A Nibbling Mechanism for Clathrin-mediated Retrieval of Secretory Granule Membrane after Exocytosis*

Received for publication, January 4, 2013, and in revised form, January 30, 2013 Published, JBC Papers in Press, February 5, 2013, DOI 10.1074/jbc.M113.450361

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Background: Retrieval of the fused secretory granule membrane is poorly understood.
Results: Granule membrane proteins remain associated after fusion, attract clathrin and dynamin, and are gradually removed together.
Conclusion: The fused membrane is a nucleation site for endocytosis, which internalizes the membrane bit-by-bit.
Significance: This work provides a fundamental mechanistic insight into a process essential for secretory cells.

Clathrin-mediated endocytosis is the major pathway for recycling of granule membrane components after strong stimulation and high exocytotic rates. It resembles “classical” receptor-mediated endocytosis but has a trigger that is unique to secretion, the sudden appearance of the secretory granule membrane in the plasma membrane. The spatial localization, the relationship to individual fusion events, the nature of the cargo, and the timing and nature of the nucleation events are unknown. Furthermore, a size mismatch between chromaffin granules (~300-nm diameter) and typical clathrin-coated vesicles (~90 nm) makes it unlikely that clathrin-mediated endocytosis internalizes as a unit the entire fused granule membrane. We have used a combination of total internal reflection fluorescence microscopy of transiently expressed proteins and time-resolved quantitative confocal imaging of endogenous proteins along with a fluid-phase marker to address these issues. We demonstrate that the fused granule membrane remains a distinct entity and serves as a nucleation site for clathrin- and dynamin-mediated endocytosis that internalizes granule membrane components in small increments.

After release of protein and other soluble contents from secretory granules in cells undergoing Ca²⁺-regulated exocytosis, the cell must recycle the fused granule membrane components and restore plasma membrane area. There are at least two pathways in neuroendocrine cells that accomplish these functions. The fused membrane can be retrieved largely intact via a rapid form of clathrin-independent endocytosis (1, 2). Alternatively, granule membrane components can be retrieved through a slower, clathrin-mediated endocytic pathway. Rapid endocytosis, sometimes called “kiss and run,” occurs within seconds of fusion and appears to be the preferred mode of recycling upon weak stimulation and low exocytotic rates (3, 4). The slower, clathrin-mediated endocytosis occurs many seconds to minutes after fusion and is thought to be the major recycling pathway upon vigorous physiological, electrical, or pharmacological stimulation (nicotinic cholinergic or elevated K⁺ stimulation). Both rapid endocytosis and slow endocytosis are dynamin-mediated, with evidence for dynamin1 being important for rapid endocytosis and dynamin2 for slow endocytosis (3, 5, 6).

Although rapid endocytosis retrieves in a single endocytotic event the entire granule membrane, the location, timing, and mechanisms underlying clathrin-mediated endocytosis of granule membrane components are not understood despite decades of investigation using a variety of techniques. The development of methods to isolate gram quantities of bovine chromaffin granule membranes enabled purification of chromaffin granule membrane proteins and the development of complementary antibodies that permitted tracking of specific granule components after fusion. Primary antibodies against the luminal domain of either dopamine-β-hydroxylase (7), a major chromaffin granule membrane protein, and glycoprotein III protein (8, 9) provided a means to label protein exposed to the extracellular environment upon secretion and then follow its fate. Both proteins appear as puncta in the plasma membrane upon stimulation; dopamine-β-hydroxylase (DBH)² puncta were shown to reflect fusion of individual granules and not aggregation of the protein (10). These data are consistent with a freeze-fracture study that suggested the fused chromaffin granule membrane remains a distinct entity in the plasma membrane (11). Immunoelectron microscopy detected formation of clathrin-coated pits and vesicles containing glycoprotein III within 15 min of stimulation, but the spatial relationship to exocytotic sites could not be determined (8).

In the following study in bovine chromaffin cells, we demonstrate that the granule membrane remains a distinct entity in the plasma membrane for many minutes after fusion, that clathrin and dynamin often accumulate within 20 s at fusion sites, and that a “nibbling” mechanism, consisting of repeated endocytic retrievals of small portions of the fused granule membrane, is the major route for the gradual, clathrin-dependent

* This work was supported, in whole or in part, by National Institutes of Health Grants R01-NS38129, R56-NS38129, and R21-NS073686.
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2 The abbreviations used are: DBH, dopamine-β-hydroxylase; TIRF, total internal reflection fluorescence; NPY, neuropeptide Y; VMAT2, vesicular monoamine transporter2; Dyn2-GFP, dynamin2 tagged with green fluorescent protein.
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removal of granule membrane components after strong stimulation.

EXPERIMENTAL PROCEDURES

Chromaffin Cell Preparation and Transfection for Live-cell Imaging—Chromaffin cell preparation from bovine adrenal medulla and transient transfection was performed as described previously (12). For live-cell imaging, cells were plated onto 25-mm coverslips (refractive index 1.51) that had been coated with poly-δ-lysine and calf skin collagen to promote cell adhesion. Cells were transfected with plasmids by calcium phosphate precipitation. Neuropeptide-Y-mCherry (NPY-mCherry) was transfected alone or with clathrin light chain-GFP. NPY-mCherry is a soluble luminal marker of chromaffin granules that is released upon exocytosis. The parent NPY plasmid was a gift from Dr. Wolfhard Almers (Vollum Institute, Oregon Health and Science University, Portland, OR). The plasmid encoding clathrin light-chain-GFP was a gift from Dr. Sanford Simon (Rockefeller University).

Immunocytochemistry for Confocal Imaging—Chromaffin cells were plated on Lab-Tek II chambered coverglasses (Nalge Nunc International, distributed by Thermo Fisher Scientific) that had been sequentially coated with poly-δ-lysine and calf skin collagen. In a typical experiment cells were either incubated with physiological saline with normal $K^+$ (145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, 15 mM HEPES, pH 7.4) or stimulated with physiological saline with elevated $K^+$ (95 mM NaCl, 56 mM KCl, 2.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, 15 mM HEPES, pH 7.4) for 15–30 s. Three different protocols were used: Protocol 1, antibodies added after endocytosis occurred, used for examining the decrease in antigen intensities on the plasma membrane (Figs. 1 and 4 (no endocytosis)), 5, and 7); Protocol 2, incubation on ice with antibody to DBH before re-warming to permit endocytosis, used to quantify internalized DBH (Fig. 8); Protocol 3, antibody to DBH and 0.5 mM Alexafluor™-cadaverine present during endocytosis, used to examine the cargo of endocytic vesicles labeled with fluid-phase marker (Figs. 9 and 10 and Table 1).

After Protocols 1 and 2, cells were fixed in 4% paraformaldehyde for 30 min. Fixed cells were rinsed and quenched in 50 mM NH$_4$Cl in PBS for 30 min. In some experiments cells were permeabilized for 7 min with ice-cold methanol or acetone and blocked for 20 min in 1% gelatin in TBS and 30 min in 4% normal donkey or goat serum before incubation with additional 1° and 2° antibodies. In experiments with cadaverine (Protocol 3), cells were fixed in 2.5% glutaraldehyde in Sorensen’s phosphate buffer for 30 min and quenched with 0.1% sodium borohydride in Sorensen’s phosphate buffer followed by permeabilization with ice-cold methanol (if necessary) and exposure to additional antibodies. Fixation with paraformaldehyde resulted in low fluorescence background; fixation with glutaraldehyde was necessary for efficient cross-linking and trapping of Alexafluor™-cadaverine in endocytic vesicles.

Antibodies and Reagents—Antibodies were from the following sources: Santa Cruz Biotechnology, Inc., Santa Cruz, CA (mouse anti-clathrin light chain (CON.1, sc-12735); goat anti-dynamin II ((C-18) sc-6400); goat anti-dopamine-β-hydroxylase); Synaptic Systems GmbH, Gottingen, Germany (mouse anti-synaptotagmin 1 (cytoplasmic domain Cl 41.1); mouse anti-synaptotagmin 1 (luminal domain, Cl 604.2); mouse anti-VAMP (cytoplasmic domain Cl 69.1)). Rabbit anti-VMAT2 (luminal domain) was a gift from Dr. Bruno Gasnier (IBP CNRS, Paris); rabbit anti-DBH was a gift from Dr. Patrick Fleming, for Georgetown University Medical Center, Washington, D.C. Alexafluor™-labeled secondary antibodies were purchased from Invitrogen. Dyngo 4a and dyngo8a were gifts from Dr. Phillip Robinson (University of Sidney, Sidney, Australia).

Confocal Microscopy—Images were acquired on an Olympus Fluoview 500 confocal microscope with a $60 \times 1.42$ numerical aperture oil objective. An argon 488-nm laser with a 505–525-nm bandpass filter, a HeNe green 543-nm laser with a 560–600 nm bandpass filter, and a HeNe red (633-nm) laser with a longpass filter were used. To minimize spillover, images with different excitations were acquired sequentially. Within an experiment, initial settings were adjusted so that the brightest pixels for each color were unsaturated, and these settings were maintained throughout. Images were analyzed with ImageJ software (National Institutes of Health), and statistics were performed with Prism 5 software from Graphpad Prism Software.

Live-cell Imaging (TIRF)—Experiments were performed in a physiological salt solution containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, 15 mM HEPES, pH 7.4, at ~28 °C. Individual cells were perfused through a pipette (100-μm inner diameter) using positive pressure from a computer-controlled perfusion system DAD-6VM (ALA Scientific Instruments, Westbury, NY). Generally, cells were perfused with physiological saline with normal $K^+$ for 5 s and then stimulated to secrete with elevated $K^+$-containing solution (95 mM NaCl, 56 mM KCl, 5 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, 15 mM HEPES, pH 7.4) for 55 s.

Transmission Electron Microscopy—Cell monolayers were rinsed in serum-free medium and then fixed in 2.5% glutaraldehyde in 0.1 M Sorensen's buffer, pH 7.4, overnight at 4 °C. After a buffer rinse, they were post-fixed for 40 min in 1% osmium tetroxide in the same buffer. After a double-distilled water rinse, they were scraped from the culture dishes and pelleted in Eppendorf tubes. For each subsequent step, the cells were resuspended in the next reagent and then pelleted. Cells were then stained en bloc with an aqueous, saturated solution of uranyl acetate for 1 h. They were then dehydrated rapidly in a graded series of ethanol, infiltrated and embedded in Epon, and polymerized. Sections (70-nm thickness) were collected onto copper grids and post-stained with uranyl acetate and lead citrate. The sections were viewed on a Philips CM100 at 60 kV. Images were recorded digitally using a Hamamatsu ORCA-ER digital camera system, which was operated using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).

Estimation of the Diameter of Clathrin-coated Vesicles from Electron Microscopy—The mean coated vesicle diameter, 77 ± 3 nm, measured in electron microscopy in unstimulated cells (Fig. 11), underestimates the true vesicle diameter because of stereological effects of thin sections. Two approaches were used to determine the true diameter. The section thickness (70 nm) is approximately equal to the diameter of the vesicle. If the
diameter measured in a section is the maximum diameter of the vesicle in that section, then purely geometric considerations indicate that half of the measured diameters should reflect the true diameter of the vesicle. The mean value of the upper half of the measurements, 89 ± 2 nm, is a plausible estimate of the average diameter. An alternative statistical approach (13) based upon the transform from a three-dimensional to a two-dimensional distribution gives a similar estimate of 86–90 nm. Upon stimulation, the mean of the measured coated vesicle diameters was 89 ± 5 nm. The statistical correction estimates a true value of 113 nm. This number likely underestimates the true diameter of the DBH-containing endocytic vesicles as it includes coated vesicles without DBH, which are on average smaller than those with DBH (see Alexafluor™-cadaverine data, Fig. 9).

RESULTS

DBH and Other Chromaffin Granule Membrane Proteins Remain Punctate and Co-localize on the Cell Surface after Fusion—Upon secretory granule fusion with the plasma membrane, lumenal domains of granule membrane proteins become exposed on the cell surface and can be visualized with antibodies (7, 9). Because DBH is a major chromaffin granule membrane protein (14) and its glycosylated lumenal domain is an excellent antigen, it is readily detected and is the primary antigen that was tracked in the following studies (there is also soluble DBH within granules, which is released into the medium upon secretion (15, 16)).

After fusion, granule membrane DBH is localized on the plasma membrane in discrete puncta, with each punctum representing a single fused granule (10). The punctate appearance is a direct consequence of fusion and not of cross-linking of DBH by bivalent antibodies (10). To examine the fate of granule membrane proteins after fusion, bovine adrenal chromaffin cells were stimulated for 30 s and then immediately placed on ice to prevent endocytosis (Fig. 1, A–C) or maintained at 34 °C for 5 min to permit endocytosis (Fig. 1, D–F). Subsequently, cells were incubated with antibodies to the lumenal domains of DBH (Fig. 1, A, D, and G), vesicular monoamine transporter 2 (VMAT2) (Fig. 1, B and E), and synaptotagmin (Fig. 1, C and F) to label the proteins on the cell surface. Confocal microscopy revealed that each of the three proteins was present as puncta and in many cases were co-localized. In cells immediately placed on ice (Fig. 1, A–C), 90% of puncta show evidence of co-localized granule membrane proteins, with 50% of puncta containing all three antigens, 40% of puncta with two antigens detectable, and 10% with only one detectable (almost invariably DBH). After 5 min at 34 °C, the three antigens retained a punctate appearance (D–F), and co-localization could still be detected in 80% of puncta, with only 20% bearing DBH alone. The data indicate that instead of diffusing away within seconds after fusion, these secretory granule membrane components remain together on the plasma membrane. The following experiments suggest that the associated granule membrane proteins reflect a distinct sorting domain that organizes clathrin- and dynamin-mediated endocytosis.

Clathrin and Dynamin Rapidly Accumulate at Fusion Sites—The prolonged presence of an identifiable fused granule membrane raises the possibility that compensatory, clathrin-mediated endocytosis occurs at fusion sites in response to secretion. Because clathrin-mediated endocytosis in chromaffin cells occurs over many minutes, we expected that accumulation of clathrin at fusion sites would occur over a similar time period. Chromaffin cells transiently expressing NPY-mCherry and clathrin light chain-GFP were stimulated with 56 mM K+ and either immediately placed on ice (0 °C, A–C) or incubated for an additional 5 min at 34 °C (D–F) before being placed on ice (Protocol 1, see Experimental Procedures). All cells were then incubated with antibodies to the lumenal domains of three granule proteins (A and D, DBH; B and E, VMAT2; C and F, synaptotagmin 1 (syn)) for 60 min on ice and then processed and imaged by confocal microscopy. Yellow arrowheads indicate instances of co-localization of the three proteins. G, unstimulated cells were processed for DBH as in A. Scale bars = 2 μm.

FIGURE 1. Co-localization of chromaffin granule membrane proteins after fusion. Cultured bovine chromaffin cells were stimulated for 30 s with 56 mM K+ at 34 °C. The solution was replaced with buffer containing 5.6 mM K+ and either immediately placed on ice (0 °C, A–C) or incubated for an additional 5 min at 34 °C (D–F) before being placed on ice (Protocol 1, see Experimental Procedures). All cells were then incubated with antibodies to the lumenal domains of three granule proteins (A and D, DBH; B and E, VMAT2; C and F, synaptotagmin 1 (syn)) for 60 min on ice and then processed and imaged by confocal microscopy. Yellow arrowheads indicate instances of co-localization of the three proteins. G, unstimulated cells were processed for DBH as in A. Scale bars = 2 μm.
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before granule fusion (as seen in C) to that seen 6 s post-fusion (when clathrin begins to accumulate) and 25 s post-fusion when it reaches a maximum (as in D). Clathrin accumulation usually did not precisely co-localize with the position of the granule immediately before fusion. The small offset (~300 nm) may reflect clathrin accumulation at the edge of the fused granule membrane (predicted radius of 300 nm). Frequently, multiple rounds of clathrin accumulation and disappearance could be observed at fusion sites. For example, in Fig. 3, three cycles of clathrin accumulation and decline were observed at precisely the same site.

Immunocytochemistry revealed that clathrin accumulation at fusion sites is not an artifact of overexpression of exogenous clathrin. After a 15-s stimulation with elevated K\(^+\), 89% of DBH puncta on the cell surface was associated with puncta of endogenous clathrin (n = 621 puncta from 12 cells).

Live-cell imaging of dynamin2 labeled with GFP (Dyn2-GFP) also revealed a significant tendency for dynamin to associate with fusion sites. Chromaffin cells co-transfected with Dyn2-GFP and NPY-mCherry were stimulated with 56 mM K\(^+\) and imaged at 10 Hz using TIRFM microscopy. The presence of Dyn2-GFP puncta at exocytotic sites was analyzed for up to 45 s after exocytosis. Of 34 exocytotic events, 19 (56%) had associated Dyn2-GFP, whereas only 2/28 (7%) of neighboring non-fusing granules had a dynamin punctum appear within 500 nm of the granule center after 13 s of stimulation (the average time to fusion in the imaging experiments).

The co-localization of endogenous dynamin2 and clathrin light chain with DBH at fusion sites was investigated with confocal microscopy. Chromaffin cells were stimulated for 15 s with 56 mM K\(^+\), incubated at 0 °C for 15 min with anti-DBH, and then fixed and permeabilized before incubation with antibodies against clathrin light chain and dynamin2 (Fig. 4). Instances of co-localization of the three antigens are indicated by yellow arrowheads. Although clathrin was again associated with the vast majority of DBH puncta, dynamin2 was present on only a small subset of those, probably because interactions of dynamin with clathrin-coated vesicles are transient (17, 18). These data raise the possibility that compensatory clathrin- and dynamin-mediated endocytosis begins within 10s of seconds of fusion and directly internalizes the fused secretory granule membrane.

The Intensities of DBH and VMAT2 Puncta Diminish Gradually Over Time—Clathrin and dynamin often arrive within seconds at sites of fusion, yet the chromaffin granule membrane proteins remain unpunctate on the cell surface for many minutes afterward. We examined the time course of the loss of DBH and VMAT2 immunoreactivity from the cell surface after stimulation to determine the manner by which clathrin-mediated endocytosis removes granule membrane components (Fig. 5). If clathrin-dependent endocytosis removes an entire granule membrane punctum all at once, then as time goes on one would expect to see fewer and fewer immunoreactive puncta, but the intensity distribution of the remaining puncta would remain unchanged. Cells were stimulated for 15 s with elevated K\(^+\)-containing solution, after which the depolarizing stimulus was removed, and the cells were maintained at 34 °C for various lengths of time before being placed on ice and incubated with antibodies to the lumenal domains of DBH (Fig. 5A) or VMAT2 (Fig. 5B) to label the remaining antigens on the cells surface.

The intensities of several hundred puncta were measured for each time point, and the intensity distributions were plotted as cumulative frequency histograms. Over the 30-min period, the number of DBH puncta per 10 \(\mu\)m (cell perimeter) dropped by only 20%, from 10.87 ± 0.39 to 8.59 ± 0.61 (Fig. 5C). It is apparent from the leftward shift in the distribution of intensities for both proteins that the individual puncta become gradually dimmer over a 30-min period. For example, initially (t = 0) more than half of the DBH puncta (54%) had an intensity greater than 40,000 arbitrary fluorescence units. This dropped to 32% of DBH puncta by 3 min and only 11% after 30 min. VMAT2 puncta showed a similar tendency to decrease in intensity over time, with 51% of puncta initially having an intensity greater than 8000 but then dropping to 31 and 10% after 3 and 10 min, respectively.

FIGURE 2. Clathrin light chain-GFP accumulates at sites of fusion within 20 s. Chromaffin cells transiently expressing NPY-mCherry and clathrin light chain-GFP were stimulated with 56 mM K\(^+\) and imaged by TIRFM at 10 Hz. Shown are surface plots (rendered by ImageJ) for a region of interest indicating a secretory granule immediately before (A) and after (B) fusion. Surface plots for clathrin fluorescence are shown just before fusion (C) and 25 s later (D), when clathrin accumulated near the site of granule disappearance. E, shown are plot profiles of fluorescence intensities through the clathrin spot immediately before granule fusion (as seen in C), 6 s post-fusion (when clathrin begins to accumulate), and 25 s post-fusion (as in D).
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The gradual loss of intensity of the population of granule membrane puncta led us to consider a pathway for endocytosis in which a fused granule membrane is retrieved bit by bit via repeated clathrin- and dynamin-mediated endocytic events (Fig. 6). This nibbling notion is attractive as the typical clathrin-coated vesicle (∼90-nm diameter) is too small to internalize as a unit the entire chromaffin granule membrane patch from the fusion of a 300-nm diameter granule. Some implications of this model are investigated in the following sections.

The Reduction in DBH Intensity of Surface Puncta after Secretion Requires Dynamin GTPase Activity—The role of dynamin GTPase activity at sites of fusion was examined using a cell-permeant inhibitor of the dynamin GTPase, dyngo4a (19). Chromaffin cells were preincubated with the inhibitor or its inactive congeners, dyngo8a, for 30 min before stimulating with

FIGURE 3. Multiple rounds of clathrin light chain-GFP accumulation and disappearance after fusion. Multiple rounds of clathrin accumulation and disappearance were often observed at fusion sites during live-cell TIRF imaging. An example of such a cycle is shown here. Chromaffin cells transiently expressing NPY-mCherry and clathrin light chain-GFP were stimulated with 56 mM K⁺ and imaged by TIRFM at 10 Hz. Plot profiles of fluorescence intensities through an accumulating clathrin punctum were generated in ImageJ. Panel A shows the initial accumulation of clathrin immediately after granule fusion. The center of the fusing granule is indicated by the line at 0.5 μm. Clathrin begins to accumulate (arrows) in the punctum on the right at 5.8 s and reaches an initial peak 11.8 s post-fusion. B, after peaking, clathrin declines (downward arrows), reaches a minimum at 14.8 s, and then regains peak values 2 s later (upward arrows). In C, the clathrin intensity of the punctum on the right peaked at 16.8 s post-fusion (last time point in B) and was maintained for an additional 3.4 s before its disappearance 500 ms later (downward arrow). Clathrin again began to accumulate within 100 ms (first upward arrow) and had regained much of its previous intensity 4.1 s later. Note that there were clathrin intensity changes at 0.25 μm that were not well synchronized with the clathrin punctum at 0.75 μm.

FIGURE 4. Co-localization of clathrin light chain, DBH, and dynamin2. Chromaffin cells were incubated with 56 mM K⁺ for 15 s at 34 °C. The incubation solution was removed, and cells were placed on ice and incubated with antibody to the lumenal domain of DBH for 15 min. Cells were subsequently permeabilized with ice-cold methanol before incubation with antibodies to clathrin light chain and dynamin2. Scale bar = 2 μm.

FIGURE 5. Time course of loss of exposed DBH and VMAT2 from the plasma membrane. Cells were incubated with ± 56 mM K⁺ for 15 s at 34 °C. The solution was replaced with buffer containing 5.6 mM K⁺, and the cells were maintained at 34 °C for times ranging from 0 to 30 min, after which they were placed on ice, incubated with antibodies to the luminal domains of DBH (A) and VMAT2 (B) for 60 min, fixed, and then imaged. A, 12–14 cells, averaging 600–800 DBH puncta, were analyzed for each time point. For unstimulated cells, there were 15 cells with 417 puncta. B, 10–12 cells, averaging 126–166 VMAT2 puncta, were analyzed for each time point. C, average number of DBH puncta/10 μm plasma membrane for the time course shown in panel A.
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FIGURE 6. Model of a nibbling mechanism for clathrin-mediated endocytosis. Upon stimulation, the membrane of fused chromaffin granules inserts into the plasma membrane. Clathrin adaptors are then rapidly recruited to sites of fusion by phosphatidylinositol-4,5-diphosphate (PIP$_2$) and granule membrane proteins (e.g. synaptotagmin-1, VMAT2) with adaptor binding motifs. Subsequent binding of clathrin permits the formation of clathrin-coated vesicles, whose scission requires dynamin GTPase activity. Because the typical clathrin-coated vesicle (90-nm diameter, 0.025-$\mu$m$^2$ surface area) is too small to internalize the entire chromaffin granule membrane patch (0.282-$\mu$m$^2$ surface area) as a unit, the process is repeated (nibbling), and the patch is internalized incrementally.

FIGURE 7. Dynamin inhibitor dyngo 4a, but not its inactive congener dyngo8a, blocks nibbling. Chromaffin cells were preincubated for 30 min with 20 $\mu$M dyngo 4a, 20 $\mu$M dyngo8a, or vehicle (DMSO, 0.2%) in serum-free medium before incubation at 34 °C for 20 s. The solutions were then replaced with 5.6 mM K$^+$, and the cells were incubated for 15 min at 0 or 34 °C in the continuing presence of inhibitors. All the cells were then placed on ice and incubated with antibody to the lumenal domain of DBH for 60 min, fixed, and then imaged. The intensities of the two populations of puncta (internalized and surface) were determined and plotted as a frequency histogram (Fig. 8). The active dynamin GTPase inhibitor dyngo 4a (A) completely blocked the loss of DBH from the surface, whereas the inactive congener dyngo8a (B) had no effect on DBH internalization.

56 mM K$^+$ for 20 s. The cells were then immediately transferred to ice or incubated for 15 min at 34 °C to permit endocytosis to occur. All groups were then incubated with anti-DBH, fixed, and analyzed by confocal microscopy. Pretreatment with the GTPase inhibitor dyngo4a almost completely blocked the reduction of the intensity of DBH puncta during the 15 min after stimulation (Fig. 7A), whereas its inactive congener, dyngo8a, had no effect (Fig. 7B). The importance of the dynamin GTPase activity for internalization of DBH was confirmed by transiently overexpressing a GTPase-defective mutant, dyn2K44A; overexpressed wild-type dynamin2 served as a control. Dyn2K44A expression completely blocked the internalization of DBH from fusion sites, whereas wild-type dynamin2 overexpression had no effect (not shown). Taken together, the data are consistent with clathrin- and dynamin-dependent endocytosis, causing the gradual decrease in intensity of DBH puncta.

Internalized Puncta Contain Much Less DBH Immunoreactivity Than Do Puncta at Initial Fusion Sites—The nibbling hypothesis leads to a strong prediction: there should be much less DBH immunoreactivity in the internalized puncta as compared with puncta on the plasma membrane before endocytosis. To test this we employed a variation on our previous protocol. Cells were stimulated for 30 s with 56 mM K$^+$ and then incubated on ice with anti-DBH for 30 min to prelabel the fused granule membranes so that the subsequent fate of DBH could be monitored. One group of cells was then warmed to 34 °C, and endocytosis was allowed to proceed for 15 min (Fig. 8B); a second group of cells was maintained on ice (Fig. 8A). The cells were then fixed and imaged. The intensities of the two populations of puncta (surface and internalized) were determined and plotted as a frequency histogram (Fig. 8C). Compared with the broad distribution of the surface puncta, the internalized puncta exhibited a distinct, much lower intensity peak.

Stimulation Elicits a New Population of Endocytosed Vesicles—We developed an alternative method for unambiguously identifying vesicles that have been internalized from the cell surface. After stimulation, cells were incubated for 15 min with a fixable fluid-phase marker (Alexafluor$^{TM}$-cadaverine) together with anti-DBH and then fixed with glutaraldehyde (a strong cross-linking fixative). Control experiments indicated that the presence of anti-DBH antibody in the medium did not alter constitutive or stimulated uptake of Alexafluor$^{TM}$-cadaverine (data not shown). The use of the fixable Alexafluor$^{TM}$-cadaverine revealed internalized Alexafluor$^{TM}$-cadaverine-containing vesicles in both unstimulated (Fig. 9A) and stimulated (Fig. 9C) cells. Alexafluor$^{TM}$-cadaverine vesicles in stimulated (Fig. 9D),
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but not in unstimulated (Fig. 9B), cells contained DBH. The latter vesicles may reflect constitutive endocytosis.

These observations were quantitated, and the results are shown in Fig. 9E. Alexafluor™-cadaverine uptake was completely inhibited at 0–4 °C. Incubation for 30 s with elevated K⁺ before introduction of Alexafluor™-cadaverine and anti-DBH doubled the number of Alexafluor™-cadaverine-containing vesicles. We quantitated the amount of Alexafluor™-cadaverine per vesicle in unstimulated and stimulated cells (Fig. 9F). The Alexafluor™-cadaverine intensity is expected to be proportional to vesicular volume. In the absence of stimulation, most of the Alexafluor™-cadaverine uptake was in a single well defined intensity peak with a mean of 20,318 ± 548. With stimulation, this peak broadened and doubled in size. Besides increasing the number of endocytic vesicles, stimulation resulted in a new population of larger vesicles containing significantly more Alexafluor™-cadaverine than those in unstimulated cells.

We compared the Alexafluor™-cadaverine intensities in vesicles with and without DBH (Fig. 9, G and H) in stimulated cells. The intensity distribution of vesicles without DBH was similar to that of vesicles from unstimulated cells (Fig. 9G). The distribution of Alexafluor™-cadaverine intensities of the DBH-containing vesicles was much broader and included vesicles of the same intensity as in unstimulated cells as well as vesicles with many-fold higher intensities (Fig. 9H). The mean intensity was significantly larger (43,282 ± 1,435).

It is possible that the broad distribution of Alexafluor™-cadaverine- and DBH-containing vesicles after secretion is not the direct product of endocytosis but reflects the appearance of new, larger vesicles caused by subsequent vesicular trafficking. The presence of clathrin on vesicles was used to identify vesicles that had recently completed endocytosis. Alexafluor™-cadaverine- and DBH-containing vesicles that formed within 5 min of stimulation were probed for endogenous clathrin light chain (Fig. 10). Alexafluor™-cadaverine vesicles from stimulated cells had a broad distribution of intensities (as in Fig. 9F). The intensities of the Alexafluor™-cadaverine puncta containing both DBH and clathrin light chain had a similarly broad distribution. Thus, the broad distribution of sizes of DBH-containing vesicles reflects the size distribution of the initial endocytic vesicles.

In summary, experiments with the fluid phase marker Alexafluor™-cadaverine permitted the investigation of vesicular uptake pathways under basal and stimulated conditions, independent of specific antigenic markers. The experiments demonstrate the appearance of DBH-containing endocytic vesicles of variety of sizes that are the immediate intracellular manifestation of the nibbling mechanism proposed above.

Size of Clathrin-coated Endocytic Vesicles in Resting and Stimulated Chromaffin Cells—The above confocal imaging of endocytosis with the fluid phase marker Alexafluor™-cadaverine revealed the appearance within 5–15 min of stimulation of vesicles that were larger than those in unstimulated cells. We used electron microscopy to determine directly whether a population of larger clathrin-coated vesicles could be detected immediately after a brief stimulation. Chromaffin cells were treated for 1 min with or without elevated K⁺ and immediately placed on ice, rinsed, fixed with glutaraldehyde, and processed for electron microscopy. Vesicles with typical clathrin coats were detected in both unstimulated and stimulated cells (Fig. 11, B and D). The mean diameters of the unstimulated and stimulated coated vesicles were 77 ± 3 and 87 ± 5 nm, respectively (F test, p < 0.002). Importantly, 34% (11/32) of the clathrin-coated vesicles in the stimulated cells had measured diameters greater than 100 nm, whereas none of the diameters of the clathrin-coated vesicles in resting cells exceeded 100 nm (Fig. 11A). An example of a large clathrin-coated vesicle is shown in Fig. 11D. The analysis may have underestimated the proportion of large coated vesicles resulting from stimulation, as fixation immediately after 1 min of stimulation likely captured coated vesicles that formed before as well as during stimulation.

Coated pits (data not shown) and clathrin-coated vesicles in the process of budding from the plasma membrane (Fig. 11C) were frequently observed in stimulated cells but not in unstimulated cells. They usually occurred along segments of the plasma membrane that were especially electron-dense (Fig. 11C), which could reflect the fused granule membrane. Larger coated vesicles sometimes contained debris (Fig. 11D) that could reflect remnants of the fused granule. Because of the almost complete absence of extruded granule cores, exocytotic sites could not be identified with certainty.

Thin-section electron microscopy underestimated the true diameter of the coated vesicles. Based upon stereological considerations, the average corrected diameters of the coated vesicles in unstimulated cells and stimulated cells were ~90 and 113 nm, respectively (see “Experimental Procedures”).

Other Granule Membrane Proteins were Endocytosed Together with DBH—If the model proposed in Fig. 6 is correct, one would expect to find other granule membrane proteins
associated specifically with the DBH-containing endocytic vesicles. We used Alexafluor™-cadaverine uptake to identify endocytic vesicles and examined the co-localization of synaptotagmin or VAMP with DBH. There were several considerations that guided the design of the protocol. To eliminate the possibility of cross-linking various epitopes by simultaneous incubation with several antibodies during the period of endocytosis, only anti-DBH was present with Alexafluor™-cadaverine in the extracellular medium. Antibodies to the other proteins were added after fixation and permeabilization. These
antibodies labeled both intact granules and endocytic vesicles, causing high background fluorescence. Nonetheless, the analysis detected a significant increment in synaptotagmin or VAMP2 associated with DBH- and Alexafluor™-cadaverine-positive vesicles compared with Alexafluor™-cadaverine-containing vesicles without detectable DBH (constitutive endocytic vesicles) (Table 1). The analysis indicates that the various granule membrane proteins remain associated not only on the cell surface but also during and after subsequent endocytosis.

DISCUSSION

Clathrin- and dynamin-mediated endocytosis is the major pathway for recycling of granule membrane components after strong stimulation and high exocytotic rates. This pathway occurs over 10s of seconds to minutes after fusion. It resembles classical receptor-mediated endocytosis but has a trigger that is unique to secretion, the sudden appearance of the secretory granule membrane in the plasma membrane. Although this slow form of granule membrane component retrieval after secretion has been recognized for decades, the spatial localization, the relationship to individual fusion events, the nature of the cargo, and the timing and nature of the nucleation events

**TABLE 1**

| Experiment 1 | Synaptotagmin (fluorescence intensity) | n (puncta) |
|--------------|---------------------------------------|------------|
| Unstimulated | 3,062 ± 144                           | 439        |
| Stimulated, no DBH | 3,393 ± 277                        | 231        |
| Stimulated, with DBH | 5,091 ± 200 p = 6.1 × 10⁻⁷ vs. stim, no DBH | 399        |

| Experiment 2 | VAMP (fluorescence intensity) | n (puncta) |
|--------------|-------------------------------|------------|
| Unstimulated | 18,003 ± 742                  | 778        |
| Stimulated, no DBH | 19,142 ± 1763         | 105        |
| Stimulated, with DBH | 25,575 ± 872 p = 1.0 × 10⁻³ vs. stim, no DBH | 420        |
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have been unknown. We have used a combination of TIRF microscopy of transiently expressed proteins and time-resolved, quantitative confocal imaging of endogenous proteins to address these issues. We demonstrate that 1) granule membrane proteins remain as a distinct punctate entity in the plasma membrane for minutes after fusion, 2) clathrin and dynamin begin to accumulate within seconds at fusion sites, 3) endogenous granule membrane proteins are gradually removed from individual surface puncta by a nibbling mechanism and appear intracellularly over a time course of minutes in a dynamin-dependent manner, and 4) stimulated endocytosis adds a new population of internalized vesicles distinct from those reflecting constitutive endocytosis.

The Fused Granule Membrane Remains a Distinct Entity in the Plasma Membrane That Is Gradually Retrieved by a Clathrin-mediated Nibbling Mechanism

An unexpected observation that motivated this study was the accumulation of fluorescent-labeled clathrin light chain or dynamin2 within 20 s at fusion sites imaged with live cell TIRF microscopy. Time-resolved immunocytochemistry of endogenous clathrin, dynamin2, and DBH confirmed this result. Importantly, endogenous chromaffin granule membrane proteins (DBH, VMAT2, and synaptotagmin) remained associated in puncta on the plasma membrane for many minutes after fusion, suggesting that the granule membrane itself is the likely nucleation site for endocytosis that follows fusion.

The manifestation of compensatory clathrin-mediated endocytosis was investigated by determining the fate of the protein puncta with quantitative immunocytochemistry. The surface frequency of DBH puncta decreased by only 20% over many minutes after 15–30 s stimulation, rendering it unlikely that the entire granule membrane undergoes endocytosis as a unit. Instead, there was a gradual decrease in the intensity of the puncta. The decrease was completely inhibited by the dynamin inhibitor dyngo4a. These data suggest that a fused granule membrane is gradually removed from the cell surface by multiple rounds of clathrin- and dynamin-dependent endocytosis (Fig. 6). Indeed, multiple rounds of clathrin accumulation and disappearance were often observed at fusion sites during live-cell TIRF imaging (Fig. 3). The gradual removal of granule membrane protein from surface puncta correctly predicts that amounts of DBH that appear in individual endocytic vesicles should be much less than those inserted in the plasma membrane at individual fusion sites (Fig. 8).

These data support the notion of retrieval bit by bit of small portions of the fused membrane. The quantitative estimates of endocytic vesicle size (1/7th the area of the fused granule membrane, see below) fully support this mechanism. A nibbling mechanism is a surprisingly logical explanation for the area mismatch between the clathrin-coated vesicles observed in electron microscopy and the fused granule membrane. It is consistent with electron micrographs of small clathrin-coated vesicles budding from identifiable sites of basal secretion in unstimulated hamster adrenal medulla (20, 21). Our data indicate that a nibbling mechanism is the major pathway for retrieval of the fused granule membrane upon strong stimulation. It is clathrin- and dynamin-mediated, begins within seconds of fusion, and proceeds for several minutes. We suspect that this nibbling mechanism is utilized for retrieval of fused secretory granule membrane by other neuroendocrine cells with relatively large (greater than an ∼200-nm diameter) granules but would be unnecessary for synaptic vesicles (∼50-nm diameter) if they are retrieved individually (22, 23).

Size of Endocytic Vesicles Responsible for Retrieval of Fused Granule Membrane

The various experiments permit three independent estimates of the size of the endocytic vesicles that account for the recycling of the granule membrane after fusion as follows.

Electron Microscopy—The average diameter of clathrin-coated vesicles in electron micrographs increased from 90 nm in unstimulated cells to 113 nm in stimulated cells (corrected for stereology). The latter value somewhat underestimates the true diameter of the DBH-containing endocytic vesicles as it includes coated vesicles without DBH, which are on average smaller than those with DBH (see Alexafluor™-cadaverine data, Fig. 9).

Volume Measurement—The average Alexafluor™-cadaverine fluorescence was 20,318 in endocytic vesicles in unstimulated cells and 43,282 in DBH-containing vesicles in stimulated cells. Because the former vesicles likely reflect the 90-nm diameter-coated vesicles in electron micrographs, the 2.1-fold increase in fluorescence indicates a mean diameter of 116 nm for the entire population of DBH-containing vesicles.

Surface Area Measurement—The average intensity of DBH in the plasma membrane puncta (individual fusion sites) and the average intensity of DBH in internalized puncta were 24,264 and 3744, respectively. The surface area of a chromaffin granule (∼300-nm diameter (24)) is ∼0.282 μm². From this we calculate the area of a DBH-containing endocytic vesicle to be 0.043 μm² and its diameter to be 118 nm.

The measurements converge on an average diameter of 116 nm for the endocytic vesicle responsible for retrieval of the fused granule membrane, thus confirming that the internalization of the fused granule membrane occurs through vesicles with surface areas much smaller (∼1/7th) than those of the fused membrane.

The Chromaffin Granule Membrane Is a Nucleation Site for Clathrin-mediated Endocytosis

Secretion-induced, clathrin-mediated endocytosis contrasts with the much more extensively studied clathrin-mediated receptor endocytosis. In the latter, endocytosis is initiated when the receptor (in some cases bound to ligand) interacts with a nucleation hotspot for endocytosis, whose localization is likely constrained by the actin cytoskeleton (25, 26). In the case of compensatory endocytosis after granule fusion, we find that the insertion of the granule membrane itself is the nucleation stimulus. Indeed, VMAT2 (27) and synaptotagmin (28, 29) either directly or indirectly bind clathrin adaptors on their cytosolic domains and thus may provide nucleation sites for clathrin accumulation.

Neither endogenous clathrin nor dynamin binds to chromaffin granules within cells (30–32), yet both proteins associate with the fused granule membrane within seconds. We suspect
that the key regulating factor is phosphatidylinositol-4,5-diphosphate, which is required for both exocytosis and endocytosis. Phosphatidylinositol-4,5-diphosphate is present in the plasma membrane but not in the granule membrane (33, 34). The lipid is a necessary cofactor for binding of numerous proteins involved in endocytosis including clathrin adaptors. Its presence at fusion sites makes it instantly accessible at the boundary of the fused granule membrane where it could initiate clathrin accumulation or from whence it could diffuse into the granule membrane. Endocytosis at the border of the fused granule membrane is supported by the observation that clathrin light chain-GFP puncta typically appeared \( \sim 300 \) nm from the granule position.

### Subsequent Fate of the Secretory Granule Membrane

This study did not address the subsequent fate of internalized granule membrane, an issue that was studied extensively in the 1970s and 1980s but never fully resolved. A variety of different experiments in protein-secreting cells indicate that soluble extracellular markers internalized by endocytosis label various intracellular compartments including the Golgi in parotid and lacrimal glands (35), large vacuolar structures in neurohypophysial neurons (36), and lysosomes in chromaffin cells (37). These experiments, however, do not provide direct information concerning the fate of the granule membrane components.

Pulse-chase experiments revealed that the turnover of the soluble protein contents of the granule is more rapid that those of the granule membrane, suggesting that granule membrane might be recycled after exocytosis (38). This conclusion was supported by two independent studies. Ultrastructural evaluation of the fate of the chromaffin granule membrane protein glycoprotein III demonstrated that it appeared in coated vesicles after fusion. After 6 h, glycoprotein III was again found in large dense core granules. The antigen was also detected in vesicles close to but not within Golgi stacks (8). Second, biotinylation of exposed DBH upon chromaffin granule fusion demonstrated that DBH had not degraded after 24 h and was associated with another granule membrane, protein cytochrome b561 (39). The demonstration in the present study of the continued association of granule membrane proteins after fusion supports the notion that the granule membrane retains its identity after fusion.

These and previous experiments suggest that endogenous granule membrane proteins have a tendency to remain associated after fusion. The tendency may be lessened for overexpressed secretory granule membrane proteins, as they can disperse rapidly into the plasma membrane upon fusion (40, 41). It is possible that the mechanisms that restrain endogenous proteins from leaving the fusion site become overloaded by overexpression.

### Related Work

A recent publication came to a similar conclusion as the present study that chromaffin granule membrane proteins remain associated and punctate after fusion and then are recaptured (42). The nibbling mechanism was not investigated, and the time resolution of the experiments was not sufficient to detect the critical processes that occur within seconds of fusion.

### Acknowledgments

We thank Dr. Arun Anantharam (Wayne State University) for preparing the chromaffin cells for electron microscopy and Dorothy Sorensen (Microscope and Image Analysis Laboratory, Univ. of Michigan) for performing the electron microscopy. We thank Dr. Anmita Ngatchou-Weiss for helpful suggestions about the manuscript. We are grateful to Dr. Bruno Gaspnier (Institut de Biologie Physico-Chimique CNRS, Paris, France) for providing the VMAT2 antibody and to Dr. Phillip Robinson (University of Sidney, Sidney, Australia) for providing dynog4a and dynogo8a. This research used the Sequencing Core of the Michigan Diabetes Research and Training Center, supported by National Institutes of Health Grant DK20572, and the Morphology Core, supported by the University of Michigan Comprehensive Cancer Center.

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