Ca\(^{2+}\)-dependent Conformational Change in Synaptotagmin I*

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Synaptotagmin I is a Ca\(^{2+}\)/phospholipid binding protein of synaptic vesicles with a proposed function as a Ca\(^{2+}\) sensor in synaptic vesicle exocytosis. Using controlled partial proteolysis as an assay, we now show that synaptotagmin I undergoes a conformational change as a function of Ca\(^{2+}\) binding. As observed for phospholipid binding, Ba\(^{2+}\) and Sr\(^{2+}\) but not Mg\(^{2+}\) substitute for Ca\(^{2+}\) in effecting this conformational change. The first C\(_2\) domain from synaptotagmin I that represents the Ca\(^{2+}\)-dependent phospholipid binding domain of synaptotagmin also undergoes a Ca\(^{2+}\)-dependent change in controlled partial proteolysis. In contrast, no effect of Ca\(^{2+}\) was observed with mutant C\(_2\) domains containing point mutations that abolish Ca\(^{2+}\) binding. The Ca\(^{2+}\) concentration dependence of the effect of Ca\(^{2+}\) on proteolysis mirrors the Ca\(^{2+}\) dependence of phospholipid binding. The conformational shift in synaptotagmin I caused by Ca\(^{2+}\)/phospholipid binding could be the basis for its Ca\(^{2+}\)-regulated function in triggering neurotransmitter release.

Synaptotagmins are abundant intrinsic membrane proteins of synaptic vesicles that are present in multiple isoforms and highly conserved in evolution (Perin et al., 1990, 1991a; Geppert et al., 1991; Wendland et al., 1991; Nonet et al., 1993; Bommert et al., 1993; Mizuta et al., 1994; Hribiush and Morgan, 1994). Synaptotagmin I, the best characterized synaptotagmin, consists of a short N-terminal intravesicular domain, a single transmembrane region, and a 335-amino acid cytoplasmic sequence (Perin et al., 1990, 1991b). The cytoplasmic sequence of synaptotagmin I comprises a highly charged N-terminal domain that mediates the multimerization of synaptotagmin I, two copies of a domain homologous to the C\(_2\) domain of protein kinase C, and a short C-terminal tail (Perin et al., 1991b). Biochemically, synaptotagmin I has been shown to bind Ca\(^{2+}\) and phospholipids in a ternary complex (Brose et al., 1992). Ca\(^{2+}\)/phospholipid binding by synaptotagmin I is probably mediated by its C\(_2\) domains since the first C\(_2\) domain exhibits Ca\(^{2+}\)/phospholipid binding properties similar to those of the whole protein (Davletov and Sudhof, 1993; Chapman and Jahn, 1994). In addition to binding Ca\(^{2+}\) and phospholipids, synaptotagmin I interacts with at least three proteins in vitro. First, it binds neurexins, synaptic cell surface receptors that include the receptor for the excitatory neurotransmitter a-latrotoxin, by a sequence-specific interaction between the C termini of these proteins (Hata et al., 1993; Perin, 1994). Second, it binds synaptotagmin, a protein involved in synaptic vesicle fusion (Bennett et al., 1992). Third, synaptotagmin I interacts with high affinity (approximately 10\(^{-10}\) M) with AP2, a protein complex that mediates clathrin assembly into coated pits (Zhang et al., 1994).

The biochemical interactions of synaptotagmin I and its specific localization to synaptic vesicles have led to the suggestion that synaptotagmin I may be a Ca\(^{2+}\)-sensing protein with a function in Ca\(^{2+}\)-triggered exocytosis and subsequent endocytosis involving multiple protein-protein interactions (Jahn and Sudhof, 1993). This hypothesis has received support from recent experiments with mutant mice carrying an inactivating mutation in the synaptotagmin I gene. Studies on hippocampal neurons in mutant mice revealed an essential function of synaptotagmin I in the fast component of Ca\(^{2+}\)-triggered neurotransmitter release that accounts for more than 80% of Ca\(^{2+}\)-dependent release but not in the docking of synaptic vesicles or in the exocytotic reaction itself.3 These experiments led to the suggestion that synaptotagmin I may be the major Ca\(^{2+}\) sensor mediating synaptic vesicle exocytosis in hippocampal neurons. It is likely that Ca\(^{2+}\) binding to synaptotagmin I is responsible for its function as the major Ca\(^{2+}\) sensor in triggering synaptic vesicle exocytosis, but all identified interactions of synaptotagmin I with other proteins are Ca\(^{2+}\)-independent. As a first step toward understanding how synaptotagmin I may function in mediating Ca\(^{2+}\)-triggered exocytosis, we have now studied the effect of Ca\(^{2+}\) binding on the conformation of synaptotagmin I using its susceptibility to partial proteolysis as an assay. Our results suggest that native synaptotagmin I and its first C\(_2\) domain undergo a Ca\(^{2+}\)-mediated conformational change whose characteristics correlate with those of Ca\(^{2+}\)/phospholipid binding and of the properties of the Ca\(^{2+}\) sensor in synaptic vesicle exocytosis.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors and Expression Purification of Recombinant Proteins—The expression vector encoding the full-length first C\(_2\) domain from rat synaptotagmin I fused to GST* (GST-SytA, residues 140-267; plasmid pGEX5-4), and the mutations in the C\(_2\) domain substituting aspartic acids at positions 178 and 230 for asparagine are described elsewhere (Davletov and Sudhof, 1993).3 Recombinant proteins were produced in bacteria and purified by affinity chromatography on glutathione-sepharose beads (Smith and Johnson, 1988).

Controlled Partial Proteolysis of Synaptotagmin I and of the Recombinant First C\(_2\) Domain from Synaptotagmin I—Rat brain membrane proteins were solubilized in 50 mM Tris-HCl, pH 7.8, 1 mM EGTA, 0.2% Triton X-100 and incubated at 1 gltiter protein for 45 min with trypsin, chymotrypsin, and V8 protease (all from Sigma) at the indicated concentrations and with the indicated additions of divalent cations and EGTA. Reactions were stopped by the addition of SDS-sample buffer (Laemmli, 1970) and analyzed by SDS-PAGE and Coomasie blue staining or immunoblotting. For the proteolysis experiments on the recombinant GST-fusion proteins, recombinant proteins were eluted from the column with glutathione and incubated at 0.2 g/liter protein with the respective proteases at the indicated concentrations in 50 mM HEPES-NaOH, pH 7.2, 0.1 mM CaCl\(_2\) with the indicated additions of Ca\(^{2+}\) and/or EGTA. For the experiments investigating the Ca\(^{2+}\) concentration dependence of trypsin digestion, free Ca\(^{2+}\) concentrations were adjusted by Ca\(^{2+}\)/EGTA buffers as described (Davletov and Sudhof, 1993) except that Ca\(^{2+}\) was replaced by Sr\(^{2+}\) or Ba\(^{2+}\) under identical conditions.


2 The abbreviations used are: GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

3 B. Davletov and T. C. Sudhof, unpublished observation.
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FIG. 1. Effect of Ca\textsuperscript{2+} on the proteolytic digestion of synaptotagmin I by trypsin, chymotrypsin, and V8 protease. Solubilized total bovine brain membrane proteins were digested with the indicated proteases in 1 mM EGTA with or without 1.1 mM Ca\textsuperscript{2+}. Proteins were analyzed by SDS-PAGE (20 \mu g/lane). Synaptotagmin I was detected by immunoblotting using enhanced chemiluminescence (top panel), and total proteins in the samples were visualized by Coomassie Blue staining (bottom panel). Immunoblotting was carried out with a polyclonal antibody raised against the complete cytoplasmic sequences of synaptotagmin I (Perin et al., 1990). Numbers on the left indicate positions of molecular weight standards.

RESULTS

In several Ca\textsuperscript{2+} binding proteins, Ca\textsuperscript{2+} binding results in a conformational change that can be detected by changes in the pattern of proteolysis by exogenous proteases (e.g. Ohnishi and Reithmeier (1987) and Orr and Newton (1994)). Changes in the rate and pattern of proteolysis as a function of ligand binding are presumably a reflection of changes in the surface exposure of particular residues in the respective proteins. Controlled partial proteolysis may be particularly well suited to analyze conformational changes in synaptotagmin I because other methods to detect conformational changes are difficult to use with this intrinsic membrane protein. Therefore we tested if Ca\textsuperscript{2+} modulates the proteolysis of synaptotagmin I mediated by low concentrations of exogenous proteases added to total brain membrane proteins. Three different proteases (trypsin, chymotrypsin, and V8 protease) with distinct cleavage specificities were used. Synaptotagmin I proteolysis was monitored as a function of protease concentration by immunoblotting with a polyclonal antibody directed against the cytoplasmic sequences of synaptotagmin I.

A dramatic Ca\textsuperscript{2+} dependence of synaptotagmin I proteolysis was observed (Fig. 1). At very low protease concentrations, synaptotagmin I is preferentially cleaved in a hypersensitive "hinge" region that is located C-terminal to the multimerization domain of synaptotagmin I and N-terminal to its first C\textsubscript{2} domain (Perin et al., 1991b). This first cleavage results in the generation of an N-terminal fragment containing the transmembrane region and a C-terminal cytoplasmic fragment consisting of the two C\textsubscript{2} domains and is not significantly affected by the presence or absence of Ca\textsuperscript{2+}. With increasing concentrations of trypsin and chymotrypsin, however, rapid degradation of the cytoplasmic fragment of synaptotagmin I is observed in the presence of EGTA whereas in the presence of Ca\textsuperscript{2+} the cytoplasmic fragment containing both C\textsubscript{2} domains is protected (Fig. 1). Coomassie Blue staining of SDS-polyacrylamide gels of the reactions shows no significant difference in overall proteolysis of proteins present in the brain membranes as a function of Ca\textsuperscript{2+}.

In the case with V8 protease, synaptotagmin I is digested more slowly than by trypsin and chymotrypsin. With this protease Ca\textsuperscript{2+} also has no effect on the initial cleavage at the hypersensitive site but also leads to the stabilization of a fragment containing both C\textsubscript{2} domains (Fig. 1). The V8 fragment stabilized by Ca\textsuperscript{2+} is slightly smaller than the corresponding fragment observed with trypsin and chymotrypsin but larger than the fragment obtained in the absence of EGTA, suggesting that the proteolysis of synaptotagmin I by V8 protease is also sensitive to Ca\textsuperscript{2+} similar to trypsin- and chymotrypsin-mediated proteolysis.

We next studied the cation specificity of the Ca\textsuperscript{2+} effect on proteolysis. Previous studies had demonstrated that Ba\textsuperscript{2+} and Sr\textsuperscript{2+} but not Mg\textsuperscript{2+} can substitute for Ca\textsuperscript{2+} in mediating phospholipid binding (Davletov and Südhof, 1992). Therefore we studied the effects of the different cations on the rate of synaptotagmin I proteolysis by trypsin. Consistent with the cation specificity of Ca\textsuperscript{2+}-dependent phospholipid binding to synaptotagmin I, Mg\textsuperscript{2+} is unable to replace Ca\textsuperscript{2+} even at high concentrations, but Sr\textsuperscript{2+} and Ba\textsuperscript{2+} are effective (Fig. 2). Proteolytic fragments of similar size are stabilized with all three divalent cations, suggesting similar mechanisms.

Four observations support the conclusion that the observed Ca\textsuperscript{2+}-dependent changes in proteolysis are caused by a Ca\textsuperscript{2+}-dependent change in the conformation of synaptotagmin I and not by an artifactual effect of Ca\textsuperscript{2+} on the proteolytic reaction. First, as demonstrated by Coomassie Blue staining (Fig. 1), Ca\textsuperscript{2+} has no detectable effect on the proteolysis of most proteins present in the sample. Second, Ca\textsuperscript{2+} should accelerate proteolysis by enzymes such as trypsin, which are themselves Ca\textsuperscript{2+} binding proteins, but we observe an inhibitory effect. Third, different proteases result in different patterns of protection, as would be expected from the different cleavage site specificities, but the size of the transiently protected fragment is similar for the three proteases. Fourth, the cation specificity of the effect on synaptotagmin I proteolysis mirrors the cation specificity of
phospholipid binding to the first C$_2$ domain of synaptotagmin I (Davletov and Südhof, 1993). Together these observations suggest that the observed Ca$^{2+}$ sensitivity of synaptotagmin I proteolysis reflects a Ca$^{2+}$-induced conformational change in synaptotagmin I.

We have previously demonstrated that the first C$_2$ domain of synaptotagmin I exhibits high affinity Ca$^{2+}$/phospholipid binding, suggesting that it represents a functional Ca$^{2+}$/phospholipid binding domain in synaptotagmin I (Davletov and Südhof, 1993). To test if a Ca$^{2+}$-dependent conformational change can also be observed with a C$_2$ domain alone, proteolysis experiments were performed with purified recombinant GST fusion protein containing the first C$_2$ domain of synaptotagmin I. A dramatic protective effect of Ca$^{2+}$ on the digestion of the C$_2$ domain by trypsin was observed that leads to the protection of the C$_2$ domain from proteolysis and is similar to the effect of Ca$^{2+}$ on native synaptotagmin I (Fig. 3). As controls, we tested the effect of Ca$^{2+}$ on the proteolysis of two mutant GST C$_2$ domain fusion proteins that are identical with the wild type C$_2$ domain fusion protein except for the presence of point mutations, which inactivate Ca$^{2+}$-dependent phospholipid binding. No effect of Ca$^{2+}$ could be observed on the proteolysis of the mutant proteins, demonstrating that Ca$^{2+}$ has no effect on the proteolytic activity of trypsin under the conditions used and that the Ca$^{2+}$-dependent effect observed is dependent on Ca$^{2+}$ binding by the C$_2$ domain (Fig. 3 and data not shown).

In a final set of experiments we studied the Ca$^{2+}$ concentration dependence of proteolysis (Fig. 4). Ca$^{2+}$-dependent phospholipid binding to the first C$_2$ domain of synaptotagmin I exhibits a steep concentration dependence with an EC$_50$ of approximately 5 $\mu$M (Davletov and Südhof, 1993). Proteolysis of the C$_2$ domain at different Ca$^{2+}$ concentrations revealed a Ca$^{2+}$ dependence that paralleled that of phospholipid binding, suggesting that both assays are measuring the same process (Fig. 4). Together these data demonstrate that a single C$_2$ domain of synaptotagmin I undergoes a Ca$^{2+}$/phospholipid-dependent conformational change that leads to a stabilization of the C$_2$ domain and its protection from trypsin digestion.

**DISCUSSION**

Synaptotagmin I is a Ca$^{2+}$ binding protein that binds Ca$^{2+}$ in a ternary complex with phospholipids (Brose et al., 1992) and is essential for physiological Ca$^{2+}$-triggered neurotransmitter release in hippocampal synapses, suggesting that its main function is that of a Ca$^{2+}$ sensor in exocytosis. The first C$_2$ domain of synaptotagmin I produced as a recombinant protein in bacteria exhibits Ca$^{2+}$-dependent phospholipid binding with an apparent cooperativity of 3–4 (Davletov and Südhof, 1993). Thus, synaptotagmin I could function as a Ca$^{2+}$ sensor in exocytosis by virtue of its Ca$^{2+}$/phospholipid binding via its first C$_2$ domain. How does synaptotagmin I perform this function? Although several protein-protein interactions have been described for synaptotagmin I (Bennett et al., 1992; Hata et al., 1993; Zhang et al., 1994), all of them are independent of Ca$^{2+}$. It is thought that Ca$^{2+}$ acts in triggering synaptic vesicle exocytosis by inducing a fusogenic conformational change in its target protein. Clearly, if synaptotagmin I is the Ca$^{2+}$ sensor, demonstration of a conformational change as a function of Ca$^{2+}$ would be the first step toward understanding its mechanism of action.

In the present study, we have addressed this question using partial proteolysis as an assay to detect conformational changes in synaptotagmin I as a function of Ca$^{2+}$/phospholipid binding.
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A major effect of Ca\(^{2+}\) was observed that leads to the relative protection of the C\(_2\) domains from proteolysis, suggesting that Ca\(^{2+}\) binding leads to a conformational change in synaptotagmin I that could form the basis for its function in triggering synaptic vesicle fusion. The following evidence supports the conclusion that the observed Ca\(^{2+}\)-dependent changes in proteolysis reflect a conformational change in the cytoplasmic C\(_2\) domains of synaptotagmin I and are not caused by a direct effect on the proteases. 1) A similar fragment of 35-40 kDa containing both C\(_2\) domains was protected in the presence of Ca\(^{2+}\) when different proteases with distinct cleavage specificities were used. 2) The cation specificity of the protective effect mirrors the cation specificity of phospholipid binding. 3) The protective effect of Ca\(^{2+}\) on proteolysis could also be observed with a recombinant functionally active C\(_2\) domain. 4) The Ca\(^{2+}\) effect has a similar Ca\(^{2+}\) concentration dependence as phospholipid binding. 5) No effect of Ca\(^{2+}\) on proteolysis was observed with a mutant synaptotagmin I that does not bind Ca\(^{2+}\)/phospholipids. Thus, although protection from partial proteolysis is an indirect assay, this evidence demonstrates that the observed Ca\(^{2+}\)-dependent changes observed reflect a conformational change in synaptotagmin I.

The Ca\(^{2+}\)-induced conformational change in a single C\(_2\) domain, characterized here for synaptotagmin I, also has implications for the regulation of protein kinase C by Ca\(^{2+}\). In a widely accepted model, activators induce protein kinase C activity by a conformational change that removes the N-terminal pseudosubstrate sequence from the active site of the enzyme (Bazzi and Nelsestuen, 1990; Orr and Newton, 1994). Interestingly, protein kinase C can also be activated by Ba\(^{2+}\) and Sr\(^{2+}\) (Wise et al., 1981). A Ca\(^{2+}\)/phospholipid-dependent conformational change in the C\(_2\) domain as observed in the current study would explain this activation. Thus, two protein families with very different functions, synaptotagmins and protein kinase C, may be activated by similar mechanisms that involve a Ca\(^{2+}\)-dependent intramolecular conformational switch.

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REFERENCES