzed in Fig. 3.4D the image of an artificial granule, represented by a Gaussian profile of constant position and of an amplitude (13 pixel units) similar

to the mean amplitude measured for post-exocytic granules before NH_4Cl was applied. When $\Delta F/F$ was determined for the artificial granule, the median value was $\Delta F/F$ = - 7% (mean -8%, n = 100). Evidently, our fitting procedure underestimated the NH_4Cl-induced brightening of post-exocytic granules. The error was larger when the artificial granule was dimmer. For an artificial granule of amplitude similar to that of the dimmest post-exocytic granules (6 pixel units), the median value of $\Delta F/F$ was -25 %, with 2 of 9 values < -50%. Evidently our analysis becomes unreliable when post-exocytic granules are dim but close neighbors brighten strongly in the presence of NH_4Cl. This fact contributes to the apparent strong dimming of some post-exocytic granules in Fig. 3.4C.

To summarize, quiescent granules never brightened by more than 22% when no NH_4Cl was applied. Virgin granules that brightened caused errors, but these tended to make $\Delta F/F$ too negative, and hence to mask rather than accentuate the brightening of post-exocytic granules. Thus we consider that any post-exocytic granule brightening by more than 25% did so because NH_4Cl collapsed its proton gradient. Indeed, in all of 23 such granules, the brightening was also visually apparent. We consider that, at least in this subset, all granules resealed. In 26 other post-exocytic granules, $\Delta F/F$ < -5% in Fig. 3.4D. We consider that these granules remained open.

Loss of FM4-64 is independent of recapture. When synaptic vesicles are found to undergo "kiss-and-run" exocytosis, the fusion pore is a barrier to the exchange of styryl dyes (Aravanis et al., 2003). To test whether this applies also to resealing granules, EGFP-phogrin expressing cells were loaded with FM4-64 and imaged in two colors simultaneously. Granules were divided into two groups based on their response to NH_4Cl, containing the granules that resealed (filled circles) and those that remained open (open circles in Fig. 3.5A,B). Both groups showed a similar loss of EGFP-phogrin fluorescence, indicating that resealing granules dim by acidification about as fast as open granules lose EGFP-phogrin. More importantly, resealing granules lost as much FM4-64 as open granules. Evidently, granules in PC12 cells lose FM4-64 before they reseal.

Granules retain more phogrin when they reseal than when they stay open. In hippocampal neurons, it was found that the membrane protein VAMP/synaptobrevin remained in synaptic vesicles that resealed after exocytosis (Gandhi and Stevens, 2003). To test how much phogrin remains in granules after exocytosis (Ohara-Imaizumi et al., 2002; Taraska et al., 2003; Tsuboi et al., 2000), phogrin was labeled with monomeric red fluorescent protein mRFP (Campbell et al., 2002) at its cytosolic end, yielding phogrin-mRFP. Insensitive to the intra-granular pH, the mRFP in this construct is expected to report the amount of phogrin remaining in the granule. Along with phogrin-mRFP, cells expressed NPY-EGFP in order to mark the moment of exocytosis. Granules lost phogrin-mRFP fluorescence to variable extents. Figs. 3.5C1, 2 show two extreme cases.

One granule lost essentially none (Fig. 5C1) and another essentially all (Fig. 3.5C2). On average, granules retained about half their phogrin-mRFP during the first 20 s after exocytosis (Fig. 3.5D. This amount is less than in earlier work (Taraska et al., 2003) that used phogrin labeled with DsRed, a protein known to form tetramers (Baird et al., 2000).

Do granules retain more phogrin when they reseal? This was tested in EGFP-phogrin expressing granules analyzed as in Figs. 3.4. We divided the fluorescence of each granule in the presence of NH_4Cl by that measured just after exocytosis. The resulting ratio is expected to be proportional to the fraction remaining in the granule. It was significantly less ($p<0.0001$) in granules that remained open (white bars) than in those that resealed (black bars). Hence the loss of phogrin-mRFP in Fig. 3.5D is mostly due to granules that remained open. The finding suggests that resealing granules lose little or no phogrin, in some ways similar to synaptic vesicles that retain VAMP/synaptobrevin when they undergo kiss-and-run (Gandhi and Stevens, 2003).

DISCUSSION

Here we used a lipid probe to test whether the bilayers of individual granules in PC12 cells merge with the plasma membrane. We found that styryl dye escaped from single granules after exocytosis, with dye loss largely being completed within the first 30 ms after fusion. As in synaptic vesicles of retinal bipolar cells (Zenisek et al., 2002), dye escaped by lateral diffusion into the plasma membrane. The finding provides evidence that the lipid bilayers of the

granule and the plasma membrane become continuous within less than 30 ms after exocytosis. We further used the granule membrane marker EGFP-phogrin to test whether individual granules reseal after exocytosis (Taraska et al., 2003). By this method, nearly half of all granules were found to do so. When EGFP-phogrin and FM4-64 were imaged simultaneously, FM4-64 was seen to escape as readily from granules that resealed as it did from those that did not. Apparently, granules merge their bilayers with the plasma membrane, but this does not prevent half of them from resealing later. As soon as the bilayers connect, the SNARE molecules that have mediated fusion find themselves in the same membrane, and are free to form *cis*-complexes. These are so stable that their formation is essentially irreversible (Hayashi et al., 1994). As a consequence, the mechanism that reseals granules is unlikely to be the molecular reverse of fusion. Instead, resealing probably requires the recruitment of fission-mediating molecules, such as dynamin (Artalejo et al., 1995; Graham et al., 2002; Holroyd et al., 2002).

It has been proposed that synaptic vesicles also can reseal after they release transmitter (Ceccarelli et al., 1973; Torri-Tarelli et al., 1985). In this model of exocytosis the vesicle does not exchange membrane components with the plasma membrane (Valtorta et al., 2001). This idea has recently gained considerable attention under the name "kiss-and-run" exocytosis (Fesce et al., 1994; Jarousse and Kelly, 2001; Ryan, 2003). Some of the strongest evidence supporting kiss-and-run has been obtained by measuring the loss of the membrane dyes such as FM1-43 from synaptic vesicles. Specifically, hippocampal synaptic terminals and the neuromuscular junction of flies have been found to release

styryl dyes from vesicles slowly and incompletely during exocytosis (Aravanis et al., 2003; Klingauf et al., 1998; Stevens and Williams, 2000; Zakharenko et al., 2002). In addition, the rate and proportion of release were dependent on the desorption rate of the dye, suggesting that dye could not leave by lateral diffusion and instead must first unbind from the vesicle lumen and escape by the same route as neurotransmitter (Aravanis et al., 2003; Klingauf et al., 1998; Ryan, 2001). These studies imply that the bilayers of the vesicles and plasma membrane do not merge, and suggest that fusion during kiss-and-run is incomplete. If so, the rapid resealing of synaptic vesicles may simply be the molecular reversal of exocytosis (Stevens and Williams, 2000).

It might be useful to distinguish two phenotypes of vesicle recapture. In the kiss-and-run model vesicles do not exchange membrane components with the plasma membrane. In contrast, granules in PC12 cells participate in a more complete form of fusion, allowing at least the lipid bilayer of granules to mix with the plasma membrane before they reseal. In other cells, even membrane proteins may escape into the plasma membrane, until only the cavity of the granule remains. We propose to call this type of resealing "*cavicapture,*" to distinguish it from "kiss-and-run" with its emphasis on complete retention of membrane components (Valtorta et al., 2001).

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Figure 3.1

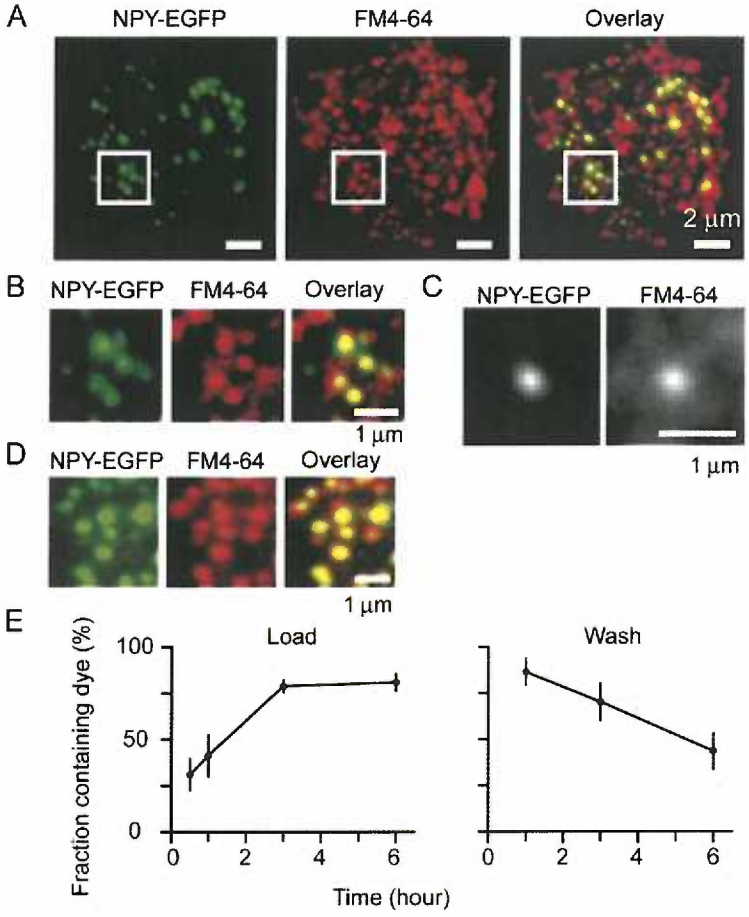

Fig. 3.1. Secretory granules accumulate the styryl dye FM4-64. (A) Evanescent field images of a PC12 cell transfected with NPY-EGFP (left) and loaded with FM4-64 (center); merged image on the right. (B) Magnified images of the regions marked with white boxes in A. (C) Left, square regions (1.5 μm x 1.5 μm) centered on single NPY-EGFP containing granules were excised from green images and averaged (30 granules, 3 cells). Right, as on the left for the same regions in the red channel. (D) NPY-EGFP expressing cells were loaded with FM4-64 as in A-C but then ruptured with a jet of buffer solution. Patches of membrane remained and a small region of one such patch is shown. (E) Left, uptake of FM4-64 by intact cells. Cells exposed to FM4-64 for various times were washed and then imaged. The fraction of granules containing FM4-64 is plotted against time in the presence of FM4-64 (left). Right, loss of dye from intact cells. Cells were loaded with a 12-hr exposure to FM4-64, then washed, incubated for various times in dye-free solution and finally imaged. Each point is the mean from at least 20 granules each in at least 5 cells

Figure 3.2

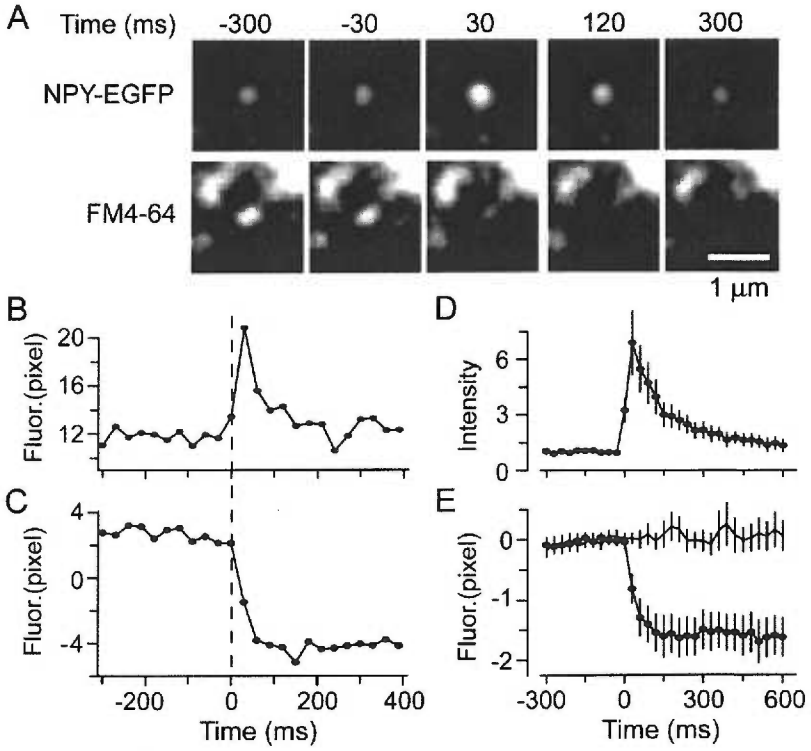

Fig. 3.2. Granules lose FM4-64 during exocytosis. (A) Images taken from a video clip of a granule containing NPY-EGFP (top) and FM4-64 (bottom) during exocytosis. The first frame wherein NPY-EGFP brightened measurably was taken to mark the start of exocytosis and the time origin. Granules usually continued to brighten, reaching maximal brightness 30 ms later. For clarity, each pixel value was multiplied by 10 and the resulting images were low-pass filtered (3 pixels), but all analysis was done before multiplication and filtering. (B) Plot of the NPY-EGFP fluorescence of the granule in A, measured in a 610 nm diameter circle. Fluorescence in a concentric annulus was subtracted as background. Fusion is taken to have occurred at the first sign of the granule getting brighter, thus defining the time origin. (C) FM4-64 fluorescence at the same site, corrected for background using a square area (see Methods). (D) NPY-EGFP fluorescence traces as in B were divided by their average value over the last 150 ms before fusion, and then averaged (25 granules, 4 cells). (E) FM4-64 fluorescence of the granules analyzed in D. Filled circles, traces as in C had their average value during the last 150 ms before exocytosis subtracted. The results were then averaged. Continuous line, fluorescence at sites of neighboring granules that did not undergo exocytosis (25 granules, 4 cells).

Figure 3.3

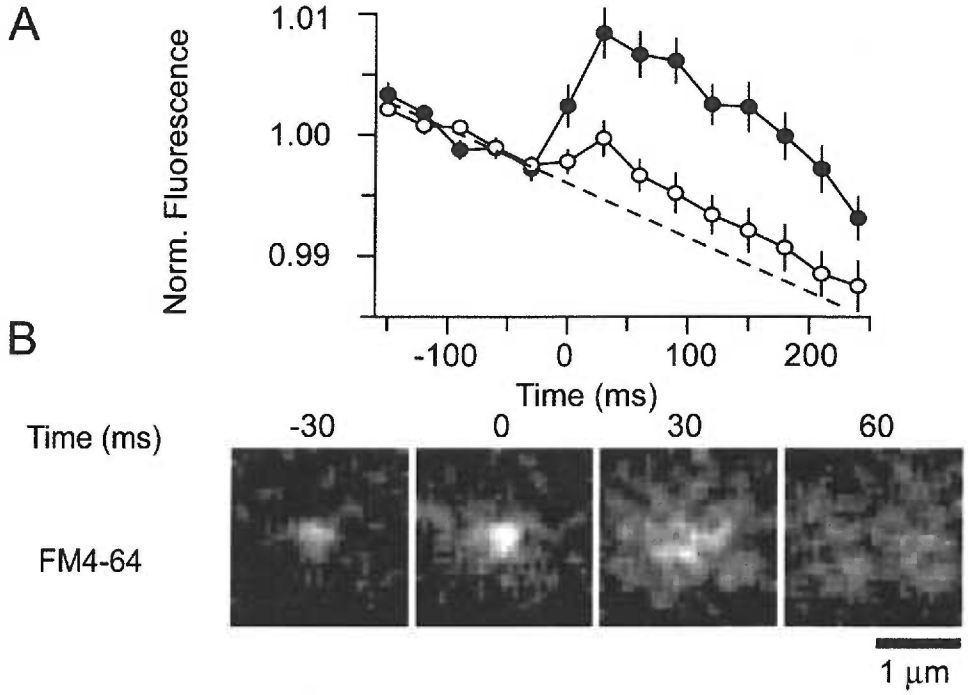

Fig. 3.3. Effect of polarization. (A) FM4-64 fluorescence in larger regions (3.11 x 3.11 μm square) centered over granules undergoing exocytosis. Illumination with either s-polarized (open circles, 25 granules, 4 cells) or p-polarized light (filled circles, 20 granules, 5 cells). Individual fluorescence traces were aligned to the moment of exocytosis as determined from the NPY-EGFP signal (see Fig. 3.2) and divided by their mean value during the last 150 ms before fusion. The results were then averaged. The dotted line was fitted to the traces 150 ms before exocytosis, and its slope is attributed to photobleaching. No background subtraction was applied to the images other than removing the electronic offset of the camera in total darkness. (B) Image sequences were acquired as in Fig. 3.2A but with p-polarized light in order to highlight dye located in the plasma membrane. To cancel out the fluorescence from neighboring structures not undergoing exocytosis, five sequential frames beginning 300 ms after exocytosis were averaged together and the result was subtracted from each sequence. The sequences thus treated were added together (20 granules, 5 cells), and subjected to 3-pixel low-pass filtering.

Figure 3.4

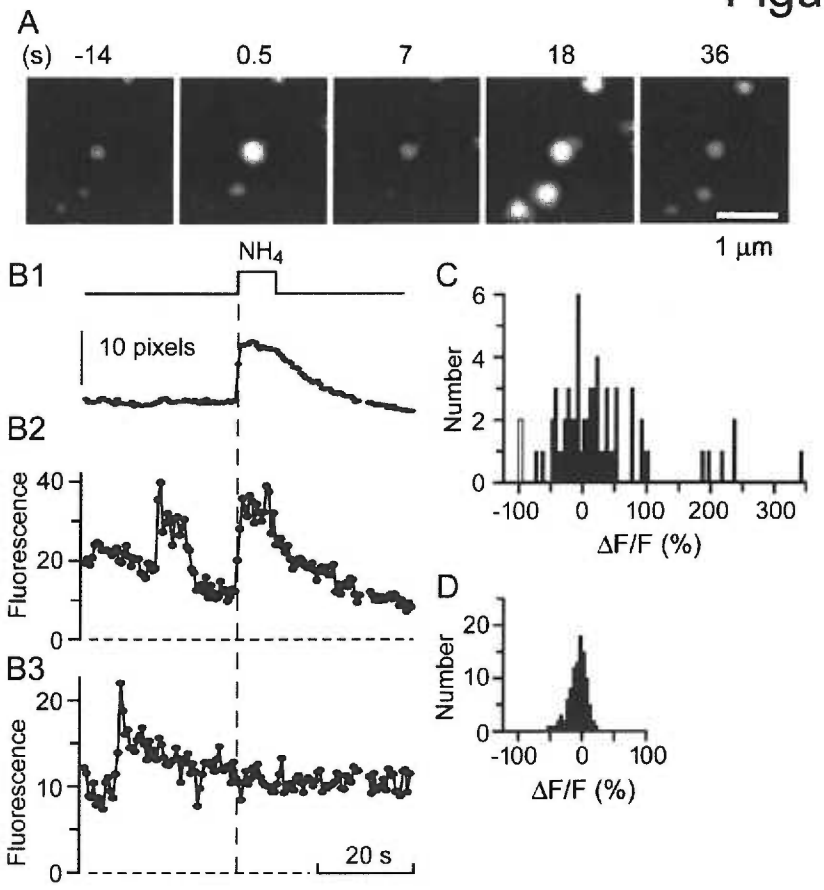

Fig. 3.4. Some granules reacidify after exocytosis. (A) EGFP-phogrin labeled granule brightens during exocytosis, dims and then re-brightens during superfusion with 50 mM NH4Cl. Images processed as in Fig. 3.2A for visual clarity. (B1) Fluorescence from granules that failed to undergo exocytosis in that cell. Background subtraction as in Fig. 3.2B; average of six granules. (B2) Fluorescence of the granule in the center of the images in A, calculated by fitting Gaussian functions (see Methods). (B3) As in B2, but in a granule that underwent exocytosis but did not brighten when NH4Cl was added. (C) NH4Cl -induced fluorescence changes were determined as in B2 and B3. Gaussians were fitted to two images of each granule, one an average over the last 2.5 s before superfusion, the other over a 2.5 s interval starting 1-2 s after NH4Cl was applied. Each value in (C) is the difference, ΔF, between the amplitudes of the two Gaussians, given as a percentage of the amplitude (F) before NH4Cl was applied. NH4Cl applied 5 to 47 s after exocytosis (64 granules in 19 cells). The two events with $\Delta F/F = -100\%$ refer to granules where the fitting routine failed for the images taken during the NH4Cl application. (D) As in (C) except that cells were not stimulated, and the buffer used for superfusion lacked NH4Cl.

Figure 3.5

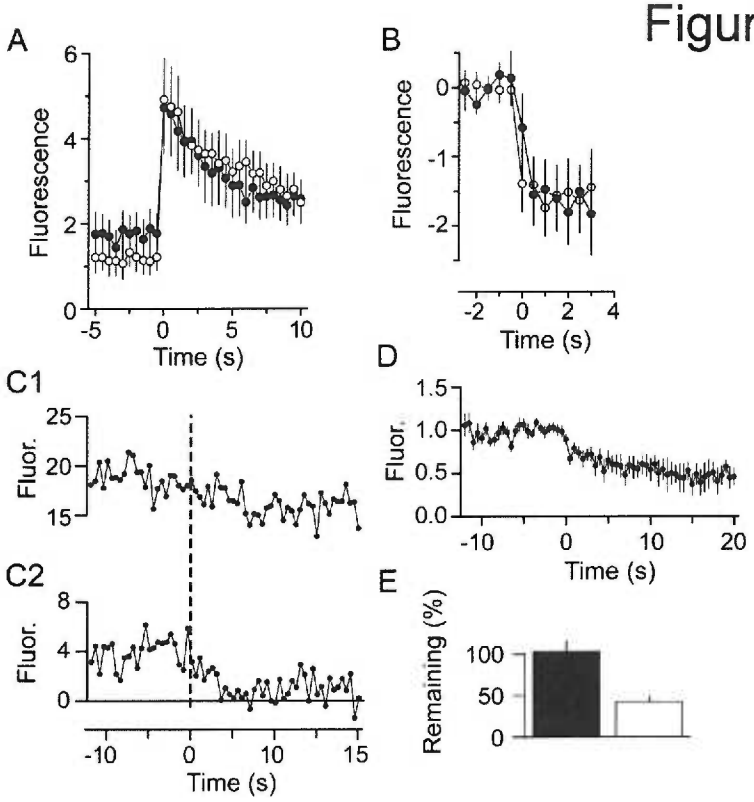

Fig. 3.5. Re-sealed granules lose FM4-64 but retain phogrin. (A, B) Fluorescence of EGFP-phogrin and of FM4-64 in granules that were grouped according to their response to NH4Cl. Open granules had $\Delta F/F < -5\%$, the median in Fig. 3.4D (open circles, 12 granules in 6 cells). Re-sealed granules passed two tests. They brightened visibly in response to NH4Cl, and had $\Delta F/F > 25\%$, a value larger than any granule in Fig. 3.4D (filled circles, 13 granules in 7 cells). In (A), fluorescence traces were analyzed as in Fig. 3.2B except that EGFP-phogrin rather than NPY-EGFP was used to mark the moment of exocytosis. The resulting traces were then averaged. In (B), FM4-64 fluorescence traces were analyzed as in Fig. 3.2 C, E. (C): Fluorescence of two granules containing mRFP, analyzed as in Fig. 3.2B in cells also expressing NPY-EGFP. Time origin is the moment of fusion as reported by NPY-EGFP. (D), traces as in C1, C2 were divided by their average value over the last 2.5 s before fusion, and the results were averaged (10 granules in 8 cells). (E) Analysis of a subset of the data shown in Fig. 3.4C. The granule fluorescence during NH4Cl perfusion was divided by that measured similarly but from images averaged over the first 1.5 s after exocytosis. Re-sealing (black bar, n = 17) of lack thereof (white bar, n = 15) was judged by the criteria used in (A, B). The dataset includes granules not stained with FM4-64, but excludes those exposed to NH4Cl >20 s after exocytosis. Values are different with $p < 0.0001$ by ANOVA.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Here we used two-color evanescent field microscopy to image the release and recapture of single dense-core granules in PC12 cells. While the small peptide NPY was lost from most granules during exocytosis in seconds, the larger protease tPA and the membrane protein phogrin were retained at the site of exocytosis for over a minute. By imaging the volume occupied by granules, we showed that granules do not collapse and flatten into the plasma membrane during exocytosis but instead maintain a volume. We further used the probe GFP-phogrin to demonstrate that close to half of these granules later reseal from the plasma membrane and re-acidify. Those granules that did reseal lost little to no phogrin. Finally, we showed that a lipid was lost into the plasma membrane within small fractions of a second during exocytosis, suggesting that the two bilayers merge completely.

Release and retention

Secretory cells are thought to control their exocytic output (the amount of product released during stimulation) simply by altering the total number of vesicles that fuse with the plasma membrane within a certain window of time. This concept originates in the quantal hypothesis of neurotransmission (Katz, 1996). The quantal hypothesis proposes that the exocytosis of individual vesicles occurs in an

all-or-nothing fashion and that the unitary release of "packets" or "quanta" from single vesicles explains the additive and discrete distribution of postsynaptic potentials (Katz, 1996). Heuser and Reese further developed this hypothesis into the full-fusion model of exocytosis (Heuser and Reese, 1973). In the full-fusion model, vesicles that are undergoing exocytosis release their quanta and then completely collapse into the presynaptic membrane, turning inside-out (Heuser and Reese, 1973). While the full-fusion model predicts aspects of exocytosis in many systems, several lines of evidence suggest that vesicles do not always collapse into the plasma membrane. Specifically, some vesicles are thought to make transient connections with the plasma membrane and release only fractions of their luminal content. Utilizing this form of fusion, cells could alter the quantal unit by controlling how much, when, and which of the vesicle's contents are released. This type of exocytosis has been named "kiss-and-run" (Valtorta et al., 2001). In our work, we show that granules in PC12 cells do not generally release their contents in an all-or-nothing fashion. Instead, some granule components are lost rapidly, some slowly, and others not at all. There are several advantages for the cell in adopting this form of exocytosis. First, cells can modulate the release of specific molecules depending on stimulation or environmental cues. This type of selective release has been seen in pituitary lactotrophes, where under normal conditions, granules' contents are not completely released during exocytosis (Angleson et al., 1999). These studies also demonstrated that granules are recaptured from the cell surface several minutes after fusion. When the cells were treated with dopamine, however, which lowers the intracellular level of cAMP,

granules were lost completely (Angleson et al., 1999). These data demonstrate that selective exocytosis of granule matrix proteins in endocrine cells can be regulated by cellular signaling cascades. Selective release also has been seen in chromaffin cells, where components of the granule membrane are retained at the granule site following exocytosis (Patzak et al., 1984; Wick et al., 1997). In these studies, patches of the membrane-associated protein dopamine-β-hydroxylase were observed to appear on the cell surface during exocytosis. These patches then were retrieved into the cytoplasm by endocytosis several minutes following fusion. Along with proteins and peptides, amperometry studies have shown that even small molecules are released incompletely during exocytosis in both endocrine cells and even neurons (Alvarez de Toledo et al., 1993; Staal et al., 2004).

Aside from its effects on signaling, the incomplete loss of granule components provides cells with another benefit. Specifically, the retention of granule components such as membrane or matrix proteins allows vesicles to be recycled rapidly. This has been demonstrated in chromaffin cells where granules can undergo a second round of exocytosis after five minutes of rest (von Grafenstein and Knight, 1992). Vesicles in neurons also have been shown to recycle and undergo many rounds of exocytosis (Aravanis et al., 2003; Rizzoli and Betz, 2004). In summary, the quantal hypothesis/full-fusion model of exocytosis, proposed over 50 years ago by Katz and colleagues, does not predict the behavior of all exocytic events in many secretory cells, including PC12 cells. Indeed, endocrine granules and even synaptic vesicles do not always release the

entirety of their contents. This process first offers cells an additional layer of control, allowing them to regulate not only the total number of vesicles that undergo exocytosis but also the quantity and content of what escapes into the extracellular space. Second, it allows for the rapid recycling of vesicles following fusion (Henkel and Almers, 1996; Meldolesi and Ceccarelli, 1981; Palfrey and Artalejo, 1998).

Recapture.

In the presented work we show that close to half of all granules reseal and re-acidify following exocytosis. Previous work by others measuring membrane capacitance and amperometry during exocytosis also has demonstrated that exocytosis is reversible in endocrine granules and even synaptic vesicles. Specifically, granules have been shown to transiently connect with the plasma membrane, flicker open and closed, and then reseal. The open time of transient fusion pores varies, yet times from a few milliseconds to several seconds have been reported (Ales et al., 1999; Alvarez de Toledo et al., 1993; Breckenridge and Almers, 1987b; Henkel et al., 2001; Wang et al., 2003). We do not know how long the fusion pore stays open in PC12 cells. Our data suggest that the pore might stay open from a few milliseconds to several seconds. In this regard, our data would agree with electrical measurements made in chromaffin and mast cells (Ales et al., 1999; Breckenridge and Almers, 1987b). A logical question that remains is how fusion pores remain open and stable for such long periods of time. Indeed, it is hard to image how a lipidic pore is maintained without collapsing.

Many cells do, however, form large pores that are stable enough to be imaged even by electron microscopy (Chandler and Heuser, 1980; Haller et al., 2001). It is possible that interactions between the granule matrix and the plasma membrane could maintain the pore. Alternatively, granule membranes might interact with the cortical cytoskeleton to maintain the shape of the granule following exocytosis. This idea has been tested in *Xenopus laevis* oocytes, where actin has been shown to mediate the resealing of exocytic granules (Sokac et al., 2003). Disrupting the ability of actin to polymerize at sites of exocytosis prevents granules from being recaptured, suggesting that a ring of actin might constrict the neck of the fusion pore. Other studies have shown that depolymerizing actin promotes exocytosis, which suggests that actin might act as a barrier to fusion (Sokac et al., 2003; Trifaro and Vitale, 1993; Vitale et al., 1995). Actin also has been shown to mediate the movement of granules below the cell surface (Lang et al., 2000). Either disrupting the actin cortex with drugs such as latrunculin or stabilizing it with jasplakinolide might have dramatic effects on the release of granule components or on the resealing of granules. A role for actin also has been found in conventional clathrin-mediated endocytosis (Merrifield et al. 2002). It is likely that actin plays a general role in many endocytic processes (Engqvist-Goldstein and Drubin, 2003). Future imaging studies of actin dynamics during stimulated exocytosis in PC12 cells should shed light on the controversial role of the actin cortex during triggered exocytosis and rapid or compensatory endocytosis.

The most well-studied endocytic protein implicated in both conventional endocytosis and rapid endocytosis of vesicles is the GTPase dynamin (De Camilli

et al., 1995; Shpetner and Vallee, 1989). Dynamin forms spiral rings, or collars, around membrane tubes. These spirals constrict when dynamin binds GTP and can cut lipid tubules (Takei et al., 1999). Blocking dynamin but not clathrin has been shown to inhibit rapid endocytosis in chromaffin cells (Artalejo et al., 1995). Furthermore, disrupting dynamin function has been shown to broaden and enlarge amperometric spikes, implying that dynamin may play a role in controlling the open time of the fusion pore (Graham et al., 2002). Preliminary work in PC12 cells has shown that dynamin and clathrin co-localize at the cell surface in small spots (Fig. 4.1). Both dynamin (Fig. 4.2) and clathrin (Fig 4.3) do not associate with granules but instead surround granule sites. This is in contrast to studies that have shown that a small number of granules (30%) are associated with dynamin prior to and after exocytosis on membrane lawns of PC12 cells (Holroyd et al., 2002). These studies also have shown that the granules invested with dynamin are more likely to reseal following exocytosis. Indeed, blocking dynamin function blocked resealing (Holroyd et al., 2002). Clearly, dynamin plays a role in the endocytosis of dense-core granules. It will be interesting to test if granules recruit dynamin either before or after triggered exocytosis in intact PC12 cells.

Figure 4.1

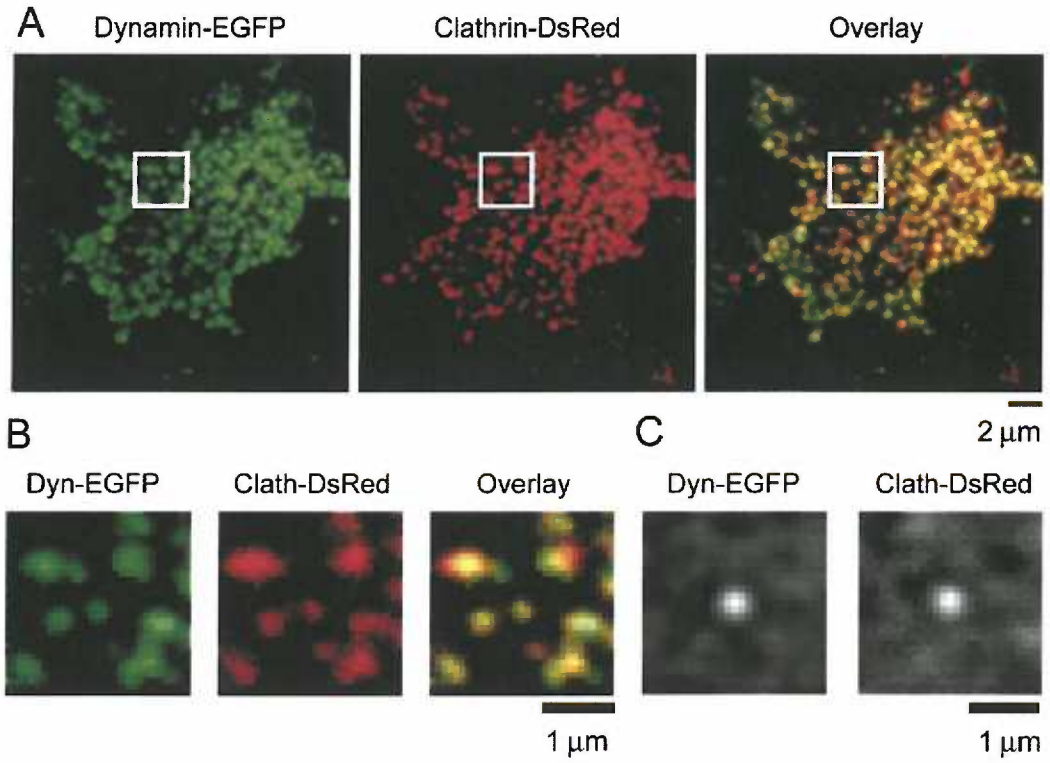

Fig. 4.1. Dynamin colocalizes with clathrin in living PC12 cells. (A) Evanescent field images of a PC12 cell transfected with dynamin-EGFP (left) and clathrin-DsRed (center); merged image on the right. (B) Magnified images of the regions marked with white boxes in A. (C) Left, square regions (2.7 μm x 2.7 μm) centered over single dynamin spots were excised from green images and averaged together (30 granules, 2 cells). Right, as on the left for the same regions in the red channel.

Figure 4.2

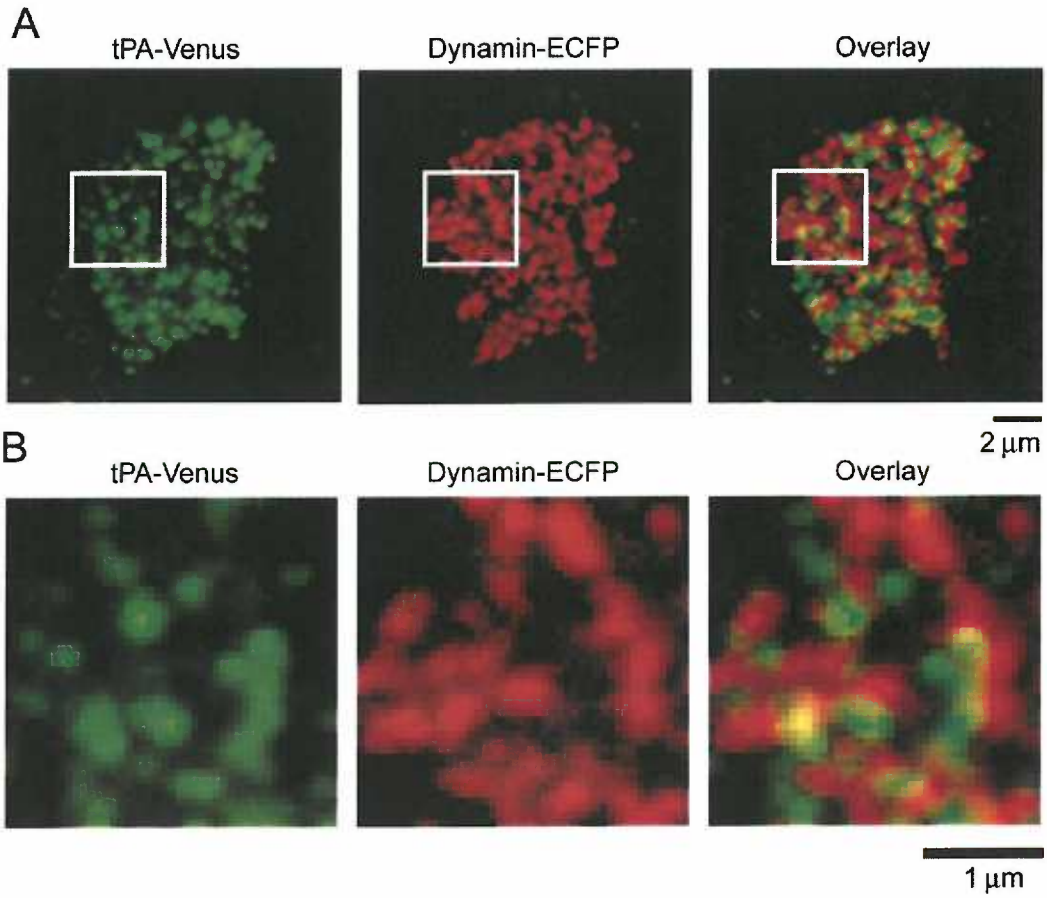

Fig. 4.2. Granules do not colocalize with dynamin in living PC12 cells. (A) Evanescent field images of a PC12 cell transfected with tPA-venus (left) and dynamin-ECFP (center); merged image on the right. (B) Magnified images of the regions marked with white boxes in A.

Figure 4.3

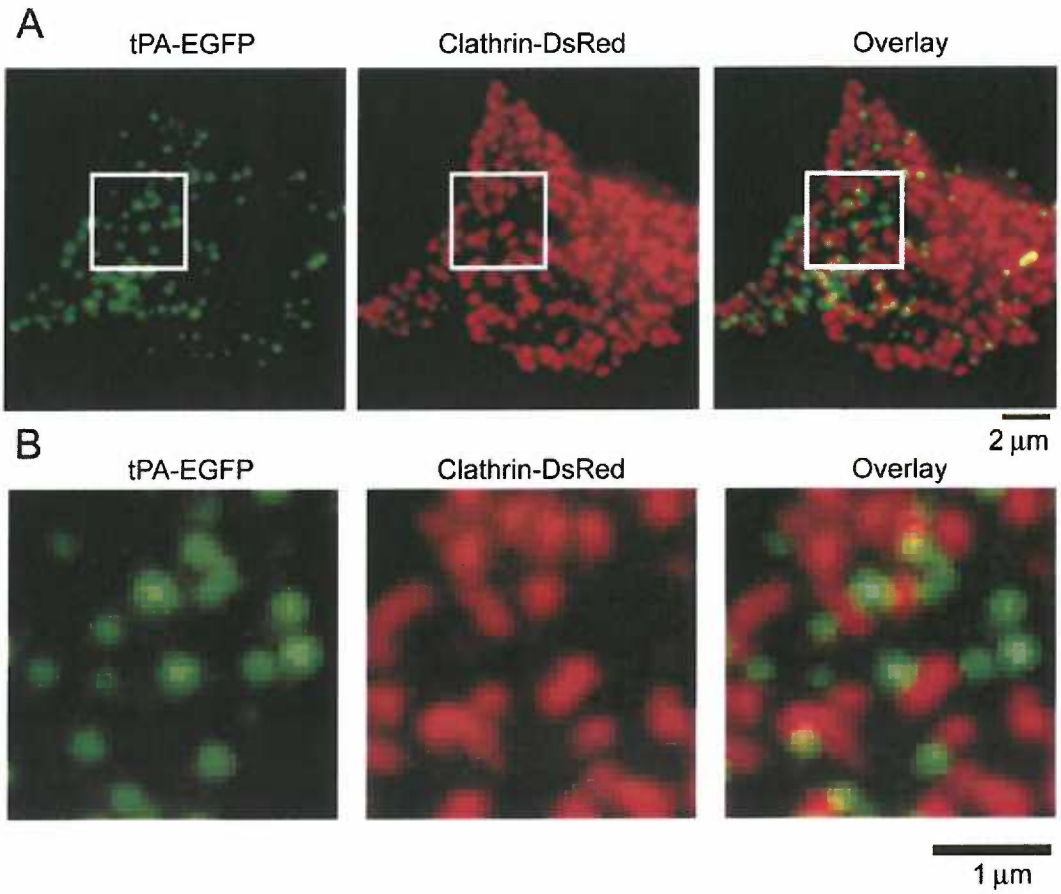

Fig. 4.3. Granules do not colocalize with clathrin in living PC12 cells. (A) Evanescent field images of a PC12 cell transfected with tPA-EGFP (left) and clathrin-DsRed (center); merged image on the right. (B) Magnified images of the regions marked with white boxes in A.

Fusion pores

Our work supports the hypothesis that granules make transient connections with the outside of the cell and remain partially intact during exocytosis in a process similar to that described by the kiss-and-run model. When granules connect, however, lipid dye contained in vesicles is able to escape into the plasma membrane in milliseconds. These findings are at odds with aspects of current kiss-and-run models. The original description of the kiss-and-run model made by Ceccarelli and colleagues over 30 years ago, however, did not suggest that vesicle components remain separate from the plasma membrane. Indeed, the fact that vesicles could be loaded with a 40 kD protein, horseradish peroxidase, in the original studies supported the idea that some molecules could enter and most likely also exit synaptic vesicles during kiss-and-run (Ceccarelli and Hurlbut, 1980b). The kiss-and-run hypothesis was later amended to include the criterion that no membrane components are exchanged during exocytosis, with vesicle components remaining completely separated from the plasma membrane (Fesce et al., 1994; Valtorta et al., 2001). This concept likely arose from imaging studies of styryl dyes in cultured hippocampal neurons, where FM1-43 was observed to leave vesicles slowly (Aravanis et al., 2003; Klingauf et al., 1998; Stevens and Williams, 2000). It has been proposed that a restrictive fusion pore keeps dye from leaving synaptic vesicles (Klingauf et al., 1998). In an earlier model, a proteinaceous channel was proposed to form between the vesicle and plasma membrane, not unlike an ion channel, that would open during exocytosis,

allowing small particles such as neurotransmitters to escape, while restricting the flow of protein and lipid (Almers and Tse, 1990). The pore would then close, resealing the vesicle. Our work does not support this model of fusion pores that form during transient exocytosis in PC12 cells. Indeed, the connection between the granule and the plasma membrane in PC12 cells appears to pass lipid within milliseconds of exocytosis. If a lipid impermeable pore exists during exocytosis, it must do so for a very short time. We feel that if styryl dyes have free access to the plasma membrane, similar lipids should be able to enter and leave the granule during exocytosis as well. Our results are similar to findings in the large synaptic terminal of goldfish bipolar neurons (Zenisek et al., 2002). In these neurons, the lipid probe FM1-43 was measured to escape from small synaptic vesicles within 10 ms of exocytosis.

In conclusion, we have shown that vesicles do not fully collapse into the plasma membrane during exocytosis but instead retain some proteins and lose lipid rapidly. These partially empty granules then reseal in a process we have named "cavicapture" for "cavity recapture." We propose that there are three exocytic modes (Fig. 4.4). In the first, kiss-and-run, vesicles briefly open a restrictive fusion pore that does not pass lipid or protein. This pore then reseals and the vesicle is captured mostly intact. Kiss-and-run might simply be the molecular reversal of fusion. In a more complete form of exocytosis, cavicapture, the bilayer of the vesicle merges with the plasma membrane. In this mode of fusion, the vesicle does not collapse into the plasma membrane but allows some components to escape while keeping others inside. The partially empty cavity of

the vesicle is then retrieved by fission-mediating machinery. In the third and most complete form of exocytosis, full-fusion, the vesicle completely collapses and spreads its components over the plasma membrane. These components then must be gathered up and retrieved by clathrin-mediated endocytosis. It will be interesting to see what proteins or lipids mediate the three forms of exocytosis. Two-color evanescent field microscopy seems well suited to test for the recruitment of proteins to sites of exocytosis or endocytosis in living PC12 cells.

Figure 4.4

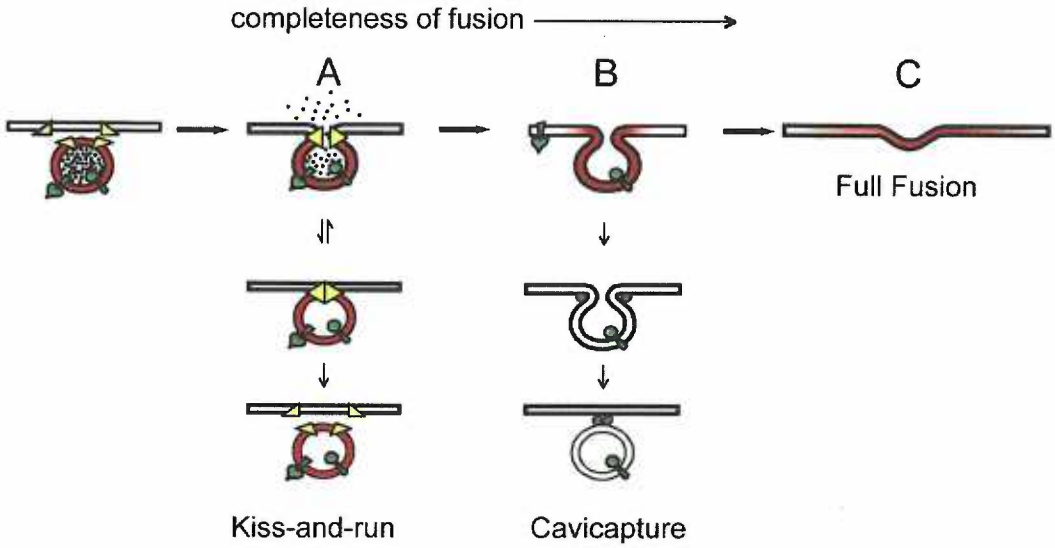

Fig. 4.4. Three modes of exocytosis. A) During kiss-and-run, a vesicle connects with the extracellular space through a restrictive fusion pore. Small soluble molecules escape while proteins and lipids do not mix with the plasma membrane. The pore then reseals and the vesicle is recaptured intact. B) During cavicapture, the bilayer of the secretory vesicle completely merges with the plasma membrane. Some membrane components are lost while others are retained in the granule cavity. Dedicated fission machinery is then recruited to reseal the partially empty vesicle. C) During full-fusion, the vesicle fully merges with the plasma membrane and collapses, spreading its components in the plasma membrane.

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