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Clustering of Syntaxin-1A in Model Membranes Is Modulated by Phosphatidylinositol 4,5-Bisphosphate and Cholesterol[†]

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ABSTRACT: Syntaxin-1A is part of the SNARE complex that forms in membrane fusion in neuronal exocytosis of synaptic vesicles. Together with SNAP-25 the single-span transmembrane protein syntaxin-1A forms the receptor complex on the plasma membrane of neuroendocrine cells. Previous studies have shown that syntaxin-1A occurs in clusters that are different from lipid rafts in neuroendocrine plasma membranes. However, the interactions that promote these clusters have been largely unexplored. Here, we have reconstituted syntaxin-1A into lipid model membranes, and we show that syntaxin cluster formation depends on cholesterol in a lipid system that lacks sphingomyelin and therefore does not form liquid-ordered phases that are commonly believed to represent lipid rafts in cell membranes. Rather, the cholesterol-induced clustering of syntaxin is found to be reversed by as little as 1−5 mol % of the regulatory lipid phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2), and PI-4,5-P_2 is shown to bind electrostatically to syntaxin, presumably mediated by the highly positively charged juxtamembrane domain of syntaxin. Possible implications of these results to the regulation of SNARE-mediated membrane fusion are discussed.

Membrane fusion is a central process of cellular life and a key element of membrane traffic in the endo- and exocytotic pathways of eukaryotic cells. Although regulated in different ways at different stages of vesicle traffic, and although variations are encountered between different cell types, the core of the intracellular fusion machinery is remarkably conserved. This core consists of a complementary set of proteins, the SNARE[1] proteins, which reside in both membranes that are to be fused. These proteins pair up to form parallel four-helix bundles to prepare for and to complete the fusion reaction (reviewed by Jahn and Scheller (*1*)). Three of the helices (termed Q-SNAREs because they contain a glutamine in the central layer of the four-helix bundle) are contributed from the target membrane SNAREs, and one helix (termed R-SNARE because it contains an arginine in the central layer of the four-helix bundle) is contributed from the vesicle membrane SNARE. That SNAREs can form the minimal fusion machinery has been demonstrated by Rothman and colleagues, who were the first to reconstitute specific, albeit physiologically slow, SNARE-mediated membrane fusion from purified components in a reconstituted liposome fusion assay (*2*).

In the case of synaptic vesicle fusion at the presynaptic membrane of neurons, the Q-SNAREs are syntaxin-1A and SNAP-25, and the R-SNARE is synaptobrevin-2. Syntaxin and synaptobrevin are single-span integral membrane proteins oriented toward the cytoplasm with single C-terminal transmembrane helices. Each contributes a single SNARE motif (heptad repeats) to the four-helix bundle and harbors a polybasic linker between its SNARE motif and transmembrane domain. SNAP-25 contributes two SNARE motifs to the core complex and is anchored in the target membrane via two palmitate chains that are posttranslationally attached to cysteines in a linker region between the two contributed helices.

Recent work has tried to understand the function of several additional factors that regulate the priming, Ca^{2+}-sensitivity, and speed of the basic fusion function of the minimal SNARE fusion machinery (*3*−*6*). Despite a much improved understanding of SNAREs and accessory proteins' role in membrane fusion, many basic questions regarding the fusion mechanism itself remain unresolved. In particular, the oligomeric state of the SNARE proteins in their respective membranes has not been addressed biochemically. Additionally, the influence of the lipid environment on the oligomerization and fusion properties of SNAREs has not been investigated.

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[1]Abbreviations: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; PI-4,5-P_2, L-α-phosphatidylinositol 4,5-bisphosphate; FRET, fluorescence resonance energy transfer; IPTG, isopropyl β-D-1-thiogalactopyranoside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NaCl, sodium chloride; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-(phospho-L-serine); Bodipy-TMR-PIP2, Bodipy−tetramethylrhodamine−phosphatidylinositol 4,5-bisphosphate with -C6, six-carbon acyl chain, and -C16, sixteen-carbon acyl chain; RB, reconstitution buffer (20 mM HEPES, 150 mM KCl, pH 7.4); KCl, potassium chloride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DQ, donor quenching; AE, acceptor emission; Chol, cholesterol.

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The oligomeric state of synaptobrevin in membranes is controversial. Several conserved residues in the transmembrane domain of synaptobrevin were found to form a helix dimerization motif (*7*). Mutagenesis and cross-linking studies on detergent-solubilized synaptobrevin appeared to confirm homodimerization of the transmembrane domain. Heterodimers between synaptobrevin and syntaxin transmembrane domains were also found (*8*). A later study using analytical ultracentrifugation revealed that the synaptobrevin interfaces for homo- and heterodimerization are different (*9*). However, another group reported that synaptobrevin exists only rarely as a dimer and that special solubilization conditions may have amplified dimerization (*10*). A site-directed spin-labeling study with the yeast syntaxin homologue Sso1p showed that it forms oligomers with three to five subunits in POPC:DOPS (85:15) membranes (*11*).

Notwithstanding the lack of biochemical evidence for specific molecular interactions between syntaxins, several studies indicate that syntaxin occurs in clusters in cell membranes. Recent superresolution fluorescence microscopy studies revealed clusters of approximately 75 syntaxin-1A molecules that are freely exchanging with monomeric syntaxins in plasma membranes of PC12 cells (*12*, *13*). Cholesterol depletion disperses these clusters (*14*), which naively could be interpreted as evidence for syntaxin clusters resulting from syntaxin partioning into cholesterol-rich ordered lipid domains ("rafts"). However, syntaxin does not copatch with typical raft markers (*14*) but rather partitions into liquid-disordered phases in lipid model membranes (*15*). Furthermore, syntaxin clusters depend on the SNARE motif and only to a lesser extent on the transmembrane domain (*12*, *13*). Yet, the presence of these clusters is dependent on cholesterol, and they appear to be the sites of exocytosis (*12*, *14*, *16*). Taken together, these results potentially lead to a model whereby syntaxin clusters depend on protein−protein interactions mediated by both the SNARE motif and transmembrane domain and the cholesterol content of the membrane. Interestingly, different syntaxin family members segregate into different clusters in the same membrane, thereby likely enhancing the sorting of different fusogenic vesicles to their cognate syntaxin clusters (*17*).

Phosphatidylinositol 4,5-bisphosphate has been long known as an important activator of Ca^{2+}-dependent regulated exocytosis (*18*−*22*). It is also well-known that phosphoinositides are not uniformly distributed in plasma membranes and that PI-4,5-P_2 and perhaps other phosphoinositides form distinct clusters in cell membranes that are regulated by specific PI-kinases (*23*). Furthermore, recent work from several laboratories has established that fusion events colocalize with PI-4,5-P_2 clusters (*18*, *24*). However, the precise molecular interactions of PI-4,5-P_2 that enhance membrane fusion remain obscure. The most prominent regulatory protein of exocytosis that interacts with PI-4,5-P_2 is synaptotagmin, interacting with the lipid via its C2B domain in a Ca^{2+}-dependent fashion (*5*, *25*). Other proteins that have been identified to upregulate exocytosis via interaction with PI-4,5-P_2 are the Ca^{2+}-dependent activator protein for secretion (CAPS) (*20*) and secretory membrane protein 2 (SCAMP2) (*26*).

Polybasic regions of membrane proteins are well-known to electrostatically bind and cluster phosphatidylinositol phosphates (*27*). Since syntaxin and synaptobrevin both have highly charged, polybasic juxtamembrane regions, the SNARE fusion machinery itself may be a target of regulation by phosphoinositides. Indeed, phosphatidic acid and several phosphoinositides have been shown to interact with syntaxin-1A via its juxtamembrane region (*28*). Furthermore, PI-4,5-P_2 retards the diffusion of syntaxin-1A/SNAP-25 heterodimers in supported model membranes (*29*).

Because of their central role in the exocytotic membrane fusion machinery, it would be of great interest to know more about the state of oligomerization and spatial organization of SNARE proteins in lipid membranes that contain known modulators of lipid phase state and effectors of membrane fusion. Specifically, what are the effects of cholesterol and phosphoinositides on the oligomerization state of syntaxin-1A? Furthermore, are their respective effects on SNARE clustering additive; i.e., are they based on common or separate mechanisms? And finally, what are these mechanisms and how could they potentially contribute to membrane fusion?

To address these questions, we have reconstituted syntaxin-1A in lipid model membranes of different lipid compositions and monitored syntaxin distribution in these membranes by fluorescence quenching and FRET assays. We have also monitored specific lipid−protein interactions in this system by lipid-to-protein FRET. Our work demonstrates that the inclusion of cholesterol promotes the clustering of syntaxin in a lipid bilayer setting that does *not* favor the formation of ordered lipid domains and that PI-4,5-P_2 disperses these clusters by binding to syntaxin, presumably at its polybasic region.

MATERIALS AND METHODS

Protein Expression and Purification. Rat neuronal syntaxin-1A, residues 183−288 lacking the regulatory H_{abc} domain and cloned in expression vector pET28a (Novagen, Darmstadt, Germany) (*30*), was expressed in BL21(DE3) cells in "Terrific Broth" at 37 °C with ∼50 μg/mL kanamycin and induced with 1 mM IPTG at an OD_{600} of 1.5 in shaker flasks for 1.5 h. The construct had an N-terminal His tag for purification and an additional C-terminal single cysteine (C289) for fluorescent labeling. The protein was extracted and purified from cell lysate as described (*31*) with minor modifications. The extraction buffer was 20 mM HEPES, pH 7.4, 0.5 M NaCl, 6 M urea, and 10 mM imidazole, with 5% (w/v) sodium cholate or 1.5% CHAPS. Purification of the lysate on Ni-NTA beads was performed in the same buffer, with elution by 400 mM imidazole. The sample was dialyzed against 20 mM HEPES, pH 7.4, 0.5 M NaCl, and 2 M urea. Thrombin was added at ∼0.03 mg/mL to cleave the His tag. After extensive dialysis against the same buffer with 0.5 M urea and 0.2 M NaCl, a final purification step was performed by monoQ anion-exchange chromatography. Fractions were eluted from the column with a NaCl gradient in 20 mM HEPES, pH 7.4, and 1.5% CHAPS or 5% sodium cholate. Pure syntaxin eluted at 0.4−0.5 M NaCl. DTT or TCEP was present through all purification steps. Protein concentrations were determined by Bradford assay and by UV absorbance.

Fluorescent Labeling. To fluorescently label syntaxin, DTT was first removed by dialyzing against 2 mM TCEP in purification buffer. The protein was then incubated with a 10-fold molar excess of Alexa546 or Alexa647 maleimide (Invitrogen, Carlsbad, CA) overnight at 4 °C. Free dye was removed by size-exclusion chromatography followed by dialysis. Typically, labeling efficiencies of 40−55% were achieved as determined by absorbance using the manufacturer's extinction coefficients.

Lipids. The following materials were purchased and used without further purification: POPC, POPS, and brain PI-4,5-P_2 (Avanti Polar Lipids, Alabaster, AL); cholesterol (Sigma-Aldrich, St. Louis, MO); and Bodipy-TMR-PI-4,5-P_2-C6 and Bodipy-TMR-PI-4,5-P_2-C16 (Echelon Biosciences, Salt Lake City, UT).

Liposomes. Unilamellar liposomes were produced by first mixing appropriate ratios of POPC, POPS, PI-4,5-P_2, Bodipy-TMR-PI-4,5-P_2, and cholesterol in chloroform. The mixtures were dried under a stream of nitrogen and desiccated under vacuum for several hours. The lipid films were dispersed with reconstitution buffer (RB), consisting of 20 mM HEPES, pH 7.4, 150 mM KCl, followed by several freeze−thaw cycles and extrusion through 100 nm polycarbonate filters using the Liposofast extruder (Avestin, Ottawa, Canada).

Reconstitution of Syntaxin in Proteoliposomes. Beginning with dried lipid films, as prepared above, appropriate amounts of protein in RB 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 against RB 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 SDS−PAGE and by quenching of the Alexa fluorescence with cobalt.

Fluorescence Spectroscopy. All fluorescence experiments were performed at 25 °C using a Fluorolog-3 spectrofluorometer (HORIBA Jobin-Yvon, Edison, NJ). Self-quenching experiments with Alexa647-labeled protein were carried out with excitation at 650 nm and emission scans between 655 and 750 nm. Protein−protein FRET experiments were performed by excitation at 556 nm and emission scans between 560 and 750 nm. For FRET between lipid and protein, samples were excited at 544 nm, and emission was recorded between 550 and 750 nm. Five nanometer slits 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 three independent experiments were averaged in all self-quenching and FRET experiments. Error bars represent single standard deviations. Absorbance measurements were performed using a Hitachi U-2000 spectrophotometer. The averages of at least three independent experiments with error bars representing three σ values are presented.

FRET Analysis. Förster resonance energy transfer experiments were analyzed by two different methods. In donor quenching (DQ), the decrease in donor fluorescence emission due to the presence of acceptors was measured to determine the FRET efficiency (*32*):

$$E_{DQ} = 1 - \frac{F_{DA}(\lambda_D^{em})}{F_D(\lambda_D^{em})} \quad (1)$$

where F_D is the fluorescence intensity of the donor-only at the wavelength of maximum donor emission, λ_D^{em}, and F_{DA} is the fluorescence intensity of the donor in the presence of acceptor evaluated at the same wavelength. This method of analysis does not require measurements of acceptor emission. In acceptor emission (AE), the increase in acceptor fluorescence emission as a result of energy transfer is measured and the FRET efficiency becomes (*32*)

$$E_{AE} = \frac{\varepsilon_A(\lambda_D^{ex})}{\varepsilon_D(\lambda_D^{ex})}\left[\frac{F_{AD}(\lambda_A^{em})}{F_A(\lambda_A^{em})} - 1\right] \quad (2)$$

where ε_D is the extinction coefficient of the donor evaluated at λ_D^{ex}, the wavelength of the maximum donor excitation, and ε_A is the extinction coefficient of the acceptor at the same wavelength. The fluorescence intensities of the acceptor-only and the acceptor in the presence of the donor, F_A and F_{AD}, are evaluated at the wavelength of maximum acceptor emission, λ_A^{em}. This analysis requires measurements of the donor and acceptor emissions in order to correct for direct excitation, as well as knowledge of the fluorophore extinction coefficients. In all cases, the same trends were observed by the DQ and AE methods. However, the AE method was better suited to show the differences in energy transfer in the protein−protein FRET experiments, and the DQ method was better suited to show lipid−protein interactions by FRET.

RESULTS

Cholesterol Clusters Syntaxin in Uncharged Membranes. We first wanted to know whether cholesterol influences the distribution of syntaxin in lipid model membranes. To test for this possibility, Alexa647-labeled syntaxin was reconstituted into POPC liposomes with increasing concentrations of cholesterol at total protein:lipid ratios of 1:1000. At this ratio we expect 16−25 syntaxin molecules to be present in each 40−50 nm vesicle. Since these vesicles do not contain a high-melting temperature lipid, no phase separation into liquid-ordered and liquid-disordered domains is expected. The vesicles should exhibit a single liquid-disordered phase (*33*).

Figure 1B shows that Alexa647 fluorescence decreased substantially as the cholesterol concentration in the proteoliposomes increased from 0 to 40 mol %. The most dramatic effect occurred between 0% and 20% cholesterol with only little further increase between 20% and 40% cholesterol (Figure 1C). The most straightforward explanation of this decrease is that it resulted from self-quenching of clustered syntaxins in the proteoliposomes as shown in Figure 1A. To exclude the possibility that fewer syntaxins may have been incorporated during the reconstitution process into the liposomes with higher than with lower cholesterol concentrations, we measured the absorbance at 650 nm of the reconstituted proteoliposomes (Figure 1D). The results indicate that roughly equal numbers of Alexa647-labeled syntaxins were incorporated in all liposomes and that the fluorescence decrease as a function of cholesterol concentration was the same when the fluorescence emission of the samples was normalized to their absorbance. This proves that the quantum yields of the fluorescent syntaxins decreased with increasing cholesterol, most likely by self-quenching of the fluorophores in locally concentrated areas of the lipid bilayers (Figure 1A). Control experiments with Alexa647-labeled lipid in bilayers with and without cholesterol showed no difference in fluorescence emission, excluding the possibility that the observed effect is due to a change of the environment of the fluorescent probe. Although these experiments were carried out with a construct of syntaxin that lacked the H_{abc} domain, similar data were obtained with the full-length syntaxin-1A (data not shown).

Acidic Lipids Relieve Cholesterol-Induced Syntaxin Clustering. Since PI-4,5-P_2 has been frequently invoked in activating regulated exocytosis and the clustering of syntaxin in plasma membranes, perhaps by binding to its polybasic juxtamembrane domain, we next asked whether PI-4,5-P_2 could also cluster syntaxins in model lipid bilayer membranes and/or whether it had any effect on the cholesterol-induced clustering of syntaxin. When the same Alexa647 self-quenching assay was

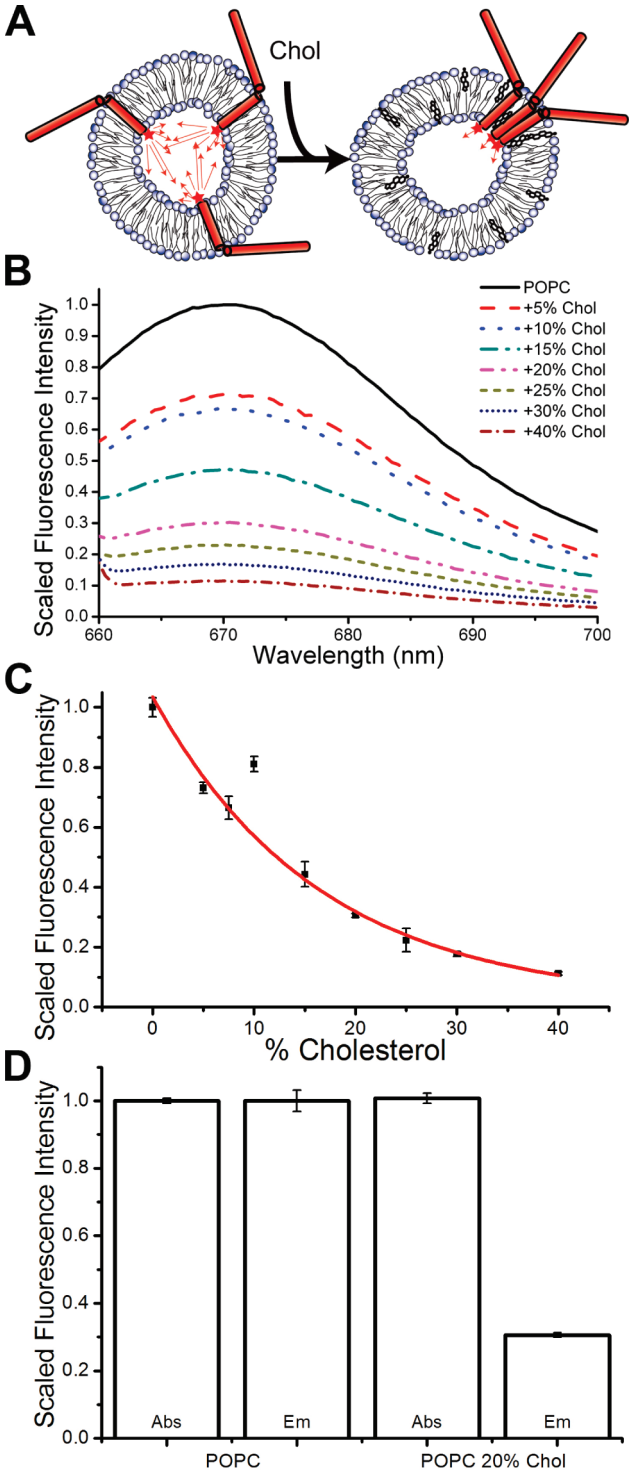

FIGURE 1: Cholesterol promotes the formation of syntaxin clusters in lipid model membranes as measured by self-quenching of fluorescently labeled syntaxin-1A. (A) Cartoon model of cholesterol-induced cluster formation of syntaxin. Arrows represent observed fluorescence. (B) Representative fluorescence emission spectra of Alexa647-labeled syntaxin-1A reconstituted in POPC liposomes with increasing concentrations of cholesterol. The protein-to-lipid ratio is 1:1000. (C) Scaled fluorescence intensities averaged from at least three experiments like the one shown in panel B as a function of cholesterol concentration. (D) Absorbance (at 650 nm) and fluorescence emission (at 671 nm) intensities of Alexa647-labeled syntaxin-1A in the presence and absence of 20 mol % cholesterol, indicating that the protein is incorporated at the same levels in POPC bilayers with and without cholesterol. All values are averages of at least three samples. Absorbance error bars represent three standard deviations for ease of observation, and emission errors are displayed as one standard deviation. Similar data were obtained at other cholesterol concentrations.

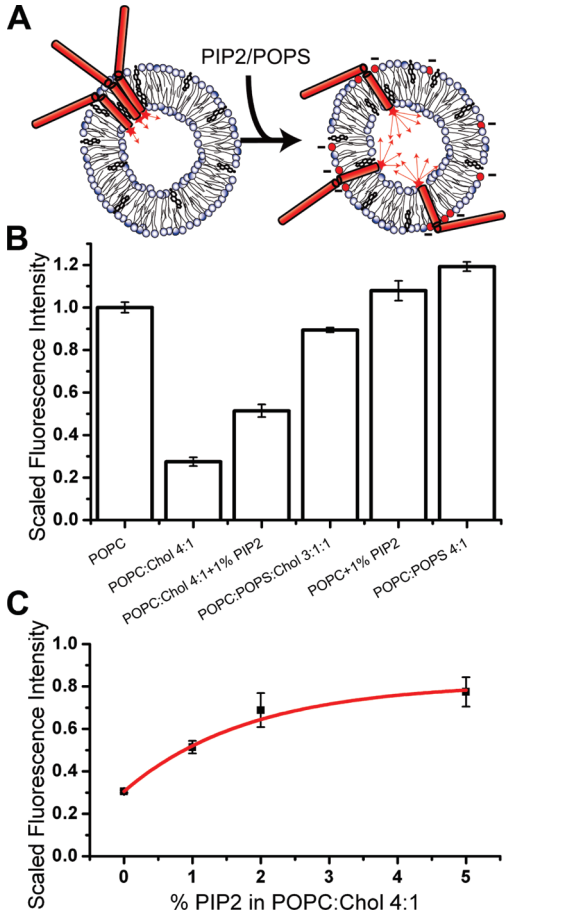

FIGURE 2: Acidic lipids disperse cholesterol-induced clusters of syntaxin in lipid model membranes as measured by self-quenching of fluorescently labeled syntaxin-1A. (A) Cartoon model of syntaxin cluster dispersion by acidic lipids (red headgroups marked with "−" charges). (B) Scaled fluorescence intensities of Alexa647-labeled syntaxin-1A in lipid bilayers of different lipid compositions. The fluorescence intensity in POPC:CHOL (4:1) is self-quenched as in Figure 1. Various additions of acidic lipids relieve this self-quenching. All intensities are scaled to the syntaxin in POPC only fluorescence. The means and standard deviations from at least three independent reconstitutions for each condition are shown. (C) Scaled fluorescence intensities of Alexa647-labeled syntaxin-1A as a function of PI-4,5-P_2 concentration in POPC lipid bilayers containing 20% cholesterol. The means and standard deviations from at least three independent reconstitutions for each condition are shown.

performed with 1 mol % PI-4,5-P_2 in cholesterol-free membranes, no significant fluorescence change, i.e., no clustering, was observed (Figure 2B). Increasing PI-4,5-P_2 to 2 or 5 mol % did not change this result (data not shown). These concentrations are believed to approximately correspond to those in average and concentrated domain regions, respectively, of the plasma membrane of neuroendocrine cells (*20*). However, when as little as 1 mol % PI-4,5-P_2 was added in the presence of cholesterol, the fluorescence self-quenching that was observed without PI-4,5,-P_2 was partially relieved and the fluorescence signal increased 2-fold (Figure 2B). A larger reversal of fluorescence quenching was observed with 2 or 5 mol % PI-4,5-P_2 (Figure 2C). As before, the reported fluorescence values were all normalized to the respective Alexa647 absorbances to correct for minor variations in protein concentrations between different reconstitutions.

We next asked whether the recovery of fluorescence in the presence of PI-4,5-P_2 was specific to this lipid or whether other acidic lipids that are also present in plasma membranes could produce similar results. Including 20 mol % POPS in the POPC:

cholesterol system resulted in a nearly complete (90%) relief of fluorescence quenching (Figure 2B). Moreover, 20% POPS in POPC in the absence of cholesterol resulted in a 20% increase in fluorescence intensity relative to that of POPC alone. This increase may be due to an increase in quantum yield of the fluorophore in an altered electrostatic environment at the membrane interface, or it may be the result of further dispersion of minimal syntaxin clusters that may still be present in pure POPC bilayers. We favor the latter explanation because the FRET results also provide additional evidence in support of a further dispersion (see below). The results of syntaxin cluster dispersion by acidic lipids in the presence of cholesterol are graphically illustrated in Figure 2A. Similar data with lower and higher POPS concentrations (1%, 5%, 10%, and 30%) show that 20−30% POPS is needed to reach saturation of unclustering (Supporting Information Figure S1). These concentrations are much higher than those needed for unclustering by PI-4,5-P_2 (Figure 1C).

FRET Confirms Lipid Modulation of Syntaxin Clustering. To obtain further proof for the lipid-modulated clustering of syntaxins, we developed a FRET assay to directly examine protein−protein interactions between syntaxins in lipid bilayers. In these experiments proteoliposomes were reconstituted with equal parts of Alexa546- and Alexa647-labeled syntaxins at 1:2000 protein:lipid ratios each (1:1000 total). Protein samples with nearly equal labeling ratios (0.45 and 0.48, respectively) were chosen. The donor-only and acceptor-only samples contained 1:2000 unlabeled protein to bring the total protein:lipid ratios to 1:1000 as in the doubly labeled samples. Representative fluorescence spectra of the relevant probes conjugated to syntaxins and reconstituted in proteoliposomes are shown in Figure 3B.

First, we sought to reproduce the effect of cholesterol-dependent clustering of syntaxin. Indeed, the protein−protein FRET efficiency in the POPC:Chol (4:1) system was nearly twice as large as protein−protein FRET in POPC only (Figure 3C). A series of experiments with variable acceptor concentrations verified that the observed FRET efficiencies were dependent on the concentration of the acceptor-labeled protein as expected for FRET (data not shown).

When 1 mol % of PI-4,5-P_2 was included in this system, the FRET efficiency was almost equal to that in POPC only, whether cholesterol was present or not. The reversal of the cholesterol-dependent clustering of syntaxin by 1 mol % PI-4,5-P_2 is even more striking in the FRET experiment than in the self-quenching experiment. As had been observed in the self-quenching experiment, POPS also completely reversed the high FRET that characterized the POPC:cholesterol system. Clearly, acidic lipids disperse clusters of syntaxin in cholesterol-containing liposomes as illustrated in Figure 3A. Apparently, 1 mol % of the triple negatively charged PI-4,5-P_2 is as effective as 20 mol % of the single negatively charged POPS. We did not seek to determine a concentration dependence on either of these lipids but rather chose concentrations that are nearly physiological for each of the components.

The protein−protein FRET efficiencies in the POPC:PI-4,5-P_2 and POPC:POPS systems in the absence of cholesterol were approximately 20−30% lower than in the pure POPC system. As noted above, this is likely due to a further dispersion by the acidic lipids of residual minimal syntaxin clusters that may persist in pure POPC. A decreased quantum yield of Alexa546 in the highly charged environments is unlikely because the self-quenching experiments described in Figure 2 are inconsistent with this interpretation.

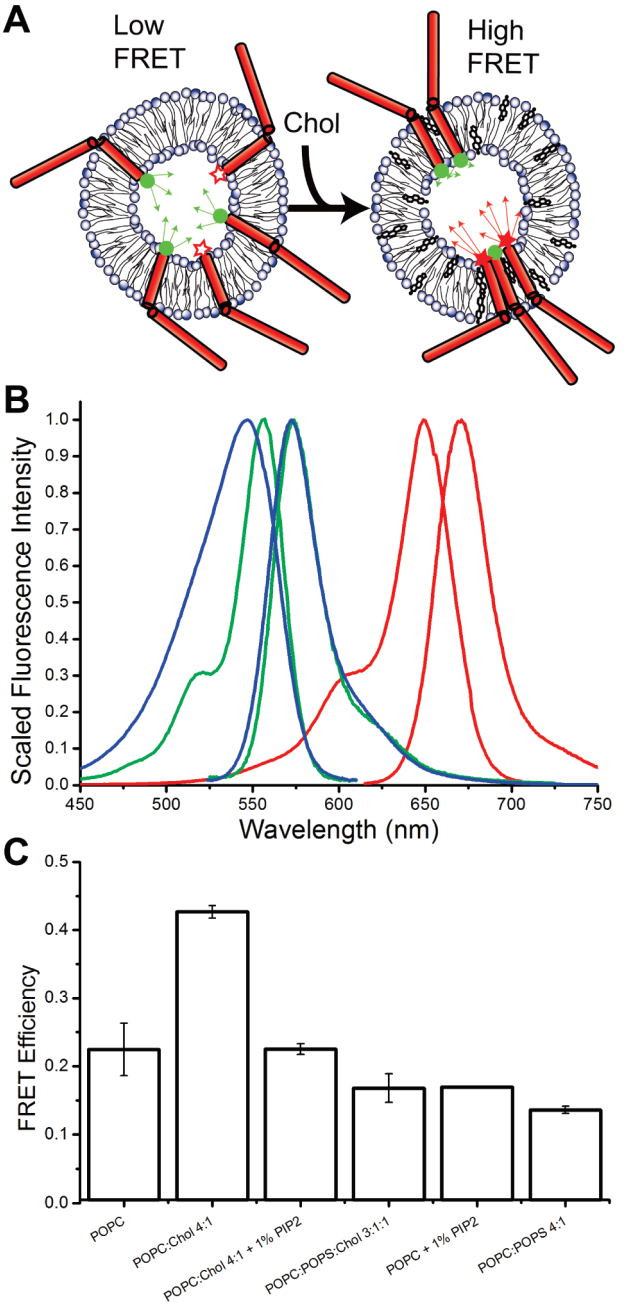

FIGURE 3: Cholesterol-induced clustering and acidic lipid-induced cluster dispersion of syntaxin in lipid model membranes as measured by FRET between two fluorescently labeled syntaxins. (A) Cartoon model of the protein−protein FRET experiments in lipid bilayers. (B) Excitation and emission fluorescence spectra of the labeled proteins and lipids reconstituted into liposomes that were used in this work: Bodipy-TMR-PIP2-C16 (blue), Alexa546-labeled syntaxin-1A (green), and Alexa647-labeled syntaxin-1A (red). (C) Mean FRET efficiencies in reconstituted lipid bilayers with Alexa546-labeled syntaxin and Alexa647-labeled syntaxin. Each protein is incorporated at a protein-to-lipid concentration of 1:2000. The presence of cholesterol significantly increases the energy transfer efficiency, and this effect is reversed by acidic lipids. The means and standard deviations from at least three independent reconstitutions for each condition are shown.

Cholesterol Promotes Syntaxin-PI-4,5-P_2 Interactions. Since according to the FRET experiments 1 mol % PI-4,5-P_2 dispersed syntaxin clusters to a similar degree as 20 mol % POPS, it is likely that PI-4,5-P_2 needs to bind directly to syntaxin to become such a powerful modulator of syntaxin clustering. To test for this possibility, we examined whether we could detect the

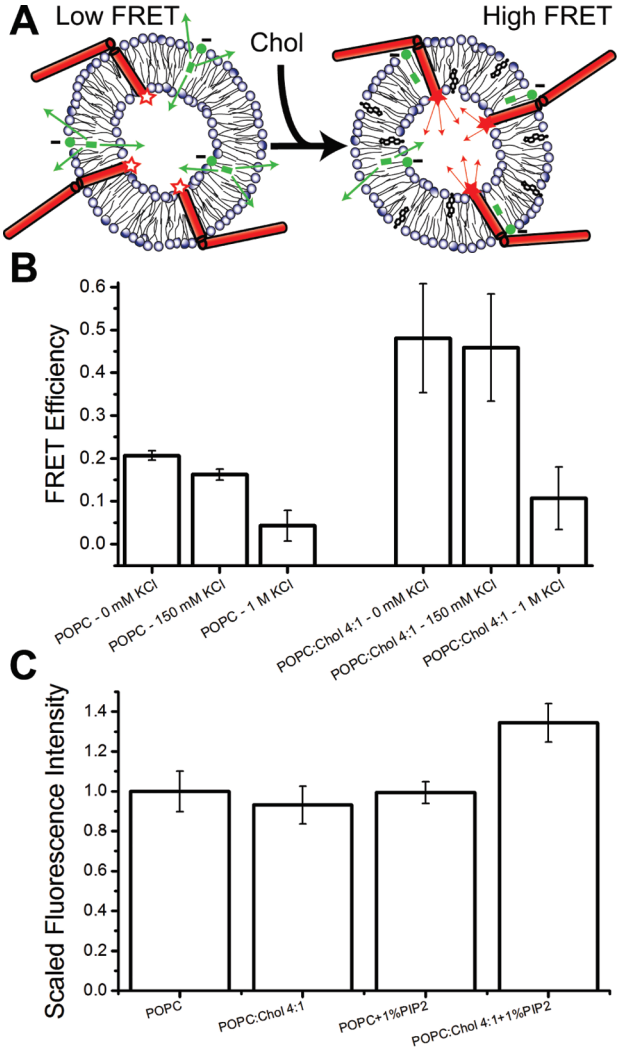

FIGURE 4: PI-4,5-P_2 associates with syntaxin in lipid bilayers containing cholesterol as measured by lipid-to-protein FRET. (A) Cartoon model of the lipid−protein FRET experiments in lipid bilayers. (B) Mean observed FRET efficiencies from BODIPY-TMR-PIP2-C16 to Alexa647-labeled syntaxin-1A in POPC bilayers with and without cholesterol and at different salt concentrations. The means and standard deviations from at least three independent reconstitutions for each condition are shown. (C) Bodipy-TMR-PIP2-C16 fluorescence intensities in protein-free liposomes of different compositions and scaled to the fluorescence in POPC showing that the fluorescent probes are approximately equally fluorescent in all lipid backgrounds, except in liposomes containing cholesterol and PI-4,5-P_2, where the fluorescence intensity is about 30% greater.

PI-4,5-P_2−syntaxin interactions directly by FRET. Bodipy-TMR-PIP2-C16 was chosen as the fluorescence donor and Alexa647-labeled syntaxin as the fluorescence acceptor (Figure 3B). (Initially we also tried Bodipy-TMR-PIP2-C6 as a donor, but in agreement with other reports (*34*−*36*) we found this analogue to be unsuitable because its shorter chains do not correctly incorporate into lipid bilayers.) One acceptor-labeled syntaxin per 1000 lipids was reconstituted into liposomes composed of POPC with 0.1 mol % Bodipy-TMR-PIP2-C16 with or without 20 mol % cholesterol. In POPC without cholesterol, we found a rather low FRET efficiency of only 16% at physiological salt concentrations (0.15 M KCl), indicating a small degree of PI-4,5-P_2−syntaxin association. This residual energy transfer was completely abolished at 1 M KCl. Reducing salt to 0 M KCl resulted in a similar amount of FRET as in physiological salt (Figure 4B, left).

In the presence of 20 mol % cholesterol, the FRET efficiency increased almost 3-fold under physiological or no additional salt conditions (Figure 4B, right). Again, under 1 M KCl conditions the energy transfer was screened to a residual level. These results provide strong evidence that PI-4,5-P_2 interacts directly and electrostatically with syntaxin in the presence, but not in the absence, of cholesterol. The salt screening results rule out the possibility that PI-4,5-P_2 cosegregates into syntaxin clusters simply because it has a higher degree of chain unsaturation or bulkier acyl chains than POPC.

To test for the possibility that the greater FRET efficiency in the cholesterol system could be due to self-quenching of Bodipy-TMR-PIP2-C16 or to a difficulty with its incorporation into lipid bilayers with high concentrations of cholesterol, we measured the fluorescence intensity of 0.1% Bodipy-TMR-PIP2-C16 in POPC and POPC:Chol (4:1) bilayers in the absence of protein. As shown in Figure 4C, no significant difference was observed, indicating that cholesterol did not cluster PI-4,5-P_2 in this system. We repeated the same experiment with 1% unlabeled PI-4,5-P_2 added in addition to 0.1% Bodipy-TMR-PIP2-C16 in POPC with and without 20% cholesterol. Increasing the total PI-4,5-P_2 concentration close to the level that was used in the syntaxin samples did not significantly change the fluorescence intensity in the POPC-only sample and increased the fluorescence intensity in the cholesterol sample only by a small amount. These results prove that there is no significant clustering of the fluorescent PI-4,5-P_2 probe in our model membranes and that the marked increase of FRET between PI-4,5-P_2 is caused by syntaxin and is almost certainly due to specific PI-4,5-P_2−syntaxin interactions (Figure 4A).

DISCUSSION

Although there is little dispute that syntaxin-1A occurs in clusters in cell membranes, the mechanisms that have been proposed in the cell biological literature responsible for this clustering are contradictory. Some have suggested that syntaxin-1A resides in lipid rafts, others have put forward cholesterol-dependent membrane domains that are different from lipid rafts as the sites of syntaxin-1A clustering, and yet others have invoked PI-4,5-P_2 domains to organize syntaxin-1A molecules into functional clusters. In order to shed more light on these diverse mechanisms and to distinguish between them, we have developed a simple biochemical system to assay for syntaxin clustering in diverse lipid backgrounds. Specifically, we sought to determine how cholesterol and different acidic lipids including PI-4,5-P_2 would affect the oligomerization of syntaxin when reconstituted into large unilamellar liposomes.

We determined by fluorescence self-quenching and FRET that the inclusion of cholesterol in POPC model membranes leads to a clustering of syntaxin-1A. Titration with cholesterol indicates that syntaxin clustering is half-maximal at about 10 mol % and saturates around 30−40 mol % cholesterol (Figure 1C). Our model membranes did not contain a high-melting temperature lipid such as sphingomyelin and therefore did not form typical lipid raft domains. Therefore, syntaxin clustering, although cholesterol-dependent, is not lipid-raft dependent. This conclusion is in agreement with the finding that syntaxin-1A does not partition into rafts when reconstituted into giant liposomes presenting such raft domains (*15*) and with the result that syntaxin-1A is concentrated in cholesterol-dependent patches in PC12 cell membranes that are different from patches that can

be visualized with typical raft markers (*14*). It has been shown previously that the cytoplasmic heptad-repeat SNARE motif is primarily responsible for the clustering of syntaxin-1A in PC12 membranes (*12*), but a contribution from the transmembrane domain of syntaxin cannot be completely excluded (*8*). How could the cholesterol-dependent clustering of syntaxin be explained? Phospholipids that are in contact with cholesterol assume more order than bulk lipid. It has been proposed that phospholipids such as POPC may form 2:1 complexes with cholesterol (*37*). Similarly, integral membrane proteins such as syntaxin prefer to surround themselves with boundary lipids that usually have less order than bulk lipids. Therefore, cholesterol and proteins both require special phospholipids for optimal solvation in the membrane. In line with these observations, we propose that cholesterol competes with syntaxin for optimal POPC solvation. If indeed POPC forms 2:1 complexes with cholesterol and these complexes solvate syntaxins much more poorly than free POPC, then a saturation of syntaxin clustering would be expected at 33 mol % cholesterol, which is close to what we have observed. Even if cholesterol is solvated by POPC without complex formation, POPC would still be withdrawn from solubilizing syntaxin, which in consequence would form clusters in the plane of the membrane. This mechanism is a two-dimensional analogue of "salting out" proteins in solution, for example by high concentrations of ammonium sulfate, in which case the salt and the protein surface compete for solvation water molecules resulting in a protein precipitate.

Using the same self-quenching and FRET assays, we found that PI-4,5-P_2 alone does not induce any detectable clustering of syntaxin-1A up to 5 mol %, i.e., close to the highest reported physiological local concentrations of this lipid in cell membranes. Aoyagi et al. (*24*) found that syntaxin coclustered with PI-4,5-P_2 clusters in PC12 cells. Since we do not observe this in our simple liposome model system, other factors must contribute to the clustering of these lipids and proteins in cell membranes. However, when PI-4,5-P_2 was added to cluster-promoting cholesterol−POPC mixtures, the clusters were dispersed at very low physiological concentrations (1 mol %) of PI-4,5-P_2. Syntaxin has a highly conserved and positively charged juxtamembrane domain consisting of three lysines and two arginines in between the SNARE and the transmembrane domains. Numerous examples in the literature describe the ability of highly basic regions of proteins to sequester acidic lipids through electrostatic interactions (for review, see ref *27*). It is therefore conceivable that the basic juxtamembrane domain of syntaxin interacts with the triple negatively charged headgroup of PI-4,5-P_2. Indeed, syntaxin was seen to bind to various phosphoinositide phosphates (but not to the single negatively charged lipids PS or PI) on nitrocellulose filters (*28*). Therefore, we propose that strongly acidic lipids such as PI-4,5-P_2 bind with relatively high affinity to syntaxin-1A via its polybasic juxtamembrane domain and that these lipid−protein complexes successfully compete with cholesterol for better solvation in POPC. The singly charged lipid PS can achieve the same effect, but at a much higher concentration of 10−20 mol %. Since PS is constituitively present in plasma membranes and a large fraction of it may be neutralized by binding numerous constituitive peripheral membrane proteins, but levels of PI-4,5-P_2 are regulated, only changing the levels of phosphoinositides may regulate the clustering of syntaxins in cells.

Our lipid−protein FRET results prove more directly that PI-4,5-P_2 binds to or at least is in close proximity to syntaxin-1A. Although there was previous indirect evidence for this interaction by filter assays (which may or may not accurately report on interactions that actually happen in membranes) (*28*), and by lateral diffusion measurements of syntaxins in supported bilayers (*29*), the present FRET measurements are to our knowledge the first that confirm these expected interactions more directly. The PI-4,5-P_2−syntaxin interaction is predominantly electrostatic because high salt (1 M KCl) is able to disrupt the interaction (Figure 4B). However, no additional salt and 150 mM KCl resulted in the same interaction, indicating that physiological salt concentrations still support the binding of PI-4,5-P_2 to syntaxin. An interesting question to further explore will be to determine whether this interaction is purely electrostatic or whether it also requires the stereospecific chemistry of PI-4,5-P_2. Either way, it would also be interesting to reveal which juxtamembrane residues are mostly responsible for the observed interaction. If the filter assays of syntaxin−phosphatidic acid interactions are an indication, mutations of the two arginines may account for most of the effect (*28*).

The effect of lipid composition on SNARE-mediated membrane fusion has not been studied in much detail. As previously mentioned, increasing the levels of PI-4,5-P_2 stimulates regulated exocytosis in cells (*18*−*22*). However, it is not clear whether this stimulation occurs mainly at the level of fusion pore opening or the regulation of priming the cells for fusion. Since depletion of either ATP or PI-4,5-P2 result in similar defective exocytosis phenotypes, it is generally assumed that PI-4,5-P_2 is mostly involved at the priming step. However, the results of the present work, combined with a requirement of the polybasic region for SNARE-mediated vesicle fusion in yeast (*38*), indicate that PI-4,5-P_2 may also have a direct effect on the fusion reaction. On the other hand, reconstitution of SNARE-mediated liposome fusion with and without PI-4,5-P_2 indicates a complex mechanism. While PI-4,5-P_2 inhibited membrane fusion presumably by adding positive curvature and charge repulsion between the two membranes to be fused, it inhibited even more when the charge in the polybasic region of syntaxin was reduced by mutation (*20*). This result suggests that binding of PI-4,5-P_2 to syntaxin actually promotes fusion relative to the inhibitory role that free PI-4,5-P_2 may have, when tested in the liposome fusion assay *in vitro*.

Lipid compositions in the reconstituted membrane fusion assays typically contain 75−85% POPC and 15−25% POPS in donor and acceptor SNARE liposomes (*2*, *39*−*41*), while others use PC:PE:PS:PI:CHOL (5:2:1:1:1) as their standard lipid compositions in liposome fusion assays (*42*). We are not aware of studies that have examined the effect of cholesterol on SNARE-mediated liposome fusion. When included, cholesterol has almost always been used in conjunction with 20 or more mol % acidic lipids, which according to our data would have dispersed possible syntaxin clusters. However, the effect of including 8 mol % PS in a lipid mixture composed of PC:PE:SM:CHOL (20 mol %) has been examined in a single study, which concluded that PS promotes PEG- and SNARE-induced liposome fusion (*43*). In fact, when PS was omitted, these authors concluded that PEG-induced fusion was inhibited by SNAREs. Based on our results, it is possible that the syntaxins were clustered in the model membranes that lacked PS but that it activated syntaxin by dispersion and/or by binding to its polybasic juxtamembrane domain in the PS-containing membranes. An additional study sought to determine the role of phosphatidic acid and PI-4,5-P_2 in reconstituted liposome fusion (*44*). Adding 10% phosphatidic acid or 10% PI-4,5-P_2 to t- and v-SNARE POPC membranes, respectively, resulted in the greatest extents of membrane fusion.

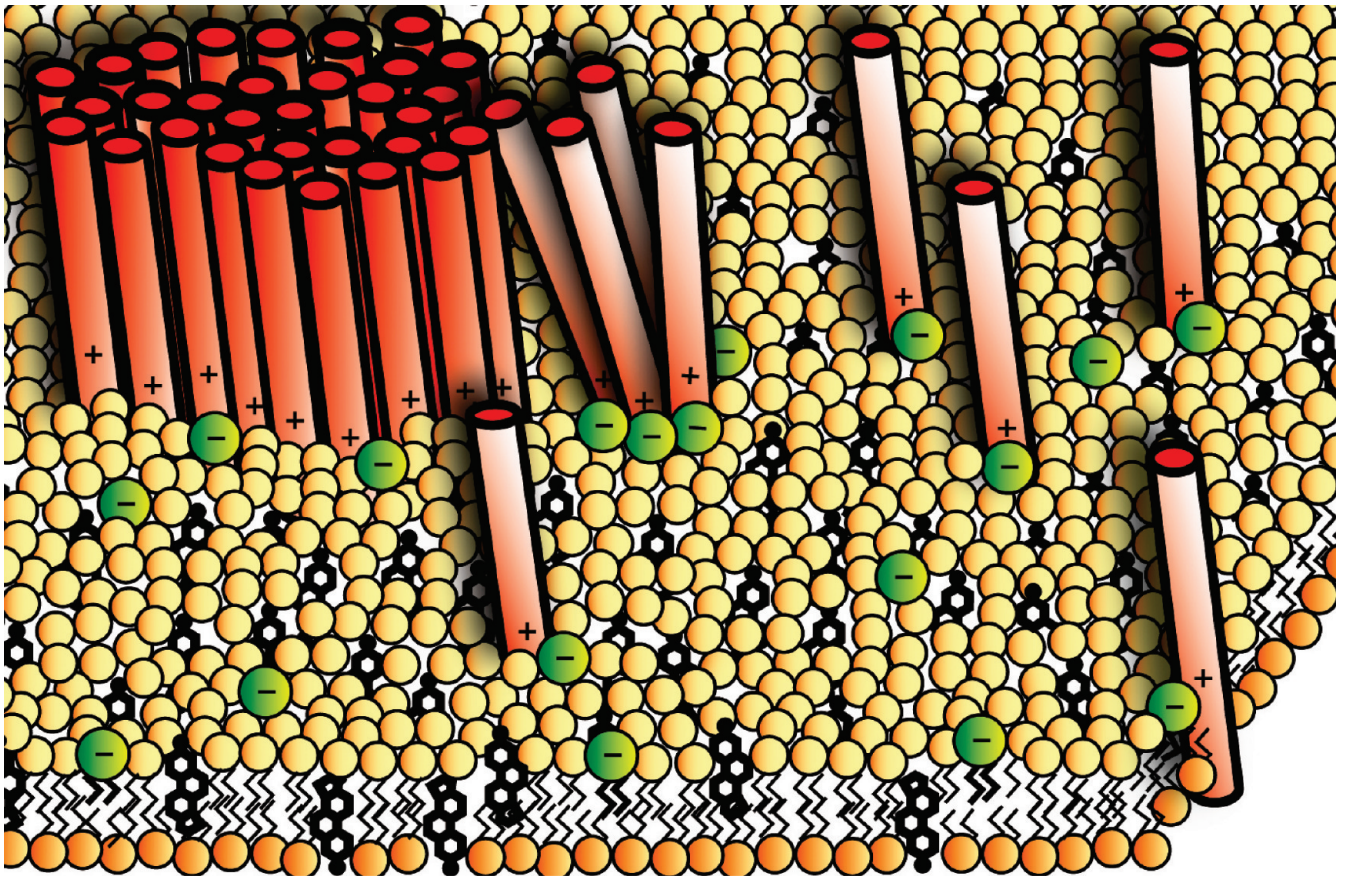

FIGURE 5: "Icebreaker" model of PI-4,5-P_2 action on cholesterol-induced syntaxin clustering and its proposed role in synaptic vesicle fusion. Synaptic vesicles are proposed to dock to nanoclusters of t-SNAREs (syntaxin and SNAP-25). Upregulation of the PI-4,5-P_2 concentration in the plasma membrane causes increased binding of this lipid to the polybasic juxtamembrane domain of syntaxin and dissociates fusogenic oligomers of t-SNAREs with docked vesicles from the nanoclusters. Lipids with green headgroups designate triple negatively charged PI-4,5-P_2. The "+" signs on the syntaxins symbolize their polybasic juxtamembrane region. Cholesterol (polycyclic lipid structures) competes with syntaxin for solvation by bulk lipid.

However, the effects of cholesterol were not addressed in this study. In summary, cholesterol and negatively charged lipids may play a greater role in fusion than previously thought. This should be taken into account when comparing fusion results from different laboratories, which may use different lipid compositions in their standard assays.

In conclusion, we propose a model in which cholesterol and acidic lipids, and particularly PI-4,5-P_2, play all distinct but essential roles in SNARE-mediated membrane fusion (Figure 5). The model invokes clusters of syntaxin as well as individual syntaxins as important contributors to the overall reaction. Since there is little doubt that secretory vesicles bind to membrane areas with about 70−80 clustered syntaxins, we propose that these clusters are primarily important for capturing vesicles from the cytoplasm (*13*, *14*). Quite obviously, regions of high t-SNARE concentration should have a much higher capture rate than other areas. Furthermore, it is likely that more than one SNARE receptor complex is needed for fusion although quantitative studies that are addressing this issue are still scarce. As shown in cell biological (*14*) and biochemical/biophysical (this work) studies, clustering of syntaxin is cholesterol-dependent but does not involve canonical lipid "rafts".

We further propose in our model that syntaxins in complex with acidic lipids simultaneously occur as monomers or perhaps smaller looser oligomers in cell membranes. Because fusion is unlikely to occur from within large, tightly packed syntaxin clusters, and because acidic lipids are known to activate fusion, we propose that t-SNAREs that have "broken off" with docked vesicles from the larger clusters are responsible for the actual fusion step of exocytosis. By binding to the polybasic juxtamembrane domain(s) of syntaxin (and perhaps synaptobrevin), acidic lipids may peel off individual or groups of syntaxins from the larger clusters. Since phosphatidylserine is the major negatively charged lipid of the inner leaflet of cytoplasmic membranes, it is unlikely to have a regulatory role in clustering. However, the highly regulated and dynamic lipid PI-4,5-P_2 could very well regulate the fusion function. Because of its much higher affinity for syntaxin, a small increase of the PI-4,5-P_2 concentration could change the balance of clustered to monomeric syntaxins by separating small fusogenic oligomers of syntaxins (with captured exocytotic vesicles) from large clusters of syntaxins that would then fuse with the help of additional regulatory proteins like complexin and synaptotagmin. In this model, PI-4,5-P_2 acts like an "icebreaker," breaking off smaller fusogenic assemblies from larger vesicle capturing clusters of syntaxin (Figure 5). While this "icebreaker" model clearly requires much further experimentation to be substantiated, we think it is a useful hypothesis subject to further testing in future experiments.

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SUPPORTING INFORMATION AVAILABLE

An additional figure (S1) depicting scaled fluorescence intensities of syntaxin in a variety of additional lipid compositions. This material is available free of charge via the Internet at http://pubs.acs.org.

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