Calcium Directly Regulates Phosphatidylinositol 4,5-Bisphosphate Headgroup Conformation and Recognition

Eva Bilkova, Š.⁴,⁷,⁸,† Roman Pleskot, §,¶,∥ Simou Sun, #,isors, Aleksander Czogalla, †,¥ Lukasz Cwiklik, & Tomasz Róg, L Vattulainen, †,.§ Paul S. Cremer, †,§,∥,⊥ Pavel Jungwirth, †,§,∥,⊥ and Unal Coskun*, †,§,∥,⊥

¹Paul Langerhans Institute Dresden of the Helmholtz Zentrum München at the University Hospital and Faculty of Medicine Carl Gustav Carus of TU Dresden, Technische Universität Dresden, Fetscher Strasse 74, 01307 Dresden, Germany
²German Center for Diabetes Research (DZD e.V.), Ingolstädter Landstraße 1, 85764 Neuperlberg, Germany
³Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo náměstí 2, 16610 Prague 6, Czech Republic
⁴Institute of Experimental Botany, Academy of Sciences of the Czech Republic, v.v.i., Rozvojová 263, 16502 Prague 6, Czech Republic
⁵Department of Physics, Tampere University of Technology, P.O. Box 692, FI-33101 Tampere, Finland
⁶Department of Chemistry and Department of Biochemistry and Molecular Biology, Penn State University, University Park, Pennsylvania 16801, United States
⁷Laboratory of Cytobiochemistry, Faculty of Biotechnology, University of Wrocław, Joliot-Curie 14a, 50-383 Wrocław, Poland
⁸A. Heyrovsky Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, v.v.i., Dolejskova 3, 18223 Prague 8, Czech Republic
⁹Department of Physics, University of Helsinki, P.O. Box 64, FI-00014, Helsinki, Finland
*MEMPHYS—Center for Biomembrane Physics, University of Southern Denmark, DK-5230 Odense, Denmark

Supporting Information

ABSTRACT: The orchestrated recognition of phosphoinositides and concomitant intracellular release of Ca²⁺ is pivotal to almost every aspect of cellular processes, including membrane homeostasis, cell division and growth, vesicle trafficking, as well as secretion. Although Ca²⁺ is known to directly impact phosphoinositide clustering, little is known about the molecular basis for this or its significance in cellular signaling. Here, we study the direct interaction of Ca²⁺ with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), the main lipid marker of the plasma membrane. Electrokinetic potential measurements of PI(4,5)P₂ containing liposomes reveal that Ca²⁺ as well as Mg²⁺ reduce the zeta potential of liposomes to nearly background levels of pure phosphatidylcholine membranes. Strikingly, lipid recognition by the default PI(4,5)P₂ lipid sensor, phospholipase C delta 1 pleckstrin homology domain (PLC δ1-PH), is completely inhibited in the presence of Ca²⁺, while Mg²⁺ has no effect with 100 nm liposomes and modest effect with giant unilamellar vesicles. Consistent with biochemical data, vibrational sum frequency spectroscopy and atomistic molecular dynamics simulations reveal how Ca²⁺ binding to the PI(4,5)P₂ headgroup and carbonyl regions leads to confined lipid headgroup tilting and conformational rearrangements. We rationalize these findings by the ability of calcium to block a highly specific interaction between PLC δ1-PH and PI(4,5)P₂ encoded within the conformational properties of the lipid itself. Our studies demonstrate the possibility that switchable phosphoinositide conformational states can serve as lipid recognition and controlled cell signaling mechanisms.

INTRODUCTION

Cell signaling pathways are largely organized via a specific recruitment of signaling effector proteins to their target membranes and a confined release of calcium ions. The quintessential example of this is the action of phospholipase C (PLC) that binds and hydrolyzes phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) in the plasma membrane to diacylglycerol (DAG) and the water-soluble inositol 1,4,5-trisphosphate (IP₃), the latter inducing the release of Ca²⁺ from the endoplasmic reticulum (ER) into the cytosol. Another prominent example is synaptotagmin-1, the main Ca²⁺ sensor of...
neuronal exocytosis in the presynaptic axon terminal. Synaptotagmin-1 binding to P(4,5)P$_2$ directly amplifies protein cooperativity and thus sensitivity to Ca$^{2+}$ by a factor of $>40$. This mutual interplay is a critical step in neurotransmitter release.$^3$

P(4,5)P$_2$ is enriched in the inner leaflet of the plasma membrane$^{5,4}$ and constitutes around 1% of the total anionic phospholipid content in cellular membranes.$^5$ In comparison with other phospholipids, it contains a rather bulky phosphorylated inositol headgroup with a negative charge ranging from $-3$ to $-5$ e, depending on the pH and the presence of proteins or ions.$^6$ P(4,5)P$_2$ and other negatively charged lipids in the cytosolic leaflet are constantly exposed to divalent cations. In resting cells, the free cytosolic Ca$^{2+}$ concentration is approximately 100 nM.$^{28}$ The cytosolic concentration of Ca$^{2+}$ upon cell signaling has been reported to span a wide range from 0.5 μM to several hundred μM, with a half-life of 500 μs to 26 ms.$^{9−14}$ Ca$^{2+}$ influx primarily originates from internal stores within the endoplasmic/sarcoplasmic reticulum or from specialized channels within the plasma membrane providing an essentially infinite supply of extracellular calcium.$^7$ In all cases, Ca$^{2+}$ is delivered as brief transients, forming microdomains at the membrane site of influx,$^{10}$ and thus, local concentrations of Ca$^{2+}$ can be expected to exceed cytosolic concentrations by orders of magnitude.$^{15}$ Meanwhile, unlike Ca$^{2+}$, the levels of free, cytosolic Mg$^{2+}$ are maintained within a fairly narrow concentration range of 0.25−1 mM.$^{16,17}$ Interestingly, calcium but not magnesium ions have been ascribed a strong propensity to promote the formation of P(4,5)P$_2$ monolayers as demonstrated in several studies, primarily by using monolayer techniques.$^{18−22}$

While the overall effects of divalent cations, including calcium, on P(4,5)P$_2$ lateral organization have been intensely studied, the mechanism of Ca$^{2+}$ and P(4,5)P$_2$ interactions at the molecular level remain unclear. Experiments with pure P(4,5)P$_2$ monolayers have suggested partial dehydration of the headgroup region as well as acyl chain region thickening.$^{24}$ In all cases, Ca$^{2+}$ is delivered as brief transients, forming microdomains at the membrane site of influx,$^{10}$ and thus, local concentrations of Ca$^{2+}$ can be expected to exceed cytosolic concentrations by orders of magnitude.$^{15}$ Meanwhile, unlike Ca$^{2+}$, the levels of free, cytosolic Mg$^{2+}$ are maintained within a fairly narrow concentration range of 0.25−1 mM.$^{16,17}$ Interestingly, calcium but not magnesium ions have been ascribed a strong propensity to promote the formation of P(4,5)P$_2$ monolayers as demonstrated in several studies, primarily by using monolayer techniques.$^{18−22}$

**RESULTS AND DISCUSSION**

**Protein−Lipid Binding Assays.** To determine the equilibrium dissociation constants ($K_D$) for divalent cation/P(4,5)P$_2$ interaction, we employed a simple fluorescent assay using a supported lipid bilayer platform$^{20−22}$ containing 5 mol % of P(4,5)P$_2$ (for details, see the Supporting Information). Significantly, the $K_D$ values differed by less than a factor of 2, with a $K_D$ of 0.6 ± 0.2 mM for Ca$^{2+}$ compared to 1.2 ± 0.2 mM for Mg$^{2+}$ (Figure S1). We therefore decided to use a cation concentration of 1 mM for all follow-up experiments, matching the free Mg$^{2+}$ concentration in the cytosol. In order to systematically study the effects of Ca$^{2+}$ on P(4,5)P$_2$, we produced 100 nm diameter large unilamellar vesicles (LUVs), facilitating the control of membrane lipid composition and properties. For quality control and physicochemical characterization, all preparations were first subjected to thin layer chromatography (TLC), dynamic light scattering (DLS), and zeta potential measurements (Figure S2). Having the opposite charge of P(4,5)P$_2$, it is not surprising that Ca$^{2+}$ and Mg$^{2+}$ equally reduce the zeta potential of POPC liposomes containing 5 mol % of P(4,5)P$_2$, the former being described previously.$^{27}$ In fact, the presence of either cation attenuates the electrokinetic potential of the membrane down to the level of POPC alone (Figure S2c).

Because of its extraordinary stereospecificity, the PLC δ1-PH domain is widely used as the canonical reporter for cellular P(4,5)P$_2$ levels at the plasma membrane as well as with in vitro assays.$^{30−34}$ We therefore used recombinant PLC δ1-PH domain to follow P(4,5)P$_2$ binding to synthetic liposomes. Size-exclusion chromatography and DLS confirmed that the purified PLC δ1-PH domain (Figure S3a,b) was monomeric in solution, even in the presence of Ca$^{2+}$ and Mg$^{2+}$ (Figure S3c,d). Next, we performed liposome flotation assays to follow PLC δ1-PH binding efficiency to POPC/P(4,5)P$_2$ vesicles. Interestingly, preincubation with 1 mM Ca$^{2+}$ but not 1 mM Mg$^{2+}$ fully inhibited liposome binding (Figure 1a,b). Moreover, PLC δ1-PH did not bind to pure POPC liposomes, highlighting its specificity to P(4,5)P$_2$.

Circular dichroism (CD) spectroscopy excluded a direct effect for cations on the secondary structure of the protein (Figure S3e,f). As such, although Ca$^{2+}$ and Mg$^{2+}$ bind to P(4,5)P$_2$ with comparable $K_D$ values and reduce electrokinetic membrane properties in an equal manner, only Ca$^{2+}$ was capable of inhibiting PLC δ1-PH binding. This indicates that P(4,5)P$_2$ recognition by proteins cannot be solely based on electrostatic interactions.

Because a concentration of 1 mM Ca$^{2+}$ corresponds to twice its $K_D$ for P(4,5)P$_2$ interaction, we performed additional flotation assays with lower Ca$^{2+}$ concentrations. Here, a significant reduction in protein binding could be observed already at a concentration of 0.6 mM Ca$^{2+}$ (Figure S4). In this context, recent data by Milovanovic and colleagues show that Ca$^{2+}$ but not Mg$^{2+}$ promotes syntaxin-1/P(4,5)P$_2$ domain formation by an underlying mechanism in which Ca$^{2+}$ clusters P(4,5)P$_2$ and syntaxin-1 independently from each other. Moreover, Ca$^{2+}$ acts as a charge bridge that merges multiple syntaxin-1/P(4,5)P$_2$ clusters into larger domains. Also here, Ca$^{2+}$ was found to be effective at a concentration of 0.5 mM while even 1 mM Mg$^{2+}$ had no effect.$^{35}$

Ca$^{2+}$ binding to membranes has been recently reported to increase with high curvature.$^{36}$ We therefore additionally followed the binding of monomeric ECFP-PLC δ1-PH fusion protein to giant unilamellar vesicles (GUVs) (Figure 1c). Despite limited control over membrane lipid composition at the individual GUV level,$^{37}$ GUVs provide the most appropriate platform to follow PLC δ1-PH binding (Figure 1d and Figure S5), demonstrating the robustness of the observed effect, irrespective of membrane curvature. Magnesium, however, also reduced ECFP-PLC δ1-PH domain binding, halfway...
Mg$^{2+}$ (green) or Ca$^{2+}$ (red) (data from two additional independent median ECFP-PLC of 1 mM Ca$^{2+}$ and Mg$^{2+}$ (Figure 2a, detailed peak assignments however, the inositol ring signal (961 cm$^{-1}$) remains constant, leading to an additional stoichiometric shift.

By contrast, protein binding in the GUV experiment is at bottom of the tube. At the same time, cation concentrations remain constant, leading to an additional stoichiometric shift. Contrast, protein binding in the GUV experiment is at equilibrium and binding events are quantified at the individual GUV level.

**Vibrational Sum Frequency Spectroscopy.** To analyze the molecular sum frequency (VSFS) was employed to study the effects of Ca$^{2+}$ and Mg$^{2+}$ on pure PI(4,5)P$_2$ monolayers at the air/water interface. The spectra were recorded over frequency ranges corresponding to the headgroup and acyl-chain portions of the lipid molecules and included the adjacent interfacial water structure.

We present VSFS spectra from the inositol ring and phosphate regions of PI(4,5)P$_2$ in the absence and presence of 1 mM Ca$^{2+}$ and Mg$^{2+}$ (Figure 2a, detailed peak assignments in Figure S6 and Table S1). In the absence of cations in the subphase, both the inositol ring vibrations and the phosphate stretches were rather weak (black data points). This is because of a relatively disordered arrangement of the PI(4,5)P$_2$ headgroups adopted in a pure buffer with a wide range of tilt angles relative to the surface normal. With 1 mM Ca$^{2+}$, however, the inositol ring signal (961 cm$^{-1}$ and 1012 cm$^{-1}$ peaks from the C–C and C–O coupled vibrations, respectively$^{18}$) increased substantially (red data points). In fact, the resonances showed 2.7- and 3.6-fold increases, respectively, in oscillator strength (Table S1). These changes reflect both reorientation of the inositol rings and a narrowing of their orientational distribution upon cation binding. Significantly, the changes were not nearly as strong upon the addition of 1 mM Mg$^{2+}$ (blue data points). In that case, the oscillator strength of the inositol ring vibrations was increased by only a factor of 1.5 and 2.1, respectively. Such results indicated that Ca$^{2+}$ rigidified the configuration of the PI(4,5)P$_2$ headgroups much more effectively than Mg$^{2+}$.

In addition to the inositol ring modes, the phosphate peaks (e.g., symPO$_3$$^-$ at 982 cm$^{-1}$, symPO$_2$$^-$ at 1086 cm$^{-1}$, asymPO$_2$$^-$ at 1115 cm$^{-1}$, symPO$_3$$^-$ at 1154 cm$^{-1}$, detailed assignments in Figure S6 and Table S1) also showed a substantial intensity increase upon the introduction of Ca$^{2+}$ to the subphase. This indicates a strong net orientation and/or ordering of the headgroup phosphates upon Ca$^{2+}$ binding. It should be noted that Ca$^{2+}$ binding may help to deprotonate the second monoesterified phosphate, which would prompt additional changes in the spectra beyond those related to ordering and tilt angle. Moreover, upon the addition of Ca$^{2+}$, the symmetric PO$_3$$^-$ stretch exhibited a relatively large 20 cm$^{-1}$ blue shift, while the asymmetric PO$_2$$^-$ and PO$_3$$^-$ stretches shifted by 6 cm$^{-1}$ and 8 cm$^{-1}$, respectively (Table S1). The shifts of both PO$_2$$^-$ peaks are consistent with phosphate dehydration upon cation binding and/or a symmetry change of the C$_{4v}$ point group. The shift of the asymmetric PO$_3$$^-$ peak also suggests headgroup phosphate deprotonation upon Ca$^{2+}$ binding.$^{30-32}$

The spectral change brought about by 1 mM Mg$^{2+}$ in the phosphate region was much less pronounced overall compared to that with 1 mM Ca$^{2+}$. The difference in the interactions of Ca$^{2+}$ and Mg$^{2+}$ with phosphate could be explained at least in part by different dehydration penalties for these two cations. It has been suggested that Ca$^{2+}$ binding to phosphate groups is favored because Ca$^{2+}$ is more easily dehydrated than Mg$^{2+}$. This difference in the hydration shell chemistry may, in turn, act to disfavor the bridging of the inositol rings of PI(4,5)P$_2$ which would weaken the ordering effect of Mg$^{2+}$.

In addition to phosphate and inositol resonances, VSFS spectra were also obtained in the carbonyl C=O symmetric stretch (1730 cm$^{-1}$) region before and after addition of 1 mM CaCl$_2$ or MgCl$_2$ (Figure 2b). Again, Ca$^{2+}$ showed a more prominent effect on the PI(4,5)P$_2$ than Mg$^{2+}$. In fact, a 1.6-fold
increase in the oscillator strength of this peak was observed upon binding of Ca\textsuperscript{2+}, while only a 1.3-fold increase was found for Mg\textsuperscript{2+} (Table S2). This oscillator strength increase should correspond to a backbone ordering effect, thus helping to reinforce a more rigid configuration of the headgroup inositol rings. Ordering of the lipid acyl chains was also observed (Figure S8 and Table S3).

Taken together, the changes in the VSFS spectra provide strong experimental evidence for distinct conformational changes within the lipid headgroup region in the presence of Ca\textsuperscript{2+}, but less with Mg\textsuperscript{2+}. Such results should be important for the PLC \delta-PH domain selectivity of PI(4,5)P\textsubscript{2} found above with liposomes and GUVs.

**Atomistic Molecular Dynamics Simulations.** With the aim of obtaining mechanistic insights into the effects of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} on PI(4,5)P\textsubscript{2} molecules at a molecular level, we employed atomistic MD simulations. In order to reduce methodological bias, we used two all-atom force fields (OPLS-AA and CHARMM36) as well as the united-atom force field from Berger (Table S4).\textsuperscript{45–47} Importantly, to further account for electronic polarization effects of charged groups in a mean field manner, for Ca\textsuperscript{2+} interacting with PI(4,5)P\textsubscript{2} phosphates we also employed the recently developed electronic continuum correction with rescaling (ECCR) method.\textsuperscript{48} This, to a large extent, dampens the unrealistically high ion pairing found when employing nonpolarizable force fields.\textsuperscript{48} It is particularly useful in the present case where strong electronic polarization can be expected in the vicinity of multiple-charged moieties.

We generated multiple sets of 1 \mu s long trajectories for different initial PI(4,5)P\textsubscript{2} distributions prior to and after the addition of Ca\textsuperscript{2+} or Mg\textsuperscript{2+}. For all simulations, consistently with all force fields used, we find that Ca\textsuperscript{2+} interacts with PI(4,5)P\textsubscript{2} and has a pronounced effect on the lipid headgroup orientation (Figure 3 and Figures S9 and S13). Moreover, control simulations with Mg\textsuperscript{2+} showed that the effects induced by magnesium are much weaker than those induced by calcium for all simulations (Figure 3c,d and S1), in full agreement with experiments.

The addition of Ca\textsuperscript{2+} or Mg\textsuperscript{2+} immediately leads to a significant reduction of the area per lipid (Figure S10 and Table S5). This macroscopic effect is in agreement with lateral condensation of the PI(4,5)P\textsubscript{2}-containing monolayers by Ca\textsuperscript{2+},\textsuperscript{20,22–24} and our VSFS analysis of the CH stretches (Figure S8). At the microscopic level, we found that each PI(4,5)P\textsubscript{2} molecule binds on average 1.6–3.1 Ca\textsuperscript{2+} molecules, depending on the force field that is employed (Table S5). This is consistent with the water peak spectral changes, which show that each lipid molecule binds more than two Ca\textsuperscript{2+} ions (Figure S8). Ca\textsuperscript{2+} binds mostly to the phosphate groups at positions 4 and 5, but it also penetrates deeper into the lipid bilayer to interact with the carbonyl groups (Figure S11). Ca\textsuperscript{2+} binding to the lipid carbonyl group is consistent with the VSFS data in the carbonyl stretch region, as documented herein (Figure 2b) and elsewhere.\textsuperscript{49–53} In agreement with previously published computational and experimental results,\textsuperscript{24,50} we observed that Ca\textsuperscript{2+} increases the order parameters of the PI(4,5)P\textsubscript{2} acyl chains (Figure S12). The acyl chain ordering is also fully in line with the effects observed in the VSFS spectra (Figure S8).

The most prominent feature observed by simulations is a pronounced headgroup reorientation, primarily caused by the ability of Ca\textsuperscript{2+} to bridge two PI(4,5)P\textsubscript{2} headgroups (Figure 3a,b). This result was found regardless of which force field was used. To quantitatively analyze the headgroup reorientation, we monitored the tilt angle between the C1−C4 atoms of the PI(4,5)P\textsubscript{2} inositol ring and the bilayer normal. The average tilt angle in the control simulation without Ca\textsuperscript{2+} was in the range of 35–41°, depending on the employed force field. This result is in agreement with previously published MD studies.\textsuperscript{51–53} In the presence of Ca\textsuperscript{2+} ions, however, the average tilt angle significantly increased for all of the force fields up to 65° (Figure 3c and Figures S9 and S13). Simulations thus consistently showed bending of the PI(4,5)P\textsubscript{2} headgroup toward the plane of the bilayer and away from bulk water (Table S5). Moreover, consistent with a narrowing of the inositol ring’s distribution as indicated by VSFS results above (Figure 2a), Ca\textsuperscript{2+} slowed PI(4,5)P\textsubscript{2} headgroup rotational diffusion as revealed by the rotational correlation function (Figure S9e). The Ca\textsuperscript{2+} effect was also manifested in the density profiles (Figure 3d), where the location of the PI(4,5)P\textsubscript{2} headgroups shifted in the presence of calcium toward the bilayer center. Moreover, Ca\textsuperscript{2+} significantly decreased the solvent accessible surface area of PI(4,5)P\textsubscript{2}, which correlated with a reduced average number of hydrogen bonds between the PI(4,5)P\textsubscript{2} headgroups and water molecules (Table S5). These data also match the experimentally observed partial dehydration of PI(4,5)P\textsubscript{2} in the presence of Ca\textsuperscript{2+} as measured here by VSFS and elsewhere.\textsuperscript{23}

The charge state of PI(4,5)P\textsubscript{2} in lipid membranes is highly sensitive to the cellular pH and the presence of proteins and ions.\textsuperscript{55} By using not only the default parametrization (CHARMM36 and OPLS-AA) but also the ECCR corrected charges for the ions and PI(4,5)P\textsubscript{2} phosphate groups (Berger, OPLS-AA), we were able to assess the potential effects of the lipid charge state. Namely, the charge used for PI(4,5)P\textsubscript{2} varied from −3.75 to −5, depending on the particular force field (for...
more details, see the SI). Reassuringly, we found semi-quantitatively the same effect of Ca²⁺ on the PL(4,5)P₂ tilt angle with Ca²⁺ in all the systems which were tested. This indicates that under the conditions of these investigations the protonation state of PL(4,5)P₂ was not particularly critical for the observed effects.

**CONCLUSION**

By means of protein–lipid binding assays and spectroscopic experiments, together with atomistic MD simulations, we have unraveled and characterized in molecular detail the pronounced effect of Ca²⁺ on PL(4,5)P₂ headgroup presentation. First, we confirmed the previously observed increase of the PL(4,5)P₂ acyl chain order and PL(4,5)P₂ cluster formation, as evidenced here by VSFG spectroscopy and MD simulations. Second, we characterized at the molecular level the interactions of Ca²⁺ with PL(4,5)P₂ headgroup phosphates, as well as the more deeply seated carbonyl groups. We observed the hitherto unrecognized consequences of Ca²⁺ binding for PL(4,5)P₂ at the molecular level. Namely, we observed a dramatic change in the PL(4,5)P₂ headgroup tilt angle. By means of lipidosome flotation and GUV binding assays, we show that Ca²⁺ has a strong propensity to render the PL(4,5)P₂ headgroup invisible to the PLC-δ1 PH domain.

Our data lead to the plausible conjecture that the calcium-induced switching of phosphoinositide conformational states may serve as a potential cellular mechanism for lipid recognition and thus play a decisive role in cell signaling and membrane trafficking. A systematic correlation of kinetics and curvature sensitivities at the nanoscale in *vivo* will be key to understanding the general applicability of our data to other proteins and to different endomembranes.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b11760.

Detailed materials and methods, additional VSFS spectra, computational and experimental controls (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**

*psc11@psu.edu*

*pavel.jungwirth@uochb.cas.cz*

*ncoskun@plid.de*

**ORCID**

Tomasz Róg: 0000-0001-6765-7013

Ilpo Vattulainen: 0000-0001-7408-3214

Paul S. Cremer: 0000-0002-8522-0438

Pavel Jungwirth: 0000-0002-6892-3288

Únaí Coskun: 0000-0003-4375-3144

**Author Contributions**

E.B., R.P., S.R., and S.S. contributed equally.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank Milena Stephan, André Nadler, and Alf Honigmann (Max Planck Institute of Molecular Cell Biology and Genetics) for support with GUV image quantification and GUV production protocol; Josef Lazar (C4Sys research infrastructure) for advice on background correction in Fiji; and the light-microscopy facility of the BIOTEC/CRTD at TU Dresden for providing excellent microscopy support and maintenance. We thank Michal Graybok for skilful experimental advice and support. P.J. thanks the Academy of Finland for the FiDiPro Award. We acknowledge generous computational resources made available by CSC-IT Centre for Science (Espoo, Finland) and the High-Performance Computing Center of the TU Dresden. Financial support was provided by the Deutsche Forschungsgemeinschaft (DFG) “Transregio 83” (Grant No. TRR83 TP18 (U.C., A.C., E.B.)), the German Federal Ministry of Education and Research grant to the German Center for Diabetes Research (DZD e.V.) (U.C.), the Academy of Finland (Center of Excellence program) (I.V., T.R., S.R.), the European Research Council (Advanced Grant CROWDED-PRO-LIPIDS) (I.V.), a FEBS Short-Term Fellowship (S.R.), the Graduate School program of Tampere University of Technology and Alfred Kordelin Foundation (S.R.), the Polish Ministry of Science and Higher Education (Inventus Plus 2015–2016 project IP2014 007373) (A.C.), the Dresden International Graduate School for Biomedicine and Bioengineering, granted by the DFG (GS879) (E.B.), the Czech Science Foundation GACR 13-19073S (R.P.), 16-01074S (P.J.), and 17-06792S (L.C.), the National Science Foundation (CHE-1413307 (P.S.C.)), and the Office of Naval Research (N00014-14-1-0792 (P.S.C.)).

**REFERENCES**

(1) Putney, J. W.; Tomita, T. Adv. Biol. Regul. 2012, 52 (1), 152–64.

(2) van den Bogaart, G.; Meyenberg, K.; Diederichsen, U.; Jahn, R. J. Biol. Chem. 2012, 287 (20), 16447–53.

(3) McLaughlin, S.; Wang, J.; Gambhir, A.; Murray, D. Annu. Rev. Biophys. Biomol. Struct. 2002, 31, 151–75.

(4) Di Paolo, G.; De Camilli, P. Nature 2006, 443 (7112), 651–7.

(5) Nasuhoglu, C.; Feng, S.; Mao, J.; Yamamoto, M.; Yin, H. L.; Earnest, S.; Barylko, B.; Albanesi, J. P.; Hilgemann, D. W. Anul Biochem. 2002, 301 (2), 243–54.

(6) Wang, Y. H.; Slochower, D. R.; Janmey, P. A. Chem. Phys. Lipids 2014, 182, 38–51.

(7) Clapham, D. E. Cell 2007, 131 (6), 1047–58.

(8) Usachev, Y. M.; Marchenko, S. M.; Sage, S. O. J. Physiol. 1995, 489, 309–17.

(9) Berridge, M. J. J. Physiol. 1997, 499, 291–306.

(10) Berridge, M. J. Cell Calcium 2006, 40 (5–6), 405–12.

(11) Heidelberger, R.; Heinemann, C.; Neher, E.; Matthews, G. Nature 1994, 371 (6547), 813–5.

(12) Llanas, R.; Sugimori, M.; Silver, R. B. Science 1992, 256 (5057), 677–9.

(13) Tengholm, A.; Gyldé, E. Mol. Cell. Endocrinol. 2009, 297 (1–2), 58–72.

(14) Ammala, C.; Eliasson, L.; Bokvist, K.; Larsson, O.; Ashcroft, F. M.; Rorsman, P. J. Physiol. 1993, 472, 665–88.

(15) Parekh, A. B. J. Physiol. 2008, 586 (13), 3043–54.

(16) Grubbs, R. D. BioMetals 2002, 15 (3), 251–9.

(17) Fujise, H.; Cruz, P.; Reo, N. V.; Lauf, P. K. Biochim. Biophys. Acta, Mol. Cell Res. 1991, 1094 (1), 51–54.

(18) Carvalho, K.; Ramos, I.; Roy, C.; Picart, C. Biochim. Biophys. J. 2008, 95 (9), 4348–60.

(19) Ellenbroek, W. G.; Wang, Y. H.; Christian, D. A.; Discher, D. E.; Janmey, P. A.; Liu, A. J. Biophys. J. 2011, 101 (9), 2178–84.

(20) Levental, I.; Christian, D. A.; Wang, Y. H.; Madara, J. J.; Discher, D. E.; Janmey, P. A. Biochemistry 2009, 48 (34), 8241–8.

(21) Sarmento, M. J.; Coutinho, A.; Fedorov, A.; Prieto, M.; Fernandes, F. Biochim. Biophys. Acta, Biomembr. 2014, 1838 (3), 822–30.
