Phosphatidylinositol 4,5-Bisphosphate Increases Ca\(^{2+}\) Affinity of Synaptotagmin-1 by 40-fold*  

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**Background:** Synaptotagmin-1, a Ca\(^{2+}\) sensor of neuronal exocytosis, interacts with the anionic phospholipid phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)).

**Results:** Microscale thermophoresis shows that PIP\(_2\) binding to the polybasic patch of synaptotagmin-1 increases the Ca\(^{2+}\) affinity by \(>40\)-fold.

**Conclusion:** PIP\(_2\) and Ca\(^{2+}\) binding to synaptotagmin-1 is strongly cooperative.

**Significance:** Understanding the interplay between Ca\(^{2+}\), synaptotagmin-1, and PIP\(_2\) is crucial for our understanding of neurotransmitter release.

Synaptotagmin-1 is the main Ca\(^{2+}\) sensor of neuronal exocytosis. It binds to both Ca\(^{2+}\) and the anionic phospholipid phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), but the precise cooperativity of this binding is still poorly understood. Here, we used microscale thermophoresis to quantify the cooperative binding of PIP\(_2\) and Ca\(^{2+}\) to synaptotagmin-1. We found that PIP\(_2\) bound to the well conserved polybasic patch of the C2B domain with an apparent dissociation constant of \(\sim 20 \mu\text{M}\). PIP\(_2\) binding reduced the apparent dissociation constant for Ca\(^{2+}\) from \(\sim 250\) to \(< 5 \mu\text{M}\). Thus, our data show that PIP\(_2\) makes synaptotagmin-1 \(>40\)-fold more sensitive to Ca\(^{2+}\). This interplay between Ca\(^{2+}\), synaptotagmin-1, and PIP\(_2\) is crucial for neurotransmitter release.

Ca\(^{2+}\) binding to synaptotagmin-1, originally demonstrated by equilibrium dialysis using native protein (2), has been characterized by isothermal titration calorimetry (3) and NMR (4–6) using a soluble fragment containing both C2 domains (C2AB fragment, residues 97–421). The C2A domain binds to three Ca\(^{2+}\) ions with affinities ranging from 50 \(\mu\text{M}\) to 10 \(\text{mm}\). The C2B domain binds two Ca\(^{2+}\) ions, both with \(\sim 200 \mu\text{M}\) affinity.

In the presence of Ca\(^{2+}\), the C2 domains of synaptotagmin-1 also bind to membranes containing anionic phospholipids, with little specificity for the phospholipid species (3, 6–14). Interestingly, binding already occurs at Ca\(^{2+}\) concentrations well below the Ca\(^{2+}\) affinity of free synaptotagmin-1. Here, anionic phospholipid headgroups complement the Ca\(^{2+}\)-binding sites, increasing the affinity of C2AB for Ca\(^{2+}\) to \(\sim 5–100 \mu\text{M}\) (3, 6–8, 11, 13). In the absence of Ca\(^{2+}\), a conserved polybasic lysine patch located on the C2B domain can also bind to anionic lipids, and this binding is strongly preferential for the polyanionic phospholipid phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) (3, 9–14). Binding of PIP\(_2\) to the polybasic patch might increase the Ca\(^{2+}\) affinity (12), although this is still controversial (3) and has hitherto not been characterized in detail.

Experimentally, measuring synaptotagmin-1 binding to PIP\(_2\) and/or Ca\(^{2+}\) is not trivial. Isothermal titration calorimetry and NMR require high (100 \(\mu\text{M}\) to 1 \(\text{mm}\)) concentrations of protein (3–5). Therefore, high affinities well below these concentrations cannot be accurately determined with these approaches. Binding of synaptotagmin to PIP\(_2\) is often inferred from binding of the C2 domains to artificial membranes containing a defined fraction of PIP\(_2\) (e.g. by FRET (3), pulldown assays (11, 13), or density flotations (3, 12)). However, it is difficult to quantitatively distinguish Ca\(^{2+}\) from PIP\(_2\) binding with these approaches. We have recently shown (10) that Ca\(^{2+}\) binding to synaptotagmin-1 can be directly measured with a new technique called microscale thermophoresis (MST) (15, 16). MST is based on the principle that molecules move along a tempera-

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4 The abbreviations used are: PIP\(_{2}\), phosphatidylinositol 4,5-bisphosphate; MST, microscale thermophoresis.
ture gradient in a capillary (the Soret effect). Upon binding to Ca\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{2} or PIP\textsubscript{2}, the surface properties of synaptotagmin-1 change, resulting in an altered thermophoretic behavior. In this study, we applied MST to study PIP\textsubscript{2} and Ca\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{2} cooperative binding to synaptotagmin-1.

**EXPERIMENTAL PROCEDURES**

The C2AB fragment of synaptotagmin-1 (rat sequence, residues 97–421) was expressed in *Escherichia coli* and purified as described (3, 10). The single cysteine mutant (C278S/S342C) was labeled with Alexa Fluor 488-maleimide (Invitrogen) as described (3, 10). Liposomes were prepared by extrusion of rehydrated lipid films through 100-nm pores (polycarbonate membranes, Avestin) (17). All lipids were from Avanti Polar Lipids. MST was measured with 50 nM fluorescently labeled C2AB in 20 mM HEPES, 150 mM KCl, and 2.5 mg/ml BSA at pH 7.4. The samples were added to hydrophobic capillaries (NanoTemper Technologies), and MST was measured with a NanoTemper Monolith NT.015 system (25% light-emitting diode, 40% IR laser power). The label-free (tryptophan) experiments were performed with 1 M wild-type C2AB, no BSA, and the NanoTemper Monolith NT.LabelFree instrument (80% UV light-emitting diode, 40% IR laser power). The MST curves were fitted with simple Michaelis-Menten kinetics to obtain the apparent dissociation constant for Ca\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{2} binding (see “Experimental Procedures”). This model does not take into account binding of multiple Ca\textsuperscript{2+} ions (or PIP\textsubscript{2} molecules; see below), and for some

**RESULTS**

We performed MST measurements on the Alexa Fluor 488-labeled C2AB fragment of synaptotagmin-1 (residues 97–421). With this technique, a glass capillary is filled with a dilute protein solution (50 nM). Fluorescence is then measured at a spot in the capillary that is heated with a focused IR laser beam. Heating by 5 °C results in the generation of a temperature gradient along the axis of the capillary (Fig. 1, A and B). The C2AB fragment thermodiffuses away from the heated spot, causing a local depletion and a drop in fluorescence. Ca\textsuperscript{2+} binding changes the thermophoretic properties of C2AB, resulting in a decreased thermodiffusion. B, MST time traces of 16 different Ca\textsuperscript{2+} concentrations (ranging from 0 to 5 mM). Note that thermodiffusion is reduced at high Ca\textsuperscript{2+} concentrations. C, dependence of the MST signal on the Ca\textsuperscript{2+} concentration (measured 30 s after turning on heating; data from B). The solid line is a fit with Michaelis-Menten kinetics, yielding an apparent dissociation constant of $K_{Ca} = 221 \mu M$. No change in the MST signal was observed in the presence of Mg\textsuperscript{2+} or when a mutant impaired in Ca\textsuperscript{2+} binding was used (D178A/D230A/D232A/D309A/D363A/D365A, called C2a*b* (a*b*)). D, same as C but using unlabeled C2AB. MST was measured using intrinsic tryptophan fluorescence and fitted, yielding $K_{Ca} = 206 \mu M$. Error bars indicate the range of data points obtained from at least two measurements.

We fitted the binding curves with simple Michaelis-Menten kinetics assuming a single binding site (see “Experimental Procedures”). This model does not take into account binding of multiple Ca\textsuperscript{2+} ions (or PIP\textsubscript{2} molecules; see below), and for some
Control experiments with Mg²⁺ called C2aⁿ⁺ (3, 10) showed that the change in the MST signal concentration was 2.5 mM, but the fraction of liposomes containing 5 mol % PIP2 varied. In the absence of Ca²⁺, the affinity increased by ~5-fold to Kₐ = 46.0 ± 5.9 µM (red). B, lipid binding as a function of the fraction of PIP₂-containing liposomes. In all cases, the total lipid concentration was 2.5 mM, but the fraction of liposomes containing 3 mol % PIP₂ varied. In the absence of Ca²⁺, C2AB bound to the PIP₂ membranes with K₋PIP₂ = 36.2 ± 7.4% (or 45.3 µM PIP₂; cyan). In the presence of 50 µM Ca²⁺, the affinity increased by 4-fold to K₋PIP₂ = 10.6 ± 2.3% (or 13.3 µM PIP₂; green). C, binding of C2AB to liposomes composed of a 5:2:1:1 molar ratio of brain isolated phosphatidylycholine, phosphatidylethanolamine, phosphatidylserine, and cholesterol. C2AB did not bind to liposomes lacking PIP₂ regardless of the presence (green) or absence (blue) of 50 µM Ca²⁺. In contrast, C2AB bound to liposomes containing 1 mol % PIP₂ already in the absence of Ca²⁺ (red). Similar to B, 50 µM Ca²⁺ increased the binding affinity (K₋PIP₂ = 50.9 ± 20.0 µM total lipid concentration; black). D, Ca²⁺ binding curve of C2AB in the presence (Kₑ = 17.7 ± 0.7 µM; pink) or absence (Kₑ = 265 ± 37.4 µM; blue) of 20 µM PIP₂ in solution. 1 mM Mg²⁺ was present to suppress potentially unspecific Ca²⁺-PIP₂ interactions. Error bars indicate the range of data points obtained from at least two measurements.

curves, this simplification may affect the quality of the fit. However, the overall quality of the data did not warrant fitting with a more sophisticated binding model. Thus, we could not differentiate between the different calcium-binding sites, and we report only the apparent dissociation constant (KᵥCa). C2AB bound to Ca²⁺ with KᵥCa = 221 ± 23 µM (n = 3). Control experiments with Mg²⁺ or a mutant with disrupted Ca²⁺ binding (D178A/D230A/D232A/D309A/D363A/D365A, called C2aⁿ⁺) (3, 10) showed that the change in the MST signal was indeed due to binding of Ca²⁺ ions to the established binding sites in the C2 domains. Furthermore, the MST measurements were not affected by the presence of the dye because a similar binding constant of KᵥCa = 206 ± 40 µM was obtained with the unlabelled C2AB fragment using the intrinsic tryptophan fluorescence as the readout (C2AB has three tryptophans) (Fig. 1D). We then set out to study the cooperativity of Ca²⁺ and PIP₂ binding.

No apparent change in the Ca²⁺-dependent thermophoretic behavior of C2AB was observed in the presence of liposomes composed of pure 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (2.5 mM total lipid concentration; KᵥCa = 226.7 ± 50.7 µM) (Fig. 2A). In contrast, the apparent affinity for Ca²⁺ increased by ~5-fold when 10% of these liposomes were replaced with a liposome population composed of 95% 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine and 5% PIP₂ (KᵥCa = 46.0 ± 5.9 µM). Accordingly, the addition of 50 µM Ca²⁺ (well below the KᵥCa of C2AB) resulted in ~4-fold stronger binding to PIP₂-containing liposomes (from K₋PIP₂ = 45.3 ± 9.25 µM to 13.3 ± 2.9 µM total PIP₂ concentration) (Fig. 2B). 50 µM Ca²⁺ also increased C2AB binding to liposomes containing a more physiological lipid composition (phosphatidylincholine/phosphatidylethanolamine/phosphatidylserine/cholesterol at a molar ratio of 5:2:1:1) but only if 1 mol % PIP₂ was present (Fig. 2C). Thus, synaptotagmin-1 binds to anionic membranes and Ca²⁺ in a cooperative manner. As reported previously (3, 6–13), we performed a set of experiments with water-solubilized PIP₂ to further characterize this cooperativity.

One of the main advantages of MST compared with alternative techniques for measuring Ca²⁺ binding is the low concentration of protein that is required: measurements could be carried out with C2AB concentrations as low as 50 nM, which is 3–4 orders of magnitude below that reported for isothermal titration calorimetry (3) or NMR (4–6). This low concentration allowed us to measure PIP₂ binding by adding PIP₂ directly to the capillary (Fig. 2D). Even PIP₂ isolated from porcine brain with long fatty acid acyl chains (dominant species C18:0 and C20:4) is water-soluble at concentrations up to ~9 mM and does poorly form micelles because of its high anionic charge (18).
Strikingly, the affinity for Ca\textsuperscript{2+} binding increased by 15-fold in the presence of 20 \(\mu\text{M} \) PIP\textsubscript{2} (from \(K_{\text{Ca}^2+} = 265.2 \pm 27.4 \text{ M} \) to 17.7 \(\pm 0.7 \text{ M} \)) (Fig. 2D). In this experiment, an excess of 1 \(\text{mM} \) Mg\textsuperscript{2+} was present to suppress potential nonspecific interactions of Ca\textsuperscript{2+} with PIP\textsubscript{2} or C2AB. At higher PIP\textsubscript{2} concentrations, the Ca\textsuperscript{2+} affinity increased even further (to \(>40\)-fold; \(K_{\text{Ca}^2+} = 3.3 \pm 1.3 \text{ M} \) at 40 – 80 \(\mu\text{M} \) PIP\textsubscript{2} compared with 221 \pm 23 \(\mu\text{M} \) without PIP\textsubscript{2}) (Fig. 3, A–C). Accordingly, the addition of Ca\textsuperscript{2+} progressively increased the binding affinity of C2AB for PIP\textsubscript{2} (from \(K_{\text{PIP}_2} = 20 \pm 5 \text{ M} \) without Ca\textsuperscript{2+} to <2 \(\mu\text{M} \) at >20 \(\mu\text{M} \) Ca\textsuperscript{2+}). This cooperativity is not specific for PIP\textsubscript{2} or the length of the acyl chains because another phosphoinositide (20 \(\mu\text{M} \) phosphatidylinositol 3,5-bisphosphate) or short-chain PIP\textsubscript{2} (20 \(\mu\text{M} \) 1,2-dioctanoyl-sn-glycero-3-phosphatidylinositol 4’,5’-bisphosphate; C8:0) also increased the apparent Ca\textsuperscript{2+} affinity (\(K_{\text{Ca}^2+} = 11 \pm 5 \text{ M} \) and 8 \(\pm 5 \text{ M} \), respectively).

PIP\textsubscript{2} binding required the well conserved polybasic patch that is located on the C2B domain because removal of two lysines from this patch (K326A/K327A, the so-called KAKA mutant (12)) (Fig. 3, D–F) almost completely abolished PIP\textsubscript{2}-dependent MST changes, even at very high Ca\textsuperscript{2+} concentrations. Accordingly, the apparent affinity for Ca\textsuperscript{2+} was increased by only \(\sim 3\)-fold in the presence of 80 \(\mu\text{M} \) PIP\textsubscript{2} (from \(K_{\text{Ca}^2+} = 195 \pm 35 \text{ M} \) to 61 \(\pm 11 \text{ M} \)). Thus, we could detect only PIP\textsubscript{2} binding to the polybasic patch and did not observe PIP\textsubscript{2} binding via the Ca\textsuperscript{2+}-binding sites on the C2A.

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and C2B domains, in contrast to previous observations by us and others (3, 10–12, 14). It is likely that, for the interaction of the Ca\(^{2+}\)/H11001-binding pockets with the membrane, hydrophobic residues surrounding these pockets must insert into the membrane (6–8, 11, 12, 14), although we cannot exclude that PIP2 binding to the Ca\(^{2+}\)/H11001 sites is silent (i.e. does not change the MST signal). Nevertheless, the Ca\(^{2+}\)/H11001-binding pocket of the C2B domain does affect PIP2 binding to the polybasic patch because disruption of Ca\(^{2+}\)/H11001 binding to the C2B domain (D309A/D363A/D365A, called C2Ab*) reduced the affinity for PIP2 by 4-fold (from \(K_{\text{PIP2}} = 20.4 \pm 5.2 \mu\text{M}\) to 70 \(\mu\text{M}\)) (Fig. 4B).

We then performed MST experiments with mutants disrupted in Ca\(^{2+}\)/H11001 binding to the C2A domain (D178A/D230A/D232A, called C2a*B). Surprisingly, only a small and insignificant PIP2-or Ca\(^{2+}\)/H11001-dependent change in the MST signal of C2a*B was observed compared with the wild type (Fig. 4, A and B). Accordingly, the combination of C2a*B with the KAKA mutation did not markedly differ from the KAKA mutant with all Ca\(^{2+}\)-binding sites intact. Apparently, Ca\(^{2+}\)/H11001 binding to the C2A domain does not result in a detectable change in the thermophoretic properties of the C2AB fragment. In contrast, Ca\(^{2+}\)/H11001 binding could no longer be detected by MST upon disruption of the C2B domain. Thus, only Ca\(^{2+}\)/H11001 binding to the C2B domain seems to change the thermophoretic properties of the C2AB fragment, indicating that the calcium-dependent changes reported above are exclusively mediated by the C2B domain. Perhaps this selectivity is related to the thermodynamically divergent modes of Ca\(^{2+}\)/H11001 binding of synaptotagmin-1: Ca\(^{2+}\)/H11001 binding to the C2A domain is endothermic, and that to the C2B domain is exothermic (3). Finally, Ca\(^{2+}\)/H11001 concentrations above 100 \(\mu\text{M}\) increased the apparent PIP2 affinity of synaptotagmin-1 even when both Ca\(^{2+}\)-binding sites were disrupted (double mutant C2a*b*) (Fig. 4B). This indicates that Ca\(^{2+}\)/H11001 was still able to bind to the double mutant at very high Ca\(^{2+}\)/H11001 concentrations in the presence of PIP2, perhaps by binding directly to PIP2 (19, 20).

**DISCUSSION**

In this work, we have shown that PIP2 binds to the polybasic patch of the C2B domain of synaptotagmin-1, in agreement with earlier studies (10–14, 21). PIP2 binding to the polybasic patch increases the apparent affinity of the C2B domain for Ca\(^{2+}\)/H11001 by >40-fold. Conversely, Ca\(^{2+}\)/H11001 binding to the C2B domain increases the affinity for PIP2 by >10-fold. Cooperative PIP2 and Ca\(^{2+}\)/H11001 binding to synaptotagmin-1 has been observed previously (12). This cooperativity is probably not caused by complementation of the Ca\(^{2+}\)-binding sites, as suggested earlier by us and others (3, 6–8), because the polybasic patch and
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the Ca\textsuperscript{2+}-binding sites are located quite far apart (Fig. 4C). Instead, PIP\textsubscript{2} may interact in a structurally less defined manner with the polybasic patch and other solvent-exposed basic residues (9, 12), and this may increase the Ca\textsuperscript{2+} affinity simply by charge screening. Alternatively, the polybasic patch may form a structurally defined complex with PIP\textsubscript{2} similar to the C2 domains of rabphilin-3A and PKCα (22–25). In fact, cooperative PIP\textsubscript{2} and Ca\textsuperscript{2+} binding has been observed for these C2 domains (22–24), very similar to our observations for the C2B domain. Moreover, the crystal structure of the C2B domain (26) can be superimposed with that of the PIP\textsubscript{2}-bound C2 domain of PKCα (25), rendering it likely that PIP\textsubscript{2} binds to the C2AB fragment of synaptotagmin-1 in a similar manner (Fig. 4, C and D). Thus, it is conceivable that such PIP\textsubscript{2} binding increases the Ca\textsuperscript{2+} affinity via a conformational change. However, how PIP\textsubscript{2} and Ca\textsuperscript{2+} precisely bind in a cooperative manner to synaptotagmin-1 remains to be elucidated.

Together, we conclude that PIP\textsubscript{2} binding to the polybasic patch of synaptotagmin-1 dramatically increases the Ca\textsuperscript{2+} sensitivity. As discussed previously (12), this explains the reduced release probability of the KAKA mutant in hippocampal neurons (12, 27) and in Drosophila (28). It also explains why in vivo already 10 μM Ca\textsuperscript{2+} is sufficient for physiological release of neurotransmitters in the calyx of Held (29). PIP\textsubscript{2} modulation of synaptotagmin-1 may well be of major physiological relevance when considering that PIP\textsubscript{2} is the predominant phospholipid species at the sites of docked vesicles in PC12 cells (30).

Finally, our work demonstrates the value of MST for measuring molecular interactions. Although we were unable to detect Ca\textsuperscript{2+} binding to the C2A domain under our conditions, MST can be extremely sensitive and allows for monitoring medium and high affinity interactions with only pico moles of material. MST has the potential to complement the limited set of techniques available to measure Ca\textsuperscript{2+} and PIP\textsubscript{2} binding to proteins under equilibrium conditions such as isothermal titration calorimetry and NMR.

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