

The C2B Domain of Synaptotagmin Is a Ca^{2+} -sensing Module Essential for Exocytosis

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Abstract. The synaptic vesicle protein synaptotagmin I has been proposed to serve as a Ca^{2+} sensor for rapid exocytosis. Synaptotagmin spans the vesicle membrane once and possesses a large cytoplasmic domain that contains two C2 domains, C2A and C2B. Multiple Ca^{2+} ions bind to the membrane proximal C2A domain. However, it is not known whether the C2B domain also functions as a Ca^{2+} -sensing module. Here, we report that Ca^{2+} drives conformational changes in the C2B domain of synaptotagmin and triggers the homo- and hetero-oligomerization of multiple isoforms of the protein. These effects of Ca^{2+} are mediated by a set of conserved acidic Ca^{2+} ligands within C2B; neutralization of

these residues results in constitutive clustering activity. We addressed the function of oligomerization using a dominant negative approach. Two distinct reagents that block synaptotagmin clustering potently inhibited secretion from semi-intact PC12 cells. Together, these data indicate that the Ca^{2+} -driven clustering of the C2B domain of synaptotagmin is an essential step in excitation-secretion coupling. We propose that clustering may regulate the opening or dilation of the exocytotic fusion pore.

Key words: oligomerization • membrane fusion • synprint • C2 domain • Ca^{2+} binding

Introduction

C2 domains are conserved motifs found in nearly a hundred proteins that function in cellular processes ranging from membrane traffic to blood coagulation. These domains are composed of ~130 amino acids and fold into compact eight-stranded distinct β -sandwich structures. Three flexible loops protrude from the tip of the domain. In some cases, two of these loops form a pocket that mediates the binding of Ca^{2+} and other divalent cations (Sutton and Sprang, 1995; Sutton et al., 1995, 1999; Grobler et al., 1996; Perisic et al., 1998; Ubach et al., 1998a,b; Pratt et al., 1999; Macedo-Ribeiro et al., 1999). Ca^{2+} binding regulates the interaction of some C2 domains with target molecules including lipids and proteins, while other C2 domains do not appear to bind Ca^{2+} . Thus, the emerging view is that C2 domains are not necessarily Ca^{2+} -binding motifs, but rather function as modules that interact with a broad range of target molecules (reviewed by Nalefski and

Falke, 1996). In most cases, the biochemical and physiological functions of C2 domains remain to be defined.

Synaptotagmin was the first integral membrane protein discovered that harbored C2 domains (Matthew et al., 1981; Perin et al., 1990). This protein is localized to synaptic vesicles and large dense core secretory vesicles (Matthew et al., 1981). It spans the vesicle membrane once and has a short luminal domain and a large cytoplasmic region largely comprised of two C2 domains, designated C2A and C2B. These tandem C2 domains prompted biochemical studies, which demonstrated that synaptotagmin I is a Ca^{2+} -binding protein (Brose et al., 1992; Davletov and Südhof, 1993; Chapman and Jahn, 1994). Subsequent gene disruption studies in mice, *Caenorhabditis elegans*, and *Drosophila* demonstrated that synaptotagmin I is essential for rapid and synchronous Ca^{2+} -triggered release of neurotransmitters (Littleton et al., 1993; Nonet et al., 1993; DiAntonio and Schwarz, 1994; Geppert et al., 1994; Littleton et al., 1994), suggesting that synaptotagmin I may function as a Ca^{2+} sensor that regulates neuronal exocytosis (Katz, 1969).

Seven isoforms of synaptotagmin have been identified in the *Drosophila* genome (Littleton et al., 1999; Littleton, J.T., and E.R. Chapman, unpublished observations), indi-

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cating that the vertebrate gene family may be much larger than the current set of 12 isoforms. Furthermore, alternatively spliced forms of synaptotagmin have been identified that lack the transmembrane anchor (Fukuda and Mikoshiba, 1999). These findings suggest that synaptotagmins may have widespread functions in intracellular membrane traffic.

A key to understanding the physiological function of synaptotagmin is to understand the biochemical properties of its C2 domains. The C2A domain of synaptotagmin I binds multiple Ca^{2+} ions (Sutton et al., 1995; Ubach et al., 1998b), and becomes partially inserted into membranes that contain anionic phospholipids (Chapman and Davis, 1998; Davis et al., 1999; Bai et al., 2000). Equilibrium and kinetic Ca^{2+} binding properties of C2A are consistent with the Ca^{2+} requirement and speed of secretion (Davis et al., 1999).

The C2A domain is connected to C2B by a short tether and the tandem C2 domains cooperate to form complexes with components of the soluble *N*-ethyl maleimide-sensitive factor attachment protein receptor (SNARE)¹ complex (Chapman et al., 1995, 1996; Davis et al., 1999; Gerona et al., 2000). This complex is composed of the target SNAREs (t-SNAREs) syntaxin and SNAP-25 and the vesicle SNARE synaptobrevin (Söllner et al., 1993); assembly of the complex is necessary (Littleton et al., 1998; Chen et al., 1999) and may be sufficient (Weber et al., 1998) for exocytotic membrane fusion. Synaptotagmin forms direct contacts with both t-SNAREs at all stages of the SNARE complex assembly (data not shown; Chapman et al., 1995; Schiavo et al., 1997; Davis et al., 1999; Gerona et al., 2000) and can bind SNAREs and interact with membranes at the same time (Davis et al., 1999). These interactions suggest that synaptotagmin may regulate SNARE-mediated membrane fusion by modulating the conformation and/or assembly of SNARE complexes.

The interactions of synaptotagmin with SNAREs and anionic phospholipids are regulated by Ca^{2+} ligands that reside within C2A (Bai et al., 2000). Here, we address the question of whether and how the C2B domain of synaptotagmin functions as a Ca^{2+} -sensing module during exocytosis. This question was prompted by the finding that mutations within the C2B domain of *Drosophila* synaptotagmin impair excitation-secretion coupling (DiAntonio and Schwarz, 1994; Littleton et al., 1993, 1994). A number of C2B-effector interactions have been identified including the following: AP-2 (Zhang et al., 1994; Jorgenson et al., 1995), SV2 (Schivell et al., 1996), β -SNAP (Schiavo et al., 1995), Ca^{2+} channels (Kim and Catterall, 1997; Sheng et al., 1997), inositol polyphosphates (Fukuda et al., 1995) and, finally, homo-oligomerization (Chapman et al., 1996; Sugita et al., 1996). Of these interactions, only oligomerization was promoted by Ca^{2+} .

While C2B was implicated in mediating Ca^{2+} -triggered synaptotagmin clustering, this domain has not been directly shown to sense Ca^{2+} or transduce Ca^{2+} binding to clustering activity. Here, we report that the C2B domains of multiple synaptotagmin isoforms undergo Ca^{2+} -driven conformational changes and can homo- and hetero-oligomerize with one another.

We have characterized the structural requirements for these Ca^{2+} effects, and have applied a dominant negative approach to determine the role of Ca^{2+} -triggered synaptotagmin clustering in Ca^{2+} -regulated exocytosis. Our findings indicate that Ca^{2+} -driven clustering of synaptotagmin constitutes a novel step in excitation-secretion coupling.

Materials and Methods

Recombinant Proteins

cDNA encoding rat synaptotagmin Ia (Perin et al., 1990) and Ib (Osborne et al., 1999) were provided by T.C. Südhof (University of Texas Southwestern Medical Center, Dallas, TX) and G. Schiavo (Imperial Cancer Research Fund, London, UK; Osborne et al., 1999), respectively. pGEX expression vectors to produce the cytoplasmic domains of synaptotagmins II–XIII (Li et al., 1995) were also provided by T.C. Südhof. pGEX expression vectors to produce the C2B domains of mouse synaptotagmins III and IV (Fukuda et al., 1995) were provided by M. Fukuda (Brain Science Institute, Ibaraki, Japan) and the C2B domains for rat synaptotagmins V–VIII were generated by PCR and were subcloned via BamHI and EcoRI sites into pGEX-2T. pGEX expression vectors to produce the C2A and C2B domain of rabphilin (Ubach et al., 1998a) were provided by J. Rizo (University of Texas Southwestern Medical Center, Dallas, TX).

The cytoplasmic (residues 96–421) and C2A domains (residues 96–265) of synaptotagmin Ia and Ib (see Fig. 1) were prepared as previously described (Chapman et al., 1995, 1996). A new C2B domain (residues 248–421) was generated from the synaptotagmin Ib sequence reported by Osborne et al. (1999) and subcloned into pGEX-2T as previously described (Chapman et al., 1996). A series of point mutations (see Fig. 3) were generated by PCR and also expressed as GST fusion proteins. Soluble synaptotagmin fragments were prepared by thrombin cleavage of GST fusion proteins using thrombin (Chapman et al., 1996). His₆-synprint (Sheng et al., 1997) was prepared as previously described (Chapman et al., 1998).

Binding Assays

All recombinant protein binding assays were carried out by immobilizing one protein on glutathione-Sepharose beads. Immobilized fusion proteins were incubated with soluble binding partners for 1–2 h at 4°C in TBS (20 mM Tris and 150 mM NaCl) plus 0.5% Triton X-100 with either 2 mM EGTA or 1 mM free Ca^{2+} , Mg^{2+} , Ba^{2+} , or Sr^{2+} . Beads were washed three times in binding buffer. Proteins were solubilized by boiling in SDS sample buffer and subjected to SDS-PAGE. In most cases, proteins were visualized by staining with Coomassie blue. However, synprint binding was detected by immunoblotting using a monoclonal antibody directed against the T7-tag (Novagen).

All GST pull-down assays using brain detergent extracts were carried out as described (Chapman et al., 1998). Bound proteins were detected by immunoblotting using mouse monoclonal antibodies directed against the luminal domain of synaptotagmin I (604.4) and α/β -SNAP (77.1; this antibody recognizes α and β SNAP equally well) that were kindly provided by S. Engers and R. Jahn (Max Planck Institute for Biophysical Chemistry, Goettingen, Germany). The antibody directed against α -adaptin (100.2) was obtained from Sigma-Aldrich.

All binding assays were carried out at least four times and representative experiments are shown in the figures.

Limited Proteolysis

Immobilized GST fusion proteins were incubated for 1 h at room temperature in TBS with the indicated concentrations of chymotrypsin or trypsin in the presence of either 2 mM EGTA or 1 mM free Ca^{2+} . Reactions were stopped by boiling in SDS sample buffer and samples were subjected to SDS-PAGE. Proteolytic fragments were visualized by staining with Coomassie blue, and samples were analyzed for the differential accumulation of proteolytic fragments in EGTA versus Ca^{2+} . Ca^{2+} dose-response assays were carried out in a similar manner using a fixed protease concentration. Free [Ca^{2+}] was determined using a Ca^{2+} electrode as previously described (Davis et al., 1999). Accumulation of a fragment that is selectively protected in the presence of Ca^{2+} was monitored by staining SDS-PAGE

¹Abbreviations used in this paper: SNARE, *N*-ethyl maleimide-sensitive factor attachment protein receptor; t-SNARE, target SNARE.

marked contrast, soluble synaptotagmin Ib exhibited a pronounced binding to immobilized synaptotagmin Ib in response to Ca^{2+} . This effect is selective for Ca^{2+} ; Mg^{2+} failed to trigger binding, and Ba^{2+} and Sr^{2+} were less potent than Ca^{2+} in driving clustering (Fig. 1 C). These data demonstrate that the inability of synaptotagmin Ia to cluster in response to Ca^{2+} is due to the presence of an aspartate residue at position 374. The precise conservation of the glycine residue at this position, with the lone exception of synaptotagmin Ia, suggests that multiple synaptotagmins may exhibit clustering activity, as borne out by data described below.

We have confirmed the expression of both the G- and D374 forms by sequencing synaptotagmin cDNA from a variety of cDNA libraries (data not shown). At present, it is unclear how the D374 form of synaptotagmin arises; the genomic sequence of the rat clone has not been reported. Future studies are required to determine whether variation occurs at position 374 via RNA editing or alternative splicing, and whether seizure activity or other factors can influence the relative levels of the Ia and Ib messages.

We reiterate that while synaptotagmin Ia cannot oligomerize with itself, it can serve as an acceptor for the clustering activity of native synaptotagmin (Chapman et al., 1996; Sugita et al., 1996). Thus, clustering appears to involve at least two separable determinants or domains, and synaptotagmin Ia is only defective in one of these domains.

Mechanism of Ca^{2+} -triggered Synaptotagmin Oligomerization

The finding that synaptotagmin Ib exhibits robust Ca^{2+} -triggered clustering activity made it possible to examine the structural features that drive oligomerization. Our first goal was to determine whether the C2B domain was necessary and sufficient to drive clustering, as inferred from earlier studies (Chapman et al., 1996; Sugita et al., 1996). To address this issue, we carried out oligomerization assays using the isolated C2 domains of synaptotagmin. Soluble C2A did not bind to immobilized C2A or C2B, and soluble C2B did not bind to immobilized C2A (Fig. 2). However, in the presence of Ca^{2+} , soluble C2B bound to immobilized C2B (Fig. 2). These data demonstrate that C2B is necessary and sufficient to drive synaptotagmin oligomerization. Furthermore, C2B functions as a Ca^{2+} -sensing module.

The putative Ca^{2+} ligands within the C2B domain of synaptotagmin are indicated in the model shown in Fig. 3 A. To assess the function of these putative ligands, they were neutralized by substitution with asparagine residues either individually or in combination, and the resulting mutant proteins were assayed for oligomerization activity as described in Fig. 1 B. As a negative control, the K326,327A mutant was assayed and failed to oligomerize (Fig. 3, B and C), which is consistent with our previous report (Chapman et al., 1998). As an additional control, the D230,232N mutant, which abolishes Ca^{2+} -triggered interactions of synaptotagmin with anionic phospholipids and the SNARE complex (Bai et al., 2000), failed to inhibit the Ca^{2+} -triggered clustering activity (Fig. 3, B and C). These data are consistent with Fig. 2, demonstrating that the C2B

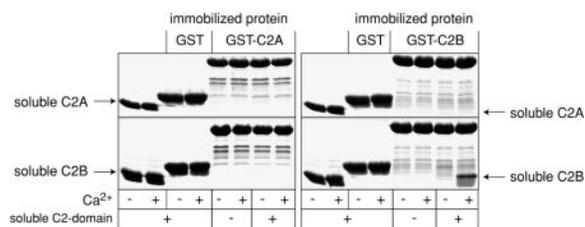


Figure 2. The C2B domain of synaptotagmin is necessary and sufficient for Ca^{2+} -triggered synaptotagmin oligomerization. C2A–C2A, C2A–C2B, and C2B–C2B interactions were assayed by incubating soluble isolated C2 domains (8 μM protein) with immobilized GST–C2 domain fusion proteins (10 μg) as described in Fig. 1 B. Samples were subjected to SDS-PAGE, and proteins were visualized by staining with Coomassie blue.

domain is both necessary and sufficient for Ca^{2+} -triggered oligomerization. Surprisingly, individual substitutions of the putative Ca^{2+} ligands within C2B had little effect on clustering activity. Rather, a slight trend toward constitutive self-association was observed among the point mutants (Fig. 3 C). Moreover, simultaneous neutralization of two (D363,365N) or four (D303,309,363,365N) of the five acidic Ca^{2+} ligands clearly resulted in Ca^{2+} -independent constitutive clustering activity (Fig. 3, B and C). These data provide strong evidence that the conserved putative Ca^{2+} ligands are functional and regulate synaptotagmin clustering. The conversion from regulated to constitutive clustering activity is addressed in the Discussion.

The inability of single point mutations to significantly disrupt Ca^{2+} -triggered clustering activity suggests that C2B binds multiple Ca^{2+} ions in a redundant manner. Loss of individual sites are tolerated since other sites remain intact. This is in striking contrast to the C2A domain of synaptotagmin, in which each of the Ca^{2+} ligands are essential for binding to anionic phospholipids (Zhang et al., 1998; Bai et al., 2000). The interaction of synaptotagmin with SNAREs exhibits an intermediate dependence on Ca^{2+} ligands within C2A: some mutations are tolerated, whereas other mutations have strong disruptive effects (Chapman et al., 1995; Bai et al., 2000). There appear to be multiple modes by which Ca^{2+} binding can regulate the interactions of the C2 domains of synaptotagmin with effector molecules.

Ca^{2+} Drives a Conformational Change in the C2B Domain of Synaptotagmin

The experiments described above provide strong evidence that the C2B domain of synaptotagmin binds Ca^{2+} , which drives clustering of the protein. We next sought to determine whether clustering is achieved via a Ca^{2+} -induced conformational change in C2B. Ca^{2+} -triggered polymerization of synaptotagmin (secondary to oligomerization) made a number of biophysical methods (NMR, CD, fluorescence measurements, etc.) inapplicable. To circumvent this problem, we applied a limited proteolysis approach to detect Ca^{2+} -driven conformational changes in the domain. For these experiments, the C2B domain of synaptotagmin Ib was immobilized as a GST fusion protein. In addition, the quadruple Ca^{2+} ligand mutant version of C2B, D303,309,363,365N, as well as the K326,327A mutant that

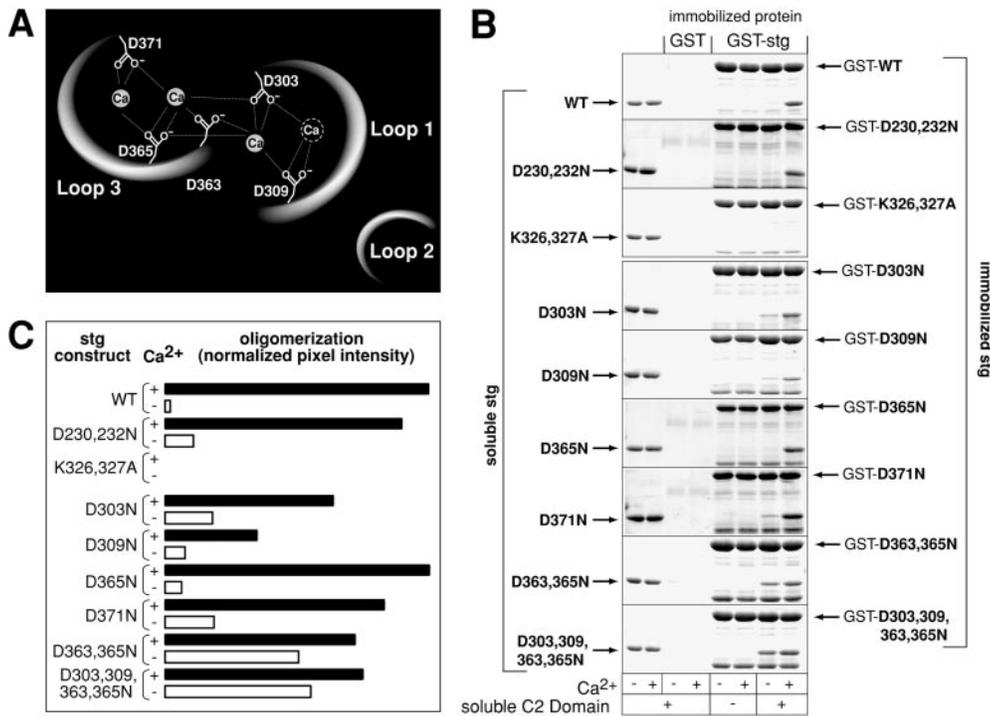


Figure 3. Effects of putative Ca²⁺ ligand mutations on Ca²⁺-triggered synaptotagmin oligomerization. (A) Schematic diagram of putative Ca²⁺ ligands within the C2B domain of synaptotagmin I. This model is based on structural studies of a variety of C2 domains (Sutton and Sprang, 1995; Sutton et al., 1995; Grobler et al., 1996; Perisic et al., 1998; Ubach et al., 1998a,b) and alignments between the C2A and C2B domains of synaptotagmin. (B) Wild-type (WT) and the indicated synaptotagmin I point mutants were analyzed for Ca²⁺-triggered oligomerization activity as described in Fig. 1 B. Neutralization of the putative Ca²⁺ ligands results in an increase in the Ca²⁺-independent component of binding. This effect is most marked for the quadruple Ca²⁺ ligand mutant

D303,309,363,365N, which oligomerizes constitutively. As a negative control, the ability of the K326,327A mutant was assayed in parallel and, consistent with our previous report (Chapman et al., 1998), failed to oligomerize in response to Ca²⁺. As a further control, the D230,232N mutant, which fails to bind Ca²⁺ via its C2A domain, was also assayed and found to exhibit Ca²⁺-triggered oligomerization activity. (C) Binding assays from the representative experiment shown in B were quantified by densitometry and plotted. The level of binding was normalized to the pixel intensity of the soluble protein in the two left lanes (corresponding to 7% if the ligand used in the binding assay).

fails to oligomerize (Fig. 3 B; Chapman et al., 1998), were analyzed in parallel. Fusion proteins were incubated with increasing concentrations of trypsin or chymotrypsin in the presence of EGTA or Ca²⁺, and the proteolysis patterns were analyzed by SDS-PAGE. As shown in Fig. 4 A, the degradation patterns of wild-type C2B in EGTA versus Ca²⁺ were distinct, suggesting that C2B undergoes a conformational change upon binding Ca²⁺. With both proteases, a protease resistant fragment accumulated in the presence of Ca²⁺, suggesting that Ca²⁺ binds to and stabilizes the domain, which is analogous to the limited proteolysis data using C2A as a substrate (Davletov and Südhof, 1994). This differential proteolysis pattern was not observed using the Ca²⁺ ligand mutant C2B domain as a substrate, providing further evidence that these acidic residues (D303,309,363,365N) constitute functional Ca²⁺ ligands (Fig. 4 A). Interestingly, the K326,327A mutant that fails to oligomerize is still capable of sensing Ca²⁺ (Fig. 4 A, bottom). Because C2 domains bind Ca²⁺ via a bipartite motif in which distal regions of the domain must come together via folding into a compact tertiary structure, this finding strongly indicated that the K326,327A mutant is correctly folded. The loss of oligomerization activity of this mutant (Chapman et al., 1998; Fig. 3 B) is not due to a loss of Ca²⁺ sensitivity, but rather is likely due to perturbation of its oligomerization interface.

We exploited the Ca²⁺-dependent accumulation of a protease-protected fragment to determine the Ca²⁺ de-

pendence of this conformational change. Limited proteolysis was carried out at a fixed protease concentration, and the [Ca²⁺]_{free} was varied. The appearance of the protected fragment was quantified by densitometry and is plotted in Fig. 4 B. The [Ca²⁺]_{1/2} was ~62 ± 20 μM, which is consistent with the Ca²⁺ dependence for exocytosis (Heidelerger et al., 1994). This value is similar to the [Ca²⁺]_{1/2} for the interaction of the C2A domain of synaptotagmin I with anionic phospholipids (Davis et al., 1999). We note that previous measurements of the Ca²⁺ dependence for synaptotagmin clustering are highly dependent on the methods and conditions in which oligomerization was measured (Chapman et al., 1996; Sugita et al., 1996; Osborne et al., 1999). While the limited proteolysis technique employed here may only report a subset of conformational changes, this assay system has the advantage that it circumvents the need to carry out the wash steps employed in C2B-synaptotagmin pull-down assays and, therefore, accurately reports the Ca²⁺ requirement for a conformational change within C2B.

We also compared the proteolysis patterns of the C2B domains of synaptotagmins V–VIII. As shown in Fig. 4 C, Ca²⁺ drove a conformational change in synaptotagmins V and VI, suggesting that the C2B domains of multiple synaptotagmin isoforms can sense Ca²⁺. The proteolysis patterns of the C2B domains of synaptotagmins VII and VIII were not influenced by Ca²⁺, indicating that these domains either do not sense Ca²⁺, or Ca²⁺-driven conformational

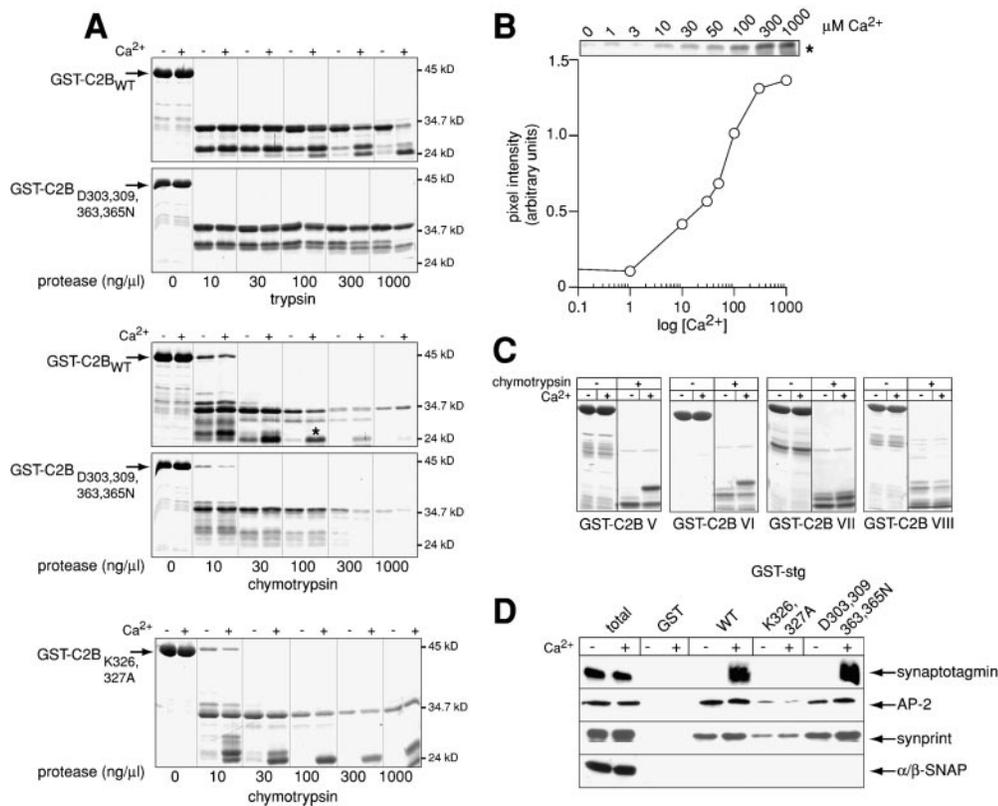


Figure 4. Ca^{2+} drives a conformational change in the C2B domain of synaptotagmin. (A) The C2B domain of synaptotagmin Ib was immobilized as a GST fusion protein subjected to limited proteolysis in the presence of Ca^{2+} or EGTA. Two mutant versions of C2B were analyzed in parallel. In one mutant, four of the five acidic Ca^{2+} ligands were neutralized by substitution with asparagine residues (D303,309,363,365N) resulting in constitutively oligomerized synaptotagmin (Fig. 3 B). In the other mutant, lysine residues 326 and 327 were substituted with alanine residues, which abolishes clustering activity (Fig. 3 B; Chapman et al., 1998). 20 μg of wild-type GST-C2B (GST-C2B_{wt}), Ca^{2+} ligand mutant (GST-C2B_{D303,309,363,365N}), or the lysine mutant GSt-C2B_{K326,327A} were incubated in TBS with the indicated concentration of trypsin or chymotrypsin for 60 min at

room temperature in either 1 mM Ca^{2+} (+ Ca^{2+}) or 2 mM EGTA ($-\text{Ca}^{2+}$). Samples were boiled in SDS sample buffer and analyzed by SDS-PAGE and staining with Coomassie blue. (B) The Ca^{2+} dependence for Ca^{2+} -driven conformational changes in C2B was assayed by monitoring the formation of a chymotrypsin-protected fragment that accumulates in the presence of Ca^{2+} (as indicated by the asterisk in A, middle panel). The generation of this fragment was assayed as a function of $[\text{Ca}^{2+}]_{\text{free}}$, quantified by densitometry and plotted. The $\text{EC}_{50} = 62 \pm 20 \mu\text{M}$ ($n = 3$). Representative data are shown and plotted. (C) Limited proteolysis of the C2B domains of synaptotagmins V–VIII. Assays were carried out, as described in A, using 300 ng/ μl chymotrypsin. (D) Effector interactions of synaptotagmin C2B mutants. 30 μg of GST alone, GST fused to the cytoplasmic domains of wild-type (wt), the K326,327A, or the D303,309,363,365N synaptotagmin Ib, were immobilized onto 20 μl glutathione–Sepharose. To assay for native synaptotagmin I, AP-2, and β -SNAP binding, beads were incubated for 2 h with 1 ml (1 mg/ml) rat brain detergent extract in the presence of 2 mM EGTA (–) or 1 mM Ca^{2+} (+) in HBS. Beads were washed three times and bound proteins were eluted by boiling in SDS sample buffer. Samples were resolved by SDS-PAGE and bound proteins were analyzed by immunoblotting. Immunoreactive bands were visualized using enhanced chemiluminescence. 10 μg of the extract (total) and 15% of the bound material were loaded onto the gel. For synprint binding assays, 20 μg of the immobilized proteins were incubated with 0.5 μM recombinant synprint in 200 μl of HBS with 0.1% Triton X-100 in the presence of either 2 mM EGTA (–) or 1 mM Ca^{2+} (+). Samples were processed as described above and bound synprint was detected using anti-T7-tag monoclonal antibody (Novagen) and enhanced chemiluminescence. 3% of the total binding reaction (total) and 12% of the bound material were loaded onto the gel.

changes within these particular domains cannot be detected using this method. We note that the C2B domain of synaptotagmin VIII lacks three of the five acidic Ca^{2+} ligands (Fig. 1 A) and would not be expected to bind Ca^{2+} (Fig. 4 C). In contrast, synaptotagmin VII possesses each of the conserved Ca^{2+} ligands. Further studies, using a different approach, are required to determine whether the C2B domain of synaptotagmin VII can sense Ca^{2+} and change conformation.

Finally, as a further control to determine the effects of the D303,309,363,365N and K326,327A mutations on the structure of synaptotagmin, we assayed for the interaction of these mutants with effectors that bind to the C2B domain. As shown in Fig. 4 D, the D303,309,363,365N mutant exhibited undiminished ability to bind native synaptotagmin in response to Ca^{2+} . This result is not surprising

since the oligomerization activity of native synaptotagmin is off in the absence of Ca^{2+} such that the native protein cannot bind the constitutively activated immobilized protein. Upon binding Ca^{2+} , the native protein is activated and binds to the oligomerization competent (albeit constitutively) immobilized mutant protein. We observed that AP-2 and synprint bound to the quadruple Ca^{2+} ligand mutant to the same extent as the wild-type protein, indicating that the mutant protein is correctly folded. In contrast to a previous report (Schiavo et al., 1995), we did not detect binding of β -SNAP to any of our immobilized synaptotagmin constructs. Consistent with our previous report, the K326,327A exhibited markedly reduced binding to native synaptotagmin, AP-2, and synprint. However, this domain appears to be correctly folded, since, as described above, this mutant still functions as a Ca^{2+} sensor.

Furthermore, this mutant is capable of efficiently coimmunoprecipitating with t-SNAREs and SNARE complexes (data not shown), an interaction that requires the C2B domain of the protein (Chapman et al., 1996; Davis et al., 1999). Finally the circular dichroism spectra of the wild-type and K326,327A mutant are identical (data not shown). Thus, both mutants appear to be correctly folded.

Ca²⁺-triggered Synaptotagmin Oligomerization Is a Conserved Property among Different Synaptotagmin Isoforms

A key issue raised by the data presented above is whether the clustering activity of synaptotagmin Ib functions in excitation-secretion coupling. First, we addressed the question of whether synaptotagmin Ia or Ib is typical of the synaptotagmin family of proteins by examining the homo- and hetero-oligomerization activity of synaptotagmins I–VIII using the approach described in Fig. 1 B. Ca²⁺-triggered homo- and hetero-oligomerization is a highly conserved function of nearly all synaptotagmins (Fig. 5). Notable exceptions appear to be synaptotagmin VII and VIII (note, because of proteolysis problems that obscured the region of interest on the gels, some of the data concerning these isoforms was omitted from Fig. 5; these data are summarized here). Synaptotagmin VIII lacks three of the five acidic Ca²⁺ ligands (Fig. 1 A) and cannot homo-oligomerize in response to Ca²⁺. However, immobilized synaptotagmin VIII can clearly serve as an acceptor for binding other synaptotagmin isoforms, as indicated by the robust binding of soluble synaptotagmin III to immobilized synaptotagmin VIII (Fig. 5). Again, these data indicate that clustering involves the interaction of two distinct sites on C2B.

The other exception, synaptotagmin VII, efficiently bound to each of the immobilized synaptotagmin isoforms tested. However, these interactions are not promoted by Ca²⁺; in some cases, Ca²⁺ triggered slight decreases in the level of binding (Fig. 5). As discussed above, the basis for this unusual behavior is not readily apparent from the primary structure of the protein. Like synaptotagmin VIII, immobilized synaptotagmin VII can serve as an acceptor for the Ca²⁺-dependent binding of soluble synaptotagmin isoforms. In summary, all synaptotagmin isoforms tested exhibit some form of homo- and/or hetero-oligomerization activity. The conservation of this biochemical function suggested that it may be tenable to carry out dominant negative experiments to investigate whether, and at what stage, synaptotagmin oligomerization functions in regulated exocytosis.

Inhibition of Synaptotagmin Clustering Blocks Ca²⁺-triggered Exocytosis

A variant PC12 cell line lacking synaptotagmin I exhibits normal-to-elevated secretory responses (Shoji-Kasai et al., 1992). However, this cell line expresses numerous other synaptotagmin isoforms including synaptotagmin III, IV, and V (Mizuta et al., 1994; Hudson and Birnbaum, 1995; Vician et al., 1995). Our findings described above, demonstrating that recombinant synaptotagmin I can homo- and hetero-oligomerize with synaptotagmins I, III, IV, and V, suggested that it may be possible to apply a dominant neg-

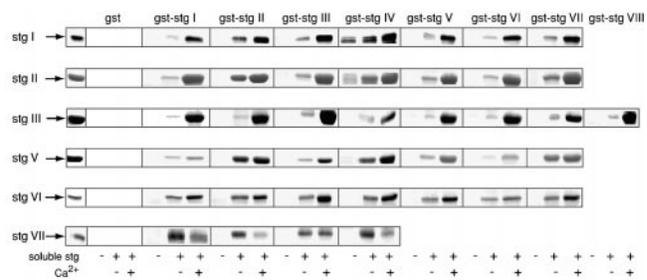
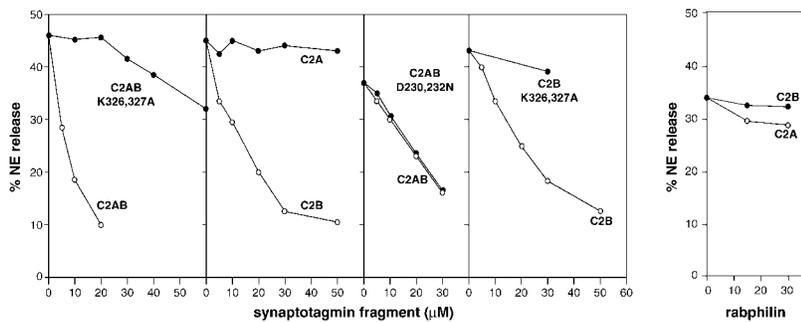


Figure 5. Ca²⁺-triggered homo- and hetero-oligomerization is conserved among multiple synaptotagmin isoforms. Fusion proteins corresponding to the cytoplasmic domains of synaptotagmin I–VIII (Li et al., 1995) were immobilized on glutathione-Sepharose (10 μg protein/assay point) and incubated with soluble versions of each synaptotagmin (1–2 μM) generated via thrombin cleavage of GST fusion proteins. Binding was carried out for 2 h at 4°C in 150 μl of TBS plus 0.5% Triton X-100 in either 2 mM EGTA (–) or 1 mM Ca²⁺ (+). Pellets were washed three times, and bound material was eluted by boiling in SDS-PAGE sample buffer for 5 min. 25% of the bound material and 7.5% of the soluble protein was subjected to SDS-PAGE and stained with Coomassie blue. Due to size considerations and clarity of presentation, gels were cropped, and only the region containing soluble synaptotagmin fragments are shown. However, the levels of immobilized proteins were carefully matched and are not a significant source of variability in the efficiency of ligand capture. In some cases, proteolysis of the immobilized fusion protein obscured the relevant region of the gel; these regions were excluded from the figure.

ative approach, using mechanically permeabilized PC12 cells, to study the function of synaptotagmin clustering in Ca²⁺-triggered exocytosis.

Secretion from permeable PC12 cells involves two stages: an ATP- and cytosol-dependent priming stage, followed by a Ca²⁺- and cytosol-dependent triggering reaction (Hay and Martin, 1992). We reasoned that recombinant fragments of synaptotagmin would, in the presence of Ca²⁺, oligomerize with native synaptotagmin in the permeable cell preparation, and thereby block native synaptotagmin oligomerization. If the Ca²⁺-triggered clustering of native copies of synaptotagmin functions in secretion, recombinant synaptotagmin should block exocytosis. As shown in Fig. 6, the cytoplasmic domain of synaptotagmin Ib (referred to as C2AB in the figure) potentially inhibited exocytosis. To determine whether inhibition was mediated by the Ca²⁺-triggered binding of recombinant C2AB to native synaptotagmin, we tested the ability of the K326,327A mutant, which only weakly clusters in response to Ca²⁺ (Fig. 3 B; Chapman et al., 1998; Davis et al., 1999), to inhibit exocytosis. As shown in Fig. 6, this mutant is not an effective inhibitor of secretion. We note that the K326,327A mutant is able to bind to SNAREs (Chapman et al., 1998), and this interaction requires both C2A and C2B (Chapman et al., 1995; Davis et al., 1999). These data indicate that C2B is not simply misfolded. The K326,327A mutant is also able to bind anionic phospholipids (Bai et al., 2000), yet fails to inhibit secretion. The results of these experiments are consistent with the hypothesis that C2AB blocks exocytosis via its C2B-mediated clustering activity.



scribed for synaptotagmin. Exocytosis was triggered by incubating with Ca^{2+} ($10 \mu\text{M}$) plus rat brain cytosol for 3 min at 30°C . Released ^3H -NE was quantified by liquid scintillation counting, and is plotted as the percentage of the total ^3H -NE content of the cells. Data shown represent the mean of duplicate determinations, with each duplicate differing by $<5\%$ of the mean value. Oligomerization-competent fragments of synaptotagmin, C2AB, C2B, and C2AB-D230,232N, potently inhibited secretion, whereas nonoligomerizing proteins, C2A and K326,327A mutant versions of C2AB and C2B, failed to inhibit secretion. Neither C2 domain of rabphilin inhibited exocytosis.

To further test this model, we compared the ability of the isolated C2 domains of synaptotagmin to inhibit release. As shown in Fig. 6, C2B inhibited release in a manner analogous to C2AB. In contrast, C2A failed to inhibit secretion. Furthermore, we generated a mutant C2B domain harboring the K326,327A mutation, and this version of C2B failed to cluster (data not shown) and failed to inhibit exocytosis (Fig. 6). Finally, we examined the ability of a mutant C2AB domain, which harbored two mutations, D230,232N, within the C2A domain that abolish Ca^{2+} -triggered C2A-effector interactions (Davis et al., 1999; Bai et al., 2000). This double mutant retained all of the inhibitory activity of wild-type C2AB (Fig. 6), despite the fact that it fails to bind either lipids or SNAREs in response to Ca^{2+} . Thus, all of the synaptotagmin fragments that exhibit Ca^{2+} -triggered clustering activity are able to inhibit exocytosis in this assay system, and all fragments that fail to cluster in response to Ca^{2+} fail to inhibit secretion despite the fact that other effector interactions remain intact. We also tested the C2A and C2B domains of rabphilin for inhibitory activity. Neither of the isolated rabphilin C2 domains bind to synaptotagmin (data not shown), and neither fragment inhibited secretion (Fig. 6), further establishing the specificity of the synaptotagmin C2B domain effect. We have tested additional C2 domains, from other proteins, and observed that some are inhibitory in our secretion assay; these C2 domains can also cluster with synaptotagmin, which is consistent with a model in which perturbation of native synaptotagmin clustering inhibits exocytosis (data not shown). Clustering may be a shared biochemical property among C2 domains from distinct proteins.

To further explore the dominant negative effect of C2AB, we determined whether inhibition occurs during the ATP-dependent priming stage or the Ca^{2+} -triggered stage of secretion. If the inhibition of exocytosis is mediated via Ca^{2+} -triggered clustering of recombinant synaptotagmin with the endogenous native protein, the inhibitory effect should be restricted to the Ca^{2+} -triggered step in secretion. Indeed, this was the case; C2AB added during priming, and then washed out before triggering, has only a

limited effect on release (Fig. 7 A). In contrast, C2AB that was added during the Ca^{2+} -triggered stage resulted in the potent inhibition of secretion, presumably because Ca^{2+} triggered the oligomerization of recombinant with endogenous synaptotagmin (Fig. 7 A). Again, C2A served as a negative control.

Figure 6. A dominant negative approach to study the function of synaptotagmin oligomerization in Ca^{2+} -triggered exocytosis from permeable PC12 cells. PC12 cells were loaded with ^3H norepinephrine (NE) and permeabilized by passage through a ball homogenizer. Permeable cells were incubated for 30 min at 30°C with ATP and rat brain cytosol to prime exocytosis. The cells were washed and incubated with the indicated concentrations of the cytoplasmic domain of synaptotagmin (C2AB), C2A, C2B, or K326,327A-substitution mutant versions of C2AB/C2B, for 45 min on ice. (right) The C2A and C2B domains of rabphilin were tested as described for synaptotagmin.

We next sought to address the function of synaptotagmin oligomerization in exocytosis using an independent approach. This was achieved by analyzing the effects of the synprint peptide on secretion. Synprint corresponds to the II-III loop of N-type Ca^{2+} channels and binds to t-SNAREs and to synaptotagmin (Kim and Catterall, 1997; Sheng et al., 1997). A critical point is that synprint binds to synaptotagmin in a Ca^{2+} -independent manner and can block Ca^{2+} -triggered synaptotagmin-clustering (Chapman et al., 1998). Thus, if clustering is critical for exocytosis, synprint should serve as an effective inhibitor akin to C2AB and C2B described above. However, in the case of synprint, the inhibitory effect should be equally effective during the ATP-dependent priming step (in the absence of Ca^{2+}) as in the Ca^{2+} -triggered stage of exocytosis, again because synprint can bind synaptotagmin equally well in the presence or absence of Ca^{2+} . Congruent with this model, synprint inhibited exocytosis with equal or greater activity during the Ca^{2+} -independent priming stage of secretion (Fig. 7 B). These data provide independent confirmation that disruption of synaptotagmin oligomerization can disrupt Ca^{2+} -triggered exocytosis.

We note that a caveat of our inhibition studies is the ability of recombinant synaptotagmin and synprint to interact with a number of distinct proteins. For example, a recent study indicated that synprint can inhibit the binding of synaptotagmin and cysteine string protein to syntaxin (Wu et al., 1999). However, synprint binds much more tightly to syntaxin in the presence, rather than the absence of Ca^{2+} (Kim and Catterall, 1997; Littleton et al., 1998; and data not shown), yet in our secretion assays, synprint does not preferentially inhibit release during the triggering phase when Ca^{2+} is present. Therefore, we view it as unlikely that synprint exerts its inhibitory effects via binding to syntaxin.

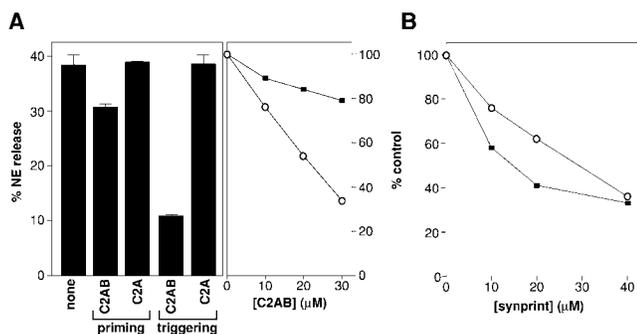


Figure 7. C2AB selectively blocks exocytosis during the Ca^{2+} -triggered stage, whereas synprint can inhibit secretion during the Ca^{2+} -independent priming stage of exocytosis. Exocytosis in permeable PC12 cells was primed in 30-min incubations with ATP and triggered in 3-min incubations with Ca^{2+} as described in Fig. 6. Released ^3H -NE was quantified by liquid scintillation counting and is plotted either as the percentage of the total ^3H -NE content of the cells, or was normalized to the maximal level of release obtained without added inhibitors and plotted as % control. Data shown represent the mean of duplicate determinations with the range indicated or within 10% of the mean value. (A, left) C2AB, or as a control, C2A, at 30 μM was incubated with the permeable PC12 cells during priming or triggering reactions. To specifically test for the effects on priming, proteins were present during 30-min incubations, and the permeable cells were washed before 3-min triggering incubations with Ca^{2+} . (A, right) The indicated concentrations of C2AB were incubated with permeable PC12 cells only during the priming (closed squares) or only during the triggering (open circles) stages of secretion. (B) The indicated concentrations of synprint were incubated with permeable PC12 cells during the priming (closed squares) or triggering (open circles) stages of secretion.

Discussion

In this study, we have demonstrated that the C2B domain of synaptotagmin functions as a Ca^{2+} -sensing module. Ca^{2+} drives a conformational change in the C2B domain of synaptotagmin I with a $[\text{Ca}^{2+}]_{1/2}$ of $62 \pm 20 \mu\text{M}$, similar to the Ca^{2+} sensitivity of the C2A domain of this isoform (Davis et al., 1999) and consistent with the Ca^{2+} dependence of exocytosis (Heidelberger et al., 1994). Furthermore, we directly demonstrate that Ca^{2+} triggers the oligomerization of the C2B domain of synaptotagmin Ib, and this property is conserved among multiple isoforms of the protein. We propose that Ca^{2+} acts by driving a conformational change that stabilizes the multimeric form of synaptotagmin. The C2A domain exhibits a similar conformational change in limited proteolysis experiments (Davletov and Südhof, 1994). These changes may reflect the movement of charged residues within or near the Ca^{2+} -binding loops (Ubach et al., 1998b; Chae et al., 1998), as indicated by structural studies of the C2 domain of phospholipase C- δ 1 (Grobler et al., 1996). Given the diffusion-limited kinetics of the C2 domains of synaptotagmin, these conformational changes are unlikely to involve large-scale structural rearrangements (Davis et al., 1999). Numerous C2 domains harbor a conserved set of five acidic residues that function as Ca^{2+} ligands. Via mutagenesis, we sought to determine whether these residues indeed function as Ca^{2+}

ligands within C2B. We observed that the simultaneous substitution of four of the five putative acidic Ca^{2+} ligands within C2B inhibited Ca^{2+} -driven conformational changes and converted the domain to a constitutively oligomerizing form. The basis for this conversion is unclear. However, structural studies of the C2 domain of phospholipase C- δ 1 indicate that these Ca^{2+} ligands participate in intramolecular ionic bonds (Grobler et al., 1996). Binding of Ca^{2+} to these acidic ligands serves to release positively charged side chains, resulting in the opening of the Ca^{2+} binding jaws or loops such that they can interact with effectors. In this model, neutralization of the acidic Ca^{2+} ligands may result in a constitutively activated domain. Clearly, however, this is not the case with C2A. In C2A, neutralization of any of the acidic Ca^{2+} ligands abolishes phospholipid binding activity (Zhang et al., 1998; Bai et al., 2000). Additional structural studies are required to understand the differential effects of Ca^{2+} ligand mutations on C2A- and C2B-effector interactions. Nonetheless, the effects of the putative Ca^{2+} ligand mutations in C2B, on both Ca^{2+} -driven conformational changes and clustering activity, demonstrate that these conserved Ca^{2+} ligands are functional.

To assess the generality of Ca^{2+} -triggered clustering activity, we assayed homo- and hetero-oligomerization of synaptotagmins I–VIII and observed that oligomerization is a highly conserved biochemical property. These findings made it possible to employ a dominant negative approach to study the function of synaptotagmin clustering in permeable PC12 cells, which harbor multiple synaptotagmin isoforms. In all cases, fragments of synaptotagmin that were capable of clustering, in response to Ca^{2+} , served as potent inhibitors of secretion. This inhibitory activity mapped to the C2B domain of recombinant synaptotagmin and required the presence of Ca^{2+} , presumably to drive oligomerization with native synaptotagmin within the permeable cells. The inhibitory activity did not involve the binding of Ca^{2+} to the C2A domain of the protein, demonstrating that Ca^{2+} -triggered binding to SNAREs or anionic phospholipids is not required for the inhibition of secretion observed in our experiments. In contrast, mutations or deletions that block the clustering activity of the C2B domain abrogated the ability of recombinant C2B to block exocytosis.

Finally, the ability of synprint to inhibit exocytosis in our assay system, when applied at either the priming or triggering stages of secretion, is also consistent with a model in which Ca^{2+} -triggered synaptotagmin clustering is critical for exocytosis. In this model, the inhibitory effects of synprint are independent of the stage of secretion because synprint can bind to synaptotagmin in a Ca^{2+} -independent manner and block clustering. Thus, synprint should be able to inhibit secretion when used to treat cells during either the Ca^{2+} -independent (i.e., ATP-dependent priming) or the Ca^{2+} -triggered stage of secretion, as borne out by the data. Others have reported that synprint has inhibitory effects on synaptic transmission. It was suggested that inhibition was due to uncoupling syntaxin from Ca^{2+} channels (Mochida et al., 1996; Rettig et al., 1997). However, chronic application of synprint (Rettig et al., 1997) can disrupt synaptotagmin–AP-2 interactions (Zhang et al., 1994; Chapman et al., 1998), resulting in a loss in the total num-

ber of vesicles (Jorgensen et al., 1995). Use of permeable PC12 cells circumvents a number of these problems since Ca^{2+} -triggered secretion does not depend upon Ca^{2+} channels nor upon endocytosis.

In an earlier report, a microinjection approach was used to assess the effects of synaptotagmin fragments on secretion from intact PC12 cells (Elferink et al., 1993). While C2AB and C2B did not appear to inhibit secretion, it was noted that these fragments had a tendency to aggregate and were difficult to quantitatively introduce into cells; from these studies, the potential ability of C2AB and C2B to inhibit secretion was not ruled out (Elferink et al., 1993). The use of permeable PC12 cells overcomes these difficulties and demonstrates that these fragments indeed inhibit release. Furthermore, recent experiments from L.A. Elferink and A.E. Hawley (personal communication), using viruses to infect PC12 cells, indicate that C2B is indeed critical for secretion from intact cells. Thus, the only apparent discrepancy between the initial PC12 cell microinjection data and the data present in our study is the inability of C2A to inhibit secretion in our assay system. One possible explanation is that C2A inhibits an upstream step in the vesicle trafficking pathway in intact cells (Elferink et al., 1993), which is not recapitulated in the permeable cells.

The function of C2B-mediated synaptotagmin clustering during exocytosis is not clear. We hypothesize that C2B-mediated oligomerization may drive the assembly of the fusion machinery into a collar or ringlike fusion pore or may alter the stability/conformation of a weakly preassembled pore. This hypothesis stems from the observation that purified SNARE-complexes do not efficiently cluster into multimeric complexes akin to viral fusion proteins (Lindau and Almers, 1995; Blumenthal et al., 1996; Danieli et al. 1996; Fasshauer et al., 1997). Also, a protein designated EEA1 recently has been reported to cluster syntaxin-13 into oligomeric structures, and this oligomerization is required for endosomal fusion (McBride et al., 1999). Indeed, genetic studies indicate that synaptotagmin functions as a multimeric complex (Littleton et al., 1994). We speculate that the Ca^{2+} -driven clustering activity of synaptotagmin may regulate the initial Ca^{2+} -triggered opening (Katz, 1969) or subsequent dilation (Fernandez-Chacon and Alvarez de Toledo, 1995) of the exocytotic fusion pore (Lindau and Almers, 1995).

The oligomerization of synaptotagmin may have other functions as well. In a previous report, we demonstrated that a seizure-induced isoform of synaptotagmin, IV, can form hetero-oligomers with synaptotagmin I. Upregulation of synaptotagmin IV reduces synaptic transmission, potentially by hetero-oligomerizing to form synaptotagmin clusters with hybrid properties (Littleton et al., 1999). Hetero-oligomerization of synaptotagmins with unique Ca^{2+} -sensing properties may provide a means to modulate release probability and, thus, modulate synaptic function.

In summary, we have identified a key function for the C2B domain of synaptotagmin in excitation-secretion coupling: C2B undergoes Ca^{2+} -driven conformational changes and assembles synaptotagmin into oligomers. In this light, we reiterate that a point mutation within this domain increases the Ca^{2+} requirement for secretion (Littleton et al., 1994). Future studies will determine

whether this phenotype results from an impaired ability of C2B to function as a Ca^{2+} -sensing oligomerization module.

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