Supplemental Data

Synaptic Vesicles Are Constitutively Active Fusion Machines that Function Independently of Ca^{2+}

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Supplemental Experimental Procedures

Protein Expression

The SNARE proteins syntaxin 1A, SNAP-25A, and synaptobrevin 2 from rat (*Rattus norvegicus*) were used. As basic expression constructs we used SNAP-25A (SN25, in which all cysteines were replaced by serines), syntaxin 1A SNARE motif (SyxH3, amino acids 180–262), syntaxin 1A SNARE motif with transmembrane domain (SyxH3, amino acids 183–288), full-length syntaxin 1A (SyxFL, amino acids 1–288), and the cytosolic domain of synaptobrevin 2 (Syb, amino acids 1–96), all of which have been described before [S1, S2].

Protein purification was by Ni^{2+}-NTA affinity chromatography, as detailed previously [S1], with the transmembrane-domain-containing proteins purified in the presence of 15 mM 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) [S2]. The binary complexes containing syntaxin 1A (amino acids 183–288 or 1–288) and SNAP-25A were assembled from purified monomers and subsequently purified by a further round of ion exchange chromatography in the presence of 15 mM CHAPS. The ΔN complex (containing syntaxin 1A [amino acids 183–288], SNAP-25A, and synaptobrevin 2 [amino acids 49–96]) was purified from BL21 (DE3) bacteria expressing all three proteins, using the pET28a vector for SNAP-25A and the pETDuet-1 vector for syntaxin 1A and synaptobrevin 2. The complex was purified by Ni^{2+}-NTA affinity chromatography, and ion exchange chromatography in the presence of 15 mM CHAPS [S3]. Protein concentrations were determined by absorption at 280 nm.

The light chains of Botulinum neurotoxin C1 (which targets syntaxin 1 on the liposome) and Tetanus toxin (which targets synaptobrevin on the synaptic vesicle), as well as the corresponding inactive mutant proteins, were expressed in bacteria and purified with Ni^{2+}-NTA chromatography [S4–S6]. Concentrations were determined with UV absorption, and proteolytic activity was tested immediately before use in experiments (see Figure S1).

Preparation of Native and Artificial Vesicles

Preparation of Proteoliposomes

For the preparation of proteoliposomes, unlabeled phospholipids were obtained from Avanti Polar Lipids. Phospholipids containing covalently linked fluorescent dyes were from Avanti or Molecular Probes. Lipids were mixed in chloroform to give our standard preparation (molar ratios): phosphatidylcholine (5), phosphatidylethanolamine (2), phosphatidylserine (1), phosphatidylinositol (1), and cholesterol (1). After drying, they were resuspended in HB100 buffer (100 mM KCl, 1 mM DTT, 25 mM HEPES [pH 7.4, KOH]), 5% (w/v) sodium cholate at a total lipid concentration of 13.5 mM. In liposomes containing phosphatidylinositol 4,5-bisphosphate, the level of phosphatidylinositol was reduced accordingly. For use in fluorescence-based assays, unlabeled phosphatidylethanolamine was substituted in part by 1.5% (n/n) N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD) phosphatidylethanolamine and 1.5% (n/n) Rhodamine phosphatidylethanolamine (lipid dequenching assay), and 5% (n/n) Oregon Green 488 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine or 5% (n/n) Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (FRET assays).

Protein complexes were incorporated at protein:lipid ratios of 1:300 [S7]. Unless explicitly stated, the syntaxin/SNAP-25 complex routinely incorporated during liposome preparation was preformed binary complex, consisting of two syntaxin 1A molecules and one SNAP-25A [S1].

For lipid dequenching experiments (NBD-Rhodamine), proteoliposomes were formed by detergent removal with size-exclusion chromatography on a SMART system with a PC 3.2/10 Fast Desalting column (GE Healthcare), equilibrated in HB100 at a constant flow rate of 50 μl/min [S2]. Alternatively, liposomes containing Oregon Green- or Texas Red-labeled lipids (FRET) were prepared with Bio-Rad EconoColumns filled with Sephadex G-50 superfine at a minimum sample-to-column volume ratio of 1:30 and equilibrated with HB100 by gravity flow [S4].

The quality of the liposome preparation was routinely tested with electron microscopy (Figure 3), which showed liposomes to have a relatively homogeneous size distribution. Liposomes were also analyzed by SDS-PAGE and Coomassie Blue staining (see Figure S1A). Nycodenz floatation of liposomes after detergent removal showed that incorporation was close to 100% for the protein densities used. For determination of protein orientation, proteoliposomes were incubated with trypsin at a molar ratio of 25:1 (protein:trypsin) at 37°C for 4 hr. Parallel incubations were performed in the presence of 0.2% Triton X-100. Reacted samples were separated by SDS-PAGE on a 10% tricine gel and visualized with Colloidal Coomassie (Invitrogen). As reported previously, approximately 80% of the syntaxin/SNAP-25 complex was correctly oriented [S4].

Purification of Synaptic Vesicles

Synaptic vesicles were purified by two different procedures for use in our experiments. The first purification method (referred to as Method A) is a modified version of a classical fractionation protocol, which was originally developed by Whittaker and coworkers. This protocol is the one used as standard in our laboratory and was the basis of a recent comprehensive characterization [S7].

In brief, 20 rat brains were homogenized in 240 ml ice-cold sucrose buffer (320 mM sucrose, 4 mM HEPES [pH 7.4, NaOH]) supplemented with 0.2 mM phenylmethylsulfonylfluoride (PMSF) and 1 μg/ml pepstatin A. The homogenate (H) was spun for 10 min at 800 g_{Av}, yielding a pellet (P1). The supernatant was centrifuged for 15 min at 12,000 g_{Av}. The resulting pellet was washed twice with sucrose buffer, yielding a clean synaptosome pellet (P2). The synaptosomal pellet was resuspended in 24 ml sucrose buffer and lysed by addition of 9 volumes distilled H_20, followed by homogenization. Five millimolar HEPES (pH 7.4, NaOH) was added, supplemented with 0.2 mM PMSF and 1 μg/ml pepstatin A. This lysate was then centrifuged for 20 min at 32,500 g_{Av} giving a pellet (LP1) and supernatant (LS1). The supernatant was centrifuged for a further 2 hr at 230,000 g_{Av}, yielding a crude synaptic vesicle pellet (LP2). This crude synaptic vesicle pellet was resuspended in 40 mM sucrose and further purified by centrifugation for 4 hr at 82,500 g_{Av} on a continuous sucrose density gradient (50–800 mM sucrose). Vesicles were collected from the gradient and subjected to size-exclusion chromatography on controlled pore glass beads (300 nm diameter), equilibrated in 300 mM glycine, 5 mM HEPES (pH 7.40, KOH), to separate synaptic vesicles from residual myelin and larger membrane contaminants. Synaptic vesicles were pelleted by centrifugation for 2 hr at 230,000 g_{Av} and resuspended in HB100 buffer by homogenization before being aliquoted into single-use fractions and snap frozen in liquid N_2. These vesicles were previously shown to be largely free of contamination, being approximately 95% pure when assayed by immunogold labeling for synaptophysin (an integral membrane protein found on the synaptic vesicle), documenting that contamination by nonsynaptic membranes is low. Synaptic vesicles purified and stored in this manner remained functional, without loss of activity, for many months, as judged by both neurotransmitter uptake and synaptobrevin activity. Typically, synaptic vesicles were used within a month of preparation for the experiments performed here.

The second method was introduced by the Davletov group (and is referred to as Method B). In brief, synaptosomes were prepared from whole brains of adult rats with Ficoll gradients as previously described [S8, S9]. Synaptic vesicles were released from synaptosomes by hypo-osmotic lysis with 9 vol H_20 to 1 vol synaptosomes, followed by homogenization. Five millimolar HEPES (pH 7.4, NaOH) was immediately added to the lysate, supplemented with 0.2 mM PMSF and 1 μg/ml pepstatin A. This lysate was then centrifuged for 20 min at 34,000 g_{Av} to remove heavy membranes from the lysate. The resulting supernatant was mixed 1:1 (v/v) with Optiprep, overlaid with 40% Optiprep containing K-gluconate buffer (adjusted to give a final concentration in mM; 140 K-gluconate, 4 $MgCl_2$, and 20 HEPES [pH 7.30, KOH]). Gradients were then centrifuged for 60 min at 420,000 g_{Av}, and synaptic vesicles were collected from the top of the upper phase of the gradient. Synaptic vesicles were stored on ice and used within 3 days. Synaptic vesicle purity was assessed immediately after preparation, according to the published criteria of Hu et al. [S9]. Experimental protocols have been described in detail elsewhere [S9, S10]. In brief, for immunogold electron microscopy, purified synaptic vesicles were adsorbed onto glow-discharged carbon grids and fixed with a solution of 2% paraformaldehyde in a 40% Optiprep buffer containing K-gluconate. Free aldehydes were subsequently quenched with 200 mM glycine. Samples were then labeled with a polyclonal

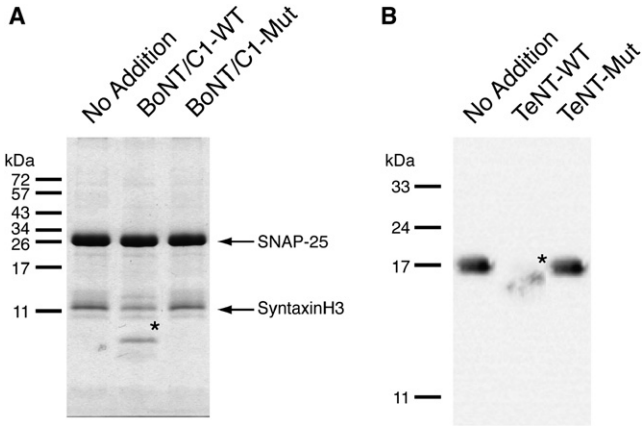

Figure S1. Cleavage of SNARE Proteins by Neurotoxins

(A) SyntaxinH3/SNAP-25 liposomes were incubated for 60 min at 37°C with HB100 buffer (No Addition), 30 nM wild-type Botulinum C1, or 30 nM mutant (inactive) Botulinum C1. Samples were separated by SDS-PAGE with a 16% tricine gel and stained with Colloidal Coomassie (Invitrogen). Consistent with previous reports, Botulinum C1 cleaves syntaxinH3 in a 2:1 binary complex with SNAP-25 [S20], with the level of syntaxinH3 obviously reduced and the main proteolytic fragment (syntaxin amino acids 188–254) clearly visible (*); consistent with previous reports, syntaxin was not fully cleaved by Botulinum C1 [S21]. In contrast, incubation with HB100 or mutant Botulinum C1 had no effect on syntaxinH3. As expected, SNAP-25 levels were also unaffected in all samples.

(B) Synaptic vesicles were incubated for 60 min at 37°C with HB100 buffer (No Addition), 40 nM wild-type Tetanus toxin, or 40 nM mutant (inactive) Tetanus toxin. Samples were separated by SDS-PAGE with a 10% tricine gel and immunoblotted with a monoclonal antibody (Cl69.1) specific for synaptobrevin. Incubation with wild-type Tetanus toxin caused almost a quantitative loss of synaptobrevin from synaptic vesicles (*). In contrast, incubation with HB100 or mutant Tetanus toxin had no effect on the amount of synaptobrevin. Note that although the antibody's epitope is still present in the Tetanus toxin cleavage product (amino acids 1–76), it is not recognized by the antibody for unknown reasons, so no cleavage products are visible. The degree of proteolytic activity correlates well with results obtained in the lipid dequenching assay (Figure 1A), in which Tetanus toxin causes a greater reduction in fusion than Botulinum C1.

antibody against synaptophysin [S11] at a dilution of 1:500 in 1% bovine serum albumin-Tris buffered saline (BSA-TBS) buffer followed by 10 nm protein A gold conjugates diluted at 1:300. The samples were further fixed for 10 min with 2% glutaraldehyde in TBS, washed with H_2O, rinsed with three drops of 1% aqueous uranyl acetate, and immediately dried. Images were taken with a Philips CM120 BioFilter electron microscope. Immunoblotting of fractions taken from the top and bottom phases of the Optiprep gradient showed that synaptic vesicles (synaptobrevin) prepared with this method were efficiently separated from a cytosolic protein (calcineurin). These results are summarized in Figure S2.

A modified Lowry procedure was used to determine the concentrations of membrane proteins [S12].

Controlling for Degradation of Synaptic Vesicle Proteins

Synaptic vesicles were either stored at −80°C (Method A) or on ice (Method B) until use. The neuronal Ca^{2+} sensor, synaptotagmin, is known to be proteolytically labile [S13]. To control for potential artifacts caused by protein degradation during our Ca^{2+} experiments, we tested for this possibility by immunoblotting.

Addition of Ca^{2+} to the reaction mixture produces a very reproducible inhibition in fusion when vesicles prepared with Method A are used. However, this inhibition was not due to vesicular synaptotagmin being degraded during the fusion reaction, because only minor amounts of proteolysis were observable that, in addition, did not change during the course of our experiments (Figure S3A). In addition, synaptotagmin immunopurified from rat brain extracts is known to be active [S14], rendering it unlikely that the synaptotagmin in our experiments was damaged during vesicle purification and consistent with the observed PIP_2 effect (Figure 5B).

Synaptic vesicles prepared according to Method B were stored on ice immediately after preparation and used for a maximum of 3 days. Samples taken at these two time points showed that no significant breakdown of synaptotagmin occurred under these conditions (Figure S3B).

Fluorescence Assays

As indicators of membrane fusion, we used lipid dequenching and FRET-based assays, which have both been previously described, albeit with minor modifications [S15, S16].

Method A

Unless stated, all reactions used liposomes lacking PIP_2 and were carried out in the absence of Ca^{2+}. The standard reaction mixture for the NBD-Rhodamine dequenching assay contained syntaxin/SNAP-25 liposomes at a final protein concentration of 750 nM with either 10 μg or 20 μg synaptic vesicles, in a total reaction volume of 75 μl HB100 buffer. The standard reaction mixture for the FRET assay contained 750 nM Oregon Green proteoliposomes with 10 μg synaptic vesicles in a total of 75 μl HB100 buffer. A saturating amount of Texas Red proteoliposomes (7500 nM) was then added to this mixture. For all experiments, reaction conditions were determined empirically to give the optimal signal:noise ratio in the assays. Ca^{2+} experiments were performed by addition of proteoliposomes and synaptic vesicles to commercial Ca^{2+} buffers of known concentration (Molecular Probes) or with a 1,3-diamino-2-propanol-N,N,N′,N′-tetraacetic acid (DPTA)-based buffer system [S17]. Final Ca^{2+} concentrations in the reaction mixtures were determined with the fluorescent dyes Mag-Fura2 and Fluo5N (Molecular Probes), against a standard curve constructed with a Molecular Probes Calibration Kit. Lysophosphatidylcholine (LPC) was dissolved in HB100 and added at the beginning of the fusion reaction, with a suitable correction made to ensure constant volume. Fluorescence anisotropy was performed essentially as described [S2], albeit slightly modified to measure the rate of vesicular synaptobrevin binding to the SNARE acceptor complex.

Method B

A modified version of our standard lipid dequenching assay was used, to account for differences in volume and buffer composition used by Hu and colleagues [S9]. NBD-Rhodamine liposomes containing full-length syntaxin 1/SNAP-25 were prepared according to our usual protocol, with gel filtration columns equilibrated in K-gluconate buffer (140 mM K-gluconate, 4 mM $MgCl_2$, 2.25 mM ethylene glycol tetraacetic acid (EGTA), 20 mM HEPES [pH 7.30, KOH]). In a typical experiment, syntaxin/SNAP-25 liposomes (in a total of 60 μl K-gluconate-EGTA buffer) were mixed with 3.5 μg synaptic vesicles (in 30 μl 40% Optiprep), giving a total reaction volume of 90 μl and a final liposomal protein concentration of 750 nM. Reactions were measured individually in a Fluoromax2 fluorimeter equilibrated to 37°C, and NBD fluorescence was monitored with monochromitor settings of 460 nm (excitation) and 538 nm (emission). Approximate Ca^{2+} concentrations were calculated with WEBMAXC (http://www.stanford.edu/~cpatton/webmaxcS.htm) and were verified with the fluorescent dye Fluo5N (Molecular Probes), against a standard curve constructed with a Molecular Probes Calibration Kit. Recombinant Tetanus toxin light-chain was dialysed against K-gluconate buffer (including EGTA), and the concentration was determined by absorption at 280 nm.

Other Methods

Cryoelectron microscopy and subsequent image analysis were performed as previously described [S4], for two independent experiments, for which the data was then combined. SDS-PAGE and immunoblotting were performed as described, with minor alterations [S4, S18, S19]. Monoclonal antibodies against synaptobrevin (clone 69.1), synaptophysin (clone 7.2), and synaptotagmin 1 (clone 41.1) were from Synaptic Systems. Peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) was from Bio-Rad and "Western Lightning" enhanced chemiluminescence kit was from Perkin Elmer. Image capture was performed with a LAS reader (Fuji) and densitometry with AIDA (Raytest). Images were contrast enhanced with the Auto Levels function of Photoshop CS1 (Adobe), to avoid pixel saturation. Data analysis was performed with Igor Pro (Wavemetrics).

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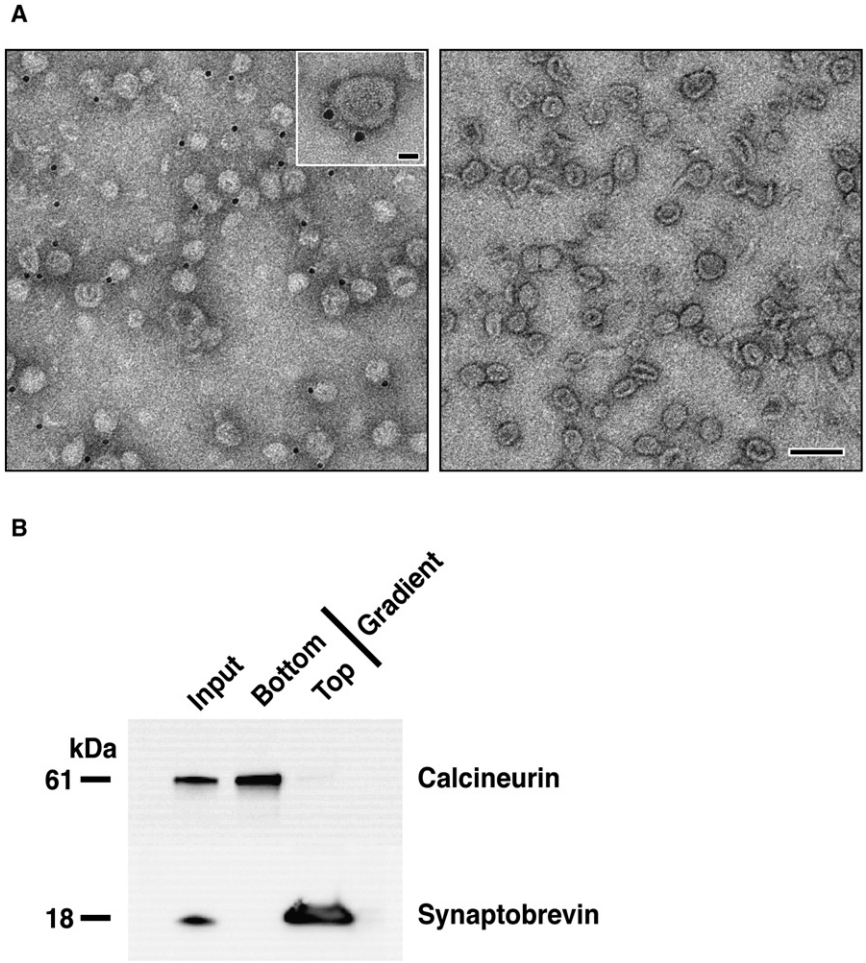

Figure S2. The Purity of Synaptic Vesicles Prepared According to Method B was Assessed with Electron Microscopy and Immunoblotting

(A) Low-magnification electron micrographs (negative staining) of vesicles purified according to Method B [S9]. The vast majority of structures were in the correct size range for synaptic vesicles (approximately 50 nm; [S7]), and 75%–80% could be labeled with an antibody against synaptophysin (left panel; c.f. our standard purification, using controlled pore-glass chromatography, which gives a purity of 95%, when assessed by these criteria). The inset shows a higher-magnification view of a labeled vesicle. Negative control, in which the primary antibody was omitted, showed no vesicle staining (right panel). The scale bars represent 100 nm (low magnification) and 20 nm (high magnification).

(B) Samples were taken from the top and bottom phases of the Optiprep gradient after centrifugation and subjected to SDS-PAGE and immunoblotting. Synaptic vesicles floated at the top (synaptobrevin) and were effectively separated away from a known cytosolic protein found at the bottom (calcineurin).

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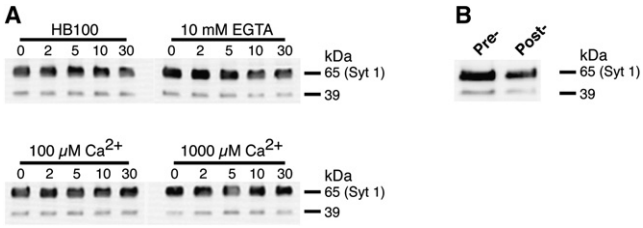

Figure S3. Controlling for Synaptotagmin Integrity

(A) Samples of fusion reactions with synaptic vesicles purified according to Method A were taken at the indicated time points of a fusion reaction (min) for the buffer conditions indicated. The reaction was stopped immediately by addition of SDS-sample buffer and analyzed by SDS-PAGE and then immunoblotting for synaptotagmin 1 (syt 1) with a monoclonal antibody specific for the cytoplasmic domain. Synaptotagmin levels remained constant for the duration of the experiment. Only a minor amount of a proteolytic fragment was observable (39 kDa) that did not increase during the experiment. (B) Synaptic vesicles prepared according to Method B were tested for synaptotagmin proteolysis with an antibody specific for the cytoplasmic domain, immediately after preparation (pre-) or after a maximum of 3 days storage on ice (post-). Minimal breakdown of synaptotagmin was seen.

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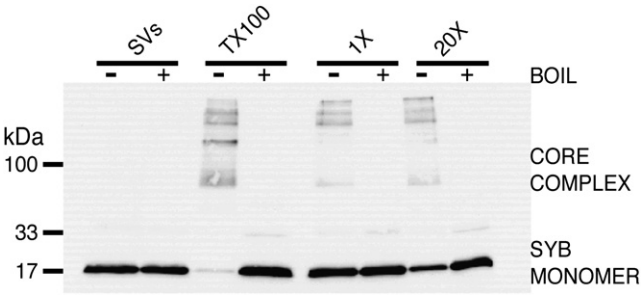

Figure S4. Fusion of Proteoliposomes and Synaptic Vesicles Prepared by Method B Was Concomitant with the Formation of SDS-Resistant, Heat-Sensitive SNARE Core Complexes

Samples were analyzed at the end of fusion reactions with SDS-PAGE and immunoblotting for synaptobrevin. As expected, only monomeric synaptobrevin was detectable when the sample was heated to 100°C before electrophoresis (+ boiled; c.f. − unboiled samples). Increasing of the amount of syntaxin/SNAP-25 proteoliposomes to 20× the standard amount quantitatively increased the amount of synaptobrevin engaged in core complexes. Note that synaptic vesicles contain low amounts of endogenous SDS-resistant complexes, but these were only detectable at longer exposure times (data not shown, see also [S22]). So that an estimate of the maximum number of SDS-resistant complexes that can form under these conditions could be obtained, proteoliposomes and synaptic vesicles were incubated at 37°C in the presence of 2% (vol:vol) of Triton X-100 (TX100).

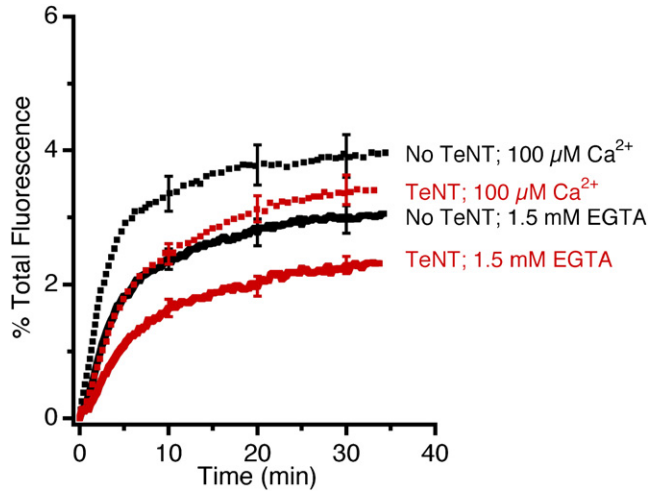

Figure S5. Effect of Ca^{2+} on Fusion between Synaptic Vesicles Prepared According to Method B and Syntaxin 1/SNAP-25 Proteoliposomes

Contrary to previous reports, reliable fusion between synaptic vesicles and proteoliposomes could be measured by lipid dequenching in the absence of Ca^{2+}, although the reported Ca^{2+}-dependent enhancement of fusion was, in fact, observed [S9]. However, in our hands, this enhancement was unaffected by Tetanus toxin (TeNT) treatment, meaning that the Ca^{2+}-effect is independent of fusion mediated by synaptic SNARE proteins. n ≥ 8 experiments ± SEMs.

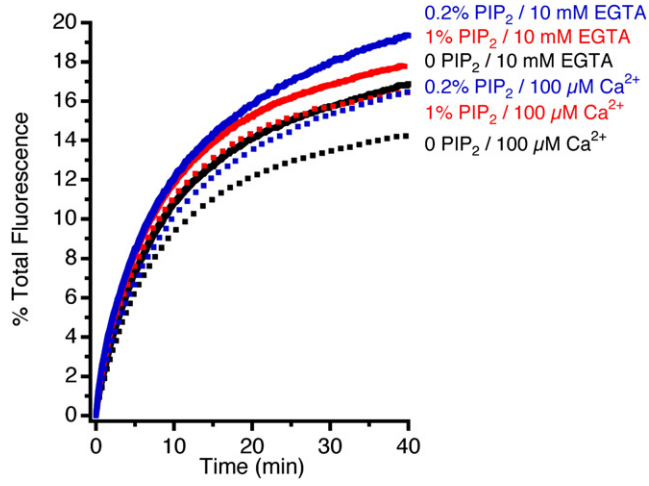

Figure S6. Variations in the Concentration of PIP_2 in the Membranes of SyntaxinH3/SNAP-25 Liposomes Had No Major Influence on the Fusion Rate

In addition to its Ca^{2+} binding properties, synaptotagmin is also known to interact with membrane phospholipids. One such interacting partner is PIP_2. Importantly, this phospholipid is localized to the plasma membrane [S23] and is essential for Ca^{2+}-triggered exocytosis [S24], interacting with synaptotagmin 1 at the elevated Ca^{2+} levels required to stimulate exocytosis [S25]. Therefore, we systematically varied the concentration of PIP_2 in the proteoliposome membrane and investigated its effects on fusion (using synaptic vesicles prepared according to Method A) in the lipid dequenching assay. Incorporation of PIP_2 into the liposome membrane (at all concentrations used) did not significantly increase the rate of fusion in the absence of Ca^{2+}. Interestingly, however, the inhibition of fusion produced by 100 μM Ca^{2+} was partially prevented as the level of PIP_2 in the proteoliposome membrane was increased. Traces are averages; 0.2% PIP_2 n = 2; all other traces n ≥ 8.

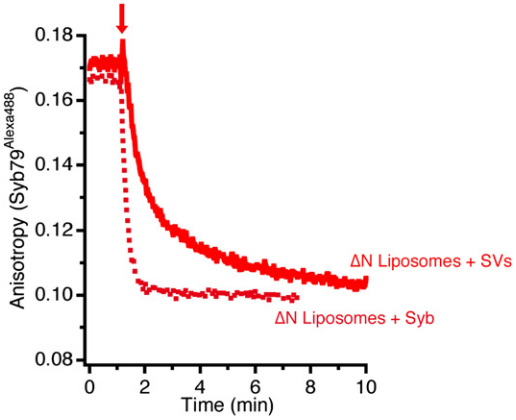

Figure S7. Binding of Vesicular Synaptobrevin to a Stabilized Acceptor Complex Can Be Measured with Fluorescence Anisotropy

A free N-terminal acceptor site for synaptobrevin can be stabilized if a SNARE complex is formed containing N-terminally truncated synaptobrevin (ΔN complex) [S3]. Use of this complex massively accelerated fusion of synaptic vesicles (prepared according to Method A) with liposomes (see Figure 6B). As expected, fusion was associated with the displacement of the N-terminally truncated peptide, as measured by fluorescence anisotropy (solid line), with this displacement occurring on a similar time scale to fusion. Hence, fusion after synaptobrevin binding was rapid (consistent with the electron microscopy data presented in Figure 3). For comparison, a soluble fragment comprising the entire cytoplasmic domain of synaptobrevin was used to quantitatively displace the fragment (dashed line). (Addition of synaptic vesicles or soluble synaptobrevin is shown by the arrow.) For this purpose, the N-terminally truncated synaptobrevin fragment was labeled at position 79 with the fluorescent dye Alexa488. The faster displacement by soluble synaptobrevin is most likely accounted for by the fact that during synaptic vesicle fusion, only a fraction of the SNARE acceptor complexes are actually involved in the initial *trans*-SNARE pairing, with the remainder of the complexes forming in *cis* after the membranes have merged.