Ca$^{2+}$ Regulates the Interaction between Synaptotagmin and Syntaxin 1*

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Edwin R. Chapman, Phyllis I. Hanson, Seong An, and Reinhard Jahn

From the Howard Hughes Medical Institute and Departments of Pharmacology and Cell Biology, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

While there is compelling evidence that the synaptic vesicle protein synaptotagmin serves as the major Ca$^{2+}$ sensor for regulated exocytosis, it is not known how Ca$^{2+}$ binding initiates membrane fusion. Here we report that Ca$^{2+}$ increases the affinity, by approximately 2 orders of magnitude, between synaptotagmin and syntaxin 1, a component of the synaptic fusion apparatus. This effect is specific for divalent cations which can stimulate exocytosis of synaptic vesicles (Ca$^{2+} > $ Ba$^{2+}$, Sr$^{2+} > $ Mg$^{2+}$). The Ca$^{2+}$-dependence of the interaction was composed of two components with EC$_{50}$ values of 0.7 and 180 $\mu$M Ca$^{2+}$. The interaction is mediated by the carboxy-terminal region of syntaxin 1 (residues 194-288) and is regulated by a novel Ca$^{2+}$-binding site(s) which does not require phospholipids and is not disrupted by mutations that abolish Ca$^{2+}$-dependent phospholipid binding to synaptotagmin. We propose that this interaction constitutes an essential step in exocytosis-secretion coupling.

Exocytosis of synaptic vesicles is strictly controlled by Ca$^{2+}$ ions (Katz, 1969; Augustine et al., 1987). Presumably, Ca$^{2+}$ ions initiate conformational changes in proteins which ultimately catalyze membrane fusion. The Ca$^{2+}$ binding properties of the synaptic vesicle protein synaptotagmin are consistent with the requirements for the exocytotic Ca$^{2+}$ receptor (Brose et al., 1992; reviewed by DeBello et al. (1993), Popov and Poo (1993), Chapman and Jahn (1994b)). Indeed, gene disruption of the expression in Drosophila abolishes evoked neurotransmission (Schulze et al., 1995). Thus, the complex containing syntaxin, SNAP-25, and synaptobrevin is thought to comprise the core of the exocytotic fusion machine. Consequently, the interaction between syntaxin 1 and synaptotagmin is of particular interest, since it could provide a direct link between the Ca$^{2+}$-sensor and the fusion apparatus. In the present study we have characterized the interaction between synaptotagmin and syntaxin 1 and report that it is regulated by Ca$^{2+}$ ions.

**EXPERIMENTAL PROCEDURES**

**Immunoprecipitation**—All manipulations were carried out on ice. Synaptosomes were prepared by homogenizing one to two rat brains in 30 ml of 320 mM sucrose with 10 strokes at 900 rpm using a Teflon glass homogenizer. The homogenate was centrifuged at 5000 rpm for 2 min in an S34 rotor, and the crude synaptosomes were collected by centrifugation of the supernatant at 11,000 rpm for 12 min in an SS34 rotor. Synaptosomes were solubilized by detergent at a detergent to protein ratio of 10:1 (w/w) with 1% Triton X-100 in 50 mM HEPES, pH 7.2, 100 mM NaCl, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 $\mu$g/ml pepstatin, 20 $\mu$g/ml apronitin). Insoluble material was removed by centrifugation at 50,000 rpm in a TL 100.3 rotor for 15 min, and samples were then supplemented with 2 mM EGTA or 0.5 mM CaCl$_2$. Immunoprecipitations were carried out by incubating aliquots of the detergent extract (1 mg of protein) with 15 $\mu$l of ascites containing monoclonal antibodies directed against synaptophysin (Cl 7.3) or synaptotagmin 1 (HPC-1 or Cl 78.2) for 2 h followed by mixing with 30 $\mu$l of protein G-Sepharose fast flow (Pharmacia Biotech Inc.) for 1 h. HPC-1 recognizes both synaptotagmin 1A and 1B and has been described previously (Barnstable et al., 1985). Cl 78.2 is a newly generated monoclonal antibody raised against recombinant full-length synaptotagmin 1A that also recognizes synaptotagmin 1A and 1B and will be described in detail elsewhere. Immunoprecipitates were washed four times with the immunoprecipitation buffer and subjected to SDS-PAGE and immunoblot analysis as described (Chapman and Jahn, 1994a). Immunoreactive bands were visualized with $^{125}$I-protein A.

Binding of recombinant proteins was also carried out by immunoprecipitation of syntaxin. Recombinant full-length syntaxin 1A and C2AB were incubated at the indicated concentrations in 20 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100 supplemented with EGTA or divalent 

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† To whom correspondence should be addressed. Tel.: 203-737-4454; Fax: 203-737-1763.

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The abbreviations used are: NSF, N-ethylmaleimide-sensitive fusion factor; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.
Ca$$^{2+}$$-regulated Binding of Synaptotagmin to Syntaxin

**RESULTS**

To determine the effect of Ca$$^{2+}$$ ions on the interaction between syntaxin and synaptotagmin, we immunoprecipitated syntaxin 1 from synaptosomal detergent extracts, using two distinct monoclonal antibodies directed against syntaxin 1, in the presence of Ca$$^{2+}$$ or excess Ca$$^{2+}$$ chelator and examined the precipitates for the presence of syntaxin 1. As shown in Fig. 1 (top panel), Ca$$^{2+}$$ dramatically increased the level of synaptotagmin associated with syntaxin 1. We did not observe the calcium-dependent recruitment of additional proteins to the syntaxin immunoprecipitates by either protein staining or immunoblot analysis using antibodies directed against other synaptic proteins (data not shown). In addition, the amount of synaptobrevin and SNAP-25, components of the SNARE complex known to interact with syntaxin was not affected by Ca$$^{2+}$$ (Fig. 1 and data not shown). Neither synaptotagmin nor syntaxin 1 was detected in control immunoprecipitations using anti-synaptophysin antibodies (Fig. 1, left lanes).

To determine whether the Ca$$^{2+}$$-dependent coprecipitation is due to a direct interaction between syntaxin 1 and synaptotagmin, we repeated the syntaxin immunoprecipitations using purified recombinant proteins (Fig. 2, left panel). Full-length syntaxin 1A was mixed with the cytoplasmic portion of synaptotagmin I (amino acids 97–421) of syntaxin 1 was assayed by coimmunoprecipitation as described in legend for the left panel in the presence of EGTA (2 mM) or EGTA (2 mM) plus Ca$$^{2+}$$ (0.5 mM) or EGTA (2 mM) plus Mg$$^{2+}$$ (5 mM). Binding was monitored by immunoprecipitation using a purified anti-syntaxin 1 monoclonal antibody (HPC-1) and analyzed by immunoblotting for synaptotagmin, syntaxin 1, synaptophysin, and synaptobrevin. Immunoreactive bands were visualized using 125I-protein A and autoradiography. C2AB was precipitated only in the presence of syntaxin 1. Coprecipitation was strongly enhanced by Ca$$^{2+}$$ and was independent of negatively charged phospholipids. Right panel, divalent cation specificity for promoting binding of synaptotagmin to syntaxin 1. Binding of synaptotagmin to syntaxin 1 was assayed by immunoprecipitation as described in legend for the left panel in the presence of EGTA (2 mM) or the following divalent cation concentrations: Mg$$^{2+}$$ alone, 2.5 mM; Ca$$^{2+}$$; Ba$$^{2+}$$; Sr$$^{2+}$$; 0.5 mM each; Mg$$^{2+}$$ (5 mM) in the presence of Ca$$^{2+}$$ (0.5 mM). Note that equal amounts of syntaxin 1 were immunoprecipitated under all conditions. As expected, synaptophysin antibodies did not precipitate syntaxin 1 or synaptotagmin but efficiently coprecipitated synaptobrevin with synaptophysin, in agreement with our earlier observations (Edelmann et al., 1995).

We next compared the effects of different divalent cations on the syntaxin 1-synaptotagmin interaction. As shown in Fig. 2 (right panel), Ba$$^{2+}$$ and Sr$$^{2+}$$ ions promoted the association of syntaxin 1 and synaptotagmin, albeit less potently than Ca$$^{2+}$$. In contrast, Mg$$^{2+}$$ was virtually inactive and, in addition, failed to antagonize the effects of Ca$$^{2+}$$, even at high concentrations (5...
Ca2+-regulated Binding of Synaptotagmin to Syntaxin

FIG. 3. Ca2+-dependence of synaptotagmin-syntaxin binding. Full-length syntaxin 1 was expressed as a GST-fusion protein and immobilized using glutathione-Sepharose. The immobilized protein (0.5 nmol) was incubated with 1 mg of the Triton X-100 rat brain synaptosomal extract (1 mg/ml) in the presence of 2 mM EGTA or 1 mM EGTA and sufficient Ca2+ to yield the indicated free Ca2+ concentration, for 4 h at 4 °C. In parallel, GST alone was incubated with the detergent extract as a control. Beads were washed three times and the association of native synaptotagmin determined by SDS-PAGE and immunoblot analysis using anti-synaptotagmin 1 monoclonal antibody Cl 41.1. Immunoreactive bands were visualized with 125I-protein A (upper panel). These data were quantified by phosphorimaging and are plotted in the lower panel. Error bars represent the standard deviation from the mean of three independent experiments. Two distinct inflection points in the binding curve corresponding to high and low affinity Ca2+-binding sites were observed. Curves were fit to each of these two components using a logistic equation (De Lean et al., 1978). From this analysis, EC50 values of 0.7 and 180 μM Ca2+ were calculated and are denoted by arrows (lower panel). E corresponds to assays carried out in 2 mM EGTA.

FIG. 4. Concentration dependence of synaptotagmin binding to syntaxin 1 in the presence and absence of Ca2+. Left panel, a fixed amount of syntaxin 1 (0.5 μM) was incubated with increasing amounts of C2AB in 2 mM EGTA or 0.5 mM Ca2+. Binding was assayed by immunoprecipitation and immunoblot analysis as described under “Experimental Procedures.” The bands immunoreactive for synaptotagmin were quantified by phosphorimaging. These data are plotted in the lower panel (closed circles, binding measured in Ca2+; open circles, binding measured in EGTA). For the estimation of the EC50 (0.5 μM in Ca2+), curves were fit to the data using a logistic equation (De Lean et al., 1978). Right panel, Coomassie Blue staining of the anti-syntaxin 1 immunoprecipitates obtained after incubating 10 μM C2AB with or without 0.5 μM syntaxin 1 in 2 mM EGTA or 0.5 mM Ca2+. Note that the staining of C2AB and syntaxin 1 is of approximately equal intensity in the presence of Ca2+. H and L denote the heavy and light chains, respectively, of the HPC-1 IgG used for immunoprecipitation.
Kee et al., 1995), suggesting that at least some of these interactions may be mediated by intermolecular coiled coils.

It is notable that the removal of the transmembrane domain of syntaxin (residues 266–288) resulted in decreased synaptotagmin binding activity and also diminished the ability of syntaxin to bind synaptobrevin (Fig. 5B) and α-SNAP (Hanson et al., 1995). In addition, insertion of the transmembrane region into membranes is required for cleavage of syntaxin by botulinum neurotoxin C1 (Blasi et al., 1993b). While it is unlikely that the transmembrane domain participates in direct contacts with other proteins, these data suggest it may be essential for oligomerization and/or folding of syntaxin into its correct conformation.

The data described above demonstrate that Ca\textsuperscript{2+} regulates the interaction between syntaxin 1 and synaptotagmin in the absence of phospholipids. As mentioned above, this contrasts with the finding that purified synaptotagmin binds Ca\textsuperscript{2+} only in the presence of phospholipids (Brose et al., 1992), a property conferred by the first C2-domain (Davletov and Südhof, 1993; Chapman and Jahn, 1994a; Fukuda et al., 1994). Within this domain, a short sequence of highly conserved residues (SDPYVK–L) has been identified that is crucial for Ca\textsuperscript{2+} binding. Deletion of, or point mutations within, this motif abolish Ca\textsuperscript{2+}-dependent phospholipid binding to the isolated first C2-domain (Davletov and Südhof, 1993; Chapman and Jahn, 1994a). In contrast, the isolated second C2-domain does not bind to phospholipids in a Ca\textsuperscript{2+}-dependent manner, even though this domain contains the SDPYVK–L motif (Fukuda et al., 1994). To determine the role of these motifs in Ca\textsuperscript{2+}-dependent syntaxin 1 binding, we prepared mutant synaptotagmins (Fig. 6A) which contained this deletion in either the first (designated C2A1B) or second C2-domain (designated C2ABΔ). The Ca\textsuperscript{2+}-dependent phospholipid and syntaxin 1 binding properties of these mutants were then compared.

Deletion of the conserved motif within the first C2-domain abolished Ca\textsuperscript{2+}-dependent phospholipid binding, whereas deletion of the corresponding motif within the second C2-domain had no effect (Fig. 6B). In contrast, binding of both deletion mutants to syntaxin 1 was stimulated by Ca\textsuperscript{2+} to the same extent as that of the wild type cytoplasmic domain of synaptotagmin (Fig. 6C). These results clearly demonstrate that Ca\textsuperscript{2+}-dependent binding of synaptotagmin to syntaxin 1 involves structural features distinct from those required for the Ca\textsuperscript{2+}-dependent interaction of synaptotagmin with phospholipids.

Therefore, Ca\textsuperscript{2+} regulates the synaptotagmin-syntaxin interaction via a novel Ca\textsuperscript{2+}-binding site within the complex.

**DISCUSSION**

Recent studies have provided insights into the sequence of events that may lead to bilayer fusion (reviewed by Rothman and Warren (1994) and Jahn and Ferro-Novick (1994)). An early step in this pathway includes assembly of the SNARE proteins synaptobrevin, syntaxin 1, and SNAP-25, linking the target membrane to the incoming carrier vesicle. The assembled SNARE complex then recruits α-SNAP, enabling NSF to bind. NSF is an ATPase and upon hydrolysis of ATP dissociates the SNARE complex, an event proposed to result in membrane fusion (Söllner et al., 1993b).

How can the Ca\textsuperscript{2+}-stimulated interaction between synaptotagmin and syntaxin 1 be integrated into this model? Söllner et al. (1993b) observed that in neuronal detergent extracts, a small (substoichiometric) amount of synaptotagmin is associated with syntaxin 1 which could be displaced by the addition of exogenous α-SNAP (Söllner et al., 1993b). It was therefore suggested that synaptotagmin interacts with the SNARE complex before α-SNAP and NSF bind and dissociate the complex. This view, however, is difficult to reconcile with the dramatic increase in affinity of synaptotagmin for syntaxin upon Ca\textsuperscript{2+} influx. Rather, we believe that in the nerve terminal the association of synaptotagmin with syntaxin 1 occurs after dissociation of the complex by NSF (O’Conner et al., 1994). In this scenario, the NSF-dependent dissociation of the complex can be viewed as an ATP-dependent priming step that is necessary but not sufficient for membrane fusion. For exocytosis to proceed, dissociation needs to be succeeded by the Ca\textsuperscript{2+}-dependent association of synaptotagmin with syntaxin 1. Such a model would provide an explanation for the apparent lack of ATP dependence in the final step of exocytosis and puts the Ca\textsuperscript{2+} sensor, synaptotagmin, closer to the fusion event (Hay and Martin, 1992; Bittner and Holz, 1992; Thomas et al., 1993; Neher and Zucker, 1993).

The effect of Ca\textsuperscript{2+} on the affinity of the synaptotagmin-syntaxin interaction is likely to reflect a conformational change in either one or both of these proteins. The significance of the high and low affinity Ca\textsuperscript{2+}-dependent components is currently under investigation. However, the component which displays a low affinity for Ca\textsuperscript{2+} (EC\textsubscript{50} = 180 µM) is the first calcium-dependent interaction which corresponds to the calcium depend-
Distinct structural determinants of synaptotagmin underlie Ca$^{2+}$-dependent syntaxin 1 and phospholipid binding. A. Coomassie Blue-stained gel of purified C2AB, C2A$\Delta$, and C2AB$\Delta$ fused to GST. A motif crucial for Ca$^{2+}$-dependent phospholipid binding to the first C2-domain (SDPYVK−L, residues 177−185 (Chapman and Jahn, 1994a) was deleted from C2AB and designated C2A$\Delta$. For comparison, the corresponding motif (amino acids 308−316) was also deleted in the second C2-domain of C2AB and is designated C2A$\Delta$. Recombinant proteins were prepared as described under "Experimental Procedures," immobilized using glutathione-Sepharose, and subjected to SDS-PAGE on 10% gels. The GST-synaptotagmin fusion proteins migrate at approximately 67 kDa; the lower molecular mass bands are proteolytic fragments. Note: the prominent ~40-kDa band in the GST-C2AB lane reflects the increased sensitivity of this deletion mutant to bacterial proteases. B. Disruption of Ca$^{2+}$-dependent phospholipid binding to synaptotagmin. GST-C2AB, GST-C2A$\Delta$, GST-C2AB$\Delta$, and GST alone were immobilized using glutathione-Sepharose (80 pmol data point) and assayed for Ca$^{2+}$-dependent phospholipid binding as described (Chapman and Jahn, 1994a), using liposomes composed of 75% phosphatidylcholine and 25% phosphatidylethanolamine and labeled with [3H]phosphatidylcholine. Phospholipid binding was measured in 50 mM Tris, pH 7.2, 100 mM NaCl with 2 mM EGTA (open bars) or 0.5 mM Ca$^{2+}$ (solid bars). The figure shows total phospholipid binding to the immobilized proteins (mean values from triplicate determinations). C. Disruption of Ca$^{2+}$-dependent phospholipid binding does not inhibit Ca$^{2+}$-dependent syntaxin 1 binding to synaptotagmin. Ca$^{2+}$-dependent binding of syntaxin 1 to wild type (C2AB) and mutant synaptotagmins (C2A$\Delta$, C2AB$\Delta$) was assayed as described in Fig. 2.

References

Augustine, G. J., Charlton, M. P., and Smith, S. J. (1987) Annu. Rev. Neurosci. 10, 633−693

Barnstable, C. J., Hofstein, R., and Akagawa, K. (1985) Dev. Brain Res. 20, 286−290

Bennett, M. K., Calakos, N., and Scheller, R. H. (1992) Science 257, 255−259

Bittner, M. A., and Hotz, R. W. (1992) J. Biol. Chem. 267, 16219−16225

Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., DeCamilli, P., Südhof, T. C., Niemann, H., and Jahn, R. (1993a) EMBO J. 12, 4821−4828

Bommert, K., Charlton, M. P., DeBello, W. M., Chin, G. J., Betz, H., and Augustine, G. J. (1993) Nature 363, 160−162

Bommert, K., Charlton, M. P., DeBello, W. M., Chin, G. J., Betz, H., and Augustine, G. J. (1993b) Nature 363, 163−165

Broaddus, K., Belen, H. J., DiAntonio, A., Littleton, J. T., and Schwarz, T. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10727−10731

Busse, M., Petrenko, A. M., Davletov, B. A., Ushkaryov, Y. A., Geppert, M., and Südhof, T. C. (1995) Nature 375, 594−599

Calakos, N., Bennett, M. K., Peterson, K. E., and Scheller, R. H. (1994) Science 263, 1146−1149

Chapman, E. R., and Jahn, R. (1994a) J. Biol. Chem. 269, 5735−5741

Chapman, E. R., and Jahn, R. (1994b) Semin. Neurosci. 6, 159−165

Chapman, E. R., An, S., Barton, N., and Jahn, R. (1994) J. Biol. Chem. 269, 27427−27432

Davletov, B. A., and Südhof, T. C. (1993) J. Biol. Chem. 268, 26386−26390

De Lean, A., Munson, P. J., and Rodbard, D. (1978) Am. J. Physiol. 235, E9−E102

DeBello, W. M., Betz, H., and Augustine, G. J. (1993) Cell 74, 947−950

Edelmann, L., Hanso, P., Chapman, E. R., and Jahn, R. (1995) EMBO J. 14, 224−231

Efferink, L. A., Peterson, M. R., and Scheller, R. H. (1993) Cell 72, 153−159

Fukuda, M., Aruga, J., Nishibe, M., Aimoto, S., and Mikoshiba, K. (1994) J. Biol. Chem. 269, 29206−29211

Geppert, M., Goda, Y., Hammer, R. E., Li, C., Rozali, T. W., Stevens, C., and Südhof, T. C. (1994) Cell 79, 717−727

Hanson, P. O., Otto, H., Barton, N., and Jahn, R. (1995) J. Biol. Chem. 270, 16955−16961

Hay, J. C., and Martin, T. F. J. (1992) J. Biol. Chem. 267, 10727−10731

Heidelberger, R., Heinemann, C., and Matthews, G. (1994) Nature 371, 513−515

Jahn, R., and Ferro-Novick, S. (1994) Nature 370, 191−193

Katz, B. (1969) Sherrington Lecture X. Charles C. Thomas, Springfield, IL

Kees, Y., Lin, R. C., Hsu, S., and Scheller, R. H. (1995) Neuron 14, 991−998

Link, E., Edelmann, L., Chou, J. H., Binz, T., Yamasaki, S., Eisell, U., Baumann, M., Südhof, T. C., Niemann, H., and Jahn, R. (1992) Biochim. Biophys. Res. Commun. 189, 1017−1023

Littleton, T. J., Stern, M., Perin, M., and Bellen, H. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10888−10892

Nehrer, E., and Zucker, R. S. (1993) Neuron 10, 21−30

Nonet, M. L., Grundahl, K., Meyer, B. J., and Randj, J. B. (1993) Cell 73, 1291−1305

O’Conner, V., Augustine, G. J., and Betz, H. (1994) Cell 76, 785−787

Perin, M., Fried, V. A., Mignery, G. A., Jahn, R., and Südhof, T. C. (1990) Nature 343, 260−263

Petrenko, A. M., Davletov, B. A., Ushkaryov, Y. A., Geppert, M., and Südhof, T. C. (1991) Nature 353, 65−68

Popov, S. V., and Poo, M. (1993) Cell 73, 1247−1249

Rothman, J. E., and Warren, G. (1994) Curr. Biol. 4, 220−233

Schíavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., Dast Gupta, B. R., and Montecucco, C. (1992) Nature 359, 832−835

Schulze, K. L., Broaddus, K., Perin, M. S., and Bellen, H. J. (1995) Cell 80, 311−320

Sheng, Z. H., Rettig, J., Takahashi, M., and Catterall, W. A. (1994) Neuron 13, 1303−1313

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) Nature 362, 318−324

Söllner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H., and Rothman, J. E. (1993b) Cell 75, 409−418

Thomas, P., Wong, J. G., Lee, A. K., and Almers, W. (1993) Neuron 11, 93−104

Yoshida, A., Ohno, C., Omori, A., Kuwahara, R., Ito, T., and Takahashi, M. (1992) J. Biol. Chem. 267, 24925−24928

Zhang, J., Davletov, B. A., Südhof, T. C., and Anderson, R. G. W. (1994) Cell 78, 751−760