Synaptotagmin-1 binds to \( \text{PIP}_2 \)-containing membrane but not to SNAREs at physiological ionic strength

Yongsoo Park\(^1\), Jong Bae Seo\(^2\), Alicia Fraind\(^3\), Angel Pérez-Lara\(^1\), Halenur Yavuz\(^1\), Kyungreem Han\(^4\), Seung-Ryoung Jung\(^2\), Iman Kattan\(^5\), Peter Jomo Walla\(^5,6\), MooYoung Choi\(^4\), David S Cafiso\(^3\), Duk-Su Koh\(^2\) & Reinhard Jahn\(^1\)

The \( \text{Ca}^{2+} \) sensor synaptotagmin-1 is thought to trigger membrane fusion by binding to acidic membrane lipids and SNARE proteins. Previous work has shown that binding is mediated by electrostatic interactions that are sensitive to the ionic environment. However, the influence of divalent or polyvalent ions, at physiological concentrations, on synaptotagmin’s binding to membranes or SNAREs has not been explored. Here we show that binding of rat synaptotagmin-1 to membranes containing phosphatidylinositol 4,5-bisphosphate (\( \text{PIP}_2 \)) is regulated by charge shielding caused by the presence of divalent cations. Surprisingly, polyvalent ions such as ATP and Mg\(^{2+}\) completely abrogate synaptotagmin-1 binding to SNAREs regardless of the presence of \( \text{Ca}^{2+} \). Altogether, our data indicate that at physiological ion concentrations \( \text{Ca}^{2+} \)-dependent synaptotagmin-1 binding is confined to \( \text{PIP}_2 \)-containing membrane patches in the plasma membrane, suggesting that membrane interaction of synaptotagmin-1 rather than \( \text{SNARE} \) binding triggers exocytosis of vesicles.

Neurotransmitters contained in synaptic vesicles and secretory granules are released by exocytotic membrane fusion in response to an elevation of intracellular \( \text{Ca}^{2+} \). Soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins are considered to be the fusion machinery. \(^1\),\(^2\) Q-SNAREs (syntaxin-1 and SNAP-25) localized in the plasma membrane assemble with an R-SNARE (synaptobrevin-2, also called VAMP-2) in the vesicle membrane, thereby bringing the vesicle and plasma membranes into proximity and initiating membrane merger.\(^3\)

Synaptotagmin-1, anchored in the vesicle membrane, is one of the main \( \text{Ca}^{2+} \) sensors mediating fast \( \text{Ca}^{2+} \)-dependent vesicle fusion. Synaptotagmin-1 consists of two \( \text{Ca}^{2+} \)-binding C2 domains, referred to as the C2A and C2B domains, which interact with acidic phospholipids upon \( \text{Ca}^{2+} \) binding.\(^5\)–\(^7\) In addition to the \( \text{Ca}^{2+} \)-binding loops, the C2B domain contains a polybasic region, enriched with lysine residues,\(^8\) that interacts with \( \text{PIP}_2 \) (refs. \(9\),\(^10\)). Recent evidence has suggested that syntaxin-1A clusters \( \text{PIP}_2 \), which in turn interacts with the polybasic region in the C2B domain, thereby positioning synaptotagmin-1 at the site of fusion before arrival of the \( \text{Ca}^{2+} \) stimulus.\(^11\),\(^12\) \( \text{PIP}_2 \) also controls the activity of multiple proteins such as ion channels and pumps,\(^13\),\(^14\) The inositol head group of \( \text{PIP}_2 \) is highly charged, owing to three negative phosphates, and therefore it generates a strong negative local electrostatic potential.\(^15\) In aqueous solution, the negative potential attracts cations and forms an ‘ion cloud’ or ‘double layer’ that was first recognized by Helmholtz in 1853 (ref. 16). This ionic double layer accounts for many surface phenomena including the behavior of colloids and other surfaces in contact with aqueous solutions.\(^17\),\(^18\)

In addition to binding to acidic phospholipids and \( \text{PIP}_2 \), synaptotagmin-1 directly binds to the \( \text{SNARE} \) proteins syntaxin-1 and SNAP-25 and to \( \text{SNARE} \) complexes containing these \( \text{SNARE} \)s. Binding is observable both in native extracts by immunoprecipitation and pulldown approaches and \textit{in vitro} by use of purified proteins. Binding can be measured in the absence of \( \text{Ca}^{2+} \) but is accelerated by \( \text{Ca}^{2+} \), and it appears to be mediated mainly by the polybasic region of the C2B domain.\(^19\)–\(^22\) Molecular dynamics simulations have suggested that multiple acidic residues of syntaxin-1A (E224, E228, D231 and E234) and the acidic residues on SNAP-25A (D51, E52 and E55) may interact with the basic residues of the C2B domain in synaptotagmin-1 (ref. 23).

The molecular mechanisms by which synaptotagmin-1 triggers exocytosis are poorly understood and highly debated.\(^2\) This is primarily because it is very difficult to integrate the diverse binding modes of synaptotagmin-1 \textit{observed \textit{in vitro} under variable experimental conditions into a coherent molecular pathway to fusion that is compatible with physiological data. In most models, the synaptotagmin-1–SNARE complex interaction has a critical role and is thought to be directly responsible for the dramatic acceleration of synaptic-vesicle

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\(^{1}\)Department of Neurobiology, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany. \(^{2}\)Department of Physiology and Biophysics, University of Washington, Seattle, Washington, USA. \(^{3}\)Department of Chemistry, Center for Membrane Biology, University of Virginia, Charlottesville, Virginia, USA. \(^{4}\)Department of Physics and Astronomy, Center for Theoretical Physics, Seoul National University, Seoul, Republic of Korea. \(^{5}\)Biomolecular Spectroscopy and Single-Molecule Detection Research Group, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany. \(^{6}\)Department of Biophysical Chemistry, Institute for Physical and Theoretical Chemistry, University of Braunschweig, Braunschweig, Germany. Correspondence should be addressed to D.S.C. (cafiso@virginia.edu), D.-S.K. (koh@uw.edu) or R.J. (jahn@gwdg.de).

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exocytosis by Ca\textsuperscript{2+} ions. Despite this emerging consensus, it has been very difficult to pinpoint the effect of synaptotagmin-1 binding on the SNARE conformational cycle and its relationship to membrane fusion. Both inhibitory and activating effects of synaptotagmin-1 on SNARE assembly have been postulated, but neither the nature of synaptotagmin-1–SNARE binding nor the effects of binding on SNARE function are understood at the molecular level.

Intriguingly, synaptotagmin-1 binding to both acidic phospholipids and SNARE proteins is highly sensitive to the presence of electrolytes, thus indicating that electrostatics plays a major role in these interactions. For instance, Ca\textsuperscript{2+}-independent binding of synaptotagmin-1 to PIP\textsubscript{2} is decreased by Mg\textsuperscript{2+} ions\textsuperscript{24}. Moreover, we have recently shown that ATP and other polyphosphates, at physiological concentrations, decrease binding to acidic phospholipids\textsuperscript{35}. Similarly, binding of synaptotagmin-1 to SNAREs and SNARE complexes, which has been investigated in many studies from different laboratories\textsuperscript{26,27}, appears to be exquisitely sensitive to the ionic strength of the medium and is scarcely detectable at physiological ion concentrations, for example, 150 mM NaCl or KCl\textsuperscript{28,29}.

For these reasons, we set out to shed light on the interactions of synaptotagmin-1 with SNARE proteins and membrane lipids in the presence of divalent and polyvalent ions in a physiologically relevant concentration range. Our data show that the affinity of synaptotagmin-1 for its binding partners is decreased by multivalent ions. Whereas Ca\textsuperscript{2+}-dependent synaptotagmin-1 binding to PIP\textsubscript{2}-containing membranes persists at physiological concentrations of monovalent ions, Mg\textsuperscript{2+} and ATP, synaptotagmin-1 binding to SNAREs is not measurable under these conditions, regardless of whether Ca\textsuperscript{2+} is present or whether the SNAREs are embedded in a membrane containing acidic phospholipids. We conclude that synaptotagmin-1 triggering requires specific binding to acidic membrane lipids, particularly PIP\textsubscript{2} clusters at the base of SNARE proteins. However, our data do not support models involving a direct effect on SNARE zipping by synaptotagmin-1 binding to SNAREs.

RESULTS



**Figure 1** High concentrations of Mg\textsuperscript{2+} ions inhibit Ca\textsuperscript{2+}-dependent and synaptotagmin-1–mediated fusion. (a) Lipid mixing between purified chromaffin granules (CG) and liposomes containing a stabilized Q-SNARE complex (Syx-SN25)\textsuperscript{3} (details in Online Methods). The liposomes contained a standard mixture of membrane lipids including 10% PS, 1% PIP\textsubscript{2}, 1.5% NBD-PE and 1.5% Rh-PE. CG fusion is shown as a percentage of maximum donor fluorescence (total fluorescence) after addition of 0.1% Triton X-100. Syb, synaptobrevin-2; Syt1, synaptotagmin-1. (b) Dose-response curve of MgCl\textsubscript{2} on Ca\textsuperscript{2+}-dependent CG fusion obtained from a. Percentage lipid mixing at 5 min after fusion reaction is normalized to control, basal fusion without any treatment of Mg\textsuperscript{2+} or Ca\textsuperscript{2+} (n = 3 independent experiments; range of values is indicated, lines within the boxes represent individual data points). (c,d) Binding of the C2AB fragment of synaptotagmin-1 (Syx\textsubscript{97–421})\textsuperscript{31} to protein-free liposomes, measured by fluorescence anisotropy (with lipidosome lipid composition as in a except that the labeled lipids were omitted). Binding was triggered by addition of 100 µM free Ca\textsuperscript{2+} at different concentrations of MgCl\textsubscript{2}. A.u., arbitrary units. (d) Dose-response curve of MgCl\textsubscript{2} on Ca\textsuperscript{2+}-dependent C2AB binding. C2AB binding is plotted as a percentage of the maximum value induced by 100 µM Ca\textsuperscript{2+} in the absence of Mg\textsuperscript{2+}. Data in d are shown as mean ± s.d. from four independent experiments. The line in d shows the fit of experimental data with our mathematical model (details in Online Methods). Anisotropy and fusion assays were performed at normal ionic strength, i.e., 140 mM K\textsuperscript{+} and 3 mM ATP.

**PI\textsubscript{P}2 shielding by Mg\textsuperscript{2+} decreases Ca\textsuperscript{2+}-dependent fusion**

It is well established that binding of synaptotagmin-1 to the membrane lipid PI\textsubscript{P}2 has an important role in Ca\textsuperscript{2+}-dependent vesicle fusion\textsuperscript{25,30–32}. Because cations such as Mg\textsuperscript{2+} electrostatically interact with the negatively charged head groups of PI\textsubscript{P}2 (ref. 33) and decrease PI\textsubscript{P}2 availability in the plasma membrane by shielding its negative charge\textsuperscript{34}, we tested whether Mg\textsuperscript{2+} interferes with the binding of synaptotagmin-1 during Ca\textsuperscript{2+}-mediated enhancement of SNARE-dependent vesicle fusion in vitro. To this end, we used an in vitro fusion assay involving fusion between purified chromaffin granules (CGs) containing endogenous synaptobrevin-2 and proteoliposomes containing a stabilized Q-SNARE acceptor complex (together with the acidic phospholipids 1% PI\textsubscript{P}2 and 10% phosphatidylinerine (PS))\textsuperscript{3,25}. Ca\textsuperscript{2+} at ~80–100 µM potently accelerated CG fusion (caused by an interaction between synaptotagmin-1 on CGs and PI\textsubscript{P}2 in the acceptor liposomes\textsuperscript{20}), whereas inclusion of soluble synaptobrevin-2 (Syb\textsubscript{1–96}) blocked the fusion, thus indicating that fusion is SNARE dependent (Fig. 1a). Intriguingly, Mg\textsuperscript{2+} inhibited Ca\textsuperscript{2+}-dependent CG fusion in a dose-dependent manner, completely inhibiting Ca\textsuperscript{2+}-dependent enhancement at 10 mM MgCl\textsubscript{2} (Fig. 1a,b). This result correlates well with a former study showing that nonphysiologically high Mg\textsuperscript{2+} concentrations (>5 mM) decrease Ca\textsuperscript{2+}-dependent exocytosis in chromaffin cells\textsuperscript{35,36}.

To test whether the Mg\textsuperscript{2+} effect is caused by an inhibition of synaptotagmin-1 binding to PI\textsubscript{P}2, we monitored the binding of synaptotagmin-1 to the membrane at different Mg\textsuperscript{2+} concentrations. We labeled a soluble fragment of synaptotagmin-1 including both C2 domains (residues 97–421, termed the C2AB fragment) with Alexa
Fluor 488 so that the binding of the C2AB fragment to PIP2-containing liposomes resulted in an increase of fluorescence anisotropy due to a decrease in mobility (details in Online Methods). Mg\(^{2+}\) at millimolar concentrations decreased Ca\(^{2+}\)-dependent C2AB binding to PIP2-containing liposomes (Fig. 1c,d), concordantly with results from the fusion experiments.

**Biphasic regulation of CG fusion by Ca\(^{2+}\)**

Electrostatic shielding is primarily determined by the valence and concentration of counterions\(^{17,18}\), thus raising the possibility that not only Mg\(^{2+}\) but also Ca\(^{2+}\) at high, nonphysiological concentrations (millimolar range) shields PIP2. Ca\(^{2+}\) promoted in vitro vesicle fusion, producing a maximal effect at a Ca\(^{2+}\) concentration of \(-80\ \mu M\) (ref. 25), and higher concentrations progressively decreased Ca\(^{2+}\)-dependent fusion and caused complete inhibition at 10 mM, thus resulting in a bell-shaped dose dependence (Fig. 2). We observed a similarly biphasic response to Ca\(^{2+}\) on exocytosis in chromaffin cells. We perfused cultured rat chromaffin cells with varying Ca\(^{2+}\) concentrations by using patch electrodes and then carried out carbon-fiber amperometry\(^{37}\). Increased free-Ca\(^{2+}\) concentrations enhanced concentrations by using patch electrodes and then carried out carbon-

![Figure 2](image-url) **Figure 2** Biphasic effect of Ca\(^{2+}\) on CG fusion in vitro and in intact cells. (a) Left, lipid mixing between CGs and SNARE-containing liposomes as described in Figure 1a, at different concentrations of Ca\(^{2+}\) in the presence of 1 mM MgCl\(_2\). Right, dose-response curve of a lipid-mixing assay at various Ca\(^{2+}\) concentrations. The degree of fusion 5 min after the start of the reaction is normalized to fusion in the absence of Ca\(^{2+}\) (control), which was set to 100% \((n = 3\) independent experiments; range of values is indicated). (b) Left, exocytotic events of rat chromaffin cells recorded by carbon-fiber electrode. The arrowheads indicate the formation of a whole-cell patch-clamping (W-C) configuration at 30 s. Right, rate of exocytosis, calculated as the number of amperometric events per 30 s (events/30 s). Average values are shown for \(n\) independent experiments (0.05 mM, \(n = 17\); 0.2 mM, \(n = 6\); 1 mM, \(n = 6\); 4 mM, \(n = 5\); 15 mM, \(n = 6\)). Data are shown as mean ± s.e.m. \(* P < 0.05; *** P < 0.001\) by two-tailed Student’s t test compared to the exocytosis triggered by 50 \(\mu M\) Ca\(^{2+}\). (c,d) Ca\(^{2+}\) dose-response curve for synaptotagmin-1 (C2AB) binding to liposomes (c, 10% PS and 1% PIP2) and purified CGs (d). 1 mM MgCl\(_2\) was included in the fusion assay, as in a. C2AB binding to liposomes or CG membranes is shown as a percentage of maximum value. Anisotropy data from c and d were fitted with the mathematical model. 140 mM K\(^+\) and 3 mM ATP for anisotropy and fusion assay. Data in c and d are shown as mean ± s.d. from four independent experiments.
binding is also decreased upon charge shielding by cations. Effective binding required higher PIP₂ concentrations (5%), and we observed no substantial binding when the membrane contained only 1% PIP₂ (Supplementary Fig. 1b,c). As expected, addition of liposomes containing 5% PIP₂ to labeled C2AB fragments resulted in binding (Supplementary Fig. 2a–c), which was mediated by the polybasic region. Importantly, high concentrations of MgCl₂ decreased Ca²⁺-independent C2AB binding to PIP₂-containing liposomes in a dose-dependent manner (Supplementary Fig. 2d,e).

To better estimate the contribution of PIP₂ shielding by divergent cations, we built a mathematical model (details in Online Methods) that considers the accumulation of divergent cations due to the local potential generated by PIP₂ and the resulting decrease of free PIP₂ available for synaptotagmin-1 binding. This model can successfully fit trans and cis interactions of the C2AB fragment (Figs. 1d and 2c,d) at different MgCl₂ and CaCl₂ concentrations, thus supporting our hypothesis that divergent cations at millimolar concentrations electrostatically shield PIP₂. The model indicates that the fusion rate drops between 100 µM and 1 mM Ca²⁺ because of the substantially increased inhibitory cis interaction (Fig. 2d), and fusion continues to decrease between 1 and 10 mM Ca²⁺, as a result of both increased cis binding and decreased trans binding of synaptotagmin-1 (Fig. 2c,d).

Finally, our hypothesis predicts that cations with higher charge density than divergent cations are more effective in shielding the negative charges of PIP₂. In fact, spermine (+4) as well as neomycin (+6) inhibited Ca²⁺-triggered exocytosis at lower concentrations than did divergent cations (Supplementary Fig. 3a,b). The only deviation from the model was the observation that Ca²⁺-triggered exocytosis was higher with 15 mM Mg²⁺ than 1 mM Mg²⁺ (Supplementary Fig. 3b). We consider this unexpected result in the discussion.

Together, the data show that the activity of synaptotagmin-1 can be modulated by divergent cations, as can be readily explained by the charge-shielding effect of these cations on acidic phospholipids including PIP₂. Our previous observations have shown that polyanions such as ATP also inhibit synaptotagmin-1 binding to acidic membrane lipids (mainly PS), although Ca²⁺-dependent binding to PIP₂ persists. Thus, charge shielding by polyvalent ions affects both binding partners: acidic membrane lipids, particularly PIP₂, are shielded by divergent cations, whereas the polybasic region of synaptotagmin-1 is shielded by polyvalent anions including ATP. In confirming the latter, we also showed that ATP indeed
ions directly to the polybasic region of synaptotagmin-1, by a fluorescence resonance energy transfer (FRET)-based assay (Supplementary Fig. 4).

**Ion dependence of synaptotagmin-1 binding to SNAREs**

Synaptotagmin-1 binding to SNARE proteins is considered to be at least as relevant for triggering exocytosis as its binding to membrane lipids. Again, synaptotagmin-1 SNARE binding has previously been shown to be sensitive to salt concentration, thus suggesting that it is primarily driven by electrostatic interactions between basic residues of synaptotagmin-1 and acidic residues of SNAP-25 and syntaxin-1A that are exposed on the surface of the SNARE complex, a notion that has recently been confirmed and extended by NMR experiments and modeling. However, the extent to which the SNARE–synaptotagmin-1 interaction is preserved under physiological ionic conditions (i.e., in the presence of monovalent as well as divalent and polyvalent ions, such as Mg$^{2+}$ and ATP), as well as the affinity of this interaction, has previously not been determined.

For this reason, we investigated how the interaction between synaptotagmin-1 and SNAREs is affected by Mg$^{2+}$-ATP. First, we used double electron–electron resonance (DEER) to characterize the association between synaptotagmin-1 and the SNARE complex in solution, using spin-labeled variants of the C2AB domain and a ternary SNARE complex lacking the membrane-anchor domains. We generated three single-cysteine mutants of the syntaxin-1A H3 segment and nine mutants of a soluble fragment of C2AB fragment to introduce a single spin-labeled side chain (R1), thereby producing 27 different spin pairs for the C2AB–SNARE complex (Fig. 3a).

Under normal ionic-strength conditions (140 mM KCl and 12 mM NaCl, pH 7.2) without polyvalent ions, the dipolar interactions yielding 27 different spin pairs for the C2AB–SNARE complex (Fig. 3a) were quenched and enhanced by 1 mM Mg$^{2+}$ and ATP, as well as the affinity of this interaction, had previously not been determined.

Addition of Mg$^{2+}$ and ATP at moderate concentrations (1 mM KCl) yields strong dipolar interactions and distances that matched predictions based on the structure (Supplementary Fig. 5b). The experiments described above were carried out with soluble protein fragments, and it is conceivable that binding could be enhanced when the SNAREs are anchored in the membrane. Therefore, we prepared liposomes containing a membrane-anchored ternary SNARE complex, using SNAP-25A labeled with Texas Red at the position 130C. We labeled the C2AB fragment (Syt97–421) with Alexa Fluor 488 at 342C (green dots). The polybasic region is shown in blue and Ca$^{2+}$ in magenta. Donor emission fluorescence spectra of Alexa Fluor 488 were recorded for C2AB in the indicated buffers, alone (black), in the presence of SNARE liposomes (red) or in the presence of SNARE liposomes and 1 mM Ca$^{2+}$ (green). (a) 50 mM KCl, no Mg$^{2+}$-ATP. (b) 150 mM KCl, no Mg$^{2+}$-ATP. (c) 50 mM KCl, plus Mg$^{2+}$-ATP. (d) 150 mM KCl, plus Mg$^{2+}$-ATP. Donor fluorescence was normalized as a percentage of the maximum value. Shown are representative donor emission fluorescence spectra from 3 to 5 independent experiments.

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Addition of Mg$^{2+}$ and ATP at moderate concentrations (1 mM and 3 mM, respectively) completely abolished the interaction of the C2AB fragment with the SNARE complex at 50 mM KCl (Fig. 4c,d), and this did not change when we used syntaxin-1A instead of a ternary SNARE complex (Supplementary Fig. 6a–d). Binding was not restored by the addition of 1 mM Ca$^{2+}$ (Fig. 4d and Supplementary Fig. 6c,d). Similarly, addition of purified complexin II, which is thought to modulate synaptotagmin-1–SNARE interactions failed to restore binding (Supplementary Fig. 6e–h). To exclude the possibility that binding does occur but is...
not reported by the FRET pair, we measured synaptotagmin-1–liposome binding by flotation gradients. Again, binding of the C2AB fragment to SNARE-containing liposomes was abolished at physiological ionic conditions (Supplementary Fig. 7a).

Although the results above show that synaptotagmin-1 does not bind to SNARE complexes under physiological ionic conditions, we cannot exclude that synaptotagmin-1 binding to membranes containing acidic phospholipids and PIP2 results in a high local concentration and/or a preferred orientation in the vicinity of membrane-anchored SNAREs, thus facilitating SNARE binding. To test this possibility, we reconstituted the SNARE complex in liposomes containing 10% PS and 1% PIP2 (Fig. 5). Ca2+ induced a FRET signal between synaptotagmin-1 and the SNARE complex (Fig. 5a). This FRET signal could result from either direct binding of C2AB to SNAREs or their proximity (without direct binding) due to the recruitment of C2AB to PIP2 surrounding syntaxin-1A clusters12. To differentiate between these possibilities, we carried out a competition experiment in which either excess soluble unlabeled SNARE complex or excess protein-free liposomes containing PS and PIP2 were added after induction of the Ca2+-dependent FRET signals. Whereas addition of protein-free liposomes reversed the FRET signals in a dose-dependent manner, we observed no effect with addition of SNARE complexes (Fig. 5b). These data suggest that synaptotagmin-1 does not directly bind to the surface of SNARE complexes but instead binds to PIP2-containing acidic membranes in the vicinity of SNAREs. To further confirm this, we compared the binding kinetics of synaptotagmin-1 to PS- and PIP2-containing liposomes that either contained SNARE complexes or were free of protein, in the presence of 100 µM Ca2+ and physiological ionic concentrations, i.e., 150 mM KCl, 1 mM MgCl2 and 3 mM ATP. If the SNARE complexes contribute to the membrane recruitment of C2AB to the PIP2-containing membrane, an enhancement of the binding rate would be expected. We measured binding kinetics by FRET between the C2AB fragment and a labeled membrane lipid (rhodamine-phosphatidylethanolamine, Rho-PE), with a stopped-flow setup (Fig. 6a). We found no difference between the two sets of liposomes (Fig. 6b,c), thus demonstrating that the presence of SNAREs does not contribute to the membrane binding of C2AB. To rule out that membrane-anchored full-length synaptotagmin-1 behaves differently with respect to SNARE binding, we reconstituted synaptotagmin-1 into liposomes and estimated binding by monitoring docking between the two vesicle populations by using fluorescence cross-correlation spectroscopy47. Again, binding to SNARE-containing liposomes devoid of acidic lipids decreased to near-background levels in the presence of physiological salt concentrations and Mg2+-ATP, both in the absence and

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presence of Ca^{2+} ions, whereas we observed Ca^{2+}-dependent binding to liposomes containing PS and PIP_{2} (Supplementary Fig. 7b,c and ref. 47). We conclude that synaptotagmin-1 binds to membranes containing acidic lipids but not to SNAREs in a physiological ionic environment.

**DISCUSSION**

In this study, we investigated the electrostatic nature of synaptotagmin-1 binding to membranes and SNARE proteins in a physiological ionic environment. In the absence of Ca^{2+}, binding to both SNAREs and membranes was abolished. In the presence of Ca^{2+}, synaptotagmin-1 specifically bound to PIP_{2}-containing membranes, thus suggesting that this interaction is likely to be mainly responsible for triggering exocytosis. Our results confirm the notion that synaptotagmin-1 operates as an electrostatic switch, as was first proposed by Rizo and colleagues^{48}. The electrostatic component clearly dominates membrane binding both in the absence and the presence of Ca^{2+}; the latter is supported by the fast kinetics of synaptotagmin-1 binding and unbinding (A.P.-L., A.T., S.B., P.H., M.T. et al., unpublished data).

Binding of synaptotagmin-1 to membranes is governed by long-range Coulombic interactions that are modulated both by the ionic strength of the medium and by the absorption of cations to the interface. This charge shielding by the counterions of PIP_{2} depends on the local potential generated by PIP_{2}, bulk concentrations of the counterions and the ionic strength of the solution, as predicted by our mathematical modeling (Supplementary Fig. 7d and Supplementary Table 1). Importantly, the intracellular concentrations of divalent and polyvalent ions are in a range at which shielding is just beginning to become substantial: cations including spermine (+4) are in the millimolar range^{49}, free Mg^{2+} ranges between 0.5 and 3 mM (refs. 43,44), and intracellular ATP concentrations range between 1 and 10 mM (ref. 42). This agrees well with our observation that spermine and neomycin (+6) suppressed exocytosis in chromaffin cells at low-millimolar concentrations (Supplementary Fig. 3b). Intriguingly, the only exception is Mg^{2+} (Supplementary Fig. 3b). We consistently observed increased exocytosis even with 15 mM Mg^{2+}, which abolished C2AB-PIP_{2} binding and vesicle fusion in vitro. Currently the reason for this is not clear, but it is conceivable that one or several steps preceding vesicle exocytosis in intact cells are promoted by millimolar concentrations of Mg^{2+}, thus compensating for the shielding effect.

Our data show that the affinity of the synaptotagmin-1–SNARE interaction is decreased beyond detection at physiological ionic conditions, thus calling its physiological relevance for regulating exocytosis into question. Several structural models based on NMR^{50}, pseudocontact shifts and MD simulations^{23}, mass spectrometry^{51} and single-molecule FRET^{28} have been proposed, and these models have revealed quite different views of the predicted conformational structure of the complex. For the NMR and MD-based models, there is close association between the charged polybasic face of C2B and the SNARE complex, but for the FRET-based model it appears that regions opposite the membrane-binding loops are in contact with the SNAREs. Importantly, our DEER data support that the interactions between synaptotagmin-1 and SNAREs are heterogeneous and non-specific (Fig. 3b and Supplementary Fig. 5a). First, in every case the distributions are extremely broad. These data indicate that a single structure does not exist for the synaptotagmin-1–SNARE interaction and that there is probably a configuration in which the polybasic face of C2B approaches close to the SNAREs as well as other configurations in which it does not. Second, addition of Ca^{2+} produces only slight changes in the distributions and does not substantially alter the complex that is formed. Third, a direct comparison between the predictions of the FRET-based model^{28}, the MD-based models^{23} and the distributions observed by EPR can be made (Fig. 3b and Supplementary Fig. 5). In general, distances predicted by the FRET-based model^{28} capture the long-distance end of the DEER distance distribution, whereas the MD-based models often reproduce the shorter end of the distribution. Finally, these DEER data are completely consistent with those from an earlier study using continuous-wave EPR, thus indicating that the synaptotagmin-1–SNARE interaction is structurally heterogeneous^{52} and also explaining why pseudocontact shifts for C2B bound to Dy^{3+}-labeled SNAREs are highly averaged^{23}. We conclude that the interaction of synaptotagmin-1 with the surface of the SNARE complex is predominantly electrostatic. The association of these proteins may be dominated by one conformation under certain low-ionic-strength conditions; however, this does not appear to take place at equilibrium under more normal conditions. It is hard to imagine that such a low-energy synaptotagmin-1–SNARE interaction has any major influence on SNAP_{25} zipper and thus on the kinetics of exocytosis^{53}.

Synaptotagmin-1 binding activities were measurable at different ionic conditions in the absence and presence of Ca^{2+} (Fig. 7). Clearly, the only Ca^{2+}-triggered binding event that has high affinity...
under physiological ionic conditions, and might contribute sufficient energy to overcome activation-energy barriers, is to PIP2-containing membranes. In the absence of Ca\(^{2+}\), synaptotagmin-1 binding seems to occur only at high PIP2 concentrations (higher than 5%)\(^\text{11}\), and we also observed decreased but measurable binding at high PIP2 concentrations under physiological ionic conditions (data not shown). Given that PIP2 forms microdomains in which PIP2 is highly enriched (up to 84%)\(^\text{2}\), and does so preferentially at the base of SNARE complexes, it is conceivable that substantial Ca\(^{2+}\)-independent binding takes place at such PIP2 microdomains.

In summary, our data demonstrate that electrostatic-charge shielding by cations, multivalent cations and ATP profoundly interferes with the various binding activities of synaptotagmin-1, thus explaining why the binding properties of synaptotagmin-1 reported in the literature are highly variable. Importantly, our data do not support an interaction of synaptotagmin-1 with SNARE proteins at physiological ionic conditions regardless of whether Ca\(^{2+}\) is present, thus suggesting that PIP2 interaction is the physiologically relevant binding event. This leaves two possibilities for the synaptotagmin-1–mediated trigger event of exocytosis.

The first possibility is that binding could perturb the local lipidic environment, thus decreasing the energy barrier that must be overcome for membrane merger. Such perturbation could be mainly confined to the membrane surface, or the molecule could penetrate deeper into the hydrophobic core of the bilayer. At the surface, synaptotagmin-1 may alter the local distribution of charged lipids (demixing)\(^\text{34}\) at the base of the SNAREs, and this effect could be enhanced by Ca\(^{2+}\). Such a mechanism would be in accordance with the primarily electrostatic nature of binding. However, side chains of membrane-bound synaptotagmin-1 have previously been shown to establish hydrophobic interactions with the bilayer core, with tryptophan residues ‘snorkeling’ at the hydrophilic–hydrophobic boundary\(^\text{40}\), and this may have effects on overall bilayer structure. Indeed, synaptotagmin-1, at least when added at saturating concentrations, has been shown to deform membranes by inducing local curvature stress\(^\text{55,56}\), which causes exocytosis triggering in several models (for example, ref. 57).

The second possibility is that synaptotagmin-1 could trigger exocytosis by shortening the distance between the membranes, thus triggering SNARE nucleation and/or zippering. This view is supported by the observation that synaptotagmin-1 is capable of cross-linking membranes and that the membrane distance is shortened upon Ca\(^{2+}\) binding\(^\text{58}\). The latter concept has recently been challenged by the topology of docked and primed synaptic vesicles, as measured by high-pressure freezing and cryo-EM tomography\(^\text{59}\), because the distance between the vesicle and plasma membrane has been found to be below 2 nm, thus leaving little room for further shortening. Clearly, more work will be required for the mechanism of calcium triggering by synaptotagmin-1 to be unraveled.

**METHODS**  
Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGMENTS**  
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28. Choi, I.B. et al. Single-molecule FRET-derived model of the synaptotagmin-1–SNARE fusion complex. Nat. Struct. Mol. Biol. 17, 318–324 (2010).
29. Tang, J. et al. A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell 126, 1175–1187 (2006).
30. Bai, J., Tucker, W.C. & Chapman, E.R. PIP2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane. Nat. Struct. Mol. Biol. 11, 36–44 (2004).
31. Radhakrishnan, A., Stein, A., Jahn, R. & Fasshauer, D. The Ca2+ affinity of synaptotagmin 1 is markedly increased by a specific interaction of its C2B domain with phosphatidylinositol 4,5-bisphosphate. J. Biol. Chem. 284, 25749–25760 (2009).
32. Lee, H.K. et al. Dynamic Ca2+-dependent stimulation of vesicle fusion by membrane-anchored synaptotagmin 1. Science 328, 760–763 (2010).
33. Toner, M., Vale, G., McLaughlin, A. & McLaughlin, S. Adsorption of cations to phosphatidylinositol 4,5-bisphosphate. Biochemistry 27, 7435–7443 (1988).
34. Suh, B.C. & Hille, B. Electrostatic interaction of internal Mg2+ with membrane PIP2 seen with KCNQ K+ channels. J. Gen. Physiol. 130, 241–256 (2007).
35. Knight, D.E. & Baker, P.F. Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. J. Membr. Biol. 68, 107–140 (1982).
36. Wengerskhi, U., Gratzl, M., Fohr, K.J. & Ahnert-Hilger, G. Millimolar concentrations of free magnesium enhance exocytosis from permeabilized rat pheochromocytoma (PC 12) cells. Neurosci. Lett. 106, 300–304 (1989).
37. Koh, D.S. Carbon fiber amperometry in the study of ion channels and secretion. Methods Mol. Biol. 337, 139–153 (2006).
38. Kuo, W., Herrick, D.Z., Ellena, J.F. & Cafiso, D.S. The calcium-dependent and calcium-independent membrane binding of synaptotagmin 1: two modes of C2B binding. J. Mol. Biol. 387, 284–299 (2009).
39. Vrlic, M. et al. Post-translational modifications and lipid binding profile of insect cell-expressed full-length mammalian synaptotagmin 1. Biochemistry 50, 9998–10012 (2011).
40. Jeschke, G. et al. A direct conversion of EPR dipolar time evolution data to distance distributions. J. Magn. Reson. 155, 72–82 (2002).
41. Pannier, M., Veit, S., Godt, A., Jeschke, G. & Spiess, H.W. Dead-time free measurement of dipole-dipole interactions between electron spins. J. Magn. Reson. 142, 331–340 (2000).
42. Beis, I. & Newsholme, E.A. The contents of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscles from vertebrates and invertebrates. Biochem. J. 152, 23–32 (1975).
43. London, R.E. Methods for measurement of intracellular magnesium: NMR and fluorescence. Annu. Rev. Physiol. 53, 241–258 (1991).
44. Hess, P., Metzger, P. & Weingart, R. Free magnesium in sheep, ferret and frog striated muscle at rest measured with ion-selective micro-electrodes. J. Physiol. ( Lond.) 333, 173–188 (1982).
45. Rickman, C. et al. Synaptotagmin interaction with the syntaxin/SNAP-25 dimer is mediated by an evolutionarily conserved motif and is sensitive to insulin hexakisphosphosphate. J. Biol. Chem. 279, 12574–12579 (2004).
46. Gaffaney, J.D., Dunning, F.M., Wang, Z., Hui, E. & Chapman, E.R. Synaptotagmin C2B domain regulates Ca2+-triggered fusion in vitro: critical residues revealed by scanning alanine mutagenesis. J. Biol. Chem. 283, 31763–31775 (2008).
47. Verneke, W. et al. cis- and trans-membrane interactions of synaptotagmin-1. Proc. Natl. Acad. Sci. USA 109, 11037–11042 (2012).
48. Shao, X. et al. Synaptotagmin-syntaxin interaction: the C2 domain as a Ca2+-dependent electrostatic switch. Neuron 18, 133–142 (1997).
49. Santhan, S. & Seiler, N. On the subcellular localization of the polyamines. Biol. Chem. Hoppe Seyler 370, 1279–1284 (1989).
50. Dai, H., Shen, N., Arac, D. & Rizo, J. A quaternary SNARE-synaptotagmin-Ca2+-phospholipid complex in neurotransmitter release. J. Mol. Biol. 367, 848–863 (2007).
51. Lynch, K.L. et al. Synaptotagmin C2A loop 2 mediates Ca2+-dependent SNARE interactions essential for Ca2+-triggered vesicle exocytosis. Mol. Biol. Cell 18, 4957–4968 (2007).
52. Lai, A.L., Huang, H., Herrick, D.Z., Epp, N. & Cafiso, D.S. Synaptotagmin 1 and SNAREs form a complex that is structurally heterogeneous. J. Mol. Biol. 405, 696–706 (2011).
53. Lai, Y., Lou, X., Diao, J. & Shin, Y.K. Molecular origins of synaptotagmin 1 activities on vesicle docking and fusion pore opening. Sci. Rep. 5, 9267 (2015).
54. Lai, A.L., Tam, L.K., Ellena, J.F. & Cafiso, D.S. Synaptotagmin 1 modulates lipid acyl chain order in lipid bilayers by demixing phosphatidylinerse. J. Biol. Chem. 286, 25291–25300 (2011).
55. Martens, S., Kozov, M.M. & McMahon, H.T. How synaptotagmin promotes membrane fusion. Science 316, 1205–1208 (2007).
56. Hui, E., Johnson, C.P., Yao, J., Dunning, F.M. & Chapman, E.R. Synaptotagmin-mediated bending of the target membrane is a critical step in Ca2+-regulated fusion. Cell 138, 709–721 (2009).
57. McMahon, H.T., Kozov, M.M. & Martens, S. Membrane curvature in synaptic vesicle fusion and beyond. Cell 140, 601–605 (2010).
58. van den Bogaart, G. et al. Synaptotagmin-1 may be a distance regulator acting upstream of SNARE nucleation. Nat. Struct. Mol. Biol. 18, 805–812 (2011).
59. Imig, C. et al. The morphological and molecular nature of synaptic vesicle priming at presynaptic active zones. Neuron 84, 416–431 (2014).
60. Jeschke, G. et al. DeerAnalysis2006: a comprehensive software package for analyzing pulsed ELDOR data. Appl. Magn. Reson. 30, 473–498 (2006).
Purification of chromaffin granules (CGs). As described previously, CGs were purified from bovine adrenal medulla tissue (obtained from the local slaughterhouse (Kassel, Germany)) by continuous sucrose-gradient centrifugation as the last purification step. CGs were resuspended in 120 mM K-glutamate, 20 mM K-acetate, and 20 mM HEPES, pH 7.4 adjusted with KOH.

Protein purification and protein labeling. All SNARE and C2AB constructs were based on Rattus norvegicus sequences, expressed in Escherichia coli strain BL21(DE3), and purified by Ni-NTA affinity chromatography and subsequent ion-exchange chromatography. The stabilized Q-SNARE acceptor complex consisting of syntaxin-1A (aa 183–288), SNAP-25A (aa 1–206, no cysteine), and a C-terminal synaptobrevin-2 fragment (aa 97–421) and the KAKA mutant (K326A K327A) were purified by a Mono S column (GE Healthcare) in the presence of 50 mM n-octyl-β-d-glucoside. The C2AB fragment of synaptotagmin-1 (1–97) and SNAP-25A (no cysteine) was purified after coexpression. The Q-SNARE complex and stabilized Q-SNARE complex were purified by ion-exchange chromatography on a Mono Q column (GE Healthcare) in the presence of 50 mM n-octyl-β-d-glucoside. The C2AB fragment of synaptotagmin-1 was purified by reverse-phase HPLC on a C18 column (GE Healthcare), eluted with 100% acetonitrile, and lyophilized. The Q-SNARE complex described previously was purified from bovine adrenal medulla tissue (obtained from the local slaughterhouse (Kassel, Germany)) by continuous sucrose-gradient centrifugation as the last purification step. CGs were resuspended in 120 mM K-glutamate, 20 mM K-acetate, and 20 mM HEPES, pH 7.4 adjusted with KOH.

Preparation of proteoliposomes. The lipid composition of liposomes (molar percentages) containing the Q-SNARE complex consisted of 45% PC, 15% PE, 10% PS, 25% chol, 4% PI, and 1% PIP2. The ternary SNARE complex consisting of syntaxin-1A (183–288), SNAP-25A (Cys130), and synaptobrevin-2 (1–96) was purified after coexpression. The Q-SNARE complex and stabilized Q-SNARE complex were purified by ion-exchange chromatography on a Mono Q column (GE Healthcare), eluted with 100% acetonitrile, and lyophilized. The Q-SNARE complex described previously was purified from bovine adrenal medulla tissue (obtained from the local slaughterhouse (Kassel, Germany)) by continuous sucrose-gradient centrifugation as the last purification step. CGs were resuspended in 120 mM K-glutamate, 20 mM K-acetate, and 20 mM HEPES, pH 7.4 adjusted with KOH.

Lipid-mixing assay. CG fusion in vitro was monitored with a lipid-mixing assay at 37 °C in 1 ml of buffer containing 120 mM K-glutamate, 20 mM K-acetate, 20 mM HEPES-KOH, pH 7.4, and 3 mM 2Na-ATP. MgCl2 was added as indicated in the legends. 30 µg CGs and proteoliposomes containing NBD-DOPE and rhodamine-DOPE as a donor and an acceptor dye, respectively, were incubated with 2% lipid solution for 20–30 min at 37 °C to dissociate single chromaffin cells. The loose medulla tissue was triturated by gentle pipetting and suspended in culture medium DMEM containing 10% FBS and 2% penicillin/streptomycin solution. The cell suspension was centrifuged at 1,200 r.p.m. for 4 min at room temperature (22–25 °C). The collected cells were resuspended in culture medium and plated on glass coverslips coated with poly-L-lysine. After 2 h, fresh culture medium was added, and the cells were maintained at 37 °C in a 5% CO2 incubator. Exocytosis was measured 1 or 2 d after cell preparation.

Dual-recording of carbon-fiber amperometry and whole-cell patch-clamping. Recordings of both carbon-fiber amperometry and whole-cell patch-clamping were simultaneously performed on the same cell. Exocytosis was measured as pulses of electric current generated by oxidation of the molecules from single secretory vesicles at the tip of a carbon-fiber electrode polarized to +400 mV. Carbon-fiber microelectrodes were fabricated with 11-µm carbon fibers and polypropylene 10-µl micropipette tips and backfilled with 3 M KCl. The amperometric current signals were recorded with an EPC 9 patch-clamp amplifier with PatchMaster software (HEKA Elektronik), filtered at 0.1 kHz, and sampled digitally at 0.5 kHz.

Whole-cell patch configuration was achieved with an EPC 9 patch-clamp amplifier. Patch electrodes had a DC resistance between 3 and 5 MΩ when filled with internal solution. Bath solution (Ringer’s) consisted of 137.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM glucose, and 10 mM HEPES (with pH adjusted to 7.3 with NaOH). The pipette solutions contained the following: 50 µM free-Ca2+ internal solution (free Ca2+ was estimated with Maxichelor simulation program, 105 mM K-glutamate, 15.7 mM CaCl2, 15 mM EGTA, 1 mM MgCl2, 5 mM Na2-ATP, 0.3 mM Na2-GTP, and 10 mM HEPES; 200 µM free-Ca2+ internal solution, 105 mM K-glutamate, 17.16 mM CaCl2, 15 mM EGTA, 1 mM MgCl2, 5 mM Na2-ATP, 0.5 mM Na2-GTP, and 10 mM HEPES; 1 mM free-Ca2+ internal solution, 100 mM K-glutamate, 19.43 mM CaCl2, 15 mM EGTA, 1 mM MgCl2, 5 mM Na2-ATP, 0.3 mM Na2-GTP, and 10 mM HEPES; 4 mM free-Ca2+ internal solution, 95 mM K-glutamate, 23.17 mM CaCl2, 15 mM EGTA, 1 mM MgCl2, 5 mM Na2-ATP, 0.3 mM Na2-GTP, and 10 mM HEPES; and 15 mM free-Ca2+ internal solution, 80 mM K-glutamate, 34.62 mM CaCl2, 15 mM EGTA, 1 mM MgCl2, 5 mM Na2-ATP, 0.3 mM Na2-GTP, and 10 mM HEPES. All pipette solutions were adjusted to pH 7.2 with KOH and...
were mixed with equal volumes of Rho-PE–containing liposomes (consisting of full-length syntaxin-1A (1–288), SNAP-25A, and soluble syntaxin-1A (180–253) and synaptobrevin-2 (1–116))66. Proteins (synaptotagmin and syntaxin) were spin-labeled with a ten-fold excess of the sulfhydryl-reactive spin label (1-oxyl-2,2,5,5-tetramethyl-π-500-400-300-200-100-0

**Stopped-flow spectroscopy.** Kinetic experiments were carried out on an Applied Pho tophysics SX.20 stopped-flow spectrophotometer at 37 °C in 150 mM KCl, 20 mM Hepes, pH 7.4 with KOH, 1 mM MgCl2, and 3 mM ATP plus 100 µM free Ca2+. Different concentrations of C2AB labeled with Alexa Fluor 488 at S342C were mixed with equal volumes of Rho-PE–containing liposomes (consisting of 49% PC, 13% PE, 10% PS, 25% chol, 1% PIP2, and 2% rhodamine-DOPC) at 500 µM under pseudo-first order conditions. The excitation wavelength was set at 495 nm, and a 590 nm–cutoff filter was used to collect data at the fluorescence emission of rhodamine. The resulting time courses were fit to a single-exponential function:

$$F(t) = F_0 + A_{obs} \times e^{-k_{obs} \times t}$$

where $F(t)$ equals the observed fluorescence at time $t$, $F_0$ is the final fluorescence, $A_{obs}$ equals the amplitude, and $k_{obs}$ is the observed rate constant. Observed rate constants were plotted as a function of protein concentration and fitted with the equation:

$$k_{obs} = k_{on}[v] + k_{off}$$

where $k_{on}$ represents the apparent association constant, and $k_{off}$ represents the apparent dissociation rate constant.

**EPR spectroscopy.** Proteins (synaptotagmin and syntaxin) were spin-labeled with a ten-fold excess of the sulphydryl-reactive spin label (1-oxyl-2,2,5,5-tetramethyl-π-500-400-300-200-100-0

$$P_x = \frac{C_X V_p e^{-\epsilon_x/k_B T}}{1 + C_X V_p e^{-\epsilon_x/k_B T}} = \frac{C_X V_p e^{-\epsilon_{x0}/k_B T}}{1 + C_X V_p e^{-\epsilon_{x0}/k_B T}}$$

where $P_x$ denotes the effective volume in which PIP2 can encounter cations of Mg2⁺ and Ca2⁺, respectively; $C_X$ and $C_C$ represent the concentrations of Mg2⁺ and Ca2⁺. The denominator, $1 + C_X V_p e^{-\epsilon_{x0}/k_B T}$, is a normalization factor that ensures that $P_x = 0$ if $P_f = 1$.

Those fractions can be written in the form of a simple Hill-type equation, which is widely used for biochemical reactions72:

$$P_x = \frac{C_X (C_y + C_C)^{-1}}{1} = \frac{C_y V_p e^{-\epsilon_x/k_B T}}{1}$$

The resulting fractions $P_x$ and $P_f$ depend on the cation concentration and are illustrated in Supplementary Figure 7d. The result provides the range of cation concentrations that screen PIP2. Millimolar concentrations of divalent cations turn out to be effective when we use the parameters obtained from fitting of the experimental data (Figs. 1d and 2c,d) to the present model. The concentration of effective (free) PIP2 then takes the form:

$$C_p^{(e)} = C_p P_f = \frac{C_p}{1 + C_X V_p e^{-\epsilon_x/k_B T}}$$

where $C_p$ represents the total concentration of PIP2.

Next, we describe the bound fraction of C2AB domains saturated by free PIP2, as a function of the free (effective) PIP2 concentration $C_p^{(e)}$:

$$P_b = \frac{1}{1 + \frac{K_{PC} C_p^{(e)}}{C_P^{(e)}}}$$

where $K_{PC}$ is the dissociation constant of the PIP2–C2AB coordination and $C_p^{(e)}$ is the effective Ca2⁺ concentration. We assume that

$$K_{PC} = \frac{[C2AB][Ca^{2+}][PIP2]}{[C2AB – Ca^{2+} – PIP2]}$$

**Mathematical model.** For screening of negatively charged PIP2 by divalent cations, the probabilities or fractions of the screened PIP2 and of the free PIP2 are described by the Poisson-Boltzmann-type70,71. The electric potential energy of a PIP2 molecule depends on both the distance from and the valence of counterions. Upon screening, the mean distance between the screened PIP2 and cation should be decreased, and, accordingly, the electric potential energy $\varepsilon_f$ of a screened PIP2 becomes lower than the energy $\varepsilon_f$ of a free PIP2. For simplicity, $\varepsilon_f$ is taken to be zero ($\varepsilon_f = \varepsilon_f = 0$) because what matters is only the energy difference between $\varepsilon_f$ and $\varepsilon_f$. The fractions $P_b$ and $P_f$ of the screened PIP2 and of the free PIP2, respectively, are thus given by

$$P_f = \frac{1}{1 + C_X V_p e^{-\epsilon_x/k_B T}}$$

$$P_x = \frac{C_X V_p e^{-\epsilon_x/k_B T}}{1 + C_X V_p e^{-\epsilon_x/k_B T}}$$

The result provides the range of cation concentrations that screen PIP2. Millimolar concentrations of divalent cations turn out to be effective when we use the parameters obtained from fitting of the experimental data (Figs. 1d and 2c,d) to the present model. The concentration of effective (free) PIP2 then takes the form:

$$C_p^{(e)} = C_p P_f = \frac{C_p}{1 + C_X V_p e^{-\epsilon_x/k_B T}}$$

where $C_p$ represents the total concentration of PIP2.

Next, we describe the bound fraction of C2AB domains saturated by free PIP2, as a function of the free (effective) PIP2 concentration $C_p^{(e)}$:

$$P_b = \frac{1}{1 + \frac{K_{PC} C_p^{(e)}}{C_P^{(e)}}}$$

where $K_{PC}$ is the dissociation constant of the PIP2–C2AB coordination and $C_p^{(e)}$ is the effective Ca2⁺ concentration. We assume that

$$K_{PC} = \frac{[C2AB][Ca^{2+}][PIP2]}{[C2AB – Ca^{2+} – PIP2]}$$
for the \textit{trans} binding is given by the product of the dissociation constants
\[
K_C = \frac{[\text{C2AB}][\text{Ca}^{2+}]}{[\text{C2AB} - \text{Ca}^{2+}]}
\]
and
\[
K_p = \frac{[\text{C2AB} - \text{Ca}^{2+}][\text{PIP}_2]}{[\text{C2AB} - \text{Ca}^{2+} - \text{PIP}_2]}
\]
of the C2AB-Ca\textsuperscript{2+} binding and of the Ca\textsuperscript{2+}-mediated C2AB-PIP\textsubscript{2} binding, respectively.

By combining equations (3) and (4), the fraction of PIP\textsubscript{2}-bound C2AB can be described as a function of the cation concentration \(C_X (X = M \text{ or } C)\):
\[
F_b(C_X) = \frac{C_C^{(e)}C_p}{C_C^{(e)}C_p + K_{PC}(1 + C_X V_p e^{-\epsilon p/5k_BT})}
\]
(5)

Now we can determine the nonlinear behavior of \textit{trans} binding of C2AB to PIP\textsubscript{2} at different Ca\textsuperscript{2+} concentrations (Fig. 2c). For simplicity, fluorescence anisotropy \(y\) is assumed to be proportional to the bound fraction \(F_b\) of C2AB domains, i.e., \(y = a_CF_b\) with an appropriate constant \(a_C\). This relationship, together with equation (5) for \(C_X = C_C\), allows us to fit the experimental data (Fig. 2c):
\[
y = a_CF_b(C_C) = \frac{a_CC_C^{(e)}C_p}{C_C^{(e)}C_p + K_{PC}(1 + C_C V_p e^{-\epsilon_p/5k_BT})}
\]
(6)

where the coordination effects of Ca\textsuperscript{2+} have been taken into account by the effective Ca\textsuperscript{2+} concentration acting on the \textit{trans} binding, \(C_C^{(e)}(\alpha = C_C^{(e)\lambda}) = C_C^{(e)}\) with the maximum effective Ca\textsuperscript{2+} concentration \(x\) displaying saturation, owing to the limited numbers of coordination counterparts. Here, \(\lambda\) measures the characteristic Ca\textsuperscript{2+} concentration at which the effective concentration reaches half the maximum, i.e., \(C_C^{(e)}(\lambda) = \lambda/2\), and the exponent \(n\) of Ca\textsuperscript{2+} cooperativity is determined from the biological experiment in ref. 73. The fitting of our experimental data with equation (6) is quite satisfactory for both the increase of PIP\textsubscript{2}-C2AB complex as mediated by Ca\textsuperscript{2+} and its decrease due to PIP\textsubscript{2} screening by Ca\textsuperscript{2+}. The parameters obtained from the fitting are listed in Supplementary Table 1.

Furthermore, the PIP\textsubscript{2} screening model successfully describes PIP\textsubscript{2}-C2AB \textit{trans} binding (Fig. 1d) at different Mg\textsuperscript{2+} concentrations. For this fitting, we have used the parameters in Supplementary Table 1.

The model has also been extended to the system of PS-mediated \textit{cis} binding (Fig. 2d). Similarly to PIP\textsubscript{2} mediated \textit{trans} binding, C2AB binding (% max) in the PS-mediated \textit{cis} binding, is given by a function of \(C_C\) with an appropriate constant \(c_C\):
\[
y = c_CF_b(C_C) = \frac{c_CC_C^{(e)}C_S}{C_C^{(e)}C_S + K_{SC}(1 + C_C V_S e^{-\epsilon_S/5k_BT})}
\]
(7)

where \(C_S\) denotes the total concentration of PS, \(K_{SC}\) the equilibrium dissociation constant for \textit{cis} binding, \(V_S\) the effective volume of PS, and \(\epsilon_S\) the electric potential energy of a screened PS. Again, considering the limited numbers of PS and C2AB domains, we have the effective Ca\textsuperscript{2+} concentration acting on the \textit{cis} binding: \(C_C^{(e)} = C_C^{(e)\mu} = m_C C_C^{(e)(n^\mu + C_C^{(e)})^{-1}}\) with the maximum (saturation) concentration \(m_C\) and the characteristic Ca\textsuperscript{2+} concentration \(\nu\) corresponding to the half-maximal effective concentration. Equation (7) could fit the nonmonotonic behavior of the \textit{cis} binding between PS and C2AB domains (line in Fig. 2d).

\section*{Statistical analysis.}

The statistical difference between two groups was evaluated by Student’s \(t\) test. Probabilities of \(P \leq 0.05\) (*) were considered significant.

61. Weber, T. \textit{et al.} SNAREpins: minimal machinery for membrane fusion. \textit{Cell} 92, 759–772 (1998).
62. Stein, A., Radhakrishnan, A., Riedel, D., Fasshauer, D. \\& Jahn, R. Synaptotagmin activates membrane fusion through a Ca\textsuperscript{2+}-dependent trans interaction with phospholipids. \textit{Nat. Struct. Mol. Biol.} 14, 904–911 (2007).
63. Park, Y. \textit{et al.} \(\alpha\)-SNAP interferes with the zippering of the SNARE protein membrane fusion machinery. \textit{J. Biol. Chem.} 289, 16326–16335 (2014).
64. Zhou, Z. \& Misler, S. Action potential-induced quantal secretion of catecholamines from rat adrenal chromaffin cells. \textit{J. Biol. Chem.} 270, 3498–3505 (1995).
65. Pannier, M., Veit, S., Godt, A., Jeschke, G. \& Spiess, H.W. Dead-time free measurement of dipole-dipole interactions between electron spins. \textit{J. Magn. Reson.} 142, 331–340 (2000).
66. Stein, A., Weber, G., Wahl, M.C. \& Jahn, R. Helical extension of the neuronal SNARE complex into the membrane. \textit{Nature} 460, 525–528 (2009).
67. Warschawski, D.T., Serbulea, L., Houk, K.N. \& Hubbell, W.L. Conformational analysis of a nitroxide side chain in an \(\alpha\)-helix with density functional theory. \textit{J. Phys. Chem. B} 115, 397–405 (2011).
68. Polychron, Y., Bordignon, E. \& Jeschke, G. Rotamer libraries of spin labelled cysteines for protein studies. \textit{Phys. Chem. Chem. Phys.} 13, 2356–2366 (2011).
69. Cypionka, A. \textit{et al.} Discrimination between docking and fusion of liposomes reconstituted with neuronal SNARE-proteins using FCS. \textit{Proc. Natl. Acad. Sci. USA} 106, 18575–18580 (2009).
70. Fegolari, F., Brigo, A. \& Molinari, H. The Poisson-Boltzmann equation for biomolecular electrostatics: a tool for structural biology. \textit{J. Mol. Recognit.} 15, 377–392 (2002).
71. Li, C., Li, L., Petukh, M. \& Alexov, E. Progress in developing Poisson-Boltzmann equation solvers. \textit{Mol. Based Math. Biol.} 1, 42–62 (2013).
72. Hill, A.V. The combinations of haemoglobin with oxygen and with carbon monoxide. \textit{J. Biol. Chem.} 247–271 (1992).