A Single C2 Domain from Synaptotagmin I Is Sufficient for High Affinity Ca2+/Phospholipid Binding*

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Synaptotagmin I is a Ca2+- and phospholipid-binding protein of synaptic vesicles with an essential function in neurotransmission. Ca2+/phospholipid binding by synaptotagmin I may be mediated by its C2 domains, sequence motifs that have been implicated in the Ca2+ regulation of a variety of proteins. However, it is currently unknown if C2 domains are sufficient for Ca2+/phospholipid binding or if they even directly participate in Ca2+/phospholipid binding. In order to address this question, we have studied the Ca2+/phospholipid-binding properties of the first C2 domain of synaptotagmin I. Our results show that this C2 domain by itself binds Ca2+ and phospholipids with high affinity (half-maximal binding at 4-6 μM free Ca2+) and exhibits strong positive cooperativity. The C2 domain is specific for negatively charged phospholipids and for those divalent cations that are known to stimulate synaptic vesicle exocytosis (Ca2+ > Sr2+, Ba2+ -> Mg2+). These studies establish that C2 domains can serve as independently folding Ca2+/phospholipid-binding domains. Furthermore, the cation specificity and the cooperativity of Ca2+ binding by the C2 domain from synaptotagmin I support a role for this protein in mediating the Ca2+ signal in neurotransmitter release.

Synaptotagmin I is an abundant synaptic vesicle membrane protein that contains a short N-terminal intravesicular sequence, a single transmembrane region, and two copies of a cytoplasmic repeat with homology to the C2 domain of PKC1 (Perin et al., 1989, 1991a, 1991b). Multiple forms of synaptotagmin are present in brain, with synaptotagmin I being the most abundant (Geppert et al., 1992; Wendland et al., 1992). Purified synaptotagmin I binds Ca2+ and phospholipids, suggesting a role for synaptotagmin I in Ca2+-dependent synaptic vesicle exocytosis (Brose et al., 1992). However, the exact function of synaptotagmin I is unclear, with studies using a variety of approaches arriving at different conclusions. These conclusions range from lack of any function for synaptotagmin in neurotransmitter release (Shoji-Kasai et al., 1992) to the demonstration that synaptotagmin is important for release (Elferink et al., 1993; DiAntonio et al., 1993; Nonet et al., 1993), and may even be essential for Ca2+-triggered synaptic vesicle exocytosis (Bommert et al., 1993; Littleton et al., 1993). In total, these experiments provide support for an important, although unidentified, function for synaptotagmin in neurotransmitter release. It is possible that this function is effected by the interaction of synaptotagmin with neuromins and the α-latrotoxin receptor (Petrenko et al., 1991; Hata et al., 1993) or with HPC-1/synactins (Bennett et al., 1992; Yoshida et al., 1992).

A variety of proteins that are regulated by Ca2+/phospholipid binding contain C2 domains, suggesting that C2 domains represent sequence motifs involved in Ca2+ signaling (Coussens et al., 1986; Knopf et al., 1986; Vogel et al., 1988; Clark et al., 1991). The possible Ca2+-binding properties of C2 domains have been investigated best in PKC where Ca2+ binding and regulation was shown to require negatively charged phospholipids (Bazzi and Nelsestuen, 1990) and to depend on the presence of the C2 domain (Kaibuchi et al., 1989; Akita et al., 1990; Luo et al., 1993). However, phospholip ester binding to the C1 domain of PKC is also dependent on Ca2+ in PKC isozymes that contain C2 domains (Ono et al., 1988), and Ca2+ binding to PKC has been shown to be dependent on sequences from the C2 domain in addition to the C2 domain (Luo et al., 1993). These latter results indicate that C2 domains may not by themselves be functionally independent Ca2+/phospholipid-binding domains but may require additional sequences for Ca2+ phospholipid binding.

In order to determine if Ca2+ binding by synaptotagmin I is mediated by the C2 domains, and in order to elucidate if the C2 domains are independently folding, autonomous Ca2+/phospholipid-binding domains, or require additional sequences, we have now investigated the Ca2+-binding properties of a single C2 domain from synaptotagmin I. Our results indicate that C2 domains are sufficient for Ca2+/phospholipid binding. Furthermore, the high affinity of the first C2 domain from synaptotagmin I for Ca2+ (4-6 μM, EC50) and its cooperativity in Ca2+ binding agree well with a C2+-dependent function in synaptic vesicle exocytosis (Littleton et al., 1993).

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors and Expression and Purification of Recombinant Proteins—For the construction of pGex-SytA that encodes the first C2 domain of synaptotagmin I fused to GST (referred to as GST-SytA), the corresponding DNA from rat synaptotagmin I (nucleotides 948-1325) (Perin et al., 1990) was amplified by PCR with oligonucleotides A and B (sequences: CGCGGATCCACCATGGTG-TCTTCCTGCTGCCTGCTGCCTGA) in combination with oligonucleotides A and B (sequences: GCGAAGCTTATGCTCAGGAGAAAATCGGGAAAGTTCCA and GCGAAGCTTATGCTCAGGAGAACGCGTGGAG). The resulting 0.4-kilobase PCR fragment was subcloned into the BamHI and HindIII sites of the bacterial expression vector pGex-KG in which the inserts are fused to GST (Guon and Dixon, 1991). pGex-SytB encoding the first C2 domain of synaptotagmin I, in which the amino acids YVK (residues 180-182) were mutated to AAA, was constructed by separately amplifying the right and left halves of the first C2 domain using oligonucleotides C and D (sequences: GCCGGATCCACCATGGTG-TCTTCCTGCTGCCTGCTGCCTGA) in combination with oligonucleotides A and B, respectively. The two resulting PCR fragments were digested with BamHI, ligated to each other, and reamplified with oligonucleotides A and B. The resulting DNA fragment encoding the mutant first C2 domain of synaptotagmin I was subcloned as above and differs from pGex-SytA only in the sequence of the three.

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amino acid residues 180–182 as confirmed by DNA sequencing. Recombinant proteins were produced in bacteria and purified by affinity chromatography on glutathione-agarose beads (Smith and Johnson, 1988). Binding experiments were carried out with the recombinant proteins still attached to glutathione-agarose beads.

Preparation of [3H]-Labeled Liposomes—The phospholipids (1.75 mg) dissolved in chloroform were mixed in a 1.5-mL tube and dried under a stream of argon. Liposomes were made from either 1.75 mg of PC or 1.25 mg of PC mixed with 0.5 mg of PS, PE, or PI (all phospholipids were from Avanti Polar Lipids, Inc.). In all experiments measuring phospholipid binding, liposomes contained 20 μCi of 1,2-dipalmitoyl-1,3-phosphatidyl[N-methyl-3H]choline from Avanti Polar Lipids, Inc.). In all experiments measuring phospholipid binding, liposomes contained 20 μCi of 1,2-dipalmitoyl-1,3-phosphatidyl[N-methyl-3H]choline from Avanti Polar Lipids, Inc.). In all experiments measuring phospholipid binding, liposomes contained 20 μCi of 1,2-dipalmitoyl-1,3-phosphatidyl[N-methyl-3H]choline from Avanti Polar Lipids, Inc.).

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Liposomes were washed three times with a stream of argon. Liposomes were made from either 20 mg of recombinant GST (lanes A–A’) and GST-synaptotagmin containing the first C2 domain of synaptotagmin I (GST-SytA; lanes B and B’). Samples (5 μg of protein) were analyzed either before (lanes A and B) or after phospholipid-binding experiments (lanes A’ and B’) to ensure that the procedure maintains the integrity of the proteins. The M, 27,000 band observed in the GST-SytA lanes represents a proteolytic protein. Primed molecular weight standards were loaded on the left lane (5) corresponding from top to bottom) to phosphorylase b (106,000), bovine serum albumin (80,000), ovalbumin (49,500), carbonic anhydride (32,500), and soybean trypsin inhibitor (27,500). B, assay for Ca2+-dependent phospholipid binding to recombinant proteins. Glutathione-agarose (Aagarose), GST bound to glutathione-agarose (GST), or GST-SytA (the first C2 domain of synaptotagmin I fused to GST) bound to glutathione-agarose were incubated with 3H-labeled PS/PC liposomes in the absence or presence of 0.1 mM Ca2+ (open and hatched bars, respectively). Phospholipid binding was measured by scintillation counting of the beads after extensive washing. Experiments were performed in triplicates with identical protein concentrations in the protein-containing incubations. Error bars indicate standard errors of the mean.

RESULTS

In order to determine if a single C2 domain of synaptotagmin is sufficient for Ca2+/phospholipid binding, a recombinant protein was produced (referred to as GST-SytA) that consists of the first C2 domain of rat synaptotagmin I (amino acid residues 140–267) (Perin et al., 1990) fused to GST (Fig. 1A). The sequence boundaries for this fusion protein correspond to the full-length sequence of the first C2 domain repeat of synaptotagmin I and flanking amino acids. Bacterial expression of shorter sequences from the first C2 domain of synaptotagmin I tended to produce insoluble products and exhibited low levels of expression (data not shown), suggesting that residues 140–267 of synaptotagmin I form an independently folding, stable domain in bacteria. Incubation of GST-SytA attached to glutathione-agarose with 3H-labeled PS/PC liposomes resulted in Ca2+-dependent binding of the 3H-labeled liposomes to GST-SytA (Fig. 1B). Incubation of the PS/PC liposome with glutathione-agarose alone or with recombinant GST bound to glutathione-agarose resulted in no Ca2+-dependent binding, indicating that the Ca2+-dependent binding observed with GST-SytA is due to its C2 domain sequence (Fig. 1B). The Ca2+-dependent binding of phospholipids to GST-SytA was linearly dependent on the concentration of GST-SytA. Binding was independent of temperature and almost complete in less than 2 min, the limit of the resolution of the current assay (data not shown). Some Ca2+-independent binding of PS/PC liposomes to glutathione-agarose with or without GST was observed and appears to be caused by nonspecific interactions of the liposomes with the agarose. Binding of 45Ca2+ to the recombinant proteins as a function of phospholipids was also measured in similar experiments, demonstrating phospholipid-dependent binding of 45Ca2+ to the GST-synaptotagmin fusion protein but not to GST or to glutathione-agarose alone (data not shown). However, due to the potential for artifacts caused by 45Ca2+ binding to phospholipids, we chose to use 3H-phospholipid binding as an assay for studying the Ca2+/phospholipid-binding properties of the isolated C2 domain of synaptotagmin.

The data in Fig. 1 suggest that the first C2 domain of synaptotagmin I in GST-SytA is capable of forming a ternary complex with Ca2+ and phospholipids. To investigate if this interaction is specific for Ca2+, the binding experiments were carried out with different cations. Ca2+ was the most potent in mediating phospholipid binding, whereas Mg2+ and K+ were without effect even at high concentrations (Fig. 2A). Sr2+ and Ba2+ did activate phospholipid binding to GST-SytA but were less efficient than Ca2+ at 0.1 mM concentrations. However, at 0.5 mM

![FIG. 1. A, SDS-PAGE analysis of recombinant GST (lanes A and A’)](image) and GST-synaptotagmin containing the first C2 domain of synaptotagmin I (GST-SytA; lanes B and B’). Samples (5 μg of protein) were analyzed either before (lanes A and B) or after phospholipid-binding experiments (lanes A’ and B’) to ensure that the procedure maintains the integrity of the proteins. The M, 27,000 band observed in the GST-SytA lanes represents a proteolytic protein. Primed molecular weight standards were loaded on the left lane (5) corresponding from top to bottom) to phosphorylase b (106,000), bovine serum albumin (80,000), ovalbumin (49,500), carbonic anhydride (32,500), and soybean trypsin inhibitor (27,500). B, assay for Ca2+-dependent phospholipid binding to recombinant proteins. Glutathione-agarose (Aagarose), GST bound to glutathione-agarose (GST), or GST-SytA (the first C2 domain of synaptotagmin I fused to GST) bound to glutathione-agarose were incubated with 3H-labeled PS/PC liposomes in the absence or presence of 0.1 mM Ca2+ (open and hatched bars, respectively). Phospholipid binding was measured by scintillation counting of the beads after extensive washing. Experiments were performed in triplicates with identical protein concentrations in the protein-containing incubations. Error bars indicate standard errors of the mean.
there was no significant difference between Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\). This suggests that Ba\(^{2+}\) and Sr\(^{2+}\) may have a lower affinity for synaptotagmin I than Ca\(^{2+}\). In earlier experiments we observed no effect of Ba\(^{2+}\) on phospholipid binding to purified synaptotagmin I (Brose et al., 1992), possibly because of the differences in the assays used.

GST alone exhibited no cation-stimulated phospholipid binding under any of the conditions used, suggesting that the binding observed is specific for the recombinant C\(_2\) domain. The relative potencies of Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\) in mediating phospholipid binding to the first C\(_2\) domain of synaptotagmin I and the lack of effect by Mg\(^{2+}\) and K\(^{+}\) agree very well with the relative actions of these cations in triggering neurotransmitter release (reviewed by Smith and Augustine (1989)).

In order to be physiologically significant, phospholipid binding to synaptotagmin should be stimulated by Ca\(^{2+}\) concentrations similar to those achieved in the nerve terminal after depolarization. In order to determine the dependence of phospholipid-binding on the Ca\(^{2+}\) concentration, a panel of Ca\(^{2+}\)-EGTA buffers was used (Fig. 3). In four independent experiments with different preparations of buffers and GST-SytA, half-maximal binding at 4–6 \(\mu\)M free Ca\(^{2+}\) was observed, with the experiment shown in Fig. 3 exhibiting an EC\(_{50}\) of 5.8 \(\mu\)M. Again, no Ca\(^{2+}\)-dependent phospholipid binding to GST alone was found. Phospholipid binding to the first C\(_2\) domain of synaptotagmin I occurs over a very narrow Ca\(^{2+}\) concentration range, suggesting cooperativity (Fig. 3). Analysis of independent binding data revealed Hill coefficients between 2.0 and 3.1 in all binding experiments, with the experiment shown in Fig. 3 having a Hill coefficient of 3.1. Thus, Ca\(^{2+}\) binding to a single C\(_2\) domain is positively cooperative, suggesting multiple interacting binding sites. Interestingly, the observed cooperativity is very similar to the cooperativity demonstrated for Ca\(^{2+}\) in triggering neurotransmitter release (reviewed by Smith and Augustine (1989) and Almers and Tse (1991)).

Activation of PKC by Ca\(^{2+}\) is dependent on negatively charged phospholipids with a limited specificity (Hannun et al., 1986; Newton and Koshland, 1989). Previous studies with synaptotagmin I indicated that its phospholipid-binding specificity was very similar to that of PKC (Perin et al., 1990; Brose et al., 1992). To test the specificity of phospholipid binding by the single C\(_2\) domain, Ca\(^{2+}\)-dependent binding of liposomes containing different phospholipid compositions was investigated (Fig. 4). These experiments revealed that liposomes containing PC in combination with either PS or PI bind to the isolated C\(_2\) domain of synaptotagmin I in a Ca\(^{2+}\)-dependent manner. On the other hand, liposomes consisting of only PC or of PE and PC do not bind in a Ca\(^{2+}\)-dependent manner, although their background binding is higher. This result suggests that Ca\(^{2+}\) phospholipid binding by the first C\(_2\) domain of synaptotagmin I requires negatively charged phospholipids.

The different experiments described above clearly establish that the Ca\(^{2+}\)/phospholipid-binding properties of a single C\(_2\) domain from synaptotagmin I can account for the binding prop-
properties of purified synaptotagmin I or of PKC. These conclusions differ from previous inferences about the Ca\textsuperscript{2+}-binding mechanisms of synaptotagmin I and PKC (Brose et al., 1992; Luo et al., 1993). Could the specific, high affinity binding of Ca\textsuperscript{2+}/phospholipids to GST-SytA be due to a nonspecific interaction caused by the highly charged synaptotagmin sequence? To rule out such a possibility, we constructed a mutated GST-synaptotagmin I fusion protein (GST-SytB). GST-SytB is identical with GST-SytA in size but carries a mutation in a sequence that is highly conserved in C\textsubscript{2} domains, changing SDPYVKVFL (residues 177–185) (Perin et al., 1990) to SDPAAAVFL. GST-SytB is expressed at 5 times lower levels than GST-SytA and is much less stable than GST-SytA (Fig. 5A).

Ca\textsuperscript{2+}/phospholipid-binding measurements comparing GST-SytB with GST alone and GST-SytA demonstrate that GST-SytB does not bind phospholipids at 0.1 mM Ca\textsuperscript{2+} and exhibits significant but reduced phospholipid binding at 1 mM Ca\textsuperscript{2+} (Fig. 5B). The absence of Ca\textsuperscript{2+}/phospholipid binding by GST-SytB is not due to the relatively lower concentrations of full-length GST-SytB protein because no binding was observed for GST-SytB at 0.1 mM Ca\textsuperscript{2+} at higher protein concentrations, whereas such binding could be observed for GST-SytA at much lower protein concentrations (data not shown). This result demonstrates that a short mutation in the C\textsubscript{2} domain abolishes its binding activity, suggesting that the binding observed for GST-SytA is not a nonspecific charge interaction but depends on a defined secondary and tertiary structure of the protein.

**DISCUSSION**

The C\textsubscript{2} domain was defined as a sequence motif in the cloning of cDNAs encoding PKC as the second sequence region conserved among different PKC isozymes (CousSENS et al., 1986; KnoPf et al., 1986). The presence of sequences homologous to this domain in synaptotagmins and a variety of other proteins suggests that this domain may represent a widely shared functional motif (Vogel et al., 1988; Ferin et al., 1990; Clark et al., 1991; Maruyama and Brenner, 1991). Transfection experiments demonstrated that the Ca\textsuperscript{2+}-dependent regulation of PKC depends on the presence of the C\textsubscript{2} domain (KAIbuChi et al., 1989; AkitA et al., 1990), and purified PKC and synaptotagmin I were shown to bind Ca\textsuperscript{2+} and phospholipids (Bazzi and Nelsestuen, 1990; Brose et al., 1992). However, the precise role of C\textsubscript{2} domains in Ca\textsuperscript{2+}/phospholipid binding remained enigmatic. Indeed, recent data suggested that sequences outside of the C\textsubscript{2} domains may be required for Ca\textsuperscript{2+}/phospholipid binding by proteins containing C\textsubscript{2} domains (Brose et al., 1992; Luo et al., 1993). The current study was initiated to determine to what extent C\textsubscript{2} domains are independently functioning Ca\textsuperscript{2+}/phospholipid-binding domains.

We present five lines of evidence that at least the first C\textsubscript{2} domain of synaptotagmin I constitutes an autonomous, fully competent Ca\textsuperscript{2+}/phospholipid-binding domain. 1) Fusion of this domain with GST conferred Ca\textsuperscript{2+}- and phospholipid-binding properties onto the fusion protein such that binding was only observed in the presence of all three partners (Ca\textsuperscript{2+}, phospholipids, and the C\textsubscript{2} domain). 2) Phospholipid binding to the C\textsubscript{2} domain was specific for Ca\textsuperscript{2+} with respect to Mg\textsuperscript{2+} and K\textsuperscript{+} but could also be promoted by Sr\textsuperscript{2+} and Ba\textsuperscript{2+}. 3) Phospholipid binding to the C\textsubscript{2} domain exhibited a high affinity, saturable Ca\textsuperscript{2+} concentration requirement with half-maximal binding observed at 4–6 μM free Ca\textsuperscript{2+}. 4) The presence of negatively charged phospholipids was required for Ca\textsuperscript{2+}/phospholipid binding. 5) Ca\textsuperscript{2+}/phospholipid binding was dependent on the presence of an intact C\textsubscript{2} domain, with a small mutation in the middle of the C\textsubscript{2} domain abolishing binding. Together, these findings constitute the first direct evidence that C\textsubscript{2} domains can serve as independent, fully functional Ca\textsuperscript{2+}-binding domains.

Three characteristics of the observed Ca\textsuperscript{2+}/phospholipid binding by the first C\textsubscript{2} domain of synaptotagmin I support a role for synaptotagmin I in neurotransmitter release. First, the high affinity of synaptotagmin I for Ca\textsuperscript{2+} in the presence of phospholipids assures that its conversion between Ca\textsuperscript{2+}-bound and -free states occurs under physiological conditions, suggesting an intracellular regulatory role. Second, the cation dependence of phospholipid binding to synaptotagmin I agrees well with the cation dependence of neurotransmitter release. Neurontransmitter release can be triggered by Ba\textsuperscript{2+}, whereas Ba\textsuperscript{2+} for example does not bind to calmodulin (reviewed by Smith and Augustin, 1988). Third, Ca\textsuperscript{2+} binding to the C\textsubscript{2} domain from synaptotagmin I exhibits a positive cooperativity very similar to that observed for neurotransmitter release as a whole (Dodge and Rahaminoff, 1965; Almers and Tse, 1990), suggesting that synaptotagmin I may be responsible for this apparent cooperativity. If synaptotagmin I is directly involved in the triggering of synaptic vesicle exocytosis by Ca\textsuperscript{2+}, it may act as a negative regulator because synaptotagmin I itself is not a fusogen (Brose et al., 1992). Furthermore, other neuronal proteins containing C\textsubscript{2} domains have recently been described that may cooperate with synaptotagmin I in synaptic functions (Maruyama and Brenner, 1991; Shirataki et al., 1993).
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