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Calcium-triggered Membrane Fusion Proceeds Independently of Specific Presynaptic Proteins*

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Complexes of specific presynaptic proteins have been hypothesized to drive or catalyze the membrane fusion steps of exocytosis. Here we use a stage-specific preparation to test the roles of SNAREs, synaptotagmin, and SNARE-binding proteins in the mechanism of Ca^{2+}-triggered membrane fusion. Excess exogenous proteins, sufficient to block SNARE interactions, did not inhibit either the Ca^{2+} sensitivity, extent, or kinetics of fusion. In contrast, despite a limited effect on SNARE and synaptotagmin densities, treatments with high doses of chymotrypsin markedly inhibited fusion. Conversely, low doses of chymotrypsin had no effect on the Ca^{2+} sensitivity or extent of fusion but did alter the kinetic profile, indicating a more direct involvement of other proteins in the triggered fusion pathway. SNAREs, synaptotagmin, and their immediate binding partners are critical to exocytosis at a stage other than membrane fusion, although they may still influence the triggered steps.

Exocytosis is a common cellular process defined by several transient, contiguous stages, enabling the secretion of cellular compounds. The mechanism of the defining stage of exocytosis, Ca^{2+}-triggered membrane fusion, has been the subject of extensive investigation, and evidence suggests that fusion proceeds via a conserved mechanism (1–4). Our understanding of the molecular mechanism has been advanced by SNARE[1] hypotheses (5–8). This model suggests that the SNARE proteins (VAMP/synaptobrevin, syntaxin, and SNAP-25) interact to form a heterotrimeric core complex consisting of a 4-stranded helical bundle (7, 9, 10), which is proposed to "zipper-up," and thereby drive or catalyze the fusion of apposed membranes. These interactions are modeled to be necessary and sufficient for membrane fusion. However, other evidence suggests that the SNARE proteins are not the minimal, essential machinery required for Ca^{2+}-triggered fusion of native membranes (11–15) and instead indicate that SNAREs function at a critical stage of exocytosis that is upstream of membrane fusion. Postulated critical upstream roles for the SNAREs in exocytosis include (*i*) membrane targeting and initial attachment (16, 17), (*ii*) the modulation of Ca^{2+} sensitivity for the triggering steps of exocytosis (11, 15, 18), and (*iii*) the regulation of Ca^{2+} channels (19–22).

Analysis of a fundamental molecular mechanism, such as Ca^{2+}-triggered fusion, is most efficiently carried out in a stage-specific native preparation; sea urchin egg cortical vesicles (CV) provide such a stage-specific model of Ca^{2+}-triggered membrane fusion (11, 12, 15, 18). By effectively isolating the triggered steps of fusion from the other concurrent and overlapping stages of the exocytotic pathway, this system permits rigorous coupling of the functional and biochemical analyses necessary to effectively dissect this molecular mechanism (11, 15, 18). This preparation allows access to the entire membrane surface of CV, ameliorating issues of access to essential proteins at fully docked sites. In addition, CV contain defined homologues of the SNARE proteins and synaptotagmin (23), consistent with their essential, conserved roles during exocytosis. Using established methods, including blockade of protein interactions (8, 24–27) and protease treatments (15), the analysis presented here firmly places the roles of identified SNARE interactions at a stage of exocytosis upstream of triggered membrane fusion.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Proteins

Syntaxin 1A (NCB accession number NP_446240; residues 1–267), syntaxin 1A "open" (residues 1–267) (28, 29), syntaxin 1B (NCB accession number B48213; residues 1–266), VAMP2 (NCB accession number NP_036795; residues 1–135), SNAP-23c (NCB accession number NP_003816; residues 134–211), SNAP-25 (NCB accession number NP_112253; residues 1–206), SNAP-29c (NCB accession number NP_004773; residues 184–258), synaptotagmin I (NCB accession number P21707; residues 77–421), nSec1 (NCB accession number AAA19246; residues 1–594), and cysteine string protein (NCB accession numbers AAA81372; residues 1–198) are glutathione *S*-transferase (GST) fusion proteins. α_{1B} II-III linker peptide of N-type Ca^{2+} channel (NCB accession number NP_671482; residues 718–963) is a His_6 fusion protein. The constructs and fusion proteins (without transmembrane regions) were expressed and isolated as described previously (29–31). The purity of recombinant SNAREs and SNARE-binding proteins identified in Table I (49–86%) was determined as described previously (32). Mock transfections and isolations of expressed GST and His_6 were carried out, and these isolates were used as background controls during functional assays.

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[1] The abbreviations used are: SNARE, soluble NSF attachment protein receptors; NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; VAMP, vesicle-associated membrane protein; CV, cortical vesicles; GST, glutathione *S*-transferase.

Preparation of CV Suspensions

CV were isolated, purified, and counted as described previously (11). All experiments were carried out in base-line intracellular media supplemented with 2.5 mM ATP and protease inhibitors (11, 15).

In Vitro Binding Assay

The capacity of endogenous CV SNAREs to bind the recombinant proteins was assessed as described previously (29). CV membrane proteins were extracted by the methods described for rat brain homogenate (29) and incubated with the recombinant proteins bound to glutathione or nickel beads; bound CV SNAREs were identified by SDS-PAGE and high sensitivity immunoblotting (32).

CV Treatments

SNARE Blocking Experiments—Based upon protein concentration (DC protein assay kit, Bio-Rad), the measured protein purities using Coomassie Brilliant Blue R stain (32), and the average density (+1 S.E.) of CV VAMP (6710 copies/CV) (32), the various recombinant proteins were added to CV suspensions at ≥75–100-fold molar excess relative to endogenous VAMP (~1200–1600-fold excess over endogenous syntaxin). To permit full access and binding of the exogenous and endogenous CV proteins, these suspensions were incubated for 1 h at 25 °C prior to assessment of fusion (end-point or kinetic assays). Affinity purified polyclonal antibodies against the cytoplasmic domain of sea urchin synaptotagmin (1:100 dilution), sea urchin VAMP (1:100) (23), and rat syntaxin 1A (1:200; Stressgen Biotechnologies Corp., Victoria, BC) were similarly tested for effects on function.

Chymotrypsin Treatments—Chymotrypsin treatments of 400 units were carried out as described previously for other broad spectrum proteases (1 h, 25 °C) (15). Chymotrypsin treatments of 50 units were carried out for 40 min at 25 °C. Following these treatments, CV were recovered and resuspended in base-line intracellular media supplemented with 2.5 mM ATP; final suspensions also contained 2 μM L-1-tosylamido-2-phenylethyl chloromethyl ketone (Sigma) in addition to the standard mixture of protease inhibitors used during the preparation of CV suspensions (11, 15).

Fusion Assays—Standard end-point fusion assays and data analysis were carried out as described (11, 15), using the Wallac VICTOR^{2}V 1420 multilabel HTS plate reader (PerkinElmer Life Sciences). Each condition was tested in sets of 3–5 repeats per experiment; data are reported as mean ± S.E. Curves were fit using SigmaPlot® (2001 for Windows) to establish the extent and Ca^{2+} sensitivity (EC_{50}) of fusion. Kinetic assays were carried out essentially as described for end-point assays, with the exception that optical density measurements were made at successive 150- or 250-ms intervals after the rapid injection of buffer containing 200 ± 27 μM $[Ca^{2+}]_{free}$ (injection time of 140 ms for 50 μl using the WALLAC 1420–251 liquid injector). Two-sample, two-tailed *t*-tests were performed to determine differences of end-point and kinetic parameters between the experimental conditions.

Western Blotting—CV proteins were isolated by organic precipitation using 1:1 acetone:ethanol (12). SDS-PAGE, electrotransfer, and immunoblotting were carried out essentially as described (32), with the exception that blots were blocked with 5% skim milk/PBST (0.05% Tween 20 in phosphate-buffered saline) solution and developed using the ECL+plus Western blotting detection system (Amersham Biosciences). Protein bands were visualized using Kodak Biomax Light film or digitized directly using the PerkinElmer ProXPRESS proteomic imaging system and quantified by densitometric analysis using ImageQuant software (Amersham Biosciences).

RESULTS

As expected for such highly conserved, essential proteins (23) (urchin and rat SNAREs are ~70–90% homologous, including the coiled-coil regions necessary for SNARE interactions), recombinant SNAREs, and other presynaptic SNARE-binding proteins also bind to the native CV membrane SNAREs (Fig. 1*A*). Urchin syntaxin binds to the recognized syntaxin binding partners VAMP2, SNAP-25, synaptotagmin, nSec1, and cysteine string protein; selectivity is confirmed by the lack of syntaxin binding to recombinant SNAP-23c and SNAP-29c (Fig. 1*A*, *top panel*). Urchin VAMP binds to syntaxin 1A, rat VAMP2, and synaptotagmin (Fig. 1*A*, *bottom panel*). Binding of all three exogenous SNAREs to the CV membrane prior to fusion assays was also confirmed (*inset*, Fig. 1*B*). As in other systems (8, 24–27), recombinant

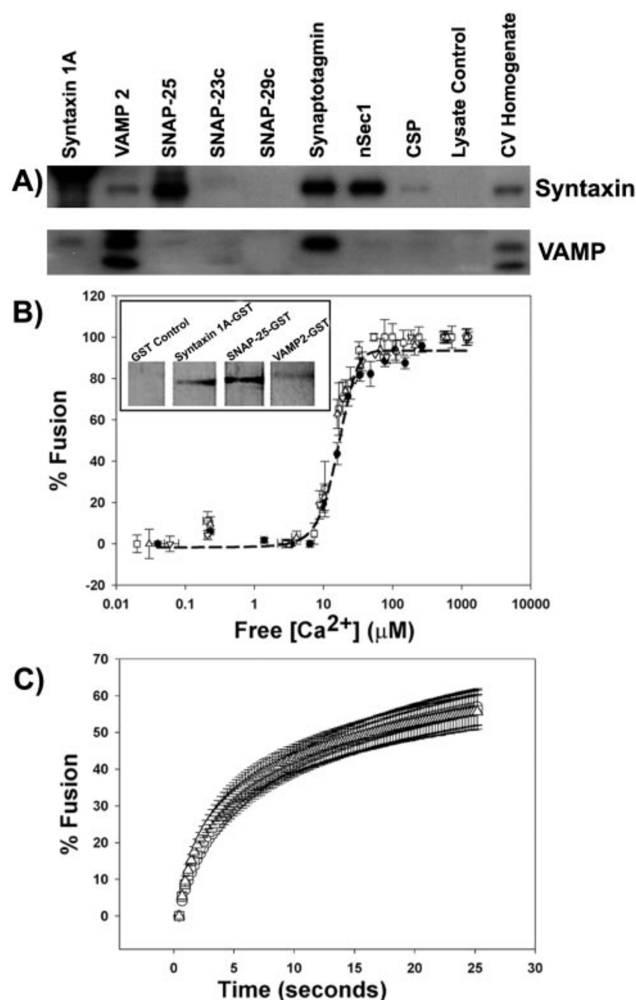

FIG. 1. **Binding of exogenous proteins to CV membrane SNAREs.** *A*, *in vitro* binding assays confirm the ability of CV SNAREs to bind to recombinant rat proteins. *B*, excess exogenous SNAREs do not affect the extent or Ca^{2+} sensitivity of fusion in a stage-specific native preparation. ●— —●, GST background controls (*n* = 14); □, syntaxin 1A (*n* = 8); ▽, SNAP-25 (*n* = 4); and △, VAMP2 (*n* = 6). *Inset*, recombinant SNAREs bind to isolated CV prior to fusion assays. *C*, exogenous SNAREs do not affect the initial fusion rate triggered by rapid perfusion with 200 ± 27 μM $[Ca^{2+}]_{free}$; symbols as in *B*.

SNAREs and SNARE-binding proteins were therefore used to block endogenous SNARE interactions (both *cis* and *trans*). The Ca^{2+} sensitivity and extent of fusion were not significantly different between buffer controls (EC_{50} = 19.0 ± 1.2 μM and extent = 96.5 ± 2.2% fusion; *n* = 22) and background controls (GST and His_6, see Table I). Data for each blocking experiment were normalized and compared with a parallel GST or His_6 background control.

At >85-fold molar excess (relative to VAMP, the most abundant CV SNARE (32)), neither syntaxin 1A, SNAP-25, nor VAMP2 had a significant effect on the extent or Ca^{2+} sensitivity of triggered fusion compared with parallel controls (Fig. 1*B* and Table I). These exogenous SNAREs also had no significant effect on the peak rate of fusion when challenged with 200 ± 27 μM $[Ca^{2+}]_{free}$ (Fig. 1*C*, Table II) or $[Ca^{2+}]_{free}$ that elicits a submaximal response (24 μM, not shown). Adding combinations of SNAP-25 and syntaxin 1A or of all three recombinant SNAREs was also without effect on end-point assays, compared with parallel controls (within the 99% confidence interval, not shown). In addition, syntaxin 1B (ΔEC_{50} = 2.0 ± 0.4 μM and Δextent = 3.2 ± 3.2% fusion) and a constitutively "open" form of syntaxin 1A (28, 29) (ΔEC_{50} = 2.6 ± 1.3 μM and Δextent =

TABLE I
The effects of recombinant proteins on CV-CV fusion

Recombinant protein added	Experiments (n)	EC_{50} ± S.E. (μM $[Ca^{2+}]_{free}$)	Average maximum % fusion ± S.E.
GST control	14	17.7 ± 1.9	96.9 ± 5.5
His_6 control	5	20.9 ± 3.7	96.6 ± 11.1
Syntaxin 1A	8	17.1 ± 1.6	100 ± 3.9
VAMP2	6	15.1 ± 1.4	99.2 ± 4.2
SNAP-25	4	16.4 ± 1.6	99.2 ± 3.1
SNAP-23c	3	17.4 ± 2.9	99.3 ± 3.1
SNAP-29c	3	12.7 ± 1.5	98.5 ± 2.2
Synaptotagmin	7	15.7 ± 1.7	100 ± 4.3
nSec1	8	17.2 ± 3.0	94.8 ± 2.9
Cysteine string protein	5	21.0 ± 2.3	98.9 ± 5.6
α_{1B} II-III linker	4	24.9 ± 1.6	100 ± 6.5

TABLE II
Peak rate of CV-CV fusion after rapid perfusion with 200 ± 27 μM $[Ca^{2+}]_{free}$

Recombinant protein added	n	Rate (% fusion/s ± S.E.)
GST control	4	15.9 ± 2.5
Syntaxin 1A	4	14.4 ± 0.9
SNAP-25	3	18.9 ± 2.8
VAMP2	4	17.3 ± 2.0

1.4 ± 0.2% fusion) had no effect (n = 2 each). An alternate assay format (11) showed no evidence for an effect of exogenous proteins on the ability of CV to contact one another (not shown). Disrupting any pre-existing *cis* SNARE complexes using Ca^{2+} pretreatments (12) to ensure full access of the exogenous proteins to the CV SNAREs also had no effect on the Ca^{2+} sensitivity or extent of fusion (not shown). Other presynaptic proteins with established SNARE binding capacities (27, 31, 33–36) were also tested for possible effects on the Ca^{2+}-triggered step(s) of fusion (at ≥75 times molar excess) (Table I) including (*i*) α_{1B} II-III linker region of N-type Ca^{2+} channel (the synprint peptide, that is known to bind urchin syntaxin (37)) (Table I); (*ii*) a combination of syntaxin 1A and nSec1 (ΔEC_{50} = 3.5 μM and Δextent = 0% fusion; n = 1); and (*iii*) affinity purified polyclonal antibodies against urchin synaptotagmin (ΔEC_{50} = 1.6 ± 0.6 μM and Δextent = 4.5 ± 4.1% fusion; n = 3), urchin VAMP (ΔEC_{50} = 2.1 μM and Δextent = 0% fusion; n = 1), and rat syntaxin 1A (ΔEC_{50} = 1.1 μM and Δextent = 0% fusion; n = 1); none of these exogenous peptides, proteins, or antibodies significantly affected the extent or Ca^{2+} sensitivity of the triggered fusion steps.

To remove proteins critical to fusion, a protease treatment paradigm (15) was applied to free-floating, non-contacting CV in suspension. This pretreatment with the serine protease chymotrypsin (400 units) significantly inhibited the extent of subsequent Ca^{2+}-triggered fusion by ~80% (n = 8; Fig. 2*A*). However, this substantial inhibition of fusion occurred despite limited effects on total SNARE densities; 52 ± 3%, 53 ± 1%, and 33 ± 5% syntaxin, SNAP-25, and VAMP, respectively, remained following the chymotrypsin treatment (*inset*, Fig. 2*A*). If the SNAREs are essential to fusion, the SNARE densities remaining after chymotrypsin treatment should be sufficient to support full fusion (15, 38). As syntaxin is the limiting native SNARE at ~330 syntaxin/CV (32), then ~172 syntaxin/CV (~52 syntaxin/μm^2) remain after chymotrypsin treatment, easily fulfilling the estimated need for three syntaxin-containing complexes/release site (38); ~1800 VAMP/CV (~550 VAMP/μm^2) and ~380 SNAP-25/CV (~115 SNAP-25/μm^2) also remained after treatment. Yet fusion is all but abolished following the treatments with chymotrypsin. Conversely, treatment of CV with 50 units of chymotrypsin does not decrease extent of fusion nor alter Ca^{2+} sensitivity (ΔEC_{50} = 1.5 μM; n = 4) (Fig. 2*B*) but does significantly reduce the peak rate of fusion from 17.1 ± 4.5%/s to 2.4 ± 0.9%/s (n = 4) (Fig. 2*C*), despite a limited effect on SNARE densities; 52 ± 7%, 64 ± 3%, and 47 ± 3% syntaxin, SNAP-25, and VAMP, respectively, remained following treatment by 50 units of chymotrypsin. In addition, synaptotagmin is not substantially affected by chymotrypsin treatment (82 ± 6%; n = 4 remained following treatment by 50 or 400 units). The altered kinetic profile of CV fusion suggests an alternate chymotrypsin target that is closer to the fusion step than are the SNAREs.

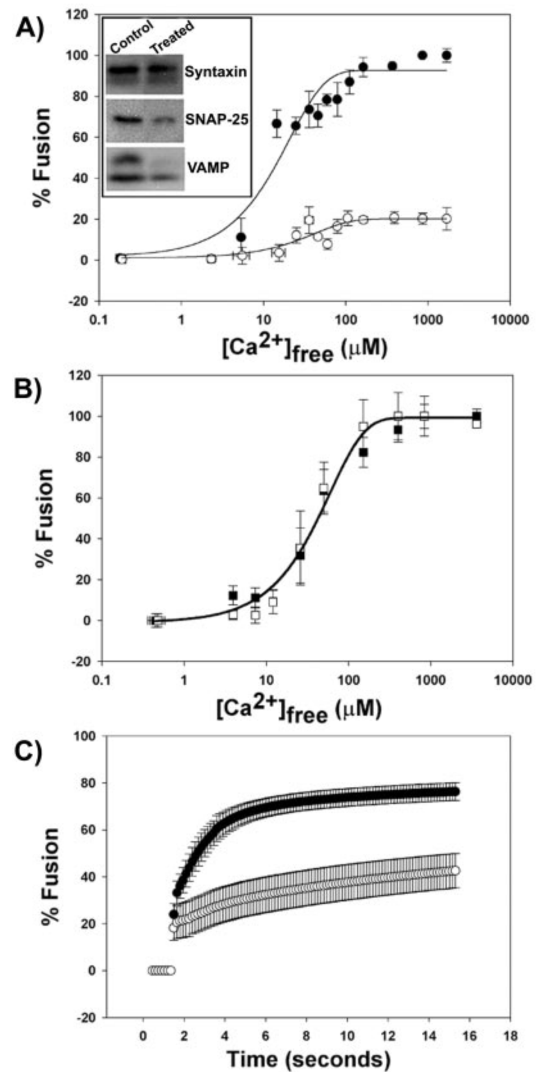

FIG. 2. **Fusion does not correlate with SNARE density.** *A*, treatment of CV with 400 units of chymotrypsin (○; n = 8) substantially inhibits fusion relative to parallel controls (●). *Inset*, quantitative immunoblotting reveals a limited effect of chymotrypsin on the density of CV SNAREs. *B*, treatment of CV with 50 units of chymotrypsin (■) does not alter the extent or Ca^{2+} sensitivity of fusion relative to parallel controls (□; n = 4). *C*, treatment with 50 units of chymotrypsin (n = 4; ○) substantially alters the kinetic profile of fusion relative to parallel controls (●).

DISCUSSION

By all available criteria, fully primed and release-ready CV are "locked" at a step in the exocytotic pathway that is downstream of critical SNARE interactions but immediately preceding Ca^{2+} triggering (11, 12, 18); essentially, this is equivalent to the rapidly (immediately) releasable pool of vesicles in neuroendocrine cells (39, 40). Even after removal from their docking sites on the plasma membrane, isolated CV remain primed

and fully fusion-competent, in the absence of cytosolic factors (11, 12, 15, 18). As any decrease in the density of a protein essential to triggered fusion is expected to inhibit the rate of fusion (41, 42), we tested the roles of different presynaptic proteins in the Ca^{2+}-triggered steps of fusion by reducing their effective concentrations (and thus critical interactions) with excess recombinant binding partners. Although SNAP-25 naturally lacks a transmembrane domain and therefore may still functionally interact with native SNAREs when added exogenously, this may be less likely as the recombinant lacks the acyl modifications necessary for membrane anchoring. There were no effects on the rate, Ca^{2+} sensitivity, or extent of triggered fusion; protease treatments directly confirmed the dissociation of fusion from SNARE densities (Fig. 2) (15). We have previously shown that fusion (at maximal $[Ca^{2+}]_{free}$) is independent of CV syntaxin density (15). Here we show that fusion kinetics can be altered despite limited changes in SNARE protein densities. The differential effects of chymotrypsin doses on function but minimal effects on synaptotagmin densities confirm the results of the blocking experiments with recombinant synaptotagmin (Fig. 1 and Table I) and synaptotagmin antibodies, suggesting that synaptotagmin is not essential to the fusion mechanism. Thus, despite isolation from their fully docked state at the plasma membrane, CV do not require additional or repeated intermembrane SNARE interactions to support Ca^{2+}-triggered fusion as rapid as seen in other secretory cell types (15, 40, 42, 43). In addition, the results argue against synaptotagmin as the Ca^{2+} sensor for triggered fusion. The data are consistent with the hypothesis presented by Voets *et al.* (43), suggesting that synaptotagmin influences the transition of a vesicle into the readily releasable pool; such a function would already have occurred in the population of fully release-ready CV.

Although not clearly defined, the state of readiness established by intermembrane SNARE binding *in vivo* or of SNARE interactions with other identified presynaptic proteins outlasts the actual physical interactions of these proteins. Although essential to exocytosis as a whole (determined by the use of clostridial toxins (44)), at pre-fusion steps, these functions are not essential to the actual molecular interactions that drive the structural rearrangements inherent to membrane merger. The results are therefore most consistent with the notion that other essential proteins function downstream of SNARE interactions to effect the actual triggered membrane fusion step(s) of exocytosis (12, 14, 15). Thus, considering the data presented here and that from a variety of other (semi)intact secretory cells, intermembrane "zippering" of SNARE complexes does not drive native fusion but may represent the final step of priming, a critical series of reactions enabling subsequent efficient triggered fusion, a function already fulfilled in fully docked and fusion-ready CV. This primed state may be analogous to previously postulated "tight" SNARE complexes (26); although this stage can be rapid and transient, as seen in secretory cells undergoing high rates of fusion, it is also clearly separated from the fusion step. Thus, identified presynaptic proteins (Table I), including synaptotagmin, have critical roles elsewhere in the exocytotic pathway, perhaps involving modulation at the plasma membrane docking site.

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