Membrane Hemifusion Is a Stable Intermediate of Exocytosis

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SUMMARY
Membrane fusion during exocytosis requires that two initially distinct bilayers pass through a hemifused intermediate in which the proximal monolayers are shared. Passage through this intermediate is an essential step in the process of secretion, but is difficult to observe directly in vivo. Here we study membrane fusion in the sea urchin egg, in which thousands of homogeneous cortical granules are associated with the plasma membrane prior to fertilization. Using fluorescence redistribution after photobleaching, we find that these granules are stably hemifused to the plasma membrane, sharing a cytoplasmic-facing monolayer. Furthermore, we find that the proteins implicated in the fusion process—the vesicle-associated proteins VAMP/synaptobrevin, synaptotagmin, and Rab3—are each immobile within the granule membrane. Thus, these secretory granules are tethered to their target plasma membrane by a static, catalytic fusion complex that maintains a hemifused membrane intermediate.

INTRODUCTION
Membrane fusion merges two phospholipid bilayers into one. This process is essential to eukaryotic cells and is utilized during vesicle trafficking between organelles, for secretion, and in cytokinesis. Membrane fusion requires significant free energy to surmount both the electrostatic repulsion of opposing membranes and the reorganization of phospholipids within the shared monolayer (Chernomordik and Kozlov, 2005; Kozlovsky and Kozlov, 2002). Enveloped viridae and eukaryotic cells alike use protein catalysts to overcome this energy barrier (Basanez, 2002). The most common eukaryotic catalysts are soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE), whose heterologous assembly into a trans-complex is believed to contribute both the membrane deformation and the free energy necessary for fusion under physiological conditions (Basanez, 2002; Bentz, 2000; Jahn and Scheller, 2006; Sorensen et al., 2006).

Three major events are required for SNARE-mediated exocytosis. (1) Secretory vesicles translocate to the plasma membrane, where (2) vesicle and target membrane SNARE proteins assemble in a calcium-dependent fashion into a ring of 3–15 ternary complexes at the future site of fusion (Hu et al., 2002; Jahn and Scheller, 2006). The assembly of this trans-SNARE complex destabilizes the membranes sufficiently to drive (3) membrane fusion through a hemifused intermediate—a structure whose proximal monolayers are shared via a low-energy stalk (Figure 1E) (Chernomordik and Kozlov, 2005; Kozlovsky and Kozlov, 2002; Zampighi et al., 2006). Any of these stages can be rate limiting in vivo (Han and Jackson, 2006; Jahn and Scheller, 2006; Kasson et al., 2006; Melia et al., 2006; Pobbati et al., 2006; Sorensen et al., 2006; Sudhof, 2004).

Exocytosis of a single vesicle is often completed in submillisecond intervals in vivo (Chernomordik and Kozlov, 2005; Sudhof, 2004), making it difficult to observe the intermediate fusion steps. Electron tomographic reconstruction of synaptic vesicles fixed at the active zone, a privileged region of the presynaptic membrane where fast-response secretory vesicles reside (Sudhof, 2004), suggests that the vesicles are hemifused to their target membrane (Zampighi et al., 2006). Live analysis of lipid dynamics and fusion processes in vivo and in vitro also supports the existence of a hemifused membrane intermediate, but such observations require alterations to the fusion machinery (Chernomordik and Kozlov, 2005; Xu et al., 2005; Zaitseva et al., 2005). Together, evidence for SNARE-dependent membrane fusion via a hemifused state is strong, but limited to fixed or perturbed systems.

The contents of over 15,000 secretory vesicles in the egg cortex are rapidly and synchronously exocytosed (Figure 1B) when sea urchin eggs are fertilized (Vacquier, 1975). These contents contribute to changes of the egg cell surface that are essential for the physical block to polyspermy, egg activation, and embryonic development (Wong and Wessel, 2006a). As in most animal oocytes, sea urchin cortical granules are synthesized throughout oogenesis (Laidlaw and Wessel, 1994; Wong and Wessel, 2006a) and are translocated to the cell cortex during the final phases of meiosis (Wessel et al., 2002), where they remain associated with the egg plasma membrane for
weeks without premature fusion (Vacquier, 1975; Walker et al., 2005). The attachment of these cortical vesicles is so tight that SNARE-specific neurotoxins (Tahara et al., 1998), mechanical shearing (Crabb and Jackson, 1985), and centrifugal force (Wong and Wessel, 2006b) do not release the granules from the plasma membrane. Yet, in vitro cortical lawns (Figure 1D)—made by shearing eggs bound to glass slides—retain fusion-competent granules attached to the plasma membrane that behave as in the intact egg (Churchward et al., 2005; Crabb and Jackson, 1985; Vacquier, 1975).

Here we used fluorescence redistribution assays to test the mobility of both the vesicle-associated proteins and lipids of sea urchin cortical granules attached to the plasma membrane. We find immobile SNARE-associated vesicle proteins within the membrane, suggesting that recruitment of these catalytic factors to the site of membrane fusion is a slow process. Surprisingly, we also find secretory vesicles attached to the plasma membrane via stable hemifused membranes. We suggest that these two characteristics together contribute to the stability of vesicles tightly associated with their target membranes, in a state that sensitizes the cortical granules for rapid, synchronous exocytosis following calcium release at fertilization.

RESULTS

Immobility of SNARE-Associated Cortical Granule Proteins
We first analyzed the mobility of proteins involved with exocytosis and associated with secretory vesicles (Conner et al., 1997). Populations of vesicle-associated membrane protein (VAMP/synaptobrevin), Rab3, and the calcium sensor, synaptotagmin, specifically accumulate in the membrane of the cortical granule (Conner et al., 1997; Leguia et al., 2006; Sudhof, 2004). These proteins were labeled with fluorophore-conjugated Fabs in lawn preparations (Vacquier, 1975), and then a region comprising one half of an attached granule was photobleached to assess protein mobility (Figure 2). All three Fab-labeled proteins were found to be essentially immobile within the plane of the vesicle membrane, whereas the general protein population was found to be highly mobile (72% ± 16% redistribution at 0.9 ± 0.2 μm² s⁻¹)(Figure 2). This differential protein mobility suggests that stable vesicle attachment to target membranes requires a limited, local population of interacting SNARE-associated proteins rather than active recruitment of such catalysts to an attachment site.

Lipid Mobility
In contrast to the immobility of VAMP/synaptobrevin, Rab3, and synaptotagmin, the lipid component of the cortical granule membrane is fluid. Overall, diffusion of aminonaphthylethenylpyridinium (ANEPPS when referring to a specific probe) (Fluhler et al., 1985) and Vybrant lipid probes is independent of aliphatic chain length or head group, as reflected in probe diffusion rates measured within the membrane of isolated granules (Figure 3A) and the plasma membrane (e.g., di-4-ANEPPS diffuses at 0.68 ± 0.11 μm² s⁻¹, whereas di-8-ANEPPS diffuses at 0.84 ± 0.11 μm² s⁻¹; P₁ test = 0.02). Unexpectedly, diffusion in the granule membrane is at least 5-fold
Figure 2. Cytoplasmically Exposed, Vesicle-Associated Proteins Are Immobile in the Plane of the Vesicle Membrane

(A) Mean fluorescence redistribution curves are shown for diffusion of total vesicle protein (AF488; reagent labeled all free amines) and Fab-labeled vesicle-associated proteins (Rab, VAMP/synaptobrevin, synaptotagmin) into one half of a bleached cortical granule attached to cortical lawns (see Figure 1D). Decay in fluorescence during the redistribution period (Rab, synaptotagmin fluorescence at 270 s) is a consequence of sample photobleaching during the time course. VAMP/synaptobrevin redistribution is calculated to be nearly zero, with a diffusion rate of 0.006 ± 0.008 μm² s⁻¹. Inset: magnification of mean redistribution of total protein associated with cortical granules. Diffusion rate of mobile protein population is listed. Note that total protein on unattached granules does not redistribute, and is thus immobile when attached to charged glass (data not shown). The time of bleaching was arbitrarily set as the origin of the time axis. All time-series data per replicate were normalized between the background (0%) and maximum fluorescence (100%) of the first prebleach frame. Numbers in brackets equal replicates per Fab. Colored dots correspond to images in (B).

(B) Snapshots of representative time series using total protein labeling (left), anti-VAMP/synaptobrevin (middle), and antisynaptotagmin Fabs (right). Prebleach and two postbleach images are shown, with corresponding time during the redistribution. The bleach area is bracketed to emphasize any retention of postbleach asymmetry over time. Images are pseudocolored according to the colorized scale, showing low (black) to high (white) fluorescence intensity.

(C) Fluorescence intensity plots averaged across synaptotagmin-probed granules. Per replicate, intensity totals were calculated for each pixel of an equatorial line drawn perpendicular to the bleach boundary (snapshot y axis; see [B]), summing the values for each pixel parallel to the bleach boundary per pixel along that line (snapshot x axis; see [B]). Data represent the mean (line) and standard deviation (shading) across all antisynaptotagmin replicates. Curves correspond to snapshot images in (B). Total width of each granule is 15 pixels (y axis); fluorescence intensity is normalized to the first prebleach data set, as in (A).
Figure 3. Cortical Granules Are Hemifused to the Plasma Membrane

(A) List of specific diffusion rates and redistribution times calculated for the five lipid probes used (see Experimental Procedures). Numbers in brackets equal replicates per treatment. nmr, no measurable redistribution.

(B) Representative pseudocolored image of the types of vesicles analyzed. Attached and isolated cortical granules and the edge of the plasma membrane (dashed line) are labeled. Colorized scale indicates lowest (black) to highest (white) fluorescence intensity.

(C) Mean fluorescence redistribution curves are shown for diffusion of lipid probes into fully bleached, attached cortical granules (solid lines) and reassociated cortical granules (dashed lines; di-4-ANEPPS, n = 3; di-8-ANEPPS, n = 4). Inset: mean redistribution of di-8-ANEPPS in isolated, half-bleached granules. The time of bleaching was arbitrarily set as the origin of the time axis. All time-series data per replicate were normalized between the background (0%) and maximum fluorescence (100%) of the first prebleach frame.

(D) Sequential snapshots from a representative fully bleached, attached and reattached cortical granule stained with di-8-ANEPPS. Images are pseudocolored (as in [A]), with each separated from its predecessor by 25 s within the time series. None of the preparations contained free probe in the media that could account for the redistribution (see Experimental Procedures).
faster than in the plasma membrane (for di-8-ANEPPS, $\rho_1 \text{test} = 10^{-9}$). Following vesicle fusion, however, the probe mobility in the now heterogeneous membrane equals the rate in the plasma membrane, as exemplified by di-8-ANEPPS mobility in calcium-treated lawns (calcium-fused rate is $0.80 \pm 0.18 \mu m^2 s^{-1}$, whereas the untreated rate is $0.84 \pm 0.11 \mu m^2 s^{-1}$; $\rho_1 \text{test} = 0.56$). Thus, the diffusion rate of lipid probes in these granules could be affected by membrane geometry (see Figure 1) and/or lipid composition (Churchward et al., 2005).

Surprisingly, cortical granules attached to the plasma membrane are hemifused. We tested fluorescence redistribution of lipid markers after photobleaching an entire vesicle bound to the plasma membrane (attached granule; Figure 3B). If the membranes remain separate, no probe will diffuse into the bleached granule membrane. Conversely, a continuous monolayer indicative of a hemifused intermediate allows probe to recover into the bleached area, as the lipids can freely diffuse from the unbleached plasma membrane (Figure 1E). Following complete photobleaching of individual vesicles, we generally observed fluorescence recover into the same granule membrane (Figures 3C and 3D). In contrast, mechanically detached vesicles allowed to stably reassociate with the plasma membrane did not regain fluorescence following photobleaching, as exhibited by the negligible (20% ± 11%) and immeasurable redistribution observed for di-4-ANEPPS and di-8-ANEPPS, respectively (Figure 3C). Results from these experiments strongly suggest that probe diffusion across an aqueous buffer alone cannot account for the extent of the probe redistribution observed in endogenously attached granules. These results are also consistent with the low aqueous solubility of the lipid probes—particularly those with longer aliphatic chains (e.g., di-8-ANEPPS, di-12-ANEPPS, and the C-18 probes dil and diO). Thus, probe redistribution into attached vesicles following photobleaching is a consequence of hemifused membranes.

Mean lipid probe redistribution into attached, hemifused vesicles ranged from 53% ± 26% (di-4-ANEPPS) to 101% ± 12% (di-8-ANEPPS), and was reproducible on individual granules following successive photobleaches (data not shown). Unlike the longer alkyl chain probes, di-4-ANEPPS has a tendency to slowly flip into the nonredistributing, luminal monolayer of the vesicle membrane (Loew, 1996). Following photobleaching then, only half of the initial di-4-ANEPPS vesicle fluorescence may be recovered through the outer, hemifused monolayer, as probe diffusion into the continuous monolayer occurs faster than probe flipping. Hence, we observe only 53% average di-4-ANEPPS redistribution into attached granules.

The redistribution times ($\tau$) per probe are dramatically longer than expected based on the diffusion coefficients of the respective probes in either membrane. $\tau$ was independent of the probe concentration used to label the samples (data not shown), suggesting that the number of membrane-associated probe molecules does not significantly affect characteristics of the lipid bilayer. With the exception of di-4-ANEPPS, we see no difference between $\tau$ and aliphatic chain length for probes with 8 to 18 alkyl carbons ($\rho_1 \text{test} > 0.06$ for all pairs). Thus, characteristics of the lipid probe alone are unlikely contributing to the slow redistribution times. Rather, differences in the extent of $\tau$ into the vesicle (e.g., dil versus diO) may include a probe’s effect on membrane curvature that could alter the size and shape of the hemifusion neck (Razinkov et al., 1998) and/or different physical or chemical interactions with protein components at the neck region that restrict probe mobility, as observed during hemagglutinin-mediated hemifusion (Leikina and Chernomordik, 2000). Although beyond the scope of the present work, further investigation of these differences may reveal important information about the structure of this stable hemifusion intermediate.

**DISCUSSION**

Sea urchin egg cortical granules have served as a paradigm for secretory vesicle biology for decades, and here we continue the tradition by showing that they are stably docked in a hemifused state. Several lines of evidence support our conclusion that these vesicles are in a hemifused—versus fully fused—state, including ultrastructural morphology of the interface (Figure 1A) (Chandler, 1991; Vacquier, 1975) and membrane capacitance traces before versus after fertilization (McCulloh and Chambers, 1992). The stability of this hemifusion is consistent with the 6.3 μs half-life of a hemifused intermediate calculated in silico (Kasson et al., 2006), as well as the membrane status of liposomes observed in the presence of limiting concentrations of the SNARE components (Xu et al., 2005) or with various permutations of trans-complexes (Lu et al., 2006). We therefore predict that, in general, secretory vesicles dock with their target membrane via stable, hemifused intermediates. Indeed, a special pool of synaptic vesicles appears stably hemifused to their target membrane, as suggested by tomographic electron microscopy in fixed neurons (Zampighi et al., 2006).

We also found that SNARE-associated vesicle proteins involved in regulating exocytosis are immobile in the plane of the vesicle membrane. This immobility may be due to anchorage by luminal domains of the transmembrane proteins VAMP/synaptobrevin and/or synaptotagmin that interact with immobile vesicle contents (Runnstrom, 1966) or due to their self-organization into a scaffold-like shell that itself is immobilized at the point of contact with the plasma membrane (Jahn and Scheller, 2006). SNARE complex immobility also suggests that continuous recruitment of SNARE-associated vesicle proteins does not contribute to stable vesicle association with the plasma membrane; rather, the initial SNARE complexes assembled at the microdomain of membrane contact are sufficient to maintain cortical granule attachment (Jahn and Scheller, 2006). Does the static nature of these surface proteins imply a specialized biological function? A uniform distribution of proteins required for exocytosis on the granules could, for example, enhance the efficiency of a vesicle
anchoring with the plasma membrane, as any surface can participate in assembling a SNAP complex. This was observed firsthand in our vesicle reattachment experiments, where cortical granules mechanically separated from the plasma membrane were able to stably reassociate with the plasma membrane—but curiously could not establish a hemifused membrane state. Evidently, cytoplasmic factors are required to achieve this state, and the hemifusion assay utilized here can be exploited to identify such regulators. The immobility of SNAP-associated vesicle proteins is also consistent with multipore exocytosis events observed in a single granule during cortical reorganization at fertilization (Chandler, 1991; Chandler and Heuser, 1979; Vacquier, 1975), a process similar to compound exocytosis used by pancreatic acinar and mast cells to rapidly release vesicle contents en masse in response to a single stimulus (Pickett and Edwardson, 2006).

Egg cortical granules are analogous to neurotransmitter vesicles at the active zones of presynaptic boutons. For example, these vesicle populations both undergo rapid fusion in response to calcium (Sudhof, 2004; Vacquier, 1975) and share hemifused membrane geometry (Zampighi et al., 2006). An extension of this analogy would suggest that the latter hemifused intermediate is regulated by complexes (synaphtins), which are necessary for the rapid, synchronous exocytosis of neurotransmitter vesicles (Tang et al., 2006) and are expressed in the sea urchin oocyte (J.L.W., unpublished data). The role of complexin in superpriming of SNAP complexes at the junction between vesicle and target membranes could also indirectly constrain SNAP-associated proteins within the vesicle membrane, perhaps by establishing a network that includes VAMP/synaptobrevin and its regulators synaptotagmin and Rab3. Only the action of calcium on the SNAP complex relieves this hemifused membrane intermediate, driving rapid granule exocytosis to completion (Crabb and Jackson, 1985; Hu et al., 2002; Sudhof, 2004; Tang et al., 2006; Vacquier, 1975).

**EXPERIMENTAL PROCEDURES**

**Animals**

Adult Strongylocentrotus purpuratus eggs were obtained from Charles Hollihan (Santa Barbara, CA, USA) and kept in 15°C artificial seawater (ASW) generated from Instant Ocean premixed salts (Aquarium Systems, Mentor, OH, USA). Eggs were collected by intraceolomic injection of 0.5 M KCl, washed once with pH 5.2 ASW-HCl, and equilibrated to calcium-free seawater.

**Reagents**

Affinity-purified antibody Fabs against sea urchin VAMP/synaptobrevin (Conner et al., 1997), Rab3 (Conner et al., 1997), and synaptotagmin (Leguia et al., 2006) were labeled with Oregon green or Alexa Fluor pyridinium (ANEP) probes (Fluhler et al., 1985; Vacquier, 1975; Wong and Wessel, 2007). Retached cortical granules were identified by a slight protrusion extending from the granule surface, morphology similar to that reported for hemifused vesicles (Zampighi et al., 2006), which we believe represents the hemifusion stalk surrounded by SNAP-associated proteins. All lipid probes and Fabs were diluted into isotonic, calcium-free buffer (CFB; 0.5 M NaCl, 0.01 M KCl, 1.5 mM NaHCO₃, 60 mM NaOH, 20 mM EGTA [pH 8.0]) immediately before use. Samples were labeled for 2 hr (Fabs) or 10 min (Alexa Fluor 488, lipid probes) on ice, washed extensively with cold CFB, and then mounted on glass slides (Wong and Wessel, 2007).

Fluorescence redistribution assays were conducted as described elsewhere (Cowen et al., 2004; Wong and Wessel, 2007). Samples were imaged on a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY, USA) using a 63×, 1.4 NA PlanApochromat objective with a 30 mW 488 nm argon (Fabs, ANEP probes, and diO) or 1 mW 543 nm helium/neon laser (diI). The pinhole was fully opened to ensure that maximal emission signal was collected from the entire vesicle. Five prebleach frames were taken, followed by 55 postbleach frames. Total time for each experiment ranged from 1.7 s to 5 min, depending on the delay between each acquisition (0, 1, or 5 s). The quality of each preparation was tested by demonstrating no fluorescence redistribution over a 5 min period following complete bleaching of individual cortical granules stuck only to the glass (unattached)—redistribution would be an artifact of excess fluorophore in the buffer (data not shown).

Redistribution into attached granules after full bleaching was calculated by measuring the average fluorescence intensity on the granule before bleaching (AVG₀) and the average fluorescence intensity on the granule as a function of time after bleaching (AVGₜ). These are transformed into a function F(t):

\[
F(t) = 1 - \frac{AVG(t)}{AVG₀}
\]

We used a Marquadt-Levenberg iterative curve-fitting algorithm to calculate:

\[
F(t) = A(e^{-t/\tau} + B)
\]

and fractional redistribution (R)

\[
R = \frac{1}{1 + B}
\]

**Analysis and Statistics**

All errors represent standard deviations. Single-factor ANOVA analysis was used to compare data for groups of fluorophores, assuming \(\alpha = 0.01\). Pairwise comparisons using two-tailed Student’s t test were conducted as a follow-up to ANOVA results. Multiple pairwise comparisons were deemed significant according to Bonferroni-adjusted criteria.

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