Phosphoinositides alter lipid bilayer properties

Radda Rusinova,[1] E. Ashley Hobart,[1] Roger E. Koeppe II,[2] and Olaf S. Andersen[1]

[1]Department of Physiology and Biophysics, Weill Cornell Medical College, New York, NY 10065
[2]Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701

Phosphatidylinositol-4,5-bisphosphate (PIP_2), which constitutes ~1% of the plasma membrane phospholipid, plays a key role in membrane-delimited signaling. PIP_2 regulates structurally and functionally diverse membrane proteins, including voltage- and ligand-gated ion channels, inwardly rectifying ion channels, transporters, and receptors. In some cases, the regulation is known to involve specific lipid–protein interactions, but the mechanisms by which PIP_2 regulates many of its various targets remain to be fully elucidated. Because many PIP_2 targets are membrane-spanning proteins, we explored whether the phosphoinositides might alter bilayer physical properties such as curvature and elasticity, which would alter the equilibrium between membrane protein conformational states—and thereby protein function. Taking advantage of the gramicidin A (gA) channels' sensitivity to changes in lipid bilayer properties, we used gA-based fluorescence quenching and single-channel assays to examine the effects of long-chain PIP_2s (brain PIP_2, which is predominantly 1-stearyl-2-arachidonyl-PIP_2, and dioleoyl-PIP_2) on bilayer properties. When premixed with dioleoyl-phosphocholine at 2 mol %, both long-chain PIP_2s produced similar changes in gA channel function (bilayer properties); when applied through the aqueous solution, however, brain PIP_2 was a more potent modifier than dioleoyl-PIP_2. Given the widespread use of short-chain dioctanoyl-phosphoinositides, we also examined the effects of diC_8-phosphoinositol (PI), PI(4,5)P_2, PI(3,5)P_2, PI(3,4)P_2, and PI(3,4,5)P_3. The diC_8 phosphoinositides, except for PI(3,5)P_2, altered bilayer properties with potencies that decreased with increasing head group charge. Nonphosphoinositide diC_8 phospholipids generally were more potent bilayer modifiers than the polyphosphoinositides. These results show that physiological increases or decreases in plasma membrane PIP_2 levels, as a result of activation of PI kinases or phosphatases, are likely to alter lipid bilayer properties, in addition to any other effects they may have. The results further show that exogenous PIP_2, as well as structural analogues that differ in acyl chain length or phosphorylation state, alters lipid bilayer properties at the concentrations used in many cell physiological experiments .

The Journal of General Physiology

INTRODUCTION

Phosphoinositides regulate the function of structurally and functionally distinct membrane proteins (Hilgemann et al., 2001; McLaughlin et al., 2002; Suh and Hille, 2008; Logothetis et al., 2010). This regulation may result from phosphoinositide binding to the target protein, and several specific phosphoinositide-binding domains have been identified. Specific binding mainly (but not exclusively; Rosenhouse-Dantsker and Logothetis, 2007) involves the interaction of positively charged residues with the negatively charged phosphatidylinositol-4,5-bisphosphate (PIP_2) phosphates (McLaughlin and Murray, 2005; Hurley, 2006; Suh and Hille, 2008; Hansen et al., 2011; Leonard and Hurley, 2011; Whorton and MacKinnon, 2011). In many cases, the regulation seems to be more general, however, in the sense that other anionic phospholipids can substitute for the phosphoinositides (Fan and Makielski, 1997; Kim and Bang, 1999; Hilgemann et al., 2001; Zhang et al., 2003; Vaithianathan et al., 2008), suggesting that the regulation involves less specific electrostatic interactions with a positively charged region—but leaving open the question of how the phosphoinositides alter function.

The unifying feature among the diverse class of membrane proteins that are regulated by PIP_2 is that many are embedded in a lipid bilayer, and changes in bilayer properties are known to alter membrane protein function (Bienvenüe and Marie, 1994; Lee, 2004; Andersen and Koeppe, 2007; Lundbæk et al., 2010a). In this context, it is important that PIP_2 has ~50% polyunsaturated fatty acids (Marsh, 2012b), which would tend to reduce the bilayer bending modulus (Rawicz et al., 2000; Bruno et al., 2013). PIP_2 constitutes ~1% of total cell lipid, varying among different cell types (Ferrell and Huestis, 1984; Nasuhoglu et al., 2002; Wenk et al., 2003; Xu et al., 2003; Horowitz et al., 2005; Li et al., 2005; Hilgemann, 2007). Because the total cell lipid profile includes intracellular membranes, and PIP_2 is localized predominantly to the inner leaflet of the plasma membrane (Balla and Várnai, 2002), the area-averaged mole percentage of PIP_2 in the plasma membrane is likely to be >1% (Hilgemann,

Correspondence to Radda Rusinova: rar2021@med.cornell.edu

Abbreviations used in this paper: ANTS, 8-aminonaphthalene-1,3,6-trisulfonate; CMC, critical micelle concentration; gA, gramicidin A; LUV, large unilamellar vesicle.

The Rockefeller University Press $30.00
J. Gen. Physiol. Vol. 141 No. 6 673–690
www.jgp.org/cgi/doi/10.1085/jgp.201310960

2007)—and local PIP_2 concentrations may be even higher (Wang and Richards, 2012). Given the lack of a clear mechanism for many of the PIP_2-mediated effects, it thus becomes important to explore whether changes in extant, or exogenously applied, PIP_2s alter bilayer properties at relevant concentrations, in which case some of PIP_2's effects may be caused by nonspecific bilayer-mediated mechanisms, as recently was proposed in the case of invertebrate phototransduction (Hardie and Franze, 2012).

Bilayer-spanning proteins are energetically coupled to the host lipid bilayer through hydrophobic interactions between the transmembrane domains and the lipid bilayer hydrophobic core, which can influence the lipid packing around the protein (Killian et al., 1989, 1996) or the protein conformation (Keller et al., 1993; Brown, 1994; Greathouse et al., 1994). This lipid reorganization (bilayer deformation) has an associated energetic cost, the bilayer deformation energy (ΔG_{def}). If protein conformational changes (e.g., from state I to state II) alter the local lipid bilayer deformations, the resulting difference in the bilayer deformation energy, $\Delta G_{bilayer}^{I\to II}$, becomes the bilayer contribution to the total free energy of the conformational transition ($\Delta G_{total}^{I\to II}$) (Nielsen and Andersen, 2000; Andersen and Koeppe, 2007; Lundbæk et al., 2010a):

$$\frac{n_{II}}{n_I} = K^{I\to II} = \exp\left\{-\frac{\Delta G_{total}^{I\to II}}{k_B T}\right\} = \exp\left\{-\frac{\Delta G_{protein}^{I\to II} + \Delta G_{bilayer}^{I\to II}}{k_B T}\right\}, \quad (1)$$

where n_I and n_{II} denote the surface densities of the protein in states I and II, $\Delta G_{protein}^{I\to II}$ and $\Delta G_{bilayer}^{I\to II}$ denote the protein and bilayer contributions to $\Delta G_{total}^{I\to II}$, and $k_B T$ consists of Boltzmann's constant and the temperature in Kelvin. Changes in lipid bilayer physical properties, which are expressed as changes in $\Delta G_{bilayer}^{I\to II}$, thus will alter the protein conformational equilibrium and function.

Amphiphiles modulate lipid bilayer properties by adsorbing to the bilayer–solution interface (Lundbæk et al., 2010a; Rusinova et al., 2011; Bruno et al., 2013), thereby altering the bilayer response to a deformation (changing ΔG_{def} and thus $\Delta G_{bilayer}$). Key bilayer properties that may be affected by amphiphile adsorption include the intrinsic curvature, as a result of the altered profile of intermolecular interactions along the bilayer normal (Seddon, 1990; Cantor, 1997; Andersen and Koeppe, 2007; Marsh, 2007), bilayer elasticity (Evans et al., 1995), lipid disorder, and possibly lipid demixing (Zhelev, 1998; Heerklotz, 2002). These amphiphile-mediated changes in bilayer properties in turn may shift the conformational equilibrium of membrane proteins. (It is given that ΔG_{def} will change, but the changes may or may not be sufficient to cause measurable changes in protein function.) We therefore set out to determine whether PIP_2 and its structural analogues alter bilayer properties and, if so, to parse out which bilayer properties are affected.

Many different probes have been used to characterize the changes in bilayer properties that are caused by drugs and other amphiphiles (Seddon et al., 2009). For the present studies, we used gramicidin A (gA) channels as probes that serve as prototypical bilayer-spanning proteins, which experience minimal interference from specific lipid–protein interactions. gA channels form by the transmembrane dimerization of nonconducting monomeric subunits residing in apposing bilayer leaflets (O'Connell et al., 1990). Channel formation is associated with a local bilayer compression (Elliott et al., 1983; Huang, 1986; Harroun et al., 1999), which makes gA channels sensitive to changes in bilayer properties (Andersen et al., 2007; Lundbæk et al., 2010a,b).

Using gA channels as probes, we have explored the effects of phosphoinositides on lipid bilayers while varying the method by which membrane PIP_2 was manipulated, the acyl chain composition, and the number and positions of phosphate groups on the inositol ring (Fig. 1). For comparison, we also examined the bilayer-perturbing effects of other related short-chain phospholipids.

Our results show that increasing or decreasing the mole percentage of long-chain PIP_2 in the plasma membrane results in altered lipid bilayer properties. Both long- and short-chain phosphoinositides, as well as nonphosphoinositide short-chain phospholipids, are bilayer-modifying agents.

MATERIALS AND METHODS

Materials

All phospholipids were purchased from Avanti Polar Lipids, Inc., except for diC_8PI (1,2-dioctanoyl-*sn*-glycero-3-phosphoinositol), which was purchased from Echelon Biosciences (Fig. 1). The diC_8 phospholipid stock solutions were 5 mM in water, except for the diC_8PE stock solution, which was 5 mM in ethanol (Pharmco-AAPER). The long-chain PIP_2s were 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-myo-inositol-4′,5′-bisphosphate) and the porcine brain extract of PIP_2, which is ∼80% $C_{18/20:4}PIP_2$. To make aqueous "solutions" of $diC_{18:1}$ and brain PIP_2, the powder was suspended in H_2O. The suspension was sonicated in an ultrasonic homogenizer (model 300 V/T; Biologics Inc.) for 30 min on ice at 90% power and 90% duty cycle using 1-s pulses. The resuspended long-chain phosphoinositides were flash frozen and stored at −40°C in a glass vial. To make $DC_{18:1}PC$ (1,2-dioleoyl-*sn*-glycero-3-phosphocholine)/PIP_2 lipid mixtures, the long-chain PIP_2 was dissolved in chloroform (Honeywell Burdick and Jackson) and mixed with $DC_{18:1}PC$ in chloroform at the appropriate mole percentage. Chloroform was evaporated under N_2, and the mixture was suspended in *n*-decane (Sigma-Aldrich). 8-Aminonaphthalene-1,3,6-trisulfonate (ANTS) was purchased from Invitrogen. For the single-channel experiments, we used a 15–amino acid analogue, [Ala^1]gA (AgA(15)), a chain-shortened 13–amino acid gA analogue of opposite helix sense, des-D-Val-Gly-gA^- (gA^-(13)), and a 15–amino acid analogue in which the NH groups on the four Trp residues are methylated, [Val^1,*N*-Me-$Trp^{9,11,13,15}$]gA (*N*-Me-gA(15)); they were synthesized and purified as described previously (Greathouse et al., 1999). For the fluorescence assay, we used the natural mixture of gramicidins

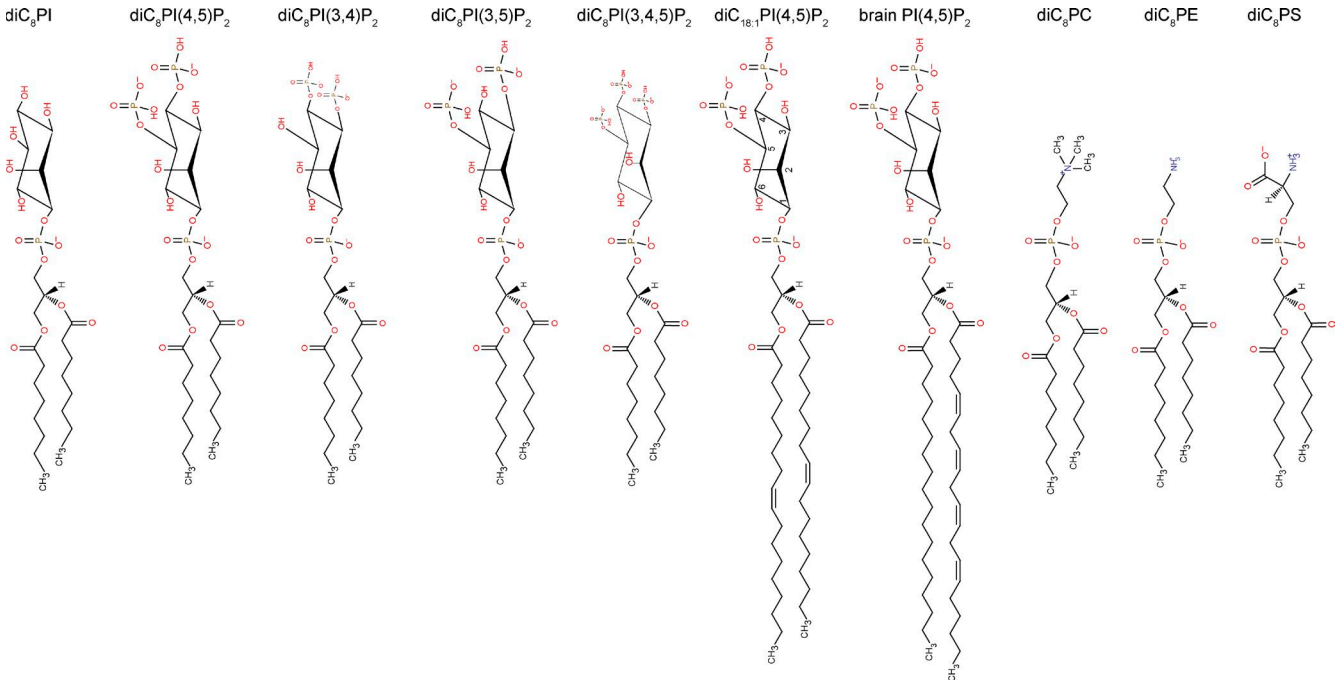

Figure 1. Structures of the phospholipids used in this study; brain PIP_2 is predominantly 1-$C_{18:0}$-2-$C_{20:4}$-PI(4,5)P_2, which is shown in the figure.

from *Bacillus brevis* (Sigma-Aldrich), which is ∼80% [Val[1]]gA (Abo-Riziq et al., 2006); the mixture is denoted as gD (for R. Dubos, who discovered the gramicidins; Dubos, 1939).

Electrophysiology

Planar lipid bilayers were formed across a 1.5-mm–diameter aperture in a Teflon partition using $DC_{18:1}PC$ or $DC_{18:1}PC$–long-chain PIP_2 suspensions in *n*-decane. The total phospholipid concentration was the same (15 mg/ml) in the pure and mixed lipid suspensions. The aqueous solution was a 1 M NaCl and 10 mM HEPES, pH 7.0, solution; the temperature was 25 ± 1°C. Single-channel measurements were performed at 200 mV using a patch clamp amplifier (model 3900A; Dagan Corp.) and the bilayer punch method (Andersen, 1983). The current signal was filtered at 5 kHz, sampled at 20 kHz, and digitally filtered at 500 Hz. After the bilayer was formed, gA analogues of different lengths and helix senses were added to both sides of the bilayer, and the solutions were stirred for 5 min (in a few experiments, only *N*-Me-gA(15) was added). After ∼30 min, to allow for gA incorporation, control current traces were recorded. In the experiments in which the lipids were added through the aqueous phase, short- or long-chain phospholipids were added to both sides of the bilayer. The solutions were stirred for 1 min and equilibrated for 10 min before recording.

Single-channel current transitions were identified using the algorithm described previously (Andersen, 1983), implemented in software written in Visual Basic (Microsoft Corp.). Single-channel lifetimes (τ) were determined by fitting single exponential distributions to survivor histograms of the lifetime distributions using Origin 6.1 (OriginLab). At least 300 channels were collected in each experiment. Changes in appearance frequency (f), the number of channels (opening followed by closing events) per unit of time, were determined only in experiments in which the bilayer remained intact after adding the phospholipid. The results were reported as means ± SD; in case there were only two observations, the results were reported as means ± range. Relative changes in appearance frequency were determined by normalizing the appearance frequency in the first two recordings right after the 10-min incubation with the compound to the appearance frequency from a recording right before compound addition. Only recordings lasting ≥300 s were used to evaluate changes in relative channel appearance frequencies.

Energetics of gA channel formation

gA in lipid bilayers exists in equilibrium between nonconducting monomers and conducting dimeric channels:

$$M + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D,$$

where k_1 and k_{-1} are the association and dissociation rate constants, respectively. The dimer dissociation rate constant can be estimated as $1/\tau$, and changes in the association rate constant can be estimated from changes in the channel appearance frequency because $k_1 = f/[M]^2$.

The channel's hydrophobic length (l), ∼2.2 nm for a 15–amino acid analogue (Elliott et al., 1983; Huang, 1986), is less than the lipid bilayer's unperturbed hydrophobic thickness (d_0), 3–4 nm (Benz et al., 1975; Lewis and Engelman, 1983; Lundbæk et al., 2010b), and gA channel formation is associated with a local bilayer compression with an associated deformation energy, ΔG_{def}. Using the theory of elastic bilayer deformations (Huang, 1986; Helfrich and Jakobsson, 1990; Dan et al., 1994; Nielsen et al., 1998; Nielsen and Andersen, 2000; Partenskii and Jordan, 2002; Rusinova et al., 2011), ΔG_{def} can be expressed as

$$\Delta G_{def} = H_B \times (l - d_0)^2 + H_X \times (l - d_0) \times c_0 + H_C \times c_0^2, \quad (2)$$

where c_0 is the intrinsic monolayer curvature and H_B, H_X, and H_C are phenomenological elastic coefficients determined by the bilayer elastic moduli, the channel radius, and d_0. The bilayer responds to the compression by imposing a disjoining force (F_{dis}) on the channel,

$$F_{dis} = -\frac{\partial \Delta G_{def}}{\partial (l - d_0)} = 2 \times H_B \times (d_0 - l) - H_X \times c_0, \quad (3)$$

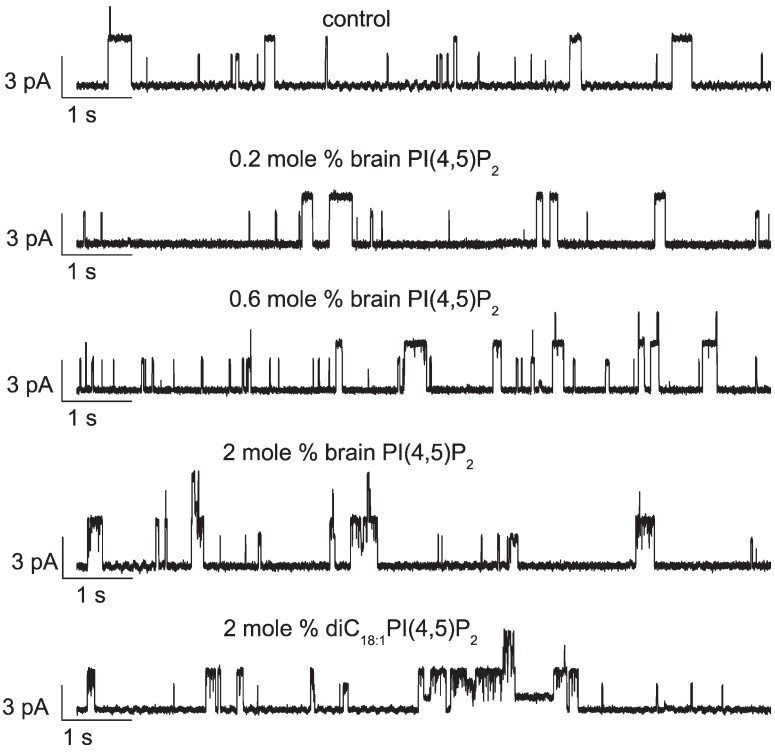

Figure 2. Effects of increasing the mole percentage of long-chain PIP_2 on gA channel activity in mixed $DC_{18:1}PC$/long-chain PIP_2 bilayers. Single-channel current traces recorded in $DC_{18:1}PC$ planar lipid bilayers containing 0 (control), 0.2, 0.6, and 2 mol % brain PIP_2 or 2 mol % $diC_{18:1}PIP_2$.

acting to dissociate the dimer. Changes in bilayer physical properties will alter the phenomenological elastic coefficients and thus ΔG_{def}, f, and τ (Lundbæk et al., 2010b; Greisen et al., 2011), which make the gA channels suitable as probes of changes in bilayer properties.

The energetics of the gA monomer↔dimer equilibrium is described by

$$\frac{[D]}{[M]^2} = K^{M\to D} = \exp\left\{-\frac{\Delta G_{tot}^{M\to D}}{k_B T}\right\} = \exp\left\{-\frac{\Delta G_{prot}^{M\to D} + \Delta G_{bil}^{M\to D}}{k_B T}\right\}, \quad (4)$$

where [M] and [D] denote the surface concentrations of gA monomers and dimers, $\Delta G_{bil}^{M\to D}$ is the change in ΔG_{def} upon dimer formation, and $\Delta G_{tot}^{M\to D}$ is the free energy contribution from protein interactions. We are interested in the amphiphile-induced changes in bilayer properties, and the associated $\Delta\Delta G_{bil}^{M\to D}$ is determined by the changes in the time-averaged gA activity in the presence relative to that in the absence of amphiphiles. From Eq. 4:

$$\Delta\Delta G_{tot}^{M\to D} = -k_B T \ln\left\{\frac{K_{amph}^{M\to D}}{K_{cntrl}^{M\to D}}\right\}, \quad (5)$$

where the subscripts denote the presence or absence of amphiphile and $\Delta\Delta G_{tot}^{M\to D}$ can be estimated as

$$\Delta\Delta G_{tot}^{M\to D} \approx -k_B T \times \ln\left\{\frac{[D]_{amph}}{[D]_{cntrl}}\right\} = -k_B T \times \ln\left\{\frac{f_{amph} \times \tau_{amph}}{f_{cntrl} \times \tau_{cntrl}}\right\} \approx \Delta\Delta G_{bil}^{M\to D}. \quad (6)$$

The first approximation holds as long as [M] is in excess and does not vary after the amphiphiles have been added—as long as $[M]_{amph} - [M]_{cntrl}/[M]_{cntrl} \approx 0$. The second approximation holds when the subunit–subunit interactions do not vary—as long as $\Delta\Delta G_{prot}^{M\to D} \approx 0$.

Additional information can be obtained through simultaneous measurements on two gA channels of different lengths (with different hydrophobic mismatch and helix sense, to prevent the formation of heterodimers), which allows for distinguishing between effects on bilayer curvature and elasticity. This is possible because, according to Eqs. 2 and 3, if the bilayer elasticity (expressed as changes in H_B) is altered, the effects will be hydrophobic mismatch dependent, with greater changes in the shorter channel's appearance frequency and lifetime (Lundbæk et al., 2005; Rusinova et al., 2011). If the relative changes in lifetime are similar for the longer and shorter channels, curvature effects dominate (Artigas et al., 2006; Ashrafuzzaman et al., 2006).

Fluorescence-based gA assay

To assay for the phosphoinositides' bilayer-modifying effects, we used a gramicidin-based fluorescence quench assay (Ingólfsson and Andersen, 2010; Ingólfsson et al., 2010; Rusinova et al., 2011) in which fluorophore-loaded lipid vesicles were mixed with a gramicidin-permeable fluorescence quencher (Tl^+) such that changes in gramicidin channel activity (the number of channels in the large unilamellar vesicle [LUV] membrane) were monitored as changes in the rate of fluorescence quenching. In brief, after solvent evaporation, $DC_{22:1}PC$ (1,2-dierucoyl-*sn*-glycero-3-phosphocholine) was rehydrated in 100 mM $NaNO_3$, 25 mM ANTS, and 10 mM HEPES, pH 7. After vesicle extrusion and desalting to remove excess ANTS, the ANTS-loaded LUVs were prepared for fluorescent experiments by diluting 1:20 in 140 mM $NaNO_3$ and 10 mM HEPES, pH 7. ANTS-loaded LUVs were doped with gD; after incubation for 24 h at 12.5°C to allow for bilayer incorporation of gD, the LUVs were incubated at 25°C with or without (control) the test phospholipid for 10 min (unless otherwise noted). The LUVs and the Tl^+-containing quench buffer were mixed in a stopped-flow spectrometer (SX.20; Applied Photophysics) with a dead time of <2 ms, and the time course of the ANTS fluorescence signal was recorded (see Figs. 8 A and 9 A). The resulting fluorescence quench curves were fit with a stretched exponential (Berberan-Santos et al., 2005) in Figs. 8 B and 9 B to

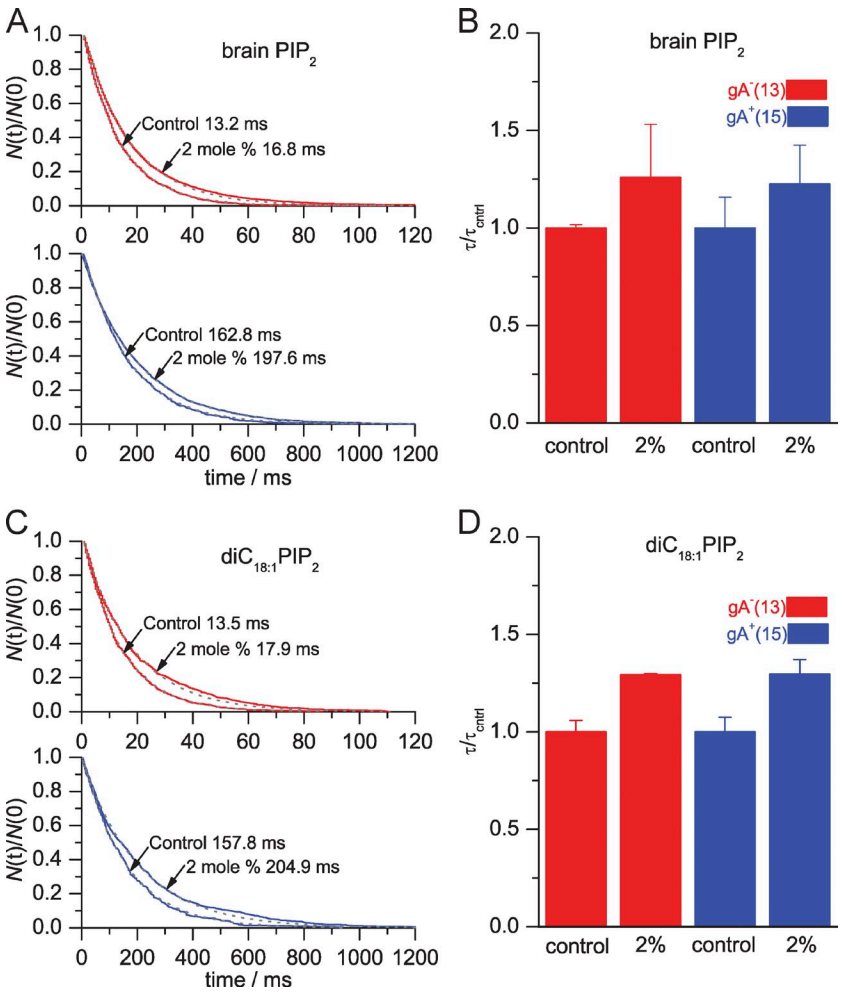

Figure 3. Effects of increasing the mole percentage of long-chain PIP_2 on single-channel lifetimes in $DC_{18:1}PC$/long-chain PIP_2 bilayers. (A and C) Lifetime distributions of $gA^-(13)$ (red lines) and AgA(15) (blue lines) channels, which are fit with single exponential distributions (dashed gray lines) in $DC_{18:1}PC$ and in bilayers with 2 mol % brain PIP_2 (A) and $DC_{18:1}PC$ and in bilayers with 2 mol % $diC_{18:1}PIP2$ (B). The τs from the fits are noted in the plots. (B and D) Changes in τ for $gA^-(13)$ (red bars) and AgA(15) (blue bars) channels in control and mixed bilayers with either 2 mol % brain PIP_2 ($n = 5$; C) or 2 mol % $diC_{18:1}PIP_2$ ($n = 2$; D). The τ values in control and mixed bilayers were normalized to the mean τ in three separate experiments in control bilayers. Error bars represent SD.

determine the fluorescence quench rate at 2 ms, which reports on the time-averaged number of gramicidin channels in the LUV membrane.

Statistical analysis

All data plots and statistical analyses were performed in Origin 6.1. One population Student's *t* test was used to establish whether means were significantly different from 1 as indicated by asterisks ($P \leq 0.05$). Two population, two-tailed, independent Student's *t* tests were used to establish whether two means were significantly different ($P \leq 0.05$). A one-way ANOVA test performed on all compared means indicated significant differences ($P \leq 0.05$).

Online supplemental materials

Fig. S1 shows the diC_8PIP_2 effect on the single-channel lifetimes, appearance frequencies, as well as the channel $\Delta\Delta G_{bil}^{M\rightarrow D}$ for *N*-Me-gA(15) channels. Online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201310960/DC1.

RESULTS

We first examine the effects of endogenous long-chain PIP_2, in which the PIP_2 was added to the lipid suspensions used to form mixed $PIP_2/DC_{18:1}PC$ bilayers; we then examine the changes in bilayer properties produced by the addition of exogenous phosphoinositides—long-chain PIP_2, as well as short-chain phosphoinositides with varying numbers (and positions) of phosphate groups in the inositol ring—through the aqueous solution; finally, we compare the effects of short-chain (diC_8) phosphoinositides with those of other diC_8 phospholipids.

Endogenous versus exogenous addition of PIP_2

To explore the mechanisms by which phosphoinositides alter lipid bilayer properties and to estimate the changes in energetic cost of protein-imposed bilayer deformations, we used single-channel electrophysiology to monitor the changes in gA channel lifetimes and appearance frequencies. These experiments were performed using gA analogues of different chirality and length, $gA^-(13)$ and AgA(15), which allows for detailed information about the phosphoinositide effects on bilayer properties (Lundbæk et al., 2005).

Effects of endogenous long-chain PIP_2s. Figs. 2 and 3 show results obtained in mixed brain $PIP_2/DC_{18:1}PC$ or $diC_{18:1}PIP_2/DC_{18:1}PC$ bilayers (Fig. 2); in each case, the PIP_2 was added at up to 2 mol %.

Using either mixture, the lifetimes of both $gA^-(13)$ and AgA(15) channels were longer than in pure $DC_{18:1}PC$

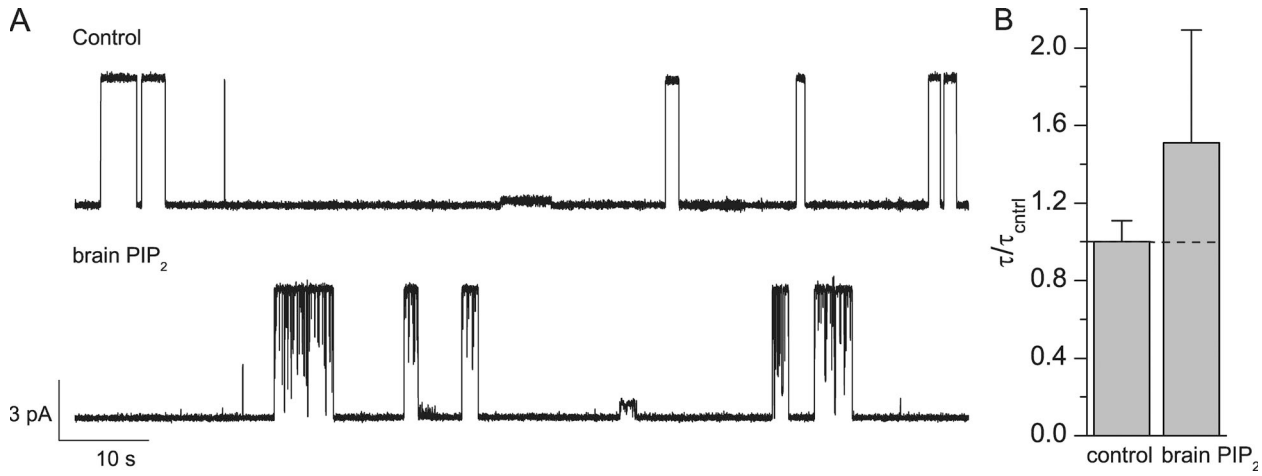

Figure 4. Effects of brain PIP_2 on *N*-Me-gA(15) channel function. (A) Current traces of *N*-Me-gA in mixed bilayers with 2 mol % brain PI(4.5)P_2. (B) The relative changes in τ in bilayers containing 2 mol % brain PIP_2 relative to $DC_{18:1}PC$ bilayers without brain PIP_2. Error bars represent SD; $n = 2–5$.

(control) bilayers (Fig. 3, A and C). Both long-chain PIP_2s thus alter lipid bilayer properties at physiologically relevant mole percentages.

In addition to altering the single-channel lifetimes, the long-chain PIP_2s caused flickering channel appearances (Fig. 2), with no change in the current through the fully conducting state. The flickers appear as rapid transitions into and out of one or more subconductance states, meaning that they reflect partial pore block rather than gA dimer dissociation. We do not understand how the long-chain PIP_2s cause these conductance transitions, but they are unlikely to result from interactions between the indole NH groups and phosphate oxygens in the inositol bisphosphate head groups (Kim et al., 2012), as 2 mol % brain PIP_2 produced similar effects in *N*-Me-gA(15) channels (Fig. 4, A and B), where the indole residues cannot function as hydrogen bond acceptors (Sun et al., 2008). These transitions were not examined further, but the overall behavior is similar for $gA^-(13)$ and AgA(15) channels and is reminiscent of the behavior induced by the green tea catechins (Ingólfsson et al., 2011).

Exogenous addition of PIP_2s. Because experiments with phosphoinositides often are done using exogenous addition, we examined the effect of brain PIP_2 and $diC_{18:1}PIP_2$ as well as diC_8PIP_2 when added through the aqueous solution. None of the PIP_2s altered the single-channel current amplitudes of $gA^-(13)$ or AgA(15) channels, indicating that they do not interact directly with, or accumulate in the vicinity of, the channels (Figs. 5 and 6, A and B).

(The small differences in the $gA^-(13)$ and gA(15) current amplitudes in the presence of the PIP_2s relative to

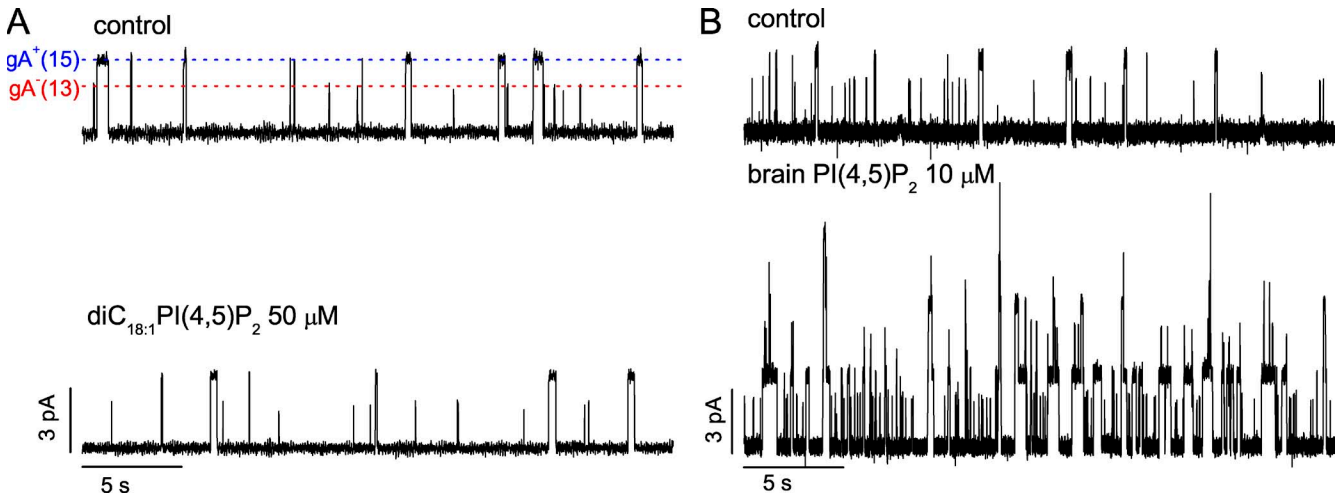

Figure 5. Testing for bilayer-modifying effects of long- and short-chain PIP_2s using a gA-based single-channel assay. (A) $gA^-(13)$ and AgA(15) single-channel current traces without (top) and with (bottom) 50 μM $diC_{18:1}PIP_2$. (B) AgA(15) and $gA^-(13)$ single-channel current traces without (top) and with (bottom) 10 μM brain PIP_2. The current amplitudes of $gA^-(13)$ and AgA(15) channels are indicated by red and blue dotted lines, respectively.

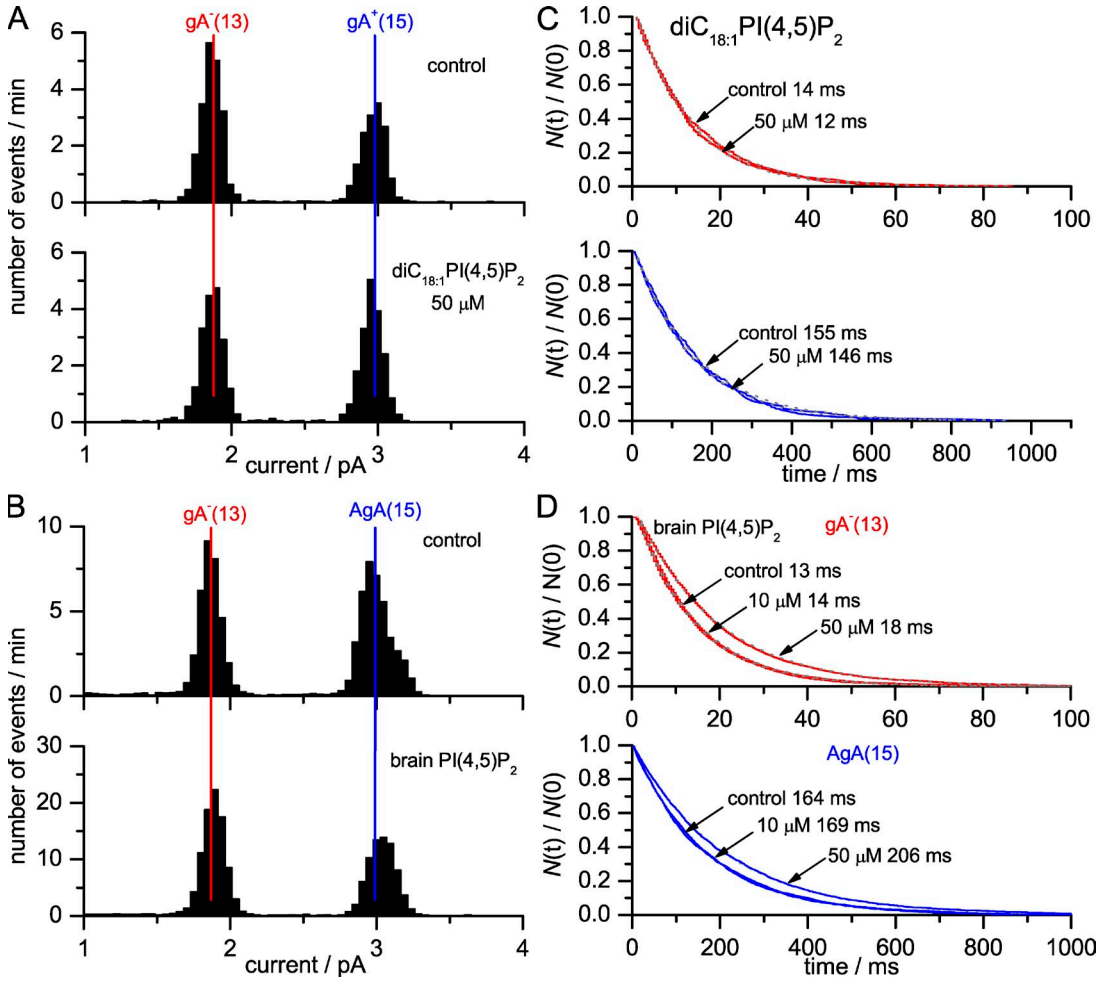

Figure 6. Effects of long- and short-chain PIP_2s on gA single-channel function. (A and B) Single-channel current transition amplitude distributions of $gA^-(13)$ and AgA(15) channels before and after additions of 50 μM $diC_{18:1}PIP_2$ (A) and 50 μM brain PIP_2 (B). The vertical red and blue lines denote the mean current transition amplitudes in the absence of PIP_2. (C and D) Lifetime distributions of $gA^-(13)$ (red lines) and AgA(15) (blue lines) channels in the presence of increasing concentrations of $diC_{18:1}PIP_2$ (C) and brain PIP_2 (D); the lifetime distributions are fit with single exponential distributions, $N(t)/N(0) = \exp\{-t/\tau\}$ (dashed gray lines) where $N(t)$ is the number of channels with lifetimes longer than time t and τ is the gA single-channel lifetime. The τs from the fits are noted in the plots.

controls are variable and not statistically significant.) Nor do the long-chain PIP_2s produce the flickering behavior that was observed in the mixed $PIP_2/DC_{18:1}PC$ bilayers (Fig. 5). Surprisingly, given the results in Figs. 2 and 3, $diC_{18:1}PIP_2$ had no effect on f or τ for either channel type (Figs. 5; 6, A and C; and 7, A and B).

Both diC_8 and brain PIP_2 increased the single-channel lifetimes, and, when added at the same nominal concentrations, the lifetime increases relative to control were similar for diC_8 and brain PIP_2 (Fig. 7 A). diC_8PIP_2 produced no changes in the appearance frequencies of $gA^-(13)$ channels and only small changes in AgA(15) channels, whereas brain PIP_2 increased f for both channel types (Fig. 7 B), with the larger changes in f being observed for the longer AgA(15) channels. It is surprising that both brain PIP_2 and diC_8PIP_2 produce larger changes in f for gA(15) as compared with $gA^-(13)$ channels, but the greater effect of brain PIP_2 on f than on τ can be understood by noting that the bilayer needs to thin by >1 nm to reach the transition state for channel formation, whereas the two subunits need to move apart by only a small distance to reach the transition state for channel dissociation (Greisen et al., 2011).

The changes in $\Delta G^{M\to D}_{bilayer}$ are modest (Fig. 7 C) but of sufficient magnitude to alter gA channel function. We also evaluated the changes in $\Delta G^{M\to D}_{bilayer}$ for *N*-Me-gA(15) channels, where $\Delta\Delta G^{M\to D}_{bilayer}$ was −2.3 kJ/mol at 50 μM $diC_8PI(4,5)P_2$ (1,2-dioctanoyl-*sn*-glycero-3-phospho-(1′-myo-inositol-4′,5′-bisphosphate; Fig. S1).

As was observed previously with other amphiphiles (Lundbæk et al., 2010b), the changes in the $gA^-(13)$ single-channel lifetimes (expressed as $\ln\{\tau_{13}/\tau_{13,cntrl}\}$) vary as a linear function of the changes in the AgA(15) single-channel lifetimes (expressed as $\ln\{\tau_{15}/\tau_{15,cntrl}\}$; Fig. 7 D). The slope is 1.30 ± 0.08 (R = 0.996) and not significantly different from a slope of 1. Thus, though

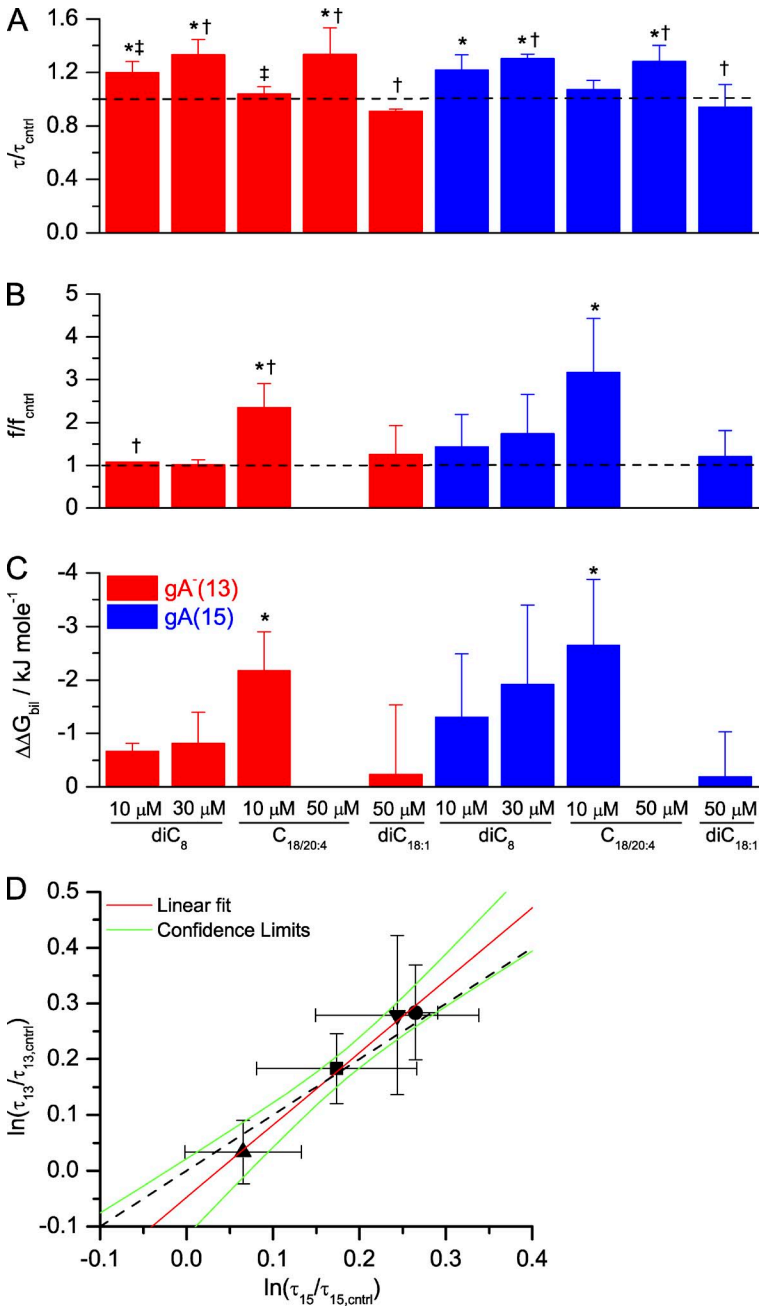

Figure 7. Effects of long- and short-chain PIP_2s on gA single-channel function. (A and B) Normalized changes in τ (A) and *f* (B) of $gA^-(13)$ (red bars) and AgA(15) (blue bars) channels produced by diC_8PIP_2, brain PIP_2, and $diC_{18:1}PIP_2$. (A) †, in the presence of 30 μM diC_8 or 50 μM brain PIP_2, τ/τ_{cntrl} is significantly >50 μM $diC_{18:1}PIP_2$ ($P \leq 0.05$) for $gA^-(13)$ as well as gA(15); ‡, $gA^-(13)$ τ/τ_{cntrl} in the presence of 10 μM diC_8PIP_2 is significantly >10 μM brain PIP_2. (B) †, $gA^-(13)$ f/f_{cntrl} in the presence of 10 μM diC_8 is significantly >10 μM brain PIP_2. (C) The resulting $\Delta\Delta G_{bil}^{M\rightarrow D}$ values are calculated from the changes in τ and *f* as described in Materials and methods (Eq. 6). We could not determine the changes in *f* at 50 μM brain PIP_2 because of bilayer instability. (A–C) All values are plotted as means ± SD or range; $n = 2$–6. *, means are significantly different from 1 ($P \leq 0.05$). Dotted lines indicate the level where there is no change relative to control (y = 1). (D) Plot of the natural logarithm of τ/τ_{cntrl} of $gA^-(13)$ as a function of τ/τ_{cntrl} of gA(15) channels in the presence of different concentrations of diC_8 and brain PIP_2 (square). The 95% confidence intervals (green lines) indicate that the slope is not significantly different from the slope of 1 (dashed line), suggesting that both short- and long-chain PIP_2s produce larger increases in the lifetimes of $gA^-(13)$ channels than of gA(15) channels.

the relative changes in single-channel appearance frequencies, *f*, and lifetimes, τ, elicited by either PIP_2 species were not significantly different for the longer AgA(15) and the shorter $gA^-(13)$ channels, which would suggest that they primarily alter intrinsic curvature, the aggregate results suggest that they also increase bilayer elasticity (Lundbæk et al., 2010b; Rusinova et al., 2011).

Fluorescence quench studies. We also explored the long-chain PIP_2s' and diC_8PIP_2's bilayer-modifying effects using a gramicidin-based fluorescence assay that takes advantage of the gramicidin channels' permeability to Tl^+, a heavy-ion quencher of the water-soluble ANTS fluorophore. In this assay, changes in the fluorescence quench rate reflect changes in the Tl^+ influx and channel activity (Ingólfsson and Andersen, 2010; Ingólfsson et al., 2010). The gA dimerization constant varies with changes in lipid bilayer properties (Andersen and Koeppe, 2007; Lundbæk et al., 2010a), meaning that one can attribute the changes in quench rate after amphiphile addition to modulation of bilayer properties, which in turn govern the gramicidin monomer↔dimer equilibrium and thus the number of channels in the LUV membrane (Ingólfsson and Andersen, 2010; Rusinova et al., 2011).

Brain PIP_2 and diC_8PIP_2 increased the fluorescence quench rates, whereas $diC_{18:1}PIP_2$ did not alter the quench rates at the tested concentrations (Fig. 8). The results in Fig. 8 are consistent with the electrophysiological

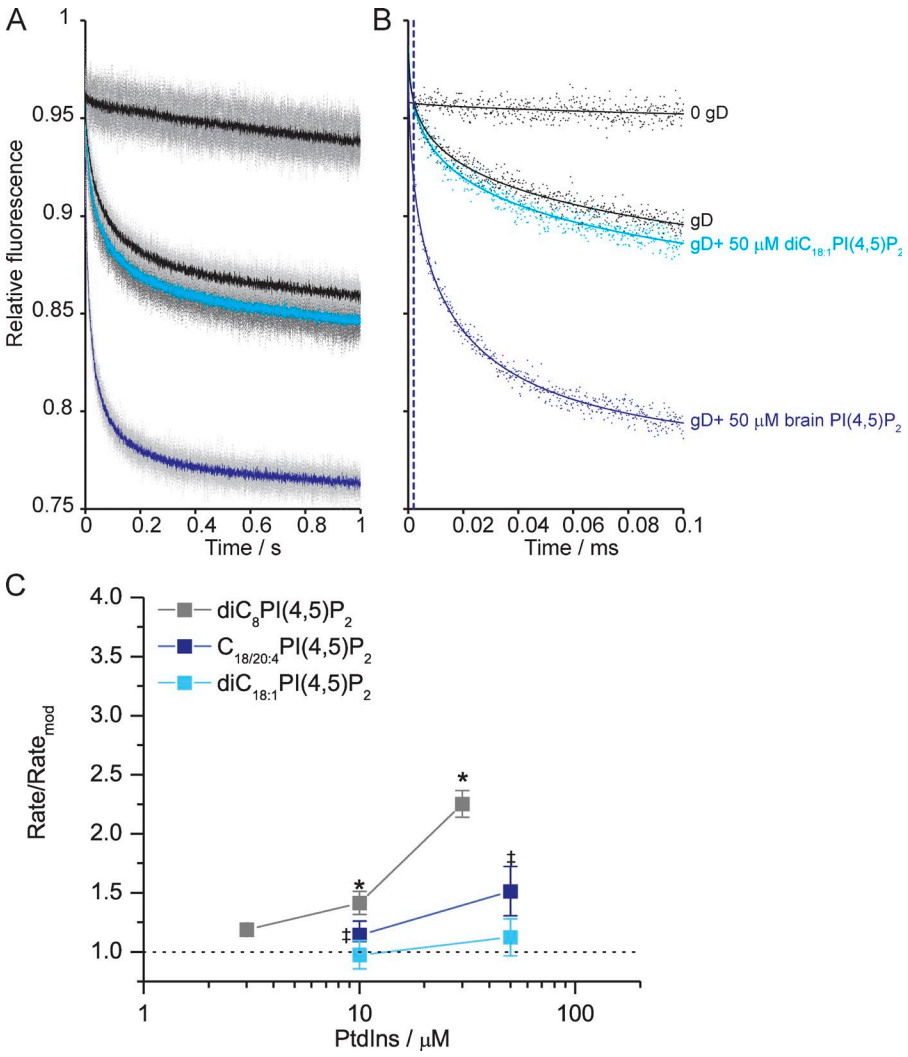

Figure 8. Testing for bilayer-modifying effects of long-chain PIP_2s and diC_8PIP_2 using a gramicidin-based fluorescence assay. (A) Time course of Tl^+ quenching of the ANTS fluorescence in the absence (top trace) and presence (bottom three traces) of gD and in the absence or presence of different PIP_2s. The trace for each condition shows data points from all mixing trials (gray points) and the corresponding mean (black lines, controls; cyan line, 50 μM $diC_{18:1}PIP_2$; navy blue line, 50 μM brain $PI(4,5)P_2$). (B) Fluorescence quench traces from a single mixing trial for each condition in A. The points are colored like the corresponding mean traces in A; the solid lines denote fits of a stretched exponential to the data,

$$F(t) = F(\infty) + (F(0) - F(\infty)) \times \exp\left\{-(t/\tau_0)^{\beta}\right\},$$

where $F(t)$ is fluorescence intensity at time t, β is a measure of sample dispersity ($0 < \beta \leq 1$), and τ_0 is a parameter with units of time (Berberan-Santos et al., 2005). (C) Normalized fluorescence quench rates in gD-doped vesicles in the presence of $diC_{18:1}$ (cyan) and brain (navy blue) $PI(4,5)P_2$ (means ± SD; n = 3–6). Data for $diC_8PI(4,5)P_2$ (gray) from Fig. 9 C is included for comparison. Quench rates for each mixing reaction was evaluated at 2 ms as $k(t) = (\beta/\tau_0) \times (t/\tau_0)^{(\beta-1)}$ (Berberan-Santos et al., 2005). The mean quench rate for each set of mixing reactions in the presence of the PIP_2s was normalized to that in the absence of phosphoinositide, and the resulting normalized quench rates are plotted. *, statistically significant difference between diC_8PIP_2 and both $diC_{18:1}$ and brain $PI(4,5)P_2$; ‡, statistically significant difference between $diC_{18:1}$ and brain $PI(4,5)P_2$. Statistical analysis was done by two-tailed, independent t test ($P < 0.05$). (B and C) Dotted lines indicate the level where there is no change relative to control (y = 1).

results (Figs. 5–7), yet the different bilayer-modifying potencies of $diC_{18:1}PIP_2$ and brain PIP_2 are surprising because bilayer partition coefficients of fatty acids increase with increasing acyl chain saturation (Yokoyama and Nakagaki, 1993; Richieri et al., 1999). One would expect that the same would apply for $diC_{18:1}PIP_2$, compared with its polyunsaturated counterpart brain PIP_2 that mostly is $C_{18:0/20:4}PIP_2$. It is not clear, however, whether this reasoning should apply to situations in which the PIP_2s are micellar because the free energy of transfer between micelles and bilayers is likely to be modest (Bullock and Cohen, 1986; Heerklotz and Seelig, 2000). The observation that brain PIP_2 was more effective than $diC_{18:1}PIP_2$ thus suggests that the exchange between micelles and the bilayer is slower for $diC_{18:1}PIP_2$ than for brain PIP_2. Nevertheless, longer incubation times of vesicles with either long-chain PIP_2 did not alter the quench rates. (The relative changes in quench rates observed with 50 μM brain PIP_2 were 2.7 ± 1 vs. 3.1 ± 1.0; with 50 μM $diC_{18:1}$ PIP_2, they were 1.0 ± 0.1 vs. 1.05 ± 0.03 after 10 and 30 min, respectively). Moreover, although the longer acyl chain lengths would suggest greater partitioning of $diC_{18:1}$ or brain PIP_2 into the bilayer, as compared with the shorter diC_8PIP_2, the dose–response curves for $diC_{18:1}$ or brain PIP_2 were right shifted compared with those of their diC_8 counterpart.

Short-chain phosphoinositides

The short-chain (diC_8) phosphoinositides are relatively water soluble, with critical micelle concentrations (CMCs) in the high micromolar range (Table 1), which makes them easy to handle in functional assays. We therefore examined the bilayer-modifying effects of diC_8 phosphoinositides with different numbers and positions of the phosphate groups on the inositol ring using the fluorescence quench method.

All of the diC_8 phosphoinositides increased gramicidin channel activity, as reflected by the increased quench

TABLE 1

Phospholipid distribution in the membrane single-channel experiments

Compound	CMC	Nominal [PL]		$[PL]_a$		$\{PL\}_m$		Mole $\%_{PL}$	
		LUV	Planar bilayer	LUV	Planar bilayer	LUV	Planar bilayer	LUV	Planar bilayer
	μM	*μM*	*μM*	*μM*	*μM*	*moles/cm²*	*moles/cm²*	*mole %*	*mole %*
diC_8PC	270[a]	30	3	15	1	2×10^{-11}	1×10^{-12}	6	0.4
diC_8PE	230[b]	30	3	10	1	2×10^{-11}	1×10^{-12}	8	0.4
diC_8PI	500[c]	30	3	20	1.5	1×10^{-11}	7×10^{-13}	4	0.3
diC_8PS	500[a]	30	3	20	1.5	1×10^{-11}	7×10^{-13}	4	0.3
brain PIP_2	30[d]	50	50	5	3	4×10^{-11}	2×10^{-11}	15	9.0

The aqueous phospholipid concentrations and their surface densities and mole percentage in the membrane ($[PL]_a$, $\{PL\}_m$, and mole $\%_{PL}$, respectively) were calculated according to Bruno et al. (2007) and Rusinova et al. (2011) using 1.2/CMC as an estimate for the (dimensionless) partition coefficient into the membrane (Bullock and Cohen, 1986); the total phospholipid concentration in the membrane was taken to be 1.2 M. This may overestimate the partition coefficient and mole $\%_{PL}$, as Heerklotz and Seelig (2000) found the partition coefficient for diC_7PC to be threefold less than predicted using the above estimator. (The difference between the mole fractions in the LUVs vs. the planar bilayer is a result of the different amount of lipids in the different systems.)

[a]Zhou et al., 1997.

[b]Estimated from the CMC for diC_6PE, 12 mM (Zhou et al., 1997), and scaling by the ratio of the CMC for diC_8PC over the CMC for diC_6PC.

[c]Wang et al., 2008.

[d]Palmer, 1981; Huang and Huang, 1991; Moens and Bagatolli, 2007.

rate, with PI being the most active and $PI(3,4,5)P_3$ and $PI(3,5)P_2$ the least bilayer active: $PI > PI(4,5)P_2 = PI(3,4)P_2 > PI(3,4,5)P_3 \geq PI(3,5)P_2$ (Fig. 9).

The concentrations at which we observed changes in bilayer properties varied from 10 μM for PI, $PI(3,4)P_2$, and $PI(4,5)P_2$ to 150 μM for $PI(3,5)P_2$ and $PI(3,4,5)P_2$. Overall, the potency decreased with increasing head group charge (compare with Fig. 1), as would be expected if the short-chain phosphoinositides' bilayer-modifying potencies depended on their ability to partition into the bilayer–solution interface. Though the formal charge on $PI(3,5)P_2$ is the same as that on $PI(4,5)P_2$ and $PI(3,4)P_2$, its bilayer-modifying potency was less than that of $PI(3,4,5)P_3$, suggesting that the phosphate configuration around the inositol ring modulates the interaction with the bilayer. Unlike the adjacent phosphates in $PI(3,4)P_2$ or $PI(4,5)P_2$, the distant phosphates in $PI(3,5)P_2$ may prevent the phosphoinositol ring from inserting as far into the bilayer (Redfern and Gericke, 2005), resulting in weaker bilayer effects.

Other short-chain phospholipids

Fluorescence quench studies. For comparison with the short-chain phosphoinositides, we examined other short-chain phospholipids—diC_8PS (1,2-dioctanoyl-*sn*-glycero-3-phospho-L-serine), -PE, and -PC. All three alter lipid bilayer properties with similar dose–response curves (Fig. 10); they have similar bilayer-modifying potencies even though their head groups vary in size and charge.

The bilayer partition coefficients of surfactants can be estimated from their CMCs (Bullock and Cohen, 1986; Heerklotz and Seelig, 2000). (In case CMCs are not available, the partition coefficient of a neutral amphiphile can be estimated from the logarithms of the calculated water/octanol partitioning ratio (clogP; Escher and Schwarzenbach, 1995; Avdeef, 2001; Seydel and Wiese, 2002). Based on the estimated mole percentage of each phospholipid in the LUV membranes (Table 1) and the dose–response curves (Fig. 10), the zwitterionic diC_8PC and -PE have similar bilayer-modifying potencies, whereas the anionic diC_8PI and -PS are more potent.

Single-channel electrophysiology. The short-chain diC_8PC (1,2-dioctanoyl-*sn*-glycero-3-phosphocholine), -PE, -PS, and -PI vary also in their effects on planar bilayers, with diC_8PS and diC_8PE eliciting the largest channel lifetime increases, and diC_8PC causing the least change (Fig. 11 A).

Although the changes are small, the linear relationship between the natural logarithm of the AgA(15) and $gA^-(13)$ single-channel lifetimes, in the presence of diC_8 phospholipids, has a slope of 1.16 ± 0.05, $R = 0.994$ (Fig. 11 D), suggesting that they do increase bilayer elasticity (Lundbæk et al., 2010b), in addition to changing the curvature (Rusinova et al., 2011). Except for the effects of diC_8PS on AgA(15) channels, the relative changes in f (Fig. 11 B) were larger than the changes in τ. At the highest concentration tested, none of the phospholipids (whether anionic or zwitterionic) altered the gA single-channel current transition amplitudes. That the anionic phospholipids do not alter the single-channel current transition amplitudes is in striking contrast to the results obtained with free fatty acids (Bruno et al., 2007, 2013).

DISCUSSION

The mechanisms by which phosphoinositides alter membrane protein function remain enigmatic in the sense that, for many proteins, it is unclear to what extent the

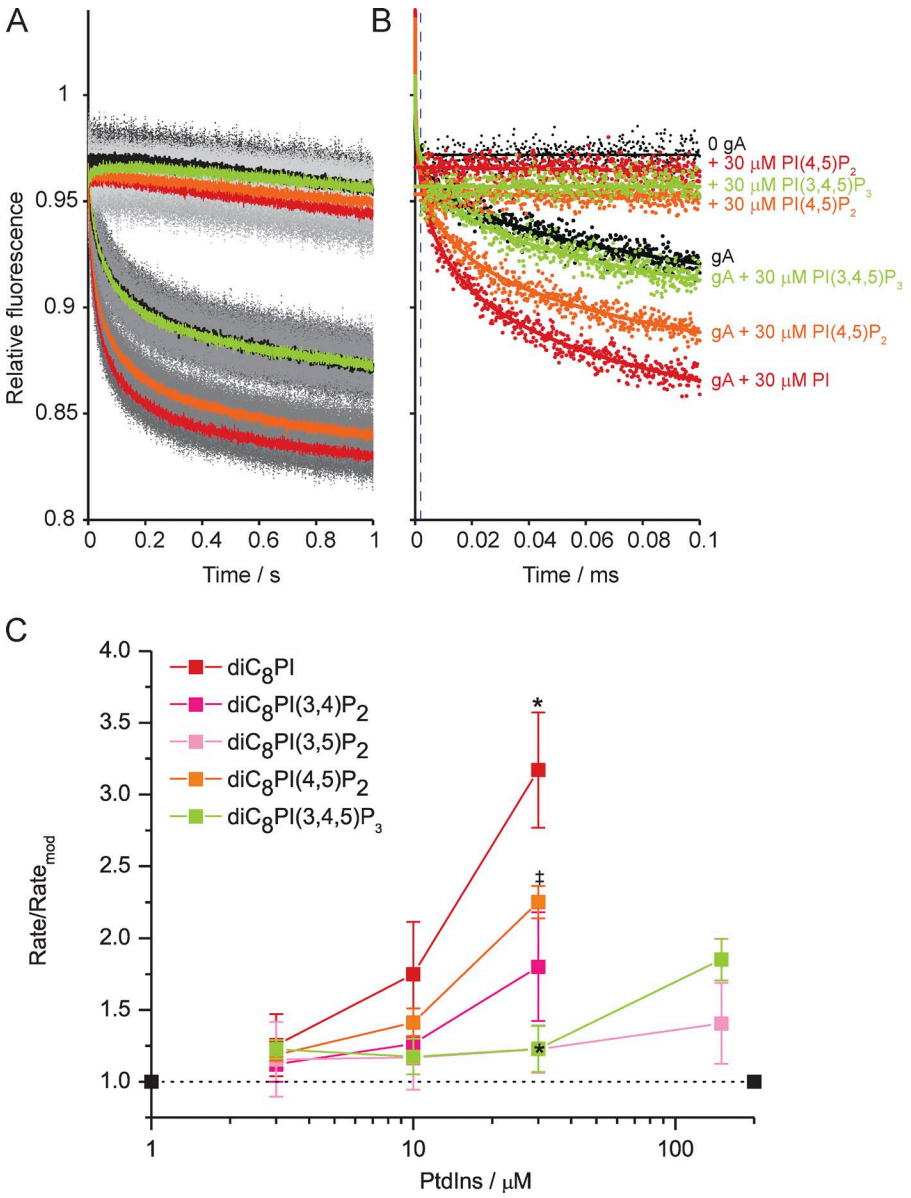

Figure 9. Testing for bilayer-modifying effects of short-chain phosphoinositides using a gA-based fluorescence quench assay. (A) Time course of Tl^+ quenching of the ANTS fluorescence in the absence (top four traces) and presence (bottom four traces) of gA and in the absence or presence of different short-chain phosphoinositides. The trace for each condition shows data points from all mixing trials (gray points) and the corresponding mean (black lines, controls; red lines, 30 μM diC_8PI; orange lines, 30 μM $diC_8PI(4,5)P_2$; green lines, 30 μM $diC_8PI(3,4,5)P_3$). (B) Fluorescence quench traces from a single mixing trial for each condition in A. The points are colored like the corresponding mean traces in A; the solid lines denote fits of a stretched exponential to the data. (C) Normalized fluorescence quench rates, evaluated at 2 ms, in gD-doped vesicles in the presence of phosphoinositides. The mean quench rate for each set of mixing reactions in the presence of phosphoinositides was normalized to that in the absence of phosphoinositide, and the resulting normalized quench rates are plotted (means ± SD; n = 3–6). *, statistically significant difference between diC_8PI or $diC_8PI(3,4,5)P_2$ and both $diC_8PI(4,5)P_2$ and $diC_8PI(3,4)P_2$ at 30 μM; ‡, statistically significant difference between $diC_8PI(4,5)P_2$ and $diC_8PI(3,4)P_2$ at 30 μM. $diC_8PI(3,4,5)P_2$ and $diC_8PI(3,5)P_2$ are not significantly different at 30 μM. (B and C) Dotted lines indicate the level where there is no change relative to control (y = 1). Statistical analysis was done by two-tailed, independent t test ($P < 0.05$).

phosphoinositides act through direct binding to their membrane protein effectors, as opposed to altering lipid bilayer properties or some other indirect mechanism that in turn will alter the energetics of protein conformational transitions. We used gramicidin-based fluorescence quenching and single-channel assays to determine whether various polyphosphoinositide isoforms alter lipid bilayer properties and found that they do so at physiologically relevant concentrations (mole fractions in the membrane).

First, we highlight that bilayer modulation by PIP_2 occurs at "relevant" concentrations and consider what bilayer properties are affected. Next, we discuss implications of our findings in view of what is known about PIP_2 and its mechanism of action. Finally, we discuss and compare the bilayer-modifying effects of PIP_2 to those of isoforms with different phosphate group organizations as well as acyl chain variants and other phospholipids.

The PIP_2 modulation of bilayer properties occurs at relevant concentrations

The PIP_2 concentrations tested here are comparable with those used in cell physiological experiments. Following Rusinova et al. (2011), we analyzed the bilayer contribution to the free energy of gA channel formation (Fig. 7 C) when the PIP_2 analogues are applied through the aqueous phase and found that the changes are modest: $\Delta\Delta G_{bil}^{M\to D} \leq k_B T$ (= 2.5 kJ mol^{-1} at 25°C). (This estimate pertains to gA channels in a relatively soft bilayer; $\Delta\Delta G_{bil}^{I\to II}$ for an integral membrane protein may be larger or smaller, depending on the protein radius, shape, and the host bilayer material properties.) Both long- and short-chain polyphosphoinositides have little effect on bilayer properties at 3 μM (nominal concentration; Figs. 7 and 9), meaning that functional effects of long-chain polyphosphoinositides that occur at or below the micromolar range are unlikely to be bilayer mediated. In contrast,

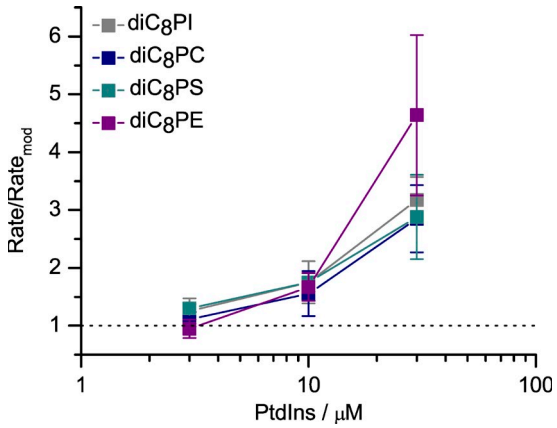

Figure 10. Testing for bilayer-modifying effects of short-chain phospholipids using the gA-based fluorescence assay. Normalized changes in quench rates produced by the diC_8 phospholipids (means ± SD; n = 3–5). Data for diC_8PI (gray line) from Fig. 2 C is included for comparison. Means of different diC_8 phospholipids were not significantly different, with the exception of diC_8PC and diC_8PE at 30 μM. The dotted line indicates the level where there is no change relative to control (y = 1).

effects that are observed only at concentrations ≥10 μM may, at least in part, be caused by altered bilayer properties. Many studies have used much higher [diC_8PIP_2] (Runnels et al., 2002; Zhang et al., 2003; Lopes et al., 2005; Rohács et al., 2005; Hernandez et al., 2008), in which case it becomes important to consider the possibility of membrane effects.

Implications for PIP_2 targets

PIP_2-sensitive channels vary considerably in their phosphate isomer specificity (Fan and Makielski, 1997; Rohács et al., 2003; Zhang et al., 2003; Rohács, 2007), and the binding pockets in PIP_2-binding membrane-spanning proteins vary greatly, meaning that the definition of binding sites for PIP_2 becomes ambiguous (Suh and Hille, 2008). Other than requirements for positive and aromatic residues, the binding sites are not clearly defined (Rosenhouse-Dantsker and Logothetis, 2007; Suh and Hille, 2008), however, and mutations of specific residues, or even entire polybasic regions, may modulate but not eliminate the PIP_2 effects (Rohács et al., 2003; Zhang et al., 2003; Rohács, 2007; Vaithianathan et al., 2008). These ambiguities suggest that some PIP_2 effects may be the result of less specific interactions, such as altered energetic coupling between the target protein and the host bilayer. Indeed, nonphosphoinositide phospholipids can alter channel function if applied at high concentrations from 0.01 to 1 mM (Fan and Makielski, 1997; Kim and Bang, 1999; Hilgemann et al., 2001; Zhang et al., 2003; Vaithianathan et al., 2008). Importantly, the PIP_2 abundance percentage in the inner leaflet of the plasma membrane is on the order of 1%, perhaps higher (Ferrell and Huestis, 1984; Balla and Várnai, 2002; Nasuhoglu et al., 2002; Wenk et al., 2003; Xu et al., 2003; Horowitz et al., 2005; Li et al., 2005; Hilgemann, 2007), and PIP_2 alters gramicidin channel stability at 2 mol % (Fig. 2). Thus, physiological depletion or enrichment of PIP_2 in the inner leaflet of the plasma membrane (Nasuhoglu et al., 2002; Xu et al., 2003; Horowitz et al., 2005; Li et al., 2005; Suh and Hille, 2005), whether global or local, is likely to be associated with changes in lipid bilayer properties.

Local PIP_2 enrichment may be considerable, as there are 70–80 phospholipids in the first two lipid shells around a bilayer-spanning protein of 3-nm radius. If one PIP_2 molecule is required to alter protein function, the local PIP_2 mole percentage would be ∼1; if four PIP_2s were needed, the mole percentage would be ≥5! PIP_2 binding to its target proteins thus may alter the local bilayer environment (the bilayer material properties), and the question becomes, what are the relative energetic contributions of specific binding and nonspecific bilayer-mediated effects on protein function?

Whether bilayer-mediated effects become important will depend on the affinity of PIP_2 (or other ligands) for the protein. Some ion channels are regulated at very low PIP_2 concentrations, whereas other PIP_2 targets require higher concentrations, as reflected in their regulation by physiological stimulation of PIP_2 hydrolysis (Huang et al., 1998; Kobrinsky et al., 2000; Runnels et al., 2002; Lopes et al., 2002; Zhang et al., 2003; Rohács et al., 2005; Rohács, 2007). In the case of Kir channels, the PIP_2 affinity is inversely correlated with the channels' susceptibility to regulation by PIP_2 isomers and other molecules (Zhang et al., 1999; Schulze et al., 2003; Lee et al., 2005; Rosenhouse-Dantsker and Logothetis, 2007). Thus, channels with lower affinity for PIP_2 are likely to be more sensitive to their environment, in which case dynamic changes in the environment (including PIP_2-induced changes in bilayer properties) may be important for function. These features may be tested by exposing the channels to short-chain nonphosphoinositide phospholipids, which modify the bilayer (Fig. 11 C) and thus allow for a positive control of sensitivity to changes in lipid bilayer properties.

Bilayer-modifying effects of different polyphosphoinositide isoforms and short-chain phospholipids

Results obtained using the gramicidin-based fluorescence assay show that all compounds tested increase the ANTS fluorescence quench rates because they shift the gA monomer↔dimer equilibrium toward the conducting dimers, indicating that each of them alters lipid bilayer properties, albeit with different potencies.

A compound's apparent bilayer-modifying potency reflects several factors: its affinity for the bilayer (its partition coefficient), which relates the concentration of monodisperse molecules in solution to the surface density or volume concentration in the bilayer; the state of the compound in solution, where micelle or other aggregate formation will influence the number of monomeric

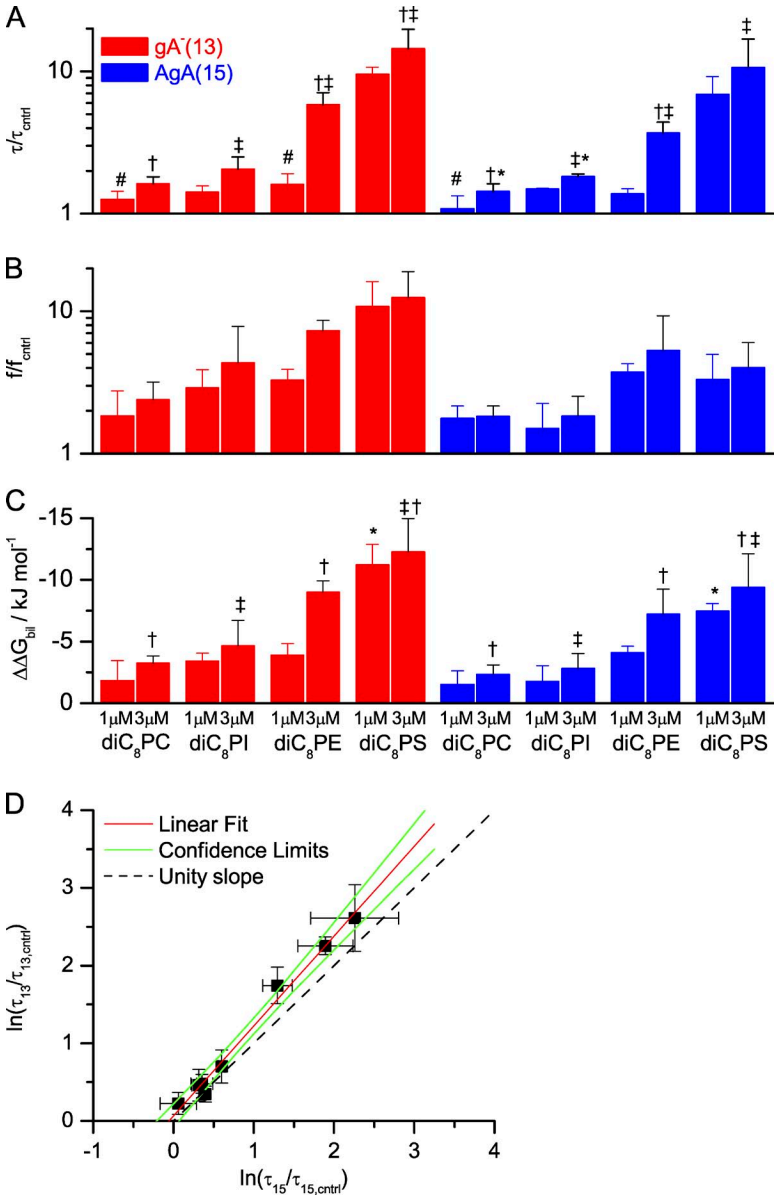

Figure 11. Effects of short-chain phospholipids on gA single-channel activity. (A and B) Normalized changes in τ (A) and *f* (B) for $gA^-(13)$ (red bars) and gA(15) (blue bars) channels in the presence of diC_8PC, diC_8PI, diC_8PE, and diC_8PS at the indicated concentrations. (A) #, not significantly different from 1. (C) The resulting $\Delta\Delta G_{bil}^{M\rightarrow D}$ values are calculated from the changes in τ and *f* as described in Materials and methods (Eq. 6). (A–C) All values are plotted as means ± SD or range (n = 2–4). *, †, and ‡ denote statistically significant differences between means ($P < 0.05$). There is no significant difference between diC_8PE and diC_8PS. (D) Plot of the natural logarithm of τ/τ_{cntrl} of $gA^-(13)$ as a function of τ/τ_{cntrl} of gA(15) channels in the presence of different concentrations of diC_8 phospholipids (squares). The 95% confidence intervals (green lines) indicate that the slope is significantly different from 1.0 (dashed line), indicating that both of the short-chain phospholipids produce relatively larger increases in the lifetimes of the $gA^-(13)$ channels as compared with the gA(15) channels.

molecules available to partition into the bilayer (see last paragraph of Discussion); and the ability of a molecule in the bilayer to alter bilayer properties. The apparent potencies of the short-chain phosphoinositides decrease with increasing charge, consistent with the expected influence of charge to lower the partition coefficient into lipid bilayers (Peitzsch and McLaughlin, 1993). In the LUV experiments, the short-chain nonphosphoinositide phospholipids and diC_8PI have similar potencies. The similar effects of the charged and zwitterionic phospholipids could be caused by the combined effect of a decreased partition coefficient (Marsh and King, 1986; Peitzsch and McLaughlin, 1993; Marsh, 2012a) and a larger effective head group size (Israelachvili et al., 1980; Kleinschmidt and Tamm, 2002) for the charged phospholipids. Thus, molecule per molecule, the charged phospholipids would be expected to have increased bilayer-modifying potency (Lundbæk et al., 1997; see also Lundbæk and Andersen, 1994; Bruno et al., 2013). In the case of the polyphosphoinositides, the decreased potencies, relative to diC_8PI (compare Fig. 9 with Fig. 10), may be the result of a dominant effect of the charge-dependent decrease in the partition coefficient.

Using isothermal titration calorimetry, we explored the partitioning of diC_8PI, diC_8PIP_2, and $diC_8PI(3,4,5)P_3$ into lipid vesicles but did not observe heats of partitioning (unpublished data), either because the partition coefficients are too low to be measured, as suggested from the CMCs (Table 1 legend), or because the partitioning is primarily entropy driven. In any case, although the head group charges make it challenging to estimate the mole fractions of PI, PIP_2, and PIP_3 in the bilayer (they preclude using clogP for the estimate), the decreasing bilayer-modifying potency with increasing charge on the

inositol ring suggests that the differences in potency are caused mainly by differences in bilayer partitioning.

The bilayer-modifying potencies of the long-chain PIP_2 and the short-chain phosphoinositides and other phospholipids varied with the experimental system, but there was overall concordance between the results obtained in planar bilayers and LUVs, indicating that bilayer-modifying effects are little affected by changes in ionic strength (and the associated changes in ionization) or the presence of decane. The key difference we observed was with the long-chain PIP_2s. When added through the aqueous solution, brain PIP_2 altered bilayer properties, whereas $diC_{18:1}PIP_2$ did not—and $diC_{18:1}PIP_2$ remained inactive after prolonged (30 min) incubation. This difference may be important for the design and interpretation of experiments in biological membranes. To gain further insight into these observations, we attempted to determine the partition coefficients of the long-chain PIP_2s by separating LUV-partitioned PIP_2 from micellar PIP_2 using ultracentrifugation and assessing the PIP_2 concentrations by phosphorus assay (Chen et al., 1956). The density of PIP_2 micelles, however, allows them to sediment with LUVs (unpublished data), precluding separation of LUVs from PIP_2 micelles by centrifugation.

To determine how the short- and long-chain phospholipids alter lipid bilayer properties, we sought to use gA single-channel recordings to explore whether the effects could be ascribed to changes in bilayer elasticity or curvature. (Long-chain PIP_2s also may alter the bilayer lateral organization [Wang and Richards, 2012], but we have discovered no evidence for lipid de-mixing.) Both long- and short-chain PIP_2s produce modest relative changes in f and τ. Even when comparing the lifetime changes observed with the $gA^-(13)$ and AgA(15) channels (Figs. 7 D and 11 D), we cannot unambiguously distinguish between PIP_2 effects on curvature and elasticity. Furthermore, it remains unclear to what extent the electrostatic interactions among the PIP_2 head groups, which would tend to increase the effective head group size (Kleinschmidt and Tamm, 2002), would influence the intrinsic curvature (Lundbæk et al., 1997) and maybe also the head group hydration. Importantly, the PIP_2s had no effect on the gA single-channel current amplitudes, indicating that their mole percentage in the first lipid shell around the channel was low (compare the contrasting results obtained with other anionic compounds; Lundbæk et al., 1997; Bruno et al., 2007). Though not conclusive evidence, this suggests that electrostatic effects are likely to be of minor importance at physiological mole percentages.

In contrast, diC_8PC, -PI, -PS, and -PE produce greater increases in f and τ for the short, as compared with the long, gA channels, indicating that short-chain nonpolyphosphoinositide phospholipids increase bilayer elasticity. The results, nevertheless, do not rule out further effects of lipid curvature (Lundbæk et al., 2010a,b). In contrast to the LUV experiments, in which the short-chain phospholipids had similar potencies, the bilayer-modifying rank-order potency in the planar bilayer experiments was PS ≥ PE > PI ≥ PC, which cannot be accounted for simply by different partitioning (Table 1). The different rank-order potencies in the LUVs and planar bilayers, as well as the differences observed for short-chain phospholipids described here and long-chain lysophospholipids from a previous study (Lundbæk and Andersen, 1994), reflect the compounds' detailed molecular properties, including the effective cross-sectional areas of the acyl chains relative to the head groups—as well as the lengths of their acyl chains relative to those of the bilayer-forming lipids in the host bilayer (Aagaard et al., 2006; Ingólfsson and Andersen, 2011). Ingólfsson and Andersen (2011), in particular, found that the sign of the effect of an *n*-alkyl alcohol on lipid bilayer properties varied with the *n*-alkyl length (short-chain alcohols increased gA channel dimerization; long-chain alcohols decreased dimerization). The chain length at which the transition occurred furthermore varied with the host bilayer phospholipid acyl chain length. The relative differences in bilayer-modifying potency observed in the stopped-flow and single-channel experiments thus may differ (see also Ingólfsson and Andersen, 2010).

The different rank-order potencies of the short- and long-chain PIP_2s in single-channel versus fluorescence assays again are likely to reflect different perturbations of the membrane acyl chains. The hydrophobic and electrostatic contributions to the free energy of adsorption at the bilayer–solution interface tend to be additive (Zaslavsky et al., 1978; Israelachvili et al., 1980; Tanford, 1980; King and Marsh, 1987; Buser et al., 1994), meaning that the difference in ionic strength between the fluorescence and single-channel experiments should have similar effects on the adsorption of the long- and short-chain PIP_2s.

The lack of effect of $diC_{18:1}PIP_2$ in the fluorescence and electrophysiological assays, when added through the aqueous phase, is in striking contrast to the effects observed when the compound is added through the lipid phase (compare Figs. 3, 6, and 7). The key difference between these experiments is the elimination of the micelle→water and water→bilayer partitioning steps, which also may have implications for the design and interpretation of cell physiological experiments. For example, the negative correlation between the PIP_2 concentration needed to elicit an effect and the length of the PIP_2 acyl chain (Rohács et al., 1999; Jin et al., 2002; Zhang et al., 2003; Vaithianathan et al., 2008) is often attributed to higher partitioning of PIP_2s with longer acyl chains. Yet, the modest differences between the dose–response curves for diC_8- and brain PIP_2, which cannot be accounted for by the difference in hydrophobicity of their acyl chains, show that the correlation between CMC and bilayer partitioning is complicated—at least in systems containing both micelles and bilayers (see beginning of this

section). The apparent differences in bilayer partitioning of the $diC_{18:1}$ and brain PIP_2 analogues in our assays also may manifest themselves in cell physiological studies, as apparent differences in potency that may not, in fact, have a functional basis.

Conclusions

Both long- and short-chain phosphoinositides are bilayer-modifying molecules in that they can alter their target protein function by a combination of direct (protein binding) and indirect (bilayer mediated) effects. The changes in bilayer properties occur at physiologically relevant concentrations (mole percentage of membrane lipids), in the case of the long-chain PIP_2s, and at pharmacologically relevant concentrations, in the case of short-chain compounds. It thus becomes important to consider the changes in membrane properties resulting from local PIP_2 depletion or enrichment, which may be a contributing mechanism through which PIP_2 alters protein function. Our results suggest a strategy for identifying, or excluding, such nonspecific bilayer effects. Effects at low micromolar concentrations of (long or short chain) PIP_2s are likely to reflect specific interactions with the target protein, whereas effects observed only at 10 µM or higher concentrations are likely to reflect, at least in part, altered bilayer properties. It is furthermore possible to test for nonspecific bilayer effects using not only short-chain phosphoinositides but also other short-chain phospholipids.

This work was supported by National Institutes of Health (NIH) grant GM021342 and American Recovery and Reinvestment Act of 2009 Supplement GM021342-35S1. The peptide facility is supported by NIH grants GM103429 and GM103450.

We thank Denise V. Greathouse for the purified gA analogues and Donald W. Hilgemann, Helgi I. Ingólfsson, Ruchi Kapoor, Jens A. Lundbæk, R. Lea Sanford, and Sidney A. Simon for helpful discussions.

Donald W. Hilgemann served as guest editor.

Submitted: 8 January 2013
Accepted: 6 May 2013

REFERENCES

Aagaard, T.H., M.N. Kristensen, and P. Westh. 2006. Packing properties of 1-alkanols and alkanes in a phospholipid membrane. *Biophys. Chem.* 119:61–68. http://dx.doi.org/10.1016/j.bpc.2005.09.003

Abo-Riziq, A., B.O. Crews, M.P. Callahan, L. Grace, and M.S. de Vries. 2006. Spectroscopy of isolated gramicidin peptides. *Angew. Chem. Int. Ed. Engl.* 45:5166–5169. http://dx.doi.org/10.1002/anie.200601516

Andersen, O.S. 1983. Ion movement through gramicidin A channels. Single-channel measurements at very high potentials. *Biophys. J.* 41:119–133. http://dx.doi.org/10.1016/S0006-3495(83)84414-2

Andersen, O.S., and R.E. Koeppe II. 2007. Bilayer thickness and membrane protein function: an energetic perspective. *Annu. Rev. Biophys. Biomol. Struct.* 36:107–130. http://dx.doi.org/10.1146/annurev.biophys.36.040306.132643

Andersen, O.S., M.J. Bruno, H. Sun, and R.E. Koeppe II. 2007. Single-molecule methods for monitoring changes in bilayer elastic properties. *Methods Mol. Biol.* 400:543–570. http://dx.doi.org/10.1007/978-1-59745-519-0_37

Artigas, P., S.J. Al'aref, E.A. Hobart, L.F. Díaz, M. Sakaguchi, S. Straw, and O.S. Andersen. 2006. 2,3-butanedione monoxime affects cystic fibrosis transmembrane conductance regulator channel function through phosphorylation-dependent and phosphorylation-independent mechanisms: the role of bilayer material properties. *Mol. Pharmacol.* 70:2015–2026. http://dx.doi.org/10.1124/mol.106.026070

Ashrafuzzaman, M., M.A. Lampson, D.V. Greathouse, R.E. Koeppe II, and O.S. Andersen. 2006. Manipulating lipid bilayer material properties using biologically active amphipathic molecules. *J. Phys. Condens. Matter.* 18:S1235–S1255. http://dx.doi.org/10.1088/0953-8984/18/28/S08

Avdeef, A. 2001. Physicochemical profiling (solubility, permeability and charge state). *Curr. Top. Med. Chem.* 1:277–351. http://dx.doi.org/10.2174/1568026013395100

Balla, T., and P. Várnai. 2002. Visualizing cellular phosphoinositide pools with GFP-fused protein-modules. *Sci. STKE.* 2002:pl3.

Benz, R., O. Fröhlich, P. Läuger, and M. Montal. 1975. Electrical capacity of black lipid films and of lipid bilayers made from monolayers. *Biochim. Biophys. Acta.* 394:323–334. http://dx.doi.org/10.1016/0005-2736(75)90287-4

Berberan-Santos, M.N., E.N. Bodunov, and B. Valeur. 2005. Mathematical functions for the analysis of luminescence decays with underlying distributions 1. Kohlrausch decay function (stretched exponential). *Chem. Phys.* 315:171–182. http://dx.doi.org/10.1016/j.chemphys.2005.04.006

Bienvenüe, A., and J.S. Marie. 1994. Modulation of protein function by lipids. *Curr. Top. Membr.* 40:319–354. http://dx.doi.org/10.1016/S0070-2161(08)60986-7

Brown, M.F. 1994. Modulation of rhodopsin function by properties of the membrane bilayer. *Chem. Phys. Lipids.* 73:159–180. http://dx.doi.org/10.1016/0009-3084(94)90180-5

Bruno, M.J., R.E. Koeppe II, and O.S. Andersen. 2007. Docosahexaenoic acid alters bilayer elastic properties. *Proc. Natl. Acad. Sci. USA.* 104:9638–9643. http://dx.doi.org/10.1073/pnas.0701015104

Bruno, M.J., R. Rusinova, N.J. Gleason, R.E. Koeppe II, and O.S. Andersen. 2013. Interactions of drugs and amphiphiles with membranes: modulation of lipid bilayer elastic properties by changes in acyl chain unsaturation and protonation. *Faraday Discuss.* 161:461–480. http://dx.doi.org/10.1039/c2fd20092a

Bullock, J.O., and F.S. Cohen. 1986. Octyl glucoside promotes incorporation of channels into neutral planar phospholipid bilayers. Studies with colicin Ia. *Biochim. Biophys. Acta.* 856:101–108. http://dx.doi.org/10.1016/0005-2736(86)90015-5

Buser, C.A., C.T. Sigal, M.D. Resh, and S. McLaughlin. 1994. Membrane binding of myristylated peptides corresponding to the NH2 terminus of Src. *Biochemistry.* 33:13093–13101. http://dx.doi.org/10.1021/bi00248a019

Cantor, R.S. 1997. Lateral pressures in cell membranes: a mechanism for modulation of protein function. *J. Phys. Chem. B.* 101:1723–1725. http://dx.doi.org/10.1021/jp963911x

Chen, P.S., T.Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* 28:1756–1758. http://dx.doi.org/10.1021/ac60119a033

Dan, N., A. Berman, P. Pincus, and S.A. Safran. 1994. Membrane-induced interactions between inclusions. *J. Phys. II (France).* 4:1713–1725. http://dx.doi.org/10.1051/jp2:1994227

Dubos, R.J. 1939. Studies on a bactericidal agent extracted from a soil bacillus: I. Preparation of the agent. Its activity in vitro. *J. Exp. Med.* 70:1–10. http://dx.doi.org/10.1084/jem.70.1.1

Elliott, J.R., D. Needham, J.P. Dilger, and D.A. Haydon. 1983. The effects of bilayer thickness and tension on gramicidin single-channel lifetime. *Biochim. Biophys. Acta.* 735:95–103. http://dx.doi.org/10.1016/0005-2736(83)90264-X

Escher, B.I., and R.P. Schwarzenbach. 1995. Partitioning of substituted phenols in liposome-water, biomembrane-water, and octanol-water systems. *Environ. Sci. Technol.* 30:260–270. http://dx.doi.org/10.1021/es9503084

Evans, E., W. Rawicz, and A.F. Hofmann. 1995. Lipid bilayer expansion and mechanical disruption in solutions of water-soluble bile acid. *In* Bile Acids in Gastroenterology: Basic and Clinical Advances. A.F. Hofmann, G. Paumgartner, and A. Stiehl, editors. Kluwer Academic Publishers, Dordrecht. 59–68.

Fan, Z., and J.C. Makielski. 1997. Anionic phospholipids activate ATP-sensitive potassium channels. *J. Biol. Chem.* 272:5388–5395. http://dx.doi.org/10.1074/jbc.272.9.5388

Ferrell, J.E. Jr., and W.H. Huestis. 1984. Phosphoinositide metabolism and the morphology of human erythrocytes. *J. Cell Biol.* 98:1992–1998. http://dx.doi.org/10.1083/jcb.98.6.1992

Greathouse, D.V., J.F. Hinton, K.S. Kim, and R.E. Koeppe II. 1994. Gramicidin A/short-chain phospholipid dispersions: chain length dependence of gramicidin conformation and lipid organization. *Biochemistry.* 33:4291–4299. http://dx.doi.org/10.1021/bi00180a025

Greathouse, D.V., R.E. Koeppe II, L.L. Providence, S. Shobana, and O.S. Andersen. 1999. Design and characterization of gramicidin channels. *Methods Enzymol.* 294:525–550. http://dx.doi.org/10.1016/S0076-6879(99)94031-4

Greisen, P. Jr., K. Lum, M. Ashrafuzzaman, D.V. Greathouse, O.S. Andersen, and J.A. Lundbæk. 2011. Linear rate-equilibrium relations arising from ion channel-bilayer energetic coupling. *Proc. Natl. Acad. Sci. USA.* 108:12717–12722. http://dx.doi.org/10.1073/pnas.1103192108

Hansen, S.B., X. Tao, and R. MacKinnon. 2011. Structural basis of PIP_2 activation of the classical inward rectifier K^+ channel Kir2.2. *Nature.* 477:495–498. http://dx.doi.org/10.1038/nature10370

Hardie, R.C., and K. Franze. 2012. Photomechanical responses in *Drosophila* photoreceptors. *Science.* 338:260–263. http://dx.doi.org/10.1126/science.1222376

Harroun, T.A., W.T. Heller, T.M. Weiss, L. Yang, and H.W. Huang. 1999. Experimental evidence for hydrophobic matching and membrane-mediated interactions in lipid bilayers containing gramicidin. *Biophys. J.* 76:937–945. http://dx.doi.org/10.1016/S0006-3495(99)77257-7

Heerklotz, H. 2002. Triton promotes domain formation in lipid raft mixtures. *Biophys. J.* 83:2693–2701. http://dx.doi.org/10.1016/S0006-3495(02)75278-8

Heerklotz, H., and J. Seelig. 2000. Correlation of membrane/water partition coefficients of detergents with the critical micelle concentration. *Biophys. J.* 78:2435–2440. http://dx.doi.org/10.1016/S0006-3495(00)76787-7

Helfrich, P., and E. Jakobsson. 1990. Calculation of deformation energies and conformations in lipid membranes containing gramicidin channels. *Biophys. J.* 57:1075–1084. http://dx.doi.org/10.1016/S0006-3495(90)82625-4

Hernandez, C.C., O. Zaika, and M.S. Shapiro. 2008. A carboxy-terminal inter-helix linker as the site of phosphatidylinositol 4,5-bisphosphate action on Kv7 (M-type) K^+ channels. *J. Gen. Physiol.* 132:361–381. http://dx.doi.org/10.1085/jgp.200810007

Hilgemann, D.W. 2007. Local $PIP_{(2)}$ signals: when, where, and how? *Pflugers Arch.* 455:55–67. http://dx.doi.org/10.1007/s00424-007-0280-9

Hilgemann, D.W., S. Feng, and C. Nasuhoglu. 2001. The complex and intriguing lives of PIP_2 with ion channels and transporters. *Sci. STKE.* 2001:re19.

Horowitz, L.F., W. Hirdes, B.C. Suh, D.W. Hilgemann, K. Mackie, and B. Hille. 2005. Phospholipase C in living cells: Activation, inhibition, Ca^{2+} requirement, and regulation of M current. *J. Gen. Physiol.* 126:243–262. http://dx.doi.org/10.1085/jgp.200509309

Huang, C.L., S. Feng, and D.W. Hilgemann. 1998. Direct activation of inward rectifier potassium channels by PIP_2 and its stabilization by Gβγ. *Nature.* 391:803–806. http://dx.doi.org/10.1038/35882

Huang, F.L., and K.P. Huang. 1991. Interaction of protein kinase C isozymes with phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* 266:8727–8733.

Huang, H.W. 1986. Deformation free energy of bilayer membrane and its effect on gramicidin channel lifetime. *Biophys. J.* 50:1061–1070. http://dx.doi.org/10.1016/S0006-3495(86)83550-0

Hurley, J.H. 2006. Membrane binding domains. *Biochim. Biophys. Acta.* 1761:805–811. http://dx.doi.org/10.1016/j.bbalip.2006.02.020

Ingólfsson, H.I., and O.S. Andersen. 2010. Screening for small molecules' bilayer-modifying potential using a gramicidin-based fluorescence assay. *Assay Drug Dev. Technol.* 8:427–436. http://dx.doi.org/10.1089/adt.2009.0250

Ingólfsson, H.I., and O.S. Andersen. 2011. Alcohol's effects on lipid bilayer properties. *Biophys. J.* 101:847–855. http://dx.doi.org/10.1016/j.bpj.2011.07.013

Ingólfsson, H.I., R.L. Sanford, R. Kapoor, and O.S. Andersen. 2010. Gramicidin-based fluorescence assay; for determining small molecules potential for modifying lipid bilayer properties. *J. Vis. Exp.* pii:2131.

Ingólfsson, H.I., R.E. Koeppe II, and O.S. Andersen. 2011. Effects of green tea catechins on gramicidin channel function and inferred changes in bilayer properties. *FEBS Lett.* 585:3101–3105. http://dx.doi.org/10.1016/j.febslet.2011.08.040

Israelachvili, J.N., S. Marcelja, and R.G. Horn. 1980. Physical principles of membrane organization. *Q. Rev. Biophys.* 13:121–200. http://dx.doi.org/10.1017/S0033583500001645

Jin, T., L. Peng, T. Mirshahi, T. Rohacs, K.W. Chan, R. Sanchez, and D.E. Logothetis. 2002. The βγ subunits of G proteins gate a K^+ channel by pivoted bending of a transmembrane segment. *Mol. Cell.* 10:469–481. http://dx.doi.org/10.1016/S1097-2765(02)00659-7

Keller, S.L., S.M. Bezrukov, S.M. Gruner, M.W. Tate, I. Vodyanoy, and V.A. Parsegian. 1993. Probability of alamethicin conductance states varies with nonlamellar tendency of bilayer phospholipids. *Biophys. J.* 65:23–27. http://dx.doi.org/10.1016/S0006-3495(93)81040-3

Killian, J.A., K.U. Prasad, D.W. Urry, and B. de Kruijff. 1989. A mismatch between the length of gramicidin and the lipid acyl chains is a prerequisite for HII phase formation in phosphatidylcholine model membranes. *Biochim. Biophys. Acta.* 978:341–345. http://dx.doi.org/10.1016/0005-2736(89)90135-1

Killian, J.A., I. Salemink, M.R. de Planque, G. Lindblom, R.E. Koeppe II, and D.V. Greathouse. 1996. Induction of nonbilayer structures in diacylphosphatidylcholine model membranes by transmembrane alpha-helical peptides: importance of hydrophobic mismatch and proposed role of tryptophans. *Biochemistry.* 35:1037–1045. http://dx.doi.org/10.1021/bi9519258

Kim, D., and H. Bang. 1999. Modulation of rat atrial G protein-coupled K^+ channel function by phospholipids. *J. Physiol.* 517:59–74. http://dx.doi.org/10.1111/j.1469-7793.1999.0059z.x

Kim, T., K.I. Lee, P. Morris, R.W. Pastor, O.S. Andersen, and W. Im. 2012. Influence of hydrophobic mismatch on structures and dynamics of gramicidin A and lipid bilayers. *Biophys. J.* 102:1551–1560. http://dx.doi.org/10.1016/j.bpj.2012.03.014

King, M.D., and D. Marsh. 1987. Head group and chain length dependence of phospholipid self-assembly studied by spin-label electron spin resonance. *Biochemistry.* 26:1224–1231. http://dx.doi.org/10.1021/bi00379a004

Kleinschmidt, J.H., and L.K. Tamm. 2002. Structural transitions in short-chain lipid assemblies studied by (31)P-NMR spectroscopy. *Biophys. J.* 83:994–1003. http://dx.doi.org/10.1016/S0006-3495(02)75225-9

Kobrinsky, E., T. Mirshahi, H. Zhang, T. Jin, and D.E. Logothetis. 2000. Receptor-mediated hydrolysis of plasma membrane messenger PIP_2 leads to K^+-current desensitization. *Nat. Cell Biol.* 2:507–514. http://dx.doi.org/10.1038/35019544

Lee, A.G. 2004. How lipids affect the activities of integral membrane proteins. *Biochim. Biophys. Acta.* 1666:62–87. http://dx.doi.org/10.1016/j.bbamem.2004.05.012

Lee, J., S.K. Cha, T.J. Sun, and C.L. Huang. 2005. PIP_2 activates TRPV5 and releases its inhibition by intracellular Mg^{2+}. *J. Gen. Physiol.* 126:439–451. http://dx.doi.org/10.1085/jgp.200509314

Leonard, T.A., and J.H. Hurley. 2011. Regulation of protein kinases by lipids. *Curr. Opin. Struct. Biol.* 21:785–791. http://dx.doi.org/10.1016/j.sbi.2011.07.006

Lewis, B.A., and D.M. Engelman. 1983. Lipid bilayer thickness varies linearly with acyl chain length in fluid phosphatidylcholine vesicles. *J. Mol. Biol.* 166:211–217. http://dx.doi.org/10.1016/S0022-2836(83)80007-2

Li, Y., N. Gamper, D.W. Hilgemann, and M.S. Shapiro. 2005. Regulation of Kv7 (KCNQ) K+ channel open probability by phosphatidylinositol 4,5-bisphosphate. *J. Neurosci.* 25:9825–9835. http://dx.doi.org/10.1523/JNEUROSCI.2597-05.2005

Logothetis, D.E., V.I. Petrou, S.K. Adney, and R. Mahajan. 2010. Channelopathies linked to plasma membrane phosphoinositides. *Pflugers Arch.* 460:321–341. http://dx.doi.org/10.1007/s00424-010-0828-y

Lopes, C.M.B., H. Zhang, T. Rohacs, T. Jin, J. Yang, and D.E. Logothetis. 2002. Alterations in conserved Kir channel-PIP_2 interactions underlie channelopathies. *Neuron.* 34:933–944. http://dx.doi.org/10.1016/S0896-6273(02)00725-0

Lopes, C.M., T. Rohács, G. Czirják, T. Balla, P. Enyedi, and D.E. Logothetis. 2005. PIP_2 hydrolysis underlies agonist-induced inhibition and regulates voltage gating of two-pore domain K^+ channels. *J. Physiol.* 564:117–129. http://dx.doi.org/10.1113/jphysiol.2004.081935

Lundbæk, J.A., and O.S. Andersen. 1994. Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers. *J. Gen. Physiol.* 104:645–673. http://dx.doi.org/10.1085/jgp.104.4.645

Lundbæk, J.A., A.M. Maer, and O.S. Andersen. 1997. Lipid bilayer electrostatic energy, curvature stress, and assembly of gramicidin channels. *Biochemistry.* 36:5695–5701. http://dx.doi.org/10.1021/bi9619841

Lundbæk, J.A., P. Birn, S.E. Tape, G.E. Toombes, R. Søgaard, R.E. Koeppe II, S.M. Gruner, A.J. Hansen, and O.S. Andersen. 2005. Capsaicin regulates voltage-dependent sodium channels by altering lipid bilayer elasticity. *Mol. Pharmacol.* 68:680–689.

Lundbæk, J.A., S.A. Collingwood, H.I. Ingólfsson, R. Kapoor, and O.S. Andersen. 2010a. Lipid bilayer regulation of membrane protein function: gramicidin channels as molecular force probes. *J. R. Soc. Interface.* 7:373–395. http://dx.doi.org/10.1098/rsif.2009.0443

Lundbæk, J.A., R.E. Koeppe II, and O.S. Andersen. 2010b. Amphiphile regulation of ion channel function by changes in the bilayer spring constant. *Proc. Natl. Acad. Sci. USA.* 107:15427–15430. http://dx.doi.org/10.1073/pnas.1007455107

Marsh, D. 2007. Lateral pressure profile, spontaneous curvature frustration, and the incorporation and conformation of proteins in membranes. *Biophys. J.* 93:3884–3899. http://dx.doi.org/10.1529/biophysj.107.107938

Marsh, D. 2012a. Thermodynamics of phospholipid self-assembly. *Biophys. J.* 102:1079–1087. http://dx.doi.org/10.1016/j.bpj.2012.01.049

Marsh, D. 2012b. Handbook of Lipid Bilayers. Second edition. CRC Press, Boca Raton, FL. 1145 pp.

Marsh, D., and M.D. King. 1986. Prediction of the critical micelle concentrations of mono- and di-acyl phospholipids. *Chem. Phys. Lipids.* 42:271–277. http://dx.doi.org/10.1016/0009-3084(86)90086-1

McLaughlin, S., and D. Murray. 2005. Plasma membrane phosphoinositide organization by protein electrostatics. *Nature.* 438:605–611. http://dx.doi.org/10.1038/nature04398

McLaughlin, S., J. Wang, A. Gambhir, and D. Murray. 2002. PIP_2 and proteins: interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struct.* 31:151–175. http://dx.doi.org/10.1146/annurev.biophys.31.082901.134259

Moens, P.D., and L.A. Bagatolli. 2007. Profilin binding to sub-micellar concentrations of phosphatidylinositol (4,5) bisphosphate and phosphatidylinositol (3,4,5) trisphosphate. *Biochim. Biophys. Acta.* 1768:439–449. http://dx.doi.org/10.1016/j.bbamem.2006.12.012

Nasuhoglu, C., S. Feng, Y. Mao, I. Shammat, M. Yamamato, S. Earnest, M. Lemmon, and D.W. Hilgemann. 2002. Modulation of cardiac PIP2 by cardioactive hormones and other physiologically relevant interventions. *Am. J. Physiol. Cell Physiol.* 283:C223–C234.

Nielsen, C., and O.S. Andersen. 2000. Inclusion-induced bilayer deformations: effects of monolayer equilibrium curvature. *Biophys. J.* 79:2583–2604. http://dx.doi.org/10.1016/S0006-3495(00)76498-8

Nielsen, C., M. Goulian, and O.S. Andersen. 1998. Energetics of inclusion-induced bilayer deformations. *Biophys. J.* 74:1966–1983. http://dx.doi.org/10.1016/S0006-3495(98)77904-4

O'Connell, A.M., R.E. Koeppe II, and O.S. Andersen. 1990. Kinetics of gramicidin channel formation in lipid bilayers: transmembrane monomer association. *Science.* 250:1256–1259. http://dx.doi.org/10.1126/science.1700867

Palmer, F.B.S.C. 1981. The phosphatidyl-myo-inositol-4,5-biphosphate phosphatase from *Crithidia fasciculata*. *Can. J. Biochem.* 59:469–476. http://dx.doi.org/10.1139/o81-065

Partenskii, M.B., and P.C. Jordan. 2002. Membrane deformation and the elastic energy of insertion: Perturbation of membrane elastic constants due to peptide insertion. *J. Chem. Phys.* 117:10768. http://dx.doi.org/10.1063/1.1519840

Peitzsch, R.M., and S. McLaughlin. 1993. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry.* 32:10436–10443. http://dx.doi.org/10.1021/bi00090a020

Rawicz, W., K.C. Olbrich, T. McIntosh, D. Needham, and E. Evans. 2000. Effect of chain length and unsaturation on elasticity of lipid bilayers. *Biophys. J.* 79:328–339. http://dx.doi.org/10.1016/S0006-3495(00)76295-3

Redfern, D.A., and A. Gericke. 2005. pH-dependent domain formation in phosphatidylinositol polyphosphate/phosphatidylcholine mixed vesicles. *J. Lipid Res.* 46:504–515. http://dx.doi.org/10.1194/jlr.M400367-JLR200

Richieri, G.V., R.T. Ogata, and A.M. Kleinfeld. 1999. The measurement of free fatty acid concentration with the fluorescent probe ADIFAB: a practical guide for the use of the ADIFAB probe. *Mol. Cell. Biochem.* 192:87–94. http://dx.doi.org/10.1023/A:1006878421990

Rohács, T. 2007. Regulation of TRP channels by PIP_2. *Pflugers Arch.* 453:753–762. http://dx.doi.org/10.1007/s00424-006-0153-7

Rohács, T., J. Chen, G.D. Prestwich, and D.E. Logothetis. 1999. Distinct specificities of inwardly rectifying K^+ channels for phosphoinositides. *J. Biol. Chem.* 274:36065–36072. http://dx.doi.org/10.1074/jbc.274.51.36065

Rohács, T., C.M. Lopes, T. Jin, P.P. Ramdya, Z. Molnár, and D.E. Logothetis. 2003. Specificity of activation by phosphoinositides determines lipid regulation of Kir channels. *Proc. Natl. Acad. Sci. USA.* 100:745–750. http://dx.doi.org/10.1073/pnas.0236364100

Rohács, T., C.M. Lopes, I. Michailidis, and D.E. Logothetis. 2005. $PI(4,5)P_2$ regulates the activation and desensitization of TRPM8 channels through the TRP domain. *Nat. Neurosci.* 8:626–634. http://dx.doi.org/10.1038/nn1451

Rosenhouse-Dantsker, A., and D.E. Logothetis. 2007. Molecular characteristics of phosphoinositide binding. *Pflugers Arch.* 455:45–53. http://dx.doi.org/10.1007/s00424-007-0291-6

Runnels, L.W., L. Yue, and D.E. Clapham. 2002. The TRPM7 channel is inactivated by PIP_2 hydrolysis. *Nat. Cell Biol.* 4:329–336.

Rusinova, R., K.F. Herold, R.L. Sanford, D.V. Greathouse, H.C. Hemmings Jr., and O.S. Andersen. 2011. Thiazolidinedione insulin sensitizers alter lipid bilayer properties and voltage-dependent sodium channel function: Implications for drug discovery. *J. Gen. Physiol.* 138:249–270. http://dx.doi.org/10.1085/jgp.201010529

Schulze, D., T. Krauter, H. Fritzenschaft, M. Soom, and T. Baukrowitz. 2003. Phosphatidylinositol 4,5-bisphosphate (PIP_2) modulation of ATP and pH sensitivity in Kir channels. A tale of an active and a silent PIP_2 site in the N terminus. *J. Biol. Chem.* 278:10500–10505. http://dx.doi.org/10.1074/jbc.M208413200

Seddon, A.M., D. Casey, R.V. Law, A. Gee, R.H. Templer, and O. Ces. 2009. Drug interactions with lipid membranes. *Chem. Soc. Rev.* 38:2509–2519. http://dx.doi.org/10.1039/b813853m

Seddon, J.M. 1990. Structure of the inverted hexagonal (H_{II}) phase, and non-lamellar phase transitions of lipids. *Biochim. Biophys. Acta.* 1031:1–69. http://dx.doi.org/10.1016/0304-4157(90)90002-T

Seydel, J.K., and M. Wiese. 2002. Drug-Membrane Interactions: Analysis, Drug Distribution, Modeling. Wiley-VCH, Weinheim.

Suh, B.C., and B. Hille. 2005. Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. *Curr. Opin. Neurobiol.* 15:370–378. http://dx.doi.org/10.1016/j.conb.2005.05.005

Suh, B.C., and B. Hille. 2008. PIP_2 is a necessary cofactor for ion channel function: How and why? *Annu. Rev. Biophys.* 37:175–195. http://dx.doi.org/10.1146/annurev.biophys.37.032807.125859

Sun, H., D.V. Greathouse, O.S. Andersen, and R.E. Koeppe II. 2008. The preference of tryptophan for membrane interfaces: insights from N-methylation of tryptophans in gramicidin channels. *J. Biol. Chem.* 283:22233–22243. http://dx.doi.org/10.1074/jbc.M802074200

Tanford, C. 1980. The Hydrophobic Effect: Formation of Micelles and Biological Membranes. Second edition. Wiley, New York.

Vaithianathan, T., A. Bukiya, J. Liu, P. Liu, M. Asuncion-Chin, Z. Fan, and A. Dopico. 2008. Direct regulation of BK channels by phosphatidylinositol 4,5-bisphosphate as a novel signaling pathway. *J. Gen. Physiol.* 132:13–28. http://dx.doi.org/10.1085/jgp.200709913

Wang, J., and D.A. Richards. 2012. Segregation of PIP_2 and PIP_3 into distinct nanoscale regions within the plasma membrane. *Biol. Open.* 1:857–862. http://dx.doi.org/10.1242/bio.20122071

Wang, Y.K., W. Chen, D. Blair, M. Pu, Y. Xu, S.J. Miller, A.G. Redfield, T.C. Chiles, and M.F. Roberts. 2008. Insights into the structural specificity of the cytotoxicity of 3-deoxyphosphatidylinositols. *J. Am. Chem. Soc.* 130:7746–7755. http://dx.doi.org/10.1021/ja710348r

Wenk, M.R., L. Lucast, G. Di Paolo, A.J. Romanelli, S.F. Suchy, R.L. Nussbaum, G.W. Cline, G.I. Shulman, W. McMurray, and P. De Camilli. 2003. Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. *Nat. Biotechnol.* 21:813–817. http://dx.doi.org/10.1038/nbt837

Whorton, M.R., and R. MacKinnon. 2011. Crystal structure of the mammalian GIRK2 K^+ channel and gating regulation by G proteins, PIP_2, and sodium. *Cell.* 147:199–208. http://dx.doi.org/10.1016/j.cell.2011.07.046

Xu, C., J. Watras, and L.M. Loew. 2003. Kinetic analysis of receptor-activated phosphoinositide turnover. *J. Cell Biol.* 161:779–791. http://dx.doi.org/10.1083/jcb.200301070

Yokoyama, S., and M. Nakagaki. 1993. Effect of double bond on the surface properties of aqueous solutions of eicosapolyenoic acids. *Colloid Polym. Sci.* 271:512–518. http://dx.doi.org/10.1007/BF00657397

Zaslavsky, B.Y., L.M. Miheeva, N.M. Mestechkina, V.M. Pogorelov, and S.V. Rogozhin. 1978. General rule of partition behaviour of cells and soluble substances in aqueous two-phase polymeric systems. *FEBS Lett.* 94:77–80. http://dx.doi.org/10.1016/0014-5793(78)80910-7

Zhang, H., C. He, X. Yan, T. Mirshahi, and D.E. Logothetis. 1999. Activation of inwardly rectifying K^+ channels by distinct $PtdIns(4,5)P_2$ interactions. *Nat. Cell Biol.* 1:183–188. http://dx.doi.org/10.1038/11103

Zhang, H., L.C. Craciun, T. Mirshahi, T. Rohács, C.M. Lopes, T. Jin, and D.E. Logothetis. 2003. PIP_2 activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron.* 37:963–975. http://dx.doi.org/10.1016/S0896-6273(03)00125-9

Zhelev, D.V. 1998. Material property characteristics for lipid bilayers containing lysolipid. *Biophys. J.* 75:321–330. http://dx.doi.org/10.1016/S0006-3495(98)77516-2

Zhou, C., Y. Wu, and M.F. Roberts. 1997. Activation of phosphatidylinositol-specific phospholipase C toward inositol 1,2-(cyclic)-phosphate. *Biochemistry.* 36:347–355. http://dx.doi.org/10.1021/bi960601w