Role of Electrostatic and Hydrophobic Interactions in Ca^{2+}-Dependent Phospholipid Binding by the C_2A-Domain From Synaptotagmin I

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Most C_2-domains bind to phospholipid bilayers as a function of Ca^{2+}. Although phospholipid binding is central for the normal functions of C_2-domain proteins, the precise mechanism of phospholipid binding is unclear. One of the key questions is whether phospholipid binding by C_2-domains is primarily governed by electrostatic or hydrophobic interactions. We have now examined this question for the C_2A-domain of synaptotagmin I, a membrane protein of secretory vesicles with an essential function in Ca^{2+}-triggered exocytosis. Our results confirm previous data showing that Ca^{2+}-dependent phospholipid binding by the synaptotagmin C_2A-domain is exquisitely sensitive to ionic strength, suggesting an essential role for electrostatic interactions. However, we find that hydrophobic interactions mediated by exposed residues in the Ca^{2+}-binding loops of the C_2A-domain, in particular methionine 173, are also essential for tight phospholipid binding. Furthermore, we demonstrate that the apparent Ca^{2+} affinity of the C_2A-domain is determined not only by electrostatic interactions as shown previously, but also by hydrophobic interactions. Together these data indicate that phospholipid binding by the C_2A-domain, although triggered by an electrostatic Ca^{2+}-dependent switch, is stabilized by a hydrophobic mechanism. As a result, Ca^{2+}-dependent phospholipid binding proceeds by a multimodal mechanism that mirrors the amphipathic nature of the phospholipid bilayer. The complex phospholipid binding mode of synaptotagmins may be important for its role in regulated exocytosis of secretory granules and synaptic vesicles. *Diabetes* 51 (Suppl. 1):S12–S18, 2002

C_2-domains are small, autonomously folded modules that are widely distributed (reviewed in references 1–3). Close to 200 C_2-domains are encoded by the vertebrate genome. C_2-domains are primarily found in signal transduction and membrane-trafficking proteins, such as phospholipases, protein kinase C, and synaptotagmins. Especially membrane-trafficking proteins often contain tandem C_2-domains, referred to as the C_2A- and C_2B-domains. As initially demonstrated for the C_2A-domain of synaptotagmin I, a membrane-trafficking protein containing two C_2-domains that is essential for fast Ca^{2+}-triggered synaptic vesicle exocytosis (4–6), many C_2-domains bind Ca^{2+} and interact with phospholipid membranes as a function of Ca^{2+}. The three-dimensional structures of several C_2-domains have been determined (see, for example, references 7–13). These structures revealed that all C_2-domains are composed of an eight-stranded β-sandwich with flexible loops emerging at the top and the bottom.

At present, the C_2-domains of synaptotagmin I, especially its C_2A-domain, are arguably the best-studied C_2-domains. Since Ca^{2+}-binding to synaptotagmin I is an essential step in regulated exocytosis (14), understanding how the C_2-domains of synaptotagmin I bind to Ca^{2+} is important for insight into the mechanism of Ca^{2+}-triggered exocytosis. The Ca^{2+}-free and Ca^{2+}-bound structures of the C_2A-domain of synaptotagmin I have been solved at atomic resolution (7,9,15), and the structure of the C_2B-domain is also likely to be available soon. Ca^{2+}-dependent and Ca^{2+}-independent interactions of these C_2-domains with phospholipids and potential target proteins have been examined in detail (5,16–22). In the synaptotagmin I C_2A-domain, Ca^{2+} binds exclusively to the top loops of the β-sandwich (15,23). These loops coordinate three Ca^{2+} ions primarily via multidentate aspartate residues (see Fig. 1*A* for a model of the Ca^{2+}-binding site). Ca^{2+} binding causes no conformational change in the domain; for example, two hydrophobic residues (methionine 173 and phenylalanine 234) are similarly exposed at the top of the C_2A-domain next to the Ca^{2+}-binding sites in the same positions in the Ca^{2+}-bound and Ca^{2+}-free structures (Fig. 1*B*) (9,15).

The intrinsic Ca^{2+}-binding affinity of the C_2A-domain is very low (~50 μmol/l to >5 mmol/l for Ca^{2+}-binding sites one to three) (14,15), presumably because the Ca^{2+} coordination spheres of the Ca^{2+}-binding sites are incomplete. The C_2A-domain binds to phospholipid bilayers in a Ca^{2+}-dependent reaction; all negatively charged phospholipids bind independently of the precise chemical nature of the headgroups (5,24). In the presence of a negatively charged phospholipid bilayer, the apparent Ca^{2+} affinity of the C_2A-domain increases dramatically (to ~10 μmol/l for all three sites), probably because the negatively charged phospholipid headgroups provide additional coordination sites for Ca^{2+}. Studies on Ca^{2+} binding by other C_2-domains have revealed properties very similar to those of

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GST, glutathione *S*-transferase; PC, phosphatidylcholine.

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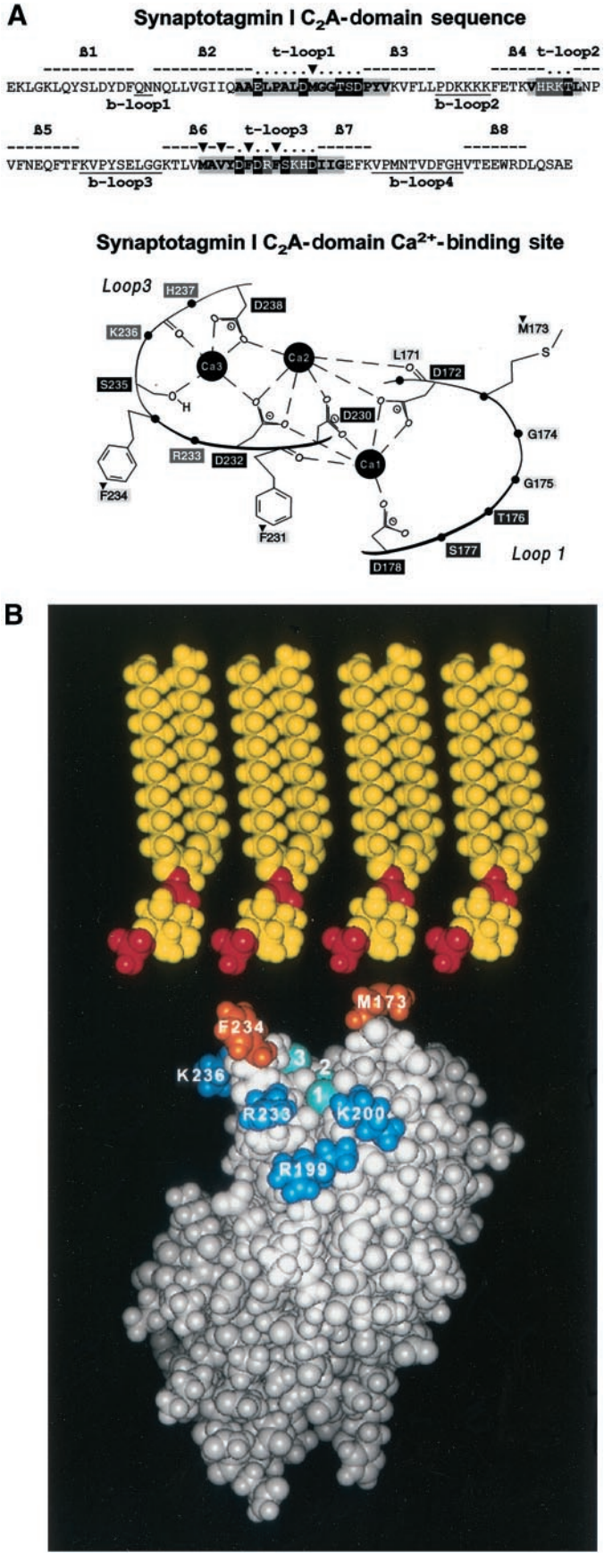

FIG. 1. *A*. **Sequence of the C_2A-domain of rat synaptotagmin I (top) and schematic structure of its Ca^{2+}-binding site (bottom). On top, the locations of the eight β-strands of the C_2A-domain β-sandwich are indicated above the sequence (β1 to β8), and the positions of the top loops (t-loop1 to t-loop3) and the bottom loops (b-loop1 to b-loop3) are identified above and below the sequence, respectively. The sequences of the top loops are shown on a colored background with the following color code: Ca^{2+}-coordinating residues, black; other negatively charged residues, blue; positively charged residues, red; hydrophilic residues, green; and hydrophobic/neutral residues, yellow. The same color scheme is applied to the drawing of the binding scheme structure at the bottom, which depicts only top loops 1 and 3 (modified from reference 15). The Ca^{2+} ions are labeled Ca1 to Ca3. In both parts of the figure, residues that were mutated in the current study are identified by triangles. B. Space-filling model of the C_2A-domain of synaptotagmin I in the Ca^{2+}-bound form shown in close proximity to phospholipids. The space-filling model of the Ca^{2+}-bound C_2A-domain depicts the three bound Ca^{2+} ions (green, labeled 1–3) surrounded by a ring of positively charged residues (R199, K200, R233, K236) and phosphatidylinositol molecules suspended above the Ca^{2+}-binding site, with the phosphate groups shown in red. The two hydrophobic residues that project from the C_2A-domain (M173 and F234) are shown in orange (modified from reference 24).**

the synaptotagmin C_2A-domain, suggesting that the various C_2-domains probably have similar Ca^{2+}-binding mechanisms (11,25–27). The only exception was observed with the C_2A-domain of piccolo/aczonin where Ca^{2+} binding causes a massive conformational change different from all other C_2-domains (28).

Based on the binding properties and structure of the synaptotagmin C_2A-domain, we proposed a model suggesting that multiple electrostatic interactions between the negatively charged phospholipid bilayer and the C_2A-domain mediate phospholipid binding (Fig. 1) (14,24). Foremost among the electrostatic interactions that promote phospholipid binding is the binding of the negatively charged phospholipid headgroups to the positively charged Ca^{2+} ions that are ligated by the top loops of the C_2A-domain, and thus serve as an electrostatic switch. In addition, a ring of positively charged residues surrounds the Ca^{2+}-binding sites of the C_2A-domain and appears to interact with the negatively charged phospholipid headgroups, thereby stabilizing the docking of the phospholipid bilayer, and lowering the apparent Ca^{2+} affinity of the C_2A-domain (14). However, the presence of hydrophobic residues that project into the solution from the tip of the C_2-domain (M173 and F234, Fig. 1) raises the possibility that hydrophobic interactions may also be involved in phospholipid binding (9,15). Understanding how precisely the C_2A-domain of synaptotagmin I and other C_2-domains bind to phospholipids is important because this activity appears to be the physiologically most important shared activity of most, if not all, C_2-domains and may be directly involved in fusion.

Several previous studies have addressed the mode of phospholipid binding by the synaptotagmin C_2A-domain (see, for example, references 5,24,29–31). Three lines of evidence revealed that, consistent with our model, electrostatic interactions are essential for phospholipid binding. First, as mentioned above, phospholipid binding is completely abolished by moderate increases in NaCl concentration (>0.3 mol/l) (24,28,29). Second, the tightness of binding and the apparent Ca^{2+} affinity of the C_2A-domain directly correlate with the density of negative charges on the bilayer surface (24). Third, substitution of one of the positively charged residues surrounding the Ca^{2+}-binding site lowers the apparent Ca^{2+} affinity of the C_2A-domain (14). However, in spite of the lack of direct evidence for a role of hydrophobic interactions in Ca^{2+}-dependent phos-

pholipid binding, fluorescence measurements have revealed that the exposed hydrophobic residues at the tip of the C_2A-domain insert into the phospholipid bilayer, suggesting that they could contribute to Ca^{2+}-dependent phospholipid binding (30). We have now used mutagenesis experiments to test the possibility that hydrophobic interactions may contribute to Ca^{2+}-dependent phospholipid binding by the synaptotagmin I C_2A-domain. Our results indicate that, although the binding reaction is driven by a switch in electrostatic surface potential induced by Ca^{2+} binding, subsequent insertion of hydrophobic residues from the C_2A-domain into the bound phospholipid bilayer is an essential component of tight binding.

RESEARCH DESIGN AND METHODS

Construction of expression vectors and protein expression. The synaptotagmin I C_2A-domain expression vector (pGEX65–4; residues 140–267) was described previously (5). Mutant synaptotagmin I C_2A-domain expression vectors were obtained using site-directed mutagenesis with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis of M173 into alanine was performed using primers A versus B and pGEX65–4 as a template (sequences: A = GCCCTGGACGCGGGGGGTACCTCCGATCCATA CG, B = CGTATGGATCGGAGGTACCCCCCGCGTCCAGGGCG). The resulting construct (pGEXSytIC2AM173A) was used for the subsequent mutagenesis of F234 into alanine using primers C versus D (sequences: C = GACT TTGATCGAGCCTCCAAGCACG, D = CGTGCTTGGAGGCTCGATCAAAGTC), resulting in pGEXSytIC2AM173A/F234A. Substitutions of M226, V228, and F231 for tryptophan were performed using primers E versus F, G versus H, and I versus J, and pGEX65–4 as a template (sequences: E = GCAAAACAC TAGTGTGGGCTGTGTATGACTTTG, F = CAAAGTCATACACAGCCCACAC TAGTGTTTTGC, G = CACTAGTGATGGCTTGGTATGACTTTGATCG, H = CGATCAAAGTCATACCAAGCCATCACTAGTG, I = GATGGCTGTGTATGAC TGGGATCGATTCTCCAAG, J = CTTGGAGAATCGATCCCAGTCATACACAG CCATC). All plasmids were verified by sequencing. Recombinant glutathione *S*-transferase (GST)-fusion proteins were purified on glutathione-agarose by standard procedures (32) and used for phospholipid-binding measurements with GST-fusion proteins immobilized on glutathione-agarose (5). Amounts, purity, and integrity of proteins used were standardized by SDS-PAGE and Coomassie blue staining.

Phospholipid binding assays. Phospholipids (1.75 mg total; Avanti Polar Lipids, Alabaster, AL) were solubilized in chloroform, mixed in the indicated weight ratios with addition of a trace of [^{3}H]-labeled phosphatidylcholine (PC) (<0.01% of total; Amersham Pharmacia Biotech, Piscataway, NJ), and dried under nitrogen. Dried lipids were resuspended in 10 ml of 50 mmol/l HEPES-NaOH, pH 7.4, 0.1 mol/l NaCl by vigorous vortexing (1 min), sonicated (5 min) in a waterbath sonicator (model G112PIG; output: 80 kc, 80 W; Laboratory Supply, Hicksville, NJ), and centrifuged (15 min) at ~5,000*g* to remove aggregates. Beads containing ~25 μg recombinant protein (1 g/l wet glutathione beads) were equilibrated in 0.1 ml of the respective binding buffers (50 mmol/l HEPES-NaOH, pH 6.8; 0.1 mol/l NaCl [if not indicated differently]; 4 mmol/l Na_2EGTA; 8.75 μg phospholipids with 0.025 μCi [^{3}H]-labeled PC). For Ca titrations, the binding buffers contained Ca/EGTA concentrations calculated using a commercial software (EqCal for Windows, Biosoft, Ferguson, MO). The mixture was incubated for 10 min at room temperature with vigorous shaking in an Eppendorf shaker, briefly centrifuged, and washed three times with 800 μl of the respective binding buffers. Phospholipid binding was quantified by scintillation counting of the beads (LS6000SC; Beckman Instruments, Fullerton, CA). All buffers were made in high-resistance MilliQ water using a 1 mol/l Ca standard solution (Fluka Chemical, Rankonkoma, NY). The half-maximal concentration (EC_{50}) and the Hill coefficient were calculated from the binding data with the GraphPad Prism program (GraphPad Software, San Diego, CA).

Miscellaneous procedures. SDS-PAGE was performed as described elsewhere (33). Protein concentrations were determined by comparison of samples run on SDS-PAGE with known amounts of bovine serum albumin standards analyzed on the same gels.

RESULTS

Two hydrophobic residues are exposed at the tip of the Ca^{2+}-free and Ca^{2+}-bound C_2A-domain, methionine 173 and phenylalanine 234 (9,15). These residues could potentially mediate hydrophobic interactions between the C_2A-domain and the phospholipid bilayer (Fig. 1*B*). To test this, we replaced these residues, individually and in combination, with alanine residues. Alanine was chosen as the substituent because this amino acid has a short hydrophobic side chain and is thus unlikely to disrupt the secondary structure of the C_2A-domain when inserted instead of methionine 173 or phenylalanine 234. The M173A single mutant and the M173A/F234A double mutant were stably expressed at high levels, consistent with efficient folding of these mutants in the bacteria. The F234A single mutant, however, was unstable, and could not be analyzed in the current experiments (data not shown). We then measured Ca^{2+}-dependent phospholipid binding by these mutants in the presence of increasing concentrations of NaCl (Fig. 2). Because increasing concentrations of NaCl disrupt electrostatic interactions but stabilize hydrophobic interactions, they can be used as a method of testing which kind of interaction predominates. In these experiments, we included a nominally "0 mol/l NaCl" condition, which contains no additional NaCl (but still has appreciable ionic strength due to the buffer components 50 mmol/l HEPES-NaOH, 4 mmol/l EGTA ± 4.5 mmol/l $CaCl_2$), to control for the possibility that the hydrophobic mutations may destabilize the binding even at physiological ionic strength.

As described previously (24,28,29), phospholipid binding to the wild-type C_2A-domain of synaptotagmin I was stable at NaCl concentrations of up to 0.3 mol/l. Phospholipid binding was completely abolished when the NaCl concentration was raised to 0.6 mol/l, indicative of an essential electrostatic interaction (Fig. 2). At nominally 0.0 mol/l NaCl, phospholipid binding to the wild type C_2A-domain was severely inhibited, presumably because of a "salt in" effect. A very different picture emerged for the M173A point mutation. Here, Ca^{2+}-dependent phospholipid binding was severely depressed at all NaCl concentrations tested (Fig. 2). The highest degree of binding was detected at nominally 0.0 mol/l NaCl, whereas at physiological ionic strength, binding was depressed by >80%. This inhibition was even stronger for the M173A/F234A double mutant, which displayed significant Ca^{2+}-dependent phospholipid binding only at nominally 0.0 mol/l NaCl. It should be noted, however, that residual Ca^{2+}-dependent binding was detected for both mutants, suggesting that the mutant C_2A-domains were still capable of Ca^{2+} binding. Together these results indicate that the hydrophobic residues projecting from the tip of the C_2A-domain are essential for tight and stable phospholipid binding.

We next examined the possibility that making the tip of the C_2A-domain more hydrophobic may increase phospholipid binding. For this purpose, three hydrophobic residues in the sixth β-strand or the third top loop (M226, V228, and F231) were individually replaced with tryptophan, the most bulky, highly hydrophobic amino acid. All three mutant proteins were produced at high levels, and were employed in phospholipid binding measurements (Fig. 3). Again, we not only measured the absolute amount of phospholipid binding, but also the effect of increasing NaCl concentrations. Substitution of M226 for tryptophan had no apparent effect on the magnitude or NaCl dependence of phospholipid binding, indicating that this residue in the middle of β6 is not directly involved in Ca^{2+}-

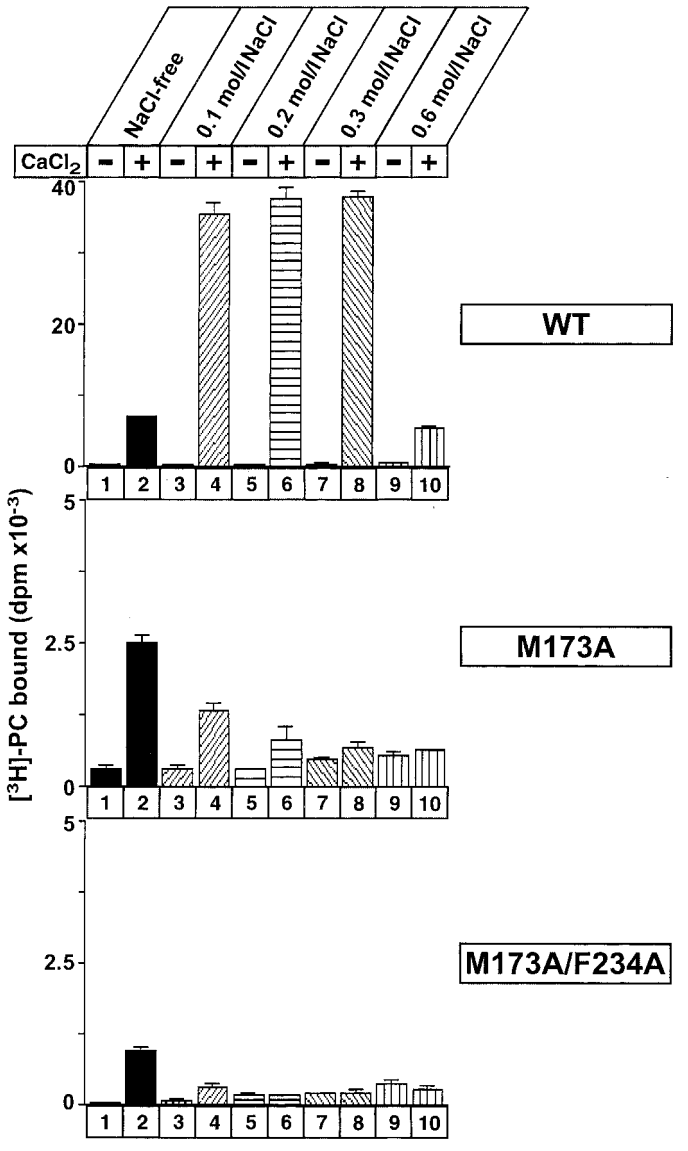

FIG. 2. Salt sensitivity of phospholipid binding to wild-type (WT) and mutant synaptotagmin C_2A-domains: effects of decreasing the hydrophobicity of the tip of the C_2A-domain. Immobilized glutathione *S*-transferase–fusion proteins of WT and mutant C_2A-domains were used to measure Ca^{2+}-dependent binding of radiolabeled liposomes in the presence of increasing NaCl concentrations. Two point mutations were examined, a single amino acid substitution replacing methionine 173 with alanine and a double substitution of both methionine 173 and phenylalanine 234 with alanine. The single amino acid substitution of phenylalanine 234 with alanine was unstable and could not be analyzed (data not shown). Phospholipid binding assays were carried out with liposomes composed of 30% phosphatidylserine/70% phosphatidylcholine (PC) in the presence and absence of 1 mmol/l Ca^{2+}. All samples contained identical amounts of recombinant protein as judged by SDS-PAGE and Coomassie staining and identical amounts of liposomes as judged by scintillation counting (not shown). Data shown are means ± SEM from a representative experiment performed in triplicate and independently repeated multiple times. Note the differences in phospholipid binding as reflected in the amount of liposomes bound.

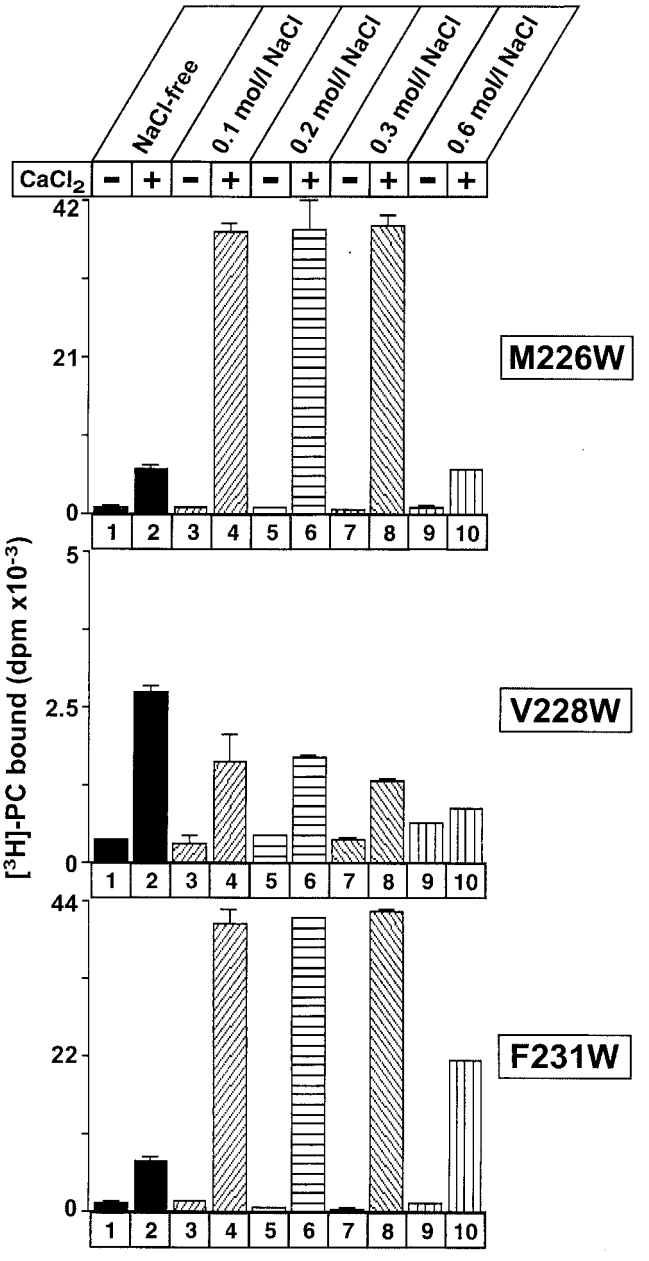

FIG. 3. Salt sensitivity of phospholipid binding to wild-type and mutant synaptotagmin C_2A-domains: effects of increasing the hydrophobicity of the tip of the C_2A-domain. Immobilized glutathione *S*-transferase–fusion proteins of mutant C_2A-domains were used to measure Ca^{2+}-dependent binding of radiolabeled liposomes in the presence of increasing NaCl concentrations as described in the legend to Fig. 2. Three point mutations in which methionine 226, valine 228, and phenylalanine 231 were individually replaced with tryptophan were analyzed. All samples contained identical amounts of recombinant protein as judged by SDS-PAGE and Coomassie staining (not shown). Data shown are means ± SEM from a representative experiment performed in triplicate and independently repeated multiple times. Note the differences in phospholipid binding as reflected in the amount of liposomes bound. PC, phosphatidylcholine.

dependent phospholipid binding (Fig. 3). The V228W mutant, however, was severely impaired in Ca^{2+}-dependent phospholipid binding. Although the hydrophobicity of this mutant domain was higher than that of the wild-type C_2A-domain, its residual phospholipid binding was more NaCl sensitive, possibly because the mutation destabilizes the domain at high NaCl concentrations. The most interesting result, however, was obtained with the F231W mutant. Here, Ca^{2+}-dependent phospholipid binding was indistinguishable from the wild-type C_2A-domain except for high NaCl concentrations. At 0.6 mol/l NaCl, phospholipid binding by the wild-type C_2A-domain was <5% of the binding obtained at physiological ionic strength (Fig. 2), whereas phospholipid binding by the F231W mutant was ~50% of that observed at physiological ionic strength (Fig.

3), suggesting that the increased hydrophobicity enhanced hydrophobic interactions. This conclusion agrees well with the exposed location of F231 on the top surface of the C_2A-domain (13,15).

To gain further insight into the changes induced into the C_2A-domain by the various mutations changing the hydrophobicity of the domain, we determined the apparent Ca^{2+} affinity of two key mutants, M173A and F231W, in the presence of phospholipids (Fig. 4). Similar to previous results (14,28), wild-type C_2A-domain exhibited an overall Ca^{2+} affinity of ~12 μmol/l Ca^{2+} (Fig. 4). Although the small amount of residual Ca^{2+}-dependent phospholipid binding was severely impaired in the M173A mutant, the residual binding was sufficient for accurate Ca^{2+} titrations, revealing a twofold lower apparent Ca^{2+} affinity. By contrast, the F231W displayed an increase in Ca^{2+} affinity. These changes were confirmed in multiple independent experiments performed to test the statistical significance of the observed changes in apparent Ca^{2+} affinity (Fig. 5). The F231W mutation (which enhances the hydrophobicity of the top loops of the C_2A-domain) increased the apparent Ca^{2+} affinity by ~25%, whereas the M173A mutation (which lowers the hydrophobicity of the top loops) decreased the apparent Ca^{2+} affinity by ~100%. Thus, hydrophobic interactions significantly contribute to the mechanism of phospholipid binding by the synaptotagmin C_2A-domain.

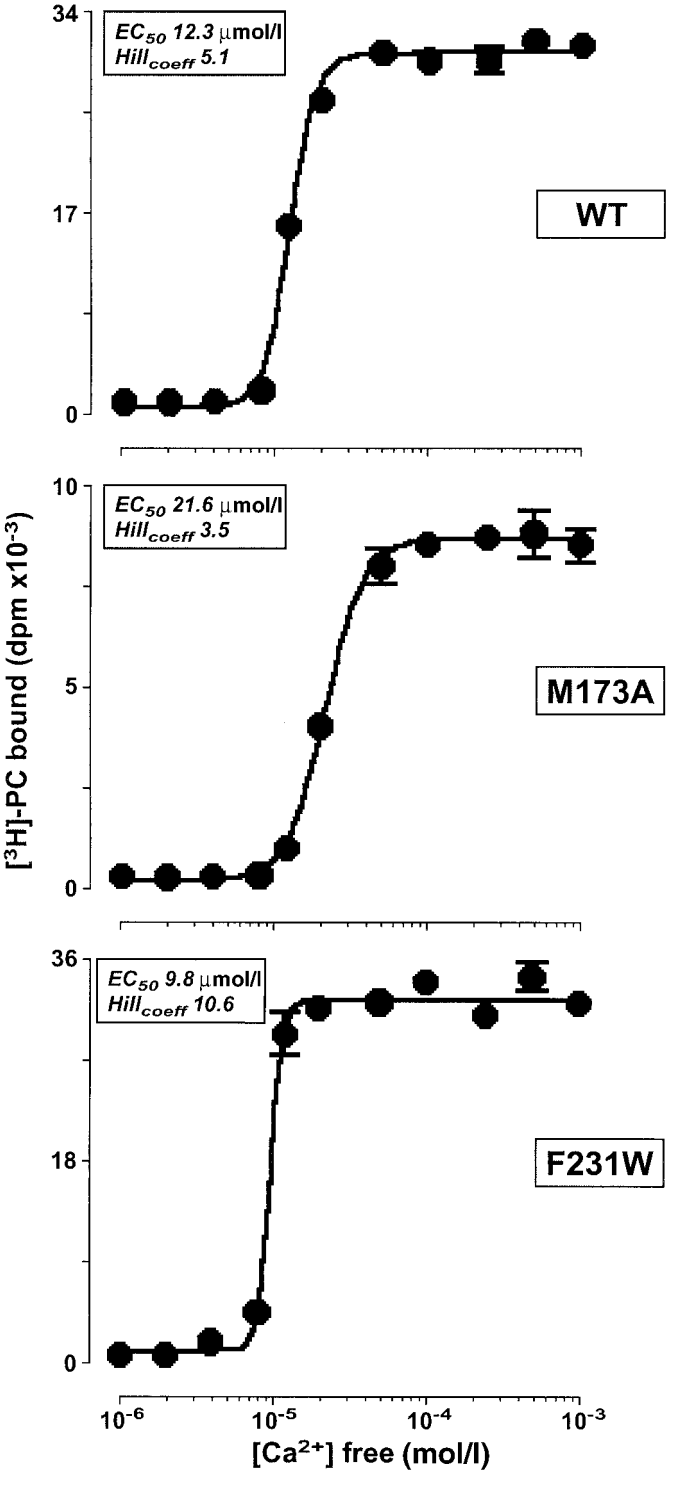

FIG. 4. Ca^{2+}-concentration dependence of phospholipid binding to the wild-type (WT) C_2A-domain of synaptotagmin I and mutant C_2A-domains carrying point mutations in methionine 173 (M173A) and phenylalanine 231 (F231): representative experiments. Binding reactions were carried out with immobilized glutathione *S*-transferase–fusion proteins in Ca^{2+}/EGTA buffers to clamp the free Ca^{2+} concentration and liposomes composed of 30% phosphatidylserine/70% phosphatidylcholine (PC). Data shown are means ± SEM from a representative experiment performed in triplicate. EC_{50}, half-maximal concentration.

DISCUSSION

Synaptotagmin I is a Ca^{2+}-binding protein that is an abundant component of synaptic vesicles in neurons and secretory granules in endocrine cells (reviewed in reference 34) and is essential for fast Ca^{2+}-dependent neurotransmitter release (6). In mice, a mutation that decreases the apparent Ca^{2+} affinity of synaptotagmin approximately twofold causes a corresponding twofold decrease in the synaptic release probability, suggesting that Ca^{2+} binding to synaptotagmin I triggers neurotransmitter release (14). The mechanism of action of synaptotagmin I in neurotransmitter release is unclear, but several lines of evidence suggest that Ca^{2+}-dependent phospholipid binding is a key component of its function. First, the Ca^{2+}-binding affinity of synaptotagmin is unphysiologically high in the absence of phospholipid membranes but closely resembles physiologically relevant concentrations in the presence of phospholipids (5,15,24). Second, the synaptotagmin mutation that decreases release probability has a selective effect on the Ca^{2+} affinity in the presence of phospholipids, but not, for example, on Ca^{2+}-dependent syntaxin binding by synaptotagmin I (14). Third, the only known physiological function of C_2-domains in other proteins, such as perforin and phospholipase A2, is to bind to phospholipids as a function of Ca^{2+} (see, for example, references 25,35–37). Together, these findings suggest that Ca^{2+}-dependent phospholipid binding by the C_2-domains of synaptotagmin is a central component of its function.

Extensive previous studies on the mechanism of Ca^{2+}-dependent phospholipid binding to the synaptotagmin I C_2A-domain have led to a model whereby the phospholipid bilayer is attached to the entire top surface of the C_2A-domain by multiple electrostatic interactions that are mediated by the bound Ca^{2+} ions and by positively charged residues surrounding the bound Ca^{2+} ions (14,24). However, this model leaves several questions unanswered, one of which is the question whether hydrophobic inter-

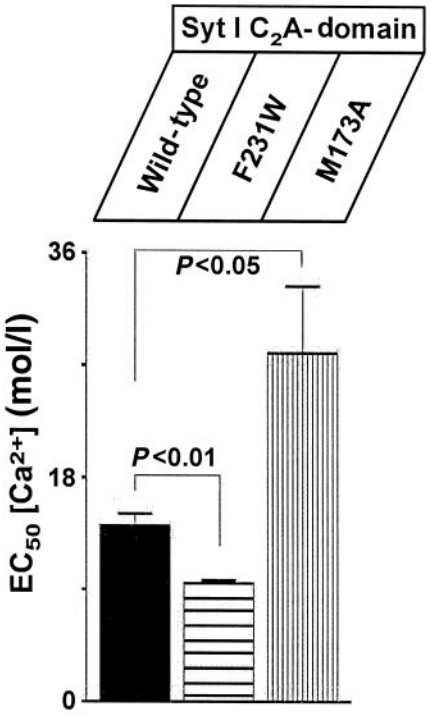

FIG. 5. Ca^{2+}-concentration dependence of phospholipid binding to the wild-type C_2A-domain of synaptotagmin I and mutant C_2A-domains carrying point mutations in methionine 173 (M173A) and phenylalanine 231 (F231): relative Ca^{2+} affinities derived from multiple experiments. Bar diagram depicts the half-maximal concentration (EC_{50}) ± SEM derived from multiple experiments ($n = 4$) as described in Fig. 4.

actions participate in phospholipid binding in addition to electrostatic interactions. We have now addressed this question by mutagenesis of key residues in the top Ca^{2+}-binding loops of the C_2A-domain. Our results reveal that changes in the hydrophobicity of the top surface of C_2A-domain have a profound influence on Ca^{2+}-dependent phospholipid binding. The observed alterations went in both directions: decreasing the apparent hydrophobicity impaired phospholipid binding, and increasing the hydrophobicity enhanced it, suggesting that phospholipid binding involves both electrostatic and hydrophobic interactions, as postulated in the initial description of phospholipid binding by synaptotagmin I (4). In evaluating these results, it is important to recall that the atomic structures of the Ca^{2+}-bound and Ca^{2+}-free forms of the C_2A-domain of synaptotagmin I revealed the same surface exposure of the hydrophobic methionine 173 and phenylalanine 234 residues at the tip of the C_2A-domain (see Fig. 1*B*) (9,15). Thus, these hydrophobic residues are not exposed on the C_2A-domain as a result of a Ca^{2+}-dependent conformational change but are always exposed on the surface of the C_2A-domain, and the Ca^{2+} trigger for phospholipid binding consists entirely of an electrostatic change, not a hydrophobic change, even though hydrophobic interactions are then required for efficient binding.

The dual mode of the Ca^{2+}-dependent interaction of the C_2A-domain with phospholipids makes maximal use of the amphipathic nature of phospholipid bilayers, which incorporate both a polyanionic surface and a hydrophobic core. As a result, the C_2A-domain interacts with phospholipid bilayers more specifically, since a simple polyanionic surface would not suffice for binding. This is biologically important in view of the role of synaptotagmin I in membrane fusion, which is, after all, a reaction involving the mixing of phospholipids from different bilayers. Furthermore, our results provide further evidence for the role of the phospholipid bilayer in shaping the Ca^{2+} affinity of synaptotagmin I. The changes in apparent Ca^{2+} affinity in the hydrophobicity mutants (Figs. 4 and 5) are best explained when one considers that these mutants change the stability with which the C_2A-domain is attached to the phospholipid bilayer. Since the phospholipid headgroups of the bilayer in turn contribute to coordinating Ca^{2+} ions, changes in the stability of phospholipid attachment obviously translate into changes in Ca^{2+} affinity. This explains why residues that are not directly involved in Ca^{2+} binding are nevertheless important in determining the apparent Ca^{2+} binding affinity.

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