SNARE-catalyzed Fusion Events Are Regulated by Syntaxin1A–Lipid Interactions

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Membrane fusion is a process that intimately involves both proteins and lipids. Although the SNARE proteins, which ultimately overcome the energy barrier for fusion, have been extensively studied, regulation of the energy barrier itself, determined by specific membrane lipids, has been largely overlooked. Our findings reveal a novel function for SNARE proteins in reducing the energy barrier for fusion, by directly binding and sequestering fusogenic lipids to sites of fusion. We demonstrate a specific interaction between Syntaxin1A and the fusogenic lipid phosphatidic acid, in addition to multiple polyphosphoinositide lipids, and define a polybasic juxtamembrane region within Syntaxin1A as its lipid-binding domain. In PC-12 cells, Syntaxin1A mutations that progressively reduced lipid binding resulted in a progressive reduction in evoked secretion. Moreover, amperometric analysis of fusion events driven by a lipid-binding-deficient Syntaxin1A mutant (5RK/A) demonstrated alterations in fusion pore dynamics, suggestive of an energetic defect in secretion. Overexpression of the phosphatidic acid-generating enzyme, phospholipase D1, completely rescued the secretory defect seen with the 5RK/A mutant. Moreover, knockdown of phospholipase D1 activity drastically reduced control secretion, while leaving 5RK/A-mediated secretion relatively unaffected. Altogether, these data suggest that Syntaxin1A–lipid interactions are a critical determinant of the energetics of SNARE-catalyzed fusion events.

INTRODUCTION

Membrane fusion is a process that underlies compartmentalization within all eukaryotic cells, and allows for the many critical and diverse physiological functions in higher organisms. Despite the essential and ubiquitous nature of this process, a considerable energetic expenditure is required to overcome the electrostatic repulsion between opposing lipid bilayers and to deform and ultimately rupture these bilayers (Chernomordik and Kozlov, 2003; Cohen and Melikyan, 2004). As a result, substantial effort has been placed on defining the molecular machinery that overcomes this energetic barrier to accomplish regulated and rapid membrane fusion.

SNARE (soluble n-ethylmaleimide-sensitive fusion factor attachment protein receptor) proteins have now been iden-

tified as the minimal protein machinery required for membrane fusion (Jahn and Scheller, 2006). Their critical role is supported by multiple lines of evidence, including that SNARE proteins are sufficient to drive membrane fusion when reconstituted into liposomes *in vitro* (Weber *et al.*, 1998) and that cleavage of SNARE proteins by clostridial toxins (Schiavo *et al.*, 1992; Blasi *et al.*, 1993a,b), as well as genetic mutations resulting in loss of SNARE protein function (Broadie *et al.*, 1995; Littleton *et al.*, 1998; Saifee *et al.*, 1998), strongly inhibit neurotransmitter release. Currently, the role of SNARE proteins in membrane fusion is believed to be predominantly mechanical. During neurotransmitter release, nucleation and zippering of a highly stable SNARE core complex formed from two plasma membrane SNARE proteins, Syntaxin1A (Syn1A) and SNAP25, and a vesicle membrane SNARE protein, VAMP2, is believed to generate the energy required to bring opposing membranes to a state of proximity that initiates the fusion event (Jahn and Scheller, 2006). As such, the majority of studies in this field have focused on characterizing SNARE protein–protein interactions, although the fusion process, by definition, must also involve lipids.

That the specific lipid composition of the membrane can have profound consequences on the energetic barrier for membrane fusion has been demonstrated both theoretically as well as in several *in vitro* membrane fusion systems (Chernomordik *et al.*, 1995; Melia *et al.*, 2006; Vicogne *et al.*, 2006; Zimmerberg and Gawrisch, 2006), although this topic has been difficult to study in the context of secretion from live cells. Local sequestration of specific bioactive lipids may provide energetic proclivity to fusion via enhancement of

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Abbreviations used: BoNT-C, botulinum neurotoxin type C; ECL, enhanced chemiluminescence; FRET, fluorescence resonance energy transfer; hGH, human growth hormone; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PC-12, pheochromocytoma-12; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI(4,5)P2, phosphatidylinositol (4,5)-bisphosphate; PLD1, phospholipase D1; PSF, prespike foot; PSS, physiological saline solution; SNAP25, synaptosomal-associated protein of 25 kDa; Syn1A, Syntaxin1A; VAMP2, vesicle-associated membrane protein-2.

protein–lipid interactions essential for priming and fusion (for instance, PI(4,5)P₂-synaptotagmin interactions) (Bai *et al.*, 2004). Outside of protein–lipid interactions, the shapes of individual lipids can also have dramatic effects on membrane curvature and on the energetics of fusion (Chernomordik *et al.*, 1993; Chernomordik and Kozlov, 2003). For example, cone-shaped lipids (e.g., phosphatidylethanolamine, diacylglycerol, or phosphatidic acid) spontaneously form negative membrane curvatures (Epand *et al.*, 1996; Leikin *et al.*, 1996; Kooijman *et al.*, 2005). The presence of these lipids in the contacting leaflets of merging membranes has been shown to favor the formation of the stalk and hemifusion intermediates that are likely to underlie membrane fusion (Kozlovsky and Kozlov, 2002), and as such, these lipids are often described as being “fusogenic.” Effects of spontaneous curvature are a highly local phenomenon (Chernomordik and Kozlov, 2003), however, and in order for fusogenic lipids to exert such effects on exocytosis, they must be directly localized to the sites at which membrane fusion occurs. As the minimal machinery for membrane fusion, SNARE proteins physically define the sites at which exocytosis occurs, and therefore, would be ideal molecular partners to bind and sequester fusogenic or bioactive lipids, thus reducing the energy barrier at exocytotic sites.

The purpose of this study was to determine whether Syn1A, a plasma membrane Q-SNARE, forms functional interactions with structural or bioactive lipids which might then exert a controlling influence over the fusion event. Syn1A (Bennett *et al.*, 1992) is a 35-kDa SNARE protein that is fully anchored to the plasma membrane via a C-terminal transmembrane domain. Several recent reports suggest that Syn1A may form key interactions with lipids. First, biochemical isolation of lipid rafts demonstrated that Syn1A localizes to cholesterol-dependent membrane microdomains; disruption of these rafts resulted in an inhibition of stimulated exocytosis in PC-12 cells (Chamberlain *et al.*, 2001; Lang *et al.*, 2001). Second, optical studies in unroofed PC-12 cells (Lang *et al.*, 2001) and TIRF (total internal reflection fluorescence) studies in intact MIN6 cells (Ohara-Imaizumi *et al.*, 2004) demonstrated that Syn1A forms cholesterol-dependent clusters within the plasma membrane, which were preferential sites at which vesicle docking and fusion occurs. Moreover, Syn1A clusters partially colocalized with PI(4,5)P₂ clusters within the plasma membrane, and vesicle fusion could be correlated with the extent of cluster colocalization (Aoyagi *et al.*, 2005). Third, analysis of single fusion events by carbon fiber amperometry demonstrated that Syn1A's transmembrane domain forms part of the fusion pore (Han *et al.*, 2004). Fourth, FRAP (fluorescence recovery after photobleaching) analysis of Syn1A in reconstituted polymer-supported lipid bilayers demonstrated an increase in the immobile fraction of Syn1A in the presence of acidic phospholipids (Wagner and Tamm, 2001). Lastly, Syn1A contains a polybasic juxtamembrane region which, although unstructured, was recently found to be inserted into the lipid bilayer (Kim *et al.*, 2002). Although these data indicate that Syn1A forms key interactions with acidic phospholipids, the specificity of these interactions and the physiological significance has not, to date, been directly evaluated.

Here, we identify a novel and specific interaction between Syn1A and the fusogenic lipid phosphatidic acid, in addition to demonstrating interactions of Syn1A with multiple phosphoinositol lipids, including PI(4,5)P₂. Progressive neutralizing mutations within the conserved, polybasic juxtamembrane region in Syn1A reduce Syn1A's affinity for acidic phospholipids in a graded manner that correlates with the

graded reduction in the secretory function of these mutants. Using carbon fiber amperometry, we demonstrate that this reduction in secretory function results from a decrease in fusion event frequency; moreover, successful fusion events that occurred demonstrated significantly longer fusion pore durations and smaller fusion pore diameters compared with control. Importantly, the secretory defect seen with a lipid-binding-deficient Syn1A (5RK/A) could be completely rescued by overexpression of the phosphatidic acid-generating enzyme, phospholipase D1 (PLD1), whereas knockdown of PLD1 activity strongly reduced control secretion without affecting secretion from Syn1A 5RK/A-expressing cells. We therefore propose that Syn1A–lipid interactions are critical in determining the energetics of SNARE-mediated fusion events. Sequence alignment across multiple SNARE protein families demonstrates a high level of conservation of the polybasic juxtamembrane region (Weimbs *et al.*, 1998), suggesting that this lipid-binding role may be a common and important one for many SNARE proteins in membrane fusion.

MATERIALS AND METHODS

Antibodies

The following antibodies were used: α -Syntaxin1A clone HPC-1 (Sigma, St. Louis, MO); α -Syntaxin1A clone 78.3 (Synaptic Systems, Göttingen, Germany), α -GST (Amersham Pharmacia Biotech, Piscataway, NJ); and α -GFP (Clontech, Palo Alto, CA), donkey α -mouse HRP and donkey α -rabbit HRP (Jackson ImmunoResearch Laboratories, West Grove, PA).

Clones

Rat Syntaxin1A was used for all syntaxin constructs. Site-directed mutagenesis was carried out using the Quickchange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The GST-Syn1A(252–265) peptide construct was made by ligating an annealed oligo (F, TCGAAAGAAGCGCCGTCAGTACAGAGCAAGGCACGCAGGAAGAAGTAG; R, AGCTCTACTTCTTCTCGCTGCCTTGCTCTGGTACTTGACGGCCTTCTT) with 5' and 3' overhangs into the pGex-KG vector cut at the XhoI and HindIII sites of the multiple cloning site (MCS) downstream of glutathione S-transferase (GST). Fluorophore-tagged proteins were constructed using the Cre-recombinase-based Creator system (Clontech), in which syntaxin1A, SNAP25, and Munc18-1 were subcloned into recipient fluorophore vectors (pEGFP-C1, or monomeric mutants of pECFP-C1, pEcYFP-C1 [citrine], and red fluorescent protein (RFP) vectors) containing LoxP sequences at the C-terminal end of the fluorophore sequence. For the mRFP-Syntaxin1A-pHluorin construct, the pHluorin (with an associated N-terminal 17 amino acid linker) was PCR subcloned from synaptotagmin and ligated into the C-terminal end of syntaxin1A (with a mutated stop codon). The PLD1 mammalian expression vector (pCGN-PLD1) encoding human wild-type (WT) PLD1 was as previously described (Hammond *et al.*, 1997). The siRNA-PLD1 construct used was a single bicistronic plasmid that expresses both human growth hormone (hGH) and a small interfering RNA (siRNA) targeted to PLD1, as previously described (Zeniou-Meyer *et al.*, 2007). The sequence fidelity of all new constructs was confirmed by DNA sequencing (University of Michigan DNA Sequencing Core).

Cell Culture

PC-12 cells were cultured in 10% CO₂, in DMEM supplemented with 10% horse serum, 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 1% gentamicin (10 μ g/ml). HEK293-S3 cells were cultured in 5% CO₂, in RPMI 1640 with L-glutamine, supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), 0.4 mg/ml hygromycin, and 0.6 mg/ml geneticin. PC-12 cells and HEK293-S3 cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Bovine adrenal chromaffin cells were isolated and cultured as described previously (Armstrong and Stuenkel, 2005).

Purification of GST-Fusion Proteins

BL21 DE3 cells were transformed with pGex KG vectors coding for the soluble forms of Syn1A (aa 1–267 or 252–267) or specific soluble mutants of Syn1A. Cells were grown to an OD₆₀₀ of 0.4–0.6, induced with 0.1 mM IPTG, and grown for 5–8 h with shaking at 23°C. Cells were harvested by centrifugation for 15 min at 5000 $\times$ g (Beckman, Fullerton, CA, JA-14 rotor), and resuspended in PBST buffer (in mM: 16 Na₂HPO₄, 4 NaH₂PO₄, 150 NaCl, 2 EDTA, 1% TX-100, pH 7.3) supplemented with protease inhibitors and 1% β -mercaptoethanol. Cells were then mechanically lysed using a French Press at 15,000 psi. GST-fusion

proteins were purified from the cell lysate using glutathione-Sepharose 4B beads (Amersham), according to the manufacturer's protocol. For select experiments, the fusion protein was cleaved from the GST moiety with 1.4 NIH units of thrombin (Amersham) for 30 min at 25°C, and the cleaved GST was removed by incubation with glutathione-Sepharose beads for 1 h at room temperature. Purity of each protein was determined by fractionation by SDS-PAGE followed by Coomassie staining and was estimated to be ~90%.

Protein Lipid Overlay

"PIP Strips" (nitrocellulose membranes prespotted with 100 pmol each of 15 defined lipids; phosphatidylinositol phosphate [PIP]) were purchased from Echelon Biosciences (Salt Lake City, UT) and binding overlay experiments were carried out according to the manufacturer's protocol. For phosphatidic acid (PA) binding overlay experiments, stock solutions of dipalmitoyl-PA (Avanti Polar Lipids, Alabaster, AL) solubilized in 2:1:0.8 MeOH:chloroform:H₂O were spotted onto Hybond C nitrocellulose and allowed to dry. The nitrocellulose was then blocked for 1 h in TBS-T + 0.1% ovalbumin before initial protein–lipid binding and then was incubated with a molar excess of specific Syn1A mutants or control protein in TBS-T + 0.1% ovalbumin overnight at 4°C. The nitrocellulose was then washed extensively with TBS-T + 0.1% ovalbumin, and the bound protein was detected using antibodies, followed by an ECL reaction. Digital images were taken using a Bio-Rad Fluor-S-Max Imager (Richmond, CA). Integrated densities at each PA spot (four replicates for each PA spot, for each mutant or control protein tested) were measured and normalized to the maximum integrated density for each protein treatment.

Liposome Flotation Binding Analysis

All lipids were purchased from Avanti (Alabaster, AL). Liposomes (small unilamellar vesicles) were made by mixing stock solutions of porcine brain phosphatidylcholine (PC), porcine brain phosphatidylethanolamine (PE), and dipalmitoyl-phosphatidic acid or oleoyl-lysophosphatidic acid (PA/LPA) in chloroform at a 3:2:1 (wt/wt) ratio (in mole % composition, equivalent to 49% PC, 33% PE, and 18% PA for PA-containing liposomes; for LPA-containing liposomes, mole % composition is 45% PC, 30% PE, and 25% LPA). Lipid mixtures were spun dry at 25°C in a tabletop centrifuge and resuspended in liposome buffer (in mM: 50 HEPES, 250 sucrose, 150 potassium acetate). The suspended liposome solution was placed in a bath sonicator for 30 min at 4°C, followed by incubation on a thermomixer to equilibrate overnight at 4°C. For assaying binding of protein to the liposomes, 100 ng of purified soluble Syn1A or specific Syn1A mutants (aa 1–267) was mixed with liposomes and liposome buffer in a total reaction volume of 20 μ L, and the reaction mixture was placed on a thermomixer at 37°C for 1 h at 800 rpm. Reaction mixtures were loaded under a 40/30/20/10% sucrose density flotation gradient and centrifuged at 390,000 $\times$ g for 1 h at 4°C (Beckman rotor TLA-100) to separate liposomes from unbound protein. Equal volume fractions were collected from the top of each flotation gradient and loaded onto an SDS-PAGE gel, and Syn1A immunoreactivity in each fraction was detected via Western blot analysis.

Coimmunoprecipitations

HEK 293-S3 cells were plated into six-well plates and transiently transfected with WT or mutant Syntaxin1A, Munc18-1, and EGFP-SNAP25. Control transfections used Munc18-1 and EGFP-SNAP25, but excluded the Syntaxin1A. After 48 h of expression, cells were rinsed in ice-cold physiological saline solution (PSS1, in mM: 140 NaCl, 5 KCl, 10 HEPES, 10 glucose, 5 NaHCO₃, 1 MgCl₂, 2.2 CaCl₂, pH 7.3), scraped into ice-cold lysis buffer (in mM: 20 Tris, pH 7.4, 1 EDTA, and 2% sucrose, supplemented with a Mammalian Protease Arrest protease inhibitor cocktail; G-Biosciences, St. Louis, MO), dounce-homogenized by 35 strokes, and centrifuged at 800 $\times$ g to remove the nuclei. Subsequently, 1.5 vol immunoprecipitation (IP) buffer (in mM: 150 Tris, pH 7.4, 1 MgCl₂, 0.1 EGTA, 2% Triton X-100, with protease inhibitors) were added to each tube, and tubes were incubated on ice for 30 min to allow solubilization of membranes. Protein concentrations and volumes of the lysates were then equalized across conditions, and IP was carried out with a 2-h incubation with α -Syntaxin1A (clone 78.3) at 4°C with rotation. Immunopure protein G beads (Pierce, Rockford, IL) were then added to each sample, and the incubation continued for 1 h. The beads were pelleted by centrifugation (1500 $\times$ g for 2 min at 4°C), washed twice in IP buffer, and washed a final time in PBS. Syntaxin1A and EGFP-SNAP25 immunoreactivity was determined in each sample using SDS-PAGE fractionation and Western blot analysis, probing with α -Syntaxin1A (clone HPC-1) and α -GFP, followed by an ECL reaction. Digital images were taken using a Bio-Rad Fluor-S-Max Imager, and integrated densities of the EGFP-SNAP25 and Syn1A signals were determined. For each transfection condition, the ratio of the integrated densities of enhanced green fluorescent protein (EGFP)-SNAP25 to Syn1A in the immunoprecipitated fraction was determined. All ratios were then normalized to the ratio from the condition containing WT Syn1A, to allow comparison and quantification across experiments.

hGH Secretion Assay

PC-12 cells were plated onto 24-well plates and cotransfected with specific constructs, including hGH, the light chain of the botulinum C neurotoxin

(BoNT-C), Munc18-1, and full-length Syntaxin1A (WT or mutant forms). The total DNA concentration was held constant across treatments, by addition of a neomycin control plasmid. At 48–72 h following transfection, cells were rinsed for 6 min in a physiological saline solution (PSS2, in mM: 145 NaCl, 5.6 KCl, 15 NaHEPES, 0.5 MgCl₂, 2.2 CaCl₂, 5.6 glucose, 0.5 NaAscorbate, 2 mg/ml BSA, pH 7.3), followed by a 6-min stimulation with 70 mM K⁺ (same as PSS2, with equimolar substitution of K⁺ for Na⁺). PSS2 containing the secreted hGH was collected, and cells were lysed (lysis buffer, in mM: 0.2 EDTA, 10 HEPES, 1% Triton X-100, pH 7.4) to determine percent of total hGH content secreted. hGH content was measured using an hGH ELISA kit (Roche Diagnostics, Alameda, CA). Each experiment was performed with quadruplicate replicates for each treatment, and each experiment was repeated a minimum of three independent times. hGH secretion experiments using lysophosphatidylcholine (LPC), PLD1 overexpression, and siRNA-PLD1 were completed in France and thus were carried out on a different strain of PC-12 cells than in all other experiments. For experiments utilizing external application of LPC, 1 μ M palmitoyl-lysophosphatidylcholine (Avanti Polar Lipids) was added during both the rinse and the stimulation period.

Imaging

Conventional Fluorescence Microscopy. Cells were transferred from media to PSS1 for imaging. Cellular fluorescence was imaged at 25°C using an Olympus 60 $\times$ 1.2 NA PlanApo water objective on an Olympus IX71 microscope (Melville, NY) coupled to a TILL Photonics (Gräfelfing, Germany) polychrome monochromator for illumination. Appropriate dichroic mirrors and emission filters were used for each fluorophore imaged. Image capture was performed using a TILL-Imago QE camera under the control of TILL software.

Confocal Imaging. Confocal images were taken on an Olympus FluoView 500 Laser Scanning Confocal Microscope, using an LD405 laser, 60 $\times$ 1.2 NA objective and a pinhole aperture of 260 μ m.

Fluorescence Resonance Energy Transfer (FRET) Imaging. A detailed description of the FRET three-cube sensitized emission imaging methodology can be found in our previous publication (Liu *et al.*, 2004). In addition to three different excitation/emission images (excitation/emission in nm): 436/465 (donor excitation, donor emission); 436/535 (donor excitation, acceptor emission), and 500/535 (acceptor excitation, acceptor emission), background and shade correction images were also taken. Image correction and analysis were performed offline using the method of FRET stoichiometry (Hoppe *et al.*, 2002; Liu *et al.*, 2004) and implemented with a custom written IGOR macro (Wave-metrics).

FURA Imaging. PC-12 cells transfected with full-length Syn1A (K253I or K253I-5RK/A), Munc18-1, BoNT-C, and RFP (to allow identification of transfected cells) were loaded for 20 min at 37°C with 3 μ M Fura2 AM (Molecular Probes) in PSS1 for calcium imaging. Cells were then rinsed with PSS1, and after 15 min, time-lapse fluorescence images were acquired with excitation fluorescence alternating between 340 and 380 nm and emission was acquired at 510 nm. Fura ratios were calculated as F340/F380.

Amperometry

At 48 h before recording, bovine chromaffin cells were transfected with Syn1A (K253I or K253I-5RK/A), BoNT-C light chain, and GFP, using biolistic bombardment (Gene Gun, Bio-Rad). Conditions for biolistic transfection, and preparation of gold beads were as suggested by the manufacturer, with 2 μ g DNA/mg gold beads. Cells were replated onto poly-L-lysine-coated glass coverslips 24 h before recording. Carbon fiber electrodes (5- μ m; ALA Scientific, Westbury, NY) held at +650 mV were used for amperometric recordings. Cells were bath perfused with PSS1. Secretion was stimulated by local application of 100 mM K⁺ (same composition as PSS1, with K⁺ substituted equimolar for Na⁺) for 60 s, and currents were recorded during these 60 s, in addition to a 5-s prestimulus baseline recording. Currents were collected using an Axopatch 200 A amplifier modified for extended voltage output (Axon Instruments, Foster City, CA), filtered at 2 kHz, and sampled at 4 kHz. No digital filtering was applied. Currents were analyzed using an Igor XOP written by Eugene Mosharov (Columbia University) and available online (<http://cumc.columbia.edu/dept/neurology/sulzer/download.html>; Mosharov and Sulzer, 2005). For spike frequency analysis, only spikes with amplitudes >10 pA above the root mean square (RMS) noise level were used. Prespike foot (PSF) analysis was limited only to those PSF whose amplitudes were <30 pA and whose durations were >1 ms (>4 $\times$ sampling frequency).

Data Analysis and Statistics

Statistical analyses were carried out using GraphPad Prism software. For most comparisons, unpaired *t* tests were used; statistical significance was designated at a *p* < 0.05.

Figure 1. Syn1A directly binds a subset of acidic phospholipids that includes the fusogenic lipid phosphatidic acid. (A) Protein-lipid overlay demonstrating concentration-dependent binding between soluble Syn1A(1-267) and multiple acidic phospholipids. Left, schematic representing the arrangement of lipids spotted on the nitrocellulose. Filled circles indicate those lipids for which an interaction was demonstrated. Right, α -Syn1A immunoreactivity from four representative protein-lipid overlay experiments, in which decreasing concentrations of Syn1A were overlaid onto nitrocellulose membranes spotted with 100 pmol each of indicated phospholipids. (B) Averaged level of interaction of Syn1A for each phospholipid relative to that of PA. Integrated immunoreactive density associated with each lipid on the overlays was averaged from three independent experiments where [Syn1A] = 3 nM and was normalized to the integrated density for PA. Bars, means $\pm$ SEM. (C) Representative Western blots of Syn1A immunoreactivity in fractions collected from liposome flotation assays. Bound Syn1A floats with liposomes to the top of the gradient.

RESULTS

Syn1A Binds with High Affinity to the Fusogenic Lipid Phosphatidic Acid

We initially set out to determine whether Syn1A specifically interacts with lipids that might decrease the energetic requirements for membrane fusion or exert other important functions in exocytosis. In these experiments, bacterially expressed soluble Syn1A (aa 1-267) was overlaid in decreasing concentrations onto PIP strips. Figure 1A demonstrates that Syn1A bound to multiple acidic phospholipids in a dose-dependent manner. Although Syn1A bound with highest apparent affinity to the fusogenic lipid, PA, interactions with several PIPs, including PI(3)P, PI(4)P, PI(5)P, PI(3,4)P2, PI(4,5)P2, and PI(3,4,5)P3, were also observed (Figure 1B). Notably, all of the lipids to which Syn1A bound were acidic, although Syn1A did not interact with every acidic phospholipid tested (e.g., phosphatidylserine [PS] or LPA). Furthermore, Syn1A exhibited a greater apparent affinity for the

monophosphate lipid PA than the polyphosphate inositol lipids, which contain a greater negative valence compared with PA. These data suggest that although electrostatic interactions have an important role in mediating Syn1A's lipid interactions, other structural features may ultimately underlie the specificity.

Although the PIP strip assay was used as an initial screen for Syn1A's lipid-binding properties, it should be noted that this assay can be prone to false positives or false negatives, and thus may not accurately report on the specificity of protein-lipid interactions (Downes *et al.*, 2005). Thus, a correlative and more physiologically relevant series of liposome flotation binding experiments was also performed, in which Syn1A was mixed with liposomes of defined composition and loaded under a sucrose density gradient. After ultracentrifugation, Syn1A bound to liposomes floats to the top of the gradient with the liposome fraction. Figure 1C shows Syn1A immunoreactivity in the collected gradient fractions of a representative experiment and demonstrates that Syn1A specifically bound to liposomes containing PA but not to those containing LPA. Furthermore, Syn1A did not bind to control liposomes (containing only PC and PE). Together, the data demonstrate that Syn1A specifically binds the fusogenic lipid, PA.

The Polybasic Juxtamembrane Region in Syn1A Comprises a Lipid Interaction Domain

Sequence analysis of Syn1A revealed a polybasic juxtamembrane region within Syn1A that is highly conserved across multiple species (Figure 2A, top). To determine if this region is responsible for Syn1A's interactions with acidic phospholipids, a series of progressive neutralizing mutations was generated within this region, in which one (R262A), two (R262A/R263A), or all five (5RK/A) basic residues were neutralized to alanines (Figure 2A, bottom). Mutant proteins were purified and tested for lipid-binding capacity using nitrocellulose blots on which PA had been spotted in increasing amounts. Binding curves were fit using the Hill equation, which allowed determination of the apparent binding affinity (EC_{50}) of each protein for PA (Figure 2B). Progressive neutralizing mutations resulted in a progressive reduction in apparent binding affinity to PA, with the 5RK/A mutant demonstrating the greatest reduction in apparent binding affinity ($EC_{50} \sim 3.2 \times 10^5$ fmol), followed by the R262A/R263A mutant ($EC_{50} \sim 6.1 \times 10^4$ fmol), and lastly, the R262A mutant ($EC_{50} \sim 2.9 \times 10^3$ fmol), which demonstrated binding similar to the WT protein ($EC_{50} \sim 2.0 \times 10^3$ fmol). Thus, complete neutralization of the polybasic juxtamembrane region in Syn1A resulted in a greater than 2 log shift in EC_{50} .

Qualitatively similar results were obtained using liposome flotation assays, in which the relative affinities of the WT and 5RK/A Syn1A proteins for PA-containing liposomes was tested. Figure 2C shows that $24.3 \pm 3.4\%$ ($n = 5$) of the total WT protein, versus $6.1 \pm 2.0\%$ ($n = 3$) of the total 5RK/A mutant protein, bound to the liposomes ($p < 0.01$). Moreover, the 5RK/A mutation eliminated binding to all acidic lipids as demonstrated by the protein-lipid overlays shown in Figure 2D. Thus, Syn1A's juxtamembrane basic residues are critical in mediating Syn1A's interactions with acidic phospholipids.

Additional Structural Determinants Underlie Syn1A's Lipid-binding Properties

To determine whether structure within the juxtamembrane basic region was important for lipid binding, we constructed a Syn1A RKRK mutant, in which the order of the juxtamem-

Figure 2. A polybasic juxtamembrane sequence in Syn1A comprises the lipid-binding domain. (A) Top, sequence alignment of juxtamembrane regions of Syn1A across species. Note the five highly conserved basic residues between positions 260–265 (NCBI accession numbers: HS: AAA53519; MMul: NP_001028037; RN: AAF23017; MMus: NP_058081; LP: AAK70494; LS: AAO83845; DM: A55673; CR: AAY54265). Bottom, sequence alignments of Syn1A juxtamembrane mutants used in this study. (B) Progressive neutralization of Syn1A juxtamembrane basic residues correlates to a progressive reduction in apparent binding affinity for PA. Binding between Syn1A mutants and PA was determined using protein–lipid overlays ($n = 4$), in which increasing amounts of PA were spotted on nitrocellulose. Soluble Syn1A(1–267) was at a fivefold molar excess to PA. Integrated Syn1A immunoreactivity was measured at each PA spot, normalized for each mutant, and the data were fitted with a Hill equation. (C) The 5RK/A Syn1A mutant exhibits significantly reduced binding to PA-containing liposomes compared with WT Syn1A. Percentage of Syn1A bound to liposomes was defined as the percentage of Syn1A immunoreactivity in the top three gradient fractions relative to the total Syn1A immunoreactivity. (D) Representative protein–lipid overlay demonstrating that Syn1A 5RK/A has reduced binding to all acidic phospholipids tested. (E) Representative protein–lipid overlay demonstrating that a peptide of Syn1A's juxtamembrane region (aa 252–265) closely recapitulates the lipid binding of the full-length soluble Syn1A.

brane basic residues was rearranged, while the overall charge was maintained. The apparent PA binding affinity of the RKRK mutant ($EC_{50} \sim 2.6 \times 10^3$ fmol) was indistinguishable from WT, indicating that Syn1A–lipid interactions can tolerate small structural changes within the juxtamembrane region (Figure 2B).

To establish whether domains outside the polybasic juxtamembrane region affect Syn1A's lipid-binding specificity, we synthesized a peptide corresponding to this region (aa 252–265), as well as the corresponding 5RK/A peptide. Protein–lipid overlays demonstrated that the WT peptide closely recapitulated Syn1A's lipid-binding profile, whereas the 5RK/A mutation abrogated the peptide's ability to bind acidic phospholipids (Figure 2E). One notable difference between the WT peptide and Syn1A (1–267) was that the WT peptide demonstrated a high apparent affinity for PS, a lipid for which Syn1A (1–267) exhibited almost no binding (compare Figure 2, D and E). Importantly, lipid binding to Syn1A (1–259), in which the polybasic juxtamembrane region had been truncated, was largely eliminated (data not shown). Thus, although other regions in Syn1A may modify its lipid-binding profile, the basic juxtamembrane residues are clearly required for these interactions.

Full-Length Syn1A Juxtamembrane Mutants Traffic Normally to the Plasma Membrane in Live Cells

To assess the relevance of Syn1A–lipid interactions in an in vivo situation, full-length, juxtamembrane mutant Syn1A proteins were next studied in living cells. Initial experiments examined whether these mutant Syn1A proteins were expressed and targeted properly. Enhanced cyan fluorescent protein (ECFP)-tagged, full-length Syn1A mutants were generated and transiently transfected into PC-12 cells. All Syn1A constructs were cotransfected with Munc18-1, which facilitated high levels of targeting of Syn1A to the plasma

membrane regions. Figure 3A demonstrates that the fluorescence signal associated with both the WT and 5RK/A ECFP-Syn1A proteins trafficked normally to the plasma membrane region, as determined by confocal microscopy.

To quantify the extent of surface labeling between mutants, we next used dual-fluorophore-labeled Syn1A constructs, which were tagged with mRFP at the N-terminus and pHluorin at the C-terminus. pHluorin is a pH-sensitive variant of GFP, whose signal is quenched within acidic intracellular compartments, but which becomes highly fluorescent upon exposure to the neutral, extracellular solution (which in this case, occurs upon insertion of Syn1A's C-terminal transmembrane domain into the plasma membrane). We thus reasoned that the pHluorin signal would report only on the pool of exogenous Syn1A that had been correctly inserted into the plasma membrane, whereas the mRFP signal would report on the entire pool of exogenous Syn1A expressed within a cell. Figure 3B shows representative epifluorescence images of PC-12 cells transiently cotransfected with the mRFP-Syn1A-pHluorin constructs and Munc18-1. The notable fluorescence of the pHluorin label at the plasma membrane region demonstrates that both the WT and 5RK/A Syn1A constructs were correctly trafficked and inserted into the plasma membrane. Importantly, measurements of the average pHluorin and mRFP fluorescence intensities across a large number of cells were comparable between the WT and 5RK/A conditions, demonstrating that both the surface levels and total expression levels of WT and 5RK/A Syn1A proteins were similar (Figure 3C). Figure 3D shows a scatterplot in which the mean pHluorin intensity was plotted against the mean mRFP intensity for each individual cell. Notably, there occurred a large overlap in the distribution of points between the WT and 5RK/A cells, and linear fits of these data for cells expressing moderate levels of the Syn1A (RFP mean intensities between 20 and 170)

Figure 3. Syn1A 5RK/A targets to plasma membrane regions in PC-12 cells. (A) Representative confocal fluorescence and corresponding DIC images of PC-12 cells transiently transfected with eCFP-tagged Syn1A(WT) and Syn1A (5RK/A) together with Munc18-1. (B) Representative epifluorescence images of PC-12 cells transiently transfected with mRFP-Syn1A-pHluorin (WT or 5RK/A) and Munc18. The pHluorin signal (left) reports only on the pool of Syn1A present at the cell surface, whereas the mRFP signal (right) reports on the entire pool of exogenous Syn1A within the cell. (C) Average pHluorin and mRFP fluorescence intensity for PC-12 cells transfected as in B. Bars, means $\pm$ SEM. Numbers above each bar represent the number of cells used to calculate the average for each condition. (D) Scatterplot of mean pHluorin intensity versus mean mRFP intensity for each individual cell. Cells demonstrating moderate Syn1A expression levels (defined as a mean RFP signal between 20 and 170, a range within which >85% of the cells analyzed fell) were used to generate linear fits for each data set, as indicated by the solid lines. Dotted lines represent the 95% confidence intervals of the linear fits, which overlap for the WT and 5RK/A data sets.

were not significantly different. Thus, the WT and 5RK/A Syn1A constructs demonstrate not only comparable expression levels, but also similar abilities to traffic and insert within the plasma membrane.

Full-Length Syn1A Juxtamembrane Mutants Demonstrate Intact Protein–Protein Interactions within Live Cells

Having determined that the full-length juxtamembrane neutralization mutants of Syn1A were capable of trafficking correctly, we next asked whether these mutants were capable of forming appropriate protein–protein interactions *in vivo*. The above data suggested that both WT and 5RK/A Syn1A constructs interacted similarly with Munc18-1, as coexpression of these constructs with Munc18-1 greatly facilitated trafficking of both constructs to the plasma membrane. To confirm the normal Munc18-1 interaction properties between these Syn1A constructs, we used a sensitized emission FRET approach to compare binding of the CFP-tagged WT Syn1A or 5RK/A mutant to citrine-Munc18-1 in live cells. That this FRET stoichiometry approach is an accurate reporter of the Syn1A-Munc18-1 interaction was established in our earlier publication (Liu *et al.*, 2004). Figure

4A demonstrates that both WT and 5RK/A proteins associated with Munc18-1 similarly, across a wide range of molar ratios. This result is quantified in Figure 4B, where at equimolar ratios (molar ratio between 0.9 and 1.1), the 5RK/A mutant exhibited a FRET efficiency (ED) with Munc18-1 that was similar to that of the WT protein (WT = 24.2 ± 0.01 , 5RK/A = 24.8 ± 0.01). In contrast, a Syn1A mutant (I233A) that was previously shown to have reduced binding to Munc18-1 (Kee *et al.*, 1995), demonstrated a reduced FRET efficiency (I233A = 5.6 ± 0.01) compared with WT. Thus, neutralizing mutations within the juxtamembrane region of Syn1A do not appear to affect Syn1A's interaction with Munc18-1.

We next asked whether Syn1A 5RK/A possessed the ability to interact normally with SNARE proteins, in particular, the Q-SNARE SNAP25. For these experiments, HEK293-S3 cells were transiently transfected with Syn1A (WT or mutant), Munc18-1, and EGFP-SNAP25. Co-IP experiments were performed to determine whether the 5RK/A mutant was capable of pulling down equal amounts of EGFP-SNAP25 compared with the WT Syn1A. Figure 4C shows a blot from a representative experiment, which demonstrates that both the WT and 5RK/A Syn1A proteins bound a similar amount of EGFP-SNAP25. In contrast, a mutant of Syn1A (L205A/E206A) which was previously shown to have reduced binding to SNARE proteins (Dulubova *et al.*, 1999), demonstrated a marked reduction in binding to EGFP-SNAP25. To quantify these results, we measured the integrated densities of the Syn1A and EGFP-SNAP25 bands and determined the ratio of EGFP-SNAP25 to Syn1A in the immunoprecipitated fractions for each condition. For each experiment, the SNAP25:Syn1A ratio for each treatment was normalized to the ratio from the WT treatment, to allow comparison of treatments across experiments. Figure 4D shows the average results for three independent experiments and again demonstrates that the 5RK/A Syn1A displays similar interactions with SNAP25 compared with the WT Syn1A.

Taken together, these data demonstrate that, despite profound differences in lipid-binding capabilities, the 5RK/A mutant Syn1A behaves nearly identically to the WT Syn1A in live cells, with respect to expression levels, membrane trafficking, and forming appropriate protein–protein interactions.

Use of BoNT-C Knockdown of Syn1A to Specifically Isolate the Functional Phenotypes of Exogenous Syn1A Constructs

To test whether disruption of Syn1A–lipid interactions would have a functional effect on regulated exocytosis, we transfected Syn1A juxtamembrane neutralization mutant constructs into live secretory cells (PC-12 cells or bovine adrenal chromaffin cells). To reduce potentially confounding effects from endogenous Syn1A in these cells, we transfected the cells with the light chain of BoNT-C, which cleaves Syn1A and precludes it from mediating membrane fusion (Schiavo *et al.*, 1995). Syntaxin 4 was previously reported to be resistant to cleavage by BoNT-C and to contain an Ile in place of Lys at residue 253 of the BoNT-C cleavage site (Schiavo *et al.*, 1995). We therefore tested whether a similar mutation (K253I) in Syn1A, would generate a BoNT-C-resistant Syn1A. For this analysis, PC-12 cells were cotransfected with an N-terminally tagged CFP-Syn1A and Munc18-1, with or without BoNT-C. Cells were then imaged using conventional fluorescence microscopy. In the absence of BoNT-C, both the WT Syn1A and Syn1A_{K253I} constructs targeted to plasma membrane regions (Figure 5A). In the presence of BoNT-C, however, the WT Syn1A signal was redistributed as a diffuse cytosolic signal within the cells, indi-

Figure 4. Syn1A 5RK/A interacts normally with Munc18-1 and SNAP25. (A) Analysis of direct CFP-Syn1A-citrine-Munc18-1 interactions by sensitized emission FRET imaging. Graph shows a pixel-by-pixel plot of FRET efficiency versus molar ratio (Syn1A:Munc18-1). Both Syn1A (WT) and Syn1A (5RK/A) bind Munc18-1 with similar FRET efficiency. By comparison, Syn1A I233A demonstrates strongly reduced FRET efficiencies with Munc18-1 compared with WT. Solid horizontal line demonstrates background FRET values determined from coexpression of CFP and citrine fluoroproteins. (B) Average FRET efficiency for each Syn1A construct, at equimolar ratios of Syn1A and Munc18-1 ($0.9 < \text{ratio} < 1.1$; see vertical lines in B). Bars, mean $\pm$ SEM. (C) Representative immunoblot demonstrating co-IP of EGFP-SNAP25 with Syn1A from HEK293-S3 cells transiently transfected with Syn1A (WT, L205A/E206A, or 5RK/A), Munc18-1, and EGFP-SNAP25. Note that Syn1A 5RK/A pulls down similar amounts of EGFP-SNAP25 compared with WT Syn1A. In contrast, a mutant of Syn1A (L205A/E206A) previously shown to have reduced binding to SNARE proteins demonstrates a reduced amount of EGFP-SNAP25 pulldown. (D) Integrated densities of Syn1A and EGFP-SNAP25 bands were measured to determine a ratio of EGFP-SNAP25 to Syn1A for each condition. Ratios within a single experiment were then normalized to that of WT Syn1A to allow for comparison across experiments. Bars, means $\pm$ SEM, from three independent experiments.

cating cleavage by BoNT-C, whereas the Syn1A_{K253I} signal distributed to the plasma membrane, indicating resistance to cleavage by BoNT-C (Figure 5A). These experiments were quantified by scoring at least 100 random cells from each condition (while blinded to the conditions) as demonstrating either a cytosolic or membrane fluorescence distribution. Importantly, in the presence of BoNT-C, the percent of cells demonstrating a cytosolic fluorescence distribution was 63% for WT Syn1A, compared with only 5% for Syn1A_{K253I}.

We next tested whether a BoNT-C resistant Syn1A_{K253I} construct could rescue secretion in BoNT-C-treated cells. hGH secretion assays (Wick *et al.*, 1993) were performed on PC-12 cells transfected with various combinations of the BoNT-C light chain, Munc18-1, and either WT Syn1A or Syn1A_{K253I}. Figure 5B shows that transfection of the BoNT-C light chain into PC-12 cells effectively reduced secretion to 30% of control secretion. Importantly, Syn1A_{K253I} rescued secretion in BoNT-C-transfected cells, to roughly 78% of control secretion, but only when cotransfected with Munc18-1. This requirement for Munc18-1 was likely the result of enhanced membrane targeting of Syn1A, as Munc18-1 itself was insufficient to rescue the BoNT-C knockdown of secretion (35% of control secretion). Importantly, WT Syn1A was also unable to rescue secretion in BoNT-C-transfected cells, even when cotransfected with Munc18-1 (29% of control secretion). This clearly indicates that rescue of the BoNT-C knockdown is specific to the expression and proper targeting of a functional BoNT-C-resistant Syn1A to the plasma membrane region in these cells. Of note, although higher expression levels of BoNT-C were sufficient to achieve a more complete knockdown of secretion (reduction to <10% of control secretion), we were often unable to rescue this phenotype by coexpression of Syn1A_{K253I} and Munc18 (data not shown). We attribute this to the fact that at higher concentrations, BoNT-C may also cleave SNAP25 in addition to Syn1A (Williamson *et al.*, 1996).

Neutralizing Mutations within Syn1A's Polybasic Juxtamembrane Region Result in a Progressive Inhibition of Syn1A's Secretory Function

To compare the ability of full-length Syn1A juxtamembrane neutralization mutants to rescue secretion, we used the BoNT-C knockdown assay in cotransfected PC-12 cells. Figure 6A demonstrates that neutralizing mutations within Syn1A's polybasic juxtamembrane domain resulted in a progressive decrease in secretory function. When normalized to the extent of rescue seen with Syn1A_{K253I}, the Syn1A_{K253I}(5RK/A) mutant was only capable of rescuing secretion to $67 \pm 3\%$ of the Syn1A_{K253I} control level ($n = 20$). The Syn1A_{K253I}(R262A/R263A) mutant rescued secretion to $77 \pm 4\%$ of the Syn1A_{K253I} control level ($n = 16$), whereas the Syn1A_{K253I}(R262A) mutant exhibited a phenotype similar to Syn1A_{K253I}, rescuing secretion to $97 \pm 4\%$ of the Syn1A_{K253I} control level. Considering that the residual baseline secretion after BoNT-C knockdown accounts for roughly 45% of the rescued control secretion (Figures 5B and 6A, left, dotted line), the actual deficit in secretion resulting from neutralization of Syn1A's juxtamembrane region was quite substantial, with the Syn1A_{K253I}(5RK/A) mutant rescuing secretion to only 43% of the Syn1A_{K253I} control (Figure 6A, right). Importantly, this decline in secretory function of the juxtamembrane Syn1A mutants correlates well with the decrease in apparent affinity of these mutants for binding phosphatidic acid (Figure 2B) and presumably, with their affinity for other acidic phospholipids as well.

Neutralizing Mutations within Syn1A's Polybasic Juxtamembrane Region Result in a Reduction in Fusion Event Frequency

We next sought to determine the mechanism by which neutralization of Syn1A's juxtamembrane region resulted in secretory inhibition. Importantly, both Syn1A_{K253I} and Syn1A_{K253I}(5RK/A) cells demonstrated comparable calcium

Figure 5. BoNT-C knockdown allows isolation of functional effects of exogenous Syn1A constructs in live secretory cells. (A) Top, representative epifluorescence images demonstrating that Syn1A (K253I) is resistant to cleavage by BoNT-C. Images compare the subcellular distribution of fluorescent signal in PC-12 cells transfected with CFP-Syn1A (WT) and CFP-Syn1A (K253I) with/without the light chain of BoNT-C. Munc18-1 was coexpressed in each condition. Bottom, normalized fluorescence intensity profiles, as determined by averaging line scans taken from the outside to the middle of each cell, corresponding to the expression conditions shown above. Line scans were normalized, the peaks were aligned, and scans were averaged for each condition. (B) Functional rescue of BoNT-C knockdown in elevated K^+ evoked (70 mM, 6 min) hGH secretion, by cotransfection with Syn1A (K253I) and Munc18-1. Note that S1A (K253I) or Munc18-1 alone did not rescue secretion, nor did Syn1A (WT) cotransfected with BoNT-C. Bars, means $\pm$ SEM.

fluxes upon depolarization, as measured using the calcium indicator Fura2 (Figure 6B). PC-12 cells were transfected with BoNT-C, Syn1A_{K253I} or Syn1A_{K253I}(5RK/A), Munc18-1, and RFP (to identify transfected cells), preloaded with the Fura2 AM ester, and depolarized using a brief, 5-s local perfusion with 100 mM K^+ . Changes in intracellular $[Ca^{2+}]$, reported by a change in F340/F380, were comparable between Syn1A_{K253I} (control) and Syn1A_{K253I}(5RK/A) cells in all parameters analyzed, including the baseline calcium levels (control: 0.91 ± 0.01 , $n = 51$; 5RK/A: 0.91 ± 0.01 , $n = 59$) and the peak change in calcium (control: 0.20 ± 0.02 , $n = 51$; 5RK/A: 0.19 ± 0.01 ; $n = 59$), as well as the kinetics of the calcium fluxes. This suggests that the decrease in evoked secretion seen in the 5RK/A cells occurs downstream of calcium influx.

Figure 6. Neutralization of Syn1A's polybasic juxtamembrane region results in a decrease in evoked secretion. (A) Progressive neutralizing mutations of Syn1A's polybasic juxtamembrane domain result in a graded reduction in evoked hGH secretion. Transfected PC-12 cells were stimulated with 70 mM K^+ for 6 min. Data were normalized to control rescue (Syn1A_{K253I}) for each experiment and pooled across at least three independent experiments for each condition. Dotted line represents the baseline level of remaining secretion following BoNT-C treatment. Bars, means $\pm$ SEM. Asterisks indicate statistical significance ($p < 0.001$) from control. Graph at right shows the same data as on the left, but with the average residual BoNT-C secretion for each experiment subtracted from each condition before normalization to control rescue. Note that when the data are corrected for residual BoNT-C secretion, the actual effect of the Syn1A neutralization mutants becomes substantially larger. (B) Evoked $[Ca^{2+}]$ dynamics reported by changes in Fura2 fluorescence intensity ratio (F340 nm/380 nm) demonstrate that depolarization-induced (100 mM K^+ for 5 s) calcium fluxes are similar between control and 5RK/A cells. Left, averaged ratio traces for control and 5RK/A cells. Horizontal bar indicates period of stimulus application. Averaged baseline (middle) and evoked change (right) in F340 nm/380 nm ratio for control and 5RK/A cells. (C) Representative amperometric recordings from bovine adrenal chromaffin cells transfected with Syn1A_{K253I} or Syn1A_{K253I}(5RK/A) together with BoNT-C. Exocytotic secretion was evoked by a 60-s application of locally perfused 100 mM K^+ . Amperometric spikes were detected and quantified by an automated analysis software program written by Eugene Mosharov (Columbia University). (D) Comparison of averaged number of spikes per cell in control and 5RK/A cells. Bars, mean $\pm$ SEM. Only spikes >10 pA in amplitude above RMS baseline were considered for analysis. (E) Cumulative spike frequency distribution, normalized for the total number of spikes for control (Syn1A_{K253I}) and Syn1A_{K253I}(5RK/A) cells. The distributions were not significantly different.

To probe the temporal resolution and analyze the dynamics of individual fusion events, we next used carbon fiber amperometry. For these single-cell experiments, bovine adrenal chromaffin cells were used rather than PC-12 cells, as we found the exocytotic responses produced by these cells to be far more robust than those of PC-12 cells. Chromaffin cells were biolistically transfected with BoNT-C, either Syn1A_{K253I} (control) or Syn1A_{K253I}(5RK/A), and GFP. Coexpression of Munc18-1 was not necessary in these experiments, as we have previously shown that Syn1A can traffic to the plasma membrane in chromaffin cells without the need for Munc18-1 overexpression (Gladycheva *et al.*, 2007). Transfected cells were stimulated to secrete by local perfusion of 100 mM K⁺ for 60 s, during which time amperometric spikes were recorded. Representative amperometric traces are displayed in Figure 6C. In agreement with the hGH data above, the average number of spikes (fusion events) per cell was substantially reduced in the 5RK/A cells compared with control (control: 33.6 ± 4.0 spikes/cell, $n = 86$; 5RK/A: 24.3 ± 2.6 , $n = 82$; Figure 6D). However, the frequency distribution of the spikes, when normalized to the total number of spikes for each condition, was identical between control and 5RK/A cells (Figure 6E). This suggests that, rather than affecting a specific subpopulation of vesicles, the decrease in fusion events observed in the 5RK/A condition results from a generalized decrease in fusogenicity.

Syn1A's Polybasic Juxtamembrane Region Regulates Fusion Pore Dynamics

We hypothesized that the generalized decrease in fusogenicity seen in 5RK/A cells might also be manifest in individual fusion events, particularly with regards to fusion pore dynamics. Many amperometric spikes contain a PSF, which is believed to represent the initial flux of catecholamine through the fusion pore, before dilation of the fusion pore and full fusion of the vesicle with the plasma membrane (Chow *et al.*, 1992; Zhou *et al.*, 1996). Analysis of PSF can thus elucidate details surrounding the late stages of vesicle fusion, especially those regarding the formation and expansion of the fusion pore. Changes in PSF amplitudes are believed to represent changes in fusion pore diameter, whereas changes in PSF duration are thought to represent changes in stability of the fusion pore and kinetics of fusion pore expansion.

Representative PSF are shown in Figure 7A. Interestingly, the Syn1A_{K253I}(5RK/A) cells demonstrated decreased PSF amplitudes, in addition to increased PSF durations, compared with control Syn1A_{K253I} (control) PSF (Figure 7, B and C). In other words, the fusion pores in the 5RK/A cells were not only smaller in diameter, but also took longer to expand to full fusion. The mean PSF amplitude for control cells was 7.66 ± 0.27 pA, which was slightly reduced to 6.18 ± 0.24 pA for 5RK/A cells ($n = 584$ control PSF, $n = 576$ 5RK/A PSF; Figure 7B, left). The mean PSF duration for control cells was 7.28 ± 0.74 ms, which was lengthened to 12.33 ± 1.05 ms in 5RK/A cells ($n = 584$ control PSF, $n = 576$ 5RK/A PSF; Figure 7C, left). Qualitatively, these results also held true under a more stringent analysis scheme, in which the median PSF parameters for each cell were first determined, and the medians then were averaged across cells. Median PSF amplitudes were 6.16 ± 0.43 and 4.42 ± 0.22 pA for control and 5RK/A cells, respectively ($n = 66$ control cells, $n = 78$ 5RK/A cells; Figure 7B, right). Median PSF durations were 5.44 ± 0.54 ms for control, compared with 8.23 ± 0.95 ms for 5RK/A cells ($n = 66$ control cells, $n = 78$ 5RK/A cells; Figure 7C, right). In all analysis schemes, the differences between control and 5RK/A PSF parameters were statistically significant ($p < 0.05$).

Figure 7. Neutralization of Syn1A's polybasic juxtamembrane region results in a decrease in fusion pore diameter and a lengthening of fusion pore duration. (A) Representative PSF for control (Syn1A_{K253I}) or Syn1A_{K253I}(5RK/A) expression conditions (+ BoNT-C) during stimulation by 60-s application of locally perfused 100 mM K⁺. (B) Left, mean PSF amplitudes for control and 5RK/A cells, from all PSF >1 ms in duration and <30 pA in amplitude ($n = 584$ control PSF analyzed, $n = 576$ 5RK/A PSF analyzed). Right, averaged median PSF amplitudes ($n = 66$ control cells analyzed, $n = 78$ 5RK/A cells analyzed). In both cases, the PSF from 5RK/A cells demonstrate statistically significant decreases in amplitude ($p < 0.05$). Bars, mean $\pm$ SEM. (C) Left, mean PSF durations for control and 5RK/A cells, from all PSF >1 ms in duration and <30 pA in amplitude ($n = 584$ control PSF analyzed, $n = 576$ 5RK/A PSF analyzed). Right, averaged median PSF durations ($n = 66$ control cells analyzed, $n = 78$ 5RK/A cells analyzed). In both cases, the PSF from 5RK/A cells demonstrate statistically significant lengthening in duration ($p < 0.05$). Bars, mean $\pm$ SEM.

icant ($p < 0.05$). Thus, neutralization of Syn1A's polybasic juxtamembrane region results in a decrease in fusion pore diameter, as well as a delay in fusion pore expansion. As formation and expansion of the fusion pore have been modeled to be among the most energetically expensive steps in the membrane fusion process (Chernomordik and Kozlov, 2003; Cohen and Melikyan, 2004), these data suggest that energetic inefficiencies in the fusion process may, in part, account for the secretory defects observed with the 5RK/A mutant.

Manipulation of Intracellular Phosphatidic Acid Levels Differentially Regulates Secretion from Control and 5RK/A Cells

Thus far, we have demonstrated that Syn1A binds multiple acidic phospholipids, including the fusogenic lipid, phosphatidic acid, and that these interactions are mediated through Syn1A's polybasic juxtamembrane domain. Moreover, we have shown that neutralization of Syn1A's polybasic juxtamembrane domain (5RK/A mutant) results in a significant decrease in stimulated secretion, in addition to effects on fusion pore amplitude and duration that are suggestive of an energetic defect in the fusion process. To de-

are normalized to the untreated Syn1A_{K253I} control rescue in each experiment to allow comparison across multiple experiments, and data were pooled from at least three independent experiments. Bars, mean $\pm$ SEM. Letters above bars indicate pairs of conditions in which the differences are statistically significant ($p < 0.05$).

Figure 8. Functional phenotypes of Syn1A_{K253I} control and 5RK/A mutant are differentially regulated by manipulation of phosphatidic acid levels in live PC-12 cells. Graph demonstrates averaged results from hGH secretion assays utilizing the BoNT-C knockdown and rescue. External application of 1 μ M LPC has no effect on control secretion, but results in a partial rescue of the 5RK/A phenotype. Overexpression of PLD1 results in near-complete rescue of the 5RK/A phenotype to control secretion levels. On the other hand, knockdown of PLD1 activity via a targeted siRNA construct results in a drastic decrease in control secretion, while having little to no effect on secretion from 5RK/A cells. Results

termine whether the functional effects seen with the 5RK/A mutant are a direct result of the inability of this mutant to bind lipids, we performed hGH secretion assays to test how various alterations in membrane lipid composition affected the abilities of the Syn1A_{K253I}(control) or Syn1A_{K253I}(5RK/A) mutant to rescue BoNT-C-mediated knockdown of secretion in PC-12 cells. As in prior experiments, all results have been normalized to the level of rescued secretion observed in the Syn1A_{K253I} control cells in the absence of any lipid manipulations.

To determine whether the secretory defect in the 5RK/A cells might be related to the energetics of fusion, we first tested the effects of externally applied LPC (1 μ M), an inverted-cone-shaped lipid that facilitates fusion pore formation and expansion by inducing positive curvature to the outer leaflet of the membrane bilayer. In these experiments, using an LPC concentration that was low enough so as not to affect stimulated secretion from the Syn1A_{K253I} control cells, we observed a statistically significant and substantial (>50%) increase in secretion from the Syn1A_{K253I}(5RK/A) cells (38.6 vs. 59.8% rescued secretion in the absence or presence of LPC, respectively; Figure 8). This result suggests that the exocytotic defect in the 5RK/A mutant occurs at a late step in fusion and is likely energetic in nature.

The partial rescue of the 5RK/A secretory phenotype by externally applied LPC (which induces positive curvature on the outer bilayer leaflet) nicely complemented our previous result demonstrating that Syn1A interacts with phosphatidic acid, a cone-shaped, fusogenic lipid (which induces negative curvature on the inner bilayer leaflet). We thus investigated whether specific Syn1A-phosphatidic acid interactions are important for regulated exocytosis. For these experiments, the BoNT-C knockdown and rescue assay was repeated, while overexpressing either phospholipase D1 (PLD1) or an siRNA construct previously demonstrated to target PLD1 (Zeniou-Meyer *et al.*, 2007). PLD1 is a stimulus-activated enzyme that cleaves phosphatidylcholine to generate free choline and phosphatidic acid. Overexpression of PLD1 has been shown to enhance regulated exocytosis, whereas knockdown of PLD1 activity results in a decrease in secretion (Humeau *et al.*, 2001; Vitale *et al.*, 2001; Hughes *et al.*, 2004; Huang *et al.*, 2005; Zeniou-Meyer *et al.*, 2007). Although the extent to which PLD1 overexpression and knockdown affected control secretion in our BoNT-C knockdown experiments (Figure 8) was slightly less than has been previously reported, this likely resulted from the BoNT-C knockdown assay's requirement for simultaneous overexpression of multiple constructs. As such, changes in PLD1

levels achieved in this system were likely more limited than when PLD1 or siRNA-PLD1 are the only constructs being overexpressed (as in prior studies).

Importantly, we found that overexpression of PLD1 resulted in a near complete phenotypic rescue of the 5RK/A mutant [117 vs. 103% rescued secretion, for PLD1-treated Syn1A_{K253I} control cells or PLD1-treated Syn1A_{K253I}(5RK/A) cells, respectively; Figure 8]. Also of substantial interest was the finding that siRNA-mediated knockdown of PLD1 drastically reduced secretion from Syn1A_{K253I} control cells, while having little effect on secretion from Syn1A_{K253I}(5RK/A) cells [74% reduction in secretion for Syn1A_{K253I} control cells, compared with a 24% reduction for Syn1A_{K253I}(5RK/A) cells, in the presence of siRNA-PLD1; Figure 8]. These data provide strong evidence that the loss of interaction between Syn1A and phosphatidic acid largely accounts for the secretory defects seen with the Syn1A 5RK/A mutation and rule out the possibility that this secretory defect resulted from disruption of untested Syn1A-protein interactions or from alterations in Syn1A structure. More importantly, these results demonstrate that Syn1A-lipid interactions are critically important in regulating the overall levels of evoked secretion in live neuroendocrine cells.

DISCUSSION

SNARE complexes comprise the minimal protein machinery required for membrane fusion; hence most studies have focused on defining the regulation of their assembly and disassembly. However, the fusion process also involves lipids, and the specific lipid composition of membranes undergoing fusion has profound consequences on the energetic barrier for fusion (Chernomordik *et al.*, 1995). A central and unresolved issue is whether specific mechanisms exist to recruit and sequester fusogenic and/or bioactive lipids at preferential sites of membrane fusion.

In this study, we establish that the Q-SNARE Syntaxin1A (Syn1A) forms functional interactions with acidic phospholipids and that these interactions facilitate membrane fusion. Syn1A specifically interacted with the fusogenic lipid, phosphatidic acid, as well as with multiple polyphosphoinositides, including PI(4,5)P₂. Neutralizing mutations within a highly conserved, polybasic juxtamembrane region of Syn1A (aa 252-265) progressively reduced Syn1A's ability to bind lipids, while leaving intact these mutants' abilities to traffic correctly to the plasma membrane and to form appropriate protein-protein interactions. Development of a novel BoNT-C knockdown and rescue assay allowed the secretory

function of exogenous mutant Syn1A constructs to be studied in isolation from endogenous WT Syn1A and demonstrated that progressive neutralization of Syn1A's juxtamembrane region resulted in a progressive decrease in secretory function. Moreover, amperometric analysis uncovered a lengthening in fusion pore duration and a decrease in fusion pore diameter in fusion events catalyzed by a lipid-binding-deficient Syn1A (5RK/A), suggesting an energetic defect in fusion. Importantly, we found that the inhibition of secretion observed with the Syn1A 5RK/A mutant could be completely rescued by overexpression of PLD1, and that knockdown of PLD1 activity strongly inhibited control secretion, while having little effect on 5RK/A secretion. Altogether, these data suggest that Syn1A–lipid interactions play a key role in regulating the energetics of membrane fusion.

We propose that Syn1A–lipid interactions function both structurally as well as electrostatically to reduce the energetic barrier for fusion specifically at sites of exocytosis. This energy barrier can be modeled as a series of membrane intermediates formed during the merging of two lipid bilayers (Chernomordik and Kozlov, 2003; Cohen and Melikyan, 2004). First, the membranes are brought within proximity to establish a region of dehydrated contact. A fusion stalk forms as the initial connection between the membranes, and radial expansion of this stalk yields a hemifusion diaphragm, where contacting leaflets of the bilayers have merged, whereas the distal leaflets remain separate. Importantly, the presence of negative curvature-favoring lipids in the contacting leaflets greatly reduces the energetic requirements for stalk and hemifusion formation (Kozlovsky and Kozlov, 2002). PA, under physiological conditions, exhibits negative spontaneous curvature approaching that of PE (Kooijman *et al.*, 2005). Thus, a first *structural* function of the Syn1A–lipid interaction may be to localize PA to sites of fusion, thereby generating negative curvature to facilitate stalk formation and stabilize the hemifusion intermediate.

Beyond hemifusion, generation of lateral tension within the hemifusion diaphragm leads to membrane rupture and formation of a fusion pore, whereby the distal leaflets of the two bilayers merge. Expansion of this fusion pore results in full membrane collapse and fusion. These steps are believed to be the most energetically expensive in membrane fusion (Chernomordik and Kozlov, 2003; Cohen and Melikyan, 2004). It has been proposed that the lateral tension required for formation and expansion of the fusion pore may be generated by electrostatic interactions between fusogenic proteins and acidic lipids (Zimmerberg and Gawrisch, 2006). Indeed, generation of the lateral tension to drive fusion pore expansion may be a second *electrostatic* function of the Syn1A–lipid interaction. This is supported by our amperometry results, which demonstrated that neutralization of Syn1A's polybasic juxtamembrane region resulted in a delay in fusion pore expansion and a decrease in fusion pore diameter.

In this study, we demonstrate a novel and specific interaction of Syn1A with PA, a lipid known to exert substantial effects on regulated exocytosis. PLD1, an enzyme that generates PA from PC, potentially enhances regulated exocytosis at a late stage in vesicle fusion, in a variety of cell types including PC12 cells, adrenal chromaffin cells, adipocytes, and neurons (Humeau *et al.*, 2001; Vitale *et al.*, 2001; Hughes *et al.*, 2004; Huang *et al.*, 2005; Zeniou-Meyer *et al.*, 2007). Whereas specific effectors for the PLD1-generated PA have remained elusive, our results strongly suggest that the facilitatory effects of PLD on secretion may be mediated in part by Syn1A–PA interactions. Namely, we found that the Syn1A 5RK/A mutant, which lacks the ability to bind PA,

demonstrated reduced levels of secretion. Overexpression of PLD1 rescued this defect, presumably because the increased local concentrations of PA generated by PLD1 compensated for the 5RK/A's inability to sequester PA. Similarly, knockdown of PLD1 activity resulted in the inhibition of control secretion, because in the absence of PA, Syn1A's function becomes similar to that of the 5RK/A mutant. Accordingly, knockdown of PLD1 activity had little effect on Syn1A 5RK/A, because this mutant cannot normally bind PA.

We have focused largely on Syn1A–PA interactions, but our finding that Syn1A directly binds PI(4,5)P₂ is also of interest. Quantitative *in vitro* binding assays suggest that a peptide of Syn1A's basic juxtamembrane region may bind to PI(4,5)P₂ with higher affinity than to PA (S. McLaughlin, personal communication). PI(4,5)P₂ is perhaps the most notable lipid signal for regulated exocytosis, given its requirement in the ATP-dependent priming of vesicles (Eberhard *et al.*, 1990; Hay *et al.*, 1995; Wiedemann *et al.*, 1996). Endogenous PI(4,5)P₂ clusters partially colocalize with Syn1A clusters in membrane sheets of PC12 cells, and exocytosis was promoted at sites of Syn1A–PI(4,5)P₂ cluster colocalization (Aoyagi *et al.*, 2005). Of substantial interest is that PA and PI(4,5)P₂ participate in a positive feed-forward cycle that results in the local generation of both lipids: PA positively regulates PIP5K1, an enzyme that generates PI(4,5)P₂ (Jenkins *et al.*, 1994), and in turn, PI(4,5)P₂ positively regulates PLD1, which generates PA (Liscovitch *et al.*, 1994). That such specific membrane microdomains can have profound implications on the energetics of SNARE-mediated fusion events is a concept conserved down through yeast. Indeed, it was shown that a yeast SNARE complex (Spo20p–Sso1p–Snc1p) that normally mediates fusion at the prospore membrane was insufficient to drive fusion at the plasma membrane (Coluccio *et al.*, 2004). This insufficiency could be overcome by overexpression of MSS4p (a yeast PI5K). Interestingly, the effect of MSS4p was mediated via recruitment of Spo14p (a yeast PLD), to the plasma membrane. It was suggested that Spo14p-mediated generation of PA at the plasma membrane reduced the energetic requirements for fusion such that the energy released by the Spo20p–Sso1p–Snc1p complex became sufficient to drive fusion at the plasma membrane.

Although the current report is the first to establish the identity of specific Syn1A–lipid interactions, map the interacting domain, and determine direct functional effects of these interactions, it is clear that SNARE–lipid interactions are emerging as significant functional interactions that underlie membrane fusion (Quetglas *et al.*, 2000; Hu *et al.*, 2002; Kweon *et al.*, 2003a,b; Vicogne *et al.*, 2006). Of substantial importance is that sequence alignment across multiple families of SNARE proteins demonstrates high conservation of the lipid-interacting polybasic juxtamembrane region (Weimbs *et al.*, 1998). Therefore, the concept of membrane fusion driven by SNARE proteins must be enlarged to encompass the possibility that SNAREs also function to spatially sequester bioactive lipids as a means to alter the energetic requirements for fusion.

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