A Novel Function for the Second C2 Domain of Synaptotagmin

Ca\(^{2+}\)-TRIGGERED DIMERIZATION*

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Synaptotagmin serves as the major Ca\(^{2+}\) sensor for regulated exocytosis from neurons. While the mechanism by which synaptotagmin regulates membrane fusion remains unknown, studies using Drosophila indicate that the molecule functions as a multimeric complex and that its second C2 domain is essential for efficient excitation-secretion coupling. Here we describe biochemical data that may account for these phenomena. We report that Ca\(^{2+}\) causes synaptotagmin to oligomerize, primarily forming dimers, via its second C2 domain. This effect is specific for divalent cations that can stimulate exocytosis of synaptic vesicles (Ca\(^{2+}\) \(>>\) Ba\(^{2+}\), Sr\(^{2+}\) \(>>\) Mg\(^{2+}\)) and occurs with an EC\(_{50}\) value of \(3-10 \mu\)M Ca\(^{2+}\). In contrast, a separate Ca\(^{2+}\)-dependent interaction between synaptotagmin and syntaxin, a component of the fusion apparatus, occurs with an EC\(_{50}\) value of \(= 100 \mu\)M Ca\(^{2+}\) and involves the synergistic action of both C2 domains of synaptotagmin. We propose that Ca\(^{2+}\) triggers two consecutive protein-protein interactions: the formation of synaptotagmin dimers at low Ca\(^{2+}\) concentrations followed by the association of synaptotagmin dimers with syntaxin at higher Ca\(^{2+}\) concentrations. Our findings, in conjunction with physiological studies, indicate that the Ca\(^{2+}\)-induced dimerization of synaptotagmin is important for the efficient regulation of exocytosis by Ca\(^{2+}\).

Rapid chemical signaling between neurons relies on the regulated secretion of neurotransmitters stored in synaptic vesicles. In the resting nerve terminal, a population of synaptic vesicles is tightly bound, or docked, to the presynaptic plasma membrane. When the nerve terminal is depolarized, Ca\(^{2+}\) enters via voltage-gated Ca\(^{2+}\) channels. High local concentrations of Ca\(^{2+}\) then rapidly trigger the fusion of a subpopulation of docked vesicles with the plasma membrane, resulting in the release of neurotransmitters into the synaptic cleft. While the mechanism(s) by which Ca\(^{2+}\) ions trigger membrane fusion remains obscure, strong evidence indicates that the synaptic vesicle protein, synaptotagmin I, plays an essential role in excitation-secretion coupling. To date, nine isoforms of synaptotagmin have been cloned and characterized (Perin et al., 1990; Geppert et al., 1991; Mizuta et al., 1994; Hilbush and Morgan, 1994; Hudson and Birnbaum, 1995; Li et al., 1995). The members of this family are integral membrane proteins that span the vesicle membrane once and possess a short amino-terminal intravesicular domain and a large cytoplasmic domain (Perin et al., 1990, 1991). The cytoplasmic domain contains two repeats homologous to the C2 domains found in Ca\(^{2+}\)-dependent but not Ca\(^{2+}\)-independent isoforms of protein kinase C, suggesting that this conserved motif comprises a Ca\(^{2+}\) binding domain (reviewed by Nishizuka (1988)). Homologous domains have also been identified in other proteins, at least some of which interact with lipids in a Ca\(^{2+}\)-dependent manner (Clark et al., 1991; Stahl et al., 1988; Vogel et al., 1988; Shiratani et al., 1993). Subsequent studies demonstrated that synaptotagmin indeed binds Ca\(^{2+}\) and negatively charged phospholipids in a mutually dependent manner (Brose et al., 1992) via its first C2 domain (Davelov and Sudhof, 1993; Chapman and J ahn, 1994; Fukuda et al., 1994).

The localization of synaptotagmin on synaptic vesicles and its ability to bind Ca\(^{2+}\) ions suggested that the molecule may serve as a Ca\(^{2+}\) sensor in exocytosis (Brose et al., 1992). This hypothesis has been the subject of numerous studies. For example, the synaptotagmin gene has been disrupted in Drosophila, Caenorhabditis elegans, and mice (Littleton et al., 1994; DiAntonio and Schwarz, 1994; Broadie et al., 1994; Nonet et al., 1993; Geppert et al., 1994). In all cases, perturbation of synaptotagmin expression results in decreased excitation-secretion coupling. In fact, disruption of the synaptotagmin I gene in mice virtually abolishes the fast component of Ca\(^{2+}\)-dependent exocytosis, while both spontaneous synaptic vesicle fusion and Ca\(^{2+}\)-independent exocytosis triggered by a-latrotoxin are unaffected (Geppert et al., 1994). Therefore, the fusion apparatus, while fully functional in the synaptotagmin I-deficient mice, is not appropriately regulated by Ca\(^{2+}\). These studies suggest that synaptotagmin either acts as the Ca\(^{2+}\) sensor for exocytosis or is essential in some other capacity to maintain efficient excitation-secretion coupling, for example by docking vesicles close to the site of Ca\(^{2+}\) influx (Neher and Penner, 1994). The former interpretation is strengthened by genetic studies in Drosophila, which demonstrated that discrete mutations in the gene encoding synaptotagmin can affect the cooperativity of the Ca\(^{2+}\) dependence of exocytosis (Littleton et al., 1994). Furthermore, the phenotypes resulting from crosses between Drosophila carrying distinct mutant synaptotagmin alleles suggest that the molecule functions as a multimeric complex and point to an essential function for its second C2 domain.

The precise mechanism by which synaptotagmin functions in excitation-secretion coupling remains ill-defined. To address this issue, efforts have been made to identify effectors of Ca\(^{2+}\)-synaptotagmin action. Synaptotagmin was shown to form Ca\(^{2+}\)-independent complexes with neurexins (Petrenko et al., 1991), a family of neuronal cell surface proteins, and with the darrhin adaptor protein AP-2 (Zhang et al., 1994). Synaptotagmin also interacts with the plasma membrane protein syntaxin 1 (Bennett et al., 1992), an essential component of the synaptic...
vesicle fusion apparatus (Söllner et al., 1993a, b; Blasi et al., 1993; Schulze et al., 1995). Recently, we and others (Chapman et al., 1995; Li et al., 1995) have reported that the interaction between syntaxin and synaptotagmin is promoted by Ca\(^{2+}\). The Ca\(^{2+}\)-dependence and divalent cation specificity of binding were consistent with those observed for exocytosis, suggesting that this interaction may comprise a step in transducing Ca\(^{2+}\)-transients to membrane fusion.

We have continued to look for additional effectors of Ca\(^{2+}\)-synaptotagmin action and report here that the second C2 domain of the protein is involved in the Ca\(^{2+}\)-triggered formation of homodimers. These findings may begin to provide the biochemical basis for the defects in synaptic transmission imparted by mutant synaptotagmins in vivo.

**EXPERIMENTAL PROCEDURES**

**Binding Assays—** Rat brain detergent extracts were prepared by solubilizing a crude synaptosome fraction using Triton X-100. Two rat brains were homogenized in 30 ml of 320 mM sucrose with 10 strokes at 300 rpm using a Teflon-glass homogenizer. All manipulations were carried out on ice. The homogenate was centrifuged at 5,000 rpm for 2 min in an SS34 rotor, and a crude synaptosome fraction was collected by centrifugation of the supernatant at 11,000 rpm for 12 min in an SS34 rotor. Synaptosomes were solubilized for 45 min at a detergent:protein ratio of 10:1 (w/w) using 1% Triton X-100 in 50 mM HEPES, pH 7.6, 100 mM NaCl containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 \(\mu\)g/ml pepstatin, 20 \(\mu\)g/ml aprotinin). Insoluble material was removed by centrifugation at 50,000 rpm for 1 h in a TLA 100.3 rotor for 15 min. One-mg aliquots of the rat brain detergent extract (1 mg/ml) were incubated with (0.5 nmol) various regions of synaptotagmin, immobilized to glutathione-Sepharose as GST fusion proteins, in EGTA or EGTA-buffered Ca\(^{2+}\), as indicated, for 2.5 h. Samples were washed three times in binding buffer, boiled in SDS-sample buffer and subjected to SDS-PAGE and immunoblot analysis as described (Chapman et al., 1995). Native synaptotagmin was detected using a monoclonal antibody directed against the luminal domain of the protein (Cl 604.1; Chapman and Jahn (1994)) that is absent from all of the recombinant immobilized synaptotagmin constructs. Monomodal antibodies directed against syntaxin (HPC-1; Barnstable et al. (1985)), synaptophysin (7.2; Jahn et al. (1985)), SNAP-25 (71.2; Hanson et al. (1994)), synaptophysin (69.1; Edelmann et al. (1995)), have been described previously.

The association of recombinant regions of synaptotagmin with recombinant syntaxin was assayed by co-immunoprecipitation using antisyntaxin antibodies. Recombinant full-length His\(_6\)-syntaxin and His\(_6\)-C2AB, or C2AB, C2A\(_{\alpha}\), and C2B, generated by thrombin cleavage of their respective GST-fusion proteins, were incubated at the indicated concentrations in 50 mM HEPES, pH 7.6, 100 mM NaCl, and 0.5% Triton X-100 supplemented with EGTA or CaCl\(_2\) for 2.5 h. His\(_6\)-syntaxin was immunoprecipitated by incubating the samples with purified HPC-1 IgG (5 \(\mu\)g) for 1.5 h and 12 \(\mu\)l of protein G-Sepharose Fast-flow (Pharmacia) bound to thrombin cleavage of the corresponding GST fusion protein as described (Chapman et al., 1995). Recombinant proteins were expressed in *Escherichia coli* and purified as described (Chapman et al., 1994, 1995). Soluble forms of the first (C2A\(_{\alpha}\)) and second (C2B\(_\beta\)) C2 domains of synaptotagmin were prepared by thrombin cleavage from the corresponding GST fusion protein as described (Chapman et al., 1995).

**Miscellaneous Procedures—** Quantitation of the immunoblots was carried out using a Molecular Dynamics Phosphorimager and Image-Quant software. For the Ca\(^{2+}\)-dose-response analyses, samples were buffered with 1 mM EGTA, and the total Ca\(^{2+}\) added to yield the indicated free Ca\(^{2+}\) concentrations was determined as described previously (Chapman and Jahn, 1994).

**RESULTS**

To determine the effect of Ca\(^{2+}\) ions on the interaction of synaptotagmin with other synaptic proteins, we immobilized the cytoplasmic domain of the protein as a GST fusion protein (GST-C2AB) bound to glutathione-Sepharose and used it as an affinity matrix. When incubated with rat brain detergent extracts in the presence of Ca\(^{2+}\) or EGTA, we observed a striking Ca\(^{2+}\)-dependent association of native synaptotagmin with GST-C2AB (Fig. 1). Binding was specific for synaptotagmin since no detectable binding of other synaptic proteins, including syntaxin, synaptophysin, SNAP-25, and synaptobrevin, was observed. To determine the region of synaptotagmin that mediates Ca\(^{2+}\)-dependent oligomerization, different domains of the molecule were immobilized as GST fusion proteins and analyzed for native synaptotagmin binding as described above.
Ca²⁺-triggered Dimerization of Synaptotagmin

**Fig. 2.** Ca²⁺-dependent synaptotagmin-syntaxin binding involves the synergistic action of both C2 domains of synaptotagmin. Soluble forms of C2AB, C2A₁, and C2B₁ were prepared by thrombin cleavage of the GST-fusion proteins described in Fig. 1 (Chapman et al., 1995). C2A₁-dependent binding of these fragments (0.5 μM) to His₆-syntaxin (0.7 μM) was analyzed by co-immunoprecipitation using anti-syntaxin antibodies in the presence of 2 mM EGTA or 0.5 mM Ca²⁺, as described under "Experimental Procedures." Bound recombinant synaptotagmins were detected using rabbit antisera directed against the individual C2 domains of the protein. Immune-reactive bands were visualized using enhanced chemiluminescence (Amersham Corp.). One-tenth of the immunoprecipitates were analyzed for recombinant synaptotagmin binding. Total corresponds to 1/100 of the starting material.

Long (with flanking sequences; GST-C2A₁) and short (without flanking sequences; GST-C2A₂) versions of the isolated first C2 domain did not bind any of the synaptic proteins analyzed. In contrast, a long version of the isolated second C2 domain (GST-C2B₁) bound native synaptotagmin as efficiently as the fragment containing most of the cytoplasmic domain of the protein (GST-C2AB). This property was preserved in the minimal second C2 domain sequence (GST-C2B₁) originally defined by its homology to protein kinase C (Perin et al. 1990; Fig. 1). Therefore, the second C2 domain mediates Ca²⁺-dependent self-association of synaptotagmin.

It has been reported previously that the first C2 domain of synaptotagmin mediates the Ca²⁺-dependent component of phospholipid binding by the molecule (Fukuda et al., 1995; Chapman et al., 1995), while the second C2 domain specifically mediates Ca²⁺-independent binding to AP-2 and IP₃ (Zhang et al., 1994; Fukuda et al., 1994). Our finding that C2B mediates Ca²⁺-dependent oligomerization demonstrates yet another functionally divergent property of the individual C2 domains of synaptotagmin and represents the first Ca²⁺-dependent interaction that directly involves the second C2 domain of the protein.

We and others have recently demonstrated that synaptotagmin binds to syntaxin in a Ca²⁺-dependent manner (Chapman et al., 1995; Li et al., 1995). Therefore, the failure of native syntaxin to bind to the immobilized GST-C2AB shown in Fig. 1 was surprising. However, when C2AB is removed from the GST-glutathione-Sepharose by thrombin cleavage it then associates with immunoprecipitated syntaxin in a Ca²⁺-dependent manner (Fig. 2). Ca²⁺ stimulates the binding of C2AB to syntaxin at 10 μM Ca²⁺. This result is not due to the Ca²⁺-dependent oligomerization of C2AB associated with syntaxin since recombinant C2AB only weakly oligomerizes in a Ca²⁺-dependent manner (described in detail below). Therefore, fusion of C2AB to GST and/or its immobilization onto glutathione-Sepharose diminishes its ability to bind syntaxin in a Ca²⁺-dependent manner. To further compare Ca²⁺-dependent synaptotagmin oligomerization and syntaxin binding, we examined the abilities of the isolated C2 domains, C2A₁ and C2B₁, to co-immunoprecipitate with syntaxin in the presence and absence of Ca²⁺. As shown in Fig. 2, under the conditions of our assay, C2A₁ did not co-immunoprecipitate with syntaxin either in the presence or absence of Ca²⁺. Ca²⁺-independent binding of C2B₁ to syntaxin was observed, which was only weakly enhanced (1.5-fold) by Ca²⁺. These results indicate that both C2 domains act in a synergistic manner to confer Ca²⁺-dependent synaptotagmin binding to syntaxin.

Our data demonstrating that the isolated C2A region of synaptotagmin does not form a stable complex with syntaxin do not agree with the findings of Li et al. (1995), who reported a Ca²⁺-dependent interaction between immobilized GST-C2A₅ and native syntaxin. We observed low levels of Ca²⁺-dependent syntaxin binding to immobilized GST-C2A₅ only after overnight incubation periods or by using very high Ca²⁺ concentrations (i.e., 2.5 mM Ca²⁺). These findings, in conjunction with the data described above, indicate that both C2 domains of synaptotagmin are required for efficient binding to syntaxin.

We next tested the ability of different divalent cations to stimulate synaptotagmin oligomerization. Binding assays using immobilized GST-C2AB and rat brain detergent extracts were carried out as described in Fig. 1 in the presence of EGTA, Mg⁡²⁺, Ca²⁺, Ba²⁺, or Sr²⁺. Self-association of synaptotagmin was specifically promoted by Ca²⁺ ions (Fig. 3). These data agree with physiological studies demonstrating that Ca²⁺ selectively triggers exocytosis (reviewed by Augustine et al. (1987)). The inability of Sr²⁺ to promote oligomerization is of particular interest since this divalent cation is unable to support the fast component of Ca²⁺-dependent exocytosis (Goda and Stevens, 1994). This finding is consistent with the notion that the Ca²⁺-dependent oligomerization of synaptotagmin may be an essential step in mediating this rapid component.

In parallel experiments, we found that recombinant His₆-C2AB also readily binds to immobilized GST-C2AB. However, this interaction was weakly (1-2-fold) and inconsistently stimulated by Ca²⁺ (data not shown). The more tightly regulated Ca²⁺-dependent interaction between native synaptotagmin derived from rat brain detergent extracts and GST-C2AB does not appear to be due to other factors (i.e., proteins or lipids) present in the extracts since the addition of the extract to the binding assay mixture does not restore Ca²⁺ responsiveness to recombinant C2AB (data not shown). We also addressed the possibility that residues 1-96 of synaptotagmin, which are not present in C2AB, are essential for Ca²⁺-dependent synaptotagmin oligomerization. However, full-length synaptotagmin derived from transfected COS-7 cells or generated by in vitro transcription and translation also failed to consistently exhibit significant Ca²⁺-dependent GST-C2AB binding (data not shown). However, these full-length forms of synaptotagmin have greater electrophoretic mobilities than the native protein, suggesting that they are not properly processed or folded. In summary, Ca²⁺-dependent oligomerization of synaptotagmin
can only be measured when one of the binding partners is brain-derived native protein.

We therefore investigated the Ca\(^{2+}\) dependence of synaptotagmin oligomerization by monitoring the binding of native synaptotagmin, derived from brain extracts, to immobilized GST-C2AB (Fig. 4, upper panel). Bound synaptotagmin was analyzed by immunoblotting as described and quantitated by PhosphorImager analysis, yielding an EC\(_{50}\) value of approximately 180 \(\mu\)M Ca\(^{2+}\). This value is in reasonable agreement with the previously reported value of 180 \(\mu\)M Ca\(^{2+}\) described above.

Therefore, synaptotagmin oligomerization (EC\(_{50} = 3–10 \mu M\) Ca\(^{2+}\)) and syntaxin binding (EC\(_{50} \sim 100 \mu M\) Ca\(^{2+}\)) are clearly
distinct Ca\textsuperscript{2+}-dependent interactions.

Finally, we addressed the stoichiometry of the Ca\textsuperscript{2+}-induced synaptotagmin oligomers using sucrose density gradient centrifugation. Previous reports had shown that synaptotagmin was broadly distributed on density gradients when solubilized in CHAPS or Zwittergent 3-10 (Brose et al., 1992; Perin et al., 1991). Using Triton X-100-solubilized rat brain membranes, Garcia et al. (1995) reported a more compact distribution, with a major peak corresponding to the dimeric form of synaptotagmin (~100 kDa). It is notable that in their study (Garcia et al., 1995), the free Ca\textsuperscript{2+} was unbuffered and can therefore be assumed to be present at \mu M levels. We therefore examined the effect of Ca\textsuperscript{2+} and EGTA on the migration of native synaptotagmin on similar gradients, using Triton X-100-solubilized rat brain membranes as a protein source. As shown in Fig. 5, synaptotagmin peaks at a position corresponding to its monomeric molecular mass in EGTA (~50 kDa) but exhibits a dramatic shift in the presence of Ca\textsuperscript{2+}, peaking at a position corresponding to the molecular mass of its dimeric form (~100 kDa). This shift was highly specific for synaptotagmin; no shift was observed for synaptophysin, rab 3A (Fig. 5), or other synaptic proteins, including the Ca\textsuperscript{2+} and phospholipid binding protein rabphilin (data not shown). Under these dilute conditions, little of the total synaptotagmin is bound to syntaxin and the interaction of synaptotagmin and the interaction of synaptotagmin with negatively charged phospholipids in either the synaptic vesicle or the plasma membrane.

**Fig. 6. Model depicting the sequential effects of Ca\textsuperscript{2+} on synaptotagmin-effector interactions.** Light shading denotes the first C2 domain of synaptotagmin (C2A), darker shading indicates the second C2 domain (C2B); syntaxin is shown in black. Arrows in the right panel denote the Ca\textsuperscript{2+}-dependent interaction of C2A and the Ca\textsuperscript{2+}-independent interaction of C2B of synaptotagmin with negatively charged phospholipids in either the synaptic vesicle or the plasma membrane.

DISCUSSION

In order to understand how the Ca\textsuperscript{2+}-binding protein synaptotagmin functions in regulated exocytosis it is essential to identify and characterize effectors for Ca\textsuperscript{2+}-synaptotagmin action. Previous work has shown that these effectors include negatively charged phospholipids (Brose et al., 1992; Davletov and Südhof, 1993; Chapman and Jahn, 1994) and syntaxin (Chapman et al., 1995; Li et al., 1995). Here we report that Ca\textsuperscript{2+} induces the formation of synaptotagmin dimers. Domain mapping analysis demonstrated that the second C2 domain (C2B) is involved in Ca\textsuperscript{2+}-dependent oligomerization, while both C2 domains are required for efficient Ca\textsuperscript{2+}-dependent synaptotagmin-syntaxin binding. Interestingly, the second C2 domain of synaptotagmin is essential for efficient Ca\textsuperscript{2+}-regulated exocytosis in Drosophila (Littleton et al., 1994). Few larvae with a mutant allele lacking the second C2 domain are viable. Of the surviving larvae, the cooperativity of excitation-secretion coupling is reduced 2-fold. In addition, an allele carrying a point mutation in the second C2 domain confers a higher requirement for extracellular Ca\textsuperscript{2+}, while the cooperativity of release remains comparable with that observed in wild type flies. These studies suggest that the Ca\textsuperscript{2+}-induced formation of synaptotagmin dimers via the second C2 domain is essential for efficient excitation-secretion coupling.

We have determined the Ca\textsuperscript{2+} dependencies for the digimerization of synaptotagmin and the interaction of synaptotagmin with syntaxin and found that they differ by an order of magnitude. These findings suggest a sequential order of events, which are summarized in the model shown in Fig. 6. As Ca\textsuperscript{2+} levels rise from resting (0.1 \mu M) to low \mu M levels (3-10 \mu M), synaptotagmin forms dimers via its second C2 domain. As Ca\textsuperscript{2+} levels rise to concentrations capable of triggering exocytosis (20-200 \mu M; Heidelberger et al. (1994)), synaptotagmin dimers bind to syntaxin via the synergistic action of both C2 domains.

As described above, nine isoforms of synaptotagmin have been identified thus far. The findings presented in this study raise the possibility that low levels of Ca\textsuperscript{2+} may trigger the association of different synaptotagmin isoforms with one another. Future experiments will be directed at determining whether such complex forms and whether these complexes exhibit distinct Ca\textsuperscript{2+}-dependent interactions with downstream effectors.

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