Calcium Can Disrupt the SNARE Protein Complex on Sea Urchin Egg Secretory Vesicles without Irreversibly Blocking Fusion*

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The homotypic fusion of sea urchin egg cortical vesicles (CV) is a system in which to correlate the biochemistry and physiology of membrane fusion. Homologues of vesicle-associated membrane protein (VAMP), syntaxin, and SNAP-25 were identified in CV membranes. A VAMP and syntaxin immunoreactive band at a higher apparent molecular mass (≈70 kDa) was detected; extraction and analysis confirmed that the band contained VAMP, SNAP-25, and syntaxin. This complex was also identified by immunoprecipitation and by sucrose gradient analysis. VAMP in the complex was insensitive to proteolysis by tetanus toxin. All criteria identify the SNARE complex as that described in other secretory systems. Complexes exist pre-formed on individual CV membranes and form between contacting CV. Most notably, CV SNARE complexes are disrupted in response to [Ca<sup>2+</sup]free that trigger maximal fusion. N-Ethylmaleimide, which blocks fusion at or before the Ca<sup>2+</sup>-triggering step, blocks complex disruption by Ca<sup>2+</sup>. However, disruption is not blocked by lysophosphatidylcholine, which transiently arrests a late stage of fusion. Since removal of lysophosphatidylcholine from Ca<sup>2+</sup>-treated CV is known to allow fusion, complex disruption occurs independently from the membrane fusion step. As Ca<sup>2+</sup> disrupts rather than stabilizes the complex, the presumably coiled-coil SNARE interactions are not needed at the time of fusion. These findings rule out models of membrane fusion in which SNARE complex formation goes to completion (“zippers-up”) after Ca<sup>2+</sup> binding removes a “fusion-clamp.”

Membrane fusion is the fundamental cellular process by which exocytotic secretion, enveloped virus entry, intracellular trafficking, and fertilization occur. Recently, conceptual advances have been made in how we think of the proteins involved in intracellular membrane trafficking and exocytosis (1–3), due in part to the discovery of a series of interactions between homologous proteins known to be required for intracellular membrane trafficking in vivo, yeast secretion in vivo, and synaptic transmission at the neuromuscular junction. These interactions are thought to contribute to the formation of a protein complex that is postulated to mediate the targeting, docking, and subsequent fusion of membranes (2). Since clostridial tox-
isolated CV can be used to reconstitute CV-plasma membrane fusion (19, 23) but will also fuse, with comparable Ca\textsuperscript{2+} sensitivities, to other CV (24) and to liposomes (25). CV-CV fusion is one of the simplest cases of membrane fusion. In particular, the fact that the isolated CV can fuse with liposomes indicates that CV have sufficient protein machinery for Ca\textsuperscript{2+} sensing and fusion.

Homologues of rat VAMP2 vesicle-associated membrane protein and syntaxin 1A genes have been cloned from Strongylocentrotus purpuratus ovary and testis cDNA libraries, and the presence of the corresponding proteins in the egg and in sperm has been demonstrated (26, 27). Moreover, there have been two reports showing involvement of SNARE components in this sea urchin exocytosis in vivo and in vitro (28, 29). Here, we have characterized the distribution and interactions of SNARE proteins on isolated CV to determine if these proteins could be elements of the molecular machinery for Ca\textsuperscript{2+}-dependent CV-CV fusion. We demonstrate that homologues of syntaxin, VAMP, and SNAP-25 exist on CV and form the heterotrimeric complexes first described in other cell types. We have found that, in the absence of cytosolic factors, SNARE complex disruption in the native membrane is triggered by free Ca\textsuperscript{2+} that over 98% of CV remained as single vesicles of 1 μm diameter. The supernatant containing the CV was retained and this centrifugation was repeated. Finally, the CV were collected by centrifugation at 2000 × g for 5 min at 4 °C. This final CV pellet consisted exclusively of vesicles approximately 1 μm in diameter.

**Preparation of Sea Urchin Egg Cortical Granules—**CV were prepared by a variation of the method of Crabb and Jackson (23). Cell surface complexes were prepared by homogenization in IM buffer (IM: 50 mM Tris-HCl, pH 6.8, 5 mM EGTA, 5 mM EDTA, 5 mM BAPTA, 2 mM DTT, 2 mM benzamidine, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 2.5% Triton X-114) for 10 min on ice. The protein was measured by the bichinchoninic acid assay (Pierce) with bovine serum albumin as standard. When CV were treated with tetanus toxin, 50 μg of tetanus holotoxin was incubated with 10 μg dithiothreitol for 30 min at 37 °C just prior to use. Isolated CV in 1 ml of IM buffer were incubated in the absence or presence of 300 mM toxin (8 μg of tetanus toxin light chain equivalent) at 30 °C for 16 h with constant rotation to prevent settling.

**MATERIALS AND METHODS**

*Reagents—* ATP, dithiothreitol (DTT), and protease inhibitors were purchased from Boehringer Mannheim, and bovine serum albumin was from ICN (Costa Mesa, CA). Polyvinylidene difluoride membranes were obtained from Millipore (Bedford, MA). Horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit IgGs and enhanced chemiluminescence (ECL) reagents were from Amersham International (Amersham, UK). Tetanus toxin was purchased from List Biological Laboratories (Campbell, CA). N-Ethylmaleimide (NEM) and high-quality calcium chloride were from Fluka (Ronkonkoma, NY). Laurylsarcophosphatidylcholine (LPC) was supplied by Avanti Polar Lipids (Alabaster, AL). All other reagents were of analytical grade and were purchased from Sigma. Antibodies were prepared as described (YAMM, Ref. 30) or were the kind gifts of Drs. Reinhard Jahn (SNAP-25, clone 71.1) and Thomas C. Südhof (syntaxin, I375).

**Obtaining and Handling Eggs—**Sea urchins of the species *S. purpuratus* were purchased from Marinus (Long Beach, CA) and maintained in aquaria in artificial sea water (435 mM NaCl, 40 mM MgCl\textsubscript{2}, 15 mM KCl, 30 mM HEPES, and 0.5 mM EDTA, pH 7.4). Eggs were obtained from Sigma. Antibodies were prepared as described (YAMM, Ref. 30) or were the kind gifts of Drs. Reinhard Jahn (SNAP-25, clone 71.1) and Thomas C. Südhof (syntaxin, I375).

**Preparation of Membrane Protein Samples—**Preparations of membrane fractions from isolated CV were prepared essentially as described previously (24). CV were suspended in IM to give an A\textsubscript{280} of 0.2—0.3 units, measured using a ThermoMax microtiter plate reader ( Molecular Devices, Menlo Park, CA). Aliquots of a suspension of CV (100 μl) were dispensed into 96-well, flat-bottom microtiter plates (Costar, Cambridge, MA) and CV-CV contact initiated by centrifugation (1000 × g, 10 min). The turbidity (A\textsubscript{405}) of the resulting sheets of CV was measured. Fusion was triggered by addition of an equal volume of Ca\textsuperscript{2+}-IM stock, designed to give the desired free Ca\textsuperscript{2+} concentration, and the plates were centrifuged again. A final turbidity measurement was made and the extent of fusion calculated as ΔA/ A\textsubscript{A\textsubscript{405}} initial, corrected for a background determined by lysing the CV with distilled water. Final free Ca\textsuperscript{2+} concentrations were verified in mock samples using a Ca\textsuperscript{2+}-sensitive electrode (World Precision Instruments, Sarasota, FL). All data were normalized to 1 mM Ca\textsuperscript{2+} in each experiment was set to 100.

**Disruption of Sea Urchin SNARE Complexes**—Sea urchin SNARE complexes were prepared by homogenization in IM buffer (IM: 50 mM Tris-HCl, pH 6.8, 5 mM EGTA, 5 mM EDTA, 5 mM BAPTA, 2 mM DTT, 2 mM benzamidine, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin and 2.5% Triton X-114) for 10 min on ice. The supernatant containing the Triton X-114-extracted CV membrane proteins was then extracted with CV treated with calcium to induce fusion, CV were resuspended in IM to give an absorbance of 0.2—0.3 at 405 nm. Aliquots (3 ml) were dispensed into six-well flat-bottomed plates (Costar, Cambridge, MA). To make certain that the Ca\textsuperscript{2+} concentrations that trigger maximal membrane fusion. This is contrary to the hypotheses in which stable SNARE complexes are needed for fusion, since calcium does not stabilize them in causing fusion.

**Electrophoresis and Western Blotting—**SDS-PAGE was performed in 12% polyacrylamide gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes in a transfer buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol. Blots were blocked for 30 min at room temperature in a solution consisting of 140 mM NaCl, 10 mM NaPO\textsubscript{4}, 0.05% Tween 20, 50 mg/ml bovine serum albumin, pH 7.4. Blots were incubated 100 nM) gave 0% fusion; as there was no significant difference in the extent of fusion triggered by 150—500 μM free Ca\textsuperscript{2+}, the mean fusion determined at these free Ca\textsuperscript{2+} concentrations in each experiment was set to 100.

**Preparation of Membrane Protein Samples—**Membrane proteins from isolated CV were disrupted by phase separation in Triton X-114 (32). Briefly, CV were dissolved in Triton X-114 buffer (20 mM PIPES, pH 6.8, 5 mM EGTA, 5 mM EDTA, 5 mM BAPTA, 2 mM DTT, 2 mM benzamidine, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin and 2.5% Triton X-114) for 10 min on ice. The sample was centrifuged at 1750 × g for 5 min at 4 °C to remove particulate material; no insoluble fraction was evident with CV but was found when cortices were extracted. After this, the solution was warmed to 30 °C to induce phase partitioning and the two phases were separated by centrifugation at 1750 × g for 10 min at room temperature. Protein was precipitated from the lower phase (containing the Triton X-114) by mixing with nine volumes of acetone/ethanol (1/1) overnight at –30 °C. The protein was concentrated by centrifugation at 1750 × g for 5 min at 4 °C and dissolved in sample buffer containing 0.5% Tris-HCl, pH 6.8, 150 mM NaCl, 5 mM EDTA, 100 mM NEM sucrose, and 0.1% bromphenol blue, with or without heating for 3 min in boiling water. Total protein was measured by the bichinchoninic acid assay (Pierce) with bovine serum albumin as standard. Parallel fusion assays were always carried out when the effects of Ca\textsuperscript{2+}, tetanus toxin, or inhibitors of fusion were being studied for their effects on SNARE complexes.
ing 1% (v/v) Triton X-100. 10 μl of syntaxin 1A antiserum or VAMP-2 antiserum was covalently coupled to protein G-agarose (100 μl of packed beads) using an Immunopure® protein G IgG orientation kit (Pierce). The beads were washed with the buffer described above, and incubated with solubilized CV samples for 5 h at 4 °C, followed by washing again with the same buffer. Bound antigens were eluted with 50 mM triethanolamine (pH 11.5). The immunoprecipitates were analyzed by immunoblotting using anti-syntaxin 1A and anti-VAMP-2 antibodies.

RESULTS

SNARE Proteins Are Present on Isolated CV—Samples of extracted CV membranes were analyzed for the presence of SNARE proteins by SDS-PAGE and immunoblotting by standard procedures. CV contained proteins that cross-reacted with polyclonal antibodies to mammalian VAMP2 at an apparent molecular mass of ~19 kDa, with antibodies to syntaxin 1A at ~40 kDa, and with a monoclonal antibody to SNAP-25 at ~35 kDa (Fig. 1). Samples of rat brain membrane proteins (5) yielded comparable results, although the mammalian VAMP ran at a slightly higher apparent molecular mass (data not shown).

Identification of a High Molecular Weight, SDS-resistant Complex of VAMP, Syntaxin, and SNAP-25 on CV Membranes—The feature of the SNARE hypothesis that accounts for the specificity of membrane interactions is the formation of a complex between pairs of t- and v-SNAREs. It is known that mammalian SNAREs can form a SDS-stable complex that is disrupted only at high temperature (33, 34). To visualize the sea urchin SNARE complex, we prepared a CV membrane protein sample dissolved in SDS sample buffer without boiling. In these samples, syntaxin and VAMP immunoreactivities now also appeared in a form with lower electrophoretic mobility (Fig. 2; about 70 kDa), in addition to the monomeric forms previously detected in boiled samples. Immunoblotting with the anti-syntaxin antibody always resulted in a broader and somewhat more diffuse band at 70 kDa than did blotting with the VAMP antibody. After boiling, the 70-kDa band was not detected by either antibody, as shown in Fig. 1. Additional studies showed that when cut and eluted from the gel, boiled, and subsequently analyzed by SDS-PAGE, the constituents of the 70-kDa band migrated as monomeric VAMP, syntaxin, and SNAP-25 (Fig. 2). Only one band of anti-VAMP-reactive protein at ~19 kDa was detected in this material derived from the complex band (data not shown). SDS-PAGE of solubilized CV protein at several different gel concentrations (7–15%) still yielded only a single band at 70 kDa, further indicating that this was a single complex (data not shown). Only a small fraction of the total amount of these proteins present in the CV is detected as a SDS-resistant complex.

To confirm that CV membrane proteins which cross-reacted with VAMP2, syntaxin 1A, and SNAP-25 antibodies interacted specifically to form a complex, like known t- and v-SNARES, CV membrane proteins were solubilized in 1% Triton X-100 and immunoprecipitated with anti-syntaxin 1A antibody or with anti-VAMP-2 antibody. Each immunoprecipitate was analyzed by immunoblotting in duplicate lanes using anti-VAMP2, anti-syntaxin 1A, and anti-SNAP-25 antibodies. A protein recognized by the VAMP2 antibody was immunoprecipitated together with syntaxin using the syntaxin 1A antibody, while syntaxin co-immunoprecipitated with VAMP using the VAMP2 antibody (Fig. 3). SNAP-25 was also detected in both immunoprecipitates (Fig. 3). Thus a stable complex containing the three putative sea urchin SNAREs was recovered from Triton X-100 extracts of CV membranes. Immunoprecipitation using antibodies to a non-SNARE protein did not result in any detectable recovery of syntaxin and only a faint band corresponding to VAMP (data not shown). We also confirmed the presence of SNARE protein complexes using sucrose gradient centrifugation according to the procedures of Schulz et al. (27). The results of these experiments were also fully consistent with the presence of a heterotrimeric SNARE protein complex on the CV membrane (data not shown). Thus, by direct SDS-PAGE (Fig. 2), as well as co-immunoprecipitation studies (Fig. 3) and sucrose gradient analysis, a fraction of the total VAMP in CV was found in a complex together with both syntaxin and SNAP-25.

Effect of Tetanus Toxin on the SNARE Complex—Another feature of the SNARE complex is the resistance of its constituent proteins to cleavage by clostridial proteases (33, 34). Tetanus toxin is a zinc endopeptidase that specifically recognizes and cleaves VAMP (35). It has been reported that exocytosis is inhibited in neuronal, neuroendocrine, and other cells treated with this toxin (28, 36–43). To determine if the SNARE complex exists in a preparation of contacting CV membranes and is thus a candidate for involvement in Ca2+-triggered CV fusion, or whether it is only formed in detergent solution after extraction, we treated intact CV with tetanus toxin. Isolated CV were incubated at 30 °C for 16 h in the absence or presence of 300 nM reduced tetanus toxin. After incubation, microscopic examination showed the CV to still exist singly in suspension. CV membrane proteins were extracted with Triton X-114, dissolved in sample buffer without boiling and analyzed by SDS-PAGE and immunoblotting with anti-VAMP2 antibody. Most of the sea urchin VAMP monomer was cleaved by tetanus toxin, whereas VAMP in the 70-kDa complex was insensitive to cleavage (Fig. 4A). However, tetanus toxin treatment did not inhibit
FIG. 3. Co-immunoprecipitation verifies the presence of a complex containing VAMP, syntaxin, and SNAP-25. To test for the presence of a SNARE protein complex, CV membrane proteins were solubilized in 1% Triton X-100 prior to immunoprecipitation with anti-VAMP or anti-syntaxin antibodies. The immunoprecipitates were analyzed by immunoblotting using anti-VAMP, anti-syntaxin, and anti-SNAP-25 antibodies. As indicated by PAGE (Fig. 2), the SDS-resistant complex contains all three SNARE proteins.

FIG. 4. Tetanus toxin cleaves CV VAMP monomers but not VAMP in the high M₁ complex. A, isolated CV in 1 ml of IM buffer were incubated for 16 h at 30 °C in the absence (−) or presence (+) of 300 nM tetanus toxin (Tex). After incubation, CV membrane proteins were extracted, dissolved in SDS sample buffer without boiling, and analyzed by SDS-PAGE on 12% gels followed by immunoblotting. Immunoblots were probed with anti-VAMP antibody. Panel is representative of four experiments. B, fusion assays of CV (open triangles) or tetanus toxin-treated CV (solid triangles), carried out in parallel with the protein analysis (A) using a portion of each preparation.

CV-CV fusion (Fig. 4B) or CV-liposome fusion. Thus the bulk of the VAMP monomer on CV is not needed for Ca²⁺-triggered fusion. It is possible that the remaining VAMP in the SNARE complex or as monomer was sufficient for fusion. Attempts to further lower the VAMP concentration on CV failed to reduce fusion, but also failed to remove all the VAMP (see “Discussion”). Since the other cross-reacting bands at ~14 kDa are not components of the complex (Fig. 2), they are unlikely to be tetanus toxin-resistant VAMPs.

SNARE Complex Disruption under Conditions That Induce CV-CV Fusion—Might disruption of the SNARE complex accompany fusion if the complex is linking two membranes early in the fusion process, as suggested by the SNARE hypothesis (2)? We tested the effect of Ca²⁺, which triggers CV-CV fusion, on SNARE complex stability. Sufficient CaCl₂ was added to contacting, layered CV to yield a final free Ca²⁺ concentration of 450 μM, a concentration known to cause maximal fusion in this system (Ref. 24; see also “Materials and Methods”). Proteins were subsequently analyzed (without boiling) by SDS-PAGE, and immunoblots were probed with antibodies to VAMP-2. After this Ca²⁺ treatment, the SNARE complex was no longer detected (Fig. 5). All the VAMP was now in the monomeric form. To check for losses of proteins from CV, proteins were also extracted from the supernatant after the addition of Ca²⁺ buffer, and from the aqueous phase after detergent extraction, but the SNARE proteins were not detected (data not shown). This complete Ca²⁺-triggered disruption of SNARE complexes was seen at all free Ca²⁺ concentrations tested (~90–500 μM; see also Figs. 6 and 7); parallel CV-CV fusion assays confirmed a maximal extent of fusion under these conditions (data not shown).

Attempts were made to mimic this disruptive effect of Ca²⁺ on SNARE complexes in the native membrane by treating detergent-extracted protein complexes with Ca²⁺. Neither SDS- nor Triton X-114-solubilized SNARE complexes were disrupted by the addition of Ca²⁺ (data not shown).

The SNARE Complex Exists on Single CV Membranes and Forms between Contacting CV—To test for the formation of SNARE complexes between interacting CV, we used a Ca²⁺ pretreatment to disrupt the already existing complexes on individual CV. Non-contacting, isolated CV in suspension were treated with or without [Ca²⁺]₀ free sufficient to cause maximal fusion of contacting CV; samples were subsequently diluted in buffer to reduce the free Ca²⁺ concentration, and finally sufficient CaCl₂ was added to the controls so that both controls and
Ca\textsuperscript{2+} disrupts sea urchin SNARE complexes

The effects of NEM and lysolipid on the SNARE complex suggest a correlation with bilayer fusion. CV were treated in suspension with 5 mM NEM for 30 min or 100 \mu M LPC for 5 min, centrifuged into contact, then treated with Ca\textsuperscript{2+} (final free concentration, 100 \mu M; see “Materials and Methods”). The resulting CV layer was extracted with Triton X-114 and the proteins analyzed by immunoblotting without boiling. Figure is representative of five experiments with NEM and three with LPC.

Ca\textsuperscript{2+}-pretreated samples had the same final Ca\textsuperscript{2+} concentration. Proteins were extracted from the CV in suspension and analyzed by immunoblotting. As shown in Fig. 6 (lane 1), pre-existing SNARE complexes were detected in control, non-contacting CV extracted from suspension. Ca\textsuperscript{2+}-pretreated CV no longer had SNARE complexes (lane 2). Thus, SNARE complexes on single CV membranes also respond to Ca\textsuperscript{2+} by disrupting. To test if SNARE proteins could form complexes between interacting CV, control and Ca\textsuperscript{2+}-pretreated CV were centrifuged on separate plates to make contacting layers, and proteins were then extracted as before and analyzed by immunoblotting. When Ca\textsuperscript{2+}-pretreated CV, devoid of complex, were diluted with IM buffer to chelate the added Ca\textsuperscript{2+} and then centrifuged into contact, SNARE complexes reappeared (Fig. 6, lane 4). As complexes do not reform on single CV in suspension over the same period of time (Fig. 6, lane 2), this suggests that SNARE proteins on opposed CV membranes can interact to form intermembrane complexes as proposed by the SNARE hypothesis (2).

Ca\textsuperscript{2+} Disrupts SNARE Complexes at a Late, Lipid-dependent Stage of Fusion prior to Bilayer Merger—To determine the stage during fusion at which complex disruption occurs, we used two inhibitors of CV-CV fusion known to act at different points in the fusion pathway. Prior treatment of CV with 5 mM NEM, which blocks fusion early at the stage of calcium sensing or initial conformational change (Refs. 24 and 31; confirmed, data not shown), prevented the Ca\textsuperscript{2+}-triggered loss of the 70-kDa complex (Fig. 7, lane 3; 100% of control, lane 1). Treatment with lysophosphatidylcholine (LPC), which reversibly blocks a late, lipid-dependent step during bilayer merger, reversibly blocks CV-CV fusion (44). In contrast to the effect of NEM, the SNARE complex was no longer detectable in immunoblots of CV treated with LPC and then Ca\textsuperscript{2+} (Fig. 7, lane 4; 0% of control, lane 1). CV-CV fusion assays were carried out in parallel and confirmed the reported inhibitory effects of LPC (44). Thus LPC cannot prevent the Ca\textsuperscript{2+}-triggered loss of the 70-kDa complex, even though removal of LPC does not prevent subsequent fusion due to calcium (44).

**Discussion**

In summary, an SDS- and tetanus toxin-resistant complex of syntaxin, VAMP, and SNAP-25 was found both on and between secretory vesicles of the sea urchin egg. This complex was disrupted by micromolar concentrations of Ca\textsuperscript{2+} when on membranes, but not when the complex was in detergent solution. When bilayer fusion of contacting vesicles was reversibly inhibited with lysophosphatidylcholine, complex disruption was still caused by Ca\textsuperscript{2+}, even though vesicles remained capable of rapid and complete fusion in response to Ca\textsuperscript{2+}. These results suggest that 1) the SNARE complex of sea urchin secretory vesicles is stable, like those in other examples of Ca\textsuperscript{2+}-triggered exocytosis and 2) SNARE complex disruption, not stabilization, is caused by the trigger for bilayer fusion: Ca\textsuperscript{2+}. It is thus unlikely that the coiled-coil stability of the SNARE complex is required for bilayer fusion per se.

**Properties of This SNARE Complex**—The membrane proteins SNAP-25, syntaxin, and VAMP (synaptobrevin) have been implicated as central elements of an exocytotic membrane fusion complex in both neuronal (35, 45) and non-neuronal cells (28, 37, 39, 46). The presence of VAMP, syntaxin, and SNAP-25 homologues in membranes of the sea urchin egg secretory organelle, as reported here (Fig. 1) and suggested by other recent work (26, 29), indicates that the molecular mechanisms controlling exocytosis are similar in neuronal cells and sea urchin eggs. Sea urchin cytosol contains a NSF homologue, NSF binding sites are present in the isolated exocytotic apparatus, and recombinant mammalian NSF and α-SNAP plus a Triton X-100 extract of CV form a 20 S complex (47). The core of the 20 S complex is the heterotrimeric, SDS-resistant complex formed by the SNAREs in the absence of NSF or α-SNAP (4, 48). As shown in Figs. 2 and 3, sea urchin homologues of VAMP, syntaxin, and SNAP-25 also form this SDS-resistant complex. Whether this exists as the 70-kDa complex on CV membranes in vivo or whether it is part of a larger complex that is disrupted in the process of sample preparation is unknown. If the SNARE complex is important for fusion, then it must already be in a fusogenic conformation, since cytosolic factors and ATP are not required for Ca\textsuperscript{2+}-triggered fusion (17–19).

In the SNARE hypothesis (2), syntaxin and SNAP-25 are postulated to be target membrane constituents, t-SNAREs, and are expected to be found only in the plasma membrane, not in secretory granules. As shown in Fig. 1, isolated CV contain syntaxin and SNAP-25 homologues. However, in unfertilized eggs, sea urchin CV are already docked. Therefore, we cannot exclude the possibility that when CV are isolated they may dissociate from the plasma membrane in such a way that the entire fusion complex, even parts originally derived from the plasma membrane, remains attached. Whether the syntaxin and SNAP-25 detected in isolated CV originate from the plasma membrane or from the CV membrane itself remains unknown. However, it has been reported that syntaxin is associated with chromaffin granules (49), purified synaptic vesicles (50, 51), and internal membranes of non-neuronal cells (52), indicating that syntaxin is not exclusively localized to the plasma membrane. Furthermore, the presence of heterotrimeric complexes has also been seen on isolated synaptic vesicles (53) and yeast vacuoles (10).

The ability of tetanus toxin to proteolytically cleave the main CV VAMP band (Fig. 3) reinforced the identification of the sea urchin cross-reactive material as a VAMP homologue. Indeed, the only sea urchin VAMP isosform so far identified using molecular cloning contains a site likely to be a substrate for tetanus toxin (26). The failure of tetanus toxin to inhibit CV-CV fusion might be explained by the fact that CV VAMP complexed with syntaxin and SNAP-25 is resistant to cleavage by tetanus toxin, as has been shown in mammalian systems (54). This indicates that the complex is already formed on single isolated CV, substantiating the direct measurement on isolated vesicles (Fig. 6). Bi et al. (28) reported that tetanus toxin injection into the unfertilized sea urchin egg prevents exocytosis of CV that had been transiently dissociated from the plasma membrane by treatment with stachyose. This seems inconsistent with our data since tetanus toxin did not inhibit fusion between CV,
which requires contact between vesicles and some form of docking. Although the reason for this difference is not clear, one possible explanation may be the procedure used to undock CV from the plasma membrane, such that undocking CV with stachyose in vivo disrupts the SNARE complex, while isolating CV with high pH buffer in vitro leaves complexes intact. CV undocked in vivo would then have only free VAMP monomer, the substrate for tetanus toxin. An alternative possibility is that the cleavage of CV VAMP monomer in vivo may trigger a degradation pathway for the CV that includes an inactivation of fusogenicity independent of toxin activity, perhaps involving cytosolic factors that are absent from our in vitro studies. It is also possible that tetanus toxin has activity in addition to the proteolysis of VAMP.

Another recent report demonstrated partial inhibition of exocytosis after tetanus toxin treatment of egg cortices from another species of urchin, *Lytechinus pictus* (29), despite apparently complete cleavage of VAMP by the toxin. This imperfect correlation between cleavage of VAMP and blockade of exocytosis has also been reported in other, non-neuronal systems (37, 40, 41) and has not been definitively explained. The discrepancy with our data, showing no inhibition of CV-CV fusion by toxin treatment, may have a simple explanation; VAMP is not absolutely required for membrane fusion, but has a priming or docking function that has already been carried out in fully primed and docked systems such as the sea urchin egg cortex. Partial inhibition of exocytosis in *L. pictus* cortices may therefore reflect transient undocking during toxin treatment due to incubation at 37 °C, an unphysiologically high temperature for the sea urchin. Redocking may require VAMP. This interpretation is consistent both with the time-dependent decay in the extent of exocytosis, even in the absence of toxin in that study (29), and with the results after undocking of CV from the plasma membrane (28). In the isolated CV-CV fusion system, where docking is at least initiated by centrifugation of the vesicles into contact, the fraction of VAMP that is in the SNARE complex and protected from toxin cleavage (carried out in vivo) would then have only free VAMP monomer, the substrate for tetanus toxin. An alternative possibility is that tetanus toxin has activity in addition to the proteolysis of VAMP.

SNARE Complex Disruption by Calcium—We have shown that the SNARE protein complex is not found on CV under conditions of maximal Ca\(^{2+}\)-induced fusion. The Ca\(^{2+}\)-induced loss of complex may coincide with protein conformational changes or with a disruption of the complex that might occur during the fusion process. We detected SNARE complexes from contacting layers of CV (Figs. 2 and 4–7) and by immunoprecipitation of CV protein extracts (Fig. 3). However, as in synaptic vesicles (53) and yeast vacuoles (10), SNARE complexes already exist on single CV membranes (Fig. 6). These complexes are also Ca\(^{2+}\)-sensitive, even in the absence of membrane-membrane contact, undergoing complete disruption at [Ca\(^{2+}\)]\(_{\text{free}}\) capable of causing maximal CV-CV fusion. This is not consistent with the original SNARE hypothesis (2). Consistent with most hypotheses is the observation that SNARE complexes form between CV brought into contact in the presence of low [Ca\(^{2+}\)]\(_{\text{free}}\) (Fig. 6). This indicates that SNARE proteins on single CV membranes form intermembrane complexes with complementary SNARE proteins on opposed membranes without the contemporary intervention of cytoplasmic factors such as NSF. These results are also consistent with reports showing that homotypic membrane fusion in yeast can use interactions between v-SNAREs on one membrane and t-SNAREs on the apposing membrane (7) but does not require NSF at the time of docking nor subsequently (8). However, unlike the yeast vacuole system, in which the ATP-dependent action of NSF and α-SNAP induces a metastable, fusion-competent state which lasts less than 90 min (8), isolated CV can be maintained as separate granules for >16 h in a primed, fusion-competent state (see Fig. 4B).

Docked and Primed Pre-fusion States Exist Downstream of Complex Disruption—This is the first report of a Ca\(^{2+}\)-triggered disruption of the SNARE complex. Since the isolated CV system preserves both the biochemical constituents and the functional Ca\(^{2+}\) sensitivity of the intact egg, we believe that this reflects a biological process. We note that strong interactions among membrane proteins may be needed for efficient membrane-membrane contact prior to fusion but could inhibit fusion pore enlargement if they persist (58). Thus, disruption of the SNARE complex, to effect its removal from the interface between two apposed membranes, may be one of the sites of Ca\(^{2+}\) action during triggered membrane fusion. To date, most characterizations of protein-protein interactions have used SNARE complexes reconstituted in detergent or recombiant proteins lacking their membrane-spanning domains, and one recent study shows disassembly of the naturally occurring SDS-resistant complex in synaptic vesicles by NSF and α-SNAP (53). The present study has focused on disruption of the SNARE complex in native membranes by micromolar [Ca\(^{2+}\)]\(_{\text{free}}\), in the complete absence of cytosolic factors. Since detergent-solubilized complexes are not disrupted by Ca\(^{2+}\), we speculate that, in the native membrane, the component proteins of the SNARE complex may assume conformations that create a Ca\(^{2+}\) binding site and/or that one or more other Ca\(^{2+}\)-binding factors (such as other proteins or lipids) are associated with the core complex but are lost during complex isolation.

The block of Ca\(^{2+}\)-triggered CV-CV fusion, but not of complex disruption, by LPC (Fig. 7) indicates that complex disassembly must occur before fusion, consistent with predictions of the original SNARE hypothesis (2) but not subsequent modifica-

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tions (11–13). The SNARE complex can form between vesicles that are docked and primed to fuse, yet disruption of the complex occurs prior to fusion, in the LPC-arrested stage. This force must be stored in the membrane after Ca²⁺ during the LPC-arrested stage is known to fuse upon subsequent washout of LPC and addition of Ca²⁺ (59, 60). If zipper-up of SNARE protein coiled-coil domains is the driving force for bilayer fusion, as has recently been suggested (11–13), this force must be stored in the membrane after Ca²⁺ treatment in the LPC-arrested stage in a novel type of pre-fusion complex. This is unlike the pre-fusion complex in HA-mediated fusion, in which HA activated by low pH during the LPC-arrested stage is still needed to obtain fusion after removal of LPC (61). Furthermore, it follows that Ca²⁺ does not remove a putative “fusion clamp” that blocks further intermembrane coiled-coil domain interactions between SNARE proteins since that suggestion predicts that Ca²⁺ would stabilize the SNARE complex, and the fact is that it disrupts the SNARE complex.

In conclusion, the combination of physiological and biochemical approaches has allowed us to narrow the field of hypotheses on the role of SNARE proteins in fusion. It is the reduced nature of the CV system that will now allow us to critically examine the role of the SNARE complex in the molecular mechanism for membrane fusion.

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