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The importance of an asymmetric distribution of acidic lipids for synaptotagmin 1 function as a Ca^{2+} sensor

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Abstract

Syt1 (synaptotagmin 1) is a major Ca^{2+} sensor for synaptic vesicle fusion. Although Syt1 is known to bind to SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) complexes and to the membrane, the mechanism by which Syt1 regulates vesicle fusion is controversial. In the present study we used *in vitro* lipid-mixing assays to investigate the Ca^{2+} -dependent Syt1 function in proteoliposome fusion. To study the role of acidic lipids, the concentration of negatively charged DOPS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine) in the vesicle was varied. Syt1 stimulated lipid mixing by 3–10-fold without Ca^{2+} . However, with Ca^{2+} there was an additional 2–5-fold enhancement. This Ca^{2+} -dependent stimulation was observed only when there was excess PS (phosphatidylserine) on the t-SNARE (target SNARE) side. If there was equal or more PS on the v-SNARE (vesicle SNARE) side the Ca^{2+} -dependent stimulation was not observed. We found that Ca^{2+} at a concentration between 10 and 50 μM was sufficient to give rise to the maximal enhancement. The single-vesicle-fusion assay indicates that the Ca^{2+} -dependent enhancement was mainly on docking, whereas its effect on lipid mixing was small. Thus for Syt1 to function as a Ca^{2+} sensor, a charge asymmetry appears to be important and this may play a role in steering Syt1 to productively *trans* bind to the plasma membrane.

Keywords

charge asymmetry; 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS); soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor (SNARE); synaptotagmin 1; vesicle fusion

AUTHOR CONTRIBUTION

Ying Lai and Yeon-Kyun Shin designed the experiments, Ying Lai carried out the experiments, and Ying Lai and Yeon-Kyun Shin wrote the paper.

INTRODUCTION

Neurotransmitter release at synapses underlies major brain functions such as cognition, emotion and memory. The precise temporal control of the release is essential for healthy brain activities. SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptors) are known to be the core fusion machinery in neuro-exocytotic pathways [1–3]. SNARE complex formation between two membranes is mediated by the cognate coiled-coil motifs of t- and v-SNAREs (target and vesicle SNAREs respectively); one such motif from the t-SNARE syntaxin 1A, two motifs from the t-SNARE SNAP-25 (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein-25) and another motif from the v-SNARE VAMP2 (vesicle-associated membrane protein 2) form a parallel four-stranded coiled-coil, which brings about apposition of two membranes [4,5]. The SNARE complex itself, however, does not appear to have the required regulatory capacity that confers the temporal on/off switching of membrane fusion. A vesicle protein Syt1 (synaptotagmin 1) is instead believed to be the key regulator for the temporal control of SNARE-dependent synaptic vesicle fusion [6–8]. Syt1 senses the spike of the Ca^{2+} level in the cytoplasm, which results from the action potential, and helps trigger fast fusion [9,10].

At the molecular level, Syt1 is known to bind SNARE complexes [11–13] and the membrane [14–16]. Syt1 has been shown to have affinity to the binary t-SNARE complex [17,18]. Consistently, several *in vivo* and *in vitro* studies have suggested that Syt1 is required for vesicle docking to the plasma membrane [10,19], most probably via its interaction with the t-SNARE complex. Also, Syt1 is known to bind to the ternary SNARE complex [11–13,20,21]. According to the fusion clamp model, complexins, a family of small soluble proteins that bind to the SNARE complex [22,23], clamp the fusion by preventing SNAREs from completing complex formation [24–28]. It has been proposed that Syt1 binding to the SNARE complex displaces complexin, allowing complete SNARE assembly and membrane fusion.

Syt1 binding to the membrane appears to be essential for membrane fusion [29–31]. For example, alanine mutations in the Ca^{2+} -binding loop of Syt1 impairs the lipid-binding activity, which results in the complete abrogation of the evoked release. But tryptophan mutations in the loop region lead to a higher lipid-binding affinity and, consequently, a higher Ca^{2+} -dependent enhancement in membrane fusion than the wild-type [30,31]. It has been suggested that Syt1 has an ability to buckle the membrane to create a locally positive curvature [31,32], which is believed to help membrane fusion [33], although such a mechanism is not fully substantiated experimentally. Interestingly, negatively charged PIP_2 (phosphatidylinositol 4,5-bisphosphate) appears to play a specific role in Syt1 function [34,35]. Syt1 may directly interact with PIP_2 , even in the absence of Ca^{2+} [34]. Moreover, PIP_2 is shown to facilitate the membrane penetration of Syt1 into the membrane and regulates the Ca^{2+} -dependent enhancement of vesicle fusion [34]. It has previously been shown that PIP_2 has the tendency to cluster near the t-SNARE complex [36], which could help the simultaneous interaction of Syt1 to both SNARE complexes and the membrane [13].

Our recent single-vesicle-fusion study indicated that for the full-length Syt1 the *trans* surface charge asymmetry with some excess charges on the t-vesicles is necessary for Ca^{2+} -dependent enhancement of vesicle fusion [10]. Alternatively, excess acidic phospholipids on the v-vesicles could cause the *cis* interaction of Syt1, which leads to the loss of the Ca^{2+} -dependent stimulatory function of Syt1 [10,37]. This result partly explains the reason why reconstituted Syt1 has consistently failed to show Ca^{2+} -dependent stimulation for SNARE-dependent lipid mixing. It turns out that in all of those previous studies of Syt1 the influence of *trans* charge asymmetry has not been explored.

In the present study, we extend our investigation of the effect of the *trans* surface charge asymmetry on Syt1 function. By changing the concentration of a negatively charged lipid, DOPS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine), on the vesicle, we verify that an excess amount of acidic phospholipids on the t-vesicles (vesicles containing t-SNAREs) compared with that on the v-vesicles (vesicles containing v-SNAREs) is important for the Ca^{2+} -dependent stimulation of lipid mixing by Syt1. Under these conditions we observed stimulation by Syt1 with a physiological level of Ca^{2+} in the range 10–50 μM . Furthermore, the single-vesicle-fusion assay, which can separate vesicle docking from lipid mixing, indicates that the stimulation by Ca^{2+} is mainly on the docking step, whereas the enhancement of the fusion step is modest.

MATERIALS AND METHODS

Protein expression and purification

Recombinant neuronal SNARE proteins from rat, syntaxin 1A (amino acids 1–288), SNAP-25 (amino acids 1–206), VAMP2 (amino acids 1–116) and Vps (soluble VAMP2, amino acids 1–96) were expressed as N-terminal GST (glutathione transferase)-fusion proteins. These fusion proteins were expressed in *Escherichia coli* Rosetta (DE3) pLysS cells (Novagene). The cells were grown at 37 °C in LB (Luria–Bertani) medium with 100 $\mu\text{g}/\text{ml}$ ampicillin until the absorbance at 600 nm reached 0.6–0.8. The cells were further grown overnight after adding IPTG (isopropyl β -D-thiogalactopyranoside, 0.5 mM final concentration) at 16 °C. Proteins were purified using glutathione–agarose chromatography. Cell pellets were resuspended in 20 ml of PBS (pH 7.4), containing 0.2 % Triton X-100, with final concentrations of 1 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride] and 2 mM DTT (dithiothreitol). Cells were broken by sonication in an ice bath and centrifuged at 27 000 g for 30 min at 4 °C. The supernatant was mixed with 2 ml of glutathione–agarose beads in PBS by rocking in a cold room (4 °C) for 2 h. The proteins were then cleaved by thrombin in cleavage buffer [50 mM Tris/HCl and 150 mM NaCl (pH 8.0)] with 0.8 g of OG (*n*-octyl-D-glucopyranoside) per 100 ml for neuronal SNAREs, or cleaved by thrombin in cleavage buffer for Vps. The full-length Syt1 (amino acids 51–421) was expressed as a C-terminal His₆-tagged protein in *E. coli* BL21 Rosetta (DE3) pLysS cells (Novagen) and purified using the same protocol as above using a Ni-NTA (Ni²⁺-nitrilotriacetate) column. His₆-tagged full-length Syt1 was eluted with elution buffer [25 mM Hepes (pH 9.0), 400 mM KCl, 500 mM imidazole and 0.8 % OG]. After removing imidazole with a PD10 desalting column (GE Healthcare), the eluted Syt1 was kept in the cleavage buffer containing 0.8 g of OG per 100 ml and 1 mM EDTA. DNA sequences were confirmed by

the Iowa State University DNA Sequencing Facility. Purified proteins were examined by SDS/PAGE (15 % gels). Purity was at least 85 % for all proteins.

Membrane reconstitution

The lipid mixture of DOPS, POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), PIP2 (from porcine brain), cholesterol, biotin-DPPE [1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamin-N-(biotinyl)], DiI [1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Invitrogen)] and DiD [1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Invitrogen)] (all lipids except DiI and DiD were obtained from Avanti Polar Lipids) in chloroform was dried in a vacuum and was resuspended in buffer [25 mM Hepes/KOH and 100 mM KCl (pH 7.4)] to make the total lipid concentration approximately 10 mM. For the bulk lipid-mixing assays, the t-vesicles contained DiI, POPC, cholesterol and DOPS at a molar ratio of 1.5:63.5:20:15, whereas the v-vesicles contained DiD, POPC and cholesterol at a molar ratio of 1.5:78.5:20. PS (phosphatidylserine) on both vesicles was varied from 0 to 35%, in expense of POPC, and 2% PIP₂ was added to t-vesicles by again replacing an equal amount of POPC. In the single-vesicle fusion assay, 0.1% biotin-DPPE was added to the v-vesicles. Protein-free large unilamellar vesicles (~100 nm in diameter) were prepared by extrusion through polycarbonate filters (Avanti Polar Lipids).

To reconstitute the membrane proteins into liposomes, syntaxin 1A and SNAP-25 at a molar ratio of 1:1.5 were premixed and the mixture was left at room temperature (25 °C) for 1 h to form the complex before reconstitution. VAMP2 and Syt1 were mixed at a molar ratio of 1:1 containing 1 mM EDTA. For membrane reconstitution, SNAREs were mixed with vesicles at a protein/lipid molar ratio of 1:200 with ~0.8 g of OG per 100 ml in cleavage buffer (50 mM Tris/HCl and 150 mM NaCl, pH 8.0) at 4 °C for 15 min. The mixture was diluted 2-fold with dialysis buffer [25 mM Hepes and 100 mM KCl (pH 8.0)], and this diluted mixture was then dialysed in 2 litres of dialysis buffer at 4 °C overnight. It is likely that Syt1 with EDTA and at a higher PH can reduce the aggregation of vesicles during dialysis.

Lipid-mixing FRET (fluorescence resonance energy transfer) assays

Reconstituted t- and v-vesicles were mixed at a ratio of 1:1. The final lipid concentration in the reaction was 0.05 mM. The fluorescence intensity was monitored in two channels with an excitation wavelength of 530 nm and emission wavelengths of 570 and 670 nm for the DiI and DiD fluorescence dye pair respectively. Fluorescence changes were recorded with the Varian Cary Eclipse model fluorescence spectrophotometer using a quartz cell of 100 μ l with a 2 mm pathlength. All measurements were performed at 35 °C.

Single-vesicle-fusion FRET assay

Details of the single-vesicle-fusion FRET assay have been described elsewhere [38]. A quartz slide was cleaned using the 5 % Alconox solution followed by 1 M potassium hydroxide, and then coated with 99:1 (mol/mol) mPEG [methoxy[poly(ethylene glycol)]]:biotin-PEG (Laysan Bio). This PEG-treated quartz slide was placed as the bottom surface of a microfluidic chamber to be used as the imaging surface of our prism-type TIRF

(total internal reflection fluorescence) microscopy (based on IX-71 microscopy, Olympus). To monitor interactions between single v- and t-vesicles, the v-vesicles (30 μ M lipid concentration) were attached on this quartz imaging surface by neutravidin (Invitrogen) which acts as molecular glue between biotin-PEG and biotinylated lipids. The t-vesicles (10 μ M lipid concentration) and the Ca^{2+} ion solution were mixed together just before the main reaction. This t-vesicle- Ca^{2+} mixture was introduced into the flow chamber for the fusion reaction, and the temperature was maintained at 37 °C during the reaction for 0.5 h.

In the docking number analysis, we used a computer algorithm which detects local Gaussian maxima in the TIRF images that were recorded by an electron-multiplying charge-coupled device (iXon DU897E, Andor technology) to count the number of the single vesicle complexes in a given area (program available at <http://bio.physics.illinois.edu/>). Our TIRF microscopy monitored the imaging area of $45 \times 90 \mu\text{m}^2$ at a time, and we calculated the ratio of docked t-vesicles and total anchored v-vesicles as the docking probability. This could be directly compared for different molecular conditions. Using the same algorithm as used for the docking number analysis, each vesicle-vesicle docking event and the subsequent fusion process were individually identified. We quantified the FRET efficiency using the equation, $I_A / (I_D + I_A)$, where I_D and I_A are the donor and acceptor fluorescence intensities respectively. The fusion percentage was calculated by the percentage of fusion events with a FRET efficiency higher than 0.5.

RESULTS

Syt1 shows both Ca^{2+} -dependent and -independent stimulation of lipid mixing

Although C2AB, a recombinant soluble version of Syt1 lacking the transmembrane segment, was often used as an alternative model for Syt1 in a variety of *in vitro* studies [31,32,39], it could not recapitulate some essential features of the full-length Syt1 functions, such as the Ca^{2+} -independent enhancement of SNARE-driven membrane fusion [10,37]. Moreover, a previous study indicated that the stimulation of SNARE-mediated lipid mixing by Ca^{2+} correlates well with the capacity of C2AB to cross-link or aggregate vesicles by simultaneous binding to two membranes [40], whereas such cross-membrane binding may not be necessary for Syt1 to stimulate vesicle fusion [10,37].

To investigate the function of Syt1 in membrane fusion, we used the *in vitro* lipid-mixing assay by reconstituting Syt1 into proteoliposomes. In our bulk fluorescence lipid-mixing assay, t-SNARE, syntaxin 1A combined with SNAP-25, was reconstituted into a population of vesicles (termed t-vesicles) which contained 1.5 mol% DiI, 63.5 mol% POPC, 15 mol% DOPS and 20 mol% cholesterol, whereas VAMP2, together with Syt1 at a molar ratio of 1:1, were reconstituted into a separate population of vesicles (termed v-vesicles), which contained 1.5 mol% DiD, 78.5 mol% POPC and 20 mol% cholesterol (Figure 1A). DiI and DiD are the fluorescence donor and acceptor lipids respectively. The molar ratio of DiI, DiD and cholesterol were kept constant, and the lipid to SNARE protein ratio was fixed at 200:1 throughout samples. To facilitate the *trans* interaction of Syt1 with the t-vesicle membrane, we removed the acidic phospholipids from the v-vesicles.

Lipid mixing was assessed by the increase of FRET between DiI and DiD when lipid mixing occurred. As expected, with Syt1, there was an obvious Ca^{2+} -independent enhancement in lipid mixing (grey line in Figure 1B), compared with the case without Syt1 (black line in Figure 1B). Moreover, with $50\ \mu\text{M}\ \text{Ca}^{2+}$, an additional enhancement was observed in the FRET signal (dashed line in Figure 1B). However, the stimulatory effect of Syt1 with Ca^{2+} on lipid mixing was blocked by adding $10\ \mu\text{M}$ recombinant Vps (amino acids 1-93) or by removing SNAP-25 (the dotted lines in black and grey respectively in Figure 1B), indicating that the stimulation by Ca^{2+} is strictly SNARE-dependent. The Ca^{2+} -independent stimulation by Syt1 of the initial lipid-mixing rate was as much as a 6-fold, which increased a further 4–5-fold with the addition of $50\ \mu\text{M}\ \text{Ca}^{2+}$ (Figure 1C).

Ca^{2+} in the range $10\text{--}50\ \mu\text{M}$ is sufficient for Ca^{2+} -dependent stimulation of lipid mixing by Syt1

It is well known that the cellular Ca^{2+} concentration is only in the nanomolar range, whereas the extracellular Ca^{2+} concentration is over $1000\ \mu\text{M}$. In response to the action potential, the cellular Ca^{2+} concentration increases from a nanomolar to a micromolar range by Ca^{2+} influx, which initiates Syt1-mediated Ca^{2+} -dependent neurotransmitter release [41]. However, the Ca^{2+} concentration range that triggers Syt1-triggered membrane fusion is still controversial. In the present study, to understand the concentration-dependence of Ca^{2+} -dependent stimulation of lipid mixing by Syt1, we measured the stimulation effect at different Ca^{2+} concentrations. We carried out the experiments at various molar ratios of PS in v-vesicles (0, 5 and 10 mol%) while keeping that in the t-vesicles fixed (15 mol%). The results show that Syt1 has the most effective Ca^{2+} -dependent stimulation at approximately $50\ \mu\text{M}$ (Figure 2A). The enhancement was approximately 4–5-fold and was similar regardless of the various molar ratios of PS in v-vesicles. As the Ca^{2+} concentration increased above $50\ \mu\text{M}$, the Syt1 stimulatory effect was gradually reduced to reach 30–60% of its maximum value at $200\ \mu\text{M}\ \text{Ca}^{2+}$, similar to what had been observed in our previous study [10]. This Ca^{2+} -dependent behaviour was successfully modelled with *cis* binding of Syt1 at high Ca^{2+} concentrations [10].

A previous study has indicated that Syt1 could interact with the membrane-containing PIP_2 [34], which could help vesicle docking in the absence of Ca^{2+} . To investigate the PIP_2 effect, 2 mol% PIP_2 was added by replacing POPC on t-vesicles. With PIP_2 , Ca^{2+} -dependent enhancement was merely approximately 2-fold, whereas the Ca^{2+} -independent enhancement was as much as 10-fold (Figure 2B). Interestingly, with PIP_2 the Ca^{2+} level that gave the maximum effect of Syt1 shifted to $\sim 20\ \mu\text{M}$. We also observed a monotonic decrease of Ca^{2+} -dependent stimulation above $20\ \mu\text{M}\ \text{Ca}^{2+}$, and when the Ca^{2+} concentration was higher than $100\ \mu\text{M}$, the Syt1 stimulatory effect was decreased to the level in the absence of Ca^{2+} .

Vectorial membrane-surface charge asymmetry may be necessary for the function of Syt1 as a Ca^{2+} sensor

Previous studies have suggested that a proper *trans* charge distribution on two membranes is critical for Syt1 to regulate Ca^{2+} -dependent stimulation of fusion [10,37]. PS on the t-vesicles seems to be required for membrane binding of Syt1 via the electrostatic interaction,

whereas PS on the v-vesicles could cause an undesirable *cis* interaction with Syt1, which would impair the Syt1 function as a Ca^{2+} sensor [10].

To further verify these results we investigated the opposite case in which there was more or equal PS on v-vesicles, which we expect to deprive the Ca^{2+} -dependent stimulatory function from Syt1. For all of the cases investigated, we still observed the Ca^{2+} -independent enhancement by Syt1 (Figure 3), although they were somewhat less than those of the previous cases of excess surface charges on t-vesicles. When the t- and v-vesicles contained an equal amount of PS, irrespective of whether the t-vesicles contained PIP_2 or not, none of them showed any Ca^{2+} -dependent enhancement by Syt1 (Figure 3). Furthermore, when v-vesicles contained more acidic phospholipids than t-vesicles, we observed a monotonic decrease in the initial rate of lipid mixing or total abrogation of the Ca^{2+} -dependent enhancement. The results show that in cases where the v-vesicles have more acidic phospholipids than the t-vesicles, Syt1- Ca^{2+} would lose its preferential binding to the t-vesicles, which leads to the loss of the Ca^{2+} -dependent stimulation function for Syt1. Overall, the results of the present study show that more acidic phospholipids on the t-vesicles than those on the v-vesicles are required for Syt1- Ca^{2+} to keep its preferential binding to the t-vesicles.

Syt1- Ca^{2+} mainly stimulates vesicle docking

Finally, to dissect Ca^{2+} -dependent stimulation by Syt1 in lipid mixing in further detail, we applied the single-vesicle-fusion assay that is capable of separating docking from lipid mixing. In our experiment, v-vesicles containing 0.1 mol% biotin and 1.5 mol% acceptor dye DiD were immobilized on the imaging PEG surface through a neutravidin-biotin conjugation, whereas t-vesicles containing 1.5 mol% donor dye DiI were mixed with Ca^{2+} and then flown into the flow cell to react with the surface-immobilized v-vesicles (Figure 4A).

Unlike the traditional lipid-mixing assay in which the overall fluorescence signal is analysed, in the single vesicle-fusion assay the fluorescence signal from individual surface-immobilized vesicles or vesicle pairs is analysed separately [10]. In this way, we can determine the percentage of v-vesicles docked by t-vesicles. Furthermore, for docked vesicle pairs, by analysing the FRET efficiency on the basis of the criteria that low FRET values in the range 0-0.5 indicates vesicle docking without fusion whereas high FRET values in the range 0.5-1 report fusion, we could determine the progression of fusion for each vesicle pair.

The docking probability was calculated by the ratio of the number of docked t-vesicles to the total number of immobilized v-vesicles. The fusion percentage was calculated by dividing the number of vesicle pairs with a FRET efficiency higher than 0.5 by the total number of docked vesicle pairs. Interestingly, we observed 3-4-fold Ca^{2+} -independent enhancement of vesicle docking. However, with 20 μM Ca^{2+} , an additional 2-3-fold stimulation of vesicle docking was observed when t-vesicles contained more acidic phospholipids than v-vesicles. We observed little increase of docking when t- and v-vesicles had an equal negative charge (Figure 4B). Without SNAP-25, Syt1- Ca^{2+} -mediated vesicle docking was inhibited by 80%, indicating that the interaction between Syt1 and the t-SNARE complex was essential for synaptic vesicle docking (Figure 4B). However, after vesicle docking, Syt1 had only a

20% Ca^{2+} -dependent enhancement in lipid mixing, regardless of whether t-vesicles contained more acidic phospholipids or not (Figure 4C). Again, without SNAP-25, lipid mixing was completely blocked, indicating that vesicle fusion was strictly SNARE-dependent (Figure 4C). Overall, the results show that an asymmetric distribution of acidic phospholipids provides a higher affinity binding site on t-vesicles for Syt1 when they dock on to the t-vesicles, which causes Ca^{2+} -dependent enhancement by Syt1 in vesicle fusion.

DISCUSSION

The results of the present study demonstrate that an excess surface charge on the t-vesicles is necessary for Ca^{2+} -triggered stimulation of SNARE-dependent lipid mixing by Syt1. Only in the presence of more acidic lipids in t-vesicles did we observe the Ca^{2+} -dependent enhancement of lipid mixing. The stimulation was at its maximum at a Ca^{2+} range of 10–50 μM , consistent with *in vivo* results [41] that synchronous neurotransmitter release is, in most cases, triggered by 10–50 μM Ca^{2+} . Interestingly, the Ca^{2+} -dependent enhancement decreases as the Ca^{2+} increases further over the optimal value. Such a decrease in Ca^{2+} -dependent enhancement at the high concentration of Ca^{2+} (above 100 μM) might stem from two possible reasons: one scenario is the *cis* interaction between Syt1 and the v-vesicle membrane [10], and the other scenario is that high Ca^{2+} can cover the negatively charged surface of t-vesicles which would reduce the *trans* binding of Syt1 to t-vesicles. In fact, it turns out that under these conditions approximately two-thirds of PS is estimated to be Ca^{2+} -bound [42]. It is well-documented that concentrations of lipid components on the synaptic vesicle are not equal to those on the plasma membrane [43,44]. In particular, the plasma membrane contains a significantly higher concentration of the acidic phospholipids, such as PIP_2 and PS, than the synaptic vesicle. Therefore such asymmetric lipid distribution with excess negative surface charges on the target membrane appears to satisfy the condition for Syt1 to function as a Ca^{2+} sensor.

We observed that the inclusion of 2 mol% PIP_2 in the t-vesicles shifted the optimum Ca^{2+} concentration from ~ 50 μM to ~ 20 μM . Our previous result from the single-vesicle-fusion study showed that it decreased further to ~ 10 μM Ca^{2+} when 6 mol% PIP_2 was included in t-vesicles [10]. On the basis of such a PIP_2 -dependent trend on the optimum Ca^{2+} level, we speculate that the spatio-temporal variation of PIP_2 in the target membrane controls the dynamic range of the Ca^{2+} sensitivity in neuro-exocytosis.

Surprisingly, we observed Ca^{2+} -dependent stimulation of lipid mixing, even in the absence of any negatively charged lipids in v-vesicles. There are two competing mechanistic models that detail the Syt1 interactions with SNAREs and two membranes. Chapman and co-workers [40] found that C2AB has the capacity to aggregate vesicles in a Ca^{2+} -dependent manner, which correlates well with its ability to stimulate lipid mixing. Thus they proposed that the cross-linking of two membranes by cross-binding of C2AB to two membranes is critical for Ca^{2+} -dependent stimulation of fusion. Alternatively, Jahn and co-workers [37] argued that Syt1 binding to t-SNARE on the target membrane and its simultaneous *cis* binding to the vesicle membrane is important for Ca^{2+} -dependent stimulation. However, both models are based on the electrostatic interaction of C2 domains to the vesicle membrane, requiring the presence of the negatively charged lipids on the vesicles. Therefore

the result of the present study that negatively charged lipid is not necessary for the Ca^{2+} -dependent stimulation of lipid mixing argues against both mechanistic models.

However, it appears that the negatively charged lipids on the t-vesicles are necessary for Syt1 functioning as a Ca^{2+} sensor. Is Syt1 then binding to the bare target membrane or is simultaneous binding to SNARE complexes required? In our single-vesicle fusion assay we found that in the absence of SNAP-25 there is no significant docking, regardless of the presence of Ca^{2+} , strongly suggesting that SNARE complexes are required for the interaction of Syt1 with the target membrane. Therefore collectively the results of the present study are consistent with the mechanistic model proposed by Rizo and co-workers [13] which predicts the simultaneous binding of Syt1 to both a SNARE complex and the target membrane.

The results of the present study show how asymmetric distribution of the acidic phospholipids between vesicle and plasma membranes can help steer Syt1 to bind the target membrane in a proper orientation with the Ca^{2+} -binding loop towards the target membrane. By changing the molar ratio of DOPS on the t- and v-vesicles, we found that vectorial asymmetric partitioning of acidic phospholipids is important for Ca^{2+} -dependent enhancement of lipid mixing by Syt1. Specifically, only when t-vesicles contain more PS than v-vesicles can Syt1 stimulate lipid mixing in a Ca^{2+} -dependent manner. Otherwise, with equal or higher DOPS in v-vesicles, Syt1 could lose its preference to bind to the t-vesicles, which could inhibit Ca^{2+} -dependent enhancement of lipid mixing.

Finally, using the single-vesicle-fusion assay, we were able to separate vesicle docking from lipid mixing. The results show that a 10-fold Ca^{2+} -independent enhancement by Syt1 in the bulk lipid-mixing assay comes from both the stimulation of vesicle docking (3–4-fold) and that of vesicle fusion (2–3-fold), whereas the 2-fold Ca^{2+} -dependent enhancement is mainly from the stimulatory effect of Syt1 on vesicle docking. Although a 2-fold enhancement of docking by Ca^{2+} in the present study is similar to our previous analysis [10], a modest increase of the fusion probability by Ca^{2+} is different from a 2-fold increase of lipid mixing observed in the previous work. The discrepancy might be due to the fact that 2 mol% PIP_2 was used in the present study, whereas 6 mol% PIP_2 was used in the previous study [10].

In summary, the results of the present study show that for Syt1 a vectorial surface charge asymmetry is necessary for Ca^{2+} -dependent enhancement of SNARE-dependent membrane docking. The charge asymmetry might play a role in steering Syt1 to bind the target membrane instead of non-productively binding to the vesicle membrane.

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Abbreviations

biotin-DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamin-N-(biotinyl)
DiD	1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbo-cyanine perchlorate
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DOPS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine
FRET	fluorescence resonance energy transfer
OG	n-octyl-D-glucopyranoside
PEG	poly(ethylene glycol)
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PIP₂	phosphatidylinositol 4,5-bisphosphate
PS	phosphatidylserine
SNAP-25	soluble N-ethylmaleimide-sensitive fusion protein-attachment protein-25
SNARE	soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor
Syt1	synaptotagmin 1
t-SNARE	target SNARE
TIRF	total internal reflection fluorescence
VAMP2	vesicle-associated membrane protein 2
Vps	soluble VAMP2
v-SNARE	vesicle SNARE

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Figure 1. Syt1 facilitates lipid mixing via both Ca^{2+} -dependent and -independent mechanisms (A) Schematic diagram for the lipid-mixing assay. Syntaxin 1A and SNAP-25 are reconstituted into t-vesicles containing DiI, whereas VAMP2 and Syt1 are reconstituted into v-vesicles containing DiD. (B) Changes of the FRET efficiency (ΔE) in time after mixing t- and v-vesicles are shown. The black line is the control with SNAREs only, without Syt1. The grey line represents lipid mixing with Syt1 and 1 mM EDTA, whereas the dashed line represents the reaction with Syt1 and 50 μ M Ca^{2+} . The dotted lines in grey and black are the negative controls without SNAP-25 (SNP) and with 10 μ M Vps respectively. (C) Relative initial rates of lipid mixing. Values are means $\pm$ S.D. from three independent measurements with three different preparations. The initial rate was measured from the slope at time near zero.

Figure 2. Ca^{2+} at 10–50 μM is sufficient to trigger Ca^{2+} -dependent enhancement of lipid mixing for Syt1

(A) Relative initial rates of lipid mixing at different Ca^{2+} concentrations with various molar ratios of DOPS on the v-vesicles. The closed circles represent the initial rates of lipid mixing with 15 mol% PS on t-vesicles and 0 mol% PS on v-vesicles. The open triangles and closed squares represent the initial rates with 5 mol% and 10 mol% PS on vesicles respectively. Values are means $\pm$ S.D. from three independent measurements with three different preparations. (B) The same initial rate analysis of lipid mixing as that in (A), but with 2 mol % PIP2 on t-vesicles.

Figure 3. Vectorial asymmetric distribution of PS is important for Ca²⁺-dependent enhancement by Syt1

(A) Relative initial rates of lipid mixing at different Ca²⁺ concentrations with a different molar ratio of DOPS on t- and v-vesicles. In the left-hand panel, the closed and open circles represent lipid mixing with 0 mol% and 15 mol% PS on both t- and v-vesicles respectively. In the right-hand panel, the closed circles represent lipid mixing with 0 mol% PS on t-vesicles and 15 mol% PS on v-vesicles. Values are means $\pm$ S.D. from three independent measurements with three different preparations. (B) The same initial rate analysis of lipid mixing as (A) with 2 mol% PIP₂ on t-vesicles. The closed circles represent the initial rate of lipid mixing with 2 mol% PIP₂ on t-vesicles and 0 mol% PS on both t- and v-vesicles. The open triangles and closed squares represent that with 15 mol% and 35 mol% PS on both t- and v-vesicles respectively. In the right-hand panel, the closed circles represent the lipid-mixing assay with 0 mol% PS on t-vesicles and 15 mol% PS on the v-vesicles, while the open circles represent the lipid-mixing assay with 15 mol% PS on t-vesicles and 35 mol% PS on v-vesicles.

Figure 4. Ca^{2+} -dependent stimulation by Syt1 is shown in both vesicle docking and fusion (A) Schematic diagram of the single-vesicle-fusion assay. The v-vesicles were immobilized on the imaging surface, whereas t-vesicles were docked on v-vesicles. (B) The docking probability at different Ca^{2+} concentrations and different molar ratios of DOPS on v-vesicles. The solid bars are the data with 0 mol% PS on v-vesicles, whereas the open bars are for v-vesicles with 15 mol% DOPS. Values are means $\pm$ S.D. from four independent measurements with four different preparations. (C) The fusion percentage among docked vesicle pairs at different Ca^{2+} concentrations. The solid and open bars represent the fusion groups with 0 mol% and 15 mol% PS on v-vesicles respectively. Values are means $\pm$ S.D. from four independent measurements with four different preparations.