

# Phosphatidylinositol 4,5-bisphosphate regulates SNARE-dependent membrane fusion

Declan J. James, Chuenchanok Khodthong, Judith A. Kowalchyk, and Thomas F.J. Martin

Department of Biochemistry, University of Wisconsin, Madison, WI 53706

**P**hosphatidylinositol 4,5-bisphosphate (PI 4,5-P<sub>2</sub>) on the plasma membrane is essential for vesicle exocytosis but its role in membrane fusion has not been determined. Here, we quantify the concentration of PI 4,5-P<sub>2</sub> as ~6 mol% in the cytoplasmic leaflet of plasma membrane microdomains at sites of docked vesicles. At this concentration of PI 4,5-P<sub>2</sub> soluble NSF attachment protein receptor (SNARE)-dependent liposome fusion is inhibited. Inhibition by PI 4,5-P<sub>2</sub> likely results from its intrinsic positive curvature-promoting properties that inhibit formation of high negative curvature membrane fusion intermediates. Mutation of juxtamembrane basic residues in the

plasma membrane SNARE syntaxin-1 increase inhibition by PI 4,5-P<sub>2</sub>, suggesting that syntaxin sequesters PI 4,5-P<sub>2</sub> to alleviate inhibition. To define an essential rather than inhibitory role for PI 4,5-P<sub>2</sub>, we test a PI 4,5-P<sub>2</sub>-binding priming factor required for vesicle exocytosis. Ca<sup>2+</sup>-dependent activator protein for secretion promotes increased rates of SNARE-dependent fusion that are PI 4,5-P<sub>2</sub> dependent. These results indicate that PI 4,5-P<sub>2</sub> regulates fusion both as a fusion restraint that syntaxin-1 alleviates and as an essential cofactor that recruits protein priming factors to facilitate SNARE-dependent fusion.

## Introduction

Phosphatidylinositol-4,5-bisphosphate (PI 4,5-P<sub>2</sub>) is a minor but key phospholipid mainly on the cytoplasmic leaflet of the plasma membrane that regulates a wide variety of processes, including exocytic and endocytic membrane traffic (Martin, 1998; Cremona and De Camilli, 2001; Ungewickell and Hinrichsen, 2007), ion channel and transporter function (Hilgemann et al., 2001; Suh and Hille, 2005), enzyme activation (McDermott et al., 2004), and protein recruitment (Lemmon, 2003; Janmey and Lindberg, 2004; Balla, 2005; Takenawa and Itoh, 2006). For some of these processes, PI 4,5-P<sub>2</sub> exerts regulation by binding known protein effectors via unstructured basic residue-rich regions or via more highly structured domains such as pleckstrin homology (PH) domains (Lemmon, 2003; Balla, 2005; McLaughlin and Murray, 2005; Takenawa and Itoh, 2006). For clathrin-dependent endocytosis, PI 4,5-P<sub>2</sub>-binding protein effectors (e.g., AP-2, AP180, epsin, and dynamin) orchestrate clathrin binding,

membrane curvature, and endosome fission (Ungewickell and Hinrichsen, 2007). Ca<sup>2+</sup>-triggered vesicle fusion also requires plasma membrane PI 4,5-P<sub>2</sub> (Hay and Martin, 1993; Hay et al., 1995; Holz et al., 2000), but the underlying basis for this requirement is not fully understood.

Regulated vesicle exocytosis is mediated by SNARE proteins with vesicle-associated membrane protein 2 (VAMP-2) on the vesicle, forming a complex with 25-kD synaptosome-associated protein (SNAP-25) and syntaxin-1 on the plasma membrane (Jahn and Scheller, 2006). SNARE regulatory proteins promote specific assembly reactions for vesicle docking, priming, and Ca<sup>2+</sup>-triggered fusion (Jahn and Scheller, 2006). PI 4,5-P<sub>2</sub> is essential for a pre-fusion step termed priming, during which the vesicle and plasma membrane develop competence for Ca<sup>2+</sup>-triggered fusion by conversion of vesicles into a ready-releasable state (Hay et al., 1995; Olsen et al., 2003; Grishanin et al., 2004; Gong et al., 2005; Milosevic et al., 2005). Several PI 4,5-P<sub>2</sub>-binding proteins potentially involved in stages of vesicle exocytosis have been identified, including rabphilin (Chung et al., 1998), Ca<sup>2+</sup>-dependent activator protein for secretion (CAPS);

Correspondence to Thomas F.J. Martin: [tfmartin@wisc.edu](mailto:tfmartin@wisc.edu)

Abbreviations used in this paper: CAPS, Ca<sup>2+</sup>-dependent activator protein for secretion; DOPC, 1,2-dioleoyl phosphatidylcholine; DOPS, 1,2-dioleoyl phosphatidylserine; LPC, lysophosphatidylcholine; NBD-PE, N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; Pf, protein-free; PH, pleckstrin homology; PI 4-P, phosphatidylinositol-4-monophosphate; PI 4,5-P<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; PS, phosphatidylserine; Rh-PE, N-[lissamine rhodamine B sulfonyl]-1,2-dipalmitoyl phosphatidylethanolamine; SNAP-25, 25-kD synaptosome-associated protein; VAMP-2, vesicle-associated membrane protein 2.

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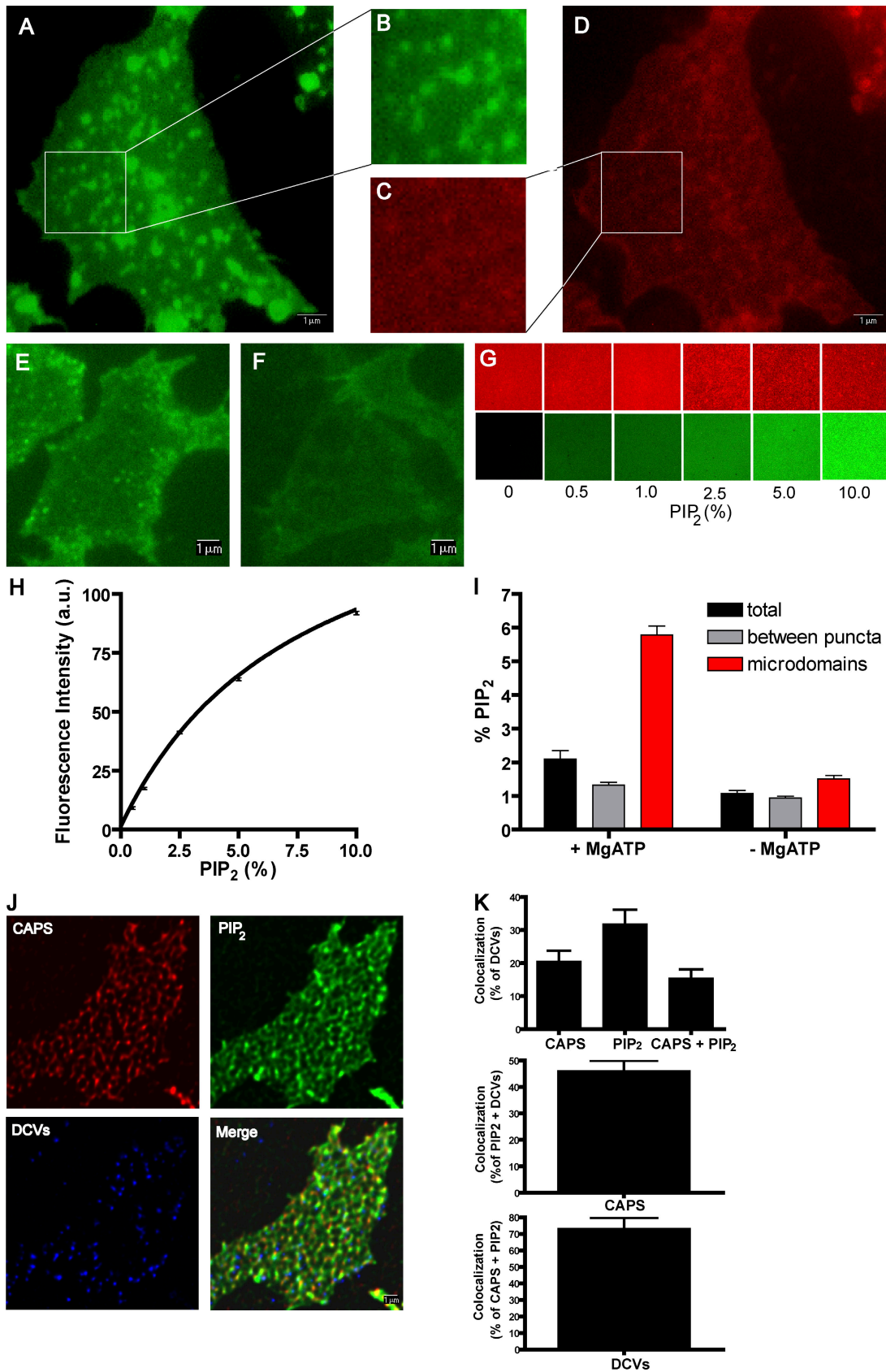


Figure 1. **Microdomains of PI 4,5-P<sub>2</sub> on the plasma membrane.** (A–F) PC12 cell membrane sheets were incubated with 2  $\mu$ M PLC $\delta_1$ -PH-GFP fusion protein and 100  $\mu$ M FM4-64 to image PI 4,5-P<sub>2</sub> (A and B) or membrane area (C and D), respectively. (E and F) Membrane sheets were incubated with PLC $\delta_1$ -PH-GFP fusion protein after incubations with or without 2 mM MgATP, respectively. (G–I) Quantification of membrane PI 4,5-P<sub>2</sub>. (G) Supported planar bilayers containing indicated mole percentage of PI 4,5-P<sub>2</sub> and 0.1 mol% rhodamine-PE were incubated with 2  $\mu$ M PLC $\delta_1$ -PH-GFP fusion protein and imaged in

Loyet et al., 1998), SCAMP2 (secretory carrier membrane protein 2; Liao et al., 2007), and synaptotagmin (Bai et al., 2004); but it is unclear which of these or other proteins might mediate the essential role of PI 4,5-P<sub>2</sub> in vesicle-priming reactions.

The physical properties of PI 4,5-P<sub>2</sub> make it an unlikely candidate for a phospholipid that exerts a positive role in membrane fusion. As an inverted cone-shaped lipid in the cytoplasmic leaflet, PI 4,5-P<sub>2</sub> is anticipated to antagonize the high negative membrane curvature required for the formation of a hemifusion intermediate that transitions to full fusion (Chernomordik and Zimmerberg, 1995). SNARE function and membrane fusion have been reconstituted in proteoliposomes (Weber et al., 1998) of various phospholipid compositions (Brugger et al., 2000; Vicogne et al., 2006) but the effects of PI 4,5-P<sub>2</sub> on fusion have not been specifically determined. In the current work, we quantified the concentrations of PI 4,5-P<sub>2</sub> at sites of vesicle docking (~6 mol%) on the plasma membrane and found that these concentrations strongly inhibited SNARE-dependent liposome fusion. However, we found that inclusion of a priming protein (CAPS) in the minimal fusion assay dramatically enhanced the rates and extent of SNARE-dependent fusion dependent on PI 4,5-P<sub>2</sub>. We conclude that PI 4,5-P<sub>2</sub>, at concentrations present in membrane microdomains, intrinsically acts to restrain membrane fusion but also serves as an essential cofactor to mediate protein recruitment, which promotes stimulation of SNARE-dependent membrane fusion.

## Results

### Sites of vesicle docking correspond to high concentration PI 4,5-P<sub>2</sub>-containing microdomains

To determine the location and size of PI 4,5-P<sub>2</sub> pools potentially required for regulated vesicle fusion, we incubated the monovalent PI 4,5-P<sub>2</sub>-binding PLCδ<sub>1</sub>-PH-GFP protein with plasma membrane sheets prepared from PC12 cells (Fig. 1). Membranes that were primed with ATP and cytosolic factors required for Ca<sup>2+</sup>-triggered vesicle exocytosis (Hay and Martin, 1992; Hay et al., 1995) exhibited a strong punctate labeling by PLCδ<sub>1</sub>-PH-GFP (Fig. 1, A and B). Puncta were not observed when the PLCδ<sub>1</sub>-PH-GFP R40L mutant, which fails to bind PI 4,5-P<sub>2</sub>, was used or when membranes were incubated with Ca<sup>2+</sup>-activated PLCδ<sub>1</sub> (not depicted), which indicated that the wild-type PLCδ<sub>1</sub>-PH-GFP probe detected PI 4,5-P<sub>2</sub> in the membrane. The punctate distribution of PLCδ<sub>1</sub>-PH-GFP contrasted with a very different pattern observed with lipophilic fluorescence stains such as FM4-64 or octadecyl rhodamine B (Fig. 1, C and D). Although some of the membrane puncta labeled with PLCδ<sub>1</sub>-PH-GFP exhibited FM4-64 staining, likely representing endocytic structures, the majority did not (Fig. 1, B vs. C). This indicated

that the majority of PI 4,5-P<sub>2</sub> puncta represent high concentration microdomains rather than membrane infoldings (van Rheenen et al., 2005). The fluorescence intensity of PLCδ<sub>1</sub>-PH-GFP in puncta was maintained in incubations containing MgATP (Fig. 1 E) but decreased in incubations that lacked MgATP (Fig. 1 F), which indicated that lipid kinase and phosphatase reactions dynamically regulate PI 4,5-P<sub>2</sub> levels in the microdomains. Incubations with MgATP in this membrane preparation were previously shown to support regulated vesicle exocytosis (Martin and Kowalchuk, 1997; Avery et al., 2000).

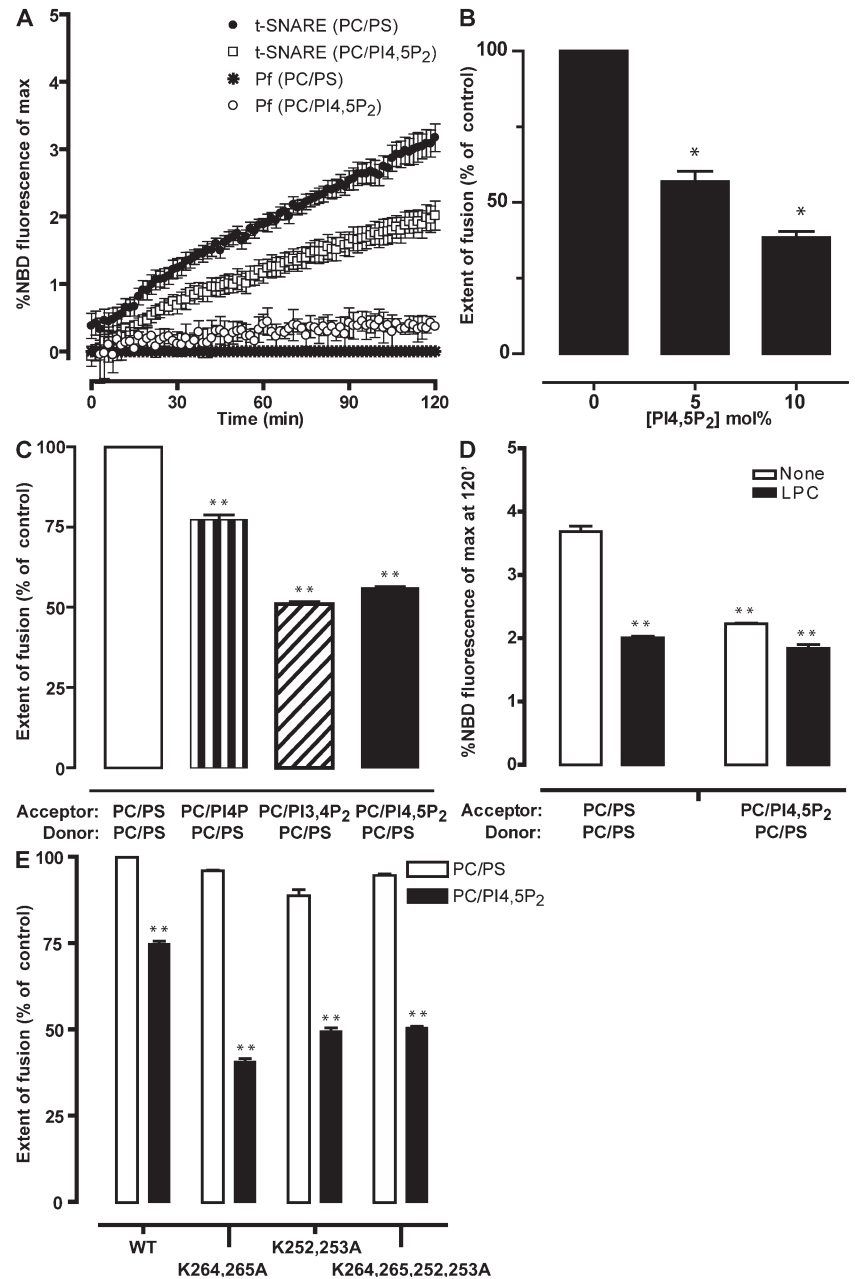
To quantify PI 4,5-P<sub>2</sub> in microdomains, a calibration curve was constructed in parallel experiments by binding PLCδ<sub>1</sub>-PH-GFP to planar-supported bilayers containing a different mole percentage of PI 4,5-P<sub>2</sub> (Fig. 1, G and H). Comparison of the fluorescence intensity per pixel for puncta on PC12 cell membrane sheets that lacked FM4-64 staining with that of calibrated supported bilayers indicated that ~6 mol% PI 4,5-P<sub>2</sub> was present in microdomains in the cytoplasmic leaflet, whereas ~1 mol% PI 4,5-P<sub>2</sub> resided in membrane regions between the microdomains (Fig. 1, H and I). Averaged total membrane concentrations in the cytoplasmic leaflet were ~2 mol% (Fig. 1 I, +MgATP), which agrees with biochemical estimates of ~1 mol% for total plasma membrane with an asymmetrical leaflet distribution of PI 4,5-P<sub>2</sub> (Ferrell and Huestis, 1984; Hagelberg and Allan, 1990; Tran et al., 1993). PI 4,5-P<sub>2</sub> concentrations within cytoplasmic leaflet microdomains are likely underestimated because the average microdomain after deconvolution of images had an apparent area of 0.03 μm<sup>2</sup> corresponding to a diameter of 195 nm, which was equivalent to the resolution limit of our microscope (~203 nm). Overestimates of microdomain area would underestimate PI 4,5-P<sub>2</sub> concentrations within the microdomains.

PI 4,5-P<sub>2</sub>-rich microdomains in PC12 cell membranes localized to sites of vesicle docking and to sites containing the priming factor CAPS (Fig. 1 J). CAPS binds PI 4,5-P<sub>2</sub> (Loyet et al., 1998) and is essential for Ca<sup>2+</sup>-triggered vesicle exocytosis after an ATP-dependent priming step that synthesizes PI 4,5-P<sub>2</sub> (Grishanin et al., 2004). Triple colocalization studies revealed that ~50% of CAPS clusters on the plasma membrane colocalized with PI 4,5-P<sub>2</sub> and docked vesicles (Fig. 1, J and K, middle). Sites containing both PI 4,5-P<sub>2</sub> and CAPS had a high likelihood (~70%) of containing a docked vesicle (Fig. 1, J and K, bottom), and ~20% of the docked vesicles were present at these sites (Fig. 1 K, top). Approximately 10–20% of the docked vesicles undergo fusion in response to Ca<sup>2+</sup> elevations (Lynch and Martin, 2007), and these fusion events were reported to occur preferentially at PI 4,5-P<sub>2</sub>-containing microdomains (Aoyagi et al., 2005). Overall, these results suggest the possibility that Ca<sup>2+</sup>-triggered vesicle fusion may occur at PI 4,5-P<sub>2</sub> microdomains on the plasma membrane containing ≥6 mol% PI 4,5-P<sub>2</sub>.

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red (top) and green (bottom) channels. (H) Fluorescence intensity per pixel was plotted for each supported bilayer to generate a calibration curve. (I) From calibration curves prepared in parallel, pixel intensity on PC12 cell membrane sheets (incubated with or without MgATP) was used to infer PI 4,5-P<sub>2</sub> concentrations in microdomains, between microdomains, and averaged across the membrane. Mean ± SEM values are shown for +MgATP (*n* = 13) and -MgATP (*n* = 5). (J and K) PI 4,5-P<sub>2</sub> microdomains overlap with clusters of CAPS and docked vesicles. (J) PI 4,5-P<sub>2</sub> microdomains in PC12 cell membrane sheets were localized by incubation with PLCδ<sub>1</sub>-PH-GFP and fixed for immunolocalization with CAPS polyclonal or chromogranin B monoclonal antibodies. (K) Triple channel colocalizations were quantified as described in Materials and methods. Mean ± SEM (*n* = 9) values are shown.

**Figure 2. PI 4,5-P<sub>2</sub> inhibits SNARE-dependent liposome fusion.** (A) Acceptor liposomes with 40 copies of syntaxin-1/SNAP-25 (t-SNARE) in 90:10 mol% PC/PI 4,5-P<sub>2</sub> or 85:15 mol% PC/PS were mixed with fluorescent PC/PS donor liposomes with 100 copies of VAMP-2. Parallel reactions with Pf acceptor liposomes at 90:10 mol% PC/PI 4,5-P<sub>2</sub> or 85:15 mol% PC/PS were conducted. The kinetics of fusion are shown as a percentage of total NBD fluorescence after correction for Pf liposomes (see Materials and methods). Points represent mean values  $\pm$  SEM for five independent experiments. (B) Acceptor liposomes reconstituted with syntaxin-1/SNAP-25 and the indicated mole percentage PI 4,5-P<sub>2</sub> were mixed with donor liposomes at a 9:1 ratio ( $\sim$ 2 mM lipid) and NBD fluorescence was monitored. The extent of fusion at 120 min is expressed as a percentage of fusion relative to that observed using acceptor liposomes with 85:15 mol% PC/PS. Bars indicate mean values  $\pm$  SEM for three independent experiments (\*,  $P < 0.01$ ). (C) Liposome fusion reactions were conducted with VAMP-2 donor liposomes incubated with syntaxin-1/SNAP-25 acceptor liposomes containing 5 mol% PI 4-P, PI 4,5-P<sub>2</sub>, or PI 3,4-P<sub>2</sub> as indicated. The extent of fusion at 120 min is expressed as in B (\*\*,  $P < 0.001$  compared with PC/PS donor-acceptor liposomes). (D) Inhibition of fusion by inverted cone lipid LPC. Acceptor liposomes with 40 copies of syntaxin-1/SNAP-25 in 85:15 mol% PC/PS or 90:5 mol% PC/PI 4,5-P<sub>2</sub> as indicated were incubated 5 min at 30°C without or with 0.2 mM LPC and mixed with donor liposomes to monitor fusion kinetics. Values (mean  $\pm$  SEM;  $n = 6$ ) of percentage of maximal NBD fluorescence at 120-min incubation times are shown (\*\*,  $P < 0.001$  compared with untreated PC/PS liposomes). (E) The extent of liposome fusion at 120 min is shown for reactions containing donor liposomes mixed with acceptor liposomes containing wild-type or mutant (K252,253A, K264,265A, and K252,253,264,265A) syntaxins in 85:15 mol% PC/PS or 90:10 mol% PC/PI 4,5-P<sub>2</sub> as indicated. Extent of fusion is shown as a percentage relative to wild-type syntaxin in PC/PS liposomes (\*\*,  $P < 0.001$  compared with WT syntaxin containing PC/PS liposomes).



### PI 4,5-P<sub>2</sub> inhibits SNARE-dependent membrane fusion

To assess the impact of local PI 4,5-P<sub>2</sub> concentrations on membrane fusion, we used a well-characterized SNARE-dependent liposome fusion assay (Weber et al., 1998; Scott et al., 2003) that we modified by using physiological SNARE densities (see Materials and methods). We incorporated PI 4,5-P<sub>2</sub> at 5–10 mol% (95–90 mol% phosphatidylcholine [PC]) into plasma membrane-like SNAP-25/syntaxin-1-containing acceptor liposomes that were tested for fusion with PC/phosphatidylserine (PS) (85:15) donor liposomes containing VAMP-2 and fluorescent phospholipids *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine (Rh-PE) at self-quenching concentrations. Parallel assays were conducted

with SNAP-25/syntaxin-1-containing acceptor liposomes composed of PC/PS (85:15). SNARE-containing PC/PS liposomes exhibited a time-dependent increase in NBD fluorescence characteristic of SNARE-dependent lipid mixing as previously reported (Weber et al., 1998; Fig. 2 A), which was reduced to background by cleaving VAMP-2 with botulinum toxin B (not depicted). In contrast, SNAP-25/syntaxin-1 acceptor liposomes that contained PI 4,5-P<sub>2</sub> exhibited decreased rates and extent of lipid mixing with donor VAMP-2-containing PC/PS liposomes. NBD fluorescence at 120 min was reduced by 25–30% in PI 4,5-P<sub>2</sub>-containing liposomes compared with PS-containing liposomes (Fig. 2 A). The extent of inhibition by 10 mol% PI 4,5-P<sub>2</sub> varied with assay conditions but reached 40–60% when a greater extent of SNARE-dependent fusion was driven at higher liposome concentrations (Fig. 2 B). The inhibition of SNARE-dependent

fusion occurred over a range of PI 4,5-P<sub>2</sub> concentrations that encompassed the 6 mol% that was measured in the cytoplasmic leaflet microdomains of PC12 cell plasma membranes (Fig. 2 B). Because protein-free (Pf) PI 4,5-P<sub>2</sub>-containing liposomes can mix phospholipids under certain conditions (Summers et al., 1996), we also tested Pf acceptor liposomes. Pf PC/PS and PC/PI 4,5-P<sub>2</sub> liposomes exhibited negligible lipid mixing (Fig. 2 A). Thus, the results indicate that PI 4,5-P<sub>2</sub> inhibits SNARE-dependent liposome fusion.

To determine whether the inhibition of fusion was selective for PI 4,5-P<sub>2</sub>, we tested other equally negatively charged (PI 3,4-P<sub>2</sub>) or less negatively charged (phosphatidylinositol-4-monophosphate [PI 4-P]) phosphoinositides. PI 4-P was about half as inhibitory as PI 4,5-P<sub>2</sub>, whereas PI 3,4-P<sub>2</sub> was as inhibitory as PI 4,5-P<sub>2</sub> (Fig. 2 C). These results indicated that the charge density and/or the size of the hydrated headgroup mediates phosphoinositide inhibition of fusion. The inhibition observed with PI 3,4-P<sub>2</sub> may not be relevant in cells where its concentrations are very low compared with PI 4,5-P<sub>2</sub> (Furutani et al., 2006). However, the results suggest that both PI 4,5-P<sub>2</sub> on the plasma membrane as well as PI 4-P on Golgi-derived vesicles, to a lesser extent, could inhibit SNARE-dependent fusion during vesicle exocytosis. Indeed, we found a similar inhibition of SNARE-dependent fusion when phosphoinositides were incorporated into either donor or acceptor liposome populations (see Fig. 3 E, open bars, for PI 4,5-P<sub>2</sub>). Overall, the results indicated that concentrations of PI 4,5-P<sub>2</sub> equivalent to those measured in cytoplasmic leaflet microdomains on the plasma membrane inhibit SNARE-mediated vesicle fusion, but the underlying mechanism of inhibition was unclear.

Two potential mechanisms for inhibition were considered. The first involves the intrinsic positive curvature-promoting properties of PI 4,5-P<sub>2</sub> as an inverted cone lipid with a large charged hydrophilic head group. Inverted cone lipids inhibit membrane fusion because packing in the contacting leaflets of bilayers (positive curvature) is energetically unfavorable for the formation of a hemifused stalk intermediate (negative curvature) necessary for full fusion (Chernomordik and Zimmerberg, 1995). This mechanism would predict that PI 3,4-P<sub>2</sub> was equally inhibitory, whereas PI 4-P was less inhibitory than PI 4,5-P<sub>2</sub> as we had shown (Fig. 2 C). Favoring this mechanism, we found that inverted cone lipids, such as lysophosphatidylcholine (LPC), inhibited SNARE-dependent fusion of PC/PS liposomes to a similar extent as inclusion of PI 4,5-P<sub>2</sub>, and that inhibition by LPC and PI 4,5-P<sub>2</sub> was nonadditive (Fig. 2 D). Other inverted cone lipids have similarly been found to inhibit SNARE-dependent liposome fusion (Chen et al., 2006; Melia et al., 2006). A mechanism for inhibition of fusion by PI 4,5-P<sub>2</sub> involving curvature promotion would not exhibit donor or acceptor membrane specificity, which is what we observed (see Fig. 3 E).

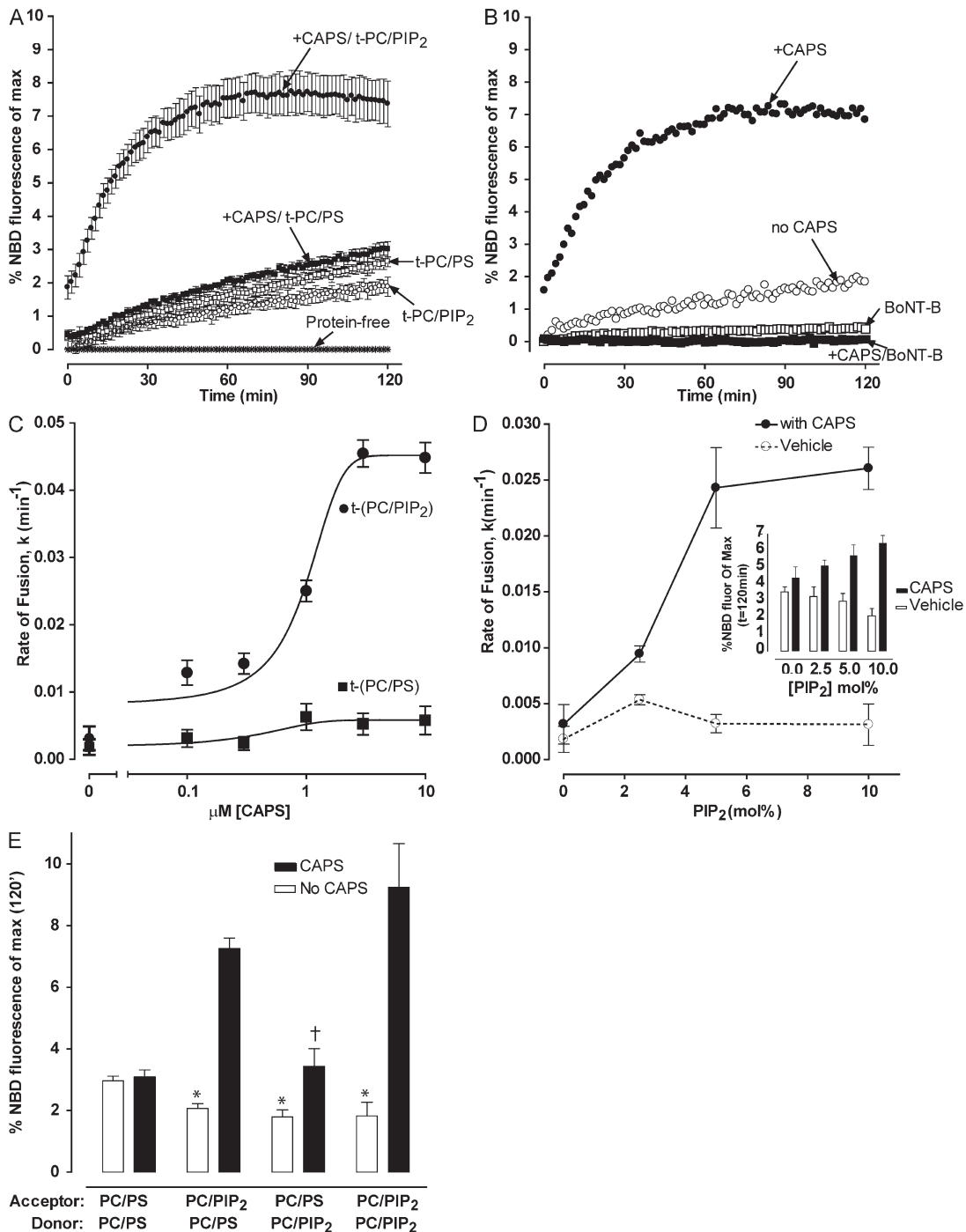
A second possible mechanism for inhibition is direct interactions between PI 4,5-P<sub>2</sub> and SNARE proteins. Syntaxin-1 and VAMP-2 possess juxtamembrane stretches of basic amino acids, which typically exhibit high affinity interactions with negatively charged PI 4,5-P<sub>2</sub> (Kweon et al., 2002; Wang et al., 2002; Lam et al., 2008). The highly basic juxtamembrane region of syntaxin-1 is positioned at the head group layer of the membrane (Kweon et al., 2002), and interactions with PI 4,5-P<sub>2</sub> are likely responsible

for the reported immobilization of SNAP-25/syntaxin-1 heterodimers in PI 4,5-P<sub>2</sub>-containing supported bilayers (Wagner and Tamm, 2001). This mechanism is based on charge interactions that would not distinguish inositide bisphosphates but would predict their greater inhibitory efficacy compared with PI 4-P, which we had observed (Fig. 2 C). To test this mechanism for inhibition by PI 4,5-P<sub>2</sub>, we generated mutant syntaxin-1 proteins with lysine to alanine substitutions in the juxtamembrane region (Stx1A-K252,253A, Stx1A-K264,265A, and Stx1A-K252,253,264,265A), which were incorporated with SNAP-25 to produce acceptor liposomes (Fig. 2 E). Wild-type and mutant syntaxins were indistinguishable in fusion assays that used PC/PS liposomes (Fig. 2 E), which indicated that properties of syntaxin-1 essential for SNARE-mediated fusion were preserved in the mutants. In contrast, wild-type and syntaxin-1 mutants exhibited different properties in fusion assays that used PC/PI 4,5-P<sub>2</sub> liposomes (Fig. 2 E). 10 mol% PI 4,5-P<sub>2</sub> inhibited SNARE-dependent liposome fusion with wild-type syntaxin-1, but this inhibition was substantially greater with the mutant syntaxins (Fig. 2 E). Thus, mutant syntaxins exhibited loss of function in fusion in PI 4,5-P<sub>2</sub>- but not in PS-containing liposomes. The results indicate that wild-type syntaxin-1 interacts with PI 4,5-P<sub>2</sub> via its membrane-proximal basic domain. The results also indicate that wild-type syntaxin-1 interactions with PI 4,5-P<sub>2</sub> function to counteract the inhibition of fusion by PI 4,5-P<sub>2</sub>. PI 4,5-P<sub>2</sub> may interfere with the formation of a high curvature membrane fusion intermediate, and syntaxin-1, by sequestering PI 4,5-P<sub>2</sub> in cis-interactions, may enable transition of this intermediate to full fusion. Similar, direct interactions of PI 4,5-P<sub>2</sub> with basic regions of VAMP-2 might also function to counteract the inhibitory effects of PI 4,5-P<sub>2</sub> in fusion.

### The stimulatory role of PI 4,5-P<sub>2</sub> on fusion mediated by CAPS

The preceding results on the inhibition of fusion by PI 4,5-P<sub>2</sub> in the minimal system for reconstituted membrane fusion are incompatible with the characterized positive essential role of PI 4,5-P<sub>2</sub> in regulated vesicle exocytosis (Hay et al., 1995). This previous study (Hay et al., 1995) found that degradation of PI 4,5-P<sub>2</sub> inhibits rather than stimulates triggered vesicle fusion. Thus, we considered the possibility that additional factors missing from the minimal system were needed to reconstitute the positive role of PI 4,5-P<sub>2</sub> in fusion. A previous study identified CAPS as an essential factor for reconstituting triggered vesicle exocytosis in permeable cells (Walent et al., 1992). CAPS binds PI 4,5-P<sub>2</sub> (Loyet et al., 1998), and the activity of CAPS is dependent on PI 4,5-P<sub>2</sub> synthesis (Grishanin et al., 2004). We found that CAPS addition to the minimal fusion assay dramatically accelerated SNARE-dependent fusion if acceptor liposomes contained PI 4,5-P<sub>2</sub> (Fig. 3 A, closed vs. open circles). In contrast, CAPS failed to affect fusion of acceptor liposomes that contained PS (Fig. 3 A, closed vs. open squares). With PI 4,5-P<sub>2</sub>-containing acceptor liposomes, CAPS enhanced fusion rates by ~10-fold ( $\tau$  of ~80 min vs.  $\tau$  of ~8 min without and with CAPS, respectively).

CAPS did not increase the basal lipid mixing observed with Pf PC/PS or PC/PI 4,5-P<sub>2</sub> liposomes, indicating that CAPS accelerated SNARE-dependent fusion (not depicted). This was



**Figure 3. CAPS accelerates liposome fusion in a PI 4,5-P<sub>2</sub>- and SNARE-dependent manner.** (A) Acceptor liposomes with 40 copies of syntaxin-1/SNAP-25 (indicated by t for t-SNAREs) in 90:10 mol% PC/PI 4,5-P<sub>2</sub> were incubated with donor liposomes with 100 copies of VAMP-2 in 85:15 mol% PC/PS in the absence or presence of 1  $\mu$ M CAPS. Similar incubations with donor and acceptor liposomes in 85:15 mol% PC/PS in the absence or presence of 1  $\mu$ M CAPS were conducted. NBD fluorescence in parallel reactions with Pf liposomes was used to correct all data. Mean values  $\pm$  SEM for five independent experiments are shown. (B) Acceptor liposomes with 40 copies of syntaxin-1/SNAP-25 in 90:10 mol% PC/PI 4,5-P<sub>2</sub> were incubated with VAMP-2-containing donor liposomes in the absence or presence of 1  $\mu$ M CAPS. Parallel incubations used botulinum neurotoxin B-treated donor liposomes incubated without or with 1  $\mu$ M CAPS. (C) Syntaxin-1/SNAP-25 acceptor liposomes (indicated by t for t-SNAREs) with 10 mol% PI 4,5-P<sub>2</sub> or with 15% PS were incubated with VAMP-2 donor liposomes in the presence of indicated CAPS concentrations. Exponential fits of time courses were used to derive rate constants ( $k$ ). Mean  $\pm$  SEM values are shown for three independent experiments. (D) Rate constants were determined for fusion reactions without or with 1  $\mu$ M CAPS in incubations that contained syntaxin-1/SNAP-25 acceptor liposomes with the indicated mole percentage PI 4,5-P<sub>2</sub>. Data in inset show the percentage of maximal NBD fluorescence at 120 min in incubations without or with CAPS. Mean  $\pm$  SEM values are shown for four independent experiments. (E) Extent of fusion in 120 min was determined in the absence or presence of 1  $\mu$ M CAPS with donor VAMP-2 or acceptor syntaxin-1/SNAP-25 liposomes that contained 85:15 mol% PC/PS or 90:10 mol% PC/PI 4,5-P<sub>2</sub> as indicated. Mean  $\pm$  SEM values are shown for three independent experiments (\*,  $P < 0.01$  for PC/PIP<sub>2</sub> compared with PC/PS; †,  $P = 0.05$  compared with parallel incubations without CAPS).

confirmed by the full inhibition of CAPS-stimulated fusion by cleavage of VAMP-2 with botulinum neurotoxin B (Fig. 3 B) or by inclusion of a soluble syntaxin-1 cytoplasmic domain protein that blocks SNARE complex formation (not depicted). These results indicated that CAPS increased liposome fusion by a PI 4,5-P<sub>2</sub>- and SNARE-dependent mechanism.

To analyze the kinetics, initial rates of fusion for either PC/PS or PC/PI 4,5-P<sub>2</sub> acceptor liposomes were measured over a range of CAPS concentrations (Fig. 3 C). CAPS increased rates of SNARE-dependent fusion in this study about fivefold with an EC<sub>50</sub> of ~1 μM in CAPS with PC/PI 4,5-P<sub>2</sub> acceptor liposomes but exerted little stimulation with PC/PS acceptor liposomes. Increased rates of SNARE-mediated fusion by CAPS were largely dependent on the presence of PI 4,5-P<sub>2</sub> with an EC<sub>50</sub> of ~3 mol% in SNAP-25/syntaxin-1 liposomes (Fig. 3 D). Maximal stimulation of rates by CAPS was observed at 5 mol% PI 4,5-P<sub>2</sub>, which corresponded closely to estimates for concentrations present in cytoplasmic leaflet microdomains at sites of docked vesicles (~6 mol%). Determinations of the final extent of liposome fusion (Fig. 3 D, inset) revealed that CAPS also counteracted the PI 4,5-P<sub>2</sub> inhibition of fusion and promoted an increased extent of fusion. Overall, the data suggest that CAPS exerts multiple PI 4,5-P<sub>2</sub>-dependent effects on fusion. Although CAPS may counteract PI 4,5-P<sub>2</sub> inhibition in a manner similar to syntaxin-1 by sequestering the phosphoinositide, it also dramatically increased rates of SNARE-dependent fusion, which suggests a direct or indirect effect of CAPS on SNARE protein function after its PI 4,5-P<sub>2</sub>-dependent membrane recruitment.

Whereas the inhibition of SNARE-dependent fusion by PI 4,5-P<sub>2</sub> was observed to be symmetric in donor and acceptor liposomes, the PI 4,5-P<sub>2</sub>-dependent stimulation of fusion by CAPS was found to be highly asymmetrical (Fig. 3 E). CAPS counteracted the inhibition observed with PI 4,5-P<sub>2</sub> in VAMP-2-containing donor liposomes (Fig. 3 E), which is consistent with a sequestration mechanism for relieving inhibition. However, CAPS failed to accelerate rates or stimulate the final extent of fusion with PI 4,5-P<sub>2</sub> in the VAMP-2-containing donor liposomes (Fig. 3 E). In contrast, CAPS accelerated rates and stimulated the extent of fusion with PI 4,5-P<sub>2</sub> in SNAP-25/syntaxin-1-containing acceptor liposomes or with PI 4,5-P<sub>2</sub> in both donor and acceptor liposomes (Fig. 3 E). The asymmetry observed for CAPS stimulation, with a requirement for PI 4,5-P<sub>2</sub> in cis with syntaxin-1 and SNAP-25, corresponds to the known dominant localization of SNAP-25, syntaxin-1, and PI 4,5-P<sub>2</sub> in the plasma membrane (Aikawa et al., 2006), to the observed PI 4,5-P<sub>2</sub>-dependent binding of CAPS to the plasma membrane (Grishanin et al., 2004), and to the colocalization of CAPS with PI 4,5-P<sub>2</sub> microdomains in the plasma membrane (Fig. 1 J).

#### **The CAPS PH domain mediates the stimulatory role of PI 4,5-P<sub>2</sub> in fusion**

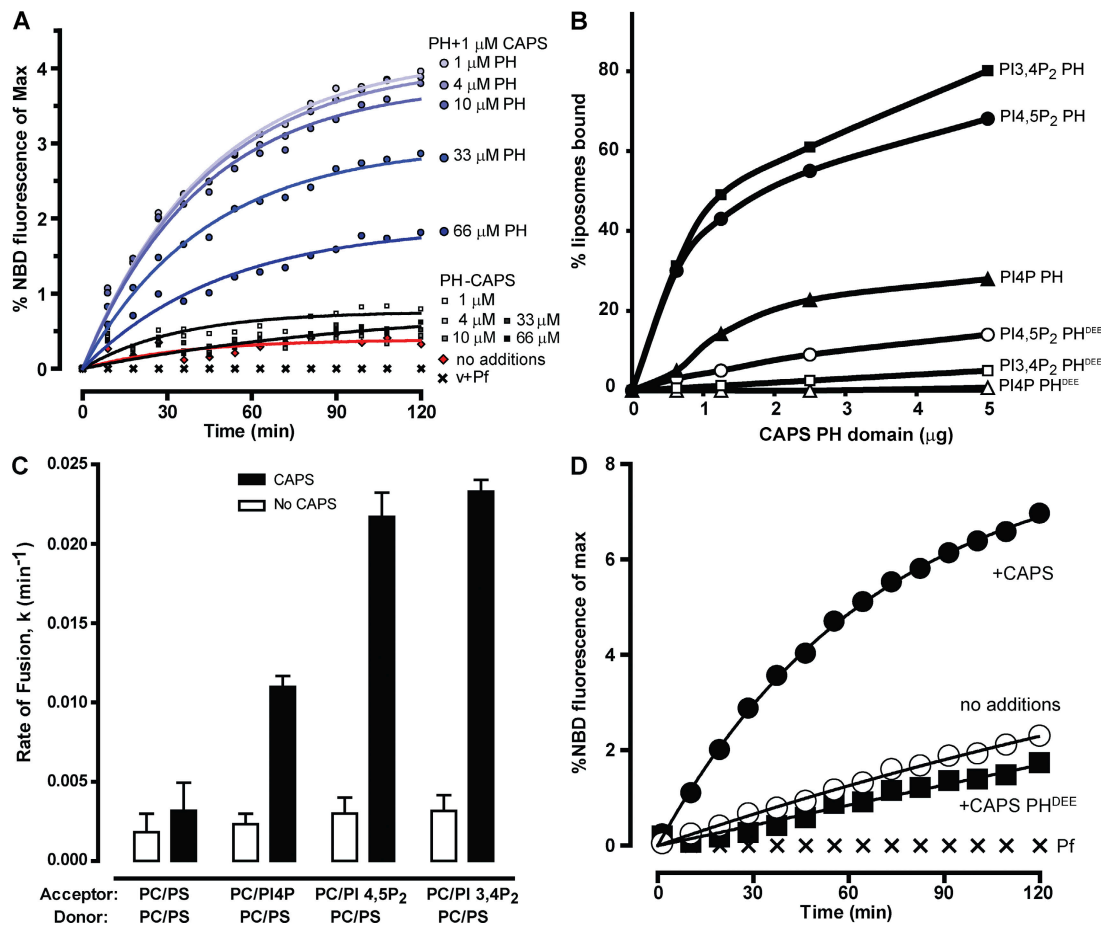
CAPS contains a PI 4,5-P<sub>2</sub>-binding PH domain that is essential for CAPS function in vesicle exocytosis (Grishanin et al., 2002). Because CAPS stimulation of SNARE-dependent liposome fusion was largely PI 4,5-P<sub>2</sub> dependent, we determined whether PI 4,5-P<sub>2</sub> binding by the PH domain was sufficient to stimulate liposome fusion. A PH domain fusion protein failed to stimulate fu-

sion even at high concentrations (Fig. 4 A), which indicates that binding to PI 4,5-P<sub>2</sub> per se is not sufficient to accelerate SNARE-dependent fusion. The PH domain fusion protein did inhibit the acceleration of SNARE-dependent fusion by CAPS (Fig. 4 A). A high molar excess of the fusion protein was required for inhibition, but this likely reflects the attenuated PI 4,5-P<sub>2</sub>-binding affinity of this GST fusion protein compared with CAPS. Nonetheless, the results suggest that binding to PI 4,5-P<sub>2</sub> is essential for CAPS stimulation of fusion. PI 4,5-P<sub>2</sub> may function by recruiting CAPS via its PH domain to the acceptor liposome membrane. Because the PH domain of CAPS also binds PI 3,4-P<sub>2</sub> and, to a lesser extent, PI 4-P (Fig. 4 B), we tested the activity of CAPS in accelerating fusion with acceptor liposomes that contained these phosphoinositides. Consistent with the phosphoinositide-binding properties of the CAPS PH domain, we found that CAPS promoted an eightfold increase in the rate of liposome fusion with 5 mol% PI 4,5-P<sub>2</sub> or PI 3,4-P<sub>2</sub> in SNAP-25/syntaxin-1 acceptor liposomes but only a 4.7-fold increase with 5 mol% PI 4-P in liposomes (Fig. 4 C). The similar phosphoinositide-binding properties of the CAPS PH domain and the CAPS acceleration of fusion are consistent with a role for the PH domain in mediating the membrane recruitment of CAPS for SNARE-dependent fusion. A lack of headgroup specificity of the CAPS PH domain for inositide bisphosphates may not be relevant in cells where PI 4,5-P<sub>2</sub> but not PI 3,4-P<sub>2</sub> is highly enriched in the plasma membrane. In contrast, binding to PI 4-P is likely relevant in cells where the priming of vesicle exocytosis by PI 4-P has been characterized (Olsen et al., 2003).

Finally, to directly assess the role of the CAPS PH domain in PI 4,5-P<sub>2</sub>-dependent liposome fusion, we used full-length CAPS with mutations that impair PI 4,5-P<sub>2</sub> binding. Charge reversal R558D/K560E/K561E mutations in the β3-β4 loop region of the CAPS PH domain (<http://www.rcsb.org/pdb/explore.do?structureId=1WI1>) strongly reduce phosphoinositide binding (Fig. 4 B) and inhibit CAPS stimulation of Ca<sup>2+</sup>-triggered vesicle exocytosis in PC12 cells (Grishanin et al., 2002). The CAPS (R558D/K560E/K561E) mutant was completely inactive in promoting SNARE-dependent liposome fusion at concentrations where wild-type CAPS is half-maximally active in fusion (Fig. 4 D). These results indicate that CAPS interactions with PI 4,5-P<sub>2</sub> are essential for the promotion of SNARE-dependent liposome fusion.

## **Discussion**

PI 4,5-P<sub>2</sub> is a minor but key phospholipid constituent in the cytoplasmic leaflet of the plasma membrane but its influence on membrane fusion has not been directly characterized. Intact PI 4,5-P<sub>2</sub> is required for Ca<sup>2+</sup>-triggered vesicle fusion with the plasma membrane (Hay et al., 1995; Grishanin et al., 2004), and PI 4,5-P<sub>2</sub> levels determine rates of vesicle priming, the size of the readily releasable vesicle pool, and ongoing rates of exocytosis in stimulated cells (Aikawa and Martin, 2003; Olsen et al., 2003; Gong et al., 2005; Milosevic et al., 2005). Mechanisms responsible for this essential role of PI 4,5-P<sub>2</sub> in triggered vesicle fusion have not been elucidated. The current studies revealed that plasma membranes from PC12 cells contain microdomains



**Figure 4. CAPS acceleration of fusion requires a functional PH domain.** (A) CAPS PH domain fails to accelerate fusion but inhibits CAPS stimulation. Fusion reactions with 5 mol% PI 4,5-P<sub>2</sub>-containing acceptor liposomes were conducted in the absence (PH –CAPS) or presence (PH +CAPS) of 1 μM CAPS, and the indicated concentrations of a CAPS GST-PH domain fusion protein. For clarity, points were selected and plotted every 7.5 min. Exponential fits of time courses are shown. (B) Phosphoinositide binding characteristics of CAPS PH domain. Indicated amounts of CAPS GST-PH domain fusion protein (either wild-type PH or mutant PH<sup>DEE</sup>) immobilized on glutathione agarose beads were incubated with liposomes containing PI 4-P, PI 4,5-P<sub>2</sub>, or PI 3,4-P<sub>2</sub> as described in Materials and methods. (C) Liposome fusion reactions were conducted with VAMP-2 donor liposomes incubated with syntaxin-1/SNAP-25 acceptor liposomes containing 5 mol% PI 4-P, PI 4,5-P<sub>2</sub>, or PI 3,4-P<sub>2</sub> as indicated. Rate constants were determined by exponential fit to time courses in the absence or presence of 1 μM CAPS. Mean ± SEM values are shown for three independent experiments. (D) The CAPS PH domain mutant R558D/K560E/K561E with impaired phosphoinositide binding fails to accelerate fusion. Fusion reactions with 10 mol% PI 4,5-P<sub>2</sub>-containing acceptor liposomes were conducted in the absence or presence of 1 μM wild-type CAPS or mutant CAPS (CAPS PH<sup>DEE</sup>).

of PI 4,5-P<sub>2</sub>, which confirms previous reports (Laux et al., 2000; Aoyagi et al., 2005; Milosevic et al., 2005). A substantial fraction of docked vesicles colocalized with PI 4,5-P<sub>2</sub>-containing (~35%) or CAPS/PI 4,5-P<sub>2</sub>-containing (~20%) microdomains, and these vesicles may correspond to the ready-release pool at which preferential triggered vesicle fusion occurs (Aoyagi et al., 2005). A direct calibrated determination of PI 4,5-P<sub>2</sub> concentrations in cytoplasmic leaflet microdomains labeled with a PLCδ<sub>1</sub>-PH-GFP protein indicated that ~6 mol% PI 4,5-P<sub>2</sub> was present at these sites, contrasted with ~1 mol% in interdomain regions. If these diffraction-limited microdomains are smaller than ~200 nm, they would contain concentrations of PI 4,5-P<sub>2</sub> >6 mol%. These determinations provide the first estimate of PI 4,5-P<sub>2</sub> concentrations that may be relevant for membrane-localized events regulated by PI 4,5-P<sub>2</sub>. However, these determinations are based on the assumption that the PLCδ<sub>1</sub>-PH domain probe binds equally well to PI 4,5-P<sub>2</sub> in the plasma membrane and in supported bilayers. Nonetheless, consistent with our estimate

for PI 4,5-P<sub>2</sub> concentrations in microdomains, reconstituted endocytic budding from liposomes was found to require ~6 mol% PI 4,5-P<sub>2</sub> (Kinuta et al., 2002), and the reconstituted CAPS stimulation of fusion described here was optimal at 5 mol% PI 4,5-P<sub>2</sub> (Fig. 3 D).

We found that concentrations of PI 4,5-P<sub>2</sub> in this range inhibited SNARE-dependent fusion in liposomes in the absence of CAPS. Vicogne et al. (2006) studied the effects of combinations of PI 4,5-P<sub>2</sub> and phosphatidic acid (PA) on SNARE-dependent fusion and reported that PI 4,5-P<sub>2</sub> in acceptor liposomes inhibited fusion with PA-containing donor liposomes. Conversely, PI 4,5-P<sub>2</sub> in donor liposomes stimulated fusion with PA-containing acceptor liposomes (Vicogne et al., 2006). In our assays, PI 4,5-P<sub>2</sub> consistently inhibited SNARE-dependent fusion symmetrically when present in either donor or acceptor liposomes. Aside from the absence of PA, our assays contained lower SNARE densities (e.g., ~100 copies of VAMP-2 per liposome), which likely accounts for differences between our results and those of Vicogne



et al. (2006). At high SNARE densities, PI 4,5-P<sub>2</sub>-containing liposomes can coaggregate in trans because of charge-mediated interactions. At the PI 4,5-P<sub>2</sub> concentrations and SNARE densities we used, liposomes did not aggregate as determined by light scattering studies (see Materials and methods).

In spite of the lack of aggregation, inclusion of PI 4,5-P<sub>2</sub> resulted in an inhibition of SNARE-dependent fusion. Because PI 4,5-P<sub>2</sub> immobilizes SNAP-25/syntaxin-1 heterodimers in supported bilayers (Wagner and Tamm, 2001), we considered the possibility that charge-mediated interactions between SNAREs and PI 4,5-P<sub>2</sub> may be responsible for the observed inhibition of fusion. However, we found that liposomes containing syntaxin mutants with a reduced number of juxtamembrane basic residues exhibited more profoundly inhibited rates of fusion. This indicates that syntaxin-1 interactions with PI 4,5-P<sub>2</sub> play a positive role in reducing inhibition. Because inverted cone lipids that exert positive curvature on the solvent-exposed leaflet are also inhibitory for SNARE-dependent fusion (Fig. 2 D), it is likely that PI 4,5-P<sub>2</sub> inhibits fusion by a similar mechanism. Consistent with this mechanism, we found that PI 3,4-P<sub>2</sub> similarly inhibited fusion whereas PI 4-P was less inhibitory. Inverted cone lipids antagonize the formation and transition of a high negative curvature stalk intermediate that is essential for full fusion (Chernomordik and Zimmerberg, 1995). Positively charged juxtamembrane regions of SNAREs may sequester PI 4,5-P<sub>2</sub> from membrane regions that undergo high curvature transitions during fusion. High curvature was also reported to be needed to fully zipper trans-SNARE complexes (Melia et al., 2006). Thus, SNAREs may help promote the curvature needed for completion of SNARE complex formation and for the transition of a hemifusion intermediate to full fusion. Interactions between PI 4,5-P<sub>2</sub> and the juxtamembrane regions of SNAREs might provide important coupling between the state of membrane curvature and trans-SNARE complex formation.

Syntaxin-1 mutants harboring charge-neutralizing substitutions in the juxtamembrane region would be anticipated to reduce evoked vesicle fusion in neuroendocrine cells because of increased PI 4,5-P<sub>2</sub> inhibition and reduced transition probabilities of hemifusion intermediates to full fusion. In accord with this, a recent study (Lam et al., 2008) showed that replacement of wild-type syntaxin with syntaxin1A-R262,263A or syntaxin1A-K260,R262,R263,K264,K265A mutants reduced evoked vesicle fusion that was partially reversed by inducing favorable membrane curvature. Vesicle exocytosis in the cells harboring syntaxin mutants also exhibited prolonged pre-spike feet in amperometric recordings of catecholamine release, which may indicate delays in fusion pore expansion (Lam et al., 2008). Although these properties are consistent with the cell-free fusion results on PI 4,5-P<sub>2</sub> reported here, there is some uncertainty about which acidic phospholipids in cells are responsible for these observed effects on fusion.

Because the intrinsic properties of PI 4,5-P<sub>2</sub> resulted in inhibition in a minimal fusion assay, we sought additional factors that might mediate the well-characterized essential positive role for PI 4,5-P<sub>2</sub> in vesicle fusion (Hay et al., 1995; Holz et al., 2000; Olsen et al., 2003; Milosevic et al., 2005). CAPS is one such factor having previously been characterized as a PI 4,5-P<sub>2</sub>-binding protein that is required for a late step in vesicle exocytosis (Grishanin

et al., 2004). Inclusion of CAPS in the minimal fusion assay containing PI 4,5-P<sub>2</sub> resulted in a marked stimulation of rates of SNARE-dependent liposome fusion. The stimulation of fusion by CAPS required its phosphoinositide-binding properties based on the dependence of its activity in fusion on inclusion of phosphoinositides in acceptor liposomes, the ability of a phosphoinositide-binding PH domain fusion protein to inhibit CAPS activity in fusion, and the strong loss of function observed for CAPS PH domain mutants. Although the CAPS PH domain interacts equally well with PI 3,4-P<sub>2</sub> and PI 4,5-P<sub>2</sub>, the selectivity for its membrane recruitment to sites of exocytosis in cells would be dictated by cytoplasmic leaflet microdomains of PI 4,5-P<sub>2</sub>. Although the concentrations and distribution of PI 4-P in the plasma membrane remain unknown, PI 4-P binding by CAPS may mediate the characterized activity of this phosphoinositide in priming vesicle exocytosis in cells (Olsen et al., 2003).

Phosphoinositide binding by CAPS appeared to be essential for its promotion of SNARE-dependent fusion, but it was not sufficient based on the lack of activity of its PH domain. We also eliminated the possibility that full-length CAPS might be a multivalent PI 4,5-P<sub>2</sub>-binding protein that could aggregate liposomes to promote fusion (see Materials and methods). Although CAPS alleviated the inhibition of fusion by PI 4,5-P<sub>2</sub> incorporated in VAMP-2 donor liposomes, it also strongly stimulated rates of fusion well beyond an alleviation of inhibition, but only when PI 4,5-P<sub>2</sub> was incorporated in SNAP-25/syntaxin-1 liposomes (Fig. 3 E). The selective effect of PI 4,5-P<sub>2</sub> in acceptor liposomes for mediating CAPS-stimulated fusion suggests that CAPS may exert additional actions on SNAP-25 and/or syntaxin-1 in cis on liposomes to accelerate SNARE-dependent fusion and that these actions are not provided by CAPS PH domain. Future studies will need to discover the full basis for CAPS regulation of SNARE-dependent fusion.

The current work suggests two mechanisms by which PI 4,5-P<sub>2</sub> regulates membrane fusion. The first of these mechanisms is inhibitory, mediated by the intrinsic positive curvature-promoting properties of PI 4,5-P<sub>2</sub>. SNAREs may counteract this restraint on membrane fusion by sequestering PI 4,5-P<sub>2</sub> via membrane proximal basic domains. In the second mechanism, PI 4,5-P<sub>2</sub> strongly facilitates rates of membrane fusion by recruitment of PI 4,5-P<sub>2</sub>-binding proteins such as CAPS that possess additional properties to promote SNARE function in fusion. Given the precedent for clathrin-dependent endocytosis in which at least six PI 4,5-P<sub>2</sub>-binding proteins function (Ungewickell and Hinrichsen, 2007), this mechanism for PI 4,5-P<sub>2</sub> in membrane fusion may extend to proteins such as synaptotagmin (Bai et al., 2004), rabphilin (Chung et al., 1998), and other PI 4,5-P<sub>2</sub>-binding proteins containing basic residue-rich regions or C2 and PH domains.

## Materials and methods

### Materials

1,2-dioleoyl phosphatidylcholine (DOPC), 1,2-dioleoyl phosphatidylserine (DOPS), NBD-PE, rhodamine-PE, PI 4,5-P<sub>2</sub>, and PI 4-P were purchased from Avanti Polar Lipids, Inc. LPC was purchased from Sigma-Aldrich.

### Plasmids, recombinant protein expression, and purification

Plasmid constructs pTW34 to coexpress rat syntaxin-1A with an N-terminally His-tagged mouse SNAP-25B and pTW2 to express C-terminal His-tagged

VAMP-2 were provided by J.E. Rothman and T. Weber (Columbia University, New York, NY; Weber et al., 1998). SNARE proteins were purified as described previously (Scott et al., 2003). Syntaxin-1A mutants were generated in the plasmid pTW34 by site-directed mutagenesis (QuikChange; Stratagene) using oligos CGTGTCTGACACCGCGGCGCCGTCAGTAC and GGCACGCAGGGCGGCATCATGATCATCATTTGC for K252,253A and K264,265A mutations, respectively. The construct encoding the PH domain of PLC $\delta_1$  fused in frame to the N-terminus of GFP-decaHis (abbreviated PLC $\delta_1$ -PH-GFP) was provided by T. Balla (National Institutes of Health, Bethesda, MD; Balla, 2005). All plasmids were transformed into *Escherichia coli* BL-21(DE3) cells for protein expression. C-terminally myc-His-tagged rat CAPS protein was produced in Sf9 insect cells or by COS-1 cell expression as previously described (Grishanin et al., 2002, 2004).

#### Preparation of plasma membrane sheets and detection of PI 4,5-P<sub>2</sub> using PLC $\delta_1$ -PH-GFP

PC12 cells were cultured as previously described (Klenchin et al., 1998). Plasma membrane sheets from PC12 cells were prepared as described previously with modification (Avery et al., 2000). Cells cultured on round glass coverslips coated with 0.1 mg/ml poly-DL-lysine (Sigma-Aldrich) were grown to confluence, washed with ice-cold KGlu buffer (20 mM Hepes, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, 0.1 mM EGTA, and 0.1% BSA), and sonicated with a brief (100 ms) ultrasonic pulse from a sonifier (Branson ultrasonics) equipped with a point probe at 10% power output. This preparation contains plasma membrane sheets with attached docked vesicles and is suitable for determining the spatial distribution of membrane proteins and vesicles. For the detection of PI 4,5-P<sub>2</sub>, membrane sheets were incubated for 15 min at 30°C with a priming mixture characterized for "cracked" PC12 cells or PC12 cell membranes (Martin and Kowalchuk, 1997; Grishanin et al., 2002, 2004) consisting of KGlu buffer with 0.5 mg/ml of rat brain cytosol, 2 mM MgCl<sub>2</sub>, and 2 mM ATP. The membrane sheets were washed with KGlu buffer, incubated with 2  $\mu$ M PLC $\delta_1$ -PH-GFP in KGlu buffer with 3% BSA for 15 min at room temperature, washed, and imaged by epifluorescence microscopy with an inverted microscope (TE2000-U; Nikon) using an objective lens (Apo TIRF 100 $\times$ , NA 1.45; Nikon). To conduct immunocytochemistry for colocalization studies, membrane sheets were further processed by fixation for 30 min in 4% formaldehyde at 4°C. Fixed membrane sheets were blocked with 10% fetal bovine serum and immunostained by incubation with CAPS polyclonal, syntaxin-1A HPC-1 monoclonal (Sigma-Aldrich), or chromogranin B monoclonal (provided by W. Huttner, Max Planck Institute, Dresden, Germany) antibodies followed by incubation with appropriate Alexa 568- or Alexa 405-conjugated secondary antibodies. Fluorescence was viewed on a confocal microscope (C1; Nikon) using an objective lens (Plan Apo 100 $\times$ , NA 1.40; Nikon), and confocal Z stack images were deconvolved with a blind 3D deconvolution algorithm using Media Cybernetics software before colocalization analysis with MetaMorph software (MDS Analytical Technologies). Triple colocalization analysis of signals corresponding to CAPS, PI 4,5-P<sub>2</sub>, and dense-core vesicles was performed using a pixel by pixel analysis algorithm, IMAJIN\_COLOC (Goucher et al., 2005). The calculated percentage of random colocalization was subtracted from colocalization values.

#### Planar-supported bilayer formation

100 nm of liposomes (consisting of DOPC/0–10 mol% PI 4,5-P<sub>2</sub>/0.1 mol% rhodamine-PE) were produced by extrusion using a mini extruder (Avanti Polar Lipids, Inc.). Liposomes were deposited at  $\sim$ 3 mM onto cleaned glass coverslips and incubated for 10 min at room temperature to form a supported bilayer. The surface was rinsed extensively with 50 mM Hepes-KOH, pH 7.4, and 100 mM KCl, and the exposed surface was incubated with 2  $\mu$ M of purified PLC $\delta_1$ -PH-GFP (see Preparation of plasma membrane sheets...).

#### Proteoliposome preparation

Proteoliposomes were formed by comicellization in the presence of either VAMP-2 or coexpressed syntaxin-1A and SNAP-25B as described previously (Scott et al., 2003). 1.67  $\mu$ g of SNAP-25/syntaxin-1 in elution buffer (25 mM Hepes-KOH, pH 7.4, 100 mM KCl, 50 mM imidazole-OAc, pH 7.4, and 1.0%  $\beta$ -octylglucoside) was used to resuspend a lipid film containing 1.5  $\mu$ mol DOPC/DOPS in an 85:15 mole ratio to generate liposomes that contained  $\sim$ 40 copies of each SNARE. For liposomes that contained either 5 or 10 mol% PI 4,5-P<sub>2</sub>, we used DOPC at 95 or 90%. For fluorescent donor liposomes,  $\sim$ 100 copies of VAMP-2 per liposome (to correspond to VAMP-2 densities on a synaptic vesicle [Takamori et al., 2006]) were incorporated with a lipid mixture of DOPC/DOPS/Rh-PE-dioleoylphosphatidylethanolamine/NBD-dioleoylphosphatidylethanolamine at an 82:15:1.5:1.5 mole ratio. For VAMP-2 liposomes containing PI 4,5-P<sub>2</sub>, 10 mol% was substituted for PS, and the DOPC mole ratio was increased to 87 mol%. Lipid mixtures were

spiked with 2  $\mu$ l [<sup>3</sup>H]1,2-dipalmitoyl phosphatidylcholine ( $\sim$ 2  $\times$  10<sup>5</sup> cpm/nmol; New England Nuclear Corp.) to determine lipid recoveries and standardization of fusion reactions. Proteoliposomes were dialyzed overnight and purified by Nycodenz gradient flotation (Weber et al., 1998; Scott et al., 2003). SNARE content, which was assessed by SDS-PAGE and visualized by Coomassie staining for SNAP-25/syntaxin-1 and amido black staining for VAMP-2, was unaffected by inclusion of PI 4,5-P<sub>2</sub> in the liposomes. Protein-containing liposomes with PC/PS were typically  $\sim$ 50  $\pm$  13 nm as measured by dynamic light scattering in an N5 submicron particle size analyzer (Beckman Coulter). SNAP-25/syntaxin-1 liposomes with 5 or 10 mol% PI 4,5-P<sub>2</sub> were typically  $\sim$ 80  $\pm$  20 nm.

#### Lipid-mixing fusion assay

The lipid-mixing assay between fluorescent donor liposomes and non-fluorescent acceptor liposomes was performed as described previously (Weber et al., 1998) with modifications. Lipid mixing was reported by the loss of fluorescence resonance energy transfer between fluorescent lipids (NBD-PE and Rh-PE) concentrated in the VAMP-2 liposomes that occurs upon fusion with the non-fluorescent SNAP-25/syntaxin-1 liposomes. The standard assay used 0.45 mM of acceptor and 0.225 mM of donor liposomes in a total volume of 75  $\mu$ l of reconstitution buffer without glycerol. CAPS protein was added at concentrations indicated in the figure legends. 90° light scattering and dynamic light scattering studies did not detect any aggregation of liposomes during the fusion reactions. Blank reactions were prepared for all conditions by substituting SNAP-25/syntaxin-1 acceptor liposomes with Pf liposomes to detect non-SNARE-mediated lipid mixing. Reactions were assembled on ice and flick mixed before addition to 96-well FluoroNunc plates. Fusion was detected by measuring dequenching of NBD fluorescence (excited at 460-nm emission at 538 nm) every 90 s (shaken before each reading) for 2 h at 35°C in a SpectraMax Gemini XS microplate spectrofluorometer (MDS Analytical Technologies). At the end of 2 h, fusion reactions were solubilized with 0.5% wt/vol dodecyl- $\beta$ -maltoside and fluorescence readings were recorded for an additional 10 min.

#### Quantification of lipid mixing

Lipid-mixing results are commonly expressed as a percentage of a maximal fluorescent signal determined by detergent solubilization (Weber et al., 1998). However, a temperature-dependent dip in NBD fluorescence from increased lipid diffusion and quenching is routinely observed during the initial 10 min, which obscures effects on fusion within this time period. We normalized raw fluorescence lipid-mixing data to eliminate non-SNARE-dependent fluorescence changes. Parallel reactions with Pf liposomes substituting for SNAP-25/syntaxin-1 liposomes provide a measure of non-SNARE-mediated lipid mixing at all time points. SNARE-dependent lipid mixing was obtained as the difference between the raw fluorescence observed for SNARE-containing liposomes and an identical lipid mixing assay containing Pf liposomes after scaling raw fluorescent values based on maximal fluorescence after detergent addition. This normalization method generates values with units that are also "percentage NBD fluorescence of max" and are comparable to conventional data but have an important background subtracted. This allowed resolution of significant fluorescence changes that occur early in time courses. Differences between treatment and control conditions were determined using a Student's *t* test with a *P* < 0.05 considered significant.

#### CAPS PH domain binding to PI 4,5-P<sub>2</sub>

A GST CAPS PH domain protein (amino acids 516–626) or a cognate mutant protein (R558D/K560E/K561E) were generated by *E. coli* expression from a pGEX4T-PHD-myc6xHis plasmid (Grishanin et al., 2002), and 0.3–5  $\mu$ g was immobilized on glutathione-agarose. 100 nm of liposomes consisting of DOPC/phosphoinositide (4:1) with trace [<sup>3</sup>H]1,2-dipalmitoyl phosphatidylcholine were produced by extrusion, incubated at  $\sim$ 0.1 mM with the immobilized CAPS PH domain protein for 1 h at room temperature, and separated by low speed centrifugation to determine bound and free <sup>3</sup>H-liposomes. We used liposomes lacking PS to compare phosphoinositide-binding specificity under conditions similar to the liposome fusion assays. The assay results (Fig. 4 B) differed from those obtained previously with liposomes containing PS (Grishanin et al., 2002).

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