The Ca\(^{2+}\) Affinity of Synaptotagmin 1 Is Markedly Increased by a Specific Interaction of Its C2B Domain with Phosphatidylinositol 4,5-Bisphosphate

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The Ca\(^{2+}\) affinity of synaptotagmin 1 is thought to convey the calcium signal onto the core secretory machinery. Its cytosolic portion mainly consists of two C2 domains, which upon calcium binding are enabled to bind to acidic lipid bilayers. Despite major advances in recent years, it is still debated how synaptotagmin controls the process of neurotransmitter release. In particular, there is disagreement with respect to its calcium binding properties and lipid preferences. To investigate how the presence of membranes influences the calcium affinity of synaptotagmin, we have now measured these properties under equilibrium conditions using isothermal titration calorimetry and fluorescence resonance energy transfer. Our data demonstrate that the acidic phospholipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\)), but not phosphatidylserine, markedly increases the calcium sensitivity of synaptotagmin. PI(4,5)P\(_2\) binding is confined to the C2B domain but is not affected significantly by mutations of a lysine-rich patch. Together, our findings lend support to the view that synaptotagmin functions by binding in a trans configuration whereby the C2A domain binds to the synaptic vesicle and the C2B binds to the PI(4,5)P\(_2\)-enriched plasma membrane.

Calcium-dependent secretion of neurotransmitter-loaded synaptic vesicles is at the heart of synaptic transmission. The underlying membrane fusion reaction between vesicle and plasma membrane has been intensively studied and found to be promoted by both protein–protein as well as protein–lipid interactions. From the multitude of proteins involved in this membrane fusion event, the Ca\(^{2+}\)-binding protein synaptotagmin 1 is one of its central regulating factors (for review, see Refs. 1 – 6). Synaptotagmin 1 is anchored in the membrane of vesicular vesicles via a single transmembrane region. Its N-terminal region comprises a short luminal domain, whereas the larger cytoplasmic C-terminal region consists of tandem C2 domains, termed C2A and C2B, tethered to each other via a short linker (7) (a schematic outline of the structural features of synaptotagmin 1 is given in Fig. 1A). Several isoforms with similar domain structure have been identified (8).

C2 domains are Ca\(^{2+}\) binding modules of ~130 amino acids, first described as the second conserved region of protein kinase C (PKC)\(^2\) (9). The C2A domain of synaptotagmin 1 was the first C2 domain structure to be determined (10). In subsequent studies other C2 domains, including the C2B domain of synaptotagmin, were shown to exhibit very similar three-dimensional structures. They have a conserved eight-stranded anti-parallel \(\beta\)-sandwich connected by surface loops. C2 modules are most commonly found in enzymes involved in lipid modifications and signal transduction (PKC, phospholipases, phosphatidylinositol 3-kinases, etc.) and proteins involved in membrane trafficking (synaptotagmins, rabphilin, DOC2, etc.) (11).

Calcium ions bind in a cup-shaped depression formed by the N- and C-terminal loops of the C2 key motifs of C2 domains. Notably, the coordination spheres for the Ca\(^{2+}\) ions are incomplete (12, 13). In canonical C2 domains, this incomplete coordination sphere can be occupied by anionic and neutral (14, 15) phospholipids, enabling the C2 domain to be attached to the membrane. Hence, it is thought that the general function of C2 domains is to mediate Ca\(^{2+}\)-triggered binding of the protein to a membrane. In fact, upon rise of the intracellular calcium level, C2 domain-containing enzymes are translocated to the membrane so that the catalytic domains can interact with lipids or membrane-anchored protein substrates (11). Yet synaptotagmin 1 does not contain such a catalytic domain, suggesting that the properties of its tandem C2 domains are the sole key to understanding its molecular function. In neurotransmission, synaptotagmin is thought to transmit the Ca\(^{2+}\) signal onto the core membrane fusion machinery, composed of the three SNARE (soluble N-ethylmaleimide sensitive factor attachment receptor) proteins syntaxin 1, SNAP-25 (Q-SNAREs, residing on the plasma membrane), and synaptobrevin 2 (also referred to as VAMP2 (vesicle-associated membrane protein)) (R-SNARE, residing on the synaptic vesicle)). So far the multifarious interplay between the SNARE machinery, the two fusing membranes, and synaptotagmin 1 is not well understood. The crystal structure of the entire cytosolic domain of synaptotagmin in the absence of Ca\(^{2+}\) has revealed an interesting domain arrangement with the two C2 domains facing in opposite directions (16), hinting at the possibility that the mol-

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2 The abbreviations used are: PKC, protein kinase C; PI(4,5)P\(_2\), phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; DPTA, diethylenetriaminepentaacetic acid; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, SNAP receptors; SNAP-25, syntaxosomosomal-associated protein of 25 kDa; ITC, isothermal titration calorimetry; FRET, fluorescence resonance energy transfer; NMR, nuclear magnetic resonance; TRPE, phosphatidylethanolamine; aa, amino acids; CHAPS, 3-[3-cholamidopropyl]dimethylammonium]-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
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A

B

C2AB (97-421)
C2A (97-273)
C2B (262-421)
C2a*B (97-421)
C2Ab* (97-421)
C2a*b* (97-421)
ncule might interact with two opposing membranes upon rise of intracellular Ca$^{2+}$.

Although the underlying processes of Ca$^{2+}$ binding and Ca$^{2+}$-dependent membrane binding of synaptotagmin 1 have been studied by a multitude of structural and biochemical investigations, they have not revealed features of synaptotagmin C2 domains that are different from those of other C2 domain-containing proteins. Calcium binding to synaptotagmin in the absence of membranes has been studied by NMR. These studies showed that the isolated C2A domain of synaptotagmin 1 binds three calcium ions with an apparent affinity of 60–75 μM, 400–500 μM, and more than 1 mM (17). The isolated C2B domain binds two calcium ions with similar calcium affinities in the range of 300–600 μM (18). The relatively low intrinsic Ca$^{2+}$ affinities of both C2 domains are difficult to reconcile with the role of synaptotagmin 1 as the Ca$^{2+}$ sensor for fast and synchronous neurotransmitter release, suggesting that interaction with phospholipids contributes to its Ca$^{2+}$ sensitivity. Indeed, Ca$^{2+}$-triggered binding of isolated C2 domains to lipid membranes was first shown in an in vitro study of synaptotagmin 1 using a fluorescence-based approach (19). Subsequent equilibrium fluorescence studies have shed more light on the molecular process underlying membrane binding of synaptotagmin 1, for example by demonstrating that the isolated C2A domain dips into the membrane bilayer upon Ca$^{2+}$ binding (20). This penetration was corroborated by electron-paramagnetic resonance (EPR) spectroscopy studies, which also showed that the penetration depth increased when both C2 domains of synaptotagmin 1 were attached to each other (21) as compared with the single domains (22, 23). However, a variety of different Ca$^{2+}$ and lipid preferences for the individual C2 domains of synaptotagmin has been reported (3, 5, 6).

To resolve these discrepancies and to shed more light on the molecular interactions of synaptotagmin 1, we have now used quantitative approaches to study the Ca$^{2+}$ concentration and the lipid composition needed for synaptotagmin to bind to membranes. We employed isothermal titration calorimetry (ITC) to measure the intrinsic calcium binding affinities of synaptotagmin 1 C2 domains both as isolated domains as well as in the context of the tandem C2AB protein. Then, we investigated whether the intrinsic calcium affinity is modulated in the presence of lipids using a newly developed fluorescence resonance energy transfer (FRET) approach. In addition, we investigated how Ca$^{2+}$ and phospholipid binding of synaptotagmin is affected when the Ca$^{2+}$ binding sites in both C2 domains and the putative phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$)-interacting site in the C2 domain are inactivated. We found that the two C2 domains bind calcium largely independently but cooperate in membrane binding. Furthermore, we confirmed that the C2B domain interacts specifically with PI(4,5)P$_2$. Remarkably, in the presence of PI(4,5)P$_2$, drastically lower amounts of calcium were needed for membrane binding.

**EXPERIMENTAL PROCEDURES**

**Protein Constructs**—All protein constructs used were from *Rattus norvegicus* and cloned into the expression vector pET28a. Expression constructs of the isolated C2A domain (aa 97–273), the C2B domain (aa 262–421), the soluble domain of synaptotagmin (aa 97–421), and of the full-length protein (aa 1–421) have been described before (24). Also the following calcium mutants of the full-length protein and of the soluble domain have been described earlier (24): C2a*B (D178A, D230A, and D232A), C2b*B (D309A, D363A, and D365A), and C2a“b* (D178A, D230A, D232A, D309A, D363A, and D365A). The constructs for the neuronal SNAREs were the SNARE motif of syntaxin 1A with its transmembrane domain (aa 183–288), a cysteine-free variant of SNAP-25A (aa 1–206), and full-length synaptobrevin 2 (aa 1–116). The synaptotagmin 1 (aa 97–421) KAKA mutant (K326A, K327A) was generated. The single cysteine variant (S342C) was obtained after first removing the single native cysteine (C278S) and then introducing a point mutation at position 342.

**Protein Purification and Labeling**—All proteins were expressed in *Escherichia coli* strain BL21 (DE3) and purified using Ni$^{2+}$-nitrilotriacetic acid beads (GE Healthcare) followed by ion exchange chromatography on the Åkta system (GE Healthcare). The protein concentrations were determined using either the Bradford assay or UV absorption. The single cysteine variant was further labeled with Alexa Fluor 488 C$_5$ maleimide. This was done by first dialyzing the proteins against the labeling buffer (50 mM HEPES, pH 7.4, 500 mM NaCl, 100 μM Tris(2-carboxyethyl)phosphine). The dialyzed protein solution was then incubated with the fluorophore for 2 h at room temperature and separated from the free dye using a Sephadex G50 superfine column. The transmembrane region containing proteins syntaxin 1A (183–288) and synaptobrevin 2 (1–116) were purified by ion-exchange chromatography in the presence of 15 mM CHAPS. The binary complex containing syntaxin 1A (183–288) and SNAP-25A was assembled from purified monomers and subsequently purified by ion-exchange chromatography in the presence of 1% CHAPS. Full-length synaptotagmin was purified in the presence of 0.03% (w/v) n-dodecyl-$\beta$-malto-side using ion exchange and size exclusion chromatography essentially as described before (24).

**Preparation of Liposomes**—All lipids were purchased from Avanti Polar Lipids except for the Texas Red-labeled phosphatidylethanolamine (TRPE), which was purchased from Invitrogen. Liposomes were prepared as previously described (24), with a few modifications. Briefly, lipid mixtures with either 0 mol % (phosphatidycholine (PC):phosphatidylethanolamine (PE):TRPE:phosphatidylserine (PS):cholesterol = 70:17:3:0:10) or 30 mol % (PC:PE:TRPE:PS:cholesterol = 40:17:3:30:10) PS stocks were first prepared. These stocks were then mixed in appropriate amounts to obtain the desired PS concentrations. In the case where PI(4,5)P$_2$ was used, 1 mol % PI(4,5)P$_2$ was

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**FIGURE 1. Structure of synaptotagmin 1.** Synaptotagmin 1 protein consists of two C2 domains, C2A and C2B, that coordinate three and two calcium ions, respectively (16). The acidic residues that coordinate calcium binding is shown schematically, with the residues mutated in the calcium binding mutants (*i.e.* C2Ab*, C2a*B, and C2a“b*) shown in red. The Lys-rich patch is represented as a ball-and-stick model colored blue with the single cysteine site for the FRET assay (S342C) colored in green (A). The different mutants and constructs used in the study are schematically depicted (B).
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added to the lipid stock solutions with the PC amount corrected accordingly. The liposomes were formed by detergent removal using the Fast Desalting PC 3.2/10 column on the SMART system (GE Healthcare). The PS concentration was calculated from the total phospholipid concentration, which was determined using the total phosphate determination method (25).

**ITC**—The protein solutions were dialyzed twice against the ITC buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 5 mM β-mercaptoethanol). The buffer was pretreated with Chelex-100 (Bio-Rad; calcium binding constant \(=4.6 \times 10^4 \text{M}^{-1}\)) to remove residual calcium ions bound with moderate affinity. This was done by first washing the Chelex-100 beads with water and then adding the beads directly to the dialysis buffer for 2 h. The Chelex-100 beads were removed by filtration through a glass filter. The buffer pH was adjusted and filtered through a 0.2-μm filter. The ITC experiment was done as previously described (26). The protein solution was loaded into the sample cell, and the calcium chloride solution was loaded in the syringe. The calcium chloride solution, in the syringe, was prepared by diluting a 1 mM stock solution with ITC buffer to the appropriate concentration. The synaptotagmin 1 protein concentration ranged from 50 to 600 μM, and the calcium chloride concentrations were between 8 and 20 mM. Calcium chloride was injected at 3-μl injections 100 times, and the heat evolved per injection was measured. To obtain the effective heat of binding, the heat of dilution, measured by injecting the calcium chloride solution into buffer, was subtracted. All ITC data were analyzed using the Microcal Origin ITC software packet.

**Assay for Synaptotagmin 1-Liposome Binding**—All measurements were carried out in a Fluorolog 3 spectrometer (Horiba Jobin Yvon) in the assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl) at 25 °C in 1-cm quartz cuvettes. Labeled synaptotagmin 1 (C342 Alexa 488) was used to a final concentration of 0.2 μM per reaction, and the spectra upon the addition of Texas Red-labeled liposomes was recorded from 500 to 700 nm with the slit widths set at 2 nm for both the excitation and emission channels. To compare the different experiments with different lipid mixes, the maximum donor fluorescence intensity \(\lambda = 520 \text{ nm}\) at different points of the experiments \((F)\) was normalized to a base-line value before the addition of liposomes \((F_0)\) to obtain the relative changes in the fluorescence intensities \((F/F_0)\). The liposome titrations were done by measuring the donor fluorescence intensity \((F)\) upon the addition of the labeled liposomes (Texas Red phosphatidylethanolamine). These intensity counts \((F)\) are then normalized similar to above to a base-line value \((F_0)\) before the first titration of liposomes \((F_0/F)\). The normalized intensity values are plotted against the PS concentration.

The calcium titration was done by mixing 0.2 μM labeled synaptotagmin with saturating amounts of liposomes (~0.4 mM) in the assay buffer containing 10 mM 1,3-diamino-2-propanol-N,N,N’,N’-tetraacetic acid (DPTA, \(K_D = 80 \mu\text{M}\)) used to buffer the free calcium. Calcium chloride stock solution was then titrated at a number of steps with the donor signal recorded at each of these steps. Using a similar normalization method as described above (in this case, \(F_0\) represents the donor intensity before calcium addition), the donor intensity was then plotted against the free calcium concentrations, which were calculated from the total calcium concentrations using the Igor Pro software.

**Liposome Fusion Assay**—Liposome preparation and fusion experiments were done as previously described (24). Briefly, liposome fusion reactions were performed at 30 °C and were followed by FRET between N-(7-nitro-2,1,3-benzoxadiazol-4-yl), the energy donor, and rhodamine, the energy acceptor. For proteoliposome containing the binary complex of syntaxin 1a and SNAP-25, lipids were mixed in the following molar ratio (in mole %): PC:PE:PS:cholesterol (60:20:10:10). To compensate for the lack of PS in synaptobrevin liposomes, a higher ratio of PC (70) was used. Fluorescence dequenching was measured \((\lambda_{ex} = 460 \text{ nm}; \lambda_{em} = 538 \text{ nm})\). For each reaction, 10 μl of labeled liposomes and 15 μl of unlabeled liposomes were mixed in 1.2 ml of buffer containing 20 mM MOPS, pH 7.4, 150 KCl, 10 mM DPTA and the appropriate amounts of calcium chloride. For experiments with precise Ca$^{2+}$ conditions, the free Ca$^{2+}$ concentrations were determined using the fluorescent dye Mag-Fura2 and a Ca$^{2+}$ calibration kit (Invitrogen).

**RESULTS**

**Calcium Binding of Synaptotagmin 1 in the Absence of Membranes**—To study the intrinsic calcium binding properties of synaptotagmin 1, we employed ITC, adapting an approach previously used for the C2 domains of classical PKCs (26, 27) and phospholipases (14, 28). The ITC approach allows for measuring the heat change associated with binding by simply titrating the ligand to the macromolecule. The heat changes are then integrated and fitted to obtain the entire set of thermodynamic parameters of the interaction. To test whether binding constants determined by ITC agree with earlier NMR studies (17, 18), we initially performed the titration on the isolated C2A (aa 97–273; see Fig. 2A) and C2B (aa 262–421; Fig. 2B) domains of synaptotagmin 1 (an overview of the constructs used for ITC measurements in given in Fig. 1B). Typically, injections of CaCl$_2$ into solution containing the individual C2 domains of synaptotagmin produced strong heat changes. With progressive injections the heat signal diminished as the Ca$^{2+}$ binding sites of synaptotagmin became gradually saturated, and eventually only background heat of dilution was observed. The integrals of the heat changes were then fitted according to the number of Ca$^{2+}$ binding sites in the individual C2 domains of synaptotagmin produced strong heat changes. With progressive injections the heat signal diminished as the Ca$^{2+}$ binding sites of synaptotagmin became gradually saturated, and eventually only background heat of dilution was observed. The integrals of the heat changes were then fitted according to the number of Ca$^{2+}$ binding sites in the individual C2 domains of synaptotagmin produced strong heat changes. With progressive injections the heat signal diminished as the Ca$^{2+}$ binding sites of synaptotagmin became gradually saturated, and eventually only background heat of dilution was observed. The integrals of the heat changes were then fitted according to the number of Ca$^{2+}$ binding sites in the individual C2 domains of synaptotagmin produced strong heat changes.
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To compare membrane binding at different conditions, quenching of donor fluorescence intensity was normalized as shown in Fig. 3A, the addition of liposomes containing TRPE to fluorescence-labeled synaptotagmin in the presence of 2 mM calcium chloride led to a strong FRET signal change, visible as a decrease in donor fluorescence intensity and an increase in acceptor fluorescence intensity, which was reversible upon chelating Ca²⁺ with EGTA, verifying that synaptotagmin is driven onto the membrane solely upon binding of Ca²⁺.

To test whether calcium binding is cooperative between the two C2 domains. Notably, because of the opposing enthalpic changes observed for the two C2 domains, the overall recorded heat changes were much smaller for the wild-type C2AB protein compared with the individual C2 domains (Fig. 2C). The binding isotherm for the C2AB was fitted using a four-site sequential model, again assuming that the two calcium binding sites of the C2B domain bound with similar affinities. According to this model, the calcium affinities were 50 μM, 140 μM, 490 μM, and 3.1 mM. These values only slightly deviate from the results obtained for the individual domains, suggesting that no major cross-talk between the calcium binding sites of the two C2 domains exists.

We also made use of previously described calcium binding mutants for either of the two domains as well as a double mutant that abolishes calcium binding in both C2 domains (24, 29). The calcium mutants are denoted as C2a*B for the C2A domain mutations, C2A*B for the C2B domain mutation, and C2a*b* for the double mutant (see “Experimental Procedures” for more details). As expected, the ITC experiment of the double calcium mutant, C2a*b*, exhibited no detectable calcium binding activity (supplemental Fig. 1C). The two Ca²⁺ binding mutants, in which only one of the two individual C2 domains was mutated, C2a*B and C2A*B, showed ITC profiles comparable with the respective single domain (supplemental Fig. 1A and B, respectively). Together, these data confirm that the introduced point mutations completely abolish Ca²⁺ binding to the mutated C2 domains of synaptotagmin and no auxiliary calcium binding sites are present in this protein.

Synaptotagmin 1 Binding to Liposomes—The calcium binding experiments enabled us to establish the intrinsic binding properties of the two C2 domains of synaptotagmin 1 for calcium in solution. The next question we had was how the intrinsic calcium binding properties are modulated when lipids are present. We attempted to carry out ITC titrations of calcium to synaptotagmin in the presence of liposomes. However, because of technical difficulties, possibly caused by aggregation, the data we obtained were not sufficiently reliable to be fitted. We, therefore, developed a robust FRET-based assay for the interaction of the soluble portion of synaptotagmin with membranes in vitro. For this assay, a variety of single cysteine variants was generated and tested for lipid binding (data not shown). Each single cysteine variant was specifically labeled with the donor fluorophore Alexa 488. Liposomes containing acceptor fluorophores were prepared by incorporating Texas Red-labeled TRPE (3 mol%). Eventually, position 342 (S342C), located in C2B domain of synaptotagmin (Fig. 1A), was chosen for further experiments due to the large and robust signal change associated with this labeling position. As shown in Fig. 3A, the addition of liposomes containing TRPE to fluorescence-labeled synaptotagmin in the presence of 2 mM calcium chloride led to a strong FRET signal change, visible as a decrease in donor fluorescence intensity and an increase in acceptor fluorescence intensity, which was reversible upon chelating Ca²⁺ with EGTA, verifying that synaptotagmin is driven onto the membrane solely upon binding of Ca²⁺.

Calcium to the wild-type C2AB fragment of synaptotagmin 1 (aa 97–421) was titrated to 594 μM C2A domain (20 mM CaCl₂) (A), 508 μM C2B domain (18 mM CaCl₂) (B), and 500 μM wild-type C2AB (20 mM CaCl₂) (C) at 25 °C in 50 mM HEPES, pH 7.4, 250 mM NaCl, 5 mM β-mercaptoethanol. The upper panels show the raw titration data, and the lower panels show the integrated heat changes after subtracting the heat of dilution. Interestingly, we observed that the two C2 domains of synaptotagmin adopt a thermodynamically divergent mechanism in calcium binding. The C2A domain bound calcium via an endothermic reaction, whereas the C2B domain exhibited an exothermic profile. The thermodynamic parameters of calcium binding are summarized in Table 1.

**TABLE 1**

| Construct | K_D (μM) | ΔH (cal mol⁻¹) | ΔS (cal K⁻¹ mol⁻¹) | ΔG (kJ mol⁻¹) |
|-----------|---------|----------------|-------------------|--------------|
| C2A       | K = 119 ± 2 | ΔH = 1841 ± 7.83 | ΔS = 24.1 | ΔG = -5.34 |
| K = 465 ± 10 | ΔH = 3309 ± 37.5 | ΔS = 26.3 | ΔG = -4.53 |
| K = 1663 ± 20 | ΔH = 4711 ± 51.0 | ΔS = 14.3 | ΔG = -3.79 |
| C2B       | K = 199 ± 4 | ΔH = 5236 ± 12.2 | ΔS = 9.27 | ΔG = -5.05 |
| C2AB      | K = 488 ± 1.3 | ΔH = 1787 ± 2.63 | ΔS = 19.1 | ΔG = -5.87 |
| K = 488 ± 10 | ΔH = 1317 ± 15.1 | ΔS = 14.7 | ΔG = -4.51 |
| K = 142 ± 2 | ΔH = 6203 ± 21.1 | ΔS = 16.2 | ΔG = -5.25 |
| K = 3122 ± 60 | ΔH = 5086 ± 30.7 | ΔS = 28.5 | ΔG = -3.41 |
| C2A*B     | K = 122 ± 2 | ΔH = 40.46 ± 17.6 | ΔS = 18.0 | ΔG = -5.32 |
| K = 427 ± 14 | ΔH = 4329 ± 47.7 | ΔS = 29.9 | ΔG = -4.58 |
| K = 3440 ± 110 | ΔH = 1176 ± 114 | ΔS = 15.2 | ΔG = -3.35 |
| C2a*b*    | K = 134 ± 3 | ΔH = -1316 ± 12.6 | ΔS = 8.45 | ΔG = -5.28 |

FIGURE 2. Calcium binding to the C2 domain of synaptotagmin 1 measured by ITC. Calcium chloride was titrated to 594 μM C2A domain (20 mM CaCl₂) (A), 508 μM C2B domain (18 mM CaCl₂) (B), and 500 μM wild-type C2AB (20 mM CaCl₂) (C) at 25 °C in 50 mM HEPES, pH 7.4, 250 mM NaCl, 5 mM β-mercaptoethanol. The upper panels show the raw titration data, and the lower panels show the integrated heat changes after subtracting the heat of dilution. Interestingly, we observed that the two C2 domains of synaptotagmin adopt a thermodynamically divergent mechanism in calcium binding. The C2A domain bound calcium via an endothermic reaction, whereas the C2B domain exhibited an exothermic profile. The thermodynamic parameters of calcium binding are summarized in Table 1.
FIGURE 3. A novel FRET assay allows the monitoring of synaptotagmin 1 binding to liposomes. Binding was studied using FRET between synaptotagmin 1 labeled with the donor dye, Alexa 488, at position 342 on the C2B domain and liposomes containing phosphatidylethanolamine labeled with Texas Red as acceptor dye. Initially the spectrum was determined for the labeled synaptotagmin (0.2 μM) in the presence of 2 mM calcium (F₀) (black (dotted line)). Upon the addition of liposomes (black (solid line)) and EGTA (gray), subsequent spectra are measured (F₁ to F₅). To compare the FRET changes for the different liposome samples, the fluorescence at 518 nm is normalized to the baseline value (F₀/F₀). This normalization was done for the different synaptotagmin mutants with liposomes containing different compositions of lipids (i.e. 0, 10, and 25% phosphatidylserine in the absence (denoted as PS) or presence (denoted as PISP) of 1% PI(4,5)P₂ (PIP₂) (B–E) (the color scheme is as in A). A.U., absorbance units. For all different liposomes tested, wild-type C2AB (B) exhibits a much stronger FRET signal than the calcium mutants C2a*B (C), C2Ab* (D), and C2a*b* (E). Note that the C2Ab* mutant appears to bind somewhat more efficiently to PI(4,5)P₂-containing membranes but only at higher PS concentrations. It seems, therefore, possible that the mutated C2B domain of the C2Ab* mutant might still be able to contribute to membrane binding by interacting to some extent with PI(4,5)P₂, hinting at a cooperative binding mechanism of calcium and PI(4,5)P₂. The C2a*b* variant, which does not bind calcium (supplemental Fig. 1), did not exhibit any detectable binding to the different liposomes.
liposomes with 25% PS regardless of whether PI(4,5)P2 was present in the liposome compositions. As expected, no detectable membrane penetration into the membrane (as reported by Herrick et al. (21) for the C2AB protein), although this is difficult to confirm in our present study. For each titration the relative fluorescence was plotted against the PS concentration. The PS concentration was calculated from the total lipid concentration, which was determined by measuring the total phosphate content of the liposome sample. Notably, the global membrane binding affinity determined for synaptotagmin is in a similar range found for classical PKC C2 domains (26, 27).

Increased Calcium Affinity of Synaptotagmin 1 in the Presence of PI(4,5)P2—To determine whether the calcium affinity of the protein is increased in the presence of PI(4,5)P2, we titrated calcium into a mix of labeled synaptotagmin and liposomes containing 25% PS in the absence or presence of 1% PI(4,5)P2. The free calcium concentration was buffered with the calcium chelator DPTA. DPTA has a much lower affinity for calcium (Kd ≈ 80 μM) than EGTA (Kd ≈ 220 μM at pH 7.40 (30)) and is, therefore, better suited for buffering the free calcium concentration in a range corresponding to the moderate Ca2+-affinities of synaptotagmin 1. The data were then fitted using the Hill equation to obtain the apparent affinity for calcium. Remarkably, for wild-type C2AB the apparent calcium affinity increased markedly when PI(4,5)P2 was present in the membrane (EC50PS = 100 μM; EC50PSP = 40 μM; Fig. 5A). As a proof
of principle, we also performed liposome titrations at lower calcium concentration (50 μM CaCl$_2$). At this calcium concentration the binding of liposomes containing only PS to synaptotagmin 1 was extremely weak when compared with the liposomes containing both PS and PI(4,5)P$_2$ (Fig. 4D). Our data corroborate the earlier report that PI(4,5)P$_2$ increases the calcium affinity of synaptotagmin (31), although no quantitative information had been provided in the earlier study. When we tested the C2a*B calcium mutant, we also found an increased Ca$^{2+}$-affinity in the presence of PI(4,5)P$_2$ (Fig. 5B), although as mentioned above, the overall membrane binding strength of this mutant is much lower than of the intact C2AB. As the C2Ab* only bound with very low affinity to membranes in the absence and presence of PI(4,5)P$_2$, we were unable to accurately estimate the calcium affinity for this variant (Fig. 5C).

A conserved lysine-rich patch in the C2B domain of synaptotagmin 1 is thought to be involved, among a variety of other processes, in binding to PI(4,5)P$_2$. This patch consists of four Lys residues in β-strand 4 (Fig. 1A). Mutation of two of the Lys residues (K326A, K327A, dubbed as KAKA mutant) has been reported to abolish the effect of PI(4,5)P$_2$ on the apparent calcium affinity of synaptotagmin (31). Employing our ITC approach, we first determined that the KAKA mutant exhibits similar Ca$^{2+}$ affinities as wild-type synaptotagmin (31). Employing our ITC approach, we first determined that the KAKA mutant exhibits similar Ca$^{2+}$ affinities as wild-type synaptotagmin, although the mutant was more prone to precipitate at higher calcium concentrations (data not shown). Hence, the two Lys residues do not contribute significantly to the intrinsic calcium binding properties of the protein. To investigate whether the two Lys residues indeed contribute to the effect of membrane binding via PI(4,5)P$_2$ interactions, we generated a KAKA mutant containing a single cysteine at position 342 for fluorescence labeling. The mutant also exhibited a strong PI(4,5)P$_2$ effect at lower PS concentrations, similar to the wild-type protein (Fig. 6A).

We then determined the calcium affinity of the KAKA mutant following the FRET approach described above. Surprisingly, in contrast to the earlier study (31), we found that the calcium affinities of the KAKA mutant were almost identical to the ones of the wild-type protein (EC$_{50}$PS = 95 μM; EC$_{50}$PS$+P_{2}$ = 50 μM; Fig. 6B); that is, the presence of PI(4,5)P$_2$ was still able to elicit a clear shift in the apparent calcium affinity of the KAKA mutant.
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suggesting that the two mutated Lys residues do not significantly contribute to the PI(4,5)P₂ binding.

Together our data demonstrate that the two C2 domains of synaptotagmin act as targeting modules that upon Ca²⁺ binding drive the protein onto a negatively charged membrane. Both domains exhibit different and mostly independent thermodynamic Ca²⁺ binding properties but cooperate during membrane binding. Remarkably, the C2B domain appears to contain a specific PI(4,5)P₂ binding site that increases its ability to bind onto membranes. The affinity for PI(4,5)P₂ is increased by Ca²⁺ binding to the C2B domain, suggesting that Ca²⁺ not only docks the acidic Ca²⁺ binding region to negatively charged phospholipids but also participates in coordinating the head group of the PI(4,5)P₂ molecule.

In the Presence of PI(4,5)P₂ Less Ca²⁺ Is Needed to Stimulate Liposome Fusion—In the final set of experiments, we tested whether the increased Ca²⁺ sensitivity of synaptotagmin 1 has an effect on the process of SNARE protein-mediated membrane fusion. For this approach, we co-reconstituted full-length synaptotagmin 1 together with synaptobrevin into one set of liposomes. This experimental setup avoids the rather unspecific effect of the soluble C2AB domain of synaptotagmin, which probably speeds up SNARE protein-mediated liposome fusion by clustering liposome membranes (for further discussion, see Ref. 24). When synaptotagmin 1 is membrane-bound, fusion is accelerated in the presence of Ca²⁺ when only the Q-SNARE liposomes, i.e. liposomes containing the co-reconstituted SNARE proteins syntaxin 1 and SNAP-25, contain the negatively charged lipid PS (24). Indeed, in agreement with our observations on the soluble C2AB domain, we found that when PI(4,5)P₂ was added to the membrane of the Q-SNARE liposomes, much less Ca²⁺ was needed to accelerate SNARE-mediated liposome fusion in the presence of full-length synaptotagmin (Fig. 7).

DISCUSSION

The synaptic vesicle protein synaptotagmin 1 is a key factor of the machinery that rapidly catalyzes Ca²⁺-dependent secretion of neurotransmitters. Synaptotagmin is thought to convey the Ca²⁺ signal onto the core membrane fusion machinery solely through its tandem C2 domains. A major goal of this work was, therefore, to study Ca²⁺ binding and Ca²⁺-mediated interaction of the two C2 domains of synaptotagmin with acidic lipid membranes by employing equilibrium methods. Here we relate these measurements to earlier observations and to structural information and also discuss broader implications of this work for the molecular role of synaptotagmin during exocytosis.

Calcium Binding to the Synaptotagmin 1 C2 Domains—Our ITC data on synaptotagmin 1 are largely in concord with earlier NMR results (17, 18). The C2A domain binds three Ca²⁺ ions with three different affinities, suggesting that the three binding sites are occupied sequentially and independently. Notably, our ITC titrations revealed a somewhat higher Ca²⁺ affinity of the two binding sites of the C2B domain compared with the NMR experiments (NMR = 500–700 μM versus ITC = 200 μM). Furthermore, we confirmed that neutralizing the Ca²⁺ binding residues in the two C2 domains abolishes Ca²⁺ binding of synaptotagmin completely. When we measured Ca²⁺ binding to the tandem C2AB region of synaptotagmin, we did not observe a major change in the global Ca²⁺ affinity, suggesting that both domains bind Ca²⁺ largely independently.

ITC provides a direct insight into the thermodynamic processes during binding. Interestingly, the synaptotagmin 1 C2 domains seem to bind calcium with markedly distinctive thermal profiles. The endothermic calcium binding of the C2A domain is coupled with a large favorable entropic change, whereas the profile of the C2B domain is dominated by exothermic enthalpies. We noted that the thermodynamic profiles of both C2 domains of synaptotagmin are different to the ones from classical PKCs, phospholipase A₂, and phospholipase D (26, 28). For classical PKCs, for example, the high affinity binding site is exothermic, and the lower affinity site binds calcium through an endothermic reaction (26). The mechanistic differences in calcium binding between the C2 domains of synaptotagmin remain elusive at the moment, but it is likely that calcium binding might involve divergent forms of solvent reorganizations or conformational changes in the two C2 domains. Previous structural investigations on both C2 domains have suggested that Ca²⁺ binding does not induce a significant conformational change but leads to an overall stabilization of the structure, in particular of the C2A domain (17, 32). Hence the large entropic change observed during Ca²⁺ binding to the C2A domain might also result from this...
Pl(4,5)P$_2$ Increases the Ca$^{2+}$ Affinity of Synaptotagmin

Described stabilization of the protein backbone. It should be noted, however, that in another study on the C2 domain of rabphilin, binding of calcium induced a conformational change on one of the calcium binding loops, leading to an enhancement of inositol 1,4,5-trisphosphate (the headgroup of Pl(4,5)P$_2$) binding (33).

Influence of the Membrane on Calcium Binding of Synaptotagmin 1—Remarkably, the Ca$^{2+}$ affinities of synaptotagmin 1 are relatively low when compared with that of classical PKC C2 domains. These enzymes are translocated to membranes at relatively low Ca$^{2+}$ concentrations, whereas synaptotagmin appears to be activated by a much higher calcium threshold (≥10 μM). As synaptotagmin is affixed to the synaptic vesicle membrane in vivo by a transmembrane domain, Ca$^{2+}$ activation does not lead to a change in its subcellular location but might influence the two C2 domains to interact in a specific spatial orientation to the membranes. It is thought that the Ca$^{2+}$ activation step occurs when the synaptic vesicle is already tethered to the plasma membrane. In this framework the C2 domains are already close to two different membranes, the synaptic vesicle membrane (“cis interaction”) and the plasma membrane (“trans interaction”). Accordingly, the C2 domains do not have to cross a larger distance upon Ca$^{2+}$ activation to interact with lipid bilayers.

The exact concentration of calcium required for neurosecretion, i.e., the concentration that activates synaptotagmin in vivo, is not easy to determine, as it is likely that fusion-competent vesicles are exposed only to a transient and local increase of calcium that enters the cell via close-by voltage-gated Ca$^{2+}$ channels. Recent Ca$^{2+}$-uncaging studies in the calyx of Held have revealed a range of 10–25 μM to be sufficient to elicit the release of physiological amounts of neurotransmitter (34), i.e., below the intrinsic Ca$^{2+}$ affinity of synaptotagmin 1. It is, thus, often assumed that the intrinsic Ca$^{2+}$ affinity of synaptotagmin 1 is increased in the presence of acidic phospholipid-containing membranes. Yet, there is so far no strong evidence for such a shift to higher Ca$^{2+}$ affinities. Furthermore, the Ca$^{2+}$ concentrations determined for binding of synaptotagmin to liposome membranes containing approximately physiological levels of PS vary widely between studies, ranging from 5 to 72 μM (19, 35–37). A reason for these differences may be that in previous studies the free calcium concentration was generally buffered with EGTA. However, due to its high Ca$^{2+}$ affinity ($K_D$ ≈ 220 nM at pH 7.40 (30)), EGTA effectively buffers free calcium concentrations only at concentrations below 1 μM.

To circumvent these problems, we used the low affinity Ca$^{2+}$ chelator DPTA ($K_D$ ≈ 80 μM), a chelator that also has been widely used in electrophysiological measurements of neuronal secretion. Using DPTA we determined the free Ca$^{2+}$ concentration needed to drive half of the synaptotagmin molecules onto liposomes containing 25% PS to be about 100 μM, close to the affinity range of the higher affinity Ca$^{2+}$ sites of synaptotagmin determined by our ITC titrations. It should be noted that the ITC measurements were carried out using a buffer with somewhat higher ionic strength (250 μM NaCl) to prevent protein precipitation compared with the liposome binding studies (150 mM NaCl). The values obtained in both cases are in a similar range, indicating that the change in the salt concentration from 150 to 250 mM is negligible, although synaptotagmin binds less tightly to membranes at higher salt concentrations (31) (supplemental Fig. 2). Consequently, our results indicate that PS-containing membranes per se do not cause a drastic increase in Ca$^{2+}$ sensitivity of synaptotagmin. Nevertheless, in contrast to the ITC titrations that confirmed several sequential binding Ca$^{2+}$ sites with a broad range of different affinities to be present on the tandem C2 domains, the membrane binding studies can only distinguish between two states, binding and non-binding, and thus cannot reveal an affinity change of individual Ca$^{2+}$ sites.

Role of the Phospholipid Pl(4,5)P$_2$ in the Activation of Synaptotagmin—The inositol phospholipid Pl(4,5)P$_2$ has long been known to have an important regulatory role in a variety of different cellular processes. Pl(4,5)P$_2$ is known to be enriched in the inner leaflet of the plasma membrane, and it has been demonstrated that Pl(4,5)P$_2$ can influence the calcium affinity of C2 domain-containing proteins (15, 26, 27). In fact, when Pl(4,5)P$_2$ was added to the lipid membrane, we found the Ca$^{2+}$ sensitivity of synaptotagmin to be markedly increased to about 40 μM. Moreover, we found that less Ca$^{2+}$ is necessary to enhance liposome fusion activity through synaptotagmin when Pl(4,5)P$_2$ is present in the Q-SNARE liposome membrane.

In agreement with previous reports (38–40), our data imply that the site of Pl(4,5)P$_2$ interaction is confined to the C2B domain of synaptotagmin. Interestingly, we observed no significant change in Pl(4,5)P$_2$ binding when two point mutations were introduced in the Lys-rich patch of the C2B domain (KAKA). This finding disagrees with a previous study (31) which reported a minor reduction in affinity of the KAKA mutant (from ~1 μM to 3 μM) using a liposome sedimentation assay. Presently, we have no explanation for this difference. On the other hand, the introduction of the KAKA mutant decreases the Ca$^{2+}$ sensitivity of transmitter release (31, 41, 42). Taken together, this might suggest a different activity at the KAKA site, e.g., SNARE binding. In fact, this has been shown in an earlier study (43) where, using a fluorescence-based phospholipid binding assay, the KAKA mutation was shown to impair to a large extent SNARE binding but not phospholipid binding. Hence, an obstruction of the interaction with SNAREs might also be a plausible explanation for the observed impairment in release properties seen in vivo.

Increases in the calcium affinity of C2 domains in the presence of Pl(4,5)P$_2$ have been reported before for rabphilin-3A (44) and classical PKC (27) C2 domains. For these C2 domains, similar to synaptotagmin, a higher Ca$^{2+}$ sensitivity was observed in the presence of Pl(4,5)P$_2$. It seems possible that Pl(4,5)P$_2$ and Ca$^{2+}$ cooperate in binding of synaptotagmin to membranes such that the head group of the bound Pl(4,5)P$_2$ molecule strengthens the Ca$^{2+}$ coordination sphere of the calcium binding site. We noticed that the Lys-rich patch is present in rabphilin and in classical PKC as well. In the case of rabphilin-3A, previous work has shown that four amino acids are involved in binding to Pl(4,5)P$_2$ (Lys-423, His-425, Lys-435, and Arg-437) (44). These residues are found on two of the β-sheets, thereby forming a positively charged patch on the C2 domains. This binding mode was confirmed by the recent crystal struc-
ture of the C2 domain of PKCa bound to PI(4,5)P$_2$ (45). The homologous residues on the C2A and C2B domains of synaptotagmin 1 are Lys-182, Phe-184, Lys-192, and Glu-194 and Lys-313, His-315, Lys-325, and Lys-327, respectively (supplemental Fig. 3). Based on these residues, the KAKA mutation in the C2B domain only hits one of the four homologous basic residues, i.e. Lys-327, found to be involved in binding in raphilin and PKCa, possibly explaining the absence of a major effect in PI(4,5)P$_2$ binding. Note that the C2A domain, which does not seem to be influenced by PI(4,5)P$_2$, contains one acidic amino acid in this patch. Although these are indeed interesting observations, more detailed experiments would be required to confirm whether this Lys-rich patch is indeed the binding site for PI(4,5)P$_2$.

**Cooperativity of Membrane Binding**—Although we observed some binding of synaptotagmin to membranes containing 1% PI(4,5)P$_2$ in the absence of PS, saturation of binding only occurred in the presence of about 25% PS. Hence, synaptotagmin can bind much more efficiently to membranes containing both PS and PI(4,5)P$_2$. In other words, each liposome containing 25% PS and 1% PI(4,5)P$_2$ is able to sequester many more Ca$^{2+}$ ions than the single isolated domains. The lipid requirement of the C2A domain appears to be less discriminating, whereas PI(4,5)P$_2$ remains a strong ligand. Indeed, recent studies have shown that the C2B domain is the first module of synaptotagmin to respond to a rise in intracellular calcium (16). The lipid requirements of the C2A domain appear to be less discriminating, allowing it to interact with the membrane of synaptic vesicle or with the plasma membrane. Also, in a previous study from our laboratory, we showed that the presence of syntaxin-SNAP-25 binary complex in the trans membrane tends to drive the synaptotagmin C2B domain to this membrane irrespective of whether calcium is present or not (24). Taken together, these observations evoke the scenario that the C2B domain, in the absence of calcium, might be first tethered on the syntaxin-SNAP-25 complex, located in the plasma membrane. Upon the influx of calcium, the C2B domain is then dislodged from the SNARE proteins and can bind directly to the PI(4,5)P$_2$-containing plasma membrane, thereby possibly contributing to membrane fusion.

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