BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2009,** *48,* 8241–8248 8241
DOI: 10.1021/bi9007879

Calcium-Dependent Lateral Organization in Phosphatidylinositol 4,5-Bisphosphate (PIP2)- and Cholesterol-Containing Monolayers†

Ilya Levental,[‡,§] David A. Christian,[‡,∥] Yu-Hsiu Wang,[⊥] Jonathan J. Madara,[⊥] Dennis E. Discher,[§,∥,£,#] and Paul A. Janmey*[,§,#,¶]

[§]*Department of Bioengineering and* [∥]*Department of Chemical Engineering and* [⊥]*Department of Chemistry and* [£]*The Biophysical Engineering Laboratory and* [#]*Department of Physics and* [¶]*Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104.* [‡]*These authors contributed equally to this work.*

Received May 7, 2009; Revised Manuscript Received July 24, 2009

ABSTRACT: Biological membrane function, in part, depends upon the local regulation of lipid composition. The spatial heterogeneity of membrane lipids has been extensively explored in the context of cholesterol and phospholipid acyl-chain-dependent domain formation, but the effects of lipid head groups and soluble factors in lateral lipid organization are less clear. In this contribution, the effects of divalent calcium ions on domain formation in monolayers containing phosphatidylinositol 4,5-bisphosphate (PIP2), a polyanionic, multifunctional lipid of the cytosolic leaflet of the plasma bilayer, are reported. In binary monolayers of PIP2 mixed with zwitterionic lipids, calcium induced a rapid, PIP2-dependent surface pressure drop, with the concomitant formation of laterally segregated, PIP2-rich domains. The effect was dependent upon head-group multivalency, because lowered pH suppressed the surface-pressure effect and domain formation. In accordance with previous observations, inclusion of cholesterol in lipid mixtures induced coexistence of two liquid phases. Phase separation strongly segregated PIP2 to the cholesterol-poor phase, suggesting a role for cholesterol-dependent lipid demixing in regulating PIP2 localization and local concentration. Similar to binary mixtures, subphase calcium induced contraction of ternary cholesterol-containing monolayers; however, in these mixtures, calcium induced an unexpected, PIP2- and multivalency-dependent decrease in the miscibility phase transition surface pressure, resulting in rapid dissolution of the domains. This result emphasizes the likely critical role of subphase factors and lipid head-group specificity in the formation and stability of cholesterol-dependent domains in cellular plasma membranes.

Membrane lipids, especially polyphosphoinositides, such as phosphatidylinositol 4,5-bisphosphate (PIP2),[1] are important effectors in many cellular processes, including apoptosis (*1*), inflammation (*2*), motility (*3*), and proliferation (*4*, *5*). In addition to the concentration and functionality of individual lipids, the spatial organization of lipids affects how they interact with proteins at the membrane/cytosol interface. The effects of acyl chain structure and cholesterol content (*6*, *7*) have been extensively studied as determinants of lateral organization in the context of the cholesterol-dependent formation of coexisting liquid phases. Head-group-mediated lipid interactions and the influence of cytosolic factors have been less thoroughly investigated, but they may be important for lipids with unusually large electrostatic charge, such as polyphosphoinositides (*8*).

Many eukaryotic cell proteins involved in cytoskeletal remodeling (*3*, *9*), membrane trafficking (*10*, *11*), transmembrane permeability (*12*, *13*), and mitogenesis (*14*, *15*) are regulated *in vitro* by PIP2, a highly anionic lipid distributed largely in the inner leaflet of the plasma membrane and within the nucleus. When PIP2-regulated proteins were first reported (*16*), a plausible hypothesis was that these proteins function in a manner analogous to the specific binding of a soluble regulator to a protein active site. However, the list of PIP2-regulated proteins of various functions has risen to the point that the cellular concentration of PIP2 ligands is greater than the cellular concentration of PIP2 (*3*, *17*). Also inconsistent with a simple mass action binding equilibrium between PIP2 and its protein ligands is the fact that cellular PIP2 distribution or turnover changes much more than total PIP2 levels (*18*, *19*) during activations of cells, where various PIP2-regulated processes are triggered. These and other findings suggest that lipid distribution and lateral organization rather than global concentration determine where and when the lipid binds its target protein.

A potentially important feature of PIP2 signaling is the varying functionality of PIP2 within regions of the membrane with different lateral organization, i.e., different roles for single, freely diffusing PIP2 molecules, small transient clusters of PIP2, and large stable PIP2 aggregates. A central tenet of this hypothesis is that multiple pools of PIP2 are spatially, structurally, and functionally distinct but rapidly convert between the different organization states depending upon membrane proximal factors and the local lipid environment. Evidence for this hypothesis

†The authors acknowledge funding from the National Science Foundation−Materials Research Science and Engineering Center (NSF−MRSEC) Grant 05-20020 and the National Institutes of Health (NIH) Grant R01HL067286.

*To whom correspondence should be addressed: 1010 Vagelos Laboratories, 3340 Smith Walk, Philadelphia, PA, 19104. Telephone: 215-573-7380. Fax: 215-573-7227. E-mail: janmey@mail.med.upenn.edu.

[1]Abbreviations: PIP2, phosphatidylinositol 4,5-bisphosphate; SOPC, 1-stearoyl-2-oleoyl phosphatidylcholine; PL, phospholipid; Chol, cholesterol.

Published on Web 07/24/2009

includes the discovery of fractions of PIP2 in the plasma membrane that are inaccessible to a PIP2-binding PH domain (*20*), as well as differential turnover of labeled PIP2 probes (*19*). Consistent with the existence of spatially segregated pools of PIP2, immunofluorescence microscopy studies of various PIP2-binding domains (*21*, *22*) and antibodies (*23*, *24*) have confirmed the non-homogeneous distribution of PIP2 in the plasma membrane. A protein-based mechanism for PIP2 oligomerization has been proposed, suggesting that PIP2 lateral heterogeneity is a function of electrostatic interactions between several neighboring lipids and an unstructured polybasic protein domain, such as found in the MARCKS protein (*21*, *25*−*27*) . In this model, the protein is required for PIP2 clustering and serves to prevent the interaction of PIP2 with other potential protein targets until the sequestering proteins dissociate from PIP2 as a result of signal-dependent post-translation modification, such as phosphorylation by protein kinase C (*28*).

An alternative or complementary mechanism for the formation of lateral PIP2 aggregates in lamellar membranes is suggested by evidence that PIP2 head groups, unlike nearly all other phospholipids (PLs), can form hydrogen-bonded networks (*29*, *30*). Additionally, it has been suggested that divalent cations can not only reduce the electrostatic repulsion between the anionic PIP2 head groups but also act as bridges between two adjacent lipids (*29*), as recently observed in model liposomes, where macromolecular aggregates of PIP2 were induced by both Ca^{2+} and Mg^{2+} (*48*). Macroscopic polycation-mediated domain formation has been observed in monolayers containing synthetic anionic lipids (*31*, *32*), but these observations have been made with singly charged lipids that contain two saturated acyl chains (DPPS/DPPA), both factors that would be expected to affect lateral organization.

In the present study, a lipid monolayer system is used to investigate the formation and electrostatic mechanisms underlying calcium-induced PIP2 domains and the effect of PIP2 clustering on phase coexistence in cholesterol-containing lipid mixtures. PIP2 clustering is shown to depend upon the multivalency of the counterion, as well as the high charge density of the lipid head group. Additionally, calcium induces a significant reduction in the phase coexistence surface pressure in PIP2-containing monolayers. These effects demonstrate that lateral organization of lipids can be modulated by cytosolic factors on several length scales and suggest that cellular control of concentrations of ions, such as Ca^{2+}, can be used to remodel lipid organization and thereby impact multiple cell-signaling pathways.

EXPERIMENTAL PROCEDURES

Reagents. Bovine liver L-α-phosphatidylinositol-4,5-bisphosphate (PIP2), cholesterol, 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC), 1-stearoyl-2-oleoyl phosphatidylserine (SOPS), 1-oleoyl-2-NBD−phosphatidylserine, and 1,2-dioleoyl-3-phosphoethanolamine-*N*-lissamine rhodamine B sulfonyl from Avanti (Alabaster, AL) and long-chain analogues of PIP2 (Bodipy FL−PIP2 and NBD−PIP2) from Echelon, Inc. (Salt Lake City, UT) were obtained as organic solutions and stored at −20 °C. The concentrations of the lipid solutions were confirmed initially with phosphate analysis following acid digestion of organic components (*33*) and, subsequently, by comparing to the measured area per lipid molecule. Subphase reagents [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), $CaCl_2$, and NaCl] were purchased from Sigma (St. Louis, MO).

Monolayer Visualization and Manipulation. A total of 30 mL of subphase solution (10 mM HEPES and 1 μM EDTA at pH 7.5 in 18.2 MΩ ddH_2O) were added to a MicroTroughX Langmuir trough (Kibron, Inc., Helsinki, Finland). For varying pH experiments, the buffer was 3.3 mM sodium phosphate, 3.3 mM sodium citrate, and 3.3 mM glycine. To avoid oxidation-induced effects, 5 mM dithiothreitol (DTT) was added to the subphase for the fluorescence visualization of cholesterol-containing monolayers. No effect of the various buffers at the same pH and ionic strength was observed. Approximately 7 nmol of premixed lipid solutions were deposited slowly on the subphase surface, and the lipids were compressed at 15 $Å^2$ $molecule^{-1}$ min^{-1} to the desired surface pressure by the barriers of the trough using a microstepping motor. The monolayer surface pressure was monitored with a surface probe using the Wilhelmy method (*33*) and the FilmWare software package (Kibron).

Subphase calcium was introduced after the monolayers were compressed to 20 mN/m (unless otherwise noted) by injection from behind one of the barriers (i.e., without disturbing the monolayer) with a gel-loading pipet tip (Sigma). The subphase was then mixed by withdrawing and expelling 300 μL of the subphase from behind each barrier 5×. Uniform mixing was verified using a soluble dye, and control experiments showed no lasting effect of mixing on the resultant surface pressure (fluctuations because of subphase movement during mixing are observed as spikes in Figure 3B).

Monolayers were imaged by epifluorescence microscopy using an inverted microscope (Leica Microsystems, Wetzlar, Germany) with the appropriate filter sets.

PIP2 Binding Markers. cDNA of a GST chimera of the PH domain from rhPLCδ1 (gift from Dr. Tobias Baumgart) was expressed in XL1-blue *Escherichia coli* and purified on glutathione-functionalized Sepharose beads using the provided protocol (Sigma). The purified GST-PH was fluorescently labeled by a reaction with fluorescein isothiocyanate (FITC) for 30 min at room temperature. The reaction time was carefully controlled because FITC reacts with solvent-accessible primary amines on the surface of proteins, typically lysines and arginines, the same residues known to make up the PIP2 binding pocket of PH-PLC (*34*). For this reason, the labeling ratio of the FITC/PH domain was minimized to maintain PIP2-binding activity and measured by optical absorbance of the protein and label to be ∼1:1. The PIP2-binding peptide (PBP10) based on the sequence of the PIP2 binding site of the actin-binding protein gelsolin functionalized with a rhodamine B fluorescent group was synthesized previously (*35*).

RESULTS

The effects of calcium ions added to the subphase of preformed unsaturated PL monolayers containing PIP2 were measured using a Langmuir film balance technique and epifluorescence microscopy. The lateral organization of PIP2-containing monolayers was visualized by the inclusion of an acyl-chain-labeled fluorescent PIP2 analogue (1% NBD−PIP2). The addition of Ca^{2+} underneath a PIP2-containing monolayer induced the formation of small, bright domains enriched in fluorescent PIP2 (Figure 1A) that persisted to physiological values of surface pressure ($\pi \sim 32$ mN/m). Coincident with domain formation, a drop in monolayer surface pressure (Π) was observed, with the magnitude of ΔΠ upon calcium addition directly related to PIP2 fraction (Figure 1B). The linear plot ($R^2 = 0.995$) of ΔΠ and [PIP2] passes through the origin, suggesting that the observed

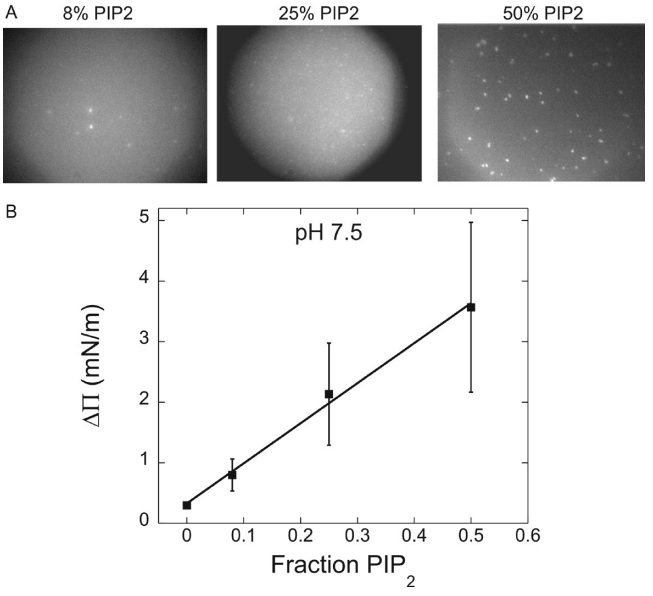

FIGURE 1: Calcium-induced change in surface pressure and domain formation linearly depends upon the PIP2 fraction. (A) Formation of calcium-induced PIP_2 domains (visualized by the inclusion of 0.5% Bodipy-PIP_2) is persistent from 8 to 50 mol % PIP_2, with the abundance of bright domains directly related to the PIP2 fraction. While the change in Π is rapid for all compositions (faster than mixing), the time required for PIP_2 domains to become microscopically visible at 8% PIP_2 is significantly longer than at 25 or 50% and likely reflects diffusion limitations. (B) Decrease in Π upon the addition of 1 mM Ca^{2+} is proportional ($R^2 = 0.995$) to the fraction of PIP_2 in the lipid monolayer (balance lipid is SOPC).

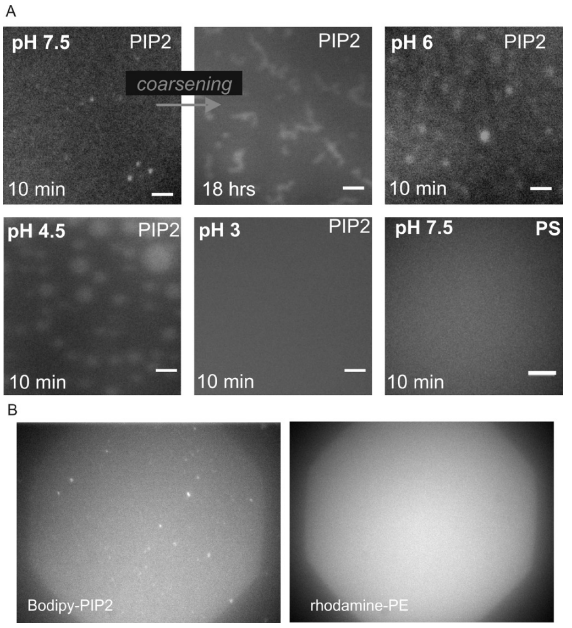

FIGURE 2: Divalent calcium induces microscopic PIP_2 domains in mixed lipid monolayers. (A) Fluorescence micrographs of SOPC/PIP2 (3:1) monolayers with 1% NBD−PIP2 or SOPC/SOPS (3:1) with 1% NBD−PS on buffered subphases at varying pH. The addition of 1 mM Ca^{2+} to the subphase induced microscopically visible PIP2 domains within minutes at conditions where PIP2 is multivalent (pH 7.5, 6, and 4.5), whereas no monolayer heterogeneity was observed at conditions under which only monovalent and zwitterionic lipids were present (PIP2 at pH 3 and PS at pH 7.5). Although the formation of domains was rapid (< 10 min), PIP2 domains at pH 7.5 coarsened to form micrometer-sized ribbon-like domains after many hours. Scale bars are 10 μm. All charged lipids added at 25 mol %. (B) To ensure that the domains observed were PIP2-enriched and not membrane defects, monolayers were also formed with 0.1% of the membrane marker, rho-PE. At conditions that result in the formation of PIP2 domains (B, left), epifluorescence micrographs of rho-PE show the monolayer to be homogeneous (B, right) and, thus, free of artifacts resulting from fluorescent lipids or monolayer collapse.

effects are present even at the small PIP2 mole fractions that would occur in cell membranes assuming random distribution of all lipids. Similarly, the abundance of bright domains increased significantly with an increasing PIP2 fraction (Figure 1A), with this effect also persisting down to lower PIP2 concentrations that are physiologically relevant.

Calcium-Induced PIP2-Enriched Domains. At pH 7.5, the domains appeared quickly (< 10 min) as abundant, sub-micrometer bright spots on a dark background and coarsened into micrometer-scale ribbons after several hours. Other shapes, from larger dots to several micrometer circles, were observed as a function of pH (Figure 2A). Once formed, PIP2-enriched domains persisted through a significant range of monolayer surface pressures (Π = 20−40 mN/m) and for the entire duration of experiments (up to 24 h).

Domains were only observed under conditions at which multivalent lipids were present in the monolayer (PIP2 at pH 7.5, 6, and 4.5). PIP2-containing monolayers at low pH (3.0) and monolayers where PIP2 was replaced by the monoanionic lipid SOPS both showed small changes in surface pressure upon calcium addition (Figure 3) but were fluorescently homogeneous (Figure 2A).

To ensure that the bright domains observed following calcium injection were PIP2-enriched membrane structures as opposed to membrane defects, surface contaminants, or dye-dependent artifacts, a different fluorescent PIP2 analogue (BodipyFL−PIP2) was visualized in parallel with a nonspecific membrane dye [rhodamine-labeled SOPE (rho-SOPE)]. The distribution of BodipyFL−PIP2 mirrored the punctate domains of NBD−PIP2, whereas no lateral heterogeneity was observed in the distribution of rho-SOPE (Figure 2B), confirming that the domains were indeed PIP2-enriched lateral domains.

Calcium-Induced PIP2-Dependent Pressure Drop. Concomitant with the observed domains, mixed monolayers of SOPC/PIP2 (3:1) show reduced surface pressures across a large range of molecular areas in the presence of subphase divalent cations (Figure 3A). This effect is divalent-cation-specific, because a much greater reduction was observed with Ca^{2+} as compared to Mg^{2+}. Similarly, injection of 1 mM $CaCl_2$ to the buffered subphase (pH 7.5) underneath monolayers of the same composition induced an immediate and substantial contraction of the monolayer, evidenced by ∼25% reduction in surface pressure (Π) (top panel in Figure 3B). The time scale of the observed surface pressure decrease was not measurable in our experiments but was shorter than the ∼10 s required for Ca^{2+} introduction and mixing. This contraction was reversible, because chelation of Ca^{2+} by excess EDTA led to an equivalent increase in Π (bottom panel in Figure 3B; spikes indicate transient changes in subphase volume induced by mixing). The concentration dependence of the calcium-induced monolayer contraction at pH 7.5 (Figure 3C, ■) showed that the effect persisted to 1 μM Ca^{2+} and could be fit by a saturable binding model with a dissociation constant of $K_d \sim 3$ μM. A similar magnitude of monolayer contraction and formation of domains were observed at increased salt concentrations (150 mM NaCl), although the calcium level required was ∼10-fold higher (data

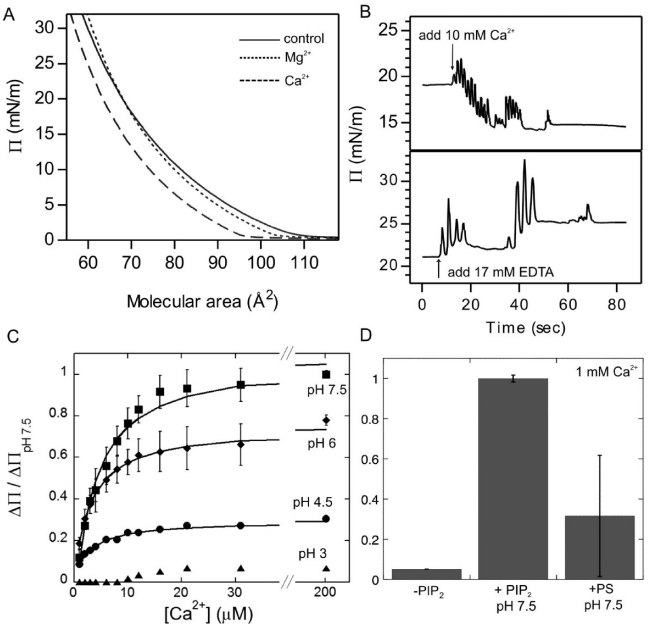

FIGURE 3: Subphase calcium induces an immediate, reversible, pH-dependent surface pressure decrease in PIP2-containing monolayers. (A) Π−molecular area isotherms of SOPC/PIP_2 (3:1) monolayers show reduced surface pressures in the presence of divalent cations, with greater reductions with Ca^{2+} compared to Mg^{2+}. (B) Surface pressure (Π) of these monolayers decreases quickly and substantially upon the addition of $CaCl_2$ to the pH 7.5 buffered subphase. The time scale (~10 s) for the pressure change reflects introduction and mixing of the subphase and is therefore an upper limit on the time required for Ca^{2+}-mediated contraction (A, top). The addition of excess EDTA to the subphase results in an immediate increase in surface pressure equal in magnitude to the calcium-induced decrease (A, bottom). (C) Change in surface pressure (ΔΠ normalized to ΔΠ with 1 mM Ca^{2+} at pH 7.5) of a SOPC/PIP2 monolayer as a function of the Ca^{2+} concentration can be modeled by first-order reversible binding with a dissociation constant (K_d) independent of subphase pH. Error bars at pH 7.5 and 6 show the typical standard deviation of three separate experiments. (D) Quantification of the total decrease in monolayer surface pressure after 1 mM Ca^{2+} addition for monolayers of varying composition and subphase pH. Monolayers containing charged lipids show a significant decrease in surface pressure in the presence of divalent cations that is strongly dependent upon total surface charge.

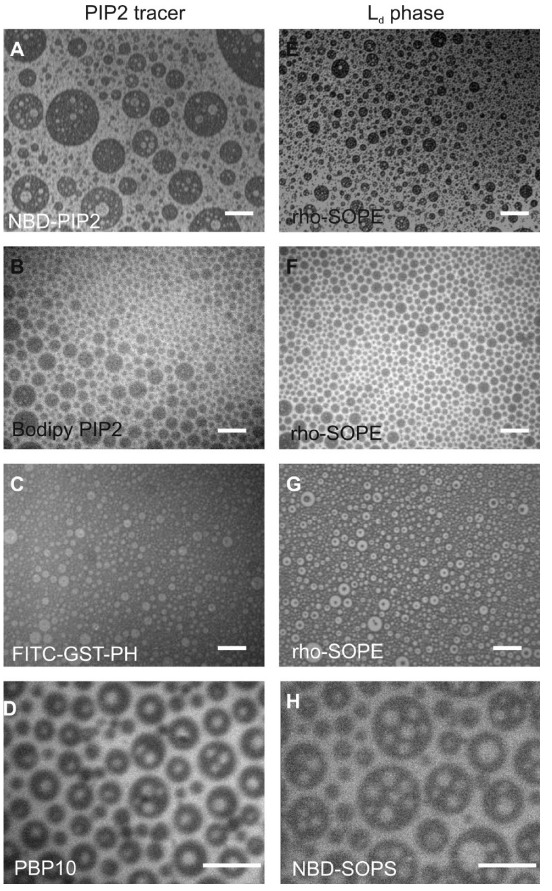

FIGURE 4: PIP2 co-localizes with PL-rich phase markers in fluid−fluid coexistence monolayers. Monolayers of PIP_2, cholesterol, and SOPC costained with PIP_2 markers (A−D) against markers of the PL-rich phase (E−H). Co-localization of PIP_2 and markers indicates the exclusion of PIP_2 from cholesterol-rich domains at Π below $Π_T$. Compositions were (A/E) DChol/SOPC/PIP2 (50:40:10) + 1% NBD−PIP2 + 0.1% rho-SOPE, (B/F) DChol/SOPC/PIP2 (50:40:10) + 0.5% BodipyFL−PIP2 + 0.1% rho-SOPE, (C/G) DChol/SOPC/PIP2 (50:40:10) + 0.1% rho-SOPE, and (D/H) DChol/SOPC/PIP2 (50:40:10) + 1% NBD−PC. Scale bars are 10 μm.

not shown) likely because of the screening of electrostatic interactions by subphase counterions.

Whereas the magnitude of the contraction was dependent upon the protonation state of the PIP2 in the monolayer (as controlled by subphase pH), the dissociation constant was nearly pH-independent (Figure 3C). The exception to this observation was at pH 3.0, where PIP2 would be expected to be monovalent (8), and no significant change in surface pressure was observed upon the addition of Ca^{2+} up to 1 mM (Figure 3C, ▲). Similarly, no change in surface pressure occurred when calcium was added to monolayers, including the monovalent lipid, SOPS, or without charged lipid (Figure 3D).

PIP2-Mediated Calcium Effects in Cholesterol-Containing Monolayers. Inclusion of cholesterol in PL monolayers results in large circular domains indicative of fluid−fluid phase coexistence that can be readily visualized by inclusion of a small concentration of a tracer lipid with a non-unity phase partition coefficient (e.g., Figure 4E; tracer lipid is 0.1% rho-SOPE). To determine the phase partitioning of PIP2 in these monolayers, the localization of a variety of PIP2 tracers was assayed with respect to PL-rich phase markers (rho-SOPE and NBD−PC). The different tracers were chosen to control for potential artifacts of each label. A comparison of fluorescent images of two distinct PIP2 analogues (NBD−PIP2, Figure 4A; BodipyFL−PIP2, Figure 4B), a fluorescently labeled PIP2 binding protein domain (FITC−PLCδ1, Figure 4C), and a rhodamine-labeled PIP2-binding peptide corresponding to the PIP2 binding domain of gelsolin (PBP10; Figure 4D) with known PL-rich phase markers (rho-SOPE and NBD−PC, panels E−H of Figure 4) clearly demonstrate the preferential partitioning of PIP2 into the Chol-poor phase of these biphasic monolayers.

In addition to the PIP2-segregating effect of cholesterol described above, PIP2 induced a change in the miscibility transition pressure of cholesterol-containing monolayers that was strongly dependent upon the subphase calcium concentration. As shown in Figure 5A and extensive previous work (*6*, *36*), cholesterol-containing PL monolayers undergo a surface-pressure-dependent transition from coexisting fluid phases at low pressure to a single homogeneous phase at a composition-dependent miscibility transition pressure ($Π_t$). Although $Π_t$ has been previously demonstrated to be strongly dependent upon the cholesterol mole fraction (*7*), PL acyl chain composition, and physical parameters, such as temperature and applied electric

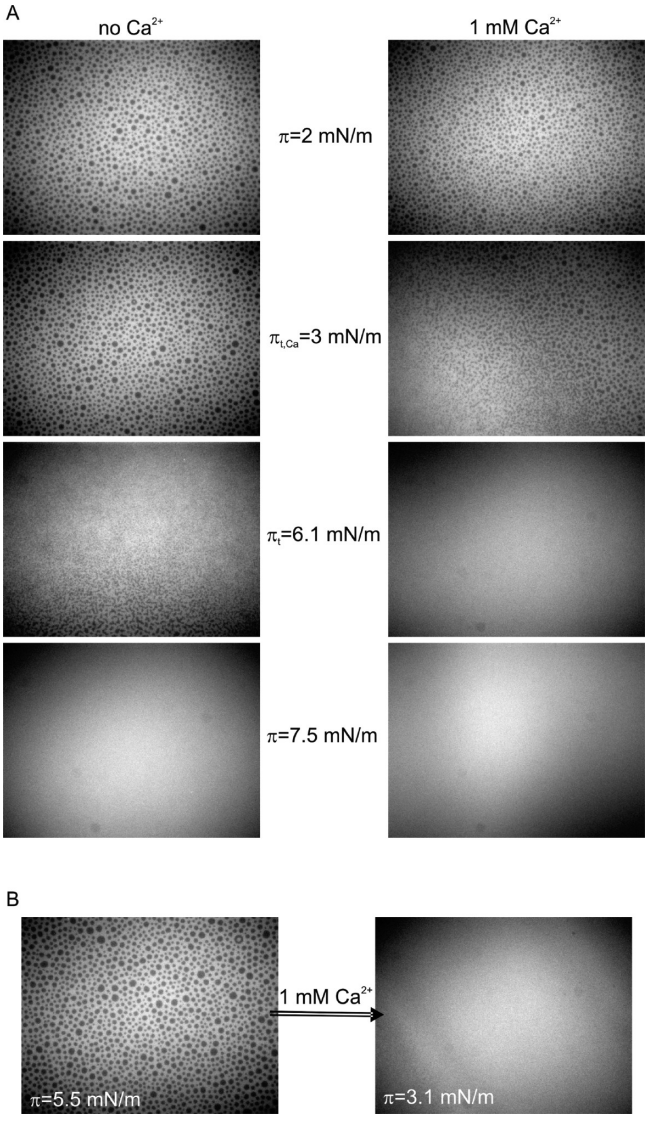

FIGURE 5: Subphase calcium decreases monolayer Π_T in cholesterol-containing monolayers. (A) Fluorescence micrographs of SOPC/PIP2/DChol (40:25:35) monolayers, which form PL-rich (bright) and Chol-rich (dark) domains at $\Pi < \Pi_T$ (visualized by the inclusion of 0.1% Rho-SOPE). As Π is increased through Π_T, the monolayer becomes mixed and homogeneously fluorescent. The addition of 1 mM Ca^{2+} to the subphase results in a decrease in Π_T from Π_T = 6.1 mN/m to $\Pi_{T,Ca}$ = 3.0 mN/m, as shown in the two series of panels. (B) Effect of calcium on Π_T was tested on demixed monolayers, where $\Pi < \Pi_T$. Ca^{2+} (1 mM) added to the subphase resulted in a rapid decrease in Π and mixing of the monolayer as $\Pi > \Pi_{T,Ca}$.

fields (*37*), there has been little evidence for a role of subphase factors in modulating the stability of fluid-phase coexistence. Figure 5 shows that the addition of 1 mM Ca^{2+} lowers the Π_t of a DChol/PIP2/SOPC (1.4:1:1.6) monolayer from 6.1 to 3.0 mN/m. The same effect was demonstrated by injecting Ca^{2+} underneath a preformed biphasic monolayer, which led to dissolution of domains, concomitant with the expected pressure drop for PIP2-containing monolayers described in Figures 1 and 3 (Figure 5B). Although the pressure drop and dissolution of domains were simultaneous, they are not causative, because a decrease in pressure would be expected to stabilize phase coexistence (*38*), as opposed to the observed monolayer homogenization.

Recalling the multivalency requirement of the calcium-induced pressure drop and PIP2-domain formation described in

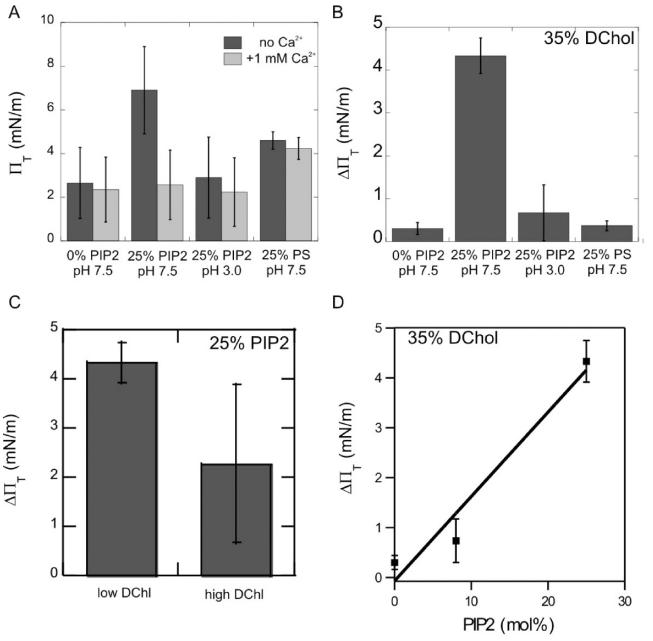

FIGURE 6: Presence of subphase Ca^{2+} affects Π_T only in monolayers containing highly charged lipids. (A) Π_T for 35% cholesterol monolayers increases significantly (p = 0.046) with the incorporation of PIP_2 at pH 7.5 in the absence of Ca^{2+} (dark bars); the effect is counteracted by the presence of 1 mM Ca^{2+} (light gray bars). This increase is dependent upon the multivalent nature of PIP2 (p < 0.001), because no significant increase in Π_T was observed with PIP2 at pH 3 (where the net charge of PIP_2 is approximately −1; p = 0.5) or with incorporation of PS (net charge −1; p = 0.6). (B) Calcium-induced decrease of Π_T ($\Delta\Pi_T = \Pi_T - \Pi_{T,Ca}$) strongly depends upon the multivalency of PIP2 at pH 7.5, where $\Delta\Pi_T = 4.3 \pm 0.4$ mN/m. For monolayers with only net neutral lipids or those with monoanionic lipids (PIP2 at pH 3 or PS at pH 7.5), the addition of Ca^{2+} induced only a small $\Delta\Pi_T$. (C) For monolayers of constant PIP2 fraction, the effect of calcium addition on Π_T is weakly dependent upon the amount of cholesterol (p = 0.1). (D) For monolayers of 35% DChol, $\Delta\Pi_T$ upon the addition of calcium linearly (R^2 = 0.952) depends upon the amount of PIP2 incorporated into the monolayer. All error bars shown are standard deviations from three separate experiments. All lipid mixtures included 0.1% rho-SOPE for fluorescent visualization, and the balance of the lipid mixture was SOPC. All charged lipids were included at 25 mol % unless otherwise noted.

Figures 1−3, the calcium-induced transition pressure effect was also dependent upon both the presence and multivalency of PIP2 in the monolayer. When no PIP2 was present in the monolayer, essentially no effect of subphase calcium was observed on 35% DChol monolayers (panels A and B of Figure 6). Inclusion of PIP2 led to a >60% decrease of Π_t (from 6.8 to 2.4 mN/m) upon the addition of 1 mM calcium (panels A and B of Figure 6). Π_t in the presence of PIP2 (without calcium) was significantly higher than that of a binary SOPC/DChol mixture (Figure 6A), and the addition of Ca^{2+} restored Π_t to the PIP2-free level. The presence of monovalent anionic lipids (PIP2 at pH 3 or SOPS at pH 7.5) had no significant effect on either the calcium-free Π_t compared to PC/DChol monolayers or the $\Delta\Pi_t$ ($\Delta\Pi_t = \Pi_t - \Pi_{t,Ca}$) following Ca^{2+} addition. Finally, the dependence of these phenomena on the cholesterol and PIP2 fractions was evaluated. The Ca^{2+}-induced decrease in Π_t was not greatly affected by varying [DChol] from 35 to 50% (Figure 6C), but varying the amount of PIP2 in the monolayer had a significant effect. $\Delta\Pi_t$ was directly related to [PIP2], with the effect persisting down to <10% PIP2, and the linear relationship (R^2 = 0.952) and zero intercept imply that the observed effects are present at

PIP2 concentrations too low to measure with this method (Figure 6D).

DISCUSSION

The data presented here demonstrate the multiple and varied effects of subphase calcium on PIP2-containing phospholipid monolayers. These effects include significant calcium-induced condensation of PIP2/SOPC monolayers, as evidenced by the reduction of monolayer surface pressure and the concomitant formation of sub-micrometer- to micrometer-scale PIP2-enriched domains. Experiments with phase-separated cholesterol-containing monolayers showed PIP2 to be strongly enriched in the Chol-depleted phase, in which the addition of subphase calcium induced a significant reduction of the miscibility transition surface pressure. These effects suggest a role for changes in membrane structure as a possible effector of calcium signaling in biological systems.

The reduction of surface pressure of anionic monolayers by subphase multivalent cations has been previously reported (*39*, *40*) and is consistent with the substantial PIP2-dependent decreases in surface pressure upon injection of Ca^{2+} beneath preformed monolayers of polyunsaturated PIP2 and SOPC (Figures 1 and 3). The magnitude of the decrease in these mixed monolayers was roughly proportional to the calcium-induced contraction observed for pure PIP2 monolayers (*29*) and was attributed to aggregation (*41*), neutralization (*42*), and dehydration (*43*) of the inositol head groups by direct electrostatic interaction between the cations and the anionic phosphomonoesters of PIP2 (*42*). Ca^{2+} had a much stronger condensing effect than Mg^{2+} (Figure 3A), suggesting a specific affinity between the bisphosphorylated head group of PIP2 and Ca^{2+}. Although the magnitude of monolayer contraction was strongly dependent upon subphase pH (and, subsequently, PIP2 charge), we measured a pH-independent binding affinity ($K_d \sim 3\ \mu M$; Figure 3C) of the calcium ions to the monolayers, suggesting surface-charge-independent binding between calcium and PIP2. Both of these observations correspond well to previous measurements of calcium binding to anionic phospholipids and bacterial polysaccharides in monolayers (*44*).

Both ion and lipid head-group multivalency is required for the observed monolayer contraction. Neither monolayers of pure zwitterionic PC nor those containing 25% of the monovalent lipid PS showed any significant decrease of surface pressure upon the addition of calcium, and the addition of monovalent cations did not induce contraction of PIP2-containing monolayers, as previously shown (*29*). Similarly, monolayers containing PIP2 showed no effect of Ca^{2+} on surface pressure at low pH, where PIP2 would not be expected to be polyvalent (although experimental pK_a values for PIP2 below pH 4.0 are unavailable, they have been estimated from PA) (*8*). These observations are consistent with a recent analysis showing that monolayers containing monovalent lipids have charge spacing that is insufficient to induce significant surface-pressure effects (*8*) as well as previous experiments showing that the binding of basic peptides to membranes is dependent upon the presence of polyvalent lipids (*45*).

While the interactions between divalent cations and anionic lipids have been investigated, the effect of calcium on PL organization in planar systems has not been clearly elucidated. Previous studies with phospholipid monolayers have indicated that the addition of calcium results in the formation of domains enriched in anionic lipid (*40*), but only recently has phase separation been microscopically visualized (*32*, *46*, *47*). Older results describe a mechanism for electrostatic-induced PL reorganization, but recent studies have opened new questions concerning the role of surface pressure (*46*). Here, it is shown that polyanionic domains of unsaturated PIP2 can be induced with micromolar calcium concentrations and maintained through a large range of surface pressures. This result is confirmed by recent observations of PIP2-rich domains in GUVs (*48*) and, coupled with the multivalent requirement for both the lipid and cation species, suggests that the mechanism for domain formation was not dependent upon acyl chain packing/ordering but rather electrostatic interactions between the cation and lipid head group.

The evidence that calcium can alter both the molecular density and the lateral organization of PIP2- and cholesterol-containing membranes suggests a role for membrane structure as a possible effector of calcium signaling. It has been previously shown that the interactions between PIP2 and unstructured polybasic protein domains (such as those of MARCKS and GAP-43) can sequester PIP2 and that those interactions are modulated by Ca^{2+}/calmodulin (*49*). One implication of the results presented here is that, in addition to releasing PIP2 from its polybasic "sinks", calcium ion flux would induce a rearrangement of the membrane and may itself sequester PIP2 in stable, multimolecular aggregates.

Both the change in monolayer surface pressure and the abundance of PIP2-enriched domains were functions of the molar fraction of PIP2, while the small and punctate appearance of the domains was composition-independent (Figure 1). Notably, PIP2 domains were observed down to 8 mol %, which, while still significantly greater than the 1% typically estimated for the unstimulated plasma membrane concentration of PIP2 (*50*), suggests that calcium-induced PIP2 clustering may be important for the low PIP2 concentrations typically observed in cellular plasma membranes.

One possibility for modulation of local PIP2 concentration/functionality in the physiological context of the plasma membrane is by physical exclusion of this lipid from cholesterol-enriched membrane microdomains, e.g., lipid rafts. Such domains are associated with phase separation into coexisting fluid phases (*6*, *7*) and segregate specific lipid components (*51*, *52*) . We observed cholesterol-dependent liquid−liquid coexistence in mixed monolayers of PIP2, SOPC, and cholesterol and found that PIP2 partitions strongly into domains enriched in PL-rich phase markers (Figure 4). Although not surprising in light of the highly unsaturated acyl chains and head-group charge of PIP2, this finding is in contrast to the enrichment of PIP2 in detergent-resistant membranes (*22*), which has led to the suggestion that PIP2 may have a preference for the more ordered environment of lipid rafts. This discrepancy might be explained by the binding of PIP2 to the GMC family of proteins (GAP43, MARCKS, and CAP23), which have been proposed to localize to lipid rafts (*21*).

In addition to its condensing and domain-inducing effect on PIP2/PC monolayers, divalent calcium also lowered the miscibility transition pressure of cholesterol-containing phase-separated monolayers (Figures 5 and 6). Liquid−liquid phase coexistence in cholesterol-containing monolayers depends upon surface pressure, and PIP2-containing monolayers had higher average miscibility transition pressures (Π_t) than SOPC/cholesterol monolayers, with the effect persisting to less than 10 mol % PIP2. The PIP2-dependent increase of Π_t was attributed to a disordering effect of the polyunsaturated acyl chains and large

head group of PIP2 on the PIP2-rich disordered phase (Figure 5). Strikingly, subphase calcium completely abrogated the increased Π_t in PIP2-containing monolayers, lowering the transition surface pressure by more than 50%. This reduction of Π_t led to dissolution of domains following the introduction of subphase calcium, which was an unexpected result because the decrease in monolayer surface pressure observed after the addition of calcium would be expected to stabilize domain coexistence. The reduction of Π_t was dependent upon lipid multivalency, because neither PS-containing monolayers nor those with PIP2 at low pH showed any change in Π_t with subphase calcium (Figure 6B). These findings suggest that the electrostatic sequestration and condensation of PIP2 by divalent calcium result in tight association of PIP2 molecules, increasing the molecular packing and ordering the more disordered phase, such that phase miscibility is achieved at lower surface pressures.

Although there has been significant investigation into the dependence of miscibility transition pressure on membrane composition (*6*, *7*, *36*), to our knowledge, this is the first demonstration of the modulation of fluid−fluid phase miscibility by strictly soluble subphase factors. This result suggests a role for lipid-interacting small molecules (e.g., metal ions, polyamines, charged or amphiphilic peptides) in regulating the stability of fluid-phase coexistence, which has been postulated to be a principle involved in the formation of lipid rafts (*53*). More specifically, the significant calcium-dependent modulation of Π_t observed in our experiments implies a role for calcium signaling in the formation/stability of lipid rafts, which have been implicated in a variety of critical cellular processes (*54*).

CONCLUSION

The dependence of lateral lipid heterogeneity upon electrostatic interactions between charged lipid head groups and soluble factors plays an important biological role but has been relatively overlooked compared to the organizing effects of cholesterol, acyl chain composition, and temperature. Here, the lateral organization of the highly anionic lipid PIP2 was shown to be sensitive to small changes in calcium ion concentration. The introduction of subphase calcium induced the formation of PIP2-rich domains that were laterally segregated from net neutral lipids in binary monolayers. The formation of calcium-induced domains required that the lipid be multivalently anionic, because monovalent PIP2 (at pH 3) and PS did not form domains. There was no evidence that Ca^{2+}-induced domain formation required a critical concentration of membrane PIP2 that might be considered non-physiological, and the linear dependence of $\Delta\Pi$ and domain abundance on [PIP2] suggested that domains form even at mole fractions of PIP2, where their clustering would produce surface pressure changes too small to measure. In addition to altering the lateral organization of PIP2 in binary monolayers, divalent calcium increased the miscibility of coexisting domains in cholesterol-containing monolayers, in a PIP2-dependent fashion. This result may have biological relevance because it demonstrates the ability of soluble factors to induce lipid reorganization in the plane of the membrane, suggesting the possibility that calcium fluxes can affect the formation of lipid rafts in PIP2-containing plasma membranes.

REFERENCES

1. Esposti, M. D. (2002) Lipids, cardiolipin and apoptosis: A greasy license to kill. *Cell Death Differ*. *9*, 234–236.
2. Lawrence, T., Willoughby, D. A., and Gilroy, D. W. (2002) Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat. Rev. Immunol. 2*, 787–795.
3. Yin, H. L., and Janmey, P. A. (2003) Phosphoinositide regulation of the actin cytoskeleton. *Annu. Rev. Physiol. 65*, 761–789.
4. Eling, T. E., and Glasgow, W. C. (1994) Cellular proliferation and lipid metabolism: Importance of lipoxygenases in modulating epidermal growth factor-dependent mitogenesis. *Cancer Metastasis Rev. 13*, 397–410.
5. Czech, M. P. (2000) PIP2 and PIP3: Complex roles at the cell surface. *Cell 100*, 603–606.
6. McConnell, H. M., and Vrljic, M. (2003) Liquid−liquid immiscibility in membranes. *Annu. Rev. Biophys. Biomol. Struct. 32*, 469–492.
7. Keller, S. L., Anderson, T. G., and McConnell, H. M. (2000) Miscibility critical pressures in monolayers of ternary lipid mixtures. *Biophys. J. 79*, 2033–2042.
8. Levental, I., Janmey, P. A., and Cebers, A. (2008) Electrostatic contribution to the surface pressure of charged monolayers containing polyphosphoinositides. *Biophys. J. 95*, 1199–1205.
9. Lassing, I., and Lindberg, U. (1985) Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature 314*, 472–474.
10. Cremona, O., and De Camilli, P. (2001) Phosphoinositides in membrane traffic at the synapse. *J. Cell Sci. 114*, 1041–1052.
11. Martin, T. F. J. (2001) PI(4,5)P2 regulation of surface membrane traffic. *Curr. Opin. Cell Biol. 13*, 493–499.
12. Hilgemann, D. W., Feng, S., and Nasuhoglu, C. (2001) The complex and intriguing lives of PIP2 with ion channels and transporters. *Sci. STKE* No. RE19.
13. Berridge, M. J., and Irvine, R. F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature 312*, 315–321.
14. Ho, K. K., Anderson, A. A., Rosivatz, E., Lam, E. W., Woscholski, R., and Mann, D. J. (2008) Identification of cyclin A2 as the downstream effector of the nuclear phosphatidylinositol 4,5-bisphosphate signaling network. *J. Biol. Chem. 283*, 5477–5485.
15. Uno, I., Fukami, K., Kato, H., Takenawa, T., and Ishikawa, T. (1988) Essential role for phosphatidylinositol 4,5-bisphosphate in yeast cell proliferation. *Nature 333*, 188–190.
16. Janmey, P. A. (1995) Protein regulation by phosphatidylinositol lipids. *Chem. Biol. 2*, 61–65.
17. Catimel, B., Schieber, C., Condron, M., Patsiouras, H., Connolly, L., Catimel, J., Nice, E. C., Burgess, A. W., and Holmes, A. B. (2008) The PI(3,5)P2 and PI(4,5)P2 interactomes. *J. Proteome Res. 7*, 5295–5313.
18. Lassing, I., and Lindberg, U. (1988) Evidence that the phosphatidylinositol cycle is linked to cell motility. *Exp. Cell Res. 174*, 1–15.
19. Varnai, P., and Balla, T. (1998) Visualization of phosphoinositides that bind pleckstrin homology domains: Calcium- and agonist-induced dynamic changes and relationship to myo-[3*H*]inositol-labeled phosphoinositide pools. *J. Cell Biol. 143*, 501–510.
20. Balla, T., Bondeva, T., and Várnai, P. (2000) How accurately can we image inositol lipids in living cells?. *Trends Pharmacol. Sci. 21*, 238–241.
21. Laux, T., Fukami, K., Thelen, M., Golub, T., Frey, D., and Caroni, P. (2000) GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *J. Cell Biol. 149*, 1455–1472.
22. Pike, L. J., and Miller, J. M. (1998) Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. *J. Biol. Chem. 273*, 22298–222304.
23. Varnai, P., Lin, X., Lee, S. B., Tuymetova, G., Bondeva, T., Spat, A., Rhee, S. G., Hajnoczky, G., and Balla, T. (2002) Inositol lipid binding and membrane localization of isolated pleckstrin homology (PH) domains. Studies on the PH domains of phospholipase C delta 1 and p130. *J. Biol. Chem. 277*, 27412–27422.
24. Huang, S., Lifshitz, L., Patki-Kamath, V., Tuft, R., Fogarty, K., and Czech, M. P. (2004) Phosphatidylinositol-4,5-bisphosphate-rich plasma membrane patches organize active zones of endocytosis and ruffling in cultured adipocytes. *Mol. Cell. Biol. 24*, 9102–9123.
25. Wang, J., Gambhir, A., McLaughlin, S., and Murray, D. (2004) A computational model for the electrostatic sequestration of PI(4,5)P2 by membrane-adsorbed basic peptides. *Biophys. J. 86*, 1969–1986.
26. Zhang, W., Crocker, E., McLaughlin, S., and Smith, S. O. (2003) Binding of peptides with basic and aromatic residues to bilayer membranes: Phenylalanine in the myristoylated alanine-rich C kinase substrate effector domain penetrates into the hydrophobic core of the bilayer. *J. Biol. Chem. 278*, 21459–21466.
27. McLaughlin, S., Wang, J., Gambhir, A., and Murray, D. (2002) PIP(2) and proteins: Interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struct. 31*, 151–175.

28. Hartwig, J. H., Thelen, M., Resen, A., Janmey, P. A., Nairn, A. C., and Aderem, A. (1992) MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature 356*, 618–622.
29. Levental, I., Cebers, A., and Janmey, P. A. (2008) Combined electrostatics and hydrogen bonding determine intermolecular interactions between polyphosphoinositides. *J. Am. Chem. Soc. 130*, 9025–9030.
30. Redfern, D. A., and Gericke, A. (2005) pH-dependent domain formation in phosphatidylinositol polyphosphate/phosphatidylcholine mixed vesicles. *J. Lipid Res. 46*, 504–515.
31. Lamberson, E. R., Cambrea, L. R., and Hovis, J. S. (2007) Controlling the charge and organization of anionic lipid bilayers: Effect of monovalent and divalent ions. *J. Phys. Chem. B 111*, 13664–13667.
32. Ross, M., Steinem, C., Galla, H.-J., and Janshoff, A. (2001) Visualization of chemical and physical properties of calcium-induced domains in DPPC/DPPS Langmuir−Blodgett layers. *Langmuir 17*, 2437–2445.
33. Kates, M. (1986) Techniques of Lipidology , 2nd ed., Elsevier Science Publishers B.V., Amsterdam, The Netherlands.
34. Harlan, J. E., Yoon, H. S., Hajduk, P. J., and Fesik, S. W. (1995) Structural characterization of the interaction between a pleckstrin homology domain and phosphatidylinositol 4,5-bisphosphate. *Biochemistry* (*Moscow*) *34*, 9859–9864.
35. Cunningham, C. C., Vegners, R., Bucki, R., Funaki, M., Korde, N., Hartwig, J. H., Stossel, T. P., and Janmey, P. A. (2001) Cell permeant polyphosphoinositide-binding peptides that block cell motility and actin assembly. *J. Biol. Chem. 276*, 43390–43399.
36. Hagen, J. P., and McConnell, H. M. (1997) Liquid−liquid immiscibility in lipid monolayers. *Biochim. Biophys. Acta, Biomembranes 1329*, 7–11.
37. Radhakrishnan, A., and McConnell, H. M. (2000) Electric field effect on cholesterol−phospholipid complexes. *Proc. Natl. Acad. Sci. U.S. A. 97*, 1073–1078.
38. Heinrich, M. C., Levental, I., Gelman, H., Janmey, P. A., and Baumgart, T. (2008) Critical exponents for line tension and dipole density difference from lipid monolayer domain boundary fluctuations. *J. Phys. Chem. B 112*, 8063–8068.
39. Shah, D. O., and Schulman, J. H. (1965) Binding of metal ions to monolayers of lecithins, plasmalogen, cardiolipin, and dicetyl phosphate. *J. Lipid Res. 6*, 341–349.
40. Papahadjopoulos, D. (1968) Surface properties of acidic phospholipids: Interaction of monolayers and hydrated liquid crystals with uni- and bi-valent metal ions. *Biochim. Biophys. Acta 163*, 240–254.
41. Flanagan, L. A., Cunningham, C. C., Chen, J., Prestwich, G. D., Kosik, K. S., and Janmey, P. A. (1997) The structure of divalent cation-induced aggregates of PIP2 and their alteration by gelsolin and tau. *Biophys. J. 73*, 1440–1447.
42. Toner, M., Vaio, G., McLaughlin, A., and McLaughlin, S. (1988) Adsorption of cations to phosphatidylinositol 4,5-bisphosphate. *Biochemistry* (*Moscow*) *27*, 7435–7443.
43. Flach, C. R., Brauner, J. W., and Mendelsohn, R. (1993) Calcium ion interactions with insoluble phospholipid monolayer films at the A/W interface. External reflection−absorption IR studies. *Biophys. J. 65*, 1994–2001.
44. Hagge, S. O., Hammer, M. U., Wiese, A., Seydel, U., and Gutsmann, T. (2006) Calcium adsorption and displacement: Characterization of lipid monolayers and their interaction with membrane-active peptides/proteins. *BMC Biochem. 7*, 15.
45. Golebiewska, U., Gambhir, A., Hangyas-Mihalyne, G., Zaitseva, I., Radler, J., and McLaughlin, S. (2006) Membrane-bound basic peptides sequester multivalent (PIP2), but not monovalent (PS), acidic lipids. *Biophys. J. 91*, 588–599.
46. Sovago, M., Wurpel, G. W., Smits, M., Muller, M., and Bonn, M. (2007) Calcium-induced phospholipid ordering depends on surface pressure. *J. Am. Chem. Soc. 129*, 11079–11084.
47. Eklund, K. K., Vuorinen, J., Mikkola, J., Virtanen, J. A., and Kinnunen, P. K. (1988) Ca^{2+}-Induced lateral phase separation in phosphatidic acid/phosphatidylcholine monolayers as revealed by fluorescence microscopy. *Biochemistry* (*Moscow*) *27*, 3433–3437.
48. Carvalho, K., Ramos, L., Roy, C., and Picart, C. (2008) Giant unilamellar vesicles containing phosphatidylinositol(4,5)bisphosphate: Characterization and functionality. *Biophys. J. 95*, 4348–4360.
49. McLaughlin, S., and Murray, D. (2005) Plasma membrane phosphoinositide organization by protein electrostatics. *Nature 438*, 605–611.
50. Ferrell, J. E., Jr., and Huestis, W. H. (1984) Phosphoinositide metabolism and the morphology of human erythrocytes. *J. Cell Biol. 98*, 1992–1998.
51. Baumgart, T., Hunt, G., Farkas, E. R., Webb, W. W., and Feigenson, G. W. (2007) Fluorescence probe partitioning between Lo/Ld phases in lipid membranes. *Biochim. Biophys. Acta, Biomembr. 1768*, 2182–2194.
52. Dietrich, C., Volovyk, Z. N., Levi, M., Thompson, N. L., and Jacobson, K. (2001) Partitioning of Thy-1, GM1, and cross-linked phospholipid analogs into lipid rafts reconstituted in supported model membrane monolayers. *Proc. Natl. Acad. Sci. U.S.A. 98*, 10642–10647.
53. Simons, K., and Vaz, W. L. (2004) Model systems, lipid rafts, and cell membranes. *Annu. Rev. Biophys. Biomol. Struct. 33*, 269–295.
54. Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol. 1*, 31–39.