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Counterion-mediated cluster formation by polyphosphoinositides

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Abstract

Polyphosphoinositides (PPI) and in particular PI(4,5)P₂, are among the most highly charged molecules in cell membranes, are important in many cellular signaling pathways, and are frequently targeted by peripheral polybasic proteins for anchoring through electrostatic interactions. Such interactions between PIP₂ and proteins containing polybasic stretches depend on the physical state and the lateral distribution of PIP₂ within the inner leaflet of the cell's lipid bilayer. The physical chemical properties of PIP₂ such as pH-dependent changes in headgroup ionization and area per molecule as determined by experiments together with molecular simulations that predict headgroup conformations at various ionization states have revealed the electrostatic properties and phase behavior of PIP₂-containing membranes. This review focuses on recent experimental and computational developments in defining the physical chemistry of PIP₂ and its interactions with counterions.

Ca²⁺-induced changes in PIP₂ charge, conformation, and lateral structure within the membrane are documented by numerous experimental and computational studies. A simplified electrostatic model successfully predicts the Ca²⁺-driven formation of PIP₂ clusters but cannot account for the different effects of Ca²⁺ and Mg²⁺ on PIP₂-containing membranes. A more recent computational study is able to see the difference between Ca²⁺ and Mg²⁺ binding to PIP₂ in the absence of a membrane and without cluster formation. Spectroscopic studies suggest that divalent cation- and multivalent polyamine-induced changes in the PIP₂ lateral distribution in model membrane are

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also different, and not simply related to the net charge of the counterion. Among these differences is the capacity of Ca^{2+} but not other polycations to induce nm scale clusters of PIP_2 in fluid membranes. Recent super resolution optical studies show that PIP_2 forms nanoclusters in the inner leaflet of a plasma membrane with a similar size distribution as those induced by Ca^{2+} in model membranes. The mechanisms by which PIP_2 forms nanoclusters and other structures inside a cell remain to be determined, but the unique electrostatic properties of PIP_2 and its interactions with multivalent counterions might have particular physiological relevance.

1. Introduction

The interface between the intracellular and extracellular environment, mediated by the cell's plasma membrane is a crucial site at which signals are generated by chemical stimuli, application of force, or formation of cell-cell and cell-matrix contacts. The signaling pathways often involve a class of multi-anionic phospholipids, polyphosphoinositides (PPIs), in the membrane lipid bilayers (Downes et al., 2005; Martin, 1998; Zhang et al., 2012) (Figure 1).

A major challenge for understanding how PPIs function *in vivo* is the sheer number of PPI (usually $\text{PI}(4,5)\text{P}_2$) binding proteins that have been well characterized biochemically as specific and high affinity ligands for these lipids. (Golebiewska et al., 2008). The variety of PPI binding proteins and the different structures that bind these lipids suggest that specificity and control within the cell might be attained by changing the physical state of the lipid within the membrane and not only its local or global concentration. An unresolved question is how PIP_2 distributes laterally within the plasma membrane and whether all PIP_2 molecules within a membrane are equally effective at binding their targets. The remaining critical issues include the relation of PIP_2 to formation of cholesterol-dependent lipid rafts, and whether PIP_2 can self-associate to form clusters independent of or at least not requiring cholesterol. The first issue of whether PIP_2 is associated with cholesterol-dependent lipid rafts remains under debate. The primary evidence of PIP_2 enrichment in lipid microdomains is based on the observation that PIP_2 is enriched in detergent insoluble fractions of the plasma membrane (Hope and Pike, 1996; Klopfenstein et al., 2002; Pike and Casey, 1996) and is delocalized when cholesterol is depleted from the cell membrane (Liu et al., 1998; Pike and Miller, 1998). This hypothesis is supported by a later observation that PIP_2 form clusters in the presence of cholesterol alone (Dasgupta et al., 2009). Paradoxically, fluorescent PIP_2 is found to be excluded from the liquidordered phase of a model membrane in the presence of cholesterol (Levental et al., 2009). Several other reports have cast doubts on the results of cyclodextrin-mediated cholesterol depletion experiments, as the cholesterol depletion by itself is found to change the structural and physical properties of the membrane (Kwik et al., 2003; van Rheenen et al., 2005). As the first issue is beyond the scope of this review and has been widely addressed in the literature, this review focuses on the latter mechanism in which PIP_2 or other PPI's form nanoscale, dynamic clusters as they interact with divalent and multivalent counterions. Both experimental and computational studies are beginning to reveal how nanodomains enriched in PPIs might form in mixed lipid membranes.

2. Physical chemical characterization of PIP₂ and other polyphosphoinositides

A lipid fraction isolated from brain and enriched in phospholipids containing inositol was isolated at least as far back as 1946 and found to be composed of a large amount of diphosphoinositide, the phospholipid now called phosphatidylinositol phosphate (Folch, 1946, 1949). This fraction was later found to contain three inositol lipid species that differed in phosphate content and from which triphosphoinositide (now called phosphatidylinositol biphosphate) could be isolated (Dawson and Dittmer, 1961; Dittmer and Dawson, 1961; Grado and Ballou, 1961). The possible isomers of triphosphoinositides were an early subject of interest, even before the three different species produced in mammalian cells, PI(4,5)P₂, PI(3,4)P₂ and PI(3,5)P₂, were identified, and in most early studies, these lipids are referred to generically as TPI or PIP₂. The potential importance of phosphoinositides was suggested by the finding that unlike other phospholipids that were thought to be mainly structural and that were relatively stable after isolation from the cell, the amount of PIP₂ that was isolated from cells and tissues depended very strongly on preparation details and the isolated lipids were rapidly degraded or modified by enzymes, often in manner that depended on divalent cations (Akhtar and Abdel-Latif, 1978; Best et al., 1982; Grove et al., 1981).

2.1. Anionic charge of PIP₂

Polyphosphoinositides are among the most highly charged molecules in the cell membrane and have often been assumed to be uniformly distributed in the plasma membrane due to the electrostatic repulsion between their highly negatively charged head groups. These negative charges arise from deprotonated phosphomonoester and phosphodiester groups, some of which have pK_a's within a biologically relevant range. The ionization state of PIP₂ affects its area within the membrane and its interaction with proteins and other ligands, and has therefore been the subject of many studies. The pH-dependent change in the net charge of PPIs has been estimated from the zeta potential of PPI-containing multilamellar vesicles as shown in Fig. 2A (Ohki et al., 2010). Similar electrophoretic mobility measurements of PIP₂ vesicles in the presence of 100 mM KCl suggest that the charge of PIP₂ is approximately -3 at pH 7.0 (McLaughlin et al., 2002; Toner et al., 1988; Wang et al., 2002), and that both a proton and a potassium ion are likely bound to PIP₂ under physiologically realistic conditions. The pH-dependent change in PIP₂ ionization has also been investigated by ³¹P-NMR using 5 mol% PIP₂ in phosphatidylcholine lipid multilamellar suspensions (Kooijman et al., 2009). The overall charge of PI(4,5)P₂, calculated from the degree of protonation on the 4- and 5-phosphate as detected from the chemical shifts in ³¹P-NMR spectra, is approximately -4.0 in a buffer containing 100 mM NaCl, 2 mM EDTA and 50 mM Tris at pH 7.0 (Fig. 2B). Alternatively, the charge per PIP₂ can be calculated from the five pK_a values as summarized by Levental et al. (Levental et al., 2008b) combining the first ionization pK_a values of PIP₂ from ³¹P-NMR studies (van Paridon et al., 1986) and second ionization pK_a values from phosphatidic acid (PA) (Abramson et al., 1964). The charge per PIP₂ calculated from the pK_a's of isolated lipids in Fig. 2B suggests that the net charge of PIP₂ at pH 7.0 is -3.7. However, as revealed by a ³¹P-NMR study, the degree of deprotonation for PIP₂ is inhibited by the presence of the anionic lipids PS and PI in the membrane due to a decreased interfacial pH as a result of the increased negative surface

potential, whereas the presence of hydrogen bond donors significantly increases the deprotonation of PIP₂ (Graber et al., 2012). Therefore, the net charge of PIP₂ is also affected by the presence of other anionic lipids as well as lipids with hydrogen bond donor capabilities in the membrane, which is subject to changes in local lipid compositions and the microenvironment of the membrane.

The correlation between the net charge and the area per molecule of PIP₂ was examined in pure monolayers at different pH values (Levental et al., 2008b). A simplified relation between surface pressure and surface charge density in the low surface potential regime can be expressed as:

$$\pi_s = 2k_B T \frac{|\sigma|}{s} \quad (1)$$

with the surface pressure π_s proportional to the surface charge density σ . An equivalent equation, which describes a linear relation between area per molecule and charge per PIP₂, is obtained by re-arranging the above equation with eq. 11 & 12 from the same report, and it gives:

$$\Sigma = \frac{2k_B T}{\pi_s} \sum_t dP_t = \frac{2k_B T q}{\pi_s} \quad (2)$$

Here, the area per molecule (Σ) at surface pressure (π_s) is linearly related to the charge per PIP₂ (q), which is the summation of the degrees of deprotonation at all protonation sites ($\sum_t dP_t$). Once the pH-dependent change in area per molecule of PIP₂ (Σ) in a pure PIP₂ monolayer is determined experimentally (Fig. 2C), the correlation between area per molecule of PIP₂ (Σ) and charge per PIP₂ (q) can be determined if the charge per PIP₂ as a function of pH values is known. A linear correlation between measured area per molecule of PIP₂ (Σ) and charge per PIP₂ (q) as shown in Fig. 2D, suggest the validity of this simple model at high surface potential, and that PIP₂ with a -4 charge ($q = -4$) has a corresponding area per molecule of 90 Å² at 30 mN/m, which is more than 25% larger than the area per molecule of phosphatidylcholine (Nagle and Tristram-Nagle, 2000).

A striking effect of the manner in which the ionization state of PIP₂ depends on surface potential is that the area per molecule increases when ionic strength is increased from 10 mM to 250 mM by adding monovalent salt, (Fig. 2C) rather than decreasing, as would be expected if the increased salt simply screened out the electrostatic repulsions between PIP₂ headgroups (Levental et al., 2008b). The expanding effect of monovalent salt is due to the fact that decreasing surface potential leads to increased deprotonation of PIP₂, and this effect is greater than the effect of salt to lessen the electrostatic repulsion within the plane of the membrane, as was pointed out for a similar effect in phosphatidic acid (Helm et al., 1986).

2.2. Early investigations of cation binding to PIP₂

The interaction of PIP₂ with divalent cations was inferred from the finding that the addition of 3 mM CaCl₂ or MgCl₂ essentially eliminated the solubility of PIP₂ in the aqueous layer formed after addition of water to brain lipids extracted in chloroform:methanol (2:1) (Folch et al., 1957). The decreased lipid partitioning into the aqueous layer was rationalized by a

model in which divalent cations, but not monovalent cations, could neutralize the anionic charge of the lipids necessary for its solubility in water. The strong interaction of PIP₂ with magnesium and calcium was also demonstrated by the effects of these ions on ion exchange chromatography of phosphoinositides (Hendrickson and Ballou, 1964). The apparent binding affinity of Ca²⁺ for PIP₂ was reported to be 2–2.5 fold higher than that of Mg²⁺ as determined by the partitioning of radioactive ⁴⁵Ca in a PIP₂-containing water-methanol-chloroform solution (Dawson, 1965) or to be 1–1.5 fold higher as determined by pH titration in the presence of divalent cations (Hendrickson and Fullington, 1965). In most initial studies of PIP₂ binding to divalent cations the PIP₂ was in a non-membrane form, and the binding of divalent cations to PIP₂ was further studied using model membranes (Hauser and Dawson, 1967; Toner et al., 1988) or red cell ghost membranes (Buckley and Hawthorne, 1972), and a lipid monolayer of PIP₂ showed a 21 fold preference for Ca²⁺ over Mg²⁺ in cation adsorption as determined by the partitioning of radioactive ⁴⁵Ca at the air-water interface (Hauser and Dawson, 1967). These early studies provided strong evidence for an effect of divalent cations on the structure, solubility and reactivity of PIP₂, and have motivated many studies to define the molecular basis and biological effects of these interactions. Some recent advances in molecular dynamics simulations and other modeling studies of PIP₂ and experimental evidence for the ability of divalent cations to form clusters of PIP₂ in membranes are summarized here.

3. Simulations of PIP₂ and its configuration in membranes

3.1. Early MD studies of PIP₂ and its interaction with peptides

The first molecular dynamics (MD) simulations of PIP₂ in 2003 investigated how a small number of PIP₂ molecules added to one leaflet of an equilibrated bilayer of DPPC altered the structure of this membrane and explored the mechanism by which a PIP₂-binding domain of the protein gelsolin might interact with phospholipid membranes. The partial atomic charges for PIP₂ were calculated using the Restrained ElectroStatic Potential (RESP) algorithm to fit the molecular electrostatic potential of methyl arachidonate, methyl stearate, and phosphoinositol diacetate to electronic structure calculations performed with the GAMESS program. After 3 ns of simulation of a membrane containing DOPC and PIP₂, a small cluster of 4 PIP₂ molecules caused fluctuations in the thickness of the membrane, and the phosphate groups on the inositol ring of PIP₂ became fully exposed to the solvent environment. The addition of the gelsolin-derived peptide to the membrane system was able to disrupt the bilayer through the formation of hydrophobic interactions between nonpolar side chains of the gelsolin peptide and the hydrocarbon tails of PIP₂. It is unclear whether proteins that localize to the plasma membrane interface in cells, but do not penetrate the membrane by inserting structural elements (such as amphipathic or transmembrane helices), are able to form close contacts with the hydrocarbon tails of phospholipids. However, some proteins have been shown to bind to membrane phospholipids only in either a liquid ordered or disordered state, implying that the fluidity of the hydrophobic hydrocarbon acyl chains is an important factor that can regulate protein binding.

These MD simulation parameters (Liepina et al., 2003) were modified to use united atom carbons for the acyl chains of PIP₂ and attach a hydrogen atom to the phosphate group on

the 5-position of the inositol ring, bringing the total charge of PIP₂ equal to -4 instead of -5 (Lorenz et al., 2008). Setting the total charge of PIP₂ to be -4 agrees with chemical shift data from NMR experiments on PIP₂-containing vesicles (Kooijman et al., 2009), which are able to report the pKa of each phosphate group separately, and a theoretical model of the relationship between the pKa of membrane phospholipids and their net charge (Levental et al., 2008b), but not with the electrophoretic mobility of PIP₂-containing vesicles in the presence of KCl as discussed in the introduction.

Lorenz, et al. (Lorenz et al., 2008) performed simulations of a 13-mer of the positively charged amino acid lysine in the presence of monolayers containing 38 phosphatidylcholine (PC) lipids, 8 phosphatidylserine (PS) lipids, and 3 PIP₂ molecules. In a 20 ns simulation, the peptide sequesters 3 PIP₂ molecules stabilized by long-lived hydrogen bonds. In a separate system without PIP₂, the authors note that sequestration does not occur with PS, which carries a negative charge of -1 , due to the short lifetime of the interactions that form between the lipids and the peptide. This result is consistent with experiments involving the tail of a myosin-I isoform (Myo1c), which contains a string of positively charged (basic) residues, and binds weakly to physiological PS concentrations ($< 40\%$ of an LUV membrane) yet binds tightly to LUVs containing only 2% PIP₂ (Hokanson and Ostap, 2006). Further, kinase associated-1 (KA1) domains, which possess the same Pleckstrin Homology (PH) domain as Myo1c, bind to immobilized membranes containing 3% PIP₂ as well as they bind to membranes containing 20% PS and almost three times as well as that to membranes containing 10% PIP₂ (Moravcevic et al., 2010).

3.2. Improvement of atomic level force fields appropriate for phosphoinositides

Development of parameters for inositol in the CHARMM all-atom additive force field began in 2009 (Hatcher et al., 2009). After adopting previously established values for bond and angle constants, dihedral parameters were fit to the free energy surface of quantum mechanical (QM) calculations. The bond, angle, dihedral, and non-bonded parameters were then optimized by matching condensed phase MD simulations to densities and diffusion coefficients from experimental solutions (Klauda et al., 2005). Later, the torsion parameters for the acyl chains were revised (force field version C27r) so that the current CHARMM lipid force field, C36, is in excellent agreement with the self-diffusivity and viscosity of heptane (Klauda et al., 2010; Pastor and MacKerell, 2011).

3.3. Headgroup conformation

Employing the C27r CHARMM force field, Li et al. (Li et al., 2009) performed MD simulations on small POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) bilayer patches containing PIP₂ and PIP₃ with 0.1 M NaCl monovalent salt in the water box. The study aimed to determine the position of phosphoinositides in a bilayer, particularly the orientation of the head group, and to apply the finite-difference Poisson-Boltzmann (FDPB) method to describe the electrostatic properties of a 'rough' membrane containing PIP₂ derived from snapshots of their MD simulations, and to compare with the electrostatics of a flat plane as in previous calculations (McLaughlin and Murray, 2005). Li et al. report that the phosphate at the 1 position (P1) of PIP₂ resides in the same plane as P1 of POPC, but the ring phosphates of PIP₂ or PIP₃ (P3, P4, or P5) extend beyond the nitrogen of POPC and

into the solvent. Experiments on monolayers containing PIP₂ have identified hydrogen bonding between PIP₂ phosphate groups and water molecules as being a key ingredient for maintaining the physical chemistry of PIP₂ with implications for sequestration or cluster formation (Levental et al., 2008a).

The tilt angle between the inositol ring and the bilayer normal was found to sample the range 0–80°, with an average of approximately 40°. Neutron diffraction experiments have reported high tilt angles for inositol that decrease with phosphorylation, albeit at very high concentrations of PIP₂, and if only the 4-position on the inositol ring is phosphorylated, then the 5-position hydroxyl extends the furthest from the bilayer (Bradshaw et al., 1997; Bradshaw et al., 1996). The twist angle oscillated between minima at ±35° and 0°, corresponding to one ring phosphate group above the other or both phosphate groups in the same plane, respectively. The tilt angle has a large impact on the solvent accessible surface area (SASA) of the phospholipids, particularly in the case of PIP₃ where the SASA changed by up to 60% depending on the orientation.

3.4. Estimating the electrostatic properties of surfaces containing phosphoinositides

Electrostatic surfaces were computed for snapshots from MD simulations and compared with the results for otherwise identical, but microscopically flat systems. The average height of the –25 mV equipotential electrostatic surface for PIP₂ was about 17 Å away from the phospholipid (Li et al., 2009), higher than established estimates of 10 Å (McLaughlin, 1989; McLaughlin and Murray, 2005), and depends more on the tilt angle than the twist angle. Theoretical calculations of the surface (zeta) potential from (Ohki et al., 2010) that are able to reproduce the electrophoretic mobility of vesicles containing PIP or PIP₂ are sensitive to the orientation, and thus the extent of exposure, of the PIP₂ head group. The orientations that produced the highest potential surfaces were close to the most probable tilt and twist angles from the MD simulations, implying the electrostatic free energy plays a role in determining the orientation of the phospholipids. The height of the –25 mV equipotential surface for the flat system, corresponding to the most probable tilt and twist angles from the MD simulations, was almost exactly the same, validating this approximation in contexts where the details of, e.g. solvent exposure are not important. Further, the interaction energy between snapshots from the MD simulations or a microscopically flat system with a positively charged peptide is the same to within a few tenths of a kcal/mol for most of the distance range tested.

A coarse-grained (CG) model of PIP₂ for MD simulations was produced in 2009 (Stansfeld et al., 2009). The six carbon inositol ring was described by three particles in the MARTINI force field version 2.1 (Marrink et al., 2007). Each phosphate group was represented as a single particle; the phosphate particles at the 4 and 5 position of the inositol ring were assigned a charge of –2, while the phosphate at the 1 position presumably carries a net charge of –1 bringing the total charge of the CG PIP₂ model to –5. The two acyl chains were represented by 4 or 5 copies of a single, neutral particle. After 0.5–1.5 μs of simulation with 8 PIP₂ molecules embedded in a POPC membrane containing three variants of the mammalian inwardly rectifying potassium channel (Kir), several stable PIP₂-protein contacts were detected. These contacts, defined with a distance cutoff of 6 Å, as in other CG

studies, are located between adjacent protein subunits and persist when the simulation was extended to 5 μ s.

An atomistic model for PIP₂ was then generated based on the CG model and the simulation continued; the lipid-protein contacts that were subsequently observed correlate well with data from channel mutants that are not activated by PIP₂ (Schmidt et al., 2013; Stansfeld et al., 2009). Another atomistic model found that phosphates P4 and P5 of PIP₂ in the presence of sodium are able to bind the phospholipase C-delta1 Pleckstrin Homology (PH) domain (Psachoulia and Sansom, 2008).

Lupyan et al. (Lupyan et al., 2010) set out to design new force field parameters for PIP₂ based on the C27 force field. A QM geometry optimization of the phosphatidylinositol (PI) head group (without phosphates) was performed at the HF/6-31+G* level of theory. Partial atomic charges were assigned to reproduce the electrostatic potential on the surface of the molecule using the RESP method, as with Liepina et al. (Liepina et al., 2003). Since all of the MD simulations performed by Lupyan et al. contain explicit monovalent salt in the form of KCl, they chose to parameterize PIP₂ with a net charge of -5 , eliminating the choice of whether or how to protonate the phosphate groups on the inositol ring. Additionally, a modular approach to the phosphate groups was established, allowing the base PI to be patched with any combination of phosphate groups at the 3, 4, or 5 position of the inositol ring, all carrying a net charge of -2 . The torsional parameters associated with the connection between the PI head group and the glycerol part of the lipid were tuned based on the results of mapping a potential energy surface computed by scanning the key dihedral angles while keeping all other atoms in the head group fixed (i.e., a rigid potential energy surface scan). Due to the use of a sizable portion of the lipid, instead of a minimal fragment, it is possible that an unfavorable dihedral angle measured in this way would correspond to overlapping inositol and glycerol atoms that are covered by other energy functions in the CHARMM force field.

A 50 ns MD simulation was performed on a patch of a DPPC (dipalmitoylphosphatidylcholine) bilayer with or without a single PIP₂ residue. When PIP₂ is present in the bilayer patch, the ordering of neighboring DPPC molecules is slightly enhanced and the angle the DPPC head group makes with respect to the bilayer is nearly normal for lipids adjacent to the PIP₂ residue and nearly parallel for all other DPPC molecules. This effect may be due to the local high concentration of counterions in the solvent surrounding the PIP₂ residue. The average angle between the bilayer normal and the axial 2-hydroxyl group (all other hydroxyl groups on the inositol ring are equatorial) is measured to be about 42° in a conformation similar to that reported by Li et al. (Li et al., 2009). P4 and P5 project about 5–6 Å farther into the solvent than P1, also consistent with Li et al. (Li et al., 2009). These results were confirmed and extended by Chen et al. who studied how binding of the GRP1 PH domain altered the PIP₂ tilt angle (in a POPC and POPS lipid background) using EPR spin labeling (Chen et al., 2012). They found that protein binding increased the tilt and twist angle of the PIP₂ head group, promoting extension of the head group towards the bilayer normal vector.

3.5 Bilayer restructuring by PIP₂

To assess the potential of PIP₂ to form microdomains, Lupyan et al. note that it takes approximately 50% more work to pull a DPPC molecule adjacent to a PIP₂ residue out of the membrane compared with the work it takes to pull a DPPC molecule out of a pure DPPC bilayer patch. The adhesion force between PIP₂ and proteins has been probed experimentally by analyzing the dissociation time of single myosin-1 molecules and beads coated with 2% PIP₂ (Pyrpassopoulos et al., 2010). The dissociation rate depends on the rate at which load between 5 and 15 pN is exerted on the myosin-1-membrane attachments. In contrast, the force required to extract a DPPC molecule from the membrane using biotinylated beads attached to neutravidin is about 27 pN, nearly twice the force of the myosin-1-membrane attachment at the same loading rate. These results suggest that PIP₂ is able to affect the organization of the lipids surrounding it and influence their packing, a result that helps explain the role of PIP₂ in membrane anchoring.

To investigate the mechanism by which N-terminal helices of BAR (Bin, Amphiphysin, Rvs) domains seed membrane curvature, and the extent to which increasing the negative charge density of the membrane affects BAR-mediated curvature, MD simulations (Blood et al., 2008) were made on membranes containing 70% DOPC and 30% DOPS (dioleoylphosphatidylserine) with and without the PIP₂ of Lupyan et al. (Lupyan et al., 2010). Each MD simulation contained 0.15 M NaCl in a water box to neutralize the total charge of the protein and bilayer system. When PIP₂ was added to the system, the fraction of negatively charged lipids in the system was kept constant; the PIP₂ head groups protrude out of the membrane further than the DOPS head groups and become tightly coordinated by residues in the protein. Crystal structures of AP180, a protein containing a PIP₂ binding motif involved in clathrin-mediated endocytosis and thus cellular processes involving the creation or sensing of high membrane curvature, highlight coordination between P4 and P5 on PIP₂ and three lysine residues and a histidine residue (Ford et al., 2001). This extension, and thus an increase in the 'roughness' of the membrane, makes it difficult to differentiate initiation of membrane bending from thermal undulation modes. That is, the natural protrusion of the PIP₂ head group may allow PIP₂ to bind positively charged amino acid side chains in proteins without requiring membrane bending.

3.6 Clustering and domain formation by PIP₂

Experimental observations discussed in more detail in section 4.3 the experimental section, show that Ca²⁺ can induce the formation of PIP₂ nano-clusters that coincides with a surface pressure drop at constant area. In order to have a better understanding of the mechanism behind this phenomenon, numerical simulations considering essentially only Coulomb interactions using charged and neutral spheres were performed to mimic calcium-induced phase segregation of PIP₂ from neutral background lipids (Ellenbroek et al., 2011) (Fig. 3C). PIP₂ molecules were represented by negatively charged spheres; neutral or zwitterionic lipids were represented by uncharged spheres. The lipid spheres were confined to diffuse in a plane, while positively charged spheres, representing counterions (such as calcium or magnesium), were allowed to diffuse in the third dimension and come into contact with the plane containing the lipid spheres. This simulation only considered the competition between electrostatics and excluded volume, given by a purely repulsive Lennard-Jones functional

form, without taking into account the atomic-level details of the lipid species. Monovalent cations were not able to cause cluster formation, whereas divalent ions were able to induce the formation of clusters given a sufficiently negative PIP₂ charge of less than or equal to -2. The ability to promote cluster formation decreases with increasing counterion sphere size, confirming the experimental result that magnesium is weaker than calcium in forming clusters.

More recent work has revisited the atomic-level details of PIP₂ residues (Slochowicz et al., 2013). Through quantum electronic structure calculations at the HF/6-31+G* level of theory on the full PIP₂ head group, with phosphate groups attached to the 4 and 5-position on the inositol ring, the stable geometry of PIP₂ was determined. To accurately incorporate the accumulating certainty that the physiological charge of PIP₂ is -4, but no knowledge about which phosphate group is protonated, a hydrogen atom was placed equidistant between the 4 and 5 phosphate groups during the geometry optimization. The most stable conformation of PIP₂ (in isolation) has a hydrogen atom shared between the 4- and 5-phosphate groups. It is nearly as stable to have a hydrogen atom bound solely to the 5-phosphate group, and this is the conformation predominantly seen in QM/MM molecular dynamics. Having a proton bound to the 4-phosphate group only is the least stable and least likely conformation for a single PIP₂ to adopt. The stable structure was used in quantum mechanics / molecular mechanics (QM/MM) MD simulations to judge the stability of protonation and the interaction with divalent cations. In pure water or in the presence of a magnesium ion, P5 is bound to a hydrogen atom (i.e., PO₄H) and carries net charge (-1.4), while the presence of calcium ion stabilizes deprotonation of P5. This has the effect of decreasing the total charge of the PIP₂ residue, but is overcompensated by the +2 charge of the tightly coordinated calcium ion. In classical simulations, it was found that calcium and magnesium ions behave similarly until approximately 6 Å away from the lipid, at which point the excess free energy necessary to desolvate the hydration shell of magnesium prevents it from coming much closer. This finding also supports the experimental result that magnesium is not able to form clusters at the same concentration as calcium.

3.7. Summary of results from simulation studies of PIP₂

Using an array of molecular simulations that span orders of magnitude in length and time, single molecule and bulk properties of PIP₂ have been elucidated. The atomistic structure of PIP₂ in isolation has been revealed, as well as the structure of PIP₂ in monolayers and bilayers that mimic the composition of the inner leaflet of the cellular plasma membrane, where PIP₂ is found in vivo. The angle the PIP₂ head group forms with the membrane and its protrusion into the solvent have been quantified by several groups, with some debate remaining. The capacity for PIP₂ to form hydrogen bonds, with water and other phospholipids, and to interact with both monovalent and divalent cations has been described with good agreement among theory, simulations, and experiments. The ability of PIP₂ to interact with several proteins has been explored, often finding very tight coordination between the phosphate groups on the 4- and 5-position of the inositol ring and positively charged amino acids, frequently lysine or arginine. Future simulation studies are likely to expand in several directions, including the extent to which PIP₂ alters the physical properties of membranes, such as the intrinsic curvature or bending modulus, and the propensity for

PIP₂ to act as targeting scaffold for protein-membrane interactions. As these simulations become able to harness better force fields, increasing the number of molecules, and lengthening the simulation time scale to biological ranges -- microseconds to milliseconds -- they will complement a wealth of accumulated experimental data described below.

4. Experimental studies of cation-mediated interactions among PPIs

In the absence of multivalent cations the lateral distribution of PIP₂ has been proposed to depend on a balance of electrostatic repulsions and attractions caused by hydrogen bond networking between PIP₂ headgroups. This hypothesis is based in part on the finding of two different phase transition temperatures for PPIs by temperature-dependent infrared spectroscopy (Redfern and Gericke, 2004) and the pH-dependent phase partitioning of PIP₂ in a PC background investigated by FRET measurements (Redfern and Gericke, 2004; Redfern and Gericke, 2005). For a homogeneous distribution of PPIs, the degrees of protonation of phosphomonoesters are expected to increase with an increasing PPI concentration, due to a reduction of the interfacial pH. The fact that the ionization state of PIP₂ does not vary with PIP₂ mole fractions in the tested range from 1 to 20 mol% PIP₂, revealed by the unchanged chemical shifts of the 4- and 5-phosphate in ³¹P-NMR, further supports the hypothesis regarding hydrogen bond-induced PIP₂ cluster formation (Kooijman et al., 2009) as it cannot be reconciled with a homogeneous distribution of PPIs.

However, some disagreements remain. A potential limitation of these first FRET studies pointed out by Fernandes et al. is the use of short chain fluorescent PPI analogs, whose membrane partition coefficients might differ from that of native PIP₂ and are therefore subject to changes in pH (Fernandes et al., 2006). In a follow up spectroscopic study, the same group concluded that PIP₂ clusters do not form in a binary fluidic PC-containing bilayer in the pH range of 4.8–8.4 in the absence of multivalent cations, since neither a change in NBDPIP₂ fluorescence intensity or anisotropy, due to clustering was observed (Fernandes et al., 2006), consistent with the results of a grazing incident X-ray scattering study, that also found a homogeneous distribution of PIP₂ in DOPC containing bilayers unless the relative humidity was decreased below 90% (Ghosh et al., 2011).

While it is still unclear whether hydrogen bond networking between PIP₂ headgroups is strong enough to induce the formation of nanoscopic PIP₂ clusters, hydrogen bonding between PIP₂ is likely to affect many aspects of its membrane distribution with or without divalent counterions. For example, PIP₂-dependent macroscopic phase separation in GUVs was observed either by incorporating 20% phosphatidylinositol (PI) into the membrane (Graber et al., 2012) or by lowering the temperature down to 10°C (Gericke et al., in this issue), and both effects are likely explained by a reinforced hydrogen bond network between lipid headgroups. The incorporation of cholesterol was also found to stabilize PIP₂ in unilamellar vesicles as indicated by time-dependent zeta potential measurements (Carvalho et al., 2008), which can, at least in part, be explained by a perturbed hydrogen bond network between PIP₂ headgroups. The addition of monovalent salt (as shown in Fig. 2C) or non-ionic chaotropes in the subphase of a PIP₂-containing monolayer leads to an expansion in PIP₂ area per molecule, which is also attributed in part to the disruption of the hydrogen bond network of PIP₂ (Levental et al., 2008a). Furthermore, the computed PIP₂ area per

molecule in a model membrane at a constant surface pressure is significantly overestimated without considering intermolecular attraction (hydrogen bonding) (Levental et al., 2008a). These results suggest that the hydrogen bond network reduces the area per molecule of PIP₂ by holding them together and creating a tighter packing of PIP₂ even when PIP₂-rich clusters are not detectable by fluorescence methods or imaging.

4.1. Counterion-mediated membrane condensation and binding affinity: Phosphoinositides and divalent counterions

The binding of monovalent and divalent cations to single-component phospholipid membranes has been treated by a mass action formulation, taking into consideration different stoichiometric ratios of phospholipids and cations assuming that divalent cations can bind to phospholipids either in 1:1 or 1:2 ratios (Cohen and Cohen, 1981). While the 1:1 or 1:2 binding of divalent cations can occur through either parallel or serial kinetic schemes, the binding isotherms derived from the two different models are essentially similar (Cohen and Cohen, 1984). Therefore a global binding constant, which assumes 1:1 binding stoichiometry, is frequently found in the literature. The intrinsic binding constants of Ca²⁺ and Mg²⁺ binding to PIP₂ were determined to be 500 M⁻¹ and 100 M⁻¹, respectively, by measuring the electrophoretic mobility of PIP₂/PC multilamellar vesicles in the presence of different divalent cations, and the intrinsic affinity is calculated based on Poisson-Boltzmann-based surface potential theory (Toner et al., 1988).

The interaction between PIP₂ and Ca²⁺ or other divalent cations has been examined to evaluate their potential to perturb PIP₂ lateral organization in the membrane. The addition of Ca²⁺ condenses PIP₂-containing membranes to produce a downshift in the monolayer pressure/area isotherm to a smaller area per molecule (Levental et al., 2008a). As the area of a monolayer is held constant, addition of Ca²⁺ leads to a surface pressure drop, which can be recovered by removing Ca²⁺ from the monolayer by adding EDTA (Levental et al., 2009). The affinity of Ca²⁺ for a PIP₂-containing monolayer can be calculated from the surface pressure change induced by titrating Ca²⁺ (Levental et al., 2009; Wang et al., 2012). The apparent and intrinsic binding constants of Ca²⁺ at a given PIP₂ mole fraction and buffer composition are determined by fitting the Ca²⁺ concentration-dependent surface pressure change to a Langmuir adsorption model coupled with a Poisson-Boltzmann-based surface potential theory based on a model reported earlier (Ohshima and Ohki, 1986; Toner et al., 1988) to investigate the binding of Ca²⁺ to phosphatidylserine (PS) (Ohki, 1982; Ohshima and Ohki, 1985) and phosphatidic acid (PA) (Ohki and Ohshima, 1985).

One advantage of using surface pressure measurements is that the competitive binding between Ca²⁺ and Mg²⁺ can be studied directly by titrating Ca²⁺ at different fixed Mg²⁺ concentration (Wang et al., 2012). Mg²⁺ competes with Ca²⁺ in binding to PIP₂-containing lipid monolayers, and its effect is observable due to the difference in the effect of the two cations on PIP₂ surface pressure. The Ca²⁺-induced surface pressure drop can be recovered not only by adding EDTA but also by adding excess Mg²⁺ to compete Ca²⁺ off the membrane. A global binding constant for divalent ion binding to PIP₂ is reported, even though the net charge of PIP₂, and thus the electrostatic attraction to counterions, might change under experimental conditions (e.g. with pH as described in the introduction). The

apparent K_{DS} of Ca^{2+} and Mg^{2+} to a 25 mol% $\text{PI}(4,5)\text{P}_2$ -containing monolayer in a low ionic strength buffer at pH 7.4 determined using this approach in a low ionic strength buffer at pH 7.4 are 4.6 ± 1.3 and 7.7 ± 1.8 μM , respectively. The intrinsic association constants of Ca^{2+} and Mg^{2+} are then calculated to be 360 and 220 M^{-1} , respectively, and are consistent with most earlier studies. Theoretically, the binding affinities of most multivalent cations, including short chain polyamines, can be attained through either direct or indirect titration.

Differences among PIP_2 isomers upon Ca^{2+} - and Mg^{2+} -adsorption are also reported (Wang et al., 2012). When the same competitive binding assay is performed on 25 mol% PIP_2 - containing monolayers, all three PIP_2 isomers show similar affinities for Ca^{2+} but different affinities for Mg^{2+} . Regardless of acyl chain composition, L- $\text{PI}(4,5)\text{P}_2$ and DO- $\text{PI}(4,5)\text{P}_2$ show similar binding selectivity ratio with greater preference in binding to Ca^{2+} , while $\text{PI}(3,5)\text{P}_2$ has a preference for Mg^{2+} over Ca^{2+} . Together with ^{31}P -NMR studies showing differences in protonation states at physiological conditions (Kooijman et al., 2009), these subtle differences among the three PIP_2 isomers might be important in understanding why all three PIP_2 isomers with apparent similar physical chemical properties have different cellular functions.

4.2. Ca^{2+} -induced structural perturbation of PIP_2 in micelles

The idea that divalent cations could induce cluster formation of anionic lipids was recognized in early studies of anionic lipids (Hauser et al., 1976), and supported by observations that Ca^{2+} induces the phase segregation of PS as detected by differential scanning calorimetry (DSC) (Chapman et al., 1974) or electron spin resonance (ESR) (Ito et al., 1975). An effect of Ca^{2+} on the macroscopic structure of PIP_2 aggregates is suggested by the observation that the addition of Ca^{2+} precipitates PIP_2 from the aqueous layer in a chloroform-methanol-water system (Dawson, 1965; Fullington and Hendrickson, 1966). In the absence of Ca^{2+} , X-ray scattering from purified PIP_2 in an aqueous dispersion suggests that PIP_2 forms spherical particles with a Stokes radius of 39 Å and a packing number of 82 (Sugiura, 1981). Calorimetric studies show that the mixing enthalpy of Ca^{2+} with an aqueous PIP_2 dispersion is strongly endothermic, suggesting a Ca^{2+} -induced dehydration of PIP_2 , while the mixing enthalpy of Ca^{2+} and PS is, in contrast, exothermic (Takizawa et al., 1988). The endothermic change is attributed to the exclusion of water that penetrates deeply into the hydrophobic spaces between PIP_2 as a result of neutralization effect of Ca^{2+} and therefore the decreased area per PIP_2 molecule (Takizawa et al., 1991). A Ca^{2+} -induced change in PIP_2 structure in the form of either large unilamellar vesicles or PIP_2 micelles was proposed to affect its interaction with proteins (Goldschmidt-Clermont et al., 1990; Lassing and Lindberg, 1985). More detailed Ca^{2+} -induced structural changes of PIP_2 in an aqueous dispersion, investigated by small angle X-ray scattering suggest that PIP_2 forms prolate ellipsoidal micelles at pH 7.2 and that the addition of Ca^{2+} in a molar ratio smaller than $\text{Ca}^{2+}/\text{PIP}_2 = 0.70$ is sufficient to induce a structural phase transition from prolate micelles to disordered lamellae. Such a phase transition can also be induced by Mg^{2+} at a higher concentration, but the transition is not as obvious (Hirai et al., 1996).

The morphology of divalent cation-induced structural transitions of PIP_2 has been imaged by light and electron microscopy. The morphology of purified PIP_2 in aqueous suspension

changes from 6 nm-diameter micelles into striated fibrils composed of stacks of discoid micelles upon the addition of 1 mM Mg^{2+} , Ca^{2+} or Ba^{2+} , independent of the presence of 120 mM NaCl or KCl (Flanagan et al., 1997). The differences among Mg^{2+} , Ca^{2+} , and Ba^{2+} in inducing PIP_2 aggregation are reflected in the diameter of the filaments, which are 19, 12 and 10 nm, respectively, and are correlated with the hydrated radii of the cations (Kjelland, 1937; Volkov et al., 1997).

4.3. Condensing effect of Ca^{2+} in binary mixed PIP_2 -containing monolayers

In contrast to the expanding effect of monovalent salt (Fig. 2C), addition of Ca^{2+} to a PIP_2 -containing monolayer decreases the area per molecule, as summarized in Fig. 4. The compression isotherm of 25 mol% PIP_2 in SOPC downshifts to a smaller area per molecule after the addition of 1 mM Ca^{2+} , but not Mg^{2+} at the same concentration as the pressure is held constant (Fig. 4A). Equivalently, Ca^{2+} induces a surface pressure drop of the membrane as the monolayer area is held constant (Levental et al., 2009). The magnitude of the surface pressure drop induced by the same amount of Ca^{2+} is PIP_2 mole fraction-dependent (Fig. 4B). The fact that the magnitude in surface pressure drop is also pH-dependent (Levental et al., 2009) is consistent with the results from earlier studies showing a positive correlation between pH and the area per molecule of PIP_2 (Fig. 2C). The effect of Ca^{2+} on PIP_2 -containing monolayers has also been investigated by X-ray reflectivity and grazing incidence X-ray diffraction (GIXD) (Ghosh et al., 2011; Ghosh et al., 2010; Ghosh et al., 2012) to determine the lateral structure of 5 mol% PIP_2 in a background of neutral PC (DOPC or DPPC) in a pH7.4 buffer with a physiological ionic strength. The X-ray studies reveal a homogeneous distribution of PIP_2 in DOPC multilamellar films since neither a second set of Bragg peaks from scattering nor splitting of the head-group electron density from its electron density profiles (EDPs) was evident at a relative humidity (RH) • 90% (Ghosh et al., 2011). This result is consistent with evidence from spectroscopic studies that 5 mol% PIP_2 does not phase segregate from the background fluidic PC in buffers containing 100 mM NaCl at pH from 4.8 to 8.1 (Fernandes et al., 2006). Non-lamellar phases and/or phase coexistence in PIP_2 /DOPC binary mixture are observed when the RH is lowered. Interestingly, two distinct lamellar phases, as indicated by two sets of Bragg peaks, are found for pure PIP_2 from porcine brain throughout a wide range of relative humidity values in the same study. In a follow up study, the same group studies the condensing effect of Ca^{2+} to PIP_2 -containing free-standing monolayers in a background of DPPC (Ghosh et al., 2012). As shown in Fig. 4C&D, the incorporation of 5 mol% PIP_2 in DPPC monolayers results in a smaller area per chain and alkyl chain tilt angle (more in parallel to the membrane normal) in the absence of Ca^{2+} compared with pure DPPC monolayers at the same surface pressure (30 mN/m). Both the area per molecule and the acyl chain tilt angle decrease with increasing Ca^{2+} concentration, as the surface pressure is held constant. The chain tilt angle decreases more in the DPPC/ PIP_2 system (25%) than in the pure DPPC system (8%) upon the addition of 30 μM Ca^{2+} , suggesting that a strong binding of Ca^{2+} and the corresponding change in structure requires PIP_2 . The positive correlation between chain tilt angle and area per molecule suggests that the reduced area per molecule due to Ca^{2+} -induced monolayer condensation is compensated by a tighter packing, and therefore a smaller chain tilt angle, of lipids in order to maintain the hydrophobic core of monolayer at a constant surface pressure.

Ca^{2+} -induced PIP_2 clustering has been visualized in Langmuir lipid monolayers doped with fluorescent PIP_2 analogs by fluorescence microscopy (Levental et al., 2009). As the Ca^{2+} -induced formation of PIP_2 clusters in the monolayer is imaged directly through the optical window underneath the trough, this setup shows coincident cluster formation and a drop in the monolayer surface pressure; adding excess EDTA to the subphase reverses both the formation of PIP_2 clusters and the change in surface pressure.

Experimental phase diagrams for cluster formation in a low ionic strength buffer with 1 mM Ca^{2+} as a function of PIP_2 fraction, charge per PIP_2 , or cation radius have been compared to numerical simulations of Ellenbroek et al., which considers only electrostatic interactions between neutral and charged spheres, as discussed above (Ellenbroek et al., 2011). The good agreement between the experimental and numerical phase diagrams, with the dielectric correction factor being the only free parameter in the numerical study, suggests that Ca^{2+} -induced PIP_2 clustering is driven mainly by electrostatic interactions. However, two aspects of this comparative study remain unresolved. First, the simulation suggests that the coarsening of the PIP_2 clusters preferentially occurs at the ends of asymmetric Ca^{2+} - PIP_2 clusters and leads to the formation of a fiber-like structure that is not observed experimentally. Second, the simulation study predicts a complete coarsening of the PIP_2 -rich clusters, which would result in the formation of a single phase-segregated PIP_2 -rich macrodomain. On the contrary, the PIP_2 clusters observed by both fluorescence and atomic force microscopy have a finite size, which depends somewhat weakly on the PIP_2 mole fraction in the lipid monolayers. The differences between simulation and experiment suggest that there might be other physical principles that govern the growth and coarsening of the PIP_2 clusters cannot be captured in a purely electrostatic 2D model.

The 2D electrostatic model also does not fully account for the difference between Mg^{2+} and Ca^{2+} to induce cluster formation by PIP_2 . Experimentally, replacing Ca^{2+} by Mg^{2+} changes the boundary of the phase diagram so that clusters form only at higher pH values, and therefore a larger net charge on PIP_2 . Simulations suggest that this difference would result from a weaker electrostatic interaction between Mg^{2+} and PIP_2 perhaps due to a larger size of Mg^{2+} compared to Ca^{2+} . However, the bare ionic diameter of Mg^{2+} is smaller than that of Ca^{2+} , and the hydrated shell of Mg^{2+} is only 4–15% larger compared to that of Ca^{2+} depending on the value determined using different methods (Kielland, 1937; Kiriukhin and Collins, 2002; Volkov et al., 1997) whereas the numerical phase diagram suggests that Mg^{2+} has to be at least more than 80% larger than Ca^{2+} to account for the differences observed in the experiments. Therefore, factors other than ionic diameter appear to be responsible for the different interaction of Mg^{2+} from Ca^{2+} with PIP_2 . These effects may be captured by the increased energy required to dehydrate Mg^{2+} over Ca^{2+} when binding to PIP_2 and could be what is seen during hybrid quantum-level simulations of a PIP_2 bound to either ion (Slochow et al., 2013).

4.4 Microscopic and spectroscopic studies of Ca^{2+} - and Mg^{2+} -induced PIP_2 clustering in supported lipid monolayers

Divalent cation-induced PIP_2 clustering was examined in greater detail by fluorescence, atomic force and electron microscopy. While the size of clusters induced by Ca^{2+} is subject

to changes in many factors, such as PIP₂ mole fraction, pH, surface pressure and Ca²⁺ concentration, the formation of Ca²⁺-induced PIP₂ clusters was detected in supported monolayers containing as low as 1 mol% of PIP₂ in the presence of 1 μM Ca²⁺ at a physiological ionic strength and surface pressure (= 35 mN/m) (Wang et al., 2012). The cluster sizes in the model membranes increase as the PIP₂ mole fraction increases, suggesting that these submicron-size clusters are rich in PIP₂. The addition of excess EDTA to monolayers containing 50 mol% PIP₂ formed with 1 mM Ca²⁺ in low ionic strength buffer dissolves PIP₂-rich clusters, as demonstrated by fluorescence microscopy (Fig. 5G&H). Intriguingly, Ca²⁺-induced PIP₂ clusters are significantly larger than those induced by Mg²⁺ at the same PIP₂ mole fraction and monovalent ionic conditions. (Wang et al., 2012). The correlation between PIP₂ cluster formation and surface pressure change was reported previously (Levental et al., 2009) and further established by showing the calcium dependence of cluster formation using tapping mode AFM (Wang et al., 2012). The size distribution of PIP₂ clusters in purified monolayers containing 10 mol% PIP₂ in the presence of 1 μM Ca²⁺ at a low ionic strength buffer is surprisingly similar to that found in cell membranes as detected by PIP₂ antibodies or photoactivatable PH domains using optical nanoscopy (Abe et al., 2012; van den Bogaart et al., 2011; Wang and Richards, 2012) (Fig. 5A–F). While this result seems to suggest a causal link between the two observations, a firm conclusion comparing these effects is so far not possible because of the still very limited information regarding the local lipid compositions and the local Ca²⁺ concentrations inside a cell.

Changes in hydration state of divalent cations bound to PIP₂ have also been studied by Fourier transform infrared spectroscopy (ATR-FTIR) using pure PIP₂ supported monolayers (Wang et al., 2012) and MD simulations (Slochowicz et al., 2013). FTIR spectra collected at different concentrations of divalent cations, are shown in Fig. 6A&B. The difference between Ca²⁺- and Mg²⁺-induced changes in the PIP₂ FTIR spectrum is evident in the water-related bands: the O-H stretching band at 3350 cm⁻¹ decreases as Ca²⁺ concentration increases; in contrast, this peak slightly increases as with increasing Mg²⁺ concentration. No detectable changes are found with the antisymmetric PO₂⁻ stretching band in the region from 1220–1250 cm⁻¹, which reflects the hydration status of the lipid headgroup. This result suggests that the loss of water comes from the hydration shell of Ca²⁺ on binding to PIP₂ and that Mg²⁺ remains hydrated. A summary scheme in which both events are taken into account is shown in Fig. 6C (Wang et al., 2012).

4.5. Ca²⁺-induced PIP₂ cluster formation in lipid bilayer vesicles

Imaging studies using confocal fluorescence microscopy show PIP₂-rich clusters in the presence of divalent cations in giant unilamellar vesicles (GUVs), which contained 4.9% PIP₂ and were doped with 0.1% fluorescent PIP₂, in a sucrose/glucose buffer (Carvalho et al., 2008). The capacity of divalent cations to cluster PIP₂ was quantified by the fluorescence intensity variation along the equatorial cross section of GUVs at different divalent cation concentrations in a low ionic strength glucose buffer. This study suggests that Ca²⁺ and Mg²⁺ cluster PIP₂ at a concentration > 25 μM for Ca²⁺ and > 300 μM for Mg²⁺. Higher concentrations of divalent cations rupture vesicles at a concentration > 300 μM for Ca²⁺ and > 1 mM for Mg²⁺. The PIP₂-rich clusters detected by fluorescence are not visible by

differential interference contrast microscopy, which suggests that the formation of these clusters is not associated with a visible aggregation of phospholipids, but only with changes in PIP₂ lateral organization.

LUVs with the same lipid composition were used to investigate the degree of PIP₂ cluster formation induced by different multivalent cations by Förster resonance energy transfer (FRET) (Wang et al., 2012). The theoretical framework of the steady-state probe-partitioning FRET in a 2D surface is well described (Buboltz, 2007). The trend in inducing PIP₂ cluster formation in a bilayer membrane and a low ionic strength buffer follows the order: Ca²⁺ $\gg$ Mg²⁺ > Zn²⁺. The addition of EDTA reverses PIP₂ clusters as evidenced by a decrease in FRET efficiency. FRET studies also show that divalent metal ions have greater capacity to cluster PIP₂ than do polyamines with higher cationic charge, in contrast to the expectation if binding of counterions was purely electrostatic. The differences between polyamines and divalent metal ions are also reflected in their surface pressure responses on addition to 25% PIP₂-containing monolayers in the same low ionic strength buffer. Polyamines with charges from +2 to +4 expand, rather than condense the membrane, in a charge-dependent manner. The apparent K_D of polyamines binding to a 25 mol% PIP₂ containing monolayer, are 71, 61, and 13 μ M for +2, +3 and +4 polyamines, respectively using the Ca²⁺ competition binding assay on surface pressure titration. The apparent K_D for Ca²⁺ and Mg²⁺ are 4.6 and 7.4 μ M respectively under the same experimental condition (Wang et al., 2012 and unpublished data).

4.6. Distinct effect of polyamines and divalent cations on membranes containing PIP₂

Since polyamines are most abundant polyvalent cations in the cytoplasm, their interactions with PIP₂ have been extensively studied (Chung et al., 1985; Coburn et al., 2002; Coburn et al., 2006; Meers et al., 1986; Sulpice et al., 1996; Tadolini and Varani, 1986; Toner et al., 1988). The tetravalent cation spermine has been reported to up-regulate the enzymatic activity of both PI4P-5K and PI4K to increase PIP₂ content *in vitro* (Coburn et al., 2002). Detailed information on the interaction of PIP₂ with polyamines and its potential role in cell function are found in an excellent recent review (Coburn, 2009). Here we restrict our focus on the differences between binding of polyamines and divalent metal ions on PIP₂.

Two fundamental differences between organic multivalent polyamines and inorganic divalent metal ions lead to different interactions with PIP₂. The positive charges of polyamines, unlike point-localized charges, are distributed at fixed lengths along a conformationally flexible carbon chain (Schuber, 1989). The length of a spermine molecule is 1.6 nm in its extended conformation and its amine groups are 4.9 and 6.1 Å apart (Liquori et al., 1967), which might permit bridging of counterion spaced at distances that are too far to bind Ca²⁺ or Mg²⁺. A different packing geometry of polyamines with anionic lipids might explain why polyamines do not condense PIP₂-containing membranes as Ca²⁺ does (Ellenbroek et al., 2011). Another difference between organic amines and divalent metal ions is in their extent of hydration. ¹H-NMR and other studies suggest that organic amines are less “hydrophilic” than divalent metal ions (Hauser et al., 1975), in agreement with the finding that NH₄⁺ has a much lower dehydration energy compared to that of Ca²⁺ (Halim et al., 2010). One further difference related to the extent of hydration, is the polarizability of

the electron cloud of inorganic divalent metal ions, which might explain why the difference between Ca^{2+} and Mg^{2+} cannot be captured in simulations that only distinguish the two ions by their size.

In model systems, polyamines do not induce vesicle fusion themselves but can modulate Ca^{2+} -triggered vesicle fusion (Hong et al., 1983; Meers et al., 1986; Ohki and Duax, 1986; Schuber et al., 1983). Polyamines promote the aggregation of vesicles by reducing the surface charge density and surface potential of the membranes. The reduced electrostatic repulsion results in the aggregation vesicles due to an increased van der Waals' attractive forces although the repulsive hydration forces prevent fusion. The presence of polyamines however largely increases the rate for fusion upon adding Ca^{2+} and reduces the concentration of Ca^{2+} required. Interestingly, it has also been noted that polyamines by themselves can induce vesicle fusion only when the vesicles contains a high PE mole fraction in addition to anionic lipids (Schuber et al., 1983).

Competition and differences in the effects of binding divalent metal ions and polyamines to PIP_2 appear to relate to differences in their effect on transmembrane lipid distribution in cell membranes. Ca^{2+} can trigger a transmembrane redistribution (scrambling) of spin-labeled phospholipids in protein-free large unilamellar vesicles (LUVs) or erythrocyte ghost membranes, only when they contain PIP_2 (Sulpice et al., 1994). This Ca^{2+} -induced scrambling process is antagonized by the addition of spermine presumably by its interaction with PIP_2 that preventing the formation of Ca^{2+} - PIP_2 complexes (Sulpice et al., 1996).

4.7 Effects of multivalent cations on the diffusion of PIP_2 in lipid bilayers and plasma membranes

The PIP_2 surface density in the cell membrane has been estimated from mammalian cell lines and cardiac tissues to range from 20,000 to 60,000 per μm^2 (Hilgemann, 2007). Therefore these lipids are spaced within nanometers of each other but still dilute compared with other membrane constituents, and their diffusion within the plane of the membrane can lead to structures and concentration gradients that affect cell signaling. The diffusion of PIP_2 in model systems in the presence of different multivalent cations was investigated by fluorescence correlation spectroscopy (FCS) (Wang et al., 2012). In GUVs with the same composition as used in LUVs for FRET studies most auto-correlation data derived from PIP_2 in the presence of multivalent cations at low ionic strength are best fit with a two-component diffusion model, suggesting that two different PIP_2 populations are present in the membrane. One fraction of PIP_2 is less sensitive to multivalent cations and has a similar diffusion correlation time (τ_D) between 0.6 and 1 ms; the other fraction increases by factors of 2 to 25 after addition of multivalent cations at their near-physiological concentrations. The effects of Ca^{2+} and polyamines on the cation-sensitive PIP_2 fraction are significantly different. A +4 polyamine (similar to spermine) at a millimolar concentration slows the diffusion of PIP_2 by about 50% at pH 7.4, whereas the diffusion of PIP_2 is 10 times slower in the presence of micromolar Ca^{2+} . The diffusion of membrane-associated fluorescently labeled Lys13 is also about 50% slower, compared to that in a PIP_2 -free membrane, when 1 mol% PIP_2 is present in the GUV (Golebiewska et al., 2006).

The size of a cation-induced PIP₂ cluster can be estimated by the Saffman-Delbrück (SD) model (Saffman and Delbruck, 1975) or its extended form, the Hughes•Pailthorpe•White (HPW) model (Hughes et al., 1981). The estimation of PIP₂ cluster is sensitive to the chosen parameter, such as membrane thickness and membrane viscosity. The PIP₂ cluster radii in 10 μM Ca²⁺, estimated from both the SD and HPW models with reference parameters (Gambin et al., 2006), are several tens of nanometers and are close to the size of Ca²⁺-induced PIP₂ clusters visualized by AFM in supported lipid monolayers (Wang et al., 2012). The PIP₂ cluster sizes calculated from both models for objects diffusing faster than 2 $\mu\text{m}^2/\text{s}$ are not significantly different from each other. The calculated PIP₂ cluster size in 1 mM TETA⁴⁺ suggests that PIP₂ is not clustered by this polycation. This result is consistent with the argument that small basic hydrophilic peptides with less than +7 basic residues do not form complexes with more than 1 PIP₂ (McLaughlin et al., 2002).

Whether PIP₂ diffuses in a cell plasma membrane as a free monomer or as an aggregated lipid cluster through reversible binding to polybasic peripheral proteins has been addressed by fluorescence recovery after photo-bleaching (FRAP) (Cho et al., 2005; Yaradanakul and Hilgemann, 2007), by FCS (Golebiewska et al., 2011; Golebiewska et al., 2008), or by a semi-quantitative measurement of lateral mobility of fluorescent PIP₂ loaded locally with a micropipette (Cho et al., 2006; Cho et al., 2005).

When the short chain BODIPY-FL PIP₂ is delivered to myocytes by incubating the cells with a phosphoinositide-Shuttle complex, FRAP studies suggest that the diffusion of PIP₂ in a whole cell patch, with cytosolic free labeled PIP₂ removed, becomes significantly slower ($D = (3.9 \pm 0.4) \times 10^{-4} \mu\text{m}^2/\text{s}$) than in intact cells ($D = 2.1 \mu\text{m}^2/\text{s}$), which is not true for PI and PIP with similar acyl chain length (Cho et al., 2005). An alternative semi-quantitative study in which NBD-C16 PIP₂ is delivered by attaching a fluorescent PIP₂-loaded micropipette to the cell surface and selectively adding cytochalasin D to disrupt actin cytoskeletal networks, suggests that the lateral mobility of PIP₂ is greatly reduced by its interaction with actin cytoskeletal proteins. In a follow up study, the same group reported that the diffusion of NBD C16 PIP₂ is restricted in some cell types (HEK), but not in others (CHO), whereas PI and PIP with the same acyl chain length diffuse rapidly within the plasma membranes of both cell lines. The diffusion retardation occurs only for the long chain, but not short chain PIP₂ variants, although the cause of a slower diffusion of long chain PIP₂ in HEK cells is unknown (Cho et al., 2006).

A similar FRAP study using NBD C16 PIP₂ and NBD C6 PC was performed on BHK and CHO cells to investigate the lipid diffusivity within unroofed cell membranes (inner leaflet) and intact cell surfaces (outer leaflet) under conditions, where protein-lipid interactions can be eliminated (Yaradanakul and Hilgemann, 2007). The diffusion coefficients determined for both probes are not significantly different from each other in either membrane systems regardless of their acyl chain length. The fact that both probes in unroofed membranes diffuse roughly three fold faster (~ 0.2 vs. $\sim 0.08 \mu\text{m}^2/\text{s}$) than they do in intact cell surfaces is attributed to the difference in lipid composition between the inner and outer leaflet of a plasma membrane.

The diffusivity of PIP₂ in the plasma membrane and plasma membrane-derived blebs, as well as in the inner and outer leaflets of a plasma membrane has been studied by FCS (Golebiewska et al., 2008). Leaflet-specific labeling was achieved by delivering exogenous fluorescent PIP₂ using PIP₂-containing micelles to a plasma or GUV membrane through either direct incubation (for outer leaflet) or microinjection (for inner leaflet). The measured diffusion coefficient of PIP₂ in the inner leaflet of a plasma membrane is only about one third of that in blebs derived from the same membrane. Three different fluorescent PIP₂ analogs showed no significant differences in diffusivity. A 2D diffusion model modified from the Fick's second law, considering the twodimensional diffusion of PIP₂ in a plasma membrane accompanied by a reversible PIP₂ sequestration, suggests that approximately two thirds of the PIP₂ in the inner leaflet of these plasma membranes is bound reversibly before the cytoskeleton is detached from the membrane. The hypothesis that an increase in the local Ca²⁺ concentration at the cell membrane increases local free PIP₂ concentration is preliminarily tested in the same report. A small but significant increase in the diffusion coefficient of PIP₂ is observed upon adding Ca²⁺ ionophores to the cells.

Evidence for a protein fence that impedes PIP₂ lateral diffusion in a way different from PIP₂ lateral sequestering is also reported (Golebiewska et al., 2011). These FCS experiments suggest that BODIPY-TMR C16 PIP₂ has similar diffusivity in both the forming phagosomes and the unengaged plasma membrane ($D \sim 1 \mu\text{m}^2/\text{s}$), while FRAP experiments suggest that the PIP₂ fluorescence does not recover after photo-bleaching the entire forming phagosome, but recovers rapidly in a comparable area of membrane outside the cup. These results are most likely explained by a protein fence model, which is not mutually exclusive with the sequestering model and therefore could work in concert with other ligands such as divalent cations in inducing PIP₂ cluster formation.

5. Conclusions

Despite their being among the most highly anionic species in the cell membrane, the distribution of PPIs, and especially PIP₂ is not dominated by electrostatic repulsions that would tend to keep them separated and uniformly distributed. Instead a range of attractive interactions involving hydrogen bonds and binding to multivalent counterions can organize them into clusters, change the orientation of their headgroups, and alter their effective area. All of these changes in physical state can impact membrane curvature, surface potential, fluidity, and docking of proteins to the cytoplasm/membrane interface.

The interaction of PIP₂ with divalent and multivalent cations does not appear to be explained by electrostatic interactions alone. A coarse-grained 2D model that accounts for repulsions between PIP₂ within the membrane and attraction to divalent cations modeled as rigid discs is in good agreement with experimental data that quantify the levels of PIP₂ net charge and counterion density required for the system to de-mix, but the model incorrectly predicts full phase transition and cannot account for the large differences in the potential of different divalent metal ions to induce PIP₂ clustering. Thermodynamic data and MD simulations suggest that at least partial dehydration of both the metal ion and the lipid also contribute to the mechanisms of PIP₂ cluster formation.

The largest effects on PIP₂-containing membrane structure are caused by Ca²⁺, compared to other divalent metal ions or to multivalent organic cations such as polyamines. Ca²⁺ strongly condenses PIP₂ containing membrane whereas Mg²⁺ has a much weaker effect and polyamines such as the tetravalent spermine expand the membrane. Similar differences among multivalent cations are observed in measurements of PIP₂ diffusivity in membranes.

Clustering of PIP₂ has been proposed to help regulate its biological activity, and three different super resolution microscopy studies have shown PIP₂-rich domains of diameter less than 100 nm and relatively narrow size distribution that closely resemble the structures formed by purified PIP₂ and Ca²⁺ in lipid monolayers. How the *in vitro* data relate to the clustering of PIP₂ in the cell remains to be determined, but the rich physical chemistry of PIP₂ and its interaction with counterions is likely to have many implications for the biological function of this phospholipid.

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Highlights

- The charge and area of PIP2 increase with increasing pH and ionic strength.
- Ca^{2+} condenses PIP2-containing membrane by inducing PIP2 cluster formation.
- Experiments and simulations show differences in hydration of Me^{2+} bound to PIP₂.
- Polycations are different from Ca^{2+} when interacting with PIP2.
- Counterion specificity involves different packing geometry and hydration enthalpy.

Figure 1.
(A) MD simulated structure and the corresponding (B) chemical structure of PI(4,5)P2.

Figure 2.

pH-dependent change in PIP₂ ionization and area per molecule. (A) Zeta potential of MLVs containing 10 mol% PIP₂ or PIP measured at different pH values. (B) PIP₂ charges are calculated base on ³¹P-NMR measurements (Kooijman et al., 2009) or five pK_as at varying pH values (Levental et al., 2008b). (C) pH-dependent change and (D) Charges-dependent change of area per molecule at 30 mN/m using pure PIP₂ monolayer at different salinities. Image (A) is adapted from *Colloids and Surfaces B: Biointerfaces*, **2010**, 79, 210–218, Copyright 2010 and Images (C) and (D) are adapted from *Biophysical Journal* **2008**, 95, 1199–1205, Copyright 2008, with permission from Elsevier.

Figure 3.

An illustration of how counterion binding to PIP₂ might work. (A) PIP₂ residues (red: oxygen, orange: phosphorus, grey: carbon, white: hydrogen) in a background of cholesterol, DOPE, and DOPS lipids, which are not shown. Ions (green) may bind tightly to the residues and facilitate packing or clustering of the PIP₂ residues. (B) A closer view of (A) that shows both intra- and inter-molecular bridging of the ions. (C) A snapshot from the simulations of Ellenbroek et al. that shows PIP₂ residues (green) clustered by counterions (red) in a background of neutral lipids (grey) (Ellenbroek et al., 2011). (D) Sensitivity of the

simulations to the charge per PIP₂ molecule. All-atom simulations of a patch of PIP₂ with a net charge of -4 (blue head groups) are planar; reducing the charge of the head group to -5 (red head groups) produces spontaneous curvature of the residues. Images (C) is adapted with permission from *Biophysical Journal* **2011**, 101, 2178–2184. Copyright 2011 Elsevier.

Figure 4.

Effect of Ca^{2+} in PIP_2 -containing monolayers: surface pressure, area per chain and chain tilt angle. (A) Compression isotherm of 25 mol% PIP_2 in SOPC at the presence or absence of divalent cations. Buffer: 10 mM HEPES and 5 mM DTT at pH7.4. (B) Decrease in upon adding Ca^{2+} is linearly proportional to the PIP_2 mole fraction in the lipid monolayer. (C&D) Grazing incident X-ray diffraction on lipid monolayers suggests that both the area per chain and the chain tilt angle of lipids decrease with increasing Ca^{2+} concentration, and this condensation is more discernible when 5 mol% PIP_2 is present in the monolayer. Image (A)

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Figure 5.

The formation of PIP₂ clusters in both cell and model membranes. (A) Nanoscale-resolution STED image of a membrane sheet of PC12 cells immunostained with a monoclonal PIP₂ antibody and a secondary antibody labeled with Alexa Fluor 488. (B) Another PC12 cell imaged using dSTORM with anti-PIP₂ antibody directly conjugated to Alexa Fluor 647. (C) The formation of PIP₂ clusters in supported lipid monolayers containing 10 mol% PIP₂ in background DOPC at the presence of 1 μM Ca²⁺. Buffer: 10 mM HEPES, 5 mM DTT, pH7.4 at room temperature. (D)-(F) The corresponding size distribution of PIP₂-rich clusters from images (A)-(C), respectively. (G) Fluorescence images of 50 mol% PIP₂ in SOPC dual labeled with rhodamine-DOPE and C16 BODIPY FL-PI(4,5)P₂ showing phase demixing in the presence of 1 mM Ca²⁺, and (H) the phase demixing is reversed by adding excess EDTA. Image (A) and (D) are reprinted by permission from Macmillan Publishers Ltd: *Nature* **2011**, 479, 552–555, copyright 2011. Image (B) and (E) are reprinted from *Biology Open* **2012**, 1, 857–862. Image (C), (F), (G)&(H) are reprinted with permission from *Journal of the American Chemical Society* **2012**, 134, 3387–3395. Copyright 2012 American Chemical Society.

Figure 6.

Dehydration upon titration by Ca^{2+} , but not Mg^{2+} . ATR-FTIR spectra are collected during optical titration with (A) Ca^{2+} and (B) Mg^{2+} using pure PIP₂ supported monolayers. (C) Cartoon pictures show the putative differences between Ca^{2+} and Mg^{2+} when interacting with PIP₂ head groups. Reprinted with permission from *Journal of the American Chemical Society* **2012**, 134, 3387–3395. Copyright 2012 American Chemical Society.