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Combined Electrostatics and Hydrogen Bonding Determine Intermolecular Interactions Between Polyphosphoinositides

Ilya Levental,[†] Andrejs Cēbers,[‡] and Paul A. Janmey*,[†]

Institute for Medicine and Engineering, Department of Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and Department of Physics, University of Latvia, Latvia

Received February 6, 2008; E-mail: janmey@mail.med.upenn.edu

Abstract: Membrane lipids are active contributors to cell function as key mediators in signaling pathways controlling cell functions including inflammation, apoptosis, migration, and proliferation. Recent work on multimolecular lipid structures suggests a critical role for lipid organization in regulating the function of both lipids and proteins. Of particular interest in this context are the polyphosphoinositides (PPI's), especially phosphatidylinositol (4,5) bisphosphate (PIP_2). The cellular functions of PIP_2 are numerous but the organization of PIP_2 in the inner leaflet of the plasma membrane, as well as the factors controlling targeting of PIP_2 to specific proteins, remains poorly understood. To analyze the organization of PIP_2 in a simplified planar system, we used Langmuir monolayers to study the effects of subphase conditions on monolayers of purified naturally derived PIP_2 and other anionic or zwitterionic phospholipids. We report a significant molecular area expanding effect of subphase monovalent salts on PIP_2 at biologically relevant surface densities. This effect is shown to be specific to PIP_2 and independent of subphase pH. Chaotropic agents (e.g., salts, trehalose, urea, temperature) that disrupt water structure and the ability of water to mediate intermolecular hydrogen bonding also specifically expanded PIP_2 monolayers. These results suggest a combination of water-mediated hydrogen bonding and headgroup repulsion in determining the organization of PIP_2, and may contribute to an explanation for the unique functionality of PIP_2 compared to other anionic phospholipids.

Introduction

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2 or PIP_2) is uniquely important among membrane-bound lipids as a regulator of cell function. Despite its structural simplicity and relative scarcity in cells (<1% of all membrane lipids [1,2]), PIP_2 is a critical mediator of a variety of cellular processes. The most widely recognized function of PIP_2 is as a substrate for hydrolytic cleavage by phospholipase C (PLC) into diacylglycerol (DAG) and inositol trisphosphate (IP_3), which are effectors of protein kinase C and calcium signaling, respectively (reviewed in ref 3) and for phosphorylation by PI 3-kinase[4] to produce the signaling lipid PIP_3. PIP_2 itself participates in several signaling pathways and is implicated in the regulation of proteins responsible for the maintenance and dynamics of the actin cytoskeleton,[5,6] attachment of these cytoskeletal structures to the plasma membrane,[7] regulation of membrane trafficking[8] and attachment,[9] ion channel activity,[10] and synaptic vesicle fusion.[11]

How a small (~1kD) membrane-bound molecule such as PIP_2 can have so many specific effects on a large number of structurally diverse binding partners is not known. Several lines of evidence suggest that control of PIP_2 signaling comes not only from enzymatic regulation of its abundance but also from regulation of its spatial organization. Some of the first evidence supporting this hypothesis was the finding that significant fractions of PIP_2 in cell membranes were unavailable for PLC hydrolysis,[12,13] as well as the dependence of PLC activity *in vitro* on PIP_2 concentration in monolayers.[12] Detergent-resistant membrane fractions were shown to be enriched in PIP_2,[14,15] possibly suggesting PIP_2 localization to membrane rafts. Imaging

[†] University of Pennsylvania.
[‡] University of Latvia.

(1) Ferrell, J. E., Jr.; Huestis, W. H. *J. Cell Biol.* **1984**, *98*, 1992.
(2) McLaughlin, S.; Wang, J.; Gambhir, A.; Murray, D. *Annu. Rev. Biophys. Biomol. Struct.* **2002**, *31*, 151–175.
(3) Berridge, M. J.; Irvine, R. F. *Nature* **1984**, *312*, 315.
(4) Chang, H. W.; Aoki, M.; Fruman, D.; Auger, K. R.; Bellacosa, A.; Tsichlis, P. N.; Cantley, L. C.; Roberts, T. M.; Vogt, P. K. *Science* **1997**, *276*, 1848–1850.
(5) Janmey, P. A.; Lindberg, U. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 658–666.
(6) Yin, H. L.; Janmey, P. A. *Annu. Rev. Physiol.* **2003**, *65*, 761–789.
(7) Gilmore, A. P.; Burridge, K. *Nature* **1996**, *381*, 531–535.
(8) Martin, T. F. J. *Curr. Op. Cell Biol.* **2001**, *13*, 493.
(9) Varnai, P.; Lin, X.; Lee, S. B.; Tuymetova, G.; Bondeva, T.; Spat, A.; Rhee, S. G.; Hajnoczky, G.; Balla, T. *J. Biol. Chem.* **2002**, *277*, 27412–27422.
(10) Hilgemann, D. W.; Feng, S.; Nasuhoglu, C. *Sci. STKE* **2001**, *2001*, RE19.
(11) Cremona, O.; De Camilli, P. *J. Cell Sci.* **2001**, *114*, 1041–1052.
(12) James, S. R.; Paterson, A.; Harden, T. K.; Demel, R. A.; Downes, C. P. *Biochemistry* **1997**, *36*, 848–855.
(13) Boguslavsky, V.; Rebecchi, M.; Morris, A. J.; Jhon, D. Y.; Rhee, S. G.; McLaughlin, S. *Biochemistry* **1994**, *33*, 3032–3037.
(14) Pike, L. J.; Miller, J. M. *J. Biol. Chem.* **1998**, *273*, 22298–22304.
(15) Laux, T.; Fukami, K.; Thelen, M.; Golub, T.; Frey, D.; Caroni, P. *J. Cell Biol.* **2000**, *149*, 1455–1472.

methods employing GFP-tagged PIP_2-binding domains[9,16] and fluorescent anti-PIP_2 antibodies[15,17] have likewise confirmed the possibility of spatially distinct PIP_2 fractions. Although the existence of these domains and their functional significance have been disputed,[18,19] spatial segregation of PIP_2 remains a plausible mechanism for regulation of this critical lipid messenger.

Despite the mounting evidence for the existence of spatially distinct pools of PIP_2, the mechanism for the formation of such domains has yet to be defined. Several studies demonstrate interaction between unstructured polybasic domains of proteins (specifically MARCKS) and multiple PIP_2 molecules, allowing concentration of this lipid through nonspecific, electrostatic attraction [2,15,20–23] and shielding of the lipid from other potential cellular targets. This hypothesis views the interactions between neighboring PIP_2 molecules as dominated by electrostatic repulsion between the charge-dense polyanionic headgroups. On the other hand, recent experiments with liposomes containing PIP_2 argue for the existence of PIP_2 domains independent of proteins, due to attractive interactions through hydrogen bonding.[24,25]

Here, we present results of experiments with monolayers of pure naturally derived PIP_2 that argue strongly for the existence of attractive interactions between adjacent PIP_2 molecules that oppose the electrostatic repulsion of the anionic headgroups. Comparison of area−pressure isotherms of PIP_2 with other acidic phospholipids over a range of subphase conditions reveals the extent to which electrostatic effects contribute to membrane surface pressure. The effects of several uncharged chaotropes preclude a strictly electrostatic interpretation and highlight the importance of hydrogen bonding or lipid headgroup hydration in maintaining the physical state of PIP_2 in planar systems. Finally, the specificity of the observed effects over other anionic and inositol-based lipids suggests that PI(4,5)P_2 may have unique ability to form hydrogen-bonded networks as a mechanism for its structural and functional sequestration.

Methods

Lipids and Reagents. Natural lipids (bovine liver L-α-phosphatidylinositol, porcine brain L-α-phosphatidylinositol-4-phosphate, porcine brain L-α-phosphatidylserine, and porcine brain L-α-phosphatidylinositol-4,5-bisphosphate) were purchased as 1 mg/mL solutions (chloroform/methanol/water 20:9:1 for PPI's; chloroform for PS) from Avanti (Alabaster, AL) and stored at −20 °C. Synthetic PIP_2 analogs (Avanti, dioleoyl phosphatidylinositol (*x*,*y*) bisphosphate) were purchased as dried 0.1 mg aliquots, dissolved in the supplied solvent, and stored at −20 °C. The concentrations of the lipid solutions were confirmed initially with phosphate analysis following acid digestion of organic components[26] and subsequently by comparing to the measured area per lipid molecule. Subphase reagents HEPES, EDTA, D-trehalose, and urea were purchased from Sigma (St. Louis, MO) and CsCl, NaCl, KCl, LiCl, $MgCl_2$, and $CaCl_2$ were purchased from Fisher (Hampton, NH).

Pressure−Area Isotherms. Monolayer subphases were prepared with 10 mM HEPES, 0.1 mM EDTA, pH 7.4 dissolved in 18.2 MΩ ddH_2O. For the low pH experiments, the buffer was 10 mM sodium phosphate; 25−30 mL of subphase solution were filtered through a 0.2 μm syringe filter (Sigma) and added to a MicroTroughX Langmuir trough (Kibron Inc. Helsinki, Finland). Approximately 7 nmol of lipid was withdrawn through a septum from a container stored at −20 °C to prevent solvent evaporation and deposited slowly on the subphase surface. After a 10 min stabilization of the monolayer, the lipids were compressed at 15 $Å^2$/molecule/min by moving the barriers of the trough using a microstepping motor. The monolayer surface pressure was monitored with a surface probe using the Wilhelmy method[26] and the FilmWare software package (Kibron). Both the low amount of lipids and the slow deposition rate were critical parameters for reproducibility of monolayer isotherms. Monolayers of pure PIP_2 could not be compressed past ∼37 mN/m in our experiments because the Teflon coated barriers of the microtrough wetted at high surface PIP_2 concentrations; hence, the collapse pressure of the PIP_2 monolayers could not be measured, but its lower bound is at least 37 mN/m. Temperature of the subphase was maintained using a circulating water bath.

Time-Course Experiment. Approximately 0.01 nmol of PIP_2 was deposited on the interface of 1 mL of filtered subphase added to a single well of a multiwell plate (Kibron). Lipid was added until the surface pressure increased to between 15−20 mN/m. The lipid was left to stabilize for ∼30 min, until the surface pressure was stable (within 1 mN/m) for several minutes. 50 μL of 5 M NaCl were added to the subphase through an injection port and the change in surface pressure was measured as a function of time.

Results

Phase Behavior of Purified, Natural PIP_2. The relationship between the surface pressure (π) and molecular area of pure naturally derived PIP_2 was investigated by compressing monolayers of PIP_2 from 250 to 50 $Å^2$/molecule and observing the effect of compression on the surface pressure of the interface. Average isotherms for 10 separate trials are shown in Figure 1a. As expected from the known composition of the acyl chains of pure PIP_2 (∼50% unsaturated, 33% arachadonic acid), these isotherms show a smooth, monotonic increase in surface pressure as the molecular area is decreased. No phase transitions were observed for monolayers of PIP_2 under any of the conditions used in these experiments. The average area of PIP_2 at a surface pressure corresponding to physiological conditions (∼30 mN/m [27]) was 73.1 ± 3.0 $Å^2$/molecule, somewhat larger than published values for SAPC (65 $Å^2$),[28] which is to be expected from the added bulk of the sugar headgroup and electrostatic repulsions. Despite the size and relatively high charge density of the PIP_2 headgroup at physiological pH, this molecule readily forms tightly compressed monolayers, as opposed to collapsing into aqueous micellar structures at higher surface pressures. Hysteresis of the monolayers due to loss of lipids through barrier leakage or monolayer collapse was negligible under all conditions, and similar to control lipids such as SOPC (data not shown).

(16) Huang, S.; Lifshitz, L.; Patki-Kamath, V.; Tuft, R.; Fogarty, K.; Czech, M. P. *Mol. Cell. Biol.* **2004**, *24*, 9102.
(17) Tran, D.; Gascard, P.; Berthon, B.; Fukami, K.; Takenawa, T.; Giraud, F.; Claret, M. *Cell Signal.* **1993**, *5*, 565–581.
(18) van Rheenen, J.; Achame, E. M.; Janssen, H.; Calafat, J.; Jalink, K. *EMBO J.* **2005**, *24*, 1664–1673.
(19) van Rheenen, J.; Jalink, K. *Mol. Biol. Cell* **2002**, *13*, 3257–67.
(20) Wang, J.; Gambhir, A.; Hangyas-Mihalyne, G.; Murray, D.; Golebiewska, U.; McLaughlin, S. *J. Biol. Chem.* **2002**, *277*, 34401–34412.
(21) Wang, J.; Gambhir, A.; McLaughlin, S.; Murray, D. *Biophys. J.* **2004**, *86*, 1969–1986.
(22) Zhang, W.; Crocker, E.; McLaughlin, S.; Smith, S. O. *J. Biol. Chem.* **2003**, *278*, 21459–21466.
(23) Gambhir, A.; Hangyas-Mihalyne, G.; Zaitseva, I.; Cafiso, D. S.; Wang, J.; Murray, D.; Pentyala, S. N.; Smith, S. O.; McLaughlin, S. *Biophys. J.* **2004**, *86*, 2188–2207.
(24) Redfern, D. A.; Gericke, A. *Biophys. J.* **2004**, *86*, 2980–2992.
(25) Redfern, D. A.; Gericke, A. *J. Lipid Res.* **2005**, *46*, 504–515.
(26) Kates, M. *Techniques of Lipidology*, 2nd ed.; Elsevier Science Publishers B.V.: Amsterdam, 1986.
(27) Demel, R. A.; Geurts van Kessel, W. S.; Zwaal, R. F.; Roelofsen, B.; van Deenen, L. L. *Biochim. Biophys. Acta* **1975**, *406*, 97–107.
(28) Brockman, H. L.; Applegate, K. R.; Momsen, M. M.; King, W. C.; Glomset, J. A. *Biophys. J.* **2003**, *85*, 2384–2396.

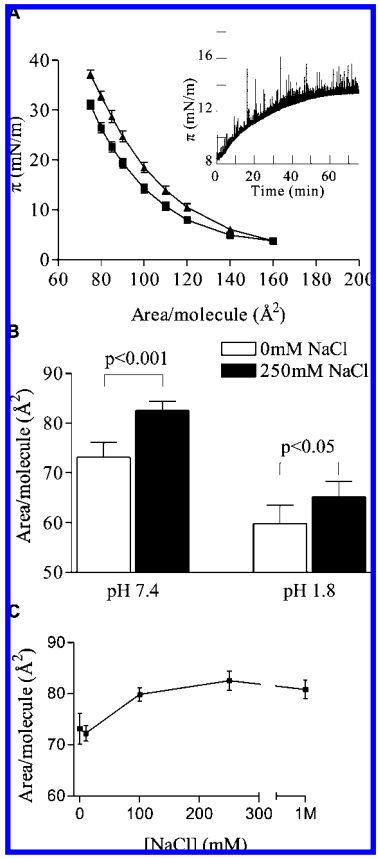

Figure 1. Expanding effect of NaCl on PIP_2 monolayers. (A) $\pi-A$ isotherms with 0 mM (■) and 250 mM NaCl (▲); (inset) change in surface pressure at constant area/molecule upon subphase injection of 250 mM NaCl (at time = 0). (B) Area per molecule at 30 mN/m at pH 1.8 ($n = 7$) and pH 7.4 ($n = 5$). (C) Dose response to subphase NaCl. Error bars are average ± SE at $n = 5$, except where indicated. All data are L-α PIP_2 on HEPES buffered subphase, pH 7.4 (unless indicated), 30 °C.

Expanding Effect of Increased Ionic Strength on Monolayers of PIP_2. To investigate the effect of ionic strength on the behavior of PIP_2 monolayers, $\pi-A$ isotherms were taken with varying concentrations of NaCl in the subphase. Addition of NaCl significantly expanded the monolayers at all surface pressures above 5 mN/m (Figure 1a). This response was also observed upon addition of NaCl to the subphase of a preformed PIP_2 monolayer. At constant molecular area, the surface pressure increased after addition of 250 mM NaCl with a magnitude commensurate to that observed in the isotherm experiments, on a diffusion-limited time scale (Figure 1a inset). At $\pi = 30$ mN/m, the area per PIP_2 molecule was increased by 13% to 82.5 $Å^2$/molecule (Figure 1b). Quantification of the dose response of this effect reveals that the effect saturates at approximately 200 mM NaCl and shows significant variation within the range of physiologically relevant salt concentrations (Figure 1c).

To test for the possibility of an electrostatic mechanism (e.g., counterion cloud repulsion) causing the monolayer expansion, the effect of 250 mM NaCl was measured on another charged lipid, L-α PS, using the same conditions as employed in the PIP_2 experiments. Monolayers of PS were not affected in the same way as those of PIP_2, instead showing a very slight contraction in response to increased subphase ionic strength (Figure 2a).

To determine whether the PIP_2-specific expansion resulted primarily from the bulky inositol ring, and at the same time

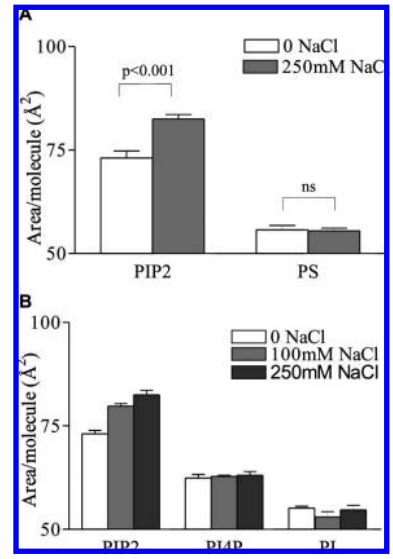

Figure 2. Specificity of salt-expanding effect to PIP_2. Area per molecule of (A) L-α PIP_2 and L-α PS; and (B) L-α PIP_2, L-α PI(4)P, and L-α PI on HEPES-buffered subphase, pH 7.4, 30 °C at $\pi = 30$mN/m. Mean ± SE, $n = 4$.

control for acyl chain composition, the pressure−area isotherms were repeated with phosphatidyl inositol 4-phosphate (L-α PI(4)P) and phosphatidyl inositol (L-α PI). Because these molecules are precursors for enzymatic PIP_2 production in cells, they have similar or identical fatty acid compositions as PIP_2, and only differ in the degree of phosphate substitution on the inositol ring. As with PIP_2, no phase transitions were observed with either inositol-based lipid, and the average molecular area increased with increased phosphate substitution, consistent with previous observations.[29] However neither PI nor PI(4)P showed a significant expansion in response to increased concentration of NaCl, although the monophosphate PI(4)P exhibited the same trend as the bisphosphate PIP_2, suggesting a similar but much smaller effect (Figure 2b). These data suggest that the mechanism involved in NaCl-induced expansion of PIP_2 monolayers is specific to PIP_2 over other anionic, as well as other inositol-based lipids.

In addition to the specificity of the expanding effect of NaCl on PIP_2 compared to other anionic phospholipids, the effect is also PIP_2 isomer dependent. Quantification of the molecular areas of synthetic PIP_2 analogs substituted at different positions on the inositol ring (3 and 5, 4 and 5, 3 and 4) shows that not only are the molecular areas dependent on the positions of the phosphate, but also that the magnitude of the NaCl-induced expansion is affected by the placement of the phosphomonoesters in the three different isomers (Figure 5a). Direct comparison of this expansion reveals the greatest difference between 0 and 250 mM NaCl for $PI(3,5)P_2$ (~22 Å), followed by $PI(4,5)P_2$ (11 $Å^2$) and $PI(3,4)P_2$ (5 $Å^2$), and that the differences between PIP_2 isomers are statistically highly significant ($p < 0.001$).

Effects of Different Counterions. To determine the ion specificity of the expanding effect of monovalent salts on PIP_2 monolayers, the effects of other cationic counterions were tested. At 250 mM, all monovalent cations tested (Na^+, K^+, Li^+, Cs^+) showed similar, statistically significant expansion of the PIP_2

(29) Patil-Sen, Y.; Tiddy, G. J. T.; Brezesinski, G.; DeWolf, C. *Phys. Chem. Chem. Phys.* **2004**, *6*, 1562–1565.

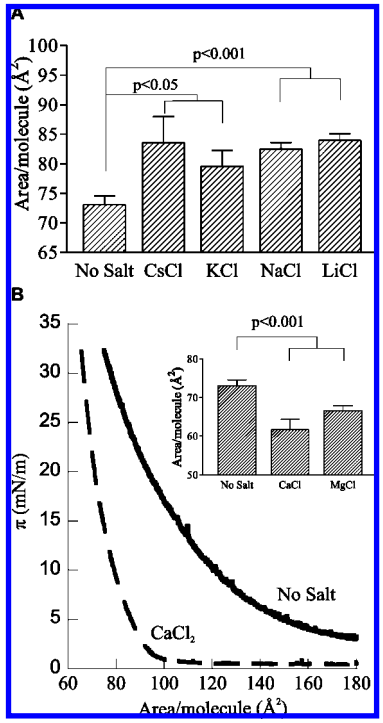

Figure 3. Effects of various counterions. (A) Area per molecule at π = 30 mN/m of L-α PIP_2 on HEPES-buffered subphase with 250 mM salt; Mean ± SE, $n = 5$. (B) π−Area isotherms of L-α PIP_2 HEPES-buffered subphase, pH 7.4, 30 °C (solid line) and same conditions plus 250 mM $CaCl_2$ (dashed line); (inset) quantification of the effects of 250 mM $CaCl_2$ and $MgCl_2$; mean ± SE, $n = 4$.

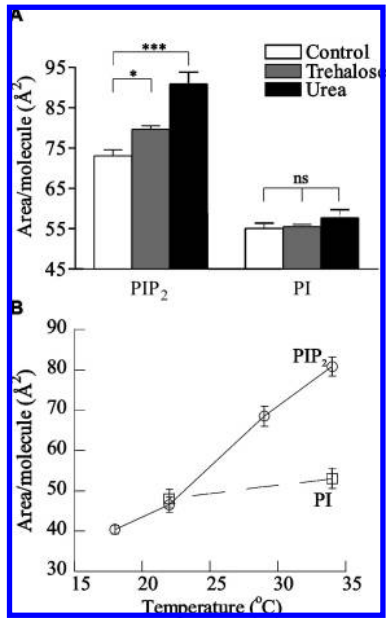

Figure 4. Evidence for water-mediated intermolecular hydrogen bonding. Area per molecule of L-α PIP_2 and L-α PI at $\pi = 30$ mN/m on HEPES buffered subphase, pH 7.4 (A) in presence of 5 mM trehalose and 5 M urea and (B) as a function of the temperature of the subphase (○ = PIP_2; □ = PI).

monolayers, with the magnitude of the effect directly related to the charge density of the ion, that is, $Li^+ > Na^+ > K^+ \approx Cs^+$ (Figure 3a, $p = 0.15-0.3$ for the differences between ions due to limited data set). The charge-density dependence observed here differs from that reported for salt-induced expansion of less highly charged anionic phospholipid monolayers, where either no cation dependence or the opposite trend was observed.[30] The magnitude of the expansion of PIP_2, in contrast to PG,[30] by the different cations appears to be directly related to the Hofmeister series describing the chaotropic nature of the ion (reviewed in ref 31). This result suggests that in addition to effects on headgroup protonation, these ions may also disrupt the structure of multimolecular water-mediated hydrogen-bonded networks within the monolayer.

Divalent counterions have a very different effect on PIP_2 compared to monovalent salts. Both $CaCl_2$ and $MgCl_2$ had a large condensing effect on pure PIP_2 monolayers (Figure 3b). The representative isotherms in Figure 3b highlight these differences, both in the area per PIP_2 at $\pi = 30$ mN/m and at lower surface pressures. The inset shows a quantification of the condensing effect of divalent cations and demonstrates that PIP_2 monolayers with 250 mM Ca^{2+} and Mg^{2+} were compressed by 15 and 9% over control, respectively. These results are consistent with the known ability of Ca^{2+} to act as a PIP_2 cross-linker by binding and dehydrating multiple phosphates with high affinity,[32,33] neutralizing their charges, and bridging headgroups to form tightly condensed monolayers,[34] even at low surface pressure.

Expanding Effect of Nonionic Chaotropes and Temperature. To test the hypothesis that monovalent salts disrupt attractive hydrogen bonding interactions among PIP_2 headgroups that partially overcome the electrostatic repulsion expected from high headgroup charge density, several nonionic chaotropic factors were tested for their ability to disrupt these putative networks and induce monolayer expansion. Urea, a protein denaturant commonly used because of its chaotropic character, and trehalose, a nonreducing glucose dimer known for its cryoprotective properties which derive from its ability to disrupt water structure, were tested for their effect on PIP_2 monolayers. Consistent with attractive interactions through hydrogen-bonding, both nonionic chaotropes had a strong expanding effect on the monolayers. At $\pi = 30$ mN/m, 5 M urea increased the area per PIP_2 molecule by almost 25% to 90.9 $Å^2$/molecule, the highest value observed for any of the conditions employed in these experiments (Figure 4b). Similarly, 5 mM trehalose significantly increased the area of the PIP_2 monolayer by 9%. These effects were specific to PIP_2, as neither treatment had a significant effect on monolayers of PI.

Finally, as confirmation of the hydrogen bonding hypothesis, the temperature-dependent behavior of PIP_2 monolayers was tested. These monolayers showed a very significant contraction as the temperature of the subphase was decreased from 34 to 17 °C, decreasing the area per molecule by almost 50% (Figure 4a). In contrast, monolayers of PI were contracted by only ∼10% over the same temperature range, consistent with a simple scaling of pressure with k_BT. Although some contraction is expected due to the decrease in kinetic energy of the lipids, the 50% difference observed for PIP_2 strongly suggests an additional mechanism, such as the disruption of a hydrogen bonded network by increased thermal energy of the subphase. Pure PIP_2 could not form compressed monolayers at subphase temperatures below ∼15 °C, instead exhibiting collapse at relatively low

(30) Sacre, M. M.; Tocanne, J. F. *Chem. Phys. Lipids* **1977**, *18*, 334–354.
(31) Cacace, M.; Landau, E.; Ramsden, J. *Q. Rev. Biophys.* **1997**, *30*, 241–277.
(32) Flanagan, L. A.; Cunningham, C. C.; Chen, J.; Prestwich, G. D.; Kosik, K. S.; Janmey, P. A. *Biophys. J.* **1997**, *73*, 1440–1447.
(33) Shah, D. O.; Schulman, J. H. *J. Lipid Res.* **1965**, *6*, 341.
(34) Papahadjopoulos, D. *Biochim. Biophys. Acta* **1968**, *163*, 240–254.

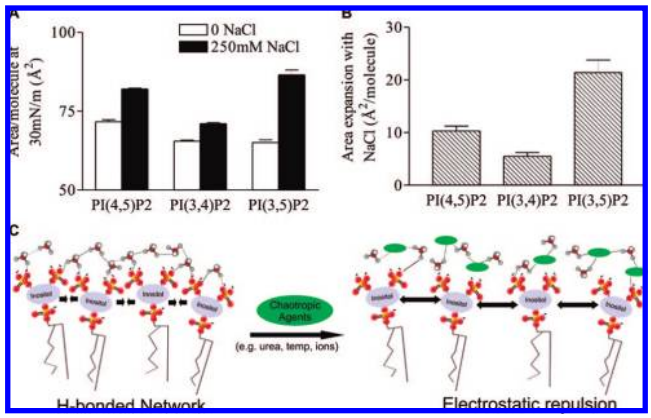

Figure 5. PIP_2 isomer specificity of subphase NaCl expansion effect. (A) Area per molecule at $\pi = 30$ mN/m of DO-PIP_2 isomers on HEPES-buffered subphase. Mean ± SE, $n = 7$. (B) Difference in area per molecule of DO-PIP_2 isomers between 250 mM NaCl and no subphase NaCl. The isomer dependence of the NaCl effect was measured to be significant to $p = 0.0001$ by two-way ANOVA. (C) Conceptual cartoon of the intermolecular interactions between PIP2 molecules. In absence of chaotropic agents (green ellipses), PIP_2 molecules form water-mediated hydrogen-bonded networks. Upon addition of chaotropes, networks are broken, and electrostatic repulsion between charged phosphates induces expansion of the monolayer.

surface pressures (<10 mN/m; data not shown). This result could be relevant to understanding temperature-induced changes in cell structure, such as cold activation of platelets, a process during which changes in PIP_2 organization at the plasma membrane trigger actin assembly.[35] The inability of PIP_2 to maintain a planar state at low temperatures may also be related to the presence of PIP_2 in detergent-resistant fractions from cellular membranes dissolved at 4 °C,[14] often identified with lipid rafts. Therefore, the presence of PIP_2 in cholesterol-rich domains at higher temperatures cannot be inferred from results below 15 °C.

Discussion

Polyphosphoinositides are well characterized as important signaling intermediates, but much more is known about the genetic regulation and expression of the enzymes that produce or degrade these lipids than about the physical chemistry that determines these lipids' distributions within the plasma membrane or their trafficking between different cellular compartments. Because of their large negative charge, it appears generally accepted that these lipids display only mutually repulsive interactions within the plane of the bilayer that keep them dispersed unless they are complexed to specific proteins.[20,23,36] Some lines of evidence suggest that PPIs are strongly sequestered under conditions that produce detergent insoluble lipid fractions (often taken as evidence of PPIs' localization to lipid rafts[14]), whereas studies using fluorescence energy transfer methods provide evidence that hydrogen bonding might stabilize PPI-rich clusters.[24,25] In this context, the present results provide quantitative estimates of the magnitude of electrostatic interactions among PPIs and show that attractive interactions, mediated by hydrogen bonding, significantly counterbalance the electrostatic repulsions.

A feature of pressure−area isotherms of PIP_2 that is well explained by purely electrostatic mechanisms is the general effect of monovalent ions on surface pressures. Although the expanding effect of monovalent salt in the subphase of PIP_2 monolayers may seem inconsistent with electrostatic repulsions between the headgroups (subphase ions might be expected to shield the anionic headgroups and allow tighter packing [37,38]), monolayer expansion by subphase cations results from the dependence of the phosphomonoester ionization potential on ionic strength, previously shown for monolayers of phosphatidic acid.[39] This effect has been shown to be important in regulating the gel−liquid transition temperature of charged monolayers,[40] although the measured magnitude of the expansion effect of subphase salts with other anionic lipids is much smaller than the expansion observed here with PIP_2.[30]

The purely electrostatic contribution to the surface pressure of PIP_2 monolayers was recently determined by modeling the system as a uniformly distributed plane of ionizable groups, the charge density of which is a function of both the pK_a's of the ionizable groups and the ionic strength of the subphase solution.[41] The surface pressure due to electrostatic repulsion, calculated by differentiating the thermodynamic potential with respect to the surface area corresponds qualitatively with some of the observed experimental results. The high pressure observed with expanded monolayers (up to 150 Å^2/molecule) at neutral pH can be explained by the repulsion of the highly charged headgroups. Additionally, both the crossing over between isotherms with low and high ionic strength and the expansion of the monolayer due to high ionic strength were confirmed with the electrostatic model at neutral pH (Figure 1a and Figure 4b in ref 41). However, many of the experimentally observed results are not compatible with a purely electrostatic treatment. Specifically, the varying effects of different monovalent ions cannot be accounted for entirely by changes in subphase ionic strength. Both the PIP_2 isomer specificity of the NaCl-induced monolayer expansion and the effects of uncharged chaotropes and temperature also point to a more complex molecular mechanism than the strictly electrostatic subphase ionic strength modulation of apparent headgroup pK_a. Additionally, the expanding effect of subphase salt at pH 1.8 (Figure 1b) is inconsistent with the model which predicts no electrostatic effects under conditions where all phosphomonoesters are protonated (Figure 4f in ref 41). Finally, in nearly all cases, the experimentally determined surface pressure of PIP_2 is significantly lower than predicted from a conservative estimate for the purely electrostatic effect.

The results of the experiments described above highlight the importance of attractive interactions, probably mediated by hydrogen bonding, that significantly counter the repulsive electrostatic interactions between PIP_2 lipids in planar systems. These attractive interactions can be disrupted by chaotropic factors such as monovalent ions, trehalose, or urea. These findings are summarized in a qualitative model presented in Figure 5c. In absence of disrupting agents, several PIP_2 molecules are shown as interacting through a water-mediated hydrogen bonded network. When either ionic factors that disrupt water−PIP_2 interactions or nonionic chaotropes are present,

(35) Hoffmeister, K. M.; Falet, H.; Toker, A.; Barkalow, K. L.; Stossel, T. P.; Hartwig, J. H. *J. Biol. Chem.* **2001**, *276*, 24751.
(36) Langner, M.; Cafiso, D.; Marcelja, S.; McLaughlin, S. *Biophys. J.* **1990**, *57*, 335–349.
(37) Lopez Cascales, J. J.; Garcia de la Torre, J. *Biochim. Biophys. Acta* **1997**, *1330*, 145–156.
(38) Pandit, S. A.; Berkowitz, M. L. *Biophys. J.* **2002**, *82*, 1818.
(39) Trauble, H. Membrane Electrostatics. In *Structure of Biological Membranes*; Abrahamsson, S., Pascher, I., Eds.; Plenum Press: New York, 1977; pp 509−550.
(40) Trauble, H.; Eibl, H. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 214–219.
(41) Levental, I.; Janmey, P. A.; Cebers, A. *Biophys. J.* **2008**, *95*.

hydrogen bonding is disrupted and electrostatic repulsion causes an increase in molecular area. This model is supported by the magnitude of the expanding effect of monovalent cations on pure PIP_2 monolayers, as well the effects of urea and trehalose (strong nonionic chaotropes). The calculated energy difference between the proposed hydrogen-bonded state and the chaotrope-disrupted expanded state (for 250 mM LiCl: ΔArea = 17.8 Å^2/molecule at 35 mN/m = ~6 kJ/mol) is commensurate with the loss of approximately one hydrogen bond per PIP_2 molecule. This energy is on the same scale as the multivalent interaction energy between PIP2 and MARCKS (~16 kJ/mol).[23] The possibility of intermolecular hydrogen bonding between PIP_2 headgroups in mixed lipid systems has been shown both experimentally[24,25] and in simulations,[42] and the data presented here confirm that possibility through experiments showing hydrogen bonding to be an important factor in intermolecular PIP_2 interactions.

The effect of temperature on PIP_2 monolayers also suggests important nonelectrostatic interactions among these lipids. The striking decrease in surface pressure with decreased temperature is far greater than observed with other charged fluid phase lipids, and does not scale simply with thermal energy. Indeed monolayers of pure PIP_2 are significantly less stable at room temperature than at 37 °C, and cannot form below 15 °C. The collapse of PIP_2 monolayers at low temperature may be related to the hypothetical clustering of PPIs at low temperature thought to trigger cold-activation of platelets and possibly other biological functions.[35]

An alternative explanation to electrostatics and hydrogen bonding for the observed effects of subphase salts involves the intercalation of the monovalent salts into the plane of the anionic headgroups to form a network lattice between the phosphates and cations. This explanation appears less likely since the expansion is greatest with the smallest, most electropositive ion (Li^+) and decreases with ion radius (Figure 3a). Also, although the formation of a rippled phase in the absence of salts could produce a more compressed monolayer, a phase transition from the liquid phase to the rippled phase was not observed with any of the isotherms (Figure 1a). Additionally, the ripple phase would only be likely to form at high surface pressures, while the differences between the high and low salt states are apparent at pressure as low as 5 mN/m (Figure 1a).

Two pieces of evidence argue for the importance of water in maintaining this network, as opposed to hydrogen bonding directly between adjacent PIP_2 molecules. The nonionic solutes urea and trehalose, which are not expected to interact with phosphate groups, have a strong expanding effect on PIP_2 monolayers, likely as a result of their disruption of water structure and subsequent disturbance of the hydrogen-bonded network (Figure 4a). Second, the significant reduction of the area per molecule of PIP_2 induced by divalent cations (Ca^{2+} and Mg^{2+}) confirms their ability to bridge neighboring lipids with resulting dehydration of the interface, and suggests that although the PIP_2 monolayers maintain a compressed state through their ability to hydrogen bond, they are not as tightly compressed as when directly cross-linked by divalent cations (Figure 3b).

Many experiments suggest that there are at least two distinct modes of interaction for the many cellular binding partners of PIP_2. Some proteins (e.g., those containing PH domains) have a specific binding site for individual PIP_2 molecules,[43–45] whereas others contain unstructured polybasic domains thought to bind several PIP_2 molecules simultaneously through nonspecific electrostatic attraction (e.g., MARCKS[23,46]). It seems reasonable to consider the possibility that a cell could regulate PIP_2-mediated signaling by influencing the balance between hydrogen-bonding and electrostatic repulsion, thereby moderating the pools of PIP_2 available for single-lipid binding protein domains versus those that bind multimolecular assemblies.

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(42) Liepina, I.; Czaplewski, C.; Janmey, P.; Liwo, A. *Biopolymers* **2003**, *71*, 49–70.
(43) Lemmon, M. A.; Ferguson, K. M.; O'Brien, R.; Sigler, P. B.; Schlessinger, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10472–10476.
(44) Harlan, J. E.; Hajduk, P. J.; Yoon, H. S.; Fesik, S. W. *Nature* **1994**, *371*, 168–170.
(45) Harlan, J. E.; Yoon, H. S.; Hajduk, P. J.; Fesik, S. W. *Biochemistry* **1995**, *34*, 9859–9864.
(46) Rauch, M. E.; Ferguson, C. G.; Prestwich, G. D.; Cafiso, D. S. *J. Biol. Chem.* **2002**, *277*, 14068–14076.