Absence of clustering of phosphatidylinositol-(4,5)-bisphosphate in fluid phosphatidylcholine

Fábio Fernandes,[1,*] Luís M. S. Loura,[*,†] Alexander Fedorov,[*] and Manuel Prieto[*]

Centro de Química-Física Molecular,* Instituto Superior Técnico, Lisbon, Portugal; and Centro de Química e Departamento de Química,† Universidade de Évora, Évora, Portugal

Abstract Phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P_2] plays a key role in the modulation of actin polymerization and vesicle trafficking. These processes seem to depend on the enrichment of PI(4,5)P_2 in plasma membrane domains. Here, we show that PI(4,5)P_2 does not form domains when in a fluid phosphatidylcholine matrix in the pH range of 4.8–8.4. This finding is at variance with the spontaneous segregation of PI(4,5)P_2 to domains as a mechanism for the compartmentalization of PI(4,5)P_2 in the plasma membrane. Water/bilayer partition of PI(4,5)P_2 is also shown to be dependent on the protonation state of the lipid.—Fernandes, F., L. M. S. Loura, A. Fedorov, and M. Prieto. **Absence of clustering of phosphatidylinositol-(4,5)-bisphosphate in fluid phosphatidylcholine. *J. Lipid Res.* 2006.** 47: **1521–1525.**

Supplementary key words PIP2 • lipid domains • fluorescence • fluorescence resonance energy transfer

Phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P_2] is found mainly in the plasma membrane, where it is a critical regulator of several cellular functions. It plays a fundamental role, particularly in actin polymerization and vesicle trafficking (1, 2). These processes seem to depend on large transient and spatially localized increases of PI(4,5)P_2 concentration, as this phospholipid constitutes only ~1% of the lipids in the plasma membrane (3).

Significant evidence indicates that the membrane patches where localized enrichment in PI(4,5)P_2 is observed are cholesterol-rich rafts (4–6), but partition of these phospholipids to liquid-ordered domains is difficult to explain, as the *sn*-2 acyl chain of PI(4,5)P_2 is mainly arachidonic acid, a polyunsaturated acyl chain that is not expected to favor partition into rafts. Local enrichment in PI(4,5)P_2 was shown to overlap with enrichment of phosphatidylinositol-4-monophosphate kinases in the same membrane patches, possibly leading to localized synthesis of the inositol (7). However, as argued previously, this effect alone cannot explain the degree of PI(4,5)P_2 compartmentalization observed, as diffusion away from the site of synthesis is faster than the synthesis itself (8). However, several proteins [myristoylated alanine-rich C-kinase substrate (MARCKS), growth associated protein 43 (GAP43), cytoskeleton-associated protein 23 (CAP23), and neuronal axonal membrane protein (NAP22)] have been shown to be responsible for the lateral sequestration of PI(4,5)P_2 to specific domains in the membrane in a process that for some cases was dependent on cholesterol (9–11). This phenomenon, together with localized synthesis, provides a plausible mechanism for PI(4,5)P_2 compartmentalization, as large concentrations of these proteins could prevent free diffusion of the phospholipid.

In a recent study, it was proposed that PI(4,5)P_2 compartmentalization can be achieved simply through hydrogen bonding between PI(4,5)P_2 head groups at or slightly above physiological pH (corresponding to partial or complete deprotonation of the phosphomonoester group) (12), without the contribution of any external agent (cholesterol or proteins). Through fluorescence resonance energy transfer (FRET) experiments in a phosphatidylcholine (PC) matrix in the fluid state, the authors claimed to have detected PI(4,5)P_2 domain formation, and similar behaviors were observed for PI(3,4)P_2 and PI(3,4,5)P_3 as well as for phosphatidylinositol monophosphates in another study (13). Because of the large biological relevance of the problem and the controversy of these conclusions, we propose in this work to investigate this issue using a detailed experimental approach. Our results show clearly that PI(4,5)P_2 does not form domains when in a PC matrix in the pH range of 4.8–8.4 and that partition of PI(4,5)P_2 to PC bilayers is largely dependent on the pH.

MATERIALS AND METHODS

POPC, 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), 1,2-dipalmitoylphosphatidycholine (DPPC), and 7-nitro-2-1,3-ben-

Manuscript received 13 March 2006 and in revised form 18 April 2006.

Published, JLR Papers in Press, April 21, 2006.
DOI 10.1194/jlr.M600121-JLR200

Abbreviations: DPH, diphenylhexatriene; DPPC, 1,2-dipalmitoylphosphatidycholine; FRET, fluorescence resonance energy transfer; NBD, 7-nitro-2-1,3-benzoxadiazol; PC, phosphatidylcholine; PI(4,5)P_2, phosphatidylinositol-(4,5)-bisphosphate; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol.

[1] To whom correspondence should be addressed.
e-mail: fernandesf@ist.utl.pt

zoxadiazol (NBD)-PC were obtained from Avanti Polar Lipids (Birmingham, AL). NBD-PI(4,5)P_2 was from Echelon Biosciences (Salt Lake City, UT). Diphenylhexatriene (DPH) was obtained from Molecular Probes (Leiden, The Netherlands). Other fine chemicals were from Merck (Darmstadt, Germany).

Liposome reconstitution procedure

The desired amounts of phospholipids were mixed in chloroform-methanol (1:2) and dried under an $N_{2(g)}$ flow. The sample was then kept in a vacuum overnight. Liposomes were prepared with 20 mM HEPES, 100 mM NaCl buffer at pH 8.4 and 7.1 and with 20 mM sodium citrate, 100 mM NaCl buffer at pH 4.8. The hydration step was performed with the addition of buffer followed by freeze-thaw cycles. Anisotropy measurements were performed with large unilamellar vesicles produced by extrusion through polycarbonate filters with a pore size of 100 nm (14).

Fluorescence measurements

Steady-state fluorescence measurements were carried out with an SLM-Aminco 8100 Series 2 spectrofluorimeter described in detail elsewhere (15). Steady-state anisotropies were determined according to Lakowicz (16). NBD-PC and NBD-PI(4,5)P_2 anisotropies were recorded with excitation and emission wavelengths of 460 and 540 nm, respectively, with spectral bandwidths of 4 nm. All measurements were performed at room temperature.

Fluorescence decay measurements of DPH (donor) were carried out with a time-correlated single-photon timing system, which is described elsewhere (15). All measurements were performed at room temperature. Excitation and emission wavelengths were 340 and 430 nm, respectively.

In the FRET experiments, the donor decays in the presence of acceptors [$i_{DA}(t)$] can be described as

$$i_{DA}(t) = i_D(t) \times \rho_{interplanar}(t) \qquad (Eq.\ 1)$$

where $i_{DA}(t)$ and $i_D(t)$ are the fluorescence decays of the donor in the presence and absence of acceptors, respectively. $\rho_{interplanar}$ is the FRET contribution arising from energy transfer to randomly distributed acceptors in two different planes from the donors (two monolayer leaflets) (17)

$$\rho_{interplanar} = \exp\left\{-4 \cdot n_2 \times \pi \times l^2 \cdot \int_0^{\sqrt{l^2 + R_c^2}} \frac{1 - \exp(-t \times b^3 \times \alpha^6)}{\alpha^3} d\alpha\right\} \qquad (Eq.\ 2)$$

where $b = (R_0^2/l)^2 \tau_D^{-1/3}$, R_0 is the Förster radius, n_2 is the acceptor density in each leaflet, and l is the distance between the plane of the donor and the two planes of acceptors. The Förster radius was calculated as described elsewhere (18).

RESULTS

NBD-PI(4,5)P_2 partition to lipid bilayers

When using a labeled phospholipid as the acceptor in a FRET study, the energy transfer efficiency will depend strongly on its concentration inside these vesicles. As PI(4,5)P_2 is known to generally form micelles in the aqueous medium (19), it is essential in the type of measurements mentioned above to determine whether all NBD-PI(4,5)P_2 incorporates in the liposomes and, if that is not the case, to quantify the effective fraction of NBD-PI(4,5)P_2 incorporated in liposomes. NBD quantum yield decreases with hydration, and clustering of NBD-PI(4,5)P_2 leads to NBD self-quenching (20). Consequently, it was not surprising that NBD-PI(4,5)P_2 in buffer (micellar state) exhibited quantum yields ~100 times smaller than when incorporated in PC liposomes (lamellar state) (**Fig. 1**). This difference allowed us to use fluorescence intensity as a tool to estimate the extent of water/bilayer partition for NBD-PI(4,5)P_2. From equation 3 (21), the extent of partition can be quantified into partition coefficients (K_p)

$$I = \frac{I_w + K_P \times \gamma_L \times [L] \times I_L}{1 + K_P \times \gamma_L \times [L]} \qquad (Eq.\ 3)$$

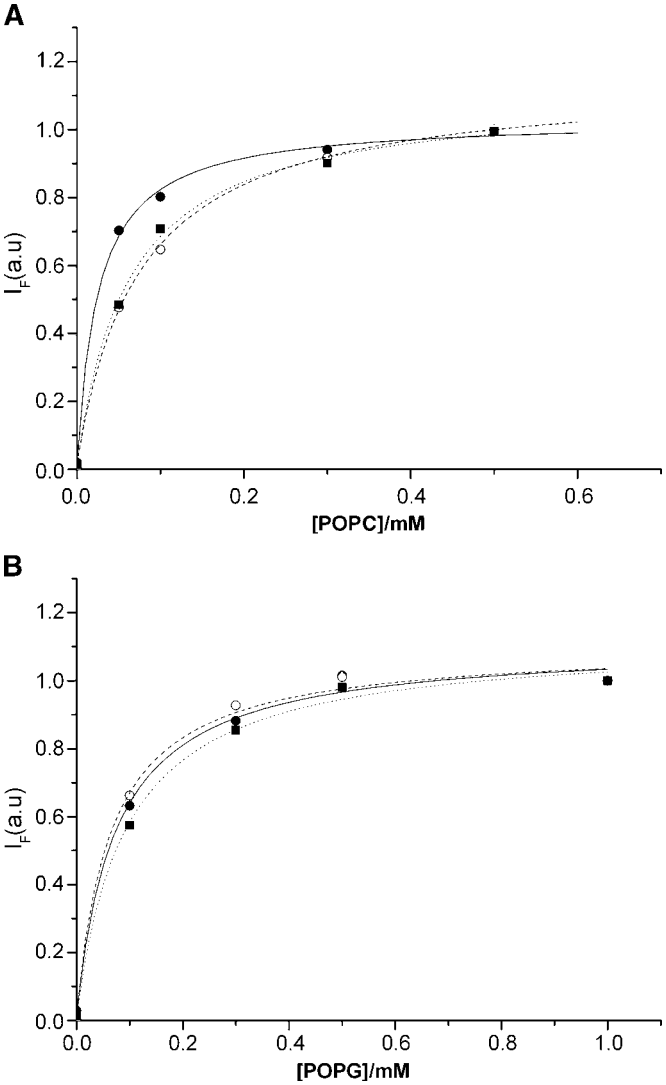

Fig. 1. Fluorescence intensity (I_F) of phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P_2] as a function of POPC (A) and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) (B) concentration. Closed circles, pH 8.4; open circles, pH 7.1; closed squares, pH 4.8. The curves are fits of equation 3 to the experimental data, allowing the determination of water/bilayer partition coefficients (K_p). 7-Nitro-2-1,3-benzoxadiazol (NBD)-PI(4,5)P_2 concentration = 1 μM. a.u., arbitrary units.

where I is the fluorescence intensity, I_W and I_l are the fluorescence intensities in buffer and in liposomes, respectively, γ_l is the lipid molar volume, and [l] is the lipid concentration.

The curves obtained when the NBD-PI(4,5)P_2 fluorescence intensities were plotted versus the lipid concentrations are shown in Fig. 1. Three different pH values (8.4, 7.1, and 4.8) corresponding to three different protonation states of PI(4,5)P_2 (for the micellar state Pk_{a2} = 6.7 for the 4′ position and 7.6 for the 5′ position) were studied. The results for the different pH values were clearly different when using POPC bilayers: a larger extent of partition was observed when pH 8.4 was used (corresponding to a total charge of −5), whereas partition for pH 7.1 (−4) was almost identical to that for pH 4.8 (−3). For POPG, the partition was much less dependent on pH. The K_p values obtained at pH 8.4, 7.1, and 4.8 for water/POPC partition were $(5.38 \pm 0.60) \times 10^4$, $(1.84 \pm 0.14) \times 10^4$, and $(2.18 \pm 0.18) \times 10^4$, respectively, whereas for micelle/POPG partition, these were $(1.84 \pm 0.33) \times 10^4$, $(2.29 \pm 0.38) \times 10^4$, and $(1.47 \pm 0.18) \times 10^3$, respectively.

Clustering of NBD-PI(4,5)P_2 in POPC

Clustering of NBD-PI(4,5)P_2 inside the POPC matrix would result in a decrease of fluorescence intensity attributable to self-quenching of NBD and in a decrease in fluorescence anisotropy attributable to energy migration (energy homotransfer) inside the clusters (20). The two parameters were studied for increasing NBD-PI(4,5)P_2 concentrations inside a POPC matrix, as shown in **Fig. 2**. NBD-PI(4,5)P_2 concentrations were corrected for the partition coefficients determined above. As a control, the fluorescence intensity and anisotropies of NBD-PC in POPC and of NBD-PI(4,5)P_2 in DPPC were compared with the data for NBD-PI(4,5)P_2 in POPC. NBD-PC in POPC is homogeneously distributed, whereas in DPPC, clustering is expected for NBD-PI(4,5)P_2 as a result of gel-fluid phase separation.

It is clear that NBD-PI(4,5)P_2 and NBD-PC have identical clustering behavior in POPC. The degree of self-quenching and energy migration of both lipids is almost identical and much smaller than for NBD-PI(4,5)P_2 in DPPC at room temperature, at which clustering is observed as a result of packing restraints in the DPPC gel matrix (22).

We also performed a FRET experiment, using for this purpose the fluorescence decay of the donor. The reason for a time-dependent approach instead of a steady-state approach is that the kinetics of the donor decay gives information on both the distribution (homogeneity/heterogeneity) and concentration of acceptors (15), whereas the steady-state approach is unable to distinguish between the two. Again, we chose POPC as the matrix lipid, the donor was DPH, which is known to have no preference between lipid phases (23), and the acceptor was NBD-PI(4,5)P_2. In this system, in the case of NBD-PI(4,5)P_2 clustering, two populations of DPH would be detectable, one residing in a NBD-PI(4,5)P_2-rich site and the other in a NBD-PI(4,5)P_2-poor area of the bilayer.

We globally fitted the FRET model for a single discrete concentration of acceptors (homogeneous distribution of acceptors) (equations 2, 3) to the data in **Fig. 3**, and for all pH values used (only results for pH 8.4 are shown), good quality fits were obtained (global Chi-square < 1.5). The acceptor concentrations were the only free parameter during these fits, and the recovered values matched closely (±10%) the bilayer concentrations expected from the partition coefficients determined above, again confirming the absence of NBD-PI(4,5)P_2 clustering.

DISCUSSION

Compartmentalization of PI(4,5)P_2 has been reported several times, but its origin had always been attributed to

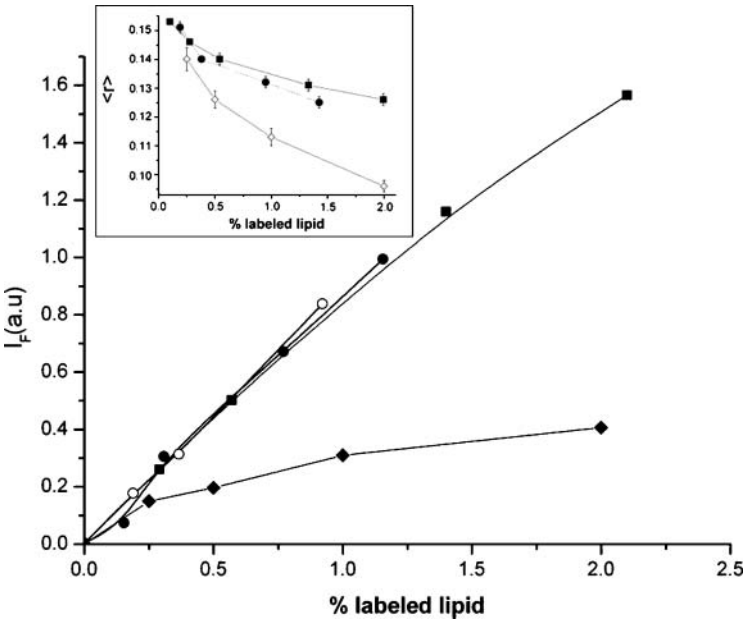

Fig. 2. Fluorescence intensity (I_F) of NBD-PI(4,5)P_2 in POPC (closed circles), POPG (open circles), and 1,2-dipalmitoylphosphatidycholine (DPPC; closed diamonds) bilayers and of phosphatidylcholine NBD-PC in POPC (closed squares). Inset: Fluorescence anisotropy (<r>) of NBD-PI(4,5)P_2 in POPC (closed circles) and DPPC (open diamonds) bilayers and of NBD-PC in POPC (closed squares). Error bars represent SD. All measurements were performed at pH 8.4. Total lipid concentration = 0.2 mM. a.u., absorbance units.

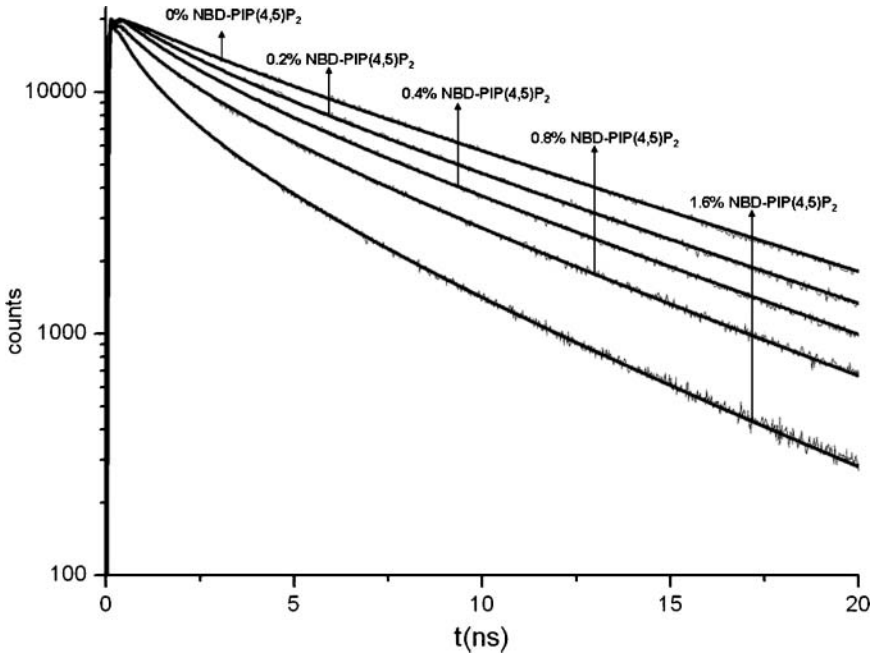

Fig. 3. Fluorescence decays of diphenylhexatriene in the absence and presence of increasing concentrations of NBD-PI(4,5)P_2 in POPC bilayers at pH 8.4 (thin lines). Global analysis of the data according to the model for homogeneous distribution of NBD-PI(4,5)P_2 (equation 3) resulted in the fitted curves (thick lines). The concentration of PI(4,5)P_2 was kept constant (5%) by the addition of unlabeled PI(4,5)P_2. Total lipid concentration = 0.2 mM. t, time.

the interaction with the cytoskeleton and/or with specific proteins exhibiting preferences for particular phases inside the membrane. The recent proposal for a spontaneous demixing of completely deprotonated PI(4,5)P_2 when in a PC matrix even at very low concentrations as a result of intermolecular hydrogen bonding (12) suggested an additional mechanism for localized enrichments of this phospholipid. In this work, we tested this hypothesis through a carefully designed approach that considered and tested the existence of possible artifacts that could go undetected in a phenomenological approach (mere observation of increases or decreases in energy transfer as a result of pH variations).

Redfern and Gericke (12) showed by differential scanning calorimetry and Fourier transform infrared spectroscopy that demixing occurred for mixtures of PI(4,5)P_2 and PC lipids in the gel phase. Nevertheless, in vivo, PI(4,5)P_2 contains polyunsaturated acyl chains, and a gel state is highly unlikely for this lipid. With this in mind, these authors used FRET as a tool to detect the immiscibility of PI(4,5)P_2 and PC in the fluid state at low PI(4,5)P_2 concentrations. They used POPC as the lipid matrix and a NBD-labeled PC as the FRET donor to a short-chain (C6) Dipyrromethaneboron difluoride labeled PI(4,5)P_2. In this system, they observed variations of energy transfer efficiency as the pH was scanned. The authors interpreted the decrease of energy transfer efficiencies at high pH [>Pk_{a2} of PI(4,5)P_2] as a demixing between PI(4,5)P_2 and POPC. However, the use of a very short-chain PI(4,5)P_2 as the acceptor in this FRET experiment is likely to have resulted in a significant fraction of acceptors not being incorporated in the membrane. The lipid/water partition coefficients of this short-chain-labeled PI(4,5)P_2 is expected to be significantly smaller than those determined here for NBD-PI(4,5)P_2, because K_p varies dramatically with the number of carbons in the acyl chains of a phospholipid (24). In this case, energy transfer efficiencies would decrease not because of the formation of PI(4,5)P_2 clusters but as a result of changes in the lipid/water partition coefficient of the probe, as the PI(4,5)P_2 group becomes fully deprotonated. Redfern and Gericke (12) acknowledge that the extent of membrane incorporation changed considerably for different labeled phosphatidylinositides.

Analysis of the kinetics of donor decay in a FRET experiment was the method chosen by us to probe the eventual clustering of PI(4,5)P_2 in a zwitterionic bilayer, because it is capable of retrieving information on the homogeneity of the fluorescently labeled PI(4,5)P_2 independently of any quantitative knowledge of the probe's bilayer concentration. The good agreement between the model considering a homogeneous distribution of NBD-PI(4,5)P_2 and the data is solid proof of the absence of PI(4,5)P_2 clustering at low PI(4,5)P_2 concentrations (<5%) even in a completely deprotonated state. The result of this experiment alone cannot exclude the formation of very small PI(4,5)P_2 clusters (~R_0 for this Förster pair = 40 Å), but, together with the NBD energy migration and self-quenching studies, it is clear that PI(4,5)P_2 has no lateral organization in a fluid POPC matrix. It is

certainly feasible that PI(4,5)P_2/PC demixing exists in the gel phase, as packing restraints are severely increased (22).

The difference in the lipid/water partition coefficients of the NBD-PI(4,5)P_2 fully and partially deprotonated states is interesting, and the same trend is expected for non-labeled PI(4,5)P_2. This difference might be the result of destabilization of the fully deprotonated PI(4,5)P_2 micellar structure. However, it is very likely that the labeling with NBD decreases the extent of partition to some extent, and further studies will be necessary to determine whether this phenomenon has physiological relevance.

F.F. acknowledges Grant SFRH/BD/14282/2003 from Fundação para a Ciência e a Tecnologia (FCT) (Portugal). A.F. acknowledges Grant SFRH/BPD/26150/2005 from FCT (Portugal). This work was funded by FCT (Portugal) under the program Programa Operacional Ciência e Inovação (POCI).

REFERENCES

1. Payrastre, B., K. Missy, S. Giuriato, S. Bodin, M. Plantavid, and M-P. Gratacap. 2001. Phosphoinositides: key players in cell signalling, in time and space. *Cell. Signal.* **13:** 377–387.
2. Czech, M. P. 2003. Dynamics of phosphoinositides in membrane retrieval and insertion. *Annu. Rev. Physiol.* **65:** 791–815.
3. Ferrell, J., Jr., and W. Huestis. 1984. Phosphoinositide metabolism and the morphology of human erythrocytes. *J. Cell Biol.* **98:** 1992–1998.
4. Pike, L. J., and L. Casey. 1996. Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains. *J. Biol. Chem.* **271:** 26453–26456.
5. Pike, L. J., and J. M. Miller. 1998. Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. *J. Biol. Chem.* **273:** 22298–22304.
6. Rozelle, A. L., L. M. Machesky, M. Yamamoto, M. H. E. Driessens, R. H. Insall, M. G. Roth, K. Luby-Phelps, G. Marriott, A. Hall, and H. L. Yin. 2000. Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. *Curr. Biol.* **10:** 311–320.
7. Botelho, R. J., M. Teruel, R. Dierckman, R. Anderson, A. Wells, J. D. York, T. Meyer, and S. Grinstein. 2000. Localized biphasic changes in phosphatidylinositol-4,5-bisphosphate at sites of phagocytosis. *J. Cell Biol.* **151:** 1353–1368.
8. McLaughlin, S., J. Wang, A. Gambhir, and D. Murray. 2002. PIP2 and proteins: interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struct.* **31:** 151–175.
9. Laux, T., K. Fukami, M. Thelen, T. Golub, D. Frey, and P. Caroni. 2000. GAP43, MARCKS, and CAP23 modulate PI(4,5)P2 at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *J. Cell Biol.* **149:** 1455–1472.
10. Rauch, M. E., C. G. Ferguson, G. D. Prestwich, and D. S. Cafiso. 2002. Myristoylated alanine-rich C kinase substrate (MARCKS) sequesters spin-labeled phosphatidylinositol 4,5-bisphosphate in lipid bilayers. *J. Biol. Chem.* **277:** 14068–14076.
11. Epand, R. M., P. Vuong, C. M. Yip, S. Maekawa, and R. F. Epand. 2004. Cholesterol-dependent partitioning of PtdIns(4,5)P2 into membrane domains by the N-terminal fragment of NAP-22 (neuronal axonal myristoylated membrane protein of 22 kDa). *Biochem. J.* **379:** 527–532.
12. Redfern, D. A., and A. Gericke. 2005. pH-dependent domain formation in phosphatidylinositol polyphosphate/phosphatidylcholine mixed vesicles. *J. Lipid Res.* **46:** 504–515.
13. Redfern, D. A., and A. Gericke. 2004. Domain formation in phosphatidylinositol monophosphate/phosphatidylcholine mixed vesicles. *Biophys. J.* **86:** 2980–2992.
14. Mayer, L. D., M. J. Hope, and P. R. Cullis. 1986. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta.* **858:** 161–168.
15. Loura, L. M. S., A. Fedorov, and M. Prieto. 2001. Fluid-fluid membrane microheterogeneity: A fluorescence resonance energy transfer study. *Biophys. J.* **80:** 776–788.
16. Lakowicz, J. R. 1999. Principles of Fluorescence Spectroscopy. Kluwer Academic/Plenum Publishers, New York.
17. Davenport, L., R. E. Dale, R. H. Bisby, and R. B. Cundall. 1985. Transverse location of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene in model lipid bilayer membrane systems by resonance energy transfer. *Biochemistry.* **24:** 4097–4108.
18. Berberan-Santos, M. N., and M. Prieto. 1987. Energy transfer in spherical geometry. *J. Chem. Soc. Faraday Trans.* **83:** 1391–1409.
19. Sugiura, Y. 1981. Structure of molecular aggregates of 1-(3-sn-phosphatidyl)-L-myo-inositol 3,4-bis(phosphate) in water. *Biochim. Biophys. Acta.* **641:** 149–159.
20. Prieto, M. J., M. Castanho, A. Coutinho, A. Ortiz, F. J. Aranda, and J. C. Gomez-Fernandez. 1994. Fluorescence study of a derivatized diacylglycerol incorporated in model membranes. *Chem. Phys. Lipids.* **69:** 75–85.
21. Santos, N. C., M. Prieto, and M. A. R. B. Castanho. 2003. Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods. *Biochim. Biophys. Acta.* **1612:** 123–135.
22. Loura, L. M. S., A. Fedorov, and M. Prieto. 2000. Membrane probe distribution heterogeneity: a resonance energy transfer study. *J. Phys. Chem. B.* **104:** 6920–6931.
23. Lentz, B. R., Y. Barenholz, and T. E. Thompson. 1976. Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. II. Two-component phosphatidylcholine liposomes. *Biochemistry.* **15:** 4529–4537.
24. Nichols, J. W. 1985. Thermodynamics and kinetics of phospholipid monomer-vesicle interaction. *Biochemistry.* **24:** 6390–6398.