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Cholesterol stabilizes fluid phosphoinositide domains

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Abstract

Local accumulation of phosphoinositides (PIPs) is an important factor for a broad range of cellular events including membrane trafficking and cell signaling. The negatively charged phosphoinositide headgroups can interact with cations or cationic proteins and this electrostatic interaction has been identified as the main phosphoinositide clustering mechanism. However, an increasing number of reports show that phosphoinositide-mediated signaling events are at least in some cases cholesterol dependent, suggesting other possible contributors to the segregation of phosphoinositides. Using fluorescence microscopy on giant unilamellar vesicles and monolayers at the air/water interface, we present data showing that cholesterol stabilizes fluid phosphoinositide-enriched phases. The interaction with cholesterol is observed for all investigated phosphoinositides (PI(4)P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂ and PI(3,4,5)P₃) as well as phosphatidylinositol. We find that cholesterol is present in the phosphoinositide-enriched phase and that the resulting phase is fluid. Cholesterol derivatives modified at the hydroxyl group (cholestenone, cholesteryl ethyl ether) do not promote formation of phosphoinositide domains, suggesting an instrumental role of the cholesterol hydroxyl group in the observed cholesterol/phosphoinositide interaction. This leads to the hypothesis that cholesterol participates in an intermolecular hydrogen bond network formed among the phosphoinositide lipids. We had previously reported that the intra- and intermolecular hydrogen bond network between the phosphoinositide lipids leads to a reduction of the charge density at the phosphoinositide phosphomonoester groups (Kooijman et al. *Biochemistry* 48, (2009) 9360). We believe that cholesterol acts as a spacer between the phosphoinositide lipids, thereby reducing the electrostatic repulsion, while participating in the hydrogen bond network, leading to its further stabilization. To illustrate the effect of phosphoinositide segregation on protein binding, we show that binding of the tumor suppressor protein PTEN to PI(5)P and PI(4,5)P₂ is enhanced in the presence of cholesterol. These results provide new insights into how phosphoinositides mediate important cellular events.

Introduction

Phosphoinositides (diacylglycerophosphatidylinositolphosphates) have been shown to mediate a large variety of important physiological functions by influencing the activity and/or localization of membrane associated proteins (Shewan et al., 2011). Several of these phosphoinositide-mediated signaling events are affected by cholesterol levels, and it has been suggested that at least some of these functions are associated with liquid-ordered (l_o) domains that are enriched in sphingolipids and cholesterol (rafts) (Johnson et al., 2008). Studies linking phosphoinositide signaling events to lipid rafts typically fall into one of these categories: cholesterol depletion studies that associate decreased cholesterol levels to an activity change of the respective pathway (Das et al., 2010; Fox et al., 2011; Hao and Bogan, 2009; Kwik et al., 2003; Peres et al., 2003; Seveau et al., 2007); studies that extract raft domains and analyze them with respect to protein and/or lipid content (Furt et al., 2010; Gao et al., 2011; Koushik et al., 2013); studies investigating the co-localization of phosphoinositides or phosphoinositide binding proteins with raft markers (Furt et al., 2010; Shen-Tu et al., 2010) or studies that highlight the localization of phosphoinositide modifying enzymes to rafts (Johnson et al., 2008; Varnai and Balla, 2007).

Physiological models suggesting raft residency of phosphoinositides, are faced with the fundamental problem that the stearyl/arachidonoyl acyl chain composition of naturally occurring phosphoinositides is believed to be unfavorable for liquid-ordered environments, such as lipid rafts (Epand et al., 2004a). Using live cell FRET experiments, van Rheenen et al. (van Rheenen et al., 2005) strongly questioned the presence of PI(4,5)P₂ in lipid rafts and showed that Triton X-100 induces PI(4,5)P₂ domain formation. In further support of this argument, *in vitro* studies showed that PI(4,5)P₂ does not partition into liquid-ordered domains (Shaw et al., 2006) unless a phosphoinositide-binding protein like GAP-43 is present in the raft (Tong et al., 2008). It is important to stress that these *in vitro* studies utilized lipid compositions characteristic for the outer leaflet of the plasma membrane, which is probably an inadequate model for the characterization of the inner leaflet phosphoinositide partitioning.

Phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) comprises only 1% of the total lipids, and the total cumulative concentration of its protein ligands exceeds its concentration in the cell (Catimel et al., 2008). This suggests that the fine-tuning of phosphoinositide-mediated signaling events requires a tight control of its lateral distribution. Even though raft association of phosphoinositides cannot be unequivocally proven or disproven at this point, lateral accumulation of phosphoinositides in response to specific cellular events has been observed in several instances (Cai and Devreotes, 2011; Calloway et al., 2011; Gao et al., 2011; Golebiewska et al., 2011; Illien et al., 2012; Rogasevskaia et al., 2012). Phosphoinositide accumulation can be triggered through interaction with cationic proteins or peptides (McLaughlin and Murray, 2005) as well as through interaction with cationic calcium (Wang et al., 2012). It has been shown in model systems that cholesterol supports PI(4,5)P₂ domain formation (Levental et al., 2009). This paper explores which underlying mechanisms lead to cholesterol-induced PI(4,5)P₂ domain formation, and whether the observed cholesterol-induced clustering is also found for the other naturally occurring

phosphoinositide derivatives. To extend our previous studies that utilized indirect methods to study phosphoinositide phase behavior (Graber et al., 2012; Kooijman et al., 2009; Redfern and Gericke, 2004, 2005), we use in this study fluorescence microscopy to visualize cholesterol dependent phosphoinositide domain formation in giant unilamellar vesicles as well as monolayers at the air/water interface. We are providing a first glimpse of the phase state of these phosphoinositide domains, and we investigate to what extent the interaction of proteins with model membranes is affected by a heterogeneous phosphoinositide distribution.

Experimental

Materials

L- α -phosphatidylinositol (PI), (Liver, Bovine), (sodium salt), L- α -phosphatidylinositol-4-phosphate (PI(4)P) (Brain, Porcine) (ammonium Salt), L- α -phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂), (Brain, Porcine) (ammonium salt), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',4'-bisphosphate), (PI(3,4)P₂), (ammonium salt), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',5'-bisphosphate), (PI(3,5)P₂), (ammonium salt), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',4',5'-trisphosphate), (PI(3,4,5)P₃) (ammonium salt), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (SAPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (RhBDOPE), 1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phosphocholine (NBD-PC), 1-oleoyl-2-{6-[4-(dipyrrrometheneboron difluoride)butanoyl] amin}hexanoyl-sn-glycero-3-phosphoinositol-4,5-bisphosphate (ammonium salt) (Topfluor PI(4,5)P₂) were obtained as powders from Avanti Polar Lipids (Alabaster, Alabama) and were used as received. Stock solutions of the lipids were obtained by dissolving the respective phosphoinositide in a 20:9:1 mixture of chloroform, methanol and water, while the less polar lipids like POPC were dissolved in a 2:1 mixture of chloroform and methanol. The concentrations of the respective stock solutions were determined using a phosphate assay (Rouser et al., 1970). All C6-BODIPY TMR labeled phosphoinositides (excitation: 542 nm, emission: 574 nm) were purchased from Echelon Biosciences Inc. (Salt Lake City, UT). 1-[2-Hydroxy-3-(N,N-di-methyl-N-hydroxyethyl) ammoniopropyl]-4-[β -[2-(di-n-butylamino)-6-naphthyl] vinyl] pyridinium dibromide (Di-4-ANEPPDHQ) was kindly provided by Dr. Robert Twieg (Kent State University, Kent, OH). Boron dipyrromethene difluoride (BODIPY) cholesterol was provided by Dr. Robert Bittman (City University of New York, NY, NY). All salts and buffers used for this study were of enzyme grade and were obtained from Fisher Scientific (Fairlawn, NJ). Chloroform (99.9%), water (HPLC grade) and methanol (99.9%) were also obtained from Fisher Scientific (Fairlawn, NJ).

Preparation of recombinant PTEN

The cDNA encoding full-length (1-403) human PTEN was cloned into the *NdeI* and *XhoI* sites of the pET30b vector (Novagen), thereby, introducing a 6-histidine tag at the C-terminus. PTEN proteins were expressed in *Escherichia coli* BL21(DE3) cells. To increase protein solubility, cells were grown at 37°C until the OD at 560 nm reached 0.5–0.6. The

culture was shifted to 21°C and after 30 min, protein expression was induced with 0.05 mM isopropyl- β -D-thiogalactoside. After 20–22 hrs, the culture was harvested. His₆-proteins were purified with a HisTrap HP Kit from GE Healthcare using buffers with 10 mM mercaptoethanol. The PTEN proteins were further purified with a Superdex 200 16/60 column in 100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM dithiothreitol. The final purification was done with a MonoQ 5/5 anion-exchange column in 10 mM Tris, pH 7.4 with a linear gradient of 50–600 mM NaCl. Fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Those fractions containing >95% pure protein were pooled. Dithiothreitol was added to 10 mM, and the proteins were stored on ice because initial experiments revealed that freeze-thawing resulted in protein aggregation. The yield of recombinant protein was 1.5–2.0 mg/l. Phosphatase activity was confirmed as described (Campbell et al., 2003).

Preparation of giant unilamellar vesicles (GUVs) using the slow rehydration and electroformation methods

With a few changes, we followed the method described in the literature (Akashi et al., 1996; Korlach et al., 1999). Lipid stock solutions were prepared as described above to yield a ~10 mM concentration. 0.2 μ moles of stock lipid solution with the desired lipid mixture (see experiments for exact lipid ratios) was mixed with 0.1 mol% of the respective fluorescently labeled lipid and diluted with 300 μ l organic solvent (chloroform/methanol 2:1 v/v). The sample was placed in a home-made test tube (internal diameter 1.5 cm) and the solvent was evaporated at 50°C with a rotary evaporator (Buchi, Rotavapor R-205) to form a thin lipid film on the surface of the lower portion of the tube (~2–3 cm height). The tube was subsequently placed in a vacuum oven for >4 h to remove remaining traces of organic solvent. The completely dried lipid film was then prehydrated at 50°C with water-saturated nitrogen gas for ~30–45 minutes. Subsequently, 2 ml N₂ purged aqueous solution (pH 7 buffer made from 100 mM NaCl, 5 mM PIPES, and 0.1 mM EDTA, NaOH and HCl were used to adjust the pH) were added gently to the test tube. The tube was sealed under nitrogen and wrapped with aluminum foil, incubated at 50°C overnight (>18h). GUVs develop spontaneously but slowly during the incubation. Subsequently, the GUV suspension was cooled slowly down to room temperature.

In general, we used the gentle hydration method to produce GUVs. However, to compare phosphoinositide phase behavior for low and high salt conditions as well as for studies involving lipid mixture mimicking lipid rafts, we also used the electroformation method to obtain GUVs in water. Briefly, a lipid mixture with the desired composition was spread onto an indium tin oxide (ITO) coated side of the glass slides. The lipid coated slides were placed in a vacuum oven for at least 2 hours to remove any trace of organic solvent. Subsequently, a custom-made Teflon spacer (0.5 cm in thickness) is placed on top of the slide; the spacer is filled with either pure water or water with sucrose and then covered with the second slide. The lipid-coated sides of the two slides (side with ITO coating) face each other with the Teflon spacer in between. An alternative current (AC) electrical field is then applied to the cell. A sinusoidal AC field with 3V amplitude and 10 HZ in frequency is applied for an hour to the cell. After one hour, the frequency is decreased 3 HZ to help vesicles detach from the slide surface. The developing sugar solution normally contains 100 mM sucrose. The vesicles were recovered in a medium containing 120 mM glucose by 10 times dilution. The

density difference between sucrose inside the vesicle and glucose outside facilitates their sedimentation and provided a better background contrast for the light microscope observation.

Preparation of large unilamellar vesicles (LUVs) and vesicles size measurements with dynamic light scattering (DLS)

Lipids were stored in organic solvent as described above. Mixed multilamellar vesicles were prepared by drying appropriate amounts of the stock solution in a stream of dry nitrogen. To avoid lipid demixing, this drying process was carried out rapidly at a slightly elevated temperature (~50°C). Subsequently, samples were kept in a high vacuum oven overnight to remove last traces of organic solvent. The lipid mixtures were re-suspended in the appropriate buffer solution. The multilamellar vesicle solution was extruded 30 times through a 100 nm diameter polycarbonate membrane (Avestin, Ottawa, ON) using an extrusion kit (Avanti Polar Lipids, Alabaster, AL). The size of the obtained unilamellar vesicles was checked using dynamic light scattering (DLS) (HPPS and Zetasizer, Malvern Instruments, Southborough, MA).

Fluorescence microscopy

For the fluorescence imaging of GUVs 0.1 mol% of a labeled lipid was added to the lipid mixture used to make the GUVs. About 400 µl of the diluted GUV suspension (10 times dilution from the original GUV suspension) was transferred to an 8-well Lab-Tek chamber (Lab-Tek, 155411) (Rochester, NY). GUVs were observed (19–23°C) with an inverted epifluorescence microscope (Olympus IX81) equipped with a water immersion objective (60X water, NA=1.4, PlanApo, Olympus) and a stage with a homebuilt temperature control device. Images of GUVs labeled with only one labeled lipid were acquired with a high-speed charge-coupled device (CCD) camera (SensiCam^{QE}, Cooke corporation) (Romulus, MI). Confocal images were taken with an Olympus Fluoview 500 confocal microscope. Acquired images were post-processed to JPEG or Tiff files with Image J.

Epifluorescence microscopy of Langmuir films

Monolayer experiments were carried out using a NIMA 601M trough (Coventry, UK) at a temperature of 20°C. The compression velocity was 15 Å² molecule⁻¹ min⁻¹. HPLC grade water was used to make the buffer solutions and the cleanliness of the water surface was checked prior to spreading the monolayer film through repeated compression/expansion of the barriers. The monolayer was spread when no increase of surface pressure was observed upon compressing the barriers. After spreading the respective monolayer, 15 minutes was allowed for film equilibration and solvent evaporation prior to starting the monolayer compression. The surface pressure was monitored using a Wilhelmy plate. For the microscopy studies 0.5 mol% of a fluorescently labeled lipid (NBD-PC) with 99.5% of the lipid of interest was spread. Experiments were carried out using an Olympus (Center Valley, PA) BX51 upright microscope equipped with a Hamamatsu (Bridgewater, NJ) EM-CCD digital camera. A 100-watt Hg HI output lamp (B&B Microscopes, Pittsburgh, PA) was used as a light source. Images were obtained using a 40x long distance working lens and a camera exposure time of 30 frames per second. The monolayer films were compressed to the desired surface pressures and were held at that pressure while images were recorded. The

monolayer is then further compressed to the next desired surface pressure and the next series of images is captured. Since the monolayer was not compressed continuously during epifluorescence imaging, separate surface pressure/area isotherms were obtained and then used as reference for the epifluorescence measurements. The images were processed using ImageJ.

Fluorescence quenching experiments using BODIPY TMR labeled phosphoinositides

Fluorescence experiments were carried out with a Varian Eclipse fluorescence spectrometer (Walnut Creek, CA) equipped with a temperature controlled sample holder. Unilamellar mixed vesicles (99.9% POPC and 0.1% BODIPY TMR PI(x,y)P₂) were made as described above, resulting in a final total lipid concentration of 0.23 mM. Half of the fluorescently labeled lipid is located on the inside of the unilamellar vesicles and, therefore, is inaccessible for the protein. These unilamellar vesicles were titrated at 20°C with PTEN protein in forty titration steps, reaching a protein to (outer leaflet) anionic lipid ratio of 0.5 (mole/mole). The fluorescence of the BODIPY TMR probe (excitation: 542 nm, emission: 574 nm) was recorded after each addition of PTEN protein. The fluorescence intensities were then corrected for dilution. These values were normalized to the initial fluorescence value in the absence of protein, which was set to 100%.

Results

Based upon FRET measurements of mixed fluorescently labeled phosphatidylcholine (PC)/phosphoinositide vesicles, we previously inferred that phosphoinositide lipids may segregate from PC and form domains in these binary mixtures (Redfern and Gericke, 2005). In a subsequent study, we found by solid-state magic angle spinning ³¹P-NMR that the concentration-dependent ionization behavior of natural chain phosphoinositides in mixtures with 1-palmitoyl-2-oleoyl-sn-3 phosphocholine (POPC) can only be explained by assuming the formation of fluid phosphoinositide domains (Kooijman et al., 2009). Considering the high phosphoinositide headgroup charge, this was a surprising result and our earlier paper attracted some criticism (Fernandes et al., 2006). In line with this criticism, fluorescence microscopy studies of SOPC/PI(4,5)P₂ monolayers at the air/water interface as well as AFM studies of transferred films revealed domain formation only in the presence of divalent cations (Ellenbroek et al., 2011; Levental et al., 2009; Wang et al., 2012). However, the results from our NMR study (Kooijman et al., 2009) suggest that PI(4,5)P₂ forms an inter- and intramolecular hydrogen bond network that dissipates the negative charge away from the PI(4,5)P₂ phosphomonoester groups, resulting in a lower charge density than what is found in cases like phosphatidic acid, where the hydrogen bond network is less developed, if it exists at all. PI(4,5)P₂ clusters in binary PC/PI(4,5)P₂ mixed vesicles are in all likelihood quite fragile; minor changes in the lipid composition (e.g., use of PI(4,5)P₂ with bulky fluorophore groups instead of native PI(4,5)P₂ (Fernandes et al., 2006) or assembly of model membranes on solid supports (Ellenbroek et al., 2011) may offset the balance between attractive hydrogen bonds and repulsive headgroup charges. Furthermore, using single point FCS as well as careful fluorophore brightness analyses Salvemini et al. recently obtained results consistent with the formation of PI(4,5)P₂ clusters that consist of only a few lipids (Salvemini et al., 2013) and are therefore too small to be detected by the methods

employed by Fernandes et al. (Fernandes et al., 2006). In another recent study, nanosized domains PI(4,5)P₂ domains were found for solid supported lipid bilayers (Braunger et al., 2013).

A drawback of our previous studies was the fact that phosphoinositide domain formation was inferred indirectly, rather than visualized by microscopy. Therefore, utilizing microscopy techniques as described in this study seems crucial to delineate phosphoinositide phase behavior. Figure 1 shows images obtained for GUVs composed of brain PI(4,5)P₂ and POPC. While at room temperature (RT) no evidence of macroscopic domain formation is found, cooling the sample to about 10°C results in a pronounced shape change of the GUV, indicating formation of two phases. At 10°C, both lipids are still in the fluid phase, therefore demixing due to a gel/liquid crystalline phase transition for either of the two lipids can be ruled out. The observed shape changes are reversible, i.e., heating of the sample to RT will lead to a disappearance of the bulging. These results in combination with our earlier findings as well as recently published results from other research groups (Braunger et al., 2013; Salvemini et al., 2013), suggest that PI(4,5)P₂ is able to form nanosized domains in mixed PC/PI(4,5)P₂ vesicles at room temperature that coalesce to macroscopic domains as the temperature is lowered to about 10°C. From a physicochemical point of view, these results are intriguing because they suggest that the electrostatic repulsion between PI(4,5)P₂ molecules can be overcome. From a physiological point of view, however, the results are of limited value since other species present in the plasma membrane will affect the mutual PI(4,5)P₂ interaction and potentially weaken or strengthen it. The physiological relevance of these data is the fact that the repulsive forces between PI(4,5)P₂ lipids are less pronounced than it was believed previously.

As noted above, a broad range of phosphoinositide-mediated signaling events have been shown to be cholesterol dependent. It is therefore a valid question whether cholesterol affects PI(4,5)P₂/PC mixing behavior. Figure 2(a – c) compares POPC/PI(4,5)P₂ GUVs for 0%, 20% and 40% cholesterol at room temperature and physiological salt concentrations. For the GUVs with 20% or 40% cholesterol two phases are observed, indicating that the presence of cholesterol results in POPC/PI(4,5)P₂ demixing even at room temperature. Variation in the PI(4,5)P₂ concentration results in smaller or larger “bulged” regions (see supplementary material Figure S1), confirming that the higher curvature region in the vesicles shown in Figure 2b and 2c are associated with the PI(4,5)P₂ enriched phase. The salt concentration is an important factor for the observed GUV shape changes. Experiments carried out at a low salt concentration in the absence (not shown) or presence of cholesterol (see supplementary material S2) revealed domain formation judged based upon the distribution of labeled lipid; however, this domain formation was not accompanied by a shape change of the vesicle. To rule out that the observed “bulging” of the GUVs is not associated with artifacts such as two vesicles lying on top of each other or the formation of multilayer membranes, single layer (Figure 2d) and multiple layer laser scanning confocal microscopy were performed. An equatorial scan and a 3D composite of several images obtained across the Z-axis are shown in Figure 2d and 2e, respectively (see the supplementary section for a movie showing the z-scan of a representative GUV). Last but not least, fluorescently labeled PI(4,5)P₂ (TopFluor PI(4,5)P₂) shows enrichment in the bulged region (Figure 2f). The use of chain labeled PI(4,5)P₂ is problematic when the

interaction that leads to PI(4,5)P₂ phase separation is weak. In such cases, the steric hindrance imposed by the large fluorescent group attached to the acyl chain is often too large to be overcome by the attractive forces that are associated with the observed phase separation. For this reason, we largely used shape changes of the respective GUVs as an indicator for phase separation. Overall, all the data confirm that the bulged region is the PI(4,5)P₂ rich phase.

To ensure that the observed phase separation can be attributed to the presence of cholesterol, we conducted cholesterol depletion experiments using β -cyclodextrin. We developed PI(4,5)P₂/PC/Chol GUVs (60%:20%:20%) and observed the phase separation described above. Upon the addition of β -cyclodextrin, we found a shrinking and eventually disappearance of the bulged region (see supplementary data Figure S3; in control experiments we ensured that β -cyclodextrin does not interact with phosphoinositides directly).

In principle, cholesterol might enhance PI(4,5)P₂ segregation by either of the following two ways: The rigid sterol ring structure is unfavorable for the interaction with highly unsaturated, disordered acyl chains like the arachidonoyl chain found at the sn-2 position of PI(4,5)P₂. This may lead to a scenario where PI(4,5)P₂ is excluded from cholesterol rich regions, leading to an “indirect” accumulation of PI(4,5)P₂ due to the limited space availability. If that were the case, one would expect a similar behavior for other lipids with stearoyl/arachidonoyl fatty acid chains. However, to the best of our knowledge, cholesterol-induced accumulation of SAPC in mixtures with POPC has not been observed.

Alternatively, cholesterol may be incorporated in PI(4,5)P₂ domains and stabilize these domains through direct interaction with PI(4,5)P₂. To test whether cholesterol co-localizes with PI(4,5)P₂, we used BODIPY labeled cholesterol (Li et al., 2006) to monitor the cholesterol distribution. We are aware of the general limitations in using fluorescently labeled cholesterol to image cholesterol distribution, however, this particular labeled cholesterol has been shown to mimic quite accurately cholesterol phase behavior (Ariola et al., 2009). Additionally, GUV test experiments mimicking raft mixtures yielded results consistent with the expected behavior of cholesterol (supplementary data Figure S4). Figure 3 shows images of POPC/PI(4,5)P₂/Chol GUVs (60%:20%:20%) labeled with 0.1 mol% BODIPYcholesterol. It is clearly evident that cholesterol is present in both phases, i.e., the phase that is enriched in PI(4,5)P₂ also contains cholesterol. This suggests that cholesterol interacts with PI(4,5)P₂ and thereby stabilizes PI(4,5)P₂ enriched phases. The GUV images shown in Figure 2 and 3 are representative of the ensemble of GUVs within the total viewing area of the microscope, however, we observed in the images GUVs with different degrees of bulging and for some vesicles it appeared as if a vesicle was about to pinch off. PI(4,5)P₂ by itself does not form vesicles, however, based upon the GUV images we hypothesized that PI(4,5)P₂ might form stable vesicles in the presence of cholesterol. Figure 4 shows the size distribution for PI(4,5)P₂ samples with varying degrees of cholesterol that were extruded through a 100 nm filter to obtain unilamellar vesicles. Extrusion through a filter with a 100 nm pore size typically yields unilamellar lipid vesicles with a diameter of 110 – 125 nm, depending on the type of lipid. In the case of PI(4,5)P₂/Chol vesicles, we find for 10 and 20 mol% cholesterol, vesicles with a diameter of about 95 nm, while for 40 and 60 mol% we observe vesicles with an average diameter of ~105 – 110 nm (Figure 4). Below

10 mol% cholesterol we observe particles with a very small diameter, indicating that vesicles are not formed. In contrast to extrusion with typical lipid mixtures (e.g., PC lipids), we observe a broadened vesicle size distribution for those cholesterol concentrations where vesicles are being formed. These results strongly suggest that cholesterol indeed stabilizes PI(4,5)P₂ rich phases.

Next, we tested whether the formation of cholesterol/phosphoinositide phases is limited to PI(4,5)P₂ or is also observed for other naturally occurring phosphoinositides. Figure 5 shows GUV images for PI, PI(4)P, PI(4,5)P₂, PI(3,4)P₂, PI(3,5)P₂ and PI(3,4,5)P₃ in the presence of cholesterol. We observed cholesterol-induced phosphoinositide segregation for all investigated phosphoinositides as well as phosphatidylinositol. The observation that also PI clusters in the presence of cholesterol, indicates that the presence of a phosphomonoester group at the inositol ring is not essential for the observed segregation. However, it is likely that the number and probably the position of the phosphomonoester groups at the inositol ring modulate the observed interaction with cholesterol.

To investigate the phase state of the phosphoinositide-enriched domains, we labeled the GUVs with Di-4-ANEPPDHQ, an environment sensitive fluorophore that shows a red shifted emission spectrum in disordered, fluid environments (*l_d*), while for an ordered environment, shorter emission wavelengths are observed (Jin et al., 2006). Figure 6 shows the images for POPC/PI(4,5)P₂/Chol (60%:20%:20%) vesicles labeled with 0.1 mol% Di-4-ANEPPDHQ. For comparison, we provide in the supplementary material (see Figure S5) corresponding images for a raft lipid mixture DOPC/DPPC/Chol (40%:40%:20%), which is known to exhibit an *l_o/l_d* phase separation (Stottrup et al., 2004). In the case of the raft mixture, the *l_o* and *l_d* phases can be clearly distinguished in the green and red channels. For the PI(4,5)P₂/Chol GUVs it is possible to identify the two phases based upon the curvature change; however, the emission wavelengths of the Di-4-ANEPPDHQ fluorophore in these two phases are indistinguishable. While we cannot rule out that there are minor spectral changes between the two lipid bilayer regions since the spectral resolution of the optical filters used for the microscopy experiment is limited, it is clear that the phosphoinositide-enriched phase has a similar fluidity as the POPC phase.

The question arises what kind of interaction between cholesterol and PI(4,5)P₂ drives the observed formation of PI(4,5)P₂/Chol domains? We have previously found that PI(4,5)P₂ is capable of forming an intra- and intermolecular hydrogen bond network that involves the hydroxyl as well as the phosphate groups (Graber et al., 2012; Kooijman et al., 2009). Since an interaction between the rigid sterol ring structure of cholesterol and the disordered acyl chain of PI(4,5)P₂ is more likely to hinder Chol/PI(4,5)P₂ cluster formation than to promote it, we hypothesized that the cholesterol hydroxyl group participates in the hydrogen bond network formed by the PI(4,5)P₂ headgroups. To test this hypothesis, we investigated PI(4,5)P₂ domain formation in Langmuir films in the presence of cholesterol as well as cholesterol derivatives with modified hydroxyl groups. Figure 7a shows an epifluorescence image for a brain PI(4,5)P₂ monolayer at the air/water interface at a surface pressure of about ~ 25 mN/m, while Figure 7b shows the corresponding image for a mixed Chol/PI(4,5)P₂ monolayer (see the supplementary material Figure S6 for several images obtained throughout the compression of the monolayer). For the PI(4,5)P₂ monolayer a homogeneous

fluid phase is observed, indicating no discernable domain formation. In contrast, the monolayer condenses in the presence of cholesterol and domains are formed (Figure 7b). This agrees with earlier observations (Levental et al., 2009). In Figures 7c and 7d, the corresponding images for PI(4,5)P₂ monolayers in the presence of cholestenone and cholesteryl ethyl ether, respectively, are shown. Cholestenone and cholesteryl ethyl ester lack the PI(4,5)P₂ monolayer condensing effect of cholesterol (Figure 7b-d). It should be noted that the ring structure of cholestenone is not planar (in contrast to the sterol ring structure of cholesterol) and cholesteryl ethyl ether exhibits a lower hydrophilicity than cholesterol; however, these data nevertheless suggest that the hydroxyl group of cholesterol is a major factor for the PI(4,5)P₂/cholesterol interaction. This will be further addressed below. We also conducted monolayer experiments involving liver PI, brain PI(4)P, SAPI(3,5)P₂ and SAPI(3,4,5)P₃. In all cases we found a homogeneous fluid film in the absence of cholesterol, while in the presence of cholesterol domain structures similar to the PI(4,5)P₂/Chol system were found (not shown).

Zeta potential measurements for PC/phosphatidylinositol bisphosphate (PI(x,y)P₂)/Chol (55%:5%:40%) vesicles (not shown) revealed an increase of the negative charge by about 5 mV in the presence of cholesterol in comparison to the vesicles without cholesterol (PC/PI(x,y)P₂ 95% : 5%). While the interpretation of zeta potentials for laterally heterogeneous lipid bilayers bears some risks, it appears that the cholesterol induced clustering of PI(4,5)P₂ results in an increased interfacial charge. Within the detection limit of the method the PC/PI(x,y)P₂ vesicles for all three bisphosphate derivatives had about the same zeta potential. The binding of proteins to phosphoinositides involves typically a combination of hydrogen bond formation as well as non-specific electrostatic interactions. An increased charge, as observed for these vesicles, should therefore give rise to enhanced binding of phosphoinositide binding proteins.

To examine this question further, we investigated the binding of the tumor suppressor protein PTEN to phosphoinositide-containing vesicles. PTEN is a phosphatase that selectively removes the phosphate group from the 3-position of phosphoinositide headgroups, thereby acting as an antagonist to PI 3-kinase signaling events (Gericke et al., 2006). PTEN's primary physiological lipid target is PI(3,4,5)P₃, which is being dephosphorylated to form PI(4,5)P₂. We have shown previously that PTEN preferentially binds to PI(4,5)P₂ (and to a lesser extent PI(5)P), which leads to a structural change and activation of the phosphatase (Redfern et al., 2008), i.e., PTEN is allosterically activated by PI(4,5)P₂ (Campbell et al., 2003). We have used in the past a simple fluorescence self-quenching assay to assess PTEN lipid binding preferences, which utilizes PC vesicles labeled with 0.1 mol% of BODIPY-phosphoinositide. Upon PTEN binding, the respective BODIPY labeled phosphoinositide will coalesce, leading to self-quenching of the BODIPY fluorophore and hence a reduction of the fluorescence emission intensity (we have confirmed using PC/PI(4,5)P₂/bodipy PI(4,5)P₂ GUVs that the addition of PTEN leads to enhanced PI(4,5)P₂ clustering, not shown). Figure 8 compares the histograms for the BODIPY fluorescence intensity at the endpoint of the titration of POPC/ BODIPY-phosphoinositide vesicles (in the absence vs. presence of cholesterol) with PTEN. The stronger PTEN binds to the respective phosphoinositide derivative, the greater is the reduction of the fluorescence intensity (for details see (Redfern et al., 2008). The data for the

titration of vesicles without cholesterol were reproduced from an earlier paper (Redfern et al., 2008). The results show that PTEN binding to PI(5)P and PI(4,5)P₂ containing vesicles is enhanced in the presence of cholesterol, while binding to vesicles with other phosphoinositides is largely unaffected or even slightly reduced (Figure 8). Both, PI(5)P and PI(4,5)P₂, are known to enhance PTEN activity (Campbell et al., 2003), and the enhanced binding of PTEN due to the presence of cholesterol is particularly evident for PI(5)P. These data further underscore our earlier finding that PTEN's N-terminal end preferentially binds PI(4,5)P₂ and to a lesser extent PI(5)P. PI(4)P and all 3-phosphorylated phosphoinositides show only minor PTEN binding. In conclusion, cholesterol enhanced phosphoinositide domain formation promotes phosphoinositide binding protein activity. The cholesterol effect on protein binding to phosphoinositides is not limited to PTEN. In the case of profilin (see supplementary Figure S7), binding to PI(3,4)P₂ is significantly enhanced in the presence of cholesterol. In the absence of cholesterol, profilin binding to PI(4,5)P₂ was strongest among all phosphoinositides, while in the presence of cholesterol, the binding appears to be slightly reduced. Profilin binding to all other naturally occurring phosphoinositides was negligible in the absence as well as the presence of cholesterol. This example illustrates that the effect of cholesterol on protein binding to phosphoinositides can be quite pronounced and in some cases even unexpected.

Discussion

The spatiotemporal control of phosphoinositides is of major importance for a broad range of membrane signaling events that are either controlled or affected by this class of lipids. While interaction with cationic protein motifs is probably the most important factor for the *in vivo* formation of phosphoinositide enriched domains, other factors are likely to contribute to the observed lateral inhomogeneities of phosphoinositides. Based upon the observation that cholesterol depletion affects many phosphoinositide-mediated signaling events, it has been suggested that phosphoinositides are enriched in liquid-ordered raft domains. While *in vitro* studies failed to show phosphoinositide accumulation in liquid-ordered phases (at least in the absence of a phosphoinositide-binding, raft resident proteins), it has to be highlighted that these studies utilized lipid mixtures that were characteristic for the outer leaflet of the plasma membrane. It is an open question at this point whether phosphoinositides accumulate in inner leaflet raft domains, whatever their composition might be.

This paper explores to what extent and how cholesterol stabilizes formation of phosphoinositide enriched domains. Several points are worth highlighting:

1. Cholesterol and phosphoinositides form domains that exhibit a fluid phase state. Despite this fluidity, cholesterol/phosphoinositide domains demix from fluid POPC phases, *i.e.*, fluid/fluid demixing is observed.
2. Phosphoinositide vesicles are not stable; however, cholesterol amounts above 10 mol% stabilize PIP vesicles. For two reasons this is an important observation: First, it shows that small amounts of cholesterol stabilize phosphoinositide-rich phases. Second, cholesterol-stabilized vesicles might be a convenient tool to study phosphoinositide/protein interactions.

3. The cholesterol hydroxyl group appears to be crucial for the observed phosphoinositide/cholesterol interaction, suggesting that the interaction between phosphoinositides and cholesterol involves hydrogen bond formation.
4. Cholesterol-induced phosphoinositide domain formation was observed for all investigated phosphoinositide derivatives as well as phosphatidylinositol. This suggests that the number and position of the phosphomonoester groups at the inositol ring may modulate the interaction between cholesterol and phosphoinositides, but the cholesterol hydroxyl group does not form hydrogen bonds with the phosphoinositide phosphomonoester groups. Such an interaction would have been surprising since it would require the cholesterol to come far out of the hydrophobic portion of the bilayer. NMR data reported in this issue support the notion that cholesterol does not interact with the phosphomonoester groups (cite Graber et al. in this issue).
5. Cholesterol enhanced phosphoinositide domain formation promotes binding of phosphoinositide-binding proteins, which might be an important factor for the spatial control of phosphoinositide mediated signaling events.

What kind of interaction is responsible for the observed stabilization of phosphoinositide-enriched phases by cholesterol? Phosphoinositides carry a significant negative charge at physiological pH, which was expected to give rise to strong repulsive forces. However, we have shown previously that the negative phosphomonoester charges delocalize through intramolecular hydrogen bond formation between the inositol ring hydroxyl and phosphomonoester groups (Graber et al., 2012; Kooijman et al., 2009). This apparently allows phosphoinositides to come close enough to form either direct or water mediated intermolecular hydrogen bonds, resulting in a further reduction of the charge density by distributing the charge across the bilayer/water interface. Because of the delicate balance between repulsive and attractive forces, this hydrogen bond network appears to be very fragile and highly dependent on the details of the experimental conditions. While in some cases experimental data were obtained that were in accordance with the formation of such a hydrogen bond network (Kooijman et al., 2009; Redfern and Gericke, 2004, 2005), computer simulations suggested that PI(4,5)P₂ indeed forms intermolecular hydrogen bonds (Lorenz et al., 2008) and recently published results support the notion of PI(4,5)P₂ clustering (Braunger et al., 2013; Salvemini et al., 2013), other studies did not show any form of domain formation in the absence of phosphoinositide clustering agents (Levental et al., 2009). The balance of repulsive forces due to the negative headgroup charge and attractive forces due to hydrogen-bond formation, likely leads to very fragile domains that can easily disintegrate. Minor differences in the experimental conditions may therefore to different results. While the observation of PI(4,5)P₂ clustering found for some experimental is of significance because it suggest that the mutual repulsive forces between PI(4,5)P₂ molecules are smaller than expected, formation of pure PI(4,5)P₂ domains is quite unlikely in real biological membranes.

From this study as well as previous studies carried out by other research groups (Levental et al., 2009), it is clear that cholesterol stabilizes phosphoinositide-rich phases and promotes phosphoinositide clustering. We hypothesize that cholesterol participates either directly or

via water in the formation of an intermolecular hydrogen bond network between the phosphoinositide headgroups. Cholesterol acts as a spacer between the phosphoinositide lipids, which should lead to a reduction of the repulsive forces. In comparison to the cholesterol-free phosphoinositide bilayer, the increased spacing of the phosphoinositide lipids may lead to a weakening of the intermolecular hydrogen bonds. However, the participation of cholesterol in the hydrogen bond network apparently compensates for this weakening. Overall, the presence of cholesterol leads to a strengthening of hydrogen bond network. The effect of the rigid sterol ring on the order of the phosphoinositide acyl chains is expected to be minor. For example, the melting transition of palmitoyl arachidonoyl phosphatidylcholine (PAPC) was essentially unaffected by the presence of moderate amounts of cholesterol (40 mol%) (Jackman et al., 1999). This study focuses on the headgroup interaction between phosphoinositides and cholesterol. To examine if phosphoinositide domain formation induced by cholesterol is modulated by the acyl chain composition, detailed additional studies will be required, however, preliminary data obtained for dioleoyl phosphoinositides suggest that the acyl chain effect is minor as long as at least one acyl chain is unsaturated.

The effect of cholesterol on the phase behavior of PI(4,5)P₂ is similar to what we have observed in the presence of PI. For mixed PC/PI(4,5)P₂/PI vesicles we also observed the formation of PI(4,5)P₂ enriched domains, presumably due to intermolecular PI/PI(4,5)P₂ hydrogen bond formation. We hypothesized that the inclusion of PI in the PI(4,5)P₂ hydrogen bond network leads to a reduction of the charge density due to the intercalation of PI between the PI(4,5)P₂ lipids, while maintaining the hydrogen bond network (Graber et al., 2012).

The increased local density of phosphoinositides due to the cholesterol induced domain formation affects protein binding, such as it is shown here for PTEN and Profilin. Similarly, it might shed light on why NAP-22 shows enhanced binding to PI(4,5)P₂ in the presence of cholesterol (Epand et al., 2004b). In general, the local accumulation of phosphoinositides in the presence of cholesterol leads apparently to an electrostatic environment that favors stronger protein binding.

Conclusions

Phosphoinositides are involved in an extraordinary array of cellular functions and their spatiotemporal control is important for the proper execution of these processes. Conclusions derived from studies like this one that involve lipid model systems obviously have limitations with respect to their transferability to *in vivo* systems. However, the results presented in this study suggest that cholesterol is likely to contribute to the lateral segregation of phosphoinositides *in vivo*. Further studies involving increasingly complex lipid mixtures that more accurately mimic the inner leaflet of the plasma membrane are required to further address this question.

Movie 1: A movie showing a multi-layer scan of a GUV (POPC/PI(4,5)P₂/Chol 60%/20%/20%) labeled with RhB DOPE. The movie was obtained by combining single layer scans

along the Z-axis of a representative GUV at RT using laser scanning confocal microscopy. The scanning depth is 0.3 μm .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Akashi K, Miyata H, Itoh H, Kinoshita K Jr. Preparation of giant liposomes in physiological conditions and their characterization under an optical microscope. *Biophys J*. 1996; 71:3242–3250. [PubMed: 8968594]
- Ariola FS, Li ZG, Cornejo C, Bittman R, Heikal AA. Membrane Fluidity and Lipid Order in Ternary Giant Unilamellar Vesicles Using a New Bodipy-Cholesterol Derivative. *Biophys J*. 2009; 96:2696–2708. [PubMed: 19348752]
- Braunger JA, Kramer C, Morick D, Steinem C. Solid Supported Membranes Doped with PIP2: Influence of Ionic Strength and pH on Bilayer Formation and Membrane Organization. *Langmuir*. 2013; 29:14204–14213. [PubMed: 24199623]
- Cai HQ, Devreotes PN. Moving in the right direction: How eukaryotic cells migrate along chemical gradients. *Semin Cell Dev Biol*. 2011; 22:834–841. [PubMed: 21821139]
- Calloway N, Owens T, Corwith K, Rodgers W, Holowka D, Baird B. Stimulated association of STIM1 and Orai1 is regulated by the balance of PtdIns(4,5)P-2 between distinct membrane pools. *J Cell Sci*. 2011; 124:2602–2610. [PubMed: 21750194]
- Campbell RB, Liu FH, Ross AH. Allosteric activation of PTEN phosphatase by phosphatidylinositol 4,5-bisphosphate. *J Biol Chem*. 2003; 278:33617–33620. [PubMed: 12857747]
- Catimel B, Schieber C, Condrón M, Patsiouras H, Connolly L, Catimel J, Nice EC, Burgess AW, Holmes AB. The PI(3,5)P2 and PI(4,5)P2 interactomes. *J Proteome Res*. 2008; 7:5295–5313. [PubMed: 19367725]
- Das S, Chakraborty S, Basu A. Critical role of lipid rafts in virus entry and activation of phosphoinositide 3' kinase/Akt signaling during early stages of Japanese encephalitis virus infection in neural stem/progenitor cells. *J Neurochem*. 2010; 115:537–549. [PubMed: 20722967]
- Ellenbroek WG, Wang YH, Christian DA, Discher DE, Janmey PA, Liu AJ. Divalent cation-dependent formation of electrostatic PIP2 clusters in lipid monolayers. *Biophys J*. 2011; 101:2178–2184. [PubMed: 22067156]
- Epand RM, Epand RF, Bain AD, Sayer BG, Hughes DW. Properties of polyunsaturated phosphatidylcholine membranes in the presence and absence of cholesterol. *Magn Reson Chem*. 2004a; 42:139–147. [PubMed: 14745793]
- Epand RM, Vuong P, Yip CM, Maekawa S, Epand RF. Cholesterol-dependent partitioning of PtdIns(4,5)P-2 into membrane domains by the N-terminal fragment of NAP-22 (neuronal axonal myristoylated membrane protein of 22 kDa). *Biochem J*. 2004b; 379:527–532. [PubMed: 14989697]
- Fernandes F, Loura LMS, Fedorov A, Prieto M. Absence of clustering of phosphatidylinositol-(4,5)-bisphosphate in fluid phosphatidylcholine. *J Lipid Res*. 2006; 47:1521–1525. [PubMed: 16632797]
- Fox TE, Young MM, Pedersen MM, Giambuzzi-Tussey S, Kester M, Gardner TW. Insulin signaling in retinal neurons is regulated within cholesterol-enriched membrane microdomains. *Am J Physiol Endocrinol Metab*. 2011; 300:E600–609. [PubMed: 21205932]

- Furt F, König S, Bessoule JJ, Sargueil F, Zallot R, Stanislas T, Noirot E, Lherminier J, Simon-Plas F, Heilmann I, Mongrand S. Polyphosphoinositides Are Enriched in Plant Membrane Rafts and Form Microdomains in the Plasma Membrane. *Plant Physiol.* 2010; 152:2173–2187. [PubMed: 20181756]
- Gao X, Lowry PR, Zhou X, Depry C, Wei Z, Wong GW, Zhang J. PI3K/Akt signaling requires spatial compartmentalization in plasma membrane microdomains. *Proc Natl Acad Sci U S A.* 2011; 108:14509–14514. [PubMed: 21873248]
- Gericke A, Munson M, Ross AH. Regulation of PTEN phosphatase. *Gene.* 2006; 374:1–9. [PubMed: 16675164]
- Golebiewska U, Kay JG, Masters T, Grinstein S, Im W, Pastor RW, Scarlata S, McLaughlin S. Evidence for a fence that impedes the diffusion of phosphatidylinositol 4,5-bisphosphate out of the forming phagosomes of macrophages. *Mol Biol Cell.* 2011; 22:3498–3507. [PubMed: 21795401]
- Graber ZT, Jiang Z, Gericke A, Kooijman EE. Phosphatidylinositol-4,5-bisphosphate ionization and domain formation in the presence of lipids with hydrogen bond donor capabilities. *Chem Phys Lipids.* 2012; 165:696–704. [PubMed: 22820347]
- Hao M, Bogan JS. Cholesterol regulates glucose-stimulated insulin secretion through phosphatidylinositol 4,5-bisphosphate. *J Biol Chem.* 2009; 284:29489–29498. [PubMed: 19729450]
- Illien F, Piao HR, Coue M, di Marco C, Ayala-Sanmartin J. Lipid organization regulates annexin A2 Ca(2+)-sensitivity for membrane bridging and its modulator effects on membrane fluidity. *Biochim Biophys Acta.* 2012; 1818:2892–2900. [PubMed: 22842545]
- Jackman C, Davis P, Morrow M, Keough K. Effect of cholesterol on the chain-ordering transition of 1-palmitoyl-2-arachidonoyl phosphatidylcholine. *J Phys Chem B.* 1999; 103:8830–8836.
- Jin L, Millard AC, Wuskell JP, Dong X, Wu D, Clark HA, Loew LM. Characterization and application of a new optical probe for membrane lipid domains. *Biophys J.* 2006; 90:2563–2575. [PubMed: 16415047]
- Johnson CM, Chichili GR, Rodgers W. Compartmentalization of Phosphatidylinositol 4,5-Bisphosphate Signaling Evidenced Using Targeted Phosphatases. *J Biol Chem.* 2008; 283:29920–29928. [PubMed: 18723502]
- Kooijman EE, King KE, Gangoda M, Gericke A. Ionization Properties of Phosphatidylinositol Polyphosphates in Mixed Model Membranes. *Biochemistry.* 2009; 48:9360–9371. [PubMed: 19725516]
- Korlach J, Schwille P, Webb WW, Feigenson GW. Characterization of lipid bilayer phases by confocal microscopy and fluorescence correlation spectroscopy. *Proc Natl Acad Sci U S A.* 1999; 96:8461–8466. [PubMed: 10411897]
- Koushik AB, Powell RR, Temesvari LA. Localization of Phosphatidylinositol 4,5-Bisphosphate to Lipid Rafts and Uroids in the Human Protozoan Parasite *Entamoeba histolytica*. *Infect Immun.* 2013; 81:2145–2155. [PubMed: 23545298]
- Kwik J, Boyle S, Fooksman D, Margolis L, Sheetz MP, Edidin M. Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc Natl Acad Sci U S A.* 2003; 100:13964–13969. [PubMed: 14612561]
- Levental I, Christian DA, Wang YH, Madara JJ, Discher DE, Janmey PA. Calcium-dependent lateral organization in phosphatidylinositol 4,5-bisphosphate (PIP₂)- and cholesterol-containing monolayers. *Biochemistry.* 2009; 48:8241–8248. [PubMed: 19630438]
- Li Z, Mintzer E, Bittman R. First synthesis of free cholesterol-BODIPY conjugates. *J Org Chem.* 2006; 71:1718–1721. [PubMed: 16468832]
- Lorenz CD, Faraudo J, Travesset A. Hydrogen bonding and binding of polybasic residues with negatively charged mixed lipid monolayers. *Langmuir.* 2008; 24:1654–1658. [PubMed: 18211111]
- McLaughlin S, Murray D. Plasma membrane phosphoinositide organization by protein electrostatics. *Nature.* 2005; 438:605–611. [PubMed: 16319880]
- Peres C, Yart A, Perret B, Salles JP, Raynal P. Modulation of phosphoinositide 3-kinase activation by cholesterol level suggests a novel positive role for lipid rafts in lysophosphatidic acid signalling. *FEBS Lett.* 2003; 534:164–168. [PubMed: 12527380]

- Redfern DA, Gericke A. Domain formation in phosphatidylinositol monophosphate/phosphatidylcholine model membrane systems. *Biophys J*. 2004; 86:2980–2992. [PubMed: 15111413]
- Redfern DA, Gericke A. pH dependent microdomain formation in phosphatidylinositol polyphosphate/phosphatidylcholine mixed vesicles. *J Lip Res*. 2005; 46:504–515.
- Redfern RE, Redfern DA, Furgason MLM, Muonson M, Ross AH, Gericke A. PTEN phosphatase selectively binds phosphoinositides and undergoes structural changes. *Biochemistry*. 2008; 47:2162–2171. [PubMed: 18220422]
- Rogasevskaia TP, Churchward MA, Coorssen JR. Anionic lipids in Ca(2+)-triggered fusion. *Cell Calcium*. 2012; 52:259–269. [PubMed: 22516687]
- Rouser G, Fkeischer S, Yamamoto A. Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids*. 1970; 5:494–496. [PubMed: 5483450]
- Salvemini IL, Gau DM, Reid J, Bagatolli LA, Macmillan A, Moens PD. Low PIP molar fractions induce nanometer size clustering in giant unilamellar vesicles. *Chem Phys Lipids*. 2013; 177C:51–63. [PubMed: 24269375]
- Seveau S, Tham TN, Payrastra B, Hoppe AD, Swanson JA, Cossart P. A FRET analysis to unravel the role of cholesterol in Rac1 and PI 3-kinase activation in the InIB/Met signalling pathway. *Cell Microbiol*. 2007; 9:790–803. [PubMed: 17140407]
- Shaw JE, Epand RF, Epand RM, Li Z, Bittman R, Yip CM. Correlated fluorescence-atomic force microscopy of membrane domains: structure of fluorescence probes determines lipid localization. *Biophys J*. 2006; 90:2170–2178. [PubMed: 16361347]
- Shen-Tu G, Schauer DB, Jones NL, Sherman PM. Detergent-resistant microdomains mediate activation of host cell signaling in response to attaching-effacing bacteria. *Lab Invest*. 2010; 90:266–281. [PubMed: 19997063]
- Shewan A, Eastburn DJ, Mostov K. Phosphoinositides in Cell Architecture. *Cold Spring Harb Perspect Biol*. 2011; 3
- Stottrup BL, Veatch SL, Keller SL. Nonequilibrium behavior in supported lipid membranes containing cholesterol. *Biophys J*. 2004; 86:2942–2950. [PubMed: 15111410]
- Tong J, Nguyen L, Vidal A, Simon SA, Skene JH, McIntosh TJ. Role of GAP-43 in sequestering phosphatidylinositol 4,5-bisphosphate to Raft bilayers. *Biophys J*. 2008; 94:125–133. [PubMed: 17827240]
- van Rheenen J, Achame EM, Janssen H, Calafat J, Jalink K. PIP2 signaling in lipid domains: a critical re-evaluation. *EMBO J*. 2005; 24:1664–1673. [PubMed: 15861130]
- Varnai P, Balla T. Visualization and manipulation of phosphoinositide dynamics in live cells using engineered protein domains. *Pflügers Archiv-European Journal of Physiology*. 2007; 455:69–82. [PubMed: 17473931]
- Wang YH, Collins A, Guo L, Smith-Dupont KB, Gai F, Svitkina T, Janmey PA. Divalent Cation-Induced Cluster Formation by Polyphosphoinositides in Model Membranes. *J Am Chem Soc*. 2012; 134:3387–3395. [PubMed: 22280226]

Figure 1.

Effect of temperature on the phase behavior of POPC/PI(4,5)P₂ (80%:20%) mixed GUVs. Left: T = 20°C; Right: T = 10°C. GUVs were labeled with RhBDOPE and were developed in a pH 7 buffer with 5 mM PIPES, 0.1 mM EDTA and 100 mM NaCl. 10 μ m scale bar is shown. Images were taken by fluorescence microscopy.

Figure 2.

Phase separation in POPC/PI(4,5)P₂/Chol GUVs. (a)-(c): Cholesterol effect on PI(4,5)P₂ domain formation. GUVs composed of POPC and PI(4,5)P₂ (20%) with different concentrations of cholesterol (a): 0%; (b): 20%; and (c): 40% Chol. GUVs were labeled with RhB DOPE and imaged with fluorescence microscopy at room temperature (RT). The POPC concentrations were adjusted as the cholesterol concentration was increased. (d) Laser scanning confocal microscopy (LSCM) image of GUVs (POPC/PI(4,5)P₂/Chol=60%:20%:20%) labeled with RhBDOPE from a single layer scanning at RT; (e) 3D composite image of the GUV in shown in (d) obtained through a multi-layer scanning along the Z-axis of the GUV. The LSCM experiment was performed at RT with a depth of 0.3 µm. (f) GUVs composed of POPC/PI(4,5)P₂/Chol (60%:20%:20%) that were labeled with TopFlour PI(4,5)P₂ and imaged with LSCM at (RT). All GUVs were developed in a pH 7 buffer with 1 mM EDTA, 5 mM PIPES and 100 mM NaCl. 10 µm scale bars are as shown.

Figure 3.

POPC/PI(4,5)P₂/Chol (60%:20%:20%) GUVs labeled with BODIPY cholesterol. The image shows that cholesterol is present in both phases. GUVs were developed in a pH 7 buffer with 5 mM PIPES, 0.1 mM EDTA and 100 mM NaCl. Image was obtained at RT using LSCM. 10 μ m scale is shown.

Figure 4.

Dynamic light scattering results for PI(4,5)P₂ LUV formation in the presence of varying amounts of cholesterol. LUVs were obtained by extrusion of the respective lipid mixture suspension (pH 7 buffer with 10 mM HEPES, 0.1 mM EDTA, 100 mM NaCl) through a 100 nm polycarbonate membrane at RT.

Figure 5.

Images of GUVs composed of POPC/PIP/Chol (60%:20%:20%). The PIPs used for the respective experiments are shown in the figure. All GUVs were labeled with RhB-DOPE and images were obtained at RT. GUVs were developed in a pH 7 buffer with 5 mM PIPES, 0.1 mM EDTA and 100 mM NaCl. Shown scale bars are 10 μm.

Figure 6.

GUVs composed of POPC/PI(4,5)P₂/Chol (60%:20%:20%) labeled with Di-4-ANEPPDHQ (0.1 mol%). The GUVs were developed in a pH 7 buffer with 0.1 mM EDTA, 5 mM PIPES and 100 mM NaCl. A 488 nm laser was used to excite the fluorescent dye. Emissions were collected at different wavelengths; green channel: 505 – 520 nm (left image), red channel: > 560 nm (center image). The right image is the overlay of both images. Images were obtained by LSCM at RT. 5 μ m scale bar is shown.

Figure 7.

Epifluorescence images of PI(4,5)P₂ monolayer at the air/water interface (~ 25 mN/m in each case). (a). pure PI(4,5)P₂; (b). PI(4,5)P₂ + 40% cholesterol; (c). PI(4,5)P₂ + 40% cholestenone; and (d). PI(4,5)P₂ + 40% cholesteryl ethylether. In each case the monolayer was spread on a buffer system consisting of 150 mM NaCl, 10 mM Tris, 0.1 mM EDTA, pH 7.2 at 20°C. See the supplementary data S7 for a complete set of images of a PI(4,5)P₂/cholesterol monolayer at different states of compression. The 25 μm scale bar shown in figure a applies to all four images.

Figure 8.

Binding of PTEN to phosphoinositides in the presence and absence of cholesterol. The extent of binding of PTEN to each of the naturally occurring phosphoinositide derivatives was monitored using a BODIPY self-quenching assay. Shown are the binding of PTEN to the phosphoinositides in the absence of cholesterol (blue) and in the presence of 40% cholesterol (red). Fluorescence intensity at the end of the titration relative to the fluorescence intensity at the beginning of the titration is given. Buffer: pH 7.0, 150 mM NaCl, 10 mM HEPES, 0.1 mM EDTA.