Electrostatic regulation of the \textit{cis}- and \textit{trans}-membrane interactions of synaptotagmin-1

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Synaptotagmin-1 is a vesicular protein and \textit{Ca}\textsuperscript{2+}-sensor for \textit{Ca}\textsuperscript{2+}-dependent exocytosis. \textit{Ca}\textsuperscript{2+} induces synaptotagmin-1 binding to its own vesicle membrane, called the \textit{cis}-interaction, thus preventing the \textit{trans}-interaction of synaptotagmin-1 to the plasma membrane. However, the electrostatic regulation of the \textit{cis}- and \textit{trans}-membrane interaction of synaptotagmin-1 was poorly understood in different \textit{Ca}\textsuperscript{2+}-buffering conditions. Here we provide an assay to monitor the \textit{cis}- and \textit{trans}-membrane interactions of synaptotagmin-1 by using native purified vesicles and the plasma membrane-mimicking liposomes (PM-liposomes). Both ATP and EGTA similarly reverse the \textit{cis}-membrane interaction of synaptotagmin-1 in free [\textit{Ca}\textsuperscript{2+}] of 10–100 μM. High PIP\textsubscript{2} concentrations in the PM-liposomes reduce the Hill coefficient of vesicle fusion and synaptotagmin-1 membrane binding; this observation suggests that local PIP\textsubscript{2} concentrations control the \textit{Ca}\textsuperscript{2+}-cooperativity of synaptotagmin-1. Our data provide evidence that \textit{Ca}\textsuperscript{2+} chelators, including EGTA and polyphosphate anions such as ATP, ADP, and AMP, electrostatically reverse the \textit{cis}-interaction of synaptotagmin-1.

Exocytosis is the process of vesicle fusion and neurotransmitter release regulated by soluble \textit{N}-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which are currently considered to be the catalysts of the fusion reaction\textsuperscript{12}. Neuronal SNARE proteins are selectively expressed in neurons and neuroendocrine cells, and regulate release of neurotransmitters and hormones\textsuperscript{3}. Neuronal SNARE proteins consist of syntaxin-1 and SNAP-25 in the plasma membrane, and vesicle-associated membrane protein-2 (VAMP-2) (also called synaptobrevin-2) in the vesicle membrane\textsuperscript{1}. Synaptotagmin-1 is a \textit{Ca}\textsuperscript{2+} sensor for fast \textit{Ca}\textsuperscript{2+}-dependent exocytosis as an electrostatic switch\textsuperscript{4}. The C2AB domain of synaptotagmin-1 coordinates \textit{Ca}\textsuperscript{2+} binding, and the \textit{Ca}\textsuperscript{2+}-bound C2AB domain penetrates negatively-charged anionic phospholipids by electrostatic interaction\textsuperscript{2}. Several different models of synaptotagmin-1 to describe the process of \textit{Ca}\textsuperscript{2+}-dependent vesicle fusion have been proposed, but the molecular mechanisms of synaptotagmin-1 remain controversial\textsuperscript{5}.

Synaptotagmin-1 is a vesicular protein and interacts with anionic phospholipids electrostatically\textsuperscript{5}. Native vesicles contain ~ 15% anionic phospholipids including phosphatidylserine (PS) and phosphatidylinositol (PI)\textsuperscript{6}, so \textit{Ca}\textsuperscript{2+} induces synaptotagmin-1 binding to its own vesicle membrane, i.e., the \textit{cis}-interaction\textsuperscript{13,14}. \textit{Ca}\textsuperscript{2+} fails and even slightly reduces vesicle fusion in the in-vitro reconstitution system, because synaptotagmin-1 preferentially interacts with vesicle membranes due to the physical proximity and this \textit{cis}-membrane interaction prevents the \textit{trans}-interaction of synaptotagmin-1 with the target membranes\textsuperscript{7–9}. We have reported that ATP reverses this inactivating \textit{cis}-interaction of synaptotagmin-1 by the electrostatic effect, and the \textit{trans}-membrane interaction of synaptotagmin-1 only occurs to trigger vesicle fusion in-vivo\textsuperscript{10}. This ATP effect on the \textit{cis}-membrane interaction of synaptotagmin-1 has been confirmed independently: in a vesicle sedimentation assay a few hundred μM ATP electrostatically prevents a \textit{cis}-configuration of synaptotagmin-1\textsuperscript{11}, and in a fusion assay using a colloidal probe microscopy and pore-spanning membranes ATP accelerates full fusion by preventing the \textit{cis}-interaction without affecting the trans-interaction of synaptotagmin-1\textsuperscript{12}. However, the electrostatic regulation of the \textit{cis}- and \textit{trans}-membrane interaction of synaptotagmin-1 to trigger \textit{Ca}\textsuperscript{2+}-dependent vesicle fusion has not been described in detail.

Although synaptotagmin-1 is a conserved \textit{Ca}\textsuperscript{2+} sensor for synchronous release of diverse vesicles including synaptic vesicles, large dense-core vesicles (LDCVs), and other secretory granules, the mechanism by which \textit{Ca}\textsuperscript{2+}-cooperativity is regulated is not clear. The Hill coefficient (n) in the \textit{Ca}\textsuperscript{2+} dose–response curves for exocytosis represents \textit{Ca}\textsuperscript{2+}-cooperativity and the Hill coefficient varies depending on cell types from 2 to 5; e.g. calyx-of-Held synapses (n, 4.2)\textsuperscript{13–15}, neuromuscular junctions (n, 3.8)\textsuperscript{16}, bipolar cells (n, 4)\textsuperscript{17}, pituitary melanotrophs (n, 2–4)\textsuperscript{18}. Synaptotagmin-1 is involved in the \textit{Ca}\textsuperscript{2+}-dependent exocytosis of these different \\

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and chromaffin cells (n, 1.8)\(^4\). The Hill coefficient is the intrinsic property of each cell type and factors that regulate Ca\(^{2+}\)-cooperativity are poorly understood.

Synaptotagmin-1 binds to anionic phospholipids by electrostatic interaction and the Ca\(^{2+}\)-binding loops of the C2 domains penetrate anionic phospholipids by reducing repulsion between anionic phospholipids and acidic residues in the C2AB domain\(^6\). The polybasic patch in the C2B domain electrostatically interacts with PIP\(_2\), in a Ca\(^{2+}\)-independent manner\(^8\), and thereby increases the Ca\(^{2+}\)-sensitivity of synaptotagmin-1 membrane binding\(^10,21\). Given that the C2AB domain has five possible Ca\(^{2+}\)-binding sites\(^22,23\) and therefore may have the Hill coefficient up to 4–5, but whether local PIP\(_2\) concentrations regulate Ca\(^{2+}\)-cooperativity is not known.

Here we provide an assay to monitor the cis- and trans-membrane interaction of synaptotagmin-1 by using native LDCVs and the plasma membrane-mimicking liposomes (PM-liposomes). Ca\(^{2+}\) chelators, including EGTA and polyphosphate anions such as ATP, ADP, and AMP, electrostatically reverse the cis-interaction of synaptotagmin-1. Both ATP and EGTA, as Ca\(^{2+}\) chelators, have a similar effect to prevent the cis-membrane interaction of synaptotagmin-1 in free [Ca\(^{2+}\)] of 10–100 μM, but ATP, which has a good buffering capacity in the range of 10–500 μM free [Ca\(^{2+}\)], is an excellent Ca\(^{2+}\) buffer to study vesicle fusion and synaptotagmin-1 membrane binding. When the trans-membrane interaction of synaptotagmin-1 only occurs, high PIP\(_2\) concentrations in the PM-liposomes decrease the Hill coefficient of vesicle fusion and synaptotagmin-1 membrane binding to ~2, suggesting that local PIP\(_2\) concentrations might control Ca\(^{2+}\)-cooperativity of synaptotagmin-1.

**Material and methods**

**Purification of large dense-core vesicles (LDCVs).** LDCVs, also known as chromaffin granules, were purified from bovine adrenal medullae by using continuous sucrose gradient, then resuspended in a solution of 120 mM K-glutamate, 20 mM K-acetate, and 20 mM HEPES-KOH, pH 7.4, as described elsewhere\(^9\).

**Protein purification.** All SNARE and the C2AB domain of synaptotagmin-1 constructs based on rat sequences were expressed in *E. coli* strain BL21 (DE3) and purified by Ni\(^{2+}\)-NTA affinity chromatography followed by ion-exchange chromatography as described elsewhere\(^10,20\). The stabilized Q-SNARE complex consists of syntaxin-1A (aa 183–288) and SNAP-25A (no cysteine, cysteines replaced by alanines) in a 1:1 ratio by the C-terminal VAMP-2 fragment (aa 49–96), and was purified as described earlier\(^9\). The C2AB domain of synaptotagmin-1 (aa 97–421) and soluble form of VAMP-2 lacking the transmembrane domain (VAMP-2\(-\text{TM}\)) were purified using a Mono S column (GE Healthcare, Piscataway, NJ) as described previously\(^26\). The stabilized Q-SNARE complex was purified by Ni\(^{2+}\)-NTA affinity chromatography followed by ion-exchange chromatography on a Mono Q column (GE Healthcare, Piscataway, NJ) in the presence of 50 mM n-octyl-β-D-glucoside (OG)\(^10\). The point mutated C2AB domain (S342C) was labelled with Alexa Fluor 488 C5 maleimide (C2ABA\(_{488}\))\(^26\).

**Lipid composition of liposomes.** All lipids were obtained from Avanti Polar lipids (Alabaster, AL). Lipid composition (mol, %) of the PM-liposomes that contain the Q-SNARE complex was 45% PC (l-α-phosphatidylcholine, Cat. 840055), 15% PE (l-α-phosphatidylethanolamine, Cat. 840042), and 1% PI (l-α-phosphatidylinositol, Cat. 840042), and 1% PI(4,5)P\(_2\) (PIP\(_2\), Cat. 840046). When PIP\(_2\) concentrations were changed, PI contents were adjusted accordingly. For FRET-based lipid-mixing assays, 1.5% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD-DOPA) as a donor dye and 1.5% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl ammonium salt (Rhodamine-DOPA) as an acceptor dye were incorporated in the PM-liposomes (accordingly 12% unlabelled PE).

**Preparation of proteoliposomes.** Incorporation of the Q-SNARE complex into large unilamellar vesicles (LUVs) was achieved by OG-mediated reconstitution, called the direct method, i.e. incorporation of proteins into preformed liposomes\(^10,20\). Briefly, lipids dissolved in a 2:1 chloroform–methanol solvent were mixed according to lipid composition. The solvent was removed using a rotary evaporator to generate lipid film on a glass flask, then lipids were resuspended in 1.5 mL diethyl ether and 0.5 mL buffer containing 150 mM KCl and 20 mM HEPES-KOH, pH 7.4. The suspension was sonicated on ice (3 × 45 s), then multilamellar vesicles were prepared by reverse-phase evaporation using a rotary evaporator as diethyl ether was removed. Multilamellar vesicles (0.5 mL) were extruded using polycarbonate membranes of pore size 100 nm (Avanti Polar lipids) to give uniformly-sized LUVs. After the preformed LUVs had been prepared, SNARE proteins were incorporated into them using OG, a mild non-ionic detergent, then the OG was removed by dialysis overnight in 1 L of buffer containing 150 mM KCl and 20 mM HEPES-KOH pH 7.4 together with 2 g SM-2 adsorbent beads. Proteoliposomes had protein-to-lipid molar ratio of 1:500.

**Vesicle fusion assay.** A FRET-based lipid-mixing assay was applied to monitor vesicle fusion in-vitro\(^10,20\). LDCV fusion reactions were performed at 37 °C in 1 mL fusion buffer containing 120 mM K-glutamate, 20 mM K-acetate, 20 mM HEPES–KOH (pH 7.4), 1 mM MgCl\(_2\), and 3 mM ATP (Fig. 4b). Fusion buffer in Fig. 3a,b contains no ATP, but EGTA; 120 mM K-glutamate, 20 mM K-acetate, 20 mM HEPES–KOH (pH 7.4), 5 mM MgCl\(_2\), and 10 μM EGTA. ATP should be made freshly before all experiments, because it is easily destroyed by freezing and thawing. Free Ca\(^{2+}\) concentration in the presence of Mg\(^{2+}\) and ATP or EGTA was calibrated using the MaxChelator simulation program.

The PM-liposomes that contain NBD-DOPA and Rhodamine-DOPA as a donor and an acceptor dye, respectively, were incubated with LDCVs, thus leading to dequenching of donor fluorescence (NBD) as a result of lipid dilution with unlabelled vesicle membrane\(^10,20\). The fluorescence dequenching signal of vesicle fusion was measured using wavelength of 460 nm for excitation and 538 nm for emission. Fluorescence values were normalized.
as a percentage of maximum donor fluorescence (i.e., total fluorescence) after addition of 0.1% Triton X-100 at the end of experiments.

**Fluorescence anisotropy measurements.** The C2AB fragments (20 nM, S342C) were labelled with Alexa Fluor 488. Anisotropy was measured at 37 °C in 1 mL of buffer containing 120 mM K-glutamate, 20 mM K-acetate, and 20 mM HEPES–KOH (pH 7.4), 5 mM MgCl2, 10 μM EGTA. First, 1 mM Ca2+ was applied, then ATP or EGTA was accordingly added to chelate Ca2+ and reverse the membrane binding of the C2AB domain; each time ATP or EGTA was uniformly mixed by pipetting and a magnetic stirring setup with dilution factor of 1:500 in 1 mL buffer. (Fig. 2). Excitation wavelength was 495 nm and emission was measured at 520 nm. Anisotropy (r) was calculated using the formula 

\[ r = \frac{(IVV - G \times IVH)}{(IVV + 2 \times G \times IVH)} \]

where IVV indicates the fluorescence intensity with vertically polarized excitation and vertical polarization on the detected emission and IVH denotes the fluorescence intensity when using a vertical polarizer on the excitation and horizontal polarizer on the emission. G is a grating factor used as a correction for the instrument’s differential transmission of the two orthogonal vector orientations. Lipid composition of the PM-liposomes (protein-free) was identical to those used in a fusion assay except labelled PE (45% PC, 15% PE, 10% PS, 25% Chol, 4% PI, and 1% PIP2).

**Ca2+ calibration.** ATP contains negatively charged oxygen atoms which bind to Mg2+, Ca2+, or Sr2+, thereby chelating divalent cations. Ca2+ concentrations were calibrated with Fluo-5N, a low-affinity Ca2+ indicator with a Kd of 90 μM. Fluo-5N (500 nM) was included in buffer containing 120 mM K-glutamate, 20 mM K-acetate, 20 mM HEPES–KOH (pH 7.4), 5 mM MgCl2, and 10 μM EGTA. 5 mM ATP, ADP, or AMP (sodium salt, Sigma-Aldrich) was added to chelate free Ca2+. The fluorescence signal was measured at 37 °C with wavelength of 494 nm for excitation and 516 nm for emission. The following equation was used to measure free Ca2+ concentrations:

\[ [\text{Ca}^{2+}]_{\text{free}} = \frac{90 \mu M (F-F_{\text{min}})}{(F_{\text{max}}-F)} \]

where Fmin is the fluorescence intensity in the absence of calcium with 10 mM EGTA, Fmax is the maximum fluorescence with 5 mM CaCl2, and F is the fluorescence of intermediate Fluo-5N. Fluo-5N experimental data with 5 mM ATP were correlated with the MaxChelator simulation program that calculates the free [Ca2+]i.

**Statistical analysis.** All quantitative data are mean ± SD from ≥ 3 independent experiments. Dose–response curves were fitted using four-parameter logistic equations (4PL) (GraphPad Prism) to calculate Hill slope and EC50.

**Results**

Calibration of free [Ca2+] using Fluo-5N and simulation program in the presence of ATP. Ca2+ is a triggering factor of vesicle fusion and intracellular Ca2+ concentration ([Ca2+]i) is typically ~100 nM, but local ([Ca2+]f) and Ca2+ microdomains at the vesicle-release sites close to voltage-gated calcium channels increase to ~300 μM. We used ATP, which is a low affinity Ca2+ buffer, to maintain ~10 ≤ free [Ca2+] ~ 300 μM for in-vitro assays. ATP has a dissociation constant (Kd) ~ 230 μM [Ca2+], so ATP is an excellent Ca2+ buffer in the range of 10–500 μM free [Ca2+]i. We used Fluo-5N to measure free [Ca2+]i in the presence of ATP to confirm the predictions of [Ca2+]i and to determine how much total [Ca2+]i is required to achieve a desired free [Ca2+]i (Fig. 1a–c). Fluo-5N is a low-affinity Ca2+ indicator with a Kd of 90 μM, which is good for measuring around 100 μM free [Ca2+]i, because Kd of Ca2+ chelators should be close to the desired free [Ca2+]i. EGTA (10 μM) was
included to remove contaminating Ca\(^{2+}\) for the calibration of free [Ca\(^{2+}\)]. An initial total 113 μM free [Ca\(^{2+}\)] was reduced to 26 μM in the presence of 5 mM ATP by its chelation of Ca\(^{2+}\) (Fig. 1a,b). Then we compared this experimental data of free [Ca\(^{2+}\)] with the MaxChelator, which is a computer simulation program\(^3\), that enables calculation of appropriate stoichiometric concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) in the presence of different Ca\(^{2+}\) chelators such as EGTA and ATP, and thereby provides detailed information to obtain the desired free [Ca\(^{2+}\)]\(^3\). The MaxChelator program included 5 mM Mg\(^{2+}\) and 10 μM EGTA, and assumed 37 °C as in the Ca\(^{2+}\) calibration experiments (Fig. 1a). Indeed the MaxChelator calculated free [Ca\(^{2+}\)] = 29 μM in the presence of 5 mM ATP with 113 μM total [Ca\(^{2+}\)] at pH 7.4. This agreement with the measured free [Ca\(^{2+}\)] = 26 μM confirms that the MaxChelator can predict free [Ca\(^{2+}\)] obtained in experiments that use a Fluo-5N fluorescent Ca\(^{2+}\) indicator (Fig. 1b).

Negatively-charged oxygen atoms of ATP chelate divalent cations such as Mg\(^{2+}\), Ca\(^{2+}\), or Sr\(^{2+}\)\(^2\). In the experiments, 5 mM ADP or 5 mM AMP chelated Ca\(^{2+}\), thereby reducing free [Ca\(^{2+}\)] from 122 to 57 μM and from 126 to 99 μM, respectively (Fig. 1c). Increasing the number of phosphate groups in Adenosine increases Ca\(^{2+}\) affinity and lowers K\(_d\) by increasing the number of Ca\(^{2+}\) ions that are bound\(^2\). ATP, ADP, and AMP have distinct ranges of Ca\(^{2+}\)-buffering capacity and distinct K\(_d\) values\(^3\), so Ca\(^{2+}\)-chelating effect is ATP > ADP > AMP (Fig. 1a–c). Altogether, the predictions of free [Ca\(^{2+}\)] in the complex buffer solutions including Mg\(^{2+}\), ATP and EGTA were confirmed using a fluorescent Ca\(^{2+}\) indicator (Fig. 1b).

Monitoring the cis- and trans-membrane interaction of synaptotagmin-1. Synaptotagmin-1 interacts with anionic phospholipids by electrostatic interaction. Native vesicles contain ~15% anionic phospholipids, including phosphatidylserine (PS) and phosphatidylinositol (PI)\(^1\). Therefore, Ca\(^{2+}\) induces synaptotagmin-1 to bind to its own vesicle membrane, i.e., cis-interaction, which prevents trans-interaction to the plasma membranes and thereby inactivates the ability of synaptotagmin-1 to trigger fusion\(^7\). Ca\(^{2+}\)-bound synaptotagmin-1 is inserted to native vesicle membranes such as synaptic vesicles and large dense-core vesicles (LDCVs) that contain anionic phospholipids\(^5\). However, ATP electrostatically prevents the cis-interaction of synaptotagmin-1, whereas the trans-interaction of synaptotagmin-1 to the plasma membrane remains active to mediate Ca\(^{2+}\)-dependent vesicle fusion, because PIP\(_2\) overcomes the inhibitory effect of ATP by increasing the membrane-binding affinity of the C2AB domain\(^10\).
exogenously-added C2AB domain of synaptotagmin-1 (Syt97-421), which was labelled with Alexa Fluor 488 at endogenous synaptotagmin-1 in native vesicle membranes is impossible, so we monitored the binding of an interaction of synaptotagmin-1 (Fig. 2). Direct measurement of the (Fig. 2a). The presence of 1 mM Ca\(^{2+}\) increased fluorescence anisotropy; this change indicates that the C2AB interaction of the C2AB domain to native vesicles or liposomes; the membrane-bound C2AB domain leads to increase of fluorescence anisotropy due to a reduction in the rotational mobility\(^{10}\) (Fig. 2a,b). It is noted that our experiments using the cytoplasmic C2AB domain are intended to shed light on the cis- and trans-interactions, but the geometry is not truly being imitated.

We first monitored the cis-membrane interaction between the C2AB domain and the LDCV membranes (Fig. 2a). The presence of 1 mM Ca\(^{2+}\) increased fluorescence anisotropy; this change indicates that the C2AB domains bind to LDCV membranes in a Ca\(^{2+}\)-dependent manner. Five sequential applications of 1 mM ATP gradually decreased the anisotropy signal by chelating Ca\(^{2+}\); this result suggests dissociation of the C2AB domain from LDCVs (Fig. 2a). 5 mM ATP in the presence of 1 mM Ca\(^{2+}\) almost completely disrupted the cis-membrane interaction of the C2AB domain with the LDCV membranes (Fig. 2a); free [Ca\(^{2+}\)] in the presence of Mg\(^{2+}\), ATP and EGTA was calibrated using the MaxChelator simulation program and free [Ca\(^{2+}\)] was 351 μM in case of 5 mM ATP and 1 mM Ca\(^{2+}\) (Table 1).

Next, we tested the trans-membrane interactions between the C2AB domain and the PM-liposomes; 10% PS, 4% PI, and 1% PIP\(_2\) were included in the PM-liposomes (Fig. 2b). The C2AB domain of synaptotagmin-1 bound to liposomes in response to 1 mM Ca\(^{2+}\), and this trans-membrane interaction was reduced by ATP, 1 mM applied thirteen times sequentially (Fig. 2b). Free [Ca\(^{2+}\)] in different ATP concentrations was summarized in Table 1. Ca\(^{2+}\)-dependent vesicle fusion is accelerated by the increase of the cis-membrane interaction of synaptotagmin-1\(^{10,20}\), so we hypothesized that 5 mM ATP in the presence of 1 mM Ca\(^{2+}\) is appropriate to observe Ca\(^{2+}\)-dependent fusion (red in Fig. 1a,b).

To test this hypothesis and examine the effect of the cis- and trans-membrane interaction of synaptotagmin-1 on vesicle fusion, we applied a reconstitution system of vesicle fusion by using native LDCVs\(^{20,25-28}\). The PM-liposomes contain the stabilized Q-SNARE complex (syntaxin-1A and SNAP-25A in a 1:1 molar ratio\(^{25}\)). Indeed, 5 mM ATP in the presence of 1 mM Ca\(^{2+}\) (i.e., 351 μM free [Ca\(^{2+}\)]) according to the MaxChelator program (Table 1) dramatically accelerated LDCV fusion, which was completely blocked by the soluble VAMP-2 (VAMP-2,106); this results indicates SNARE-dependent vesicle fusion (Fig. 2c). We have previously shown that 300–400 μM free [Ca\(^{2+}\)] in the absence of ATP fails to enhance vesicle fusion, but rather slightly inhibits fusion, because the cis-membrane interaction of the C2AB domain to native vesicle membranes becomes robust from 100 μM up to 3 mM\(^{10}\). ATP prevents this cis-membrane interaction by charge screening and competing with the vesicle membrane, thus allowing synaptotagmin-1 to interact in trans with the plasma membrane\(^{10}\).

Polyporphates such as ATP reverse an inactivating cis-interaction of synaptotagmin-1 by an electrostatic effect (Fig. 2a–c). Next, we tested whether other Ca\(^{2+}\) chelators, e.g., EGTA, can have a similar inhibitory effect on the cis-membrane interaction. Anisotropy measurement was performed to monitor the cis- and trans-membrane interaction of the C2AB domain (Fig. 2a,b). EGTA was applied 10 times (100 μM each in the presence of 1 mM Ca\(^{2+}\)) to reverse the cis-interaction of the C2AB domain to LDCVs (Fig. 2d). Application of 800 μM EGTA dramatically disrupted the cis-interaction in the presence of total 1 mM Ca\(^{2+}\) (red in Fig. 2d); free [Ca\(^{2+}\)] was 200 μM (Table 1). However, the trans-membrane interactions of the C2AB domain to the PM-liposomes remained robust in the presence of 800 μM EGTA with 1 mM Ca\(^{2+}\) (200 μM free [Ca\(^{2+}\)], Fig. 2e), whereas 1 mM EGTA significantly disrupted both the cis- and trans-membrane interactions of the C2AB domain (Fig. 2d,e); free [Ca\(^{2+}\)] was 12 μM (Table 1).

Anisotropy measurement is useful to find a Ca\(^{2+}\)-buffering condition to observe Ca\(^{2+}\)-dependent vesicle fusion, where the cis-membrane interaction is prevented and the trans-interaction remains active. The presence of 800 μM EGTA with 1 mM Ca\(^{2+}\) (200 μM free [Ca\(^{2+}\)], Table 1) significantly reversed the cis-interaction (Fig. 2d), but had a minor effect on the trans-interaction (Fig. 2e). Indeed, 800 μM EGTA with 1 mM Ca\(^{2+}\) reproduced Ca\(^{2+}\)-dependent LDCV fusion (Fig. 2f). 1 mM EGTA with 1 mM Ca\(^{2+}\) (12 μM free [Ca\(^{2+}\)], Table 1) failed to accelerate fusion, because the trans-interaction of the C2AB domain was dramatically disrupted by 1 mM EGTA (red in Fig. 2e); it is mainly because of low free [Ca\(^{2+}\)]. Taken together, we established an anisotropy assay to monitor the cis- and trans-membrane interaction of synaptotagmin-1 by using native LDCVs and the PM-liposomes. Our data suggest that Ca\(^{2+}\) chelators such as EGTA, in addition to polyporphates such as ATP, can prevent the cis-membrane interaction of synaptotagmin-1 by the electrostatic effect in a certain range of free [Ca\(^{2+}\)].

**Table 1.** Calibration of free Ca\(^{2+}\) concentration in the presence of ATP or EGTA. a, The MaxChelator simulation program was used to calculate free Ca\(^{2+}\) concentration in the presence of ATP or EGTA. b, 1 mM Ca\(^{2+}\), 5 mM Mg\(^{2+}\), and 10 μM EGTA.

| ATP concentration [mM] | Free Ca\(^{2+}\) [μM] (total 1 mM Ca\(^{2+}\)) | EGTA concentration [μM] | Free Ca\(^{2+}\) [μM] (total 1 mM Ca\(^{2+}\)) |
|------------------------|-------------------------------|------------------------|-------------------------------|
| 1                      | 896                           | 100                    | 900                           |
| 2                      | 785                           | 200                    | 800                           |
| 4                      | 508                           | 300                    | 700                           |
| 5                      | 351                           | 800                    | 200                           |
| 13                     | 31                            | 1000                   | 12                            |

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120 mM K-glutamate, 20 mM K-acetate, 20 mM HEPES–KOH (pH 7.4), 5 mM MgCl2, and 10 μM EGTA. The Ca2+ dose–response of the 300 μM to 1 mM (Fig. 3a,b). Ca2+ dose–response curves of vesicle fusion and the numbers of Ca2+ ions coordinated to one synaptotagmin-1 might be reduced to 2–3 (see section “Discussion”).

The presence of 10 μM EGTA, instead of ATP. (b) Dose–response curve of LDCV fusion at various free [Ca2+]i. Fusion is normalized as a percentage of control (No Ca2+). (c) Ca2+ dose–response curve for C2AB binding to LDCVs in the presence of 10 μM EGTA using anisotropy as described in Fig. 2a. Data in (bc) are mean ± SD from three independent experiments (n = 3).

Free [Ca2+]i were calibrated using the MaxChelator simulation program. (a–c) ATP was not included in buffer: 120 mM K-glutamate, 20 mM K-acetate, 20 mM HEPES–KOH (pH 7.4), 5 mM MgCl2, and 10 μM EGTA.

EGTA reproduces the biphasic regulation of Ca2+ on LDCV fusion. We have previously reported the biphasic regulation of Ca2+ on LDCV fusion; 10–100 μM free Ca2+ exponentially accelerates native vesicle fusion, but > 300 μM free [Ca2+]i progressively reduces Ca2+-dependent fusion, showing biphasic regulation of Ca2+ on LDCV fusion in a bell-shaped dose-dependence. ATP was used for Ca2+-buffering to maintain free [Ca2+]i in the range of 10–500 μM. We examined whether EGTA reproduces this biphasic regulation of Ca2+ on LDCV fusion (Fig. 3a,b). Instead of ATP, 10 μM EGTA was included in fusion buffer and free [Ca2+]i was calculated using the MaxChelator program. As expected, biphasic regulation of Ca2+ on LDCV fusion was observed, where Ca2+-dependent fusion progressively increased until [Ca2+]i = ~ 100 μM, and gradually decreased at [Ca2+]i from 300 μM to 1 mM (Fig. 3a,b).

The Ca2+ dose–response of the cis-interaction of synaptotagmin-1 20, because ATP effectively buffers free [Ca2+]i in the range of 10–500 μM, but EGTA cannot efficiently buffer free [Ca2+]i in this range.

PIP2 concentration regulates Ca2+ cooperativity of synaptotagmin-1. Synaptotagmin-1 binds to anionic phospholipids by electrostatic interaction and the Ca2+-binding loops of the C2 domains are inserted to anionic phospholipids in a Ca2+-dependent manner; aspartate residues of the Ca2+-binding loops in the C2-domains together with anionic membrane lipids coordinate Ca2+-ions12,36. PIP2 enhances Ca2+-sensitivity of synaptotagmin-1 by interacting with the polybasic patch in the C2B domain10,21. Ca2+-cooperativity of synaptotagmin-1 varies among cell types, with the Hill coefficients ranging from ~ 2 to ~ 5. We tested that PIP2 also regulates Ca2+-cooperativity of synaptotagmin-1 for membrane binding (Fig. 4a, Table 2) and vesicle fusion (Fig. 4b, Table 2). Increases of PIP2 concentration from 1 to 5% in the PM-liposomes shifted Ca2+ titration curves for membrane binding to the left side; this change indicates increased Ca2+ sensitivity, but reduced Ca2+ cooperativity (Fig. 4a, Table 2).

Next, we observed that Ca2+-cooperativity of synaptotagmin-1 for vesicle fusion was also reduced by increasing PIP2 concentration, correlating with the Ca2+-cooperativity of synaptotagmin-1 for membrane binding. The Ca2+ dose–response curve for LDCV fusion was shifted leftward as PIP2 concentration was increased in the PM-liposomes (Fig. 4b, Table 2). Taken together, high PIP2 concentration increases the sensitivity of synaptotagmin-1 to Ca2+, but lowers Ca2+ cooperativity. These changes imply that increasing the negative electrostatic potential in the plasma membranes attracts Ca2+-bound synaptotagmin-1 with low Ca2+ cooperativity, in which the total numbers of Ca2+ ions coordinated to one synaptotagmin-1 might be reduced to 2–3 (see section “Discussion”).

Discussion

The cis-binding of synaptotagmin-1 occurs in native vesicles such as LDCVs and synaptic vesicles, and inactivates Ca2+-dependent vesicle fusion by preventing the trans-interaction of synaptotagmin-1. Independent groups have confirmed that ATP at physiological concentrations disrupts such cis-interaction of synaptotagmin-111,12,37.

Figure 3. EGTA reproduces ATP effect on Ca2+-dependent LDCV fusion and the C2AB binding to LDCVs. (a,b) LDCV fusion using a lipid-mixing assay as described in Fig. 2c at different concentrations of Ca2+ in the presence of 10 μM EGTA, instead of ATP. (a) Representative trace of dequenching of donor fluorescence (NBD). (b) Dose–response curve of LDCV fusion at various free [Ca2+]i. Fusion is normalized as a percentage of control (No Ca2+). (c) Ca2+ dose–response curve for C2AB binding to LDCVs in the presence of 10 μM EGTA using anisotropy as described in Fig. 2a. Data in (bc) are mean ± SD from three independent experiments (n = 3).

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Here we show that Ca^{2+} chelators, including EGTA and polyphosphate anions such as ATP, ADP, and AMP, electrostatically reverse the cis-interaction of synaptotagmin-1. We propose that Ca^{2+} chelators compete with vesicle membranes that contain anionic phospholipids in binding to Ca^{2+} and disrupt the cis-interaction of synaptotagmin-1 by charge screening. However, PIP_2 overcomes this inhibitory effect of ATP, because PIP_2 dramatically enhances the Ca^{2+}-binding affinity of synaptotagmin-1; this high Ca^{2+} affinity of the C2AB domain to PIP_2-containing membranes is not affected by ATP. EGTA and 1,2-bis(o-aminophenoxy)ethane-N,N,N0,N0-tetraacetic acid (BAPTA) are well-known and reliable Ca^{2+} buffers in the range of 10 nM–1 μM [Ca^{2+}] at the typical intracellular pH of 7.2. Given that EGTA and BAPTA have a K_d of 67 nM and 192 nM [Ca^{2+}] at pH 7, respectively, and have a higher affinity for Ca^{2+} than for Mg^{2+}, both EGTA and BAPTA effectively buffer free [Ca^{2+}] only at concentrations < 1 μM, which is close to intracellular free [Ca^{2+}]. However, EGTA is sensitively dependent on pH, and BAPTA family has a strong dependence on ionic strength; importantly, because EGTA and BAPTA have nanomolar-level K_d, they poorly buffer free [Ca^{2+}] in the range of 10–500 μM. In contrast, ATP has K_d 230 μM and is an excellent buffer for free [Ca^{2+}] in the range of 10–500 μM.

Synaptotagmin-1 is a low-affinity Ca^{2+} sensor; 10–100 μM [Ca^{2+}] exponentially induce synaptotagmin-1 binding to membrane that contain PS and PIP_2 with K_d ~ 50 μM. Therefore, ATP is an appropriate and better Ca^{2+} buffer than EGTA or BAPTA to study the synaptotagmin-1 activity to bind membrane and trigger vesicle fusion. Indeed, we observed that ATP and EGTA result in different kinetics of the Ca^{2+} dose–response curves of vesicle fusion and of the cis-interaction of synaptotagmin-1 (Fig. 3b,c), because ATP has a different Ca^{2+}-buffering capacity than EGTA.

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**Figure 4.** PIP_2 concentration regulates Ca^{2+} sensitivity and cooperativity of synaptotagmin-1. (a) Membrane binding of the C2AB domain of synaptotagmin-1 was monitored using anisotropy as in Fig. 2b. Ca^{2+} dose–response curve for C2AB binding to the PM-liposomes that include PS and PIP_2. C2AB binding is presented as a percentage of maximum C2AB binding. (b) Ca^{2+} dose–response curve for LDCV fusion with the PM-liposomes containing different PIP_2 concentrations. Fusion is normalized as a percentage of maximum fusion. Data in (a,b) are mean ± SD from three independent experiments (n = 3). 3 mM MgCl_2 and 1 mM ATP were included in buffer, and free [Ca^{2+}] was calibrated using the MaxChelator simulation program.

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**Table 2.** Hill slope and EC_{50} of Ca^{2+} dose–response curve. Hill slope and EC_{50} of Ca^{2+} dose–response curve were calculated using four-parameter logistic equations in GraphPad Prism. Data in the table are means ± SE (standard error) from three to five independent experiments. All experiments were carried out in buffer containing 3 mM ATP and 1 mM MgCl_2 (section "Material and methods").

| Methods                     | Synaptotagmin-1 | Hill slope† | EC_{50} (μM) † | Anionic phospholipids (%)‡ |
|-----------------------------|-----------------|-------------|----------------|--------------------------|
| Anisotropy                   | C2AB            | 3.39 ± 1.29 | 37.7 ± 2.9     | PIP_2 (1), PS (15), PI (4) |
|                             | C2AB            | 1.92 ± 0.7  | 9.7 ± 1.7      | PIP_2 (5), PS (15)       |
| Fusion (LDCV and liposomes)  | Full length     | 4.57 ± 1.14 | 59.4 ± 2.47    | PIP_2 (0.5), PS (15), PI (4.5) |
|                             | Full length     | 2.69 ± 0.08 | 27.1 ± 0.29    | PIP_2 (1), PS (15), PI (4) |
|                             | Full length     | 2.16 ± 0.18 | 6.96 ± 0.29    | PIP_2 (5), PS (15)       |

† Hill slope and EC_{50} of Ca^{2+} dose–response curve were calculated using four-parameter logistic equations in GraphPad Prism. Data in the table are means ± SE (standard error) from three to five independent experiments.

‡ Lipid compositions of anionic phospholipids in liposomes. a Endogenous synaptotagmin-1 from purified native LDCVs.
The $K_d$ of low-affinity Ca$^{2+}$ indicator dyes can vary depending on ionic strength and is changed by anions such as ATP$^{81}$; e.g., the $K_d$ of low-affinity Ca$^{2+}$ indicator dyes is increased by ATP and slightly decreased by excess Mg$^{2+}$. The $K_d$ of Fluo-5N can be altered by the presence of ATP/Mg$^{2+}$, which makes it difficult to accurately measure free [Ca$^{2+}$]. ATP binds both Ca$^{2+}$ and Mg$^{2+}$ with a different affinity$^{73,74}$, so computer simulation programs$^{32,33}$ like the MaxChelator are useful to calibrate free [Ca$^{2+}$] in the presence of Mg$^{2+}$, ATP or EGTA by calculating free [Mg$^{2+}$], [Ca-ATP], and [Mg-ATP]$^{35}$. We confirmed the MaxChelator-based predictions using a Fluo-5N fluorescent Ca$^{2+}$ indicator (Fig. 1b).

Both the C2A and C2B domains of synaptotagmin-1 have highly cooperative Ca$^{2+}$-dependent binding to membranes that contain anionic phospholipids$^{36,42–46}$. Furthermore, synaptotagmin-1 contains a polybasic region within the C2B domain that binds to PIP$_2$, in an Ca$^{2+}$-independent manner$^{46,47}$ and enhances Ca$^{2+}$ sensitivity of synaptotagmin-1 membrane binding$^{48}$ and exocytosis$^{49}$. The C2AB domain has five possible Ca$^{2+}$-binding sites$^{22,23}$; negatively charged oxygen atom from acidic aspartate residues in the C2AB domain and negatively charged oxygen atom from anionic phospholipids provide complete coordination sites for Ca$^{2+}$, which cooperativity of the C2AB domain seems reasonable when the Hill coefficient is ~4 to 5, but what regulates Ca$^{2+}$ cooperativity remains poorly understood, e.g., low Hill coefficient (n, 2–3) in neuroendocrine cells such as pituitary melanotrophs (n, 2.5)$^{48}$ and chromaffin cells (n, 1.8)$^{35}$, but high Hill coefficient in synapses including calyx-of-Held synapses (n, 4.2)$^{11–13}$, neuromuscular junctions (n, 3.8)$^{49}$, and bipolar cells (n, 4)$^{50}$. We oversaw that increasing PIP$_2$ concentration reduces the Hill coefficient, which represents Ca$^{2+}$ cooperativity (Fig. 4). Our data support that local PIP$_2$ concentration might control Ca$^{2+}$ cooperativity by allosterically-stabilized dual binding of synaptotagmin-1 to Ca$^{2+}$ and PIP$_2$.$^{48}$.

In this study, we investigate the electrostatic regulation of C2AB binding to vesicle membrane and the PM-liposomes. We have previously observed that Ca$^{2+}$-independent interactions of the C2AB domain with the PM-liposomes containing anionic phospholipids (10% PS/1% PIP$_2$) is significantly disrupted in the presence of physiological concentration of ATP/Mg$^{2+}$, but this Ca$^{2+}$-independent interaction remains strong when the PM-liposomes contain high PIP$_2$ (10% PS/5% PIP$_2$), suggesting that high PIP$_2$ concentrations are required for Ca$^{2+}$-independent binding of the C2AB domain in physiological ionic strength$^{30}$. Here, we have used 10% PS/1% PIP$_2$ in the PM-liposomes to selectively examine the Ca$^{2+}$-dependent membrane interaction and binding of the C2AB domain. However, in the pre-fusion state for vesicle docking and priming, the C2AB domain of synaptotagmin-1 is most likely bound to the plasma membrane through the PIP$_2$-interacting polybasic region of the C2B domain$^{30}$ or the SNARE complex$^{48}$ in a Ca$^{2+}$-independent manner. Ca$^{2+}$ can induce a re-orientation of the C2AB domain on the plasma membrane by changing the binding mode with the SNARE complex$^{49}$ or PIP$_2$. This change in orientation may act as a switch to trigger synaptotagmin-1-dependent vesicle fusion in neurons and neuroendocrine cells. Our results do not rule out the possibility for Ca$^{2+}$-independent interactions of synaptotagmin-1 with the SNARE complex despite extremely weak interaction$^{49}$ and it remains a topic of further study to include Ca$^{2+}$-independent interactions of synaptotagmin-1 in our system for physiological relevance.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable requests.

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Y.P. and H.Y.A.M. purified vesicles and performed experiments. Y.P. collected and analyzed data. Y.P. wrote the manuscript and all authors read and provided their comments.

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