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Molecular Mechanism of Cholesterol- and Polyphosphoinositide-Mediated Syntaxin Clustering

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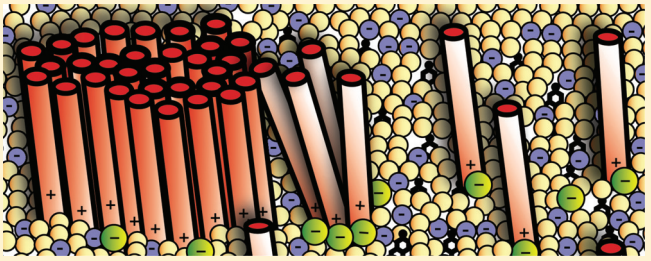
ABSTRACT: The neuronal acceptor SNARE complex that functions as the receptor for synaptic vesicle docking and fusion at the presynaptic membrane is composed of the single-span transmembrane protein syntaxin-1A and the palmitoylated soluble protein SNAP-25. Previously, we explored interactions that promote the formation of syntaxin-1A clusters in membranes. Cholesterol activates clustering in native and model membranes, and its depletion in neuroendocrine cells results in a homogeneous distribution of the protein. However, as little as 1 mol % phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2) or 20 mol % phosphatidylserine was found to disperse syntaxin-1A clusters [Murray, D. H., and Tamm, L. K. (2009) *Biochemistry 48*, 4617−4625]. Strong evidence suggests that syntaxin-1A and its synaptic vesicle cognate synaptobrevin both interact directly with PI-4,5-P_2 and that this interaction activates fusion. However, the molecular details of this interaction and its relationship to the partial dispersion of syntaxin-1A clusters remain largely unexplored. Hence, we mutated the polybasic juxtamembrane motif of syntaxin-1A and found several residues that partially or fully abrogate the electrostatic interaction with PI-4,5-P_2. We further show that even in the presence of physiological concentrations of phosphatidylserine, the PI-4,5-P_2−syntaxin interaction is sufficiently strong to disrupt syntaxin-1A clustering. The stereochemistry of PI-4,5-P_2 is not critical for this interaction as other polyphosphoinositides have similar effects. Forming an acceptor SNARE complex between syntaxin-1A and SNAP-25 weakens but does not abrogate cholesterol/PI-4,5-P_2-controlled cluster formation. Potential consequences of these interactions with respect to synaptic vesicle fusion are discussed.



The neuronal integral plasma membrane protein syntaxin-1A, along with SNAP-25, forms the acceptor complex for the synaptic vesicle membrane protein synaptobrevin. In calcium-triggered membrane fusion in neurons, these proteins contribute SNARE motifs to form a four-helix bundle known as the core complex.[1] SNARE recognition is thought to be responsible for docking of synaptic vesicles to the presynaptic membrane, and the energy gained from the assembly and folding of the SNARE core complex is believed to drive the membrane fusion reaction.[2] Following fusion, the SNAREs are in a four-helical bundle with both the syntaxin and synaptobrevin SNARE motifs continuing as helices to the transmembrane domains in the plasma membrane.[3] For recycling of the SNAREs, this highly stable *cis* complex is disassembled by NSF and α-SNAP.[2] The disassembly of the complex is followed by recycling of synaptobrevin back to the synaptic vesicles via clathrin-mediated endocytosis.[4] Concurrently, Syx is redistributed in the plasma membrane, achieving an equilibrium of monomers and large oligomers with ∼85% of Syx assembling into nanoclusters of 70−90 subunits, with the remainder in a monomeric state.[5] The plasma membrane-associated protein SNAP-25 colocalizes with syntaxin at a level of >90% in chromaffin cells, indicating a cosegregation of these components of the acceptor complex within the membrane.[6] Although there is ample evidence that Syx and SNAP-25 form the active acceptor complex for synaptic vesicle binding, the dynamics and equilibrium of the syntaxin−SNAP-25 association are not well understood.

The oligomeric state of syntaxin is dependent on the cholesterol concentration in several cell types.[7−10] Along with cholesterol, residues in the N-terminal segment of the SNARE motif are critical for clustering.[11] Cholesterol-dependent clustering of syntaxin has also been observed in reconstituted model membranes in vitro.[12] On the basis of these studies, it is thought that the oligomeric state of syntaxin depends on a balance of the interactions mediated by membrane cholesterol concentration, self-affinity through the SNARE motif, and possibly interactions between the polybasic juxtamembrane domain and anionic lipids.

A variety of membrane trafficking processes are regulated by polyphosphoinositides.[13,14] Among the most important consequences of plasma membrane PI-4,5-P_2 misregulation in vivo are the inhibition of SNARE-mediated exocytosis in both mice and primary cultured neurons and defects in synaptic vesicle recycling due to weakened clathrin-mediated endocytosis.[13,15,16] Furthermore, mutations in PI kinases and phosphatases responsible for generating PI-4,5-P_2 and other PIPs are genetically linked to bipolar disorder in humans, in addition to PI misregulation in other syndromes.[15] Interactions between

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SNAREs and the highly negatively charged PIPs play an important role in membrane fusion upstream of the fusion event, and thus likely in the membrane organization and/or formation of the acceptor SNARE complex.[17−19] Besides the aforementioned clusters of Syx, PI-4,5-P_2 also exists in the plasma membrane in clusters at densities of ≥6 mol % of the total lipid. These PI-4,5-P_2 clusters colocalize with ∼30% of docked dense-core vesicles at the plasma membrane.[20] Despite these elegant cellular studies clarifying the activating role of PIPs in exocytosis, the mechanism and role of specific PIP−SNARE interactions are still only poorly understood. In an effort to shed more light on these interactions, we previously showed that PI-4,5-P_2 interacts directly with syntaxin-1A and that this interaction is electrostatically controlled.[12]

The SNARE motif in syntaxin is connected to the transmembrane domain by a highly conserved 10-residue juxtamembrane region that contains a total of six basic residues (Figure 1). Two additional lysines occur between the +7 and

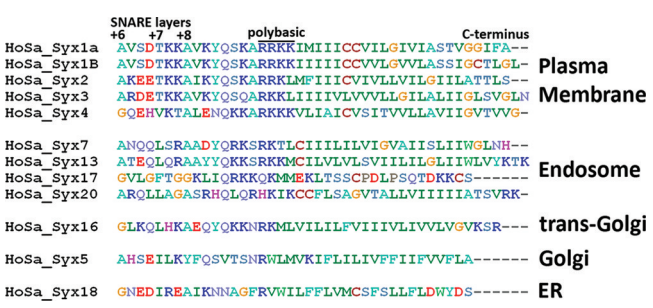

Figure 1. Sequence alignment of the juxtamembrane domain regions of human syntaxins. The alignment (generated in BioEdit[44] using a database of SNAREs[26]) reveals a conserved polybasic region that is most pronounced in family members that are targeted to the plasma membrane. The final layers of the four-helix bundle making up the SNARE core complex and all the transmembrane domains concluding with the C-termini are also shown flanking the juxtamembrane regions.

+8 heptad repeat layers at the C-terminal end of the SNARE motif. Indeed, these residues were identified as potential effectors of fusion. For example, a mutation of the RRKK motif in the juxtamembrane region (residues 262−265 of syntaxin-1A) to all alanines results in a fusion defect that manifests as fewer fusion events, and a greater duration and decreased diameter of the fusion pore.[17] A separate study, in which upstream lysines 252 and 253 and lysines 264 and 265 of the RRKK motif were replaced with alanines, leaving only arginines 262 and 263 unperturbed, revealed a decrease in the extent of fusion compared with that of the wild type when liposomes containing PI-4,5-P_2 (charge of −3 to −4) but not liposomes containing POPS (charge of −1) were used in an in vitro fusion assay.[20] The yeast syntaxin-1A homologue sso1p similarly required the polybasic region for fusion in vivo.[21] However, intriguingly, a mutation of the six basic juxtamembrane residues of yeast Syx to alanine was permitted in fusion assays with liposomes containing 15% POPS in POPC, with the extent of fusion decreased to only ∼84% of that of the wild type.

Synaptobrevin has a similar juxtamembrane region, with five basic residues in the 10-residue linker between the SNARE motif and the transmembrane domain, plus an additional lysine between the +7 and +8 heptad repeat layers. This region also interacts with anionic lipids in a manner that facilitates fusion.[22] Binding studies with a soluble peptide comprising this region binding to anionic lipid model membranes provide evidence of a strong electrostatic component of the interaction. A nearly 50% decrease in the level of granule secretion was observed as a consequence of replacing two of these basic residues with acidic residues.[22] Taken together, the experimental evidence suggests that the juxtamembrane domains of both SNARE fusion partners influence and perhaps even control membrane fusion and that this influence likely occurs through interaction with acidic lipids in either of the two fusing membranes.

We sought to delineate the molecular mechanism that is responsible for Syx−PIP interactions and to define the determinants of the related clustering of Syx in membranes. Single and cumulative mutations of the RRKK motif of the Syx juxtamembrane domain to alanines gradually abrogate the dispersive effect of anionic lipids on Syx clusters and identify the residues that are most important for this interaction. General electrostatics dominate these interactions, and there is a lack of stereochemical specificity for different phosphate headgroup positions in the mammalian PIP repertoire. Together, these results explain the role of these signaling lipids in the lateral organization of syntaxin in model membranes, a role that they very likely also play in plasma membranes.

MATERIALS AND METHODS

Protein Expression and Purification. Syntaxin-1A residues 183−288 with an additional C-terminal cysteine and SNAP-25 were cloned in the pET28 expression vector. The deletion of the regulatory H_{abc} domain did not significantly affect the clustering behavior of Syx in lipid model membranes as was noted previously.[12] The proteins were expressed and purified as previously described with minor modifications.[12] All mutagenesis was performed using the Quickchange site-directed mutagenesis kit (Qiagen, Hilden, Germany). Briefly, syntaxin was affinity purified on nickel resin under denaturing conditions in the presence of 5% (w/v) sodium cholate. Following extensive on column washing with buffer containing 20 mM HEPES (pH 7.4), 500 mM NaCl, 5% sodium cholate, 8 M urea, 10% glycerol, and 30−50 mM imidazole, the protein was eluted in the presence of the same buffer also containing 10 mM EDTA and 400 mM imidazole. The sample was next dialyzed against 20 mM HEPES (pH 7.4), 0.5 M NaCl, 1.5% CHAPS, and 2 M urea. Thrombin was added at a concentration of ∼0.03 mg/mL to cleave the His tag. The protein was dialyzed in buffer containing 0.5 M urea, and then buffer lacking denaturant. Thrombin was removed with benzamidine agarose. All buffers contained 1 mM DTT. Protein concentrations were determined by the Bradford assay and by UV absorbance.

Fluorescent Labeling. To fluorescently label syntaxin, samples were freshly incubated with 2−5 mM DTT for 1 h at room temperature, followed by the removal of DTT by desalting against 2 mM tris(2-carboxyethyl)phosphine (PD-10, Invitrogen). The protein was immediately incubated in a 2−10-fold molar excess of Alexa Fluor 647 maleimide (Invitrogen, Carlsbad, CA) for 1 h at room temperature. No change in efficiency was observed between the 2- and 10-fold molar excess of the fluorophore. Free label was removed by size-exclusion chromatography followed by dialysis. Labeling efficiencies between 55 and 100% were determined by dye absorbance using manufacturer's extinction coefficients and the protein concentration determined by the Bradford assay.

Lipids. The following materials were purchased and used without further purification: POPC, POPS, PI, PI-3,4-P_2, PI-3,5-P_2, PI-4,5-P_2, PI-3,4,5-P_2, all with dioleoyl chains, and brain

PI-4,5-P_2 (Avanti Polar Lipids, Alabaster, AL). Cholesterol was from Sigma-Aldrich (St. Louis, MO).

Reconstitution of Syntaxin in Liposomes. Proteoliposomes were produced by first mixing appropriate ratios of POPC, POPS, PIPs, and cholesterol in chloroform. The mixtures were dried under a stream of nitrogen and desiccated under vacuum for several hours. For vesicles containing PIPs, drying under nitrogen took place at 37 °C. Beginning with dried lipid films, prepared as described above, appropriate amounts of protein in buffer containing 1.5% CHAPS or 5% sodium cholate (w/v) were added and incubated for 1−2 h to solubilize the lipids and to form mixed protein/lipid/detergent micelles. Samples were diluted 3-fold and dialyzed extensively with ∼1 g/L SM-2 BioBeads (Bio-Rad, Hercules, CA). All buffers contained 1 mM DTT. Syntaxin was found to be oriented ∼90% right-side-out with the C-termini facing the lumen of the liposomes. This topology was determined by trypsin digestion and subsequent sodium dodecyl sulfate−polyacrylamide gel electrophoresis. We also confirmed by absorbance and fluorescence spectroscopy of proteoliposomes dissolved in TX-100 that all proteins (mutants) were reconstituted into lipid bilayers with similar efficiencies and that large differences in reconstitution efficiency cannot be a potential source of the increased level of fluorescence self-quenching.

Fluorescence Spectroscopy. All fluorescence experiments were performed essentially as described previously[12] and at 25 °C (unless otherwise indicated) using a Fluorolog-3 spectrofluorimeter (HORIBA Jobin-Yvon, Edison, NJ). Self-quenching experiments with Alexa Fluor 647-labeled protein were conducted with excitation at 650 nm and emission scans between 655 and 750 nm. When SNAP-25 was included, incubation lasted for more than 1 h at 25 °C. Slits (5 nm) were used in the excitation and emission paths in all experiments. Peak fluorescence emission intensities were evaluated in all experiments. In some cases, we also used integrated intensities and found the results to be quantitatively very similar to those using peak intensities. The results of at least four independent experiments were averaged in all self-quenching experiments. Error bars represent single standard deviations.

RESULTS

PI-4,5-P_2 Is Able To Disperse Syntaxin Clusters in an Anionic Lipid Background. We showed previously that phosphatidylserine and PI-4,5-P_2 each dispersed cholesterol-induced clusters of syntaxin-1A in lipid model membranes.[12] However, physiological concentrations of just PS or just PI-4,5-P_2 were not sufficient to completely weaken the cholesterol-dependent clustering effect. To investigate whether PI-4,5-P_2 could further potentiate the dispersion of syntaxin clusters in model membranes containing physiological amounts of PS and cholesterol, we utilized a fluorescent self-quenching assay to observe the clustering and dispersion of Syx in more complex lipid mixtures.[12]

If Syx interacts with acidic lipids by electrostatics, then that interaction should be far stronger with multivalent anionic lipids. POPS carries a net charge of −1 and constitutes approximately 20% of all inner leaflet lipids of the plasma membrane.[23] PI-4,5-P_2 carries a net charge of −3 to −4 and occurs in the inner leaflet of plasma membranes at levels ranging from 1 to 5%, although local concentrations in cells can be higher.[20,24] Thus, our strategy was to use a fluorescence quenching assay to assess dequenching by PI-4,5-P_2 in liposomes containing 20% POPS and 20% cholesterol.

We first sought to recapitulate the cholesterol-dependent clustering of Syx (Figure 2B). In these preparations, the

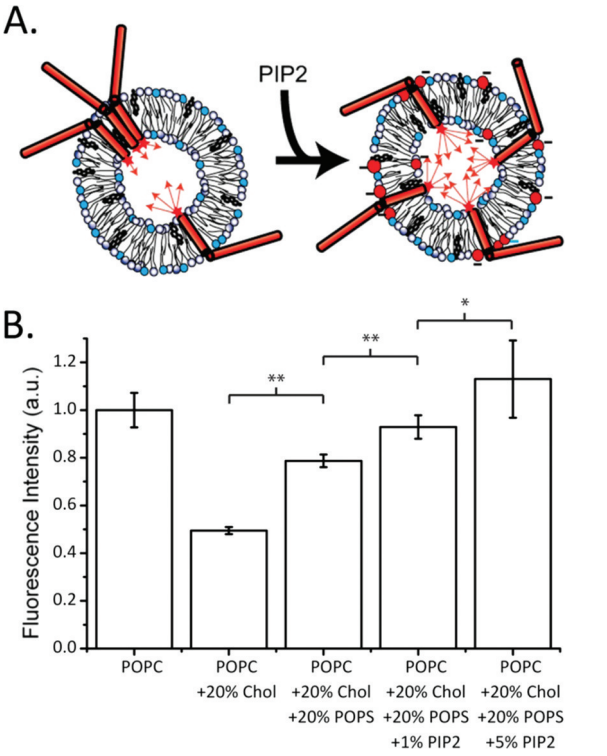

Figure 2. Cholesterol-dependent clustering of syntaxin-1A in lipid model membranes is relieved by the anionic lipids POPS and PI-4,5-P_2 as measured by self-quenching of Alexa Fluor 647-labeled syntaxin. (A) Cartoon model of syntaxin clustering and the resulting self-quenching that is weakened in the presence of anionic lipids. (B) Scaled fluorescence intensities of labeled syntaxin reconstituted into liposomes with different lipid compositions at protein:lipid ratios of 1:1000. The data represent averages of four or more independent experiments with error bars indicating single standard deviations. Paired bars are statistically significantly different at $p < 0.05$ (one asterisk) and $p < 0.01$ (two asterisks) confidence levels.

presence of 20% cholesterol in the POPC vesicles resulted in an ∼50% decrease in fluorescence, which we attribute to the self-quenching of the Alexa Fluor 647 fluorophore on the C-terminus of Syx due to Syx clustering.[12] [We observed that the quenching may vary between 30 and 50%, depending on the sample preparation (this work and ref 12). The reasons for this variability are not fully understood. These differences affect only the dynamic range of each experiment but have no consequences on any of the results reported here. Vesicles prepared from identical lipid and fluorescently labeled protein stocks produced more precise results with relatively small error bars.] Incorporating 20% POPS into the POPC/Chol vesicles produced a significant, though not complete, increase and recovery of the original fluorescence intensity. However, the recoveries could be further increased with the addition of 1 or 5% PI-4,5-P_2 to the POPC/POPS/Chol vesicles. At 5% PI-4,5-P_2, we observed a complete recovery of the original fluorescence observed in the absence of cholesterol. Thus, relatively high physiological local PI-4,5-P_2 concentrations[20] are

sufficient to completely disperse cholesterol-induced Syx clusters. This result is illustrated in Figure 2A.

Syntaxin Has No Specificity for the Phosphate Position on the Inositol Headgroup. We reported previously that Syx interacts with PI-4,5-P_2 in an electrostatic manner.[12] To further test if the interaction is purely electrostatic or also includes a stereospecific chemical component, we employed the self-quenching assay to determine the fraction of fluorescence recovery for four different synthetic PIPs and, as a control, unphosphorylated PI. Because the length and degree of acyl chain saturation of phospholipids are known to result in differences in membrane packing and hence protein interactions,[25] we compared the effects of the different PIPs with identical chain compositions, namely, oleoyl chains in the *sn*-1 and *sn*-2 positions. Thus, these lipids should all have comparable phase and chain packing properties.

Like PS, PI is singly negatively charged. Including 1% of either of these lipids had no effect on the self-quenched fluorescence intensity of Alexa Fluor 647-labled Syx (not shown). The fluorescence in these experiments is scaled to the range given by concomitantly measured POPC/Chol and pure POPC control samples defining 0 and 1 fraction recoveries, respectively. Thus, with 1% PI in POPC/Chol vesicles, we observed 0 fraction recovery (Figure 3).

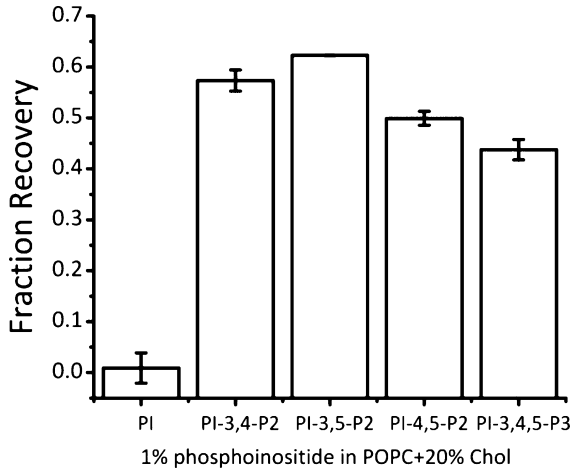

Figure 3. Dispersion of syntaxin-1A clusters by different polyphosphoinositides in lipid model membranes is not specific for the phosphate position on the lipid headgroup. Clustering was measured by self-quenching of labeled syntaxin in POPC/Chol (4:1) bilayers containing the indicated phosphoinositide at 1 mol %. The fluorescence is scaled (fraction recovery) to control samples with syntaxin in POPC and POPC/Chol bilayers without phosphoinositides that were measured in each experimental series to establish 1 and 0 fraction recovery, respectively. Data represent averages of four or more independent experiments with error bars indicating single standard deviations.

When 1% PI-3,4-P_2 or PI-3,5-P_2 was incorporated into the POPC/Chol vesicles, similar fraction recoveries of approximately 0.5−0.6 were observed as with PI-4,5-P_2 (Figure 3). Thus, the configuration and stereochemistry of the attachment of phosphate to the PI headgroup did not seem to have an influence on the interaction and dispersing power of PIP_2s on Syx. Surprisingly, including 1% of the tris-phosphoinositide PI-3,4,5-P_3 in POPC/Chol vesicles also produced ∼0.5 fraction recovery, i.e., no more than the recovery observed with the bis-phosphoinositides. The reason for this is not entirely clear but may be attributed to a shifted pK_a, or electrostatic or chemical shielding of the third phosphate group. Regardless, the PI-3,4,5-P_3 concentration used here is greater than that present in plasma membranes at any time.[13] Therefore, and considering the much higher concentrations of PIP_2s in cells, it is unlikely that PI-3,4,5-P_3 has any regulatory power over the degree of clustering of Syx in plasma membranes.

The Polybasic Juxtamembrane Region of Syntaxin Is Responsible for the Interaction with PI-4,5-P_2. Because previous results clearly demonstrated a role for the juxtamembrane domain of Syx in fusion, we wanted to know whether this domain is also responsible for the previously described electrostatic interactions with anionic lipids.[12] A sequence analysis of known human syntaxin family members shows that the polybasic motif is most prevalent in the members that are targeted to the plasma membrane, which includes syntaxin-1A studied here and syntaxin 4, which functions in glucose transport[26,27] (Figure 1). The positively charged region is least prevalent in syntaxins that are involved early in cellular membrane trafficking pathways, i.e., in syntaxin 18 of the endoplasmic reticulum or syntaxin 5 of the Golgi.[28,29] Interestingly, the most positively charged syntaxins localize to cellular membranes with the highest negative charge density and the most cholesterol.[23,30] In syntaxins that are localized to the plasma membrane, two arginines in positions 262 and 263 and a lysine in position 264 are highly conserved in mammals. A further lysine is also frequently found in position 265 in plasma membrane syntaxins.

Lam et al.[17] showed previously by nitrocellulose dot blot analysis that various PIPs and phosphatidic acid interact with syntaxin-1A. To ask whether these four basic residues constitute a binding site for PI-4,5-P_2 also in a lipid bilayer setting, we prepared an Alexa Fluor 647-labeled mutant of Syx, in which all four basic residues were replaced with alanines. We call this mutant Syx-AAAA. We chose to use 40% Chol and 3% PI-4,5-P_2 in these clustering and unclustering experiments to optimize the dynamic range for all experiments in this series with a range of mutants that will be described below. Figure 4A shows that 3% PI-4,5-P_2 recovered clustering of wild-type Syx at a level of 90% (green bars), but the recovery of clustering of Syx-AAAA was only 60% (red bars). We next asked whether the two arginines or the two lysines were mostly responsible for this effect. Mutants Syx-RRAA (two lysines replaced with alanines) and Syx-AAKK (two arginines replaced with alanines) were prepared to answer this question. Figure 4A shows that the arginine replacements had no effect (orange bars) but the lysine replacements caused a partial defect in the ability of PI-4,5-P_2 to uncluster Syx (yellow bars).

To test if specific individual residues were responsible for the electrostatic interaction, we prepared mutants retaining only one of each of the polybasic residues in the RRKK motif, with the remainder mutated to alanines. The specific mutants are Syx-RAAA, Syx-ARAA, Syx-AAKA, and Syx-AAAK. We observed that these mutants did not cluster in the presence of cholesterol to the same extent as the wild type or the previously described mutants, resulting in a limited dynamic range for analysis of the dispersion by PI-4,5-P_2. Because of this limited dynamic range, the single-mutant syntaxins recovered only very slightly, with none of the mutants capable of a clear strong interaction with PI-4,5-P_2 and none of them more effective than another (Figure 4B).

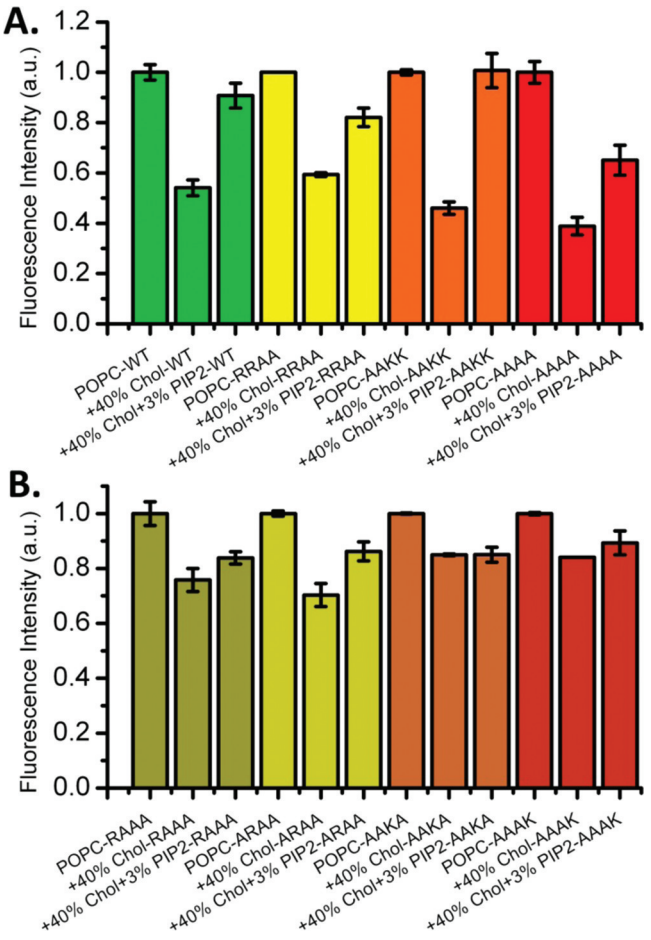

Figure 4. Role of individual basic residues of the polybasic juxtamembrane motif in dispersion of cholesterol-induced syntaxin-1A clusters by binding of PI-4,5-P_2. For each mutant, fluorescence self-quenching experiments were performed in POPC, POPC/Chol (3:2), and POPC/Chol (3:2) vesicles with 3 mol % PI-4,5-P_2. (A) Experiments with the wild type and RRAA, AAKK, and AAAA mutants. (B) Experiments with single basic residue mutants RAAA, ARAA, AAKA, and AAAK. The two lysines seem mostly responsible for the cluster dispersion interaction with PI-4,5-P_2, although additional cumulative effects of all basic residues in this region are also observed. Protein:lipid ratios were 1:1000, and measurements represent averages of four or more experiments with error bars indicating single standard deviations.

The Syntaxin–SNAP-25 Acceptor SNARE Complex Is Less Clustered in Cholesterol-Containing Membranes Than Syntaxin Alone. Although there is now ample evidence that Syx clusters in plasma and model membranes, little is known about how this clustering is affected by Syx forming an acceptor SNARE complex with SNAP-25. Clusters of Syx are thought to be relatively densely packed with their SNARE motifs interacting and standing upright on the membrane.[5] Because SNAP-25 contributes two additional helices to the acceptor SNARE complex, one might expect that SNAP-25 could have an effect on the Syx clustering density in membranes. To test this hypothesis, we prepared Syx–SNAP-25 complexes in membranes by two different methods, namely, by titration of SNAP-25 to pre-reconstituted Syx in proteoliposomes or by first forming complexes and then reconstituting the preformed complexes into proteoliposomes by removal of the dialytic detergent.

As controls, we first tested whether SNAP-25 would have an effect on the Alexa Fluor 647-labeled Syx fluorescence in POPC vesicles, i.e., in the absence of Syx clustering. We observed no change in fluorescence intensity by titration or co-reconstitution of SNAP-25 in proteoliposomes without cholesterol (data not shown). When SNAP-25 was titrated up to a 10-fold excess over pre-reconstituted Syx in POPC/Chol vesicles, we observed no significant change in fluorescence intensity (Figure 5). However, when Syx and SNAP-25 were first assembled into

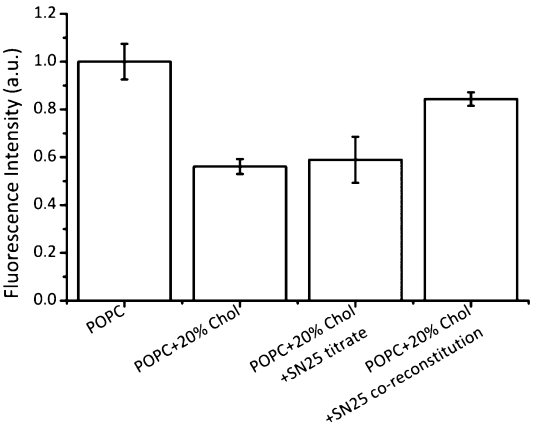

Figure 5. SNAP-25 partially disrupts clustering of syntaxin-1A in cholesterol-containing lipid model membranes. Disruption is observed only when SNAP-25 is co-reconstituted as a complex with syntaxin. Titration of SNAP-25 up to 10-fold excess over syntaxin to preformed syntaxin clusters in cholesterol-containing bilayers has no effect, i.e., does not disrupt these clusters. Mean scaled fluorescence intensities of labeled syntaxin at protein:lipid ratios of 1:1000 are shown for each lipid composition and SNAP-25 condition.

a complex (10-fold excess of SNAP-25 over Syx to minimize formation of a 2:1 complex) before reconstitution into POPC/Chol vesicles, we observed a significant increase in fluorescence intensity compared to that of Syx alone in the same kind of vesicles (Figure 5). The simplest explanation for this result is that SNAP-25 does not bind to clustered Syx in cholesterol-containing liposomes and is not able to disrupt such clusters after they have formed, a result that we also confirmed in supported bilayers (M. K. Domanska, D. H. Murray, and L. K. Tamm, unpublished results). However, when SNAP-25–Syx complexes are formed before reconstitution into model membranes, SNAP-25 prevents the formation of high-order Syx clusters by chemically shielding the open SNARE motif that is thought to contribute to cluster formation[11] and/or by sterically hindering the formation of laterally dense Syx aggregates.

Syntaxin Clustering Is Weakened but Not Abolished at Higher Temperatures. All experiments described so far were performed at 25 °C. We wanted to know if the clustering of Syx persists at higher temperatures, for example, at the body temperature of 37 °C, which is often used in membrane fusion assays. It is well-known that fluorescence emission of many fluorophores decreases as the temperature is increased because of molecular dynamics and an increased level of solvent relaxation.[31,32] Therefore, to establish a baseline of temperature effects intrinsic to the photophysics of our probe, we first measured the fluorescence of Alexa Fluor 647-labeled Syx in Triton X-100. The sample was diluted to a concentration producing a fluorescence intensity similar to that of Syx in

vesicles (approximately 1 μM). The fluorescence decreased linearly as the temperature increased, with a total decrease of approximately 20% over the 5−35 °C interval (Figure 6A). We then used this curve as a standard to normalize fluorescence intensities obtained from Syx reconstituted into liposomes.

Syx proteoliposomes with and without 20% cholesterol were cooled to 5 °C; the temperature was increased in steps to 40 °C, and fluorescence intensities were determined at each temperature. When the fluorescence intensity was normalized to the standard curve determined in Triton X-100, no significant change was observed for Syx in POPC vesicles with an increase in temperature (filled symbols in Figure 6B). However, the relative fluorescence of Syx in POPC/Chol vesicles was significantly decreased compared to that of the POPC vesicles at all temperatures, and a significant fluorescence increase occurred for Syx in the POPC/Chol model membranes as the temperature was increased (empty symbols in Figure 6B). To ensure that the same amounts of protein were present in all experiments, we disrupted the vesicles at the end of each experiment and solubilized all Syx by adding Triton X-100 to a final concentration of 2% (v/v). The resulting fluorescence showed no significant difference between the POPC and POPC/Chol samples, indicating that the reconstitution process resulted in equal incorporation of protein at either lipid composition (Figure 6C).

DISCUSSION

It is well established that polyphosphoinositides are directly implicated in neuronal exocytosis and specifically in SNARE-mediated membrane fusion. However, the nature and determinants of the responsible interactions have been understood only poorly. Depending on the study, syntaxin has been proposed to be in lipid rafts, excluded from lipid rafts, or associated with special nanodomains enriched with polyphosphoinositides.[19,33,34] Our own previous studies showed that Syx forms cholesterol-dependent clusters that can be disrupted with acidic lipids in uniform (nonraft) lipid model membranes.[12] High concentrations of PS or low concentrations of PI-4,5-P_2 could disrupt these cholesterol-induced clusters. Our interpretation of these effects is that Syx requires free phospholipid (e.g., POPC) to be fully solvated in the lipid bilayer. In the presence of a high level of cholesterol, there is not enough free phospholipid to fully solvate the protein because much is needed to solvate cholesterol. Similar to chaotropic salts reversibly precipitating proteins in solution, cholesterol is able to drive Syx into clusters in membranes. However, anionic lipids with a high affinity for the polybasic motif of the juxtamembrane domain of Syx will bind and thereby solvate Syx even in the presence of a high level of cholesterol. Starting from this model, we now ask if PI-4,5-P_2 could be a regulatory factor of Syx clustering even in a background of physiological concentrations of PS in a presynaptic plasma membrane. We also ask whether the cluster-disrupting power is unique to PI-4,5-P_2 or whether other PIPs can achieve the same effect.

To answer these questions, we used our previously developed reconstituted membrane model system to measure the disruption of Syx clusters by relief of fluorescence self-quenching. Our data show that 20% PS and 1% PI-4,5-P_2, which are typical average lipid concentrations in the inner leaflets of plasma membranes, are each not sufficient to completely disrupt the Syx clusters in cholesterol-containing lipid model membranes. However, when combined with 20%

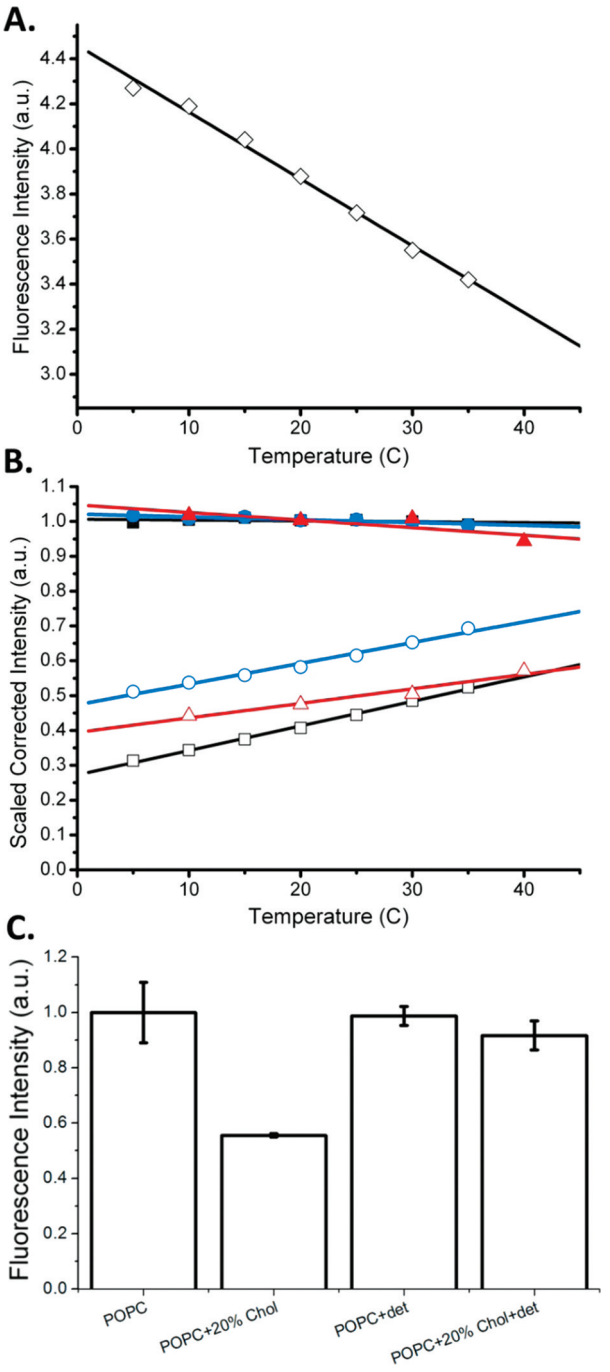

Figure 6. Clustering of syntaxin-1A in cholesterol-containing lipid model membranes weakens with an increase in temperature as revealed by relief of self-quenching of fluorescently labeled syntaxin. (A) Fluorescence intensities of 1 μM Alexa Fluor 647-labeled syntaxin in 2% (v/v) Triton X-100 as a function of temperature. (B) Scaled fluorescence intensities of labeled syntaxin reconstituted into either POPC (filled symbols) or POPC/Chol (empty symbols) bilayers at protein:lipid ratios of 1:1000. Results from three representative experiments are shown for each lipid composition. The data are normalized with the values in POPC bilayers at 20 °C set to 1 and corrected with the fluorophore temperature coefficient determined from panel A as described in the text. (C) Mean scaled fluorescence intensities of labeled syntaxin in different environments from three or more experiments as indicated. Triton X-100 (2%, v/v) was added to the samples labeled with det.

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PS, 1% and especially 5% PI-4,5-P_2 were able to completely disperse the Syx clusters (Figure 2). Because plasma membranes of secretory cells contain localized patches of PI-4,5-P_2 of at least this concentration[20] and because these patches have been identified to be at or near the locations of exocytosis, it is likely that PI-4,5-P_2 could dissociate Syx from clusters in preparation for fusion. This function appears not to be unique to PI-4,5-P_2 because other PIPs can exert the same effect on the clusters (Figure 3). Even with this being the case, PI-4,5-P_2 still is the most likely Syx-liberating lipid molecule in secretory cells, because this species is the most abundant PIP in plasma membranes near secretory granules or synaptic vesicles.[13] This lipid is also the PIP that is the most regulated by phosphoinositide kinases and phosphatases, which likely provide another layer of Syx cluster regulation in these specialized membranes.

Once pried loose from a cluster, Syx may associate with SNAP-25 to form an acceptor complex that is ready to receive an R-SNARE (i.e., a SNARE containing an arginine in the central heptad repeat layer of the SNARE complex, such as synaptobrevin 2 of synaptic vesicles) for docking and *trans* SNARE complex formation. Syx and SNAP-25 are both nonuniformly distributed and colocalize in plasma membranes to a high degree.[6,7] Our results shown in Figure 5 show that SNAP-25 cannot bind to Syx in clusters, but once SNAP-25 is bound to dispersed monomeric Syx, it prevents the re-formation of Syx clusters to a large degree. Therefore, acceptor SNARE complexes consisting of Syx and SNAP-25 are likely ready to engage in vesicle docking and fusion and are unlikely to revert back to the clustered state. These clustering–unclustering and SNAP-25 binding equilibria clearly happen at 25 °C, i.e., the temperature at which most of our experiments were conducted, but they very likely also persist at physiological temperatures of 37 °C as demonstrated by the data shown in Figure 6.

Lipid bilayers composed of POPC, 20% cholesterol, and 20% POPS have an average charge density per lipid of −0.2. If the bilayers contain an additional 1% PI-4,5-P_2, the charge density increases only marginally to −0.23. However, with 5% PI-4,5-P_2, the charge density becomes −0.35, i.e., almost twice that of the PC/Chol/PS bilayers. We previously showed that fluorescently labeled PI-4,5-P_2 is evenly distributed and does not separate into any kind of domain distinct from the lipid background of POPC and POPC/Chol vesicles.[12] An evenly distributed charge of −0.35 per lipid creates a −25 mV surface potential ∼10 Å from the membrane surface.[35] However, the positive potential formed by the highly charged juxtamembrane region is expected to further attract the more negatively charged PIPs and thereby demix this lipid from the bulk lipid even in the presence of 20% PS.[14] PS is unlikely to demix under these conditions.[14] We thus expect PI-4,5-P_2 to be enriched in the vicinity of Syx, which further contributes to the dispersion of Syx clusters even in a uniform background of 20% monovalent anionic lipid.

If we consider that the Syx SNARE motif is mostly disordered in the absence of other SNAREs, then it likely samples the environment both parallel and perpendicular to the membrane. Electron paramagnetic resonance studies favor a conformation that places the bulk of the juxtamembrane region at or below the lipid–water interface.[36] After formation of a complex with SNAP-25 and synaptobrevin, the complex may assume a more perpendicular alignment,[3,37] yet even in the most extreme perpendicular alignment, the negative membrane surface potential would still reach all basic residues of the juxtamembrane region. Thus, complexes between Syx and PI-4,5-P_2 are likely also present in the Syx–SNAP-25 acceptor complex, being thermodynamically and electrostatically favorable, and may very well persist in the postfusion *cis* SNARE complex.[3] Because the calcium sensor synaptotagmin through its C2B domain also has a high affinity for PI-4,5-P_2, PI-4,5-P_2 may actually function as an organizing platform, bringing SNAREs and synaptotagmin together and thus helping to drive the assembly of the neuronal fusion machinery.[6,19,20,22,38,39]

In model peptides, there is no preference for lysine or arginine to bind to negatively charged lipid bilayers.[40] Moreover, the ability of the polybasic peptides to bind anionic lipids scales linearly with the number of basic residues using a series of equivalent lysine- or arginine-containing model peptides.[41] This seems not to be the case for the Syx–PI-4,5-P_2 interactions studied here in the context of a larger transmembrane protein. The results depicted in Figure 4 show that lysines 264 and 265 are more effective in binding PI-4,5-P_2 than arginines 262 and 263. Even if the lysines are anchored deeper in the membrane interface than the arginines,[36] they may still snorkel up to approximately the same plane at the membrane surface as the arginines. If that is the case, lysines could be more effective interacting with the PI phosphates because their positive charge is localized on the terminal amine grouping of lysine whereas the positive charge is delocalized on the guanidinium grouping of arginine. Previously, it was shown that mutation of these arginines is responsible for the weakened binding to phosphatidic acid in nitrocellulose blots and that the Syx-AAKK mutant fails to rescue secretion.[17] However, the converse mutant Syx-RRAA was not studied in this previous work. Therefore, it is not clear whether our findings are consistent or inconsistent with these earlier studies. We also found that sequential mutation of the polybasic region showed a biphasic behavior on Syx clustering. In the absence of acidic lipids, single mutations seem to disrupt clustering more than the wild type or double mutants. Although this observation does not affect any of our conclusions, the reason for this behavior is at present unclear. Although sequence position specificity is rare in electrostatic surface interactions of peptides on membranes, it is not without precedent. For example, an arginine within SCAMP2, a protein also involved in the regulation of exocytosis, is critical, but a nearby lysine is disposable for binding of this peptide to PI-4,5-P_2.[42]

In addition to generating quite strongly negative electrostatic sinks, polyphosphoinositides have an inverted-conical shape, i.e., a polar headgroup larger than the hydrophobic tail cross section. Lipids of this shape generally inhibit fusion at an early stage, namely at the hemifusion stage where lipids communicate between two membranes, but no aqueous pore is formed yet.[43] Indeed, PI-4,5-P_2 has been observed to inhibit fusion in an in vitro liposome fusion assay.[20] Thus, the presence of and requirement for PI-4,5-P_2 to promote calcium-dependent membrane fusion seem contradictory. A recent study describing evidence of interaction of synaptobrevin with anionic lipids proposes a solution to this dilemma.[22] The authors suggest that the polybasic juxtamembrane region may provide a screen for the negatively charged membrane. This charge screening may allow the vesicle to better approach the plasma membrane where the local electrostatic effects of anionic lipids, synaptobrevin, and syntaxin together may pin the two membranes in close contact. It remains to be seen how these

various properties of PI-4,5-P_2 balance with SNARE organization, docking, and fusion to ultimately allow SNARE complex zippering and coupling to calcium release in neuronal exocytosis.

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ABBREVIATIONS

CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Chol, cholesterol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PI-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment receptor; Syx, syntaxin-1A; TM, transmembrane.

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