Mechanism of Phospholipid Binding by the C_2A-Domain of Synaptotagmin I†

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ABSTRACT: Synaptotagmin I is a synaptic vesicle membrane protein that probably functions as a Ca^{2+} sensor in neurotransmitter release and contains two C_2-domains which bind Ca^{2+}. The first C_2-domain of synaptotagmin I (the C_2A-domain) binds phospholipids in a Ca^{2+}-dependent manner similar to that of the C_2-domains of protein kinase C, cytoplasmic phospholipase A_2, and phospholipase Cδ1. Although the tertiary structure of these C_2-domains is known, the molecular basis for their Ca^{2+}-dependent interactions with phospholipids is unclear. We have now investigated the mechanisms involved in Ca^{2+}-dependent phospholipid binding by the C_2A-domain of synaptotagmin I. Our data show that the C_2A-domain binds negatively charged liposomes in an electrostatic interaction that is determined by the charge density of the liposome surface but not by the phospholipid headgroup. At the tip of the C_2A-domain, three tightly clustered Ca^{2+}-binding sites are formed by five aspartates and one serine. Mutations in these aspartate and serine residues demonstrated that all three Ca^{2+}-binding sites are required for phospholipid binding. The Ca^{2+} binding sites at the top of the C_2A-domain are surrounded by positively charged amino acids that were shown by mutagenesis to be also involved in phospholipid binding. Our results yield a molecular picture of the interactions between a C_2-domain and phospholipids. Binding is highly electrostatic and occurs between the surfaces of the phospholipid bilayer and of the tip of the C_2A-domain. The data suggest that the negatively charged phospholipid headgroups interact with the basic side chains surrounding the Ca^{2+}-binding sites and with bound Ca^{2+} ions, thereby filling empty coordination sites and increasing the apparent affinity for Ca^{2+}. In addition, insertion of hydrophobic side chains may contribute to phospholipid binding. This model is likely to be general for other C_2-domains, with the relative contributions of electrostatic and hydrophobic interactions dictated by the exposed side chains surrounding the Ca^{2+}-binding region.

C_2-domains represent widespread protein modules that are found in more than 50 proteins, mainly proteins involved in signal transduction or membrane traffic (reviewed in refs *1*−*5*). Most C_2-domains function as Ca^{2+}-binding domains, with Ca^{2+}-dependent phospholipid binding as their most common activity. For example, protein kinase C, $cPLA_2$,[1] ubiquitin ligase Nedd4, and perforin are all activated by Ca^{2+} in a reaction that involves the Ca^{2+}-dependent binding of these proteins to membranes mediated by their C_2-domains (*6*−*12*). Synaptotagmin I is probably the best studied protein with C_2-domains that functions in membrane trafficking (reviewed in ref *5*). Synaptotagmin I contains two C_2-domains. The first C_2-domain (the C_2A-domain) binds to phospholipids as a function of Ca^{2+} in a reaction similar to that of protein kinase C, $cPLA_2$, ubiquitin ligase Nedd4, and perforin (*13*, *14*). In addition, the C_2A-domain also binds to syntaxin in the presence of Ca^{2+} (*15*, *16*). In contrast, the second C_2-domain of synaptotagmin I does not interact with phospholipids or syntaxin but instead mediates the Ca^{2+}-dependent binding of synaptotagmin I to itself (*17*, *18*). Physiologically, synaptotagmin I is essential for Ca^{2+}-dependent neurotransmitter release but not for Ca^{2+}-independent release or membrane fusion (*19*). The function of synaptotagmin I in the Ca^{2+}-evoked step of release and its Ca^{2+}-binding properties led to the proposal that it represents the Ca^{2+} sensor for synaptic vesicle exocytosis (*20*).

Studies on the Ca^{2+}-dependent phospholipid binding by the C_2-domains from synaptotagmins, protein kinase C, perforin, and $cPLA_2$ showed that the C_2-domains from the first three proteins exhibited a similar preference for negatively charged phospholipids (*8*, *12*, *14*, *15*). In contrast, the C_2-domain from $cPLA_2$ only interacted with neutral lipids (*10*). All C_2-domains exhibit a similar Ca^{2+} dependence for phospholipid binding despite differences in phospholipid specificity, with a steeply cooperative binding curve and half-maximal binding at 5−10 μM free Ca^{2+} as first observed for the C_2A-domain of synaptotagmin I (*14*). The interaction of $cPLA_2$ with neutral lipids and the binding of protein kinase C to negatively charged phospholipids were proposed to involve hydrophobic components (*10*, *21*), but the mechanisms of these binding reactions are unclear.

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[1] Abbreviations: CD, circular dichroism; $cPLA_2$, cytoplasmic phospholipase A_2; EST, expressed sequence tag; GST, glutathione *S*-transferase; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP_2, phosphatidylinositol bisphosphate; PS, phosphatidylserine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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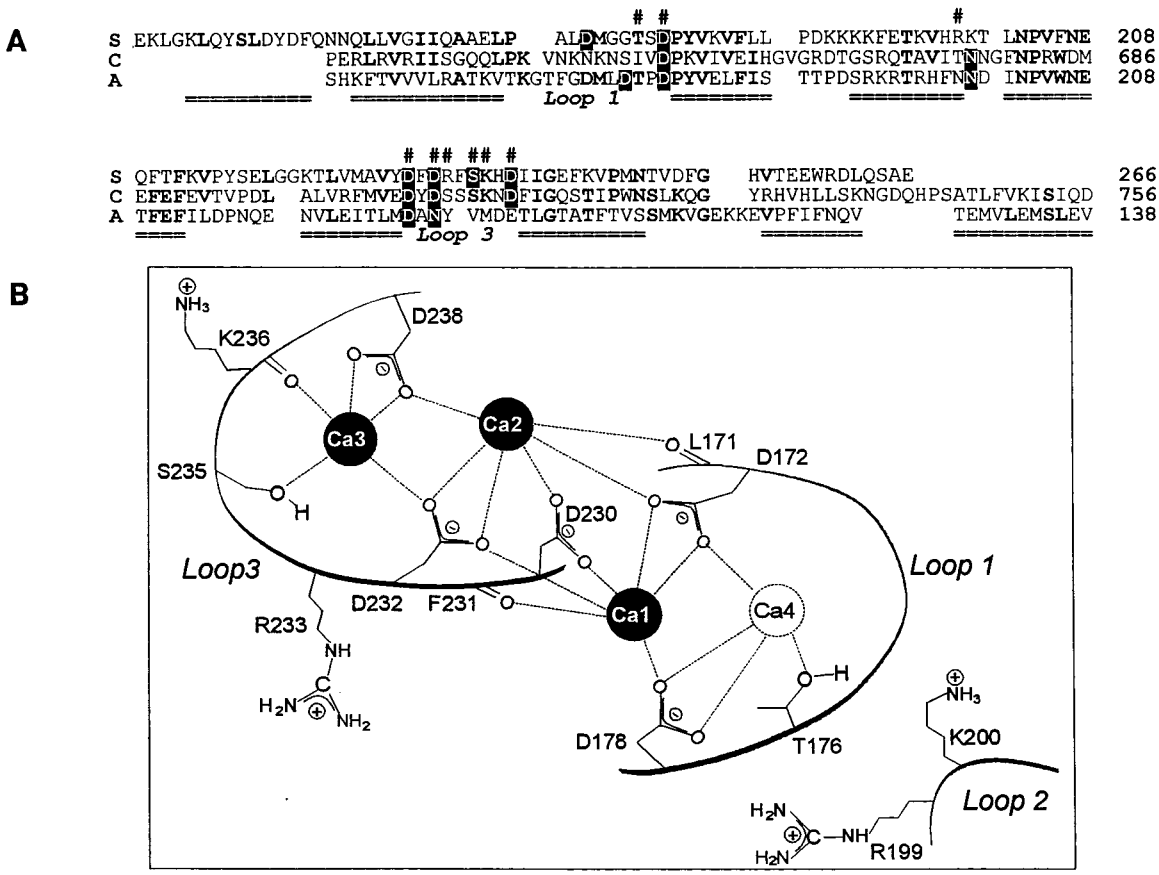

FIGURE 1: (A) Sequences of C_2-domains with known tertiary structures. The sequences of the C_2A-domain of synaptotagmin I (S) and the C_2-domains from phospholipase Cδ1 (C) and cytoplasmic phospholipase A_2 (A) are aligned with each other on the basis of their structures (*22*−*26*). Identical residues are shown in bold type. Double underlined regions correspond to β-strands. All C_2-domains have eight β-strands but exhibit two topologies; as a result, the first β-strand of the synaptotagmin C_2A-domain corresponds to the eighth β-strand in the C_2-domains from phospholipase Cδ1 and cytoplasmic phospholipase A_2. Residues whose side chains are involved in the coordination of Ca^{2+} ions in the C_2-domains (in synaptotagmin I: D^{172}, D^{178}, D^{230}, D^{232}, S^{235}, and D^{238} located in loop 1 and loop 3 at the top of the domain) are shown in white on a black background. Positions corresponding to residues that were mutated in the C_2A-domain of synaptotagmin I for the current study are identified by # signs above the alignment. Sequences are numbered on the right. (B) Diagram of the Ca^{2+}-binding sites in the C_2A-domain of synaptotagmin and location of positive charges surrounding it. The three loops at the top of the C_2A-domain are shown with the three demonstrated Ca^{2+}-binding sites (Ca1, Ca2, and Ca3) and the fourth potential Ca^{2+}-binding site (Ca4). Amino acids coordinating the Ca^{2+} ions either via side chains (D^{172}, D^{178}, D^{230}, D^{232}, S^{235}, and D^{238}) or carbonyl groups (F^{231}, L^{171}, and K^{236}) are shown, as are the locations of positively charged residues in the loops (R^{199}, K^{200}, R^{233}, and K^{236}) (modified from refs *1* and *23*).

The tertiary structures of the C_2-domains from synaptotagmin I, phospholipase Cδ1, and $cPLA_2$ were studied by X-ray crystallography and by NMR spectroscopy (*22*−*27*). Structures were generated from Ca^{2+}-free molecules and from domains complexed with Ca^{2+} or Ca^{2+} analogues. These studies have given us a detailed picture of the structures of C_2-domains and their Ca^{2+}-binding modes. In all C_2-domains, two β-sheets form a stable core β-sandwich that is extensively hydrogen bonded and almost identical among C_2-domains. The β-sandwich serves as a central scoffold from which variable loops emerge at the top and bottom. Ca^{2+} binds only to the top loops in the C_2-domains. Multiple Ca^{2+} ions bind to all C_2-domains in a cluster, with recent studies demonstrating that the C_2A-domain from synaptotagmin I binds three Ca^{2+} ions coordinated by five aspartate side chains and one serine side chain in addition to three carbonyl groups (Figure 1; refs *8* and *23*). The residues that form these Ca^{2+}-binding sites are present on two of the top three sequence loops. Although three Ca^{2+} ions bind, no major conformational changes were observed. Studies on the binding of syntaxin to the C_2A-domain have shown that binding occurs exclusively at the sequences of the C_2A-domain that surround the Ca^{2+}-binding site (*28*). Positively charged residues in the vicinity of the Ca^{2+}-binding sites are required for syntaxin binding. Surprisingly, all three Ca^{2+} ions are necessary for syntaxin binding; even disruption of the binding of a single Ca^{2+} ion abolishes binding (*23*). Together, these data led to a model whereby syntaxin binds to the C_2A-domain by an electrostatic mechanism, and Ca^{2+} triggers binding by serving as an electrostatic switch instead of inducing a Ca^{2+}-dependent conformational change that exposes a syntaxin binding site (*28*).

Although we now have a detailed understanding of Ca^{2+} and syntaxin binding to the C_2A-domain of synaptotagmin, we know little about the mechanisms involved in phospholipid binding. Since phospholipids and syntaxin both participate in membrane fusion, their binding to the C_2A-domain is likely to be physiologically relevant. Furthermore, the general phenomenon of Ca^{2+}-dependent phospholipid binding to C_2-domains is not understood, despite our detailed knowledge of their structures and Ca^{2+}-binding modes. In the current study, we have addressed this question with the C_2A-domain of synaptotagmin I. Our results support the model that the C_2A-domain functions as an electrostatic

switch also for phospholipids, thereby generalizing our previous results with syntaxin I (*28*).

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors and Purification of Recombinant Proteins. Wild type or mutant constructs of the C_2A-domain of synaptotagmin I were generated in pGEX-KG (*29*) as described (*14*, *28*), resulting in the following expression plasmids encoding residues 140−267 of rat synaptotagmin I: pGex65-4, wild type, and mutants pGEX65-4^{D178N}, pGEX65-4^{D230N}, pGEX65-4^{D232N}, pGEX65-4^{D238N}, pGEX65-4^{D238A}, pGEX65-4^{D238R}, pGEX65-4^{R199Q}, pGEX65-4^{R233Q}, pGEX65-4^{K236Q}, pGEX65-4^{R199Q,R233Q}, pGEX65-4^{R199Q,K236Q}, pGEX65-4^{F231W}, pGEX65-4^{S235A}, and pGEX65-4^{T176A}. All plasmids were verified by sequencing. Recombinant GST-fusion proteins were purified on glutathione−agarose and used for phospholipid binding measurements immobilized on glutathione−agarose without elution. Amounts of proteins used were standardized on the basis of Coomassie blue stained SDS gels.

Phospholipid Binding Measurements. Phospholipids (3.5 mg total; obtained from Avanti Polar Lipids) were dissolved in chloroform, mixed in the indicated weight ratios with a trace amount of ^{3}H-labeled PC (<0.01% of total; Amersham), and dried under a stream of nitrogen. Dried lipids were resuspended in 20 mL of 50 mM HEPES−NaOH, pH 7.4, and 0.1 M NaCl (buffer A) by vigorous shaking for 1 min. Suspensions were sonicated for 5 min in a water bath sonicator (model G112SP1G; Laboratory Supply Co. Inc.) at an intensity setting of 5 and centrifuged for 20 min at approximately 5000*g* to remove aggregates. The standard binding assay contained approximately 25 μg of recombinant protein with 1 μg of protein/μL of wet glutathione beads. Beads were prewashed and resuspended in the respective incubation buffers (0.1 mL of buffer A containing 1 mM EGTA, 17.5 μg of phospholipids with 0.025 μCi of ^{3}H-labeled PC, and either no additions or 1.10 mM Ca^{2+} as stated in the figure legends). The mixture was incubated for 10 min at room temperature with vigorous shaking, briefly centrifuged, and washed twice with 1.0 mL of the respective incubation buffers. Phospholipid binding was quantified by scintillation counting.

RESULTS

Effect of Phospholipid Composition on Ca^{2+}-Dependent Binding to the C_2A-Domain. Phosphatidylinositols have been implicated in a number of membrane trafficking reactions in eukaryotic cells (reviewed in refs *31* and *32*); thus their binding to a protein that functions in membrane traffic, such as synaptotagmin I, may be important (*33*). In addition, the phosphatidylinositols PI, PIP, and PIP_2 only differ from each other in the number of phosphates on the inositol ring, resulting in an increasing number of negative charges on the phospholipid headgroup (PI = 1, PIP = 2, and PIP_2 = 3 negative charges). To evaluate the binding of phosphatidylinositols to the C_2A-domain of synaptotagmin I and to test the effect of charge on binding, we generated ^{3}H-labeled liposomes composed of increasing amounts of PI, PIP, and PIP_2, with the balance made up by PC. The liposomes were then used in Ca^{2+}-dependent phospholipid binding experiments to the immobilized C_2A-domain of synaptotagmin I using standard techniques (Figure 2; refs 14, 15, 30).

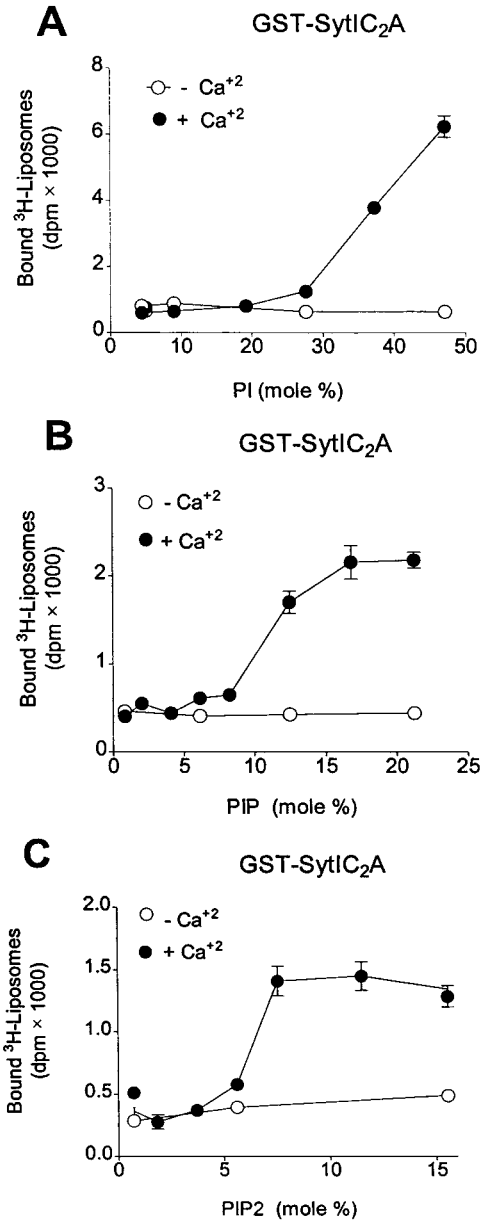

FIGURE 2: Ca^{2+}-dependent binding of liposomes composed of phosphatidylinositols and PC to the C_2A-domain of synaptotagmin I. Liposomes containing the indicated mole percent of PI (A), PIP (B), or PIP_2 (C) balanced with PC were incubated with immobilized GST-fusion proteins of the C_2A-domain of synaptotagmin I in 1.0 mM EGTA buffer without (open circles) or with addition of 1.1 mM Ca^{2+} (filled circles). Liposomes bound to the GST-fusion proteins were quantified by scintillation counting using a trace amount of ^{3}H-labeled PC incorporated into the liposomes. Error bars indicate SEMs from triplicate determinations.

In agreement with previous studies (*14*), we observed no binding to liposomes composed of only PC (Figure 2). For PI, liposomes required at least 30 mol % of PI in the liposomes in order for Ca^{2+} to trigger binding, for PIP approximately 10−15 mol % was necessary, and for PIP_2, less than 10 mol % was sufficient for Ca^{2+}-dependent binding (Figure 2). No specific binding was observed in the absence of Ca^{2+} or with GST alone (Figure 2 and data not shown). Thus there was a clear inverse correlation between the number of negative charges in the phospholipid headgroups and the concentration of the acidic phospholipid required

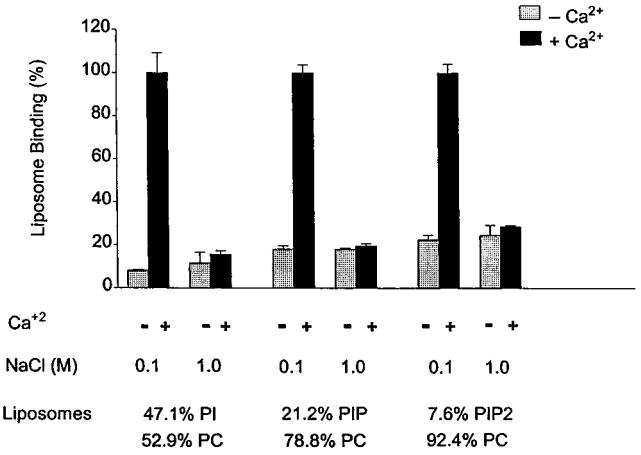

FIGURE 3: Ca^{2+}-dependent phospholipid binding is salt sensitive. Liposomes composed of PC mixed with PI, PIP, or PIP_2 in the indicated mole percentages were bound to the C_2A-domain GST-fusion protein of synaptotagmin I as described above, except that the incubations were carried out in either 0.1 or 1.0 M NaCl. Bound liposomes were quantified by scintillation counting using a trace amount of ^3H-labeled PC incorporated into the liposomes. Error bars indicate SEMs from triplicate determinations.

for Ca^{2+}-dependent binding. This result demonstrates that the C_2A-domain promiscuously binds negatively charged phospholipids in a reaction that depends on the concentration of negative charges formed by lipid headgroups on the surface of the liposomes.

Phospholipid Binding to the C_2A-Domain Depends on Electrostatic Interactions. The finding that not the structure of the phospholipid headgroups in a liposome but the density of negative charges determines Ca^{2+}-dependent binding suggests that binding is mediated by an electrostatic mechanism. To test this directly, we used liposomes composed of PC mixed with PI, PIP, and PIP_2 at concentrations at which robust Ca^{2+}-dependent binding is observed. We then measured the effect of NaCl on the Ca^{2+}-dependent binding of the C_2A-domain to these liposomes. Each type of liposome bound in 0.1 M NaCl, but no binding was observed in the presence of 1.0 M NaCl (Figure 3). These data show that binding involves primarily electrostatic mechanisms similar to the binding of syntaxin to the C_2A-domain.

Positively Charged Residues Surrounding the Ca^{2+}-Binding Site Are Important for Phospholipid Binding. The three-dimensional structure of the C_2A-domain from synaptotagmin I showed that its Ca^{2+}-binding sites are formed by clusters of aspartate residues that are located on two loops at the top of the domain (loops 1 and 3; Figure 1). The Ca^{2+}-binding sites are surrounded by a ring of positively charged residues. Binding of negatively charged liposomes to the C_2A-domain is triggered by Ca^{2+} which neutralizes the negatively charged Ca^{2+}-binding site, but binding may also involve the ring of positively charged residues. To test this, we used point mutants substituting three of these positively charged residues (R^{199}, R^{233}, and K^{236}) for glutamine. In addition, we designed double mutants in which $R^{199}Q$ is combined with either $R^{233}Q$ or $K^{236}Q$. All of these mutants were previously shown to bind Ca^{2+} with an enhanced affinity compared to the wild-type C_2A-domain (*28*).

We tested the effect of these mutations on Ca^{2+}-dependent binding of liposomes composed of PC and relatively high concentrations of PS, PI, PIP, and PIP_2 to achieve a robust response (Figure 4). Under these conditions, the binding of the single mutants was similar to that of the wild-type C_2A-domain, with only the $R^{199}Q$ mutation having a significant effect (panels A−D of Figure 4). Double mutants of $R^{199}Q$ with $R^{233}Q$, however, exhibited a more severe decrease in binding whereas the $R^{199}Q/K^{236}Q$ double mutant was similar to the $R^{199}Q$ single mutant (panels E and F of Figure 4). These data suggest that two of the positively charged residues surrounding the Ca^{2+}-binding site, R^{199} and R^{233}, may contribute to Ca^{2+}-dependent phospholipid binding but these residues are clearly not essential for binding.

Since the experiments performed in Figure 4 used saturating levels of negatively charged phospholipids, it is possible that an important role for the residues that were mutated may not be apparent. Therefore, we used the same mutations in experiments in which the Ca^{2+}-dependent binding of liposomes containing increasing amounts of PIP was compared between wild-type and mutant C_2A-domains (Figure 5). Now two single point mutants, $R^{199}Q$ and $R^{233}Q$, exhibited major defects in that liposome binding was depressed at lower PIP concentrations. This became more apparent in the double mutant of these two residues, which was greatly depressed in its ability to bind PIP-containing liposomes at concentrations at which binding to the wild-type C_2A-domain was saturated (panel B of Figure 5). The $K^{236}Q$ mutation, however, again had no measurable effect on phospholipid binding, indicating that not all positively charged residues at the top of the C_2A-domain are involved. These data demonstrate that if one or two positive charges are removed from the vicinity of the Ca^{2+}-binding site in the C_2A-domain, more negatively charged phospholipids are required for binding. Thus, although each individual positively charged residue is not required, together they form part of a positively charged binding site that provides a general binding surface for phospholipid bilayers on the C_2A-domain.

Relation of the Different Ca^{2+}-Binding Sites to Phospholipid Binding. NMR spectroscopy studies revealed that the C_2A-domain contains three Ca^{2+}-binding sites formed by residues present on two top loops (Figure 1; ref *23*). In the order of affinities, the first Ca^{2+} ion (Ca1) is coordinated by D^{172}, D^{178}, D^{230}, and D^{232}; the second Ca^{2+} ion (Ca2) by D^{172}, D^{230}, D^{232}, and D^{238}; and the third Ca^{2+} ion (Ca3) by D^{232}, S^{235}, and D^{238} (*8*, *23*). In addition, a fourth Ca^{2+}-binding site could potentially exist in a cavity formed by the side chains of D^{172}, T^{176}, and D^{178} (site Ca4 in Figure 1B) at a position similar to one of the Ca^{2+} binding sites of the PLCδ1 and $cPLA_2$ C_2-domains (*25*, *34*). While no Ca^{2+} binding was observed at this site of the C_2A-domain (*23*), binding could occur in the presence of phospholipids. To explore if occupancy of only some or all of the Ca^{2+}-binding sites are essential for Ca^{2+}-dependent phospholipid binding, we constructed mutants in the coordinating residues. We then measured the effect of these mutations on Ca^{2+}-dependent phospholipid binding using a variety of negatively charged phospholipids (Figure 6). Generally, mutations were made as aspartate to asparagine or as serine/threonine to alanine substitutions in order to prevent structure perturbations. Furthermore, one aspartate (D^{238}) was additionally mutated to alanine and arginine to exclude the possibility that asparagine could still serve as a Ca^{2+} coordinator and to probe the potential of making dominant positive mutants by introducing a positive charge into the Ca^{2+}-binding site.

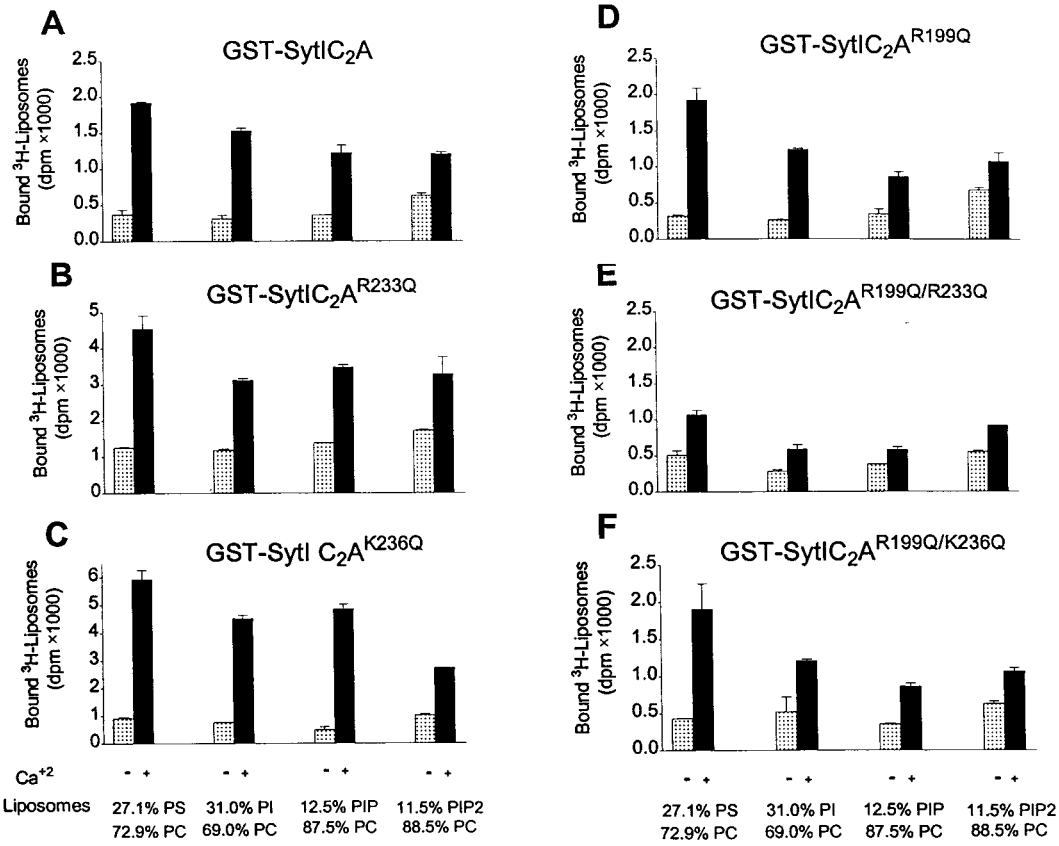

FIGURE 4: Effects of mutations in positively charged residues surrounding the Ca^{2+}-binding site on phospholipid binding. Liposomes containing relatively high concentrations of negatively charged phospholipids (see Figure 2) were tested for Ca^{2+}-dependent binding to the wild-type C_2A-domain and to C_2A-domains carrying point mutations in basic residues surrounding the Ca^{2+}-binding sites (see Figure 1B). Error bars indicate SEMs from triplicate determinations. For the positions of the mutations in the Ca^{2+}-binding loops, see Figure 1.

Of the mutations in the Ca^{2+}-binding site, Ca^{2+}-dependent phospholipid binding was completely abolished by the $D^{178}N$ and the $D^{230}N$ substitutions. This correlates well with the NMR data which show that $D^{178}N$ has the strongest effect on Ca^{2+} binding among the mutants tested. The substitution of $T^{176}A$, on the other hand, had no effect (Figure 6), indicating that if a fourth Ca^{2+} does bind to this site, it is not involved in phospholipid binding. Conversely, the $S^{235}A$ mutation severely suppressed Ca^{2+}-dependent phospholipid binding. This mutation only interferes with binding of the third Ca^{2+}, which binds with very low affinity (K_D > 1.0 mM) in the absence of phospholipids (*23*). The fact that this mutation interferes with Ca^{2+}-dependent phospholipid binding but that Ca^{2+}-dependent phospholipid binding requires only low micromolar concentrations of Ca^{2+} (*14*) provides strong support for the notion that, in the presence of phospholipids, the affinity of this Ca^{2+}-binding site must be much higher than in the absence of phospholipids.

Substituting D^{232} for asparagine also suppressed Ca^{2+}-dependent phospholipid binding, in agreement with its effect on overall Ca^{2+} binding, as did the $D^{238}N$ mutation (panels E and G of Figure 6). However, with both of these aspartate-to-asparagine substitutions, some Ca^{2+}-dependent phospholipid binding was retained. This raises the possibility that Ca^{2+} may still be coordinated by asparagine. To exclude this possibility, we also analyzed substitutions of D^{238} for alanine and arginine. There was no significant difference between the three types of substitutions ($D^{238}N$, $D^{238}A$, and $D^{238}R$) except that the background Ca^{2+}-independent binding was slightly enhanced by the alanine and arginine mutants (Figure 6). This result demonstrates that the residual Ca^{2+}-dependent binding observed with the $D^{238}N$ substitution is not due to a Ca^{2+}-coordinating activity of the asparagine residue introduced by the mutation. We observed no significant changes in the binding of liposomes with different phospholipid composition in the different mutants. This suggests that the various mutations affect phospholipid binding equally independent of the phospholipid headgroup, confirming that the binding mode of the different lipids is analogous.

DISCUSSION

A large number of C_2-domains in a variety of proteins have been described (reviewed in refs *1*−*4*). Although the various C_2-domains may perform distinct biological roles, their most frequent and best characterized function is to bind to phospholipids in a Ca^{2+}-regulated manner (see, for example, refs 6 and 9−12). The three-dimensional structures of C_2-domains from synaptotagmin I, PLCδ1, and cPLA$_2$ have been elucidated, and the mechanism of Ca^{2+} binding to these C_2-domains has been studied in detail (Figure 1; refs *8*, *22*−*27*, and *34*). All of these C_2-domains bind phospholipids as a function of Ca^{2+} and have multiple Ca^{2+}-binding sites that are localized to the tip of the domains (reviewed in ref *1*). As a result of these studies, we now have an advanced understanding of how C_2-domains bind Ca^{2+}, but little is known about how they interact with phospholipids. The C_2A-domains of synaptotagmins and the C_2-domains of PKC, perforin, and Nedd4 interact with negatively charged liposomes whereas the C_2-domain of

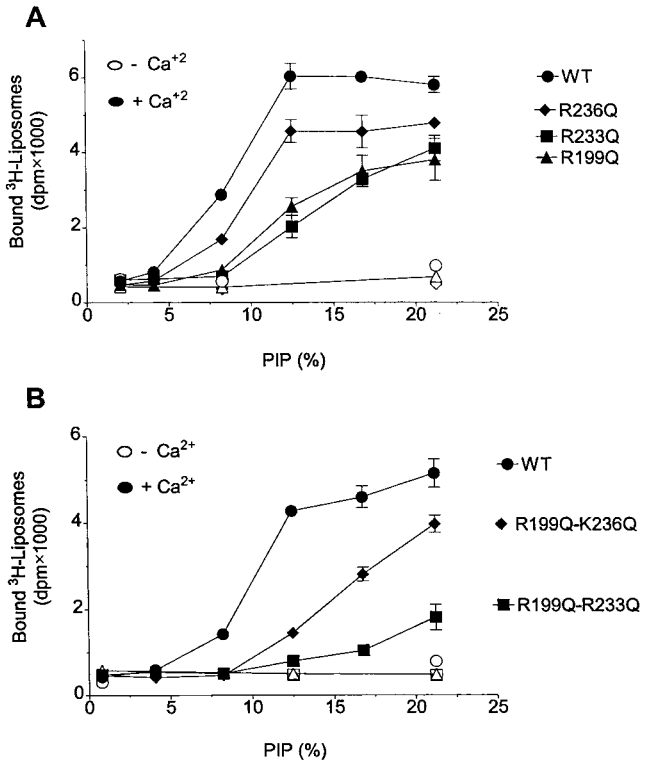

FIGURE 5: Substitutions in positively charged residues surrounding the Ca^{2+}-binding sites suppress phospholipid binding at low densities of negatively charged headgroups. Liposomes containing the indicated mole percent of PIP were used in Ca^{2+}-dependent binding assays with the wild type and mutant C_2A-domains shown. Note that, for all mutations, binding is suppressed at low PIP concentrations. Error bars indicate SEMs from triplicate determinations.

$cPLA_2$ binds neutral lipids (*8*, *10*−*12*, *14*, *15*). Recent data on PKC and on $cPLA_2$ suggested that their C_2-domains bind phospholipids via hydrophobic interactions (*10*, *21*). In contrast, Ca^{2+} binding to the C_2A-domain of synaptotagmin I was proposed to mediate primarily electrostatic and not hydrophobic binding reactions (*28*). In the current study, we have investigated how the C_2A-domain of synaptotagmin I, arguably the best studied C_2-domain, binds to phospholipids. We have focused on four questions: (1) Does the C_2A-domain bind to phospholipids primarily via hydrophobic or electrostatic interactions? (2) Is there any specificity in phospholipid binding beyond the physicochemical characteristics of the phospholipids? (3) What amino acid residues in the C_2A-domain are involved in phospholipid binding? (4) Which of the three Ca^{2+}-binding sites in the C_2A-domain are required for phospholipid binding?

(1) Our data demonstrate that the primary mode of interaction of the C_2A-domain with phospholipids is electrostatic, although hydrophobic interactions probably contribute. This is based on two observations: First, there is a direct correlation between the density of negative charges on the surface of the liposomes and the amount of Ca^{2+}-dependent binding of the C_2A-domain. Second, binding can be completely suppressed by moderate increases in ionic strength. Although we have not tested the mechanisms of binding of other C_2-domains, the similarities in phospholipid-binding properties between the C_2-domains from synaptotagmins, PKC, and perforin suggest that these will have similar mechanisms.

(2) Our results show that the C_2A-domain has no preference for a particular phospholipid. Polyphosphorylated phosphoinositides have been implicated in exo- and endocytosis, membrane trafficking reactions in which synaptotagmin I may function (*31*, *32*). Differential binding of specific phosphoinositides by the C_2A-domain would thus have been important, but our data suggest that there is little specificity, with charge being the guiding factor in binding. Furthermore, relatively high concentrations of PIP and PIP_2 were required in order to achieve binding. Although it is possible that local enrichment of phosphatidylinositols could lead to such high concentrations, this would require a clustering of phosphatidylinositols. Other protein modules, such as PTB and PH domains, have a much higher affinity for phosphatidylinositols (*35*−*38*). Thus, if phospholipid binding by the C_2A-domain is functionally important, it occurs with little specificity.

(3) Since phospholipid binding to the C_2A-domain is primarily electrostatic, we studied if positively charged residues that surround the Ca^{2+}-binding sites are involved. Mutations in two of these residues decreased Ca^{2+}-dependent phospholipid binding by requiring a higher density of negative charges on the liposome surface for binding (Figure 5). This is in contrast to the fact that the same mutants bind Ca^{2+} with a higher affinity than wild-type C_2A-domains (*28*), presumably because the local charge density in the Ca^{2+}-binding site becomes more negative. The data suggest that, analyzed separately, none of the individual positively charged residues is required for phospholipid binding, but together they form an essential component of the binding site. Furthermore, these results localize the phospholipid binding site to the top of the C_2-domain.

(4) Finally, we studied the role of the three Ca^{2+}-binding sites in phospholipid binding using mutations in residues involved in Ca^{2+} sites (Figure 1B). The data suggest that occupation of all three Ca^{2+}-binding sites is required for phospholipid interactions. The $D^{178}N$ mutation completely abolished Ca^{2+}-dependent phospholipid binding. This correlates with the NMR data which indicate that the $D^{178}N$ mutation has the largest effect on Ca^{2+} binding to the C_2A-domain, especially to site Ca1 (*23*). Phospholipid binding was also strongly decreased by the $S^{235}A$ mutation which prevents Ca^{2+} binding to site Ca3 but still allows binding to sites Ca1 and Ca2. Thus occupation of site Ca3 is required for Ca^{2+}-dependent phospholipid binding. The $D^{232}N$ and D^{238} mutations, which affect sites Ca2 and Ca3, also had substantial effects on phospholipid binding. Residual Ca^{2+} binding to sites Ca2 and/or Ca3 is observed by NMR (*23*) for the $D^{232}N$ and D^{238} mutations and agrees well with the fact that these mutants exhibit residual phospholipid binding. It is likely that phospholipids enhance the residual Ca^{2+} binding by filling empty coordination sites in the Ca^{2+} ions. Direct coordination of Ca^{2+} by phospholipids probably occurs also in the wild-type C_2A-domain since all the bound Ca^{2+} ions have empty coordination sites, and their intrinsic affinities are considerably lower than the apparent Ca^{2+} affinities observed in phospholipid binding ($\approx 5\ \mu M$). This is particularly conspicuous for the Ca3 site, which has the most empty coordination sites and an affinity that is more than 2 orders of magnitude lower in the absence of phospholipids than in the presence of phospholipids. Coordination of Ca^{2+} by phospholipids explains why the mere

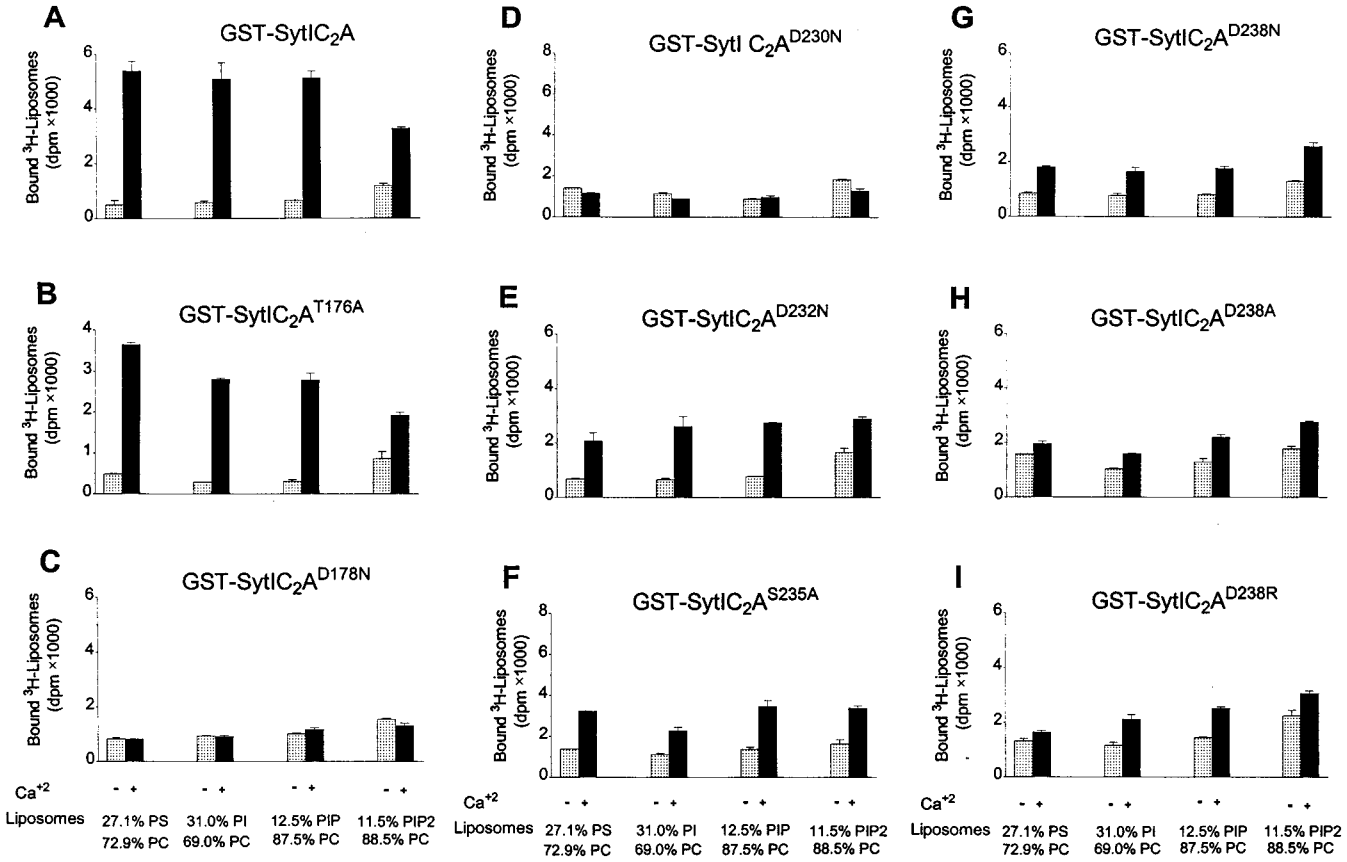

FIGURE 6: Effect of mutations in the Ca^{2+}-binding sites of the C_2A-domain from synaptotagmin I on Ca^{2+}-dependent phospholipid binding. Liposomes composed of PS, PI, PIP, or PIP_2 and PC in the compositions listed at the bottom of the figure were used in binding experiments with recombinant GST-fusion proteins of wild type and mutant forms of the C_2A-domain of synaptotagmin I. For a localization of the mutations in the Ca^{2+}-binding loops, see Figure 1.

reversal of charge involved in aspartate-to-arginine mutations (*21*) does not result in constitutive phospholipid binding. It is also interesting that, in the Ca^{2+}-binding sites of the C_2A-domain, asparagine apparently cannot substitute for aspartates since the effects of the $D^{238}N$, $D^{238}A$, and $D^{238}R$ substitutions were of similar severity.

On the basis of our data, we would like to propose a mechanism of Ca^{2+}-dependent phospholipid binding to the C_2A-domain that is illustrated by the space-filling models of the C_2A-domain and phospholipids shown in Figure 7. Since Ca^{2+} does not promote major conformational changes in the C_2A-domain (*24*), there is no reason to assume a Ca^{2+}-induced change in affinity for phospholipids in a region of the C_2A-domain distant from the Ca^{2+}-binding sites, such as the cluster of lysine residues at the bottom of the domain (K^{189}, K^{190}, K^{191}, and K^{192}). Furthermore, there is no deep pocket for phospholipid binding in the structure of the C_2A-domain. Thus binding between the phospholipids and C_2A-domain must occur at an interface formed by the bilayer surface and the area of the C_2A-domain surrounding the Ca^{2+}-binding sites, as confirmed by the effects of mutations in this region on phospholipid binding.

Ca^{2+} binding to the top of the C_2A-domain has two major consequences: the electrostatic potential changes dramatically, and the three Ca^{2+} ions bound contain empty coordination sites that can be filled by ligands, such as phospholipid phosphate groups. The fact that all three Ca^{2+} ions are essential for phospholipid binding and that the apparent Ca^{2+} affinity of the C_2A-domain in the presence of phospholipids is much higher than in the absence of phospholipids strongly supports the notion that phospholipids directly interact with the bound Ca^{2+} ions and fill their empty coordination sites. Furthermore, the Ca^{2+}-binding sites are surrounded by four basic side chains that can provide additional interaction points for the negative charges of the phospholipids. This was confirmed with the substitution experiments demonstrating that at least two of the basic residues are involved in phospholipid binding. The volumes of the phospholipid molecules indicate that a maximum of four or five negatively charged phospholipids can bind at the top of the C_2A-domain. Thus, lipids with a single negative charge such as PI need to cluster in a tight region of space within the bilayer to maximize the number of interactions with the available coordination sites in the Ca^{2+} ions and the basic side chains. For lipids with more than one negative charge such as PIP or PIP_2, a fewer number of lipid molecules have to colocalize to maximize the number of interactions, explaining why binding occurs with a lower percentage of such lipids in the vesicles. Note that the proximity between the Ca^{2+} ions and the basic side chains (Figure 7) allows the establishment of multiple interactions with a given headgroup and the flexibility of the basic side chains facilitates adaptation to different headgroup structures.

As shown in Figure 7, the two most exposed residues at the tips of the Ca^{2+}-binding loops (M173 and F234) are hydrophobic. Because of their orientation and surface exposure, these residues could easily insert into the lipid bilayer. Thus, hydrophobic interactions probably contribute

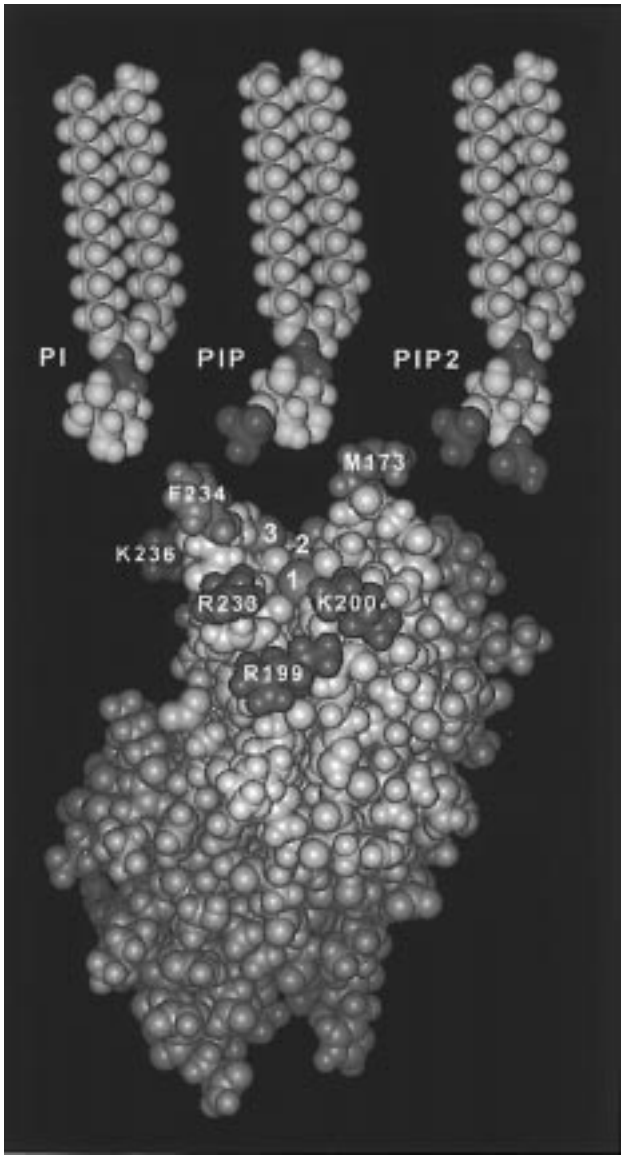

FIGURE 7: Model of phospholipid binding to the C_2A-domain of synaptotagmin I. Space-filling models of dipalmitoyl-PI, PIP, and PIP_2 are shown on top in yellow with the headgroups pointing down and the phosphate groups colored red. PI, PIP, and PIP_2 are compared with the solution structure of the Ca^{2+}-bound C_2A-domain. The C_2A-domain is shown in white with the four basic side chains surrounding the Ca^{2+}-binding sites in blue (R199, K200, R233, K236), the two hydrophobic side chains exposed at the tip of the C_2A-domain in orange (M173 and F234), and the three Ca^{2+} ions in cyan blue (labeled 1, 2, and 3). The structures illustrate the relative sizes of the phospholipid headgroups and the C_2A-domain. Note that only a few lipids can potentially bind to the tip of the C_2A-domain and that lipid headgroups with more than one phosphate moiety can easily establish multiple ionic interactions with the Ca^{2+} ions and/or basic side chains. The model also reveals that the two hydrophobic side chains (M173 and F234) could easily insert into the bilayer upon binding of the lipid headgroups to the Ca^{2+} ions and basic side chains.

to phospholipid binding. In C_2-domains with a larger number of exposed hydrophobic side chains such as the C_2-domain of $cPLA_2$, hydrophobic interactions are likely to play a more important role (*25*). This correlates with the observation that high salt concentrations can induce Ca^{2+}-independent phospholipid binding for this C_2-domain (*10*), although electrostatic interactions and partial coordination of bound Ca^{2+} ions by phospholipid headgroups are probably still responsible for the Ca^{2+}-dependent changes in affinity for phospholipids. Hydrophobic interactions are likely to play an indirect, general role in phospholipid binding to C_2-domains in the maintenance of a bilayer structure for the phospholipids, helping to orient lipid headgroups in a small region of space. The importance of a bilayer lipid structure is emphasized by the failure to observe binding of phospholipid headgroups in crystals of the $cPLA_2$ even at millimolar concentrations (*25*). Thus Ca^{2+}-dependent phospholipid binding to C_2-domains requires the cooperation of a variety of interactions, with the relative contributions of electrostatic and hydrophobic forces dictated by the surface-exposed side chains in the Ca^{2+}-binding region, and Ca^{2+} providing at the same time an electrostatic switch and coordination sites for the lipid headgroups.

In summary, our data expand the model of the C_2A-domain of synaptotagmin I as an electrostatic switch in neurotransmitter release. Our results suggest that phospholipids bind to the top of the C_2-domain after Ca^{2+} binding because Ca^{2+} binding leads to a charge switch, allowing the negatively charged phospholipids to bind to the now positively charged surface. This binding site is a nonspecific binding surface involving multiple positively charged residues in addition to the Ca^{2+} coordination sites.

NOTE ADDED IN PROOF

Direct evidence that F^{234} inserts into the lipid bilayer as predicted in the structure in Figure 7 was recently published in a mutagenesis study (*39*).

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