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Diffusion Coefficient of Fluorescent Phosphatidylinositol 4,5-bisphosphate in the Plasma Membrane of Cells

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Phosphatidylinositol 4,5-bisphosphate (PIP_2) controls a surprisingly large number of processes in cells. Thus, many investigators have suggested that there might be different pools of PIP_2 on the inner leaflet of the plasma membrane. If a significant fraction of PIP_2 is bound electrostatically to unstructured clusters of basic residues on membrane proteins, the PIP_2 diffusion constant, D, should be reduced. We microinjected micelles of Bodipy TMR-PIP_2 into cells, and we measured D on the inner leaflet of fibroblasts and epithelial cells by using fluorescence correlation spectroscopy. The average ± SD value from all cell types was D = 0.8 ± 0.2 $\mu m^2/s$ (n = 218; 25°C). This is threefold lower than the D in blebs formed on Rat1 cells, D = 2.5 ± 0.8 $\mu m^2/s$ (n = 26). It is also significantly lower than the D in the outer leaflet or in giant unilamellar vesicles and the diffusion coefficient for other lipids on the inner leaflet of these cell membranes. The simplest interpretation is that approximately two thirds of the PIP_2 on inner leaflet of these plasma membranes is bound reversibly.

INTRODUCTION

Phosphatidylinositol 4,5 bisphosphate (PIP_2) directly regulates many processes at the inner leaflet of the plasma membrane, such as the activation of ion channels, endocytosis, and exocytosis (Di Paolo and De Camilli, 2006). It is also the source of three second messengers: how does one lipid do so much? Many investigators have considered the possibility there are different pools of PIP_2 in the plasma membrane (Hinchliffe *et al*., 1998; Aikawa and Martin, 2003; Janmey and Lindberg, 2004). Little is known, however, about how these separate pools come about and how they are regulated. One hypothesis is that membrane-bound clusters of basic residues on peripheral (e.g., myristoylated alanine rich C kinase substrate [MARCKS], K-Ras, and gravin) and integral (e.g., epidermal growth factor receptor [EGFR] and polymeric immunoglobulin receptor [pIgR]) proteins concentrate electrostatically (sequester) a significant fraction of the PIP_2 in the membrane; when the local concentration of Ca^{2+} rises and calcium/calmodulin (Ca/CaM) binds to these basic clusters, the sequestered PIP_2 is released, increasing its local free concentration (McLaughlin and Murray, 2005).

Theoretical calculations and experimental measurements on model systems support this hypothesis. Specifically, electrostatic theory predicts a membrane-bound cluster of basic residues produces a local positive potential that concentrates multivalent acidic lipids such as PIP_2 (Wang *et al*., 2004; McLaughlin and Murray, 2005). Fluorescence resonance energy transfer, electron paramagnetic resonance, and phospholipase C (PLC) activity measurements confirm that basic clusters on purified proteins or peptides do indeed laterally concentrate PIP_2 in phospholipid vesicles (Murray *et al*., 2002; Gambhir *et al*., 2004). These calculations and model studies also suggest that if the number of membrane-bound basic clusters is equal to or greater than the number of PIP_2 on the inner leaflet of a plasma membrane, a significant fraction of PIP_2 will be sequestered. Testing this prediction requires direct experiments on living cells, however, because the exact concentration of these basic clusters is not known with certainty for any cell type.

Studies of microvesicles released from human erythrocytes under different conditions (Hagelberg and Allan, 1990) provided evidence that ~50% of the PIP_2 is bound to cytoskeletal proteins (e.g., Band III and glycophorin) that are depleted in the microvesicles. Specifically, the microvesicles and native membranes contained identical mole fractions of most lipids, but not polyphosphoinositides (e.g., PIP_2). The higher mole fraction of PIP_2 in the native membranes suggests ~50% of their PIP_2 is not free to diffuse into the nascent microvesicles.

One independent way to estimate the fraction of free PIP_2 in the plasma membrane is to measure directly the diffusion coefficient, D, of PIP_2 on the inner leaflet in a living cell. A simple analysis (see Supplemental Material) reveals that if R is the ratio of reversibly bound or sequestered (S) to free (C) PIP_2, the diffusion constant will be scaled by a factor $1/(R + 1)$.

Specifically, combining the diffusion equation (Fick's second law) with a linear adsorption isotherm (Henry's law) produces a diffusion equation with an apparent diffusion constant, D:

$$D = \frac{D_{free}}{R + 1}, \quad (1)$$

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Abbreviations used: FCS, fluorescence correlation spectroscopy; GUV, giant unilamellar vesicle; MARCKS, myristoylated alanine rich C kinase substrate.

where

$$S = RC \quad (2)$$

where S is the concentration of sequestered PIP_2 (reversibly bound or immobilized), C is the concentration of PIP_2 free to diffuse, and D_{free} is the diffusion coefficient of free PIP_2. If the total concentration of PIP_2 $T = S + C$, then $C = T/(R + 1)$.

If the erythrocyte result can be extrapolated to other cells, such as fibroblasts, a significant fraction of PIP_2 in these plasma membranes is also bound reversibly to proteins. Equation 1 then predicts that PIP_2 will diffuse less rapidly in the inner leaflet of a fibroblast plasma membrane than in a "bleb" formed on that membrane. It also predicts that PIP_2 will diffuse less rapidly in the inner than in the outer leaflet of the plasma membrane and less rapidly on the inner leaflet than either neutral or monovalent lipids that are not bound to proteins.

Experiments on giant unilamellar vesicles (GUVs) showed monomers of fluorescent PIP_2 can be incorporated into the outer leaflet of preformed vesicles by exposing them to micelles of PIP_2; fluorescence correlation spectroscopy (FCS) measurements showed PIP_2 diffuses with a D characteristic of other lipids in the GUVs (Golebiewska *et al*., 2006). Here, we report FCS measurements of the D of fluorescent PIP_2 in plasma membranes after microinjecting fluorescent PIP_2 micelles into fibroblasts (Rat1, Cos1, REF52, and NIH3T3) and epithelial cells (human embryonic kidney [HEK]293 and Fisher rat thyroid [FRT]).

Our most important result is that Bodipy TMR-PIP_2 in the inner leaflet of plasma membranes diffuses significantly more slowly than the same lipid in blebs, in the outer leaflet of plasma membranes, or in GUVs. These observations are consistent with the Hagelberg and Allan (1990) erythrocyte microvesicle partitioning results. Hence, both the partitioning and diffusion experiments suggest a significant fraction of the PIP_2 on the inner leaflet is bound reversibly.

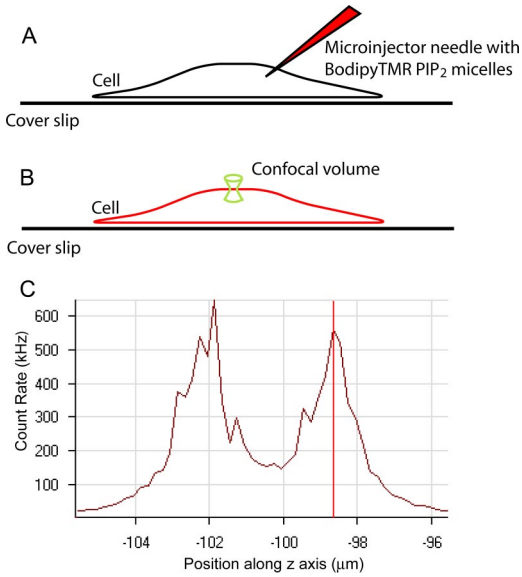

Figure 1. Incorporation of Bodipy TMR-PI(4,5)P_2 into the inner leaflet of a plasma membrane. (A) Cartoon depicting a Rat1 fibroblast with an adjacent microinjector needle filled with micelles that contain Bodipy TMR-PIP_2. (B) One minute after injecting the micelles, much of the fluorescent PIP_2 incorporates as monomers into the inner leaflet of the plasma membrane. We use the confocal microscope to scan from the bottom to the top of the cell both to ensure incorporation of the fluorescent PIP_2 and to determine the location of the upper membrane (C). We then focus on the upper membrane, as illustrated, and use the FCS function to record the fluctuations in fluorescence and compute the autocorrelation functions illustrated in Figure 2. (C) Fluorescence intensity scan in the z-direction of a Rat 1 cell after injection with arachidoyl-lysoPC/Bodipy TMR-PIP_2 micelles. The peaks correspond to the positions of the plasma membrane; the data in Figure 2 are from the same cell. The simplest interpretation is that a significant fraction of the fluorescent PIP_2 has incorporated into the plasma membrane.

MATERIALS AND METHODS

The Supplemental Material includes details of the experimental protocols. In brief, we performed confocal imaging and FCS measurements using an LSM 510 Meta/Confocor 2 apparatus (Carl Zeiss, Jena, Germany) with standard configurations. FCS has been reviewed previously (Elson, 2001; Rigler and Elson, 2001; Marguet *et al*., 2006; Haustein and Schwille, 2007). We microinjected the micelles using an InjectMan NI2 with FemtoJet pump from Eppendorf, typically mounted on an Axiovert 200M microscope (Carl Zeiss) fitted with a long working distance 40× phase contrast objective.

We obtained similar results with three different types of Bodipy TMR-phosphatidylinositol 4,5-biphosphate [PI(4,5)P_2], by using three different types of micelles, as discussed in Supplemental Material. One major advantage of FCS over fluorescence recovery after photobleaching (FRAP) is that the fluorescent PIP_2 we introduce into the membrane does not perturb significantly the endogenous level of PIP_2 in the plasma membrane. Specifically, the illuminated (~0.2-μm radius) portion of the plasma membrane contains ~10^5 lipids and ~10^3 PIP_2, and our technique incorporated 1–100 fluorescent PIP_2 into this area.

We made most of our measurements on Rat1 fibroblasts or HEK293 epithelial cells, but we also report data from FCS measurements on REF52, Cos1, NIH3T3 fibroblasts, and FRT epithelial cells. The diffusion coefficient of PIP_2 on the inner leaflet did not differ significantly between the cell types or lines, and we pooled all data. All measurements were made at 25°C.

RESULTS

PIP_2 in Inner Leaflet of Rat1 Plasma Membranes Diffuses Threefold More Slowly than PIP_2 in Blebs

Direct physical measurements (Hirai *et al*., 1996; Flanagan *et al*., 1997) and our FCS data (not shown) indicate both native PIP_2 and long-chain Bodipy TMR-PIP_2 [see figure 2 of Gambhir *et al*. (2004) for structure] form micelles, rather than bilayer vesicles, in solution; this is because of the disparity in the cross sectional areas of the PIP_2 head group and hydrocarbon tails. Earlier work showed adding Bodipy TMR-PIP_2 micelles (as well as mixed micelles containing this lipid: see Supplemental Material methods) to preformed GUVS produces spontaneous transfer of fluorescent PIP_2 into the exposed bilayer leaflet, where they have a diffusion constant characteristic of other phospholipids (Fernandes *et al*., 2006; Golebiewska *et al*., 2006). Thus, we simply microinjected micelles containing Bodipy TMR-PIP_2 into cells (Figure 1A), where they diffused rapidly through the cytoplasm and incorporated PIP_2 monomers into the plasma membrane (Figure 1B). We then used the confocal microscope to scan from the bottom to the top of the cell and record the Bodipy TMR-PIP_2 fluorescence. Figure 1C shows a typical result for a Rat1 fibroblast; the fluorescence peaks correspond to the locations of the plasma membrane at the bottom and top of the cell. The width at half maximum is similar to that observed in GUVs with fluorescent PIP_2, where the fluorescence is confined to a bilayer (Golebiewska *et al*., 2006). The resolution is not sufficient to distinguish between PIP_2 incorporated into the plasma membrane bilayer and PIP_2 adsorbed to cytoskeletal elements. We consider this issue in the Supplemental Material, where we also discuss experiments that suggest why the micelles deliver PIP_2 primarily to plasma membrane versus internal membranes.

The FCS measurements were made on the upper portion of the plasma membrane (Figure 1B). The instrument re-

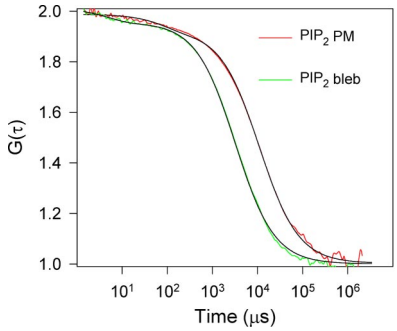

Figure 2. Autocorrelation function, $G(\tau)$, of Bodipy TMR-PIP_2 diffusing in the inner leaflet of a Rat1 cell (red curve) and a large bleb (green curve) on a different Rat1 cell from the same dish. The black curves represent the fit of Eq. S3 in Supplemental Material to the data. The correlation time (approximate midpoint of curve) for PIP_2 in the native plasma membrane is 10.2 ms, which corresponds to a $D = 0.9\ \mu m^2/s$. The correlation time for PIP_2 in the large bleb is 3.2 ms, which corresponds to a $D = 3\ \mu m^2/s$. There are eight Bodipy TMR-PIP_2 diffusing in the confocal volume and $\sim 10^3$ native PIP_2 in the ~200-nm-radius area.

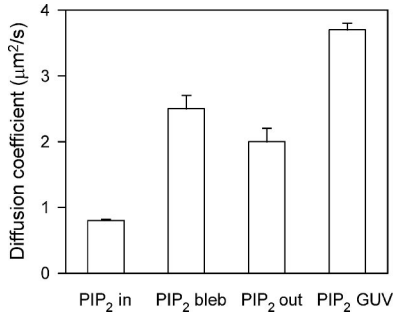

Figure 3. Average diffusion coefficients of Bodipy TMR-PIP_2 in the inner leaflet of epithelial and fibroblast plasma membranes (PIP_2 in), in large blebs formed on Rat1 cells (PIP_2 bleb), in the outer leaflet of cells (PIP_2 out), and in a giant unilamellar phospholipid vesicle formed from a mixture of palmitoyl-oleoyl phosphatidylcholine and palmitoyl-oleoyl phosphatidylserine (PIP_2 GUV). Note that PIP_2 diffuses threefold less rapidly in the inner leaflet than the bleb; the result is consistent (Eq. 1) with the hypothesis that two thirds of the PIP_2 on the inner leaflet is bound reversibly to cytoskeletal elements that are absent in the bleb. All measurements were made at 25°C; vertical bars indicate SE. We calculated diffusion coefficients from autocorrelation curves similar to those illustrated in Figure 2. The difference between the values of D in the bleb and inner leaflet is statistically significant. See text for details.

corded fluctuations in fluorescence for 10-s intervals 10 times, and then it calculated the autocorrelation function from the experimental results. The micelles we microinjected in these experiments were formed from a mixture of Bodipy TMR-PIP_2 and lysophosphatidylcholine (lysoPC) for technical reasons discussed in the Supplemental Material. Because the injections cause some cells to form large blebs (Sheetz *et al*., 2006; Charras *et al*., 2006; Baumgart *et al*., 2007), we were able to compare the D of PIP_2 on blebs, which presumably lack much of the cytoskeleton and intrinsic membrane proteins associated with the cytoskeleton, with the D of PIP_2 in native Rat1 plasma membranes.

Figure 2 shows autocorrelation functions computed from the signals recorded from a large bleb (green) on a Rat1 cell and from the plasma membrane (red) of a Rat1 cell that lacked blebs; both cells were in the same dish, and they were injected at about the same time. The FCS measurements determine the correlation times of the fluorescent PIP_2 diffusing in the inner leaflet of the plasma membrane. (Equations S1 and S3 in Supplemental Material show the correlation time is approximately the midpoint of the curves in Figure 2. If the confocal volume contains one diffusing fluorescent PIP_2, this corresponds to the residence time, or average diffusion time required for the lipid to exit the illuminated region, radius $r = 0.19\ \mu m$). We calculate the diffusion coefficient, D, from the Einstein relation, $r^2 = 4Dt$, where r is the radius of the confocal volume (calibrated daily by measuring the correlation time of rhodamine, which has a known diffusion coefficient) and t is the correlation time (Rigler and Elson, 2001). For the data in Figure 2, we calculated the correlation time for Bodipy TMR-PIP_2 in the Rat1 bleb as 3.2 ms and in the plasma membrane as 10.2 ms; the diffusion constants corresponding to these correlation times are $D = 2.8$ and $0.9\ \mu m^2/s$, respectively. Thus, these two measurements indicate PIP_2 diffuses threefold faster in the bleb than in the normal membrane.

The average ± SD D value for Bodipy TMR-PIP_2 in an unperturbed inner leaflet of the plasma membrane of Rat1 cells was $0.9 \pm 0.2\ \mu m^2/s$ ($n = 40$), similar to the average value we measured in all experiments with the four fibroblast and two epithelial cell lines, $0.8 \pm 0.2\ \mu m^2/s$ ($n = 218$); the experiments used three different types of micelles containing three different chemical forms of Bodipy TMR-PIP_2 (see Supplemental Material). The average D in the Rat1 blebs, $2.5 \pm 0.8\ \mu m^2/s$ ($n = 26$), was 2.8-fold higher than D in the intact Rat1 plasma membrane. Figure 3 is a bar graph summarizing these results. The simplest interpretation of the results (Eq. 1) is that two thirds of the PIP_2 in a Rat1 plasma membrane is not free to diffuse, possibly because it is bound reversibly to cytoskeletal proteins that are present at lower concentration in the blebs than in the unperturbed membrane.

Comparison with Model Membranes

We also measured the diffusion coefficient of fluorescent PIP_2 incorporated from LysoPC/Bodipy TMR-PIP_2 micelles into palmitoyl-oleoyl phosphatidylcholine (POPC)/palmitoyl-oleoyl phosphatidylserine (POPS) (9:1) GUVs using the same FCS technique and temperature (25°C); Figure 3 shows $D = 3.7 \pm 0.8\ \mu m^2/s$ (average ± SD; $n = 6$) under these conditions. (As expected, this is slightly faster than the D measured at 21°C: $D = 3.3 \pm 0.8\ \mu m^2/s$ [$n = 23$] using same FCS technique; Golebiewska *et al*., 2006). Thus, the D of PIP_2 in blebs formed on the plasma membranes of Rat1 cells is comparable with the value observed on pure phospholipid membranes, where the PIP_2 diffuses freely (Figure 3). The lower D in the blebs (2.5 vs. 3.7 $\mu m^2/s$) could be due to presence of cholesterol, which is known to reduce the D of lipids in a fluid membrane (Almeida and Vaz, 1995; Korlach *et al*., 1999; Filippov *et al*., 2003), although the mechanism by which this occurs is controversial (Almeida *et al*., 2005). We confirmed with FCS measurements that incorporating physiological mole fractions of cholesterol can decrease D in a POPC/POPS GUV to the value of D measured in blebs (data not shown).

PIP_2 on the Outer Leaflet of Fibroblast and Epithelial Cells Diffuses 2.2-Fold Faster than PIP_2 on the Inner Leaflet

We measured D in the outer leaflets of Rat1, REF52, and HEK293 cells by adding Bodipy-TMR PIP_2 micelles to the solution bathing the cells; Figure 3 shows $D = 2 \pm 1.3\ \mu m^2/s$

(average ± SD; n = 65). Our data agree with FRAP measurements of fluorescent PIP_2 diffusion on the outer leaflet of N1E-115 neuroblastoma cells (van Rheenen and Jalink, 2002). The 2.2-fold disparity in PIP_2 diffusion on the inner and outer leaflets of plasma membranes again suggests that much of the PIP_2 on the inner leaflet is not free to diffuse, presumably because it is bound or sequestered in some manner.

One possible explanation for why PIP_2 diffuses significantly more slowly on the inner leaflet than either the outer leaflet or blebs is its reversible electrostatic association with basic clusters on proteins. As discussed below, many transmembrane proteins contain clusters with four or more basic residues on their cytoplasmic juxtamembrane regions (Nilsson *et al*., 2005), and many peripheral membrane proteins use clusters of basic residues to bind to the plasma membrane. Our experiments, however, do not exclude other possibilities. For example, PIP_2 and other lipids on the inner leaflet could have more tortuous diffusion pathways than lipids on the outer leaflet or in blebs. We tested this possibility by measuring the diffusion of the lipid DiD on the inner leaflet of REF52 cells. DiD (also known as DiI-C18) has a single positive charge, so it is unlikely to interact electrostatically with basic clusters on proteins. The charge is delocalized, and there is good experimental evidence this molecule rapidly flip-flops across bilayers (Melikyan *et al*., 1996). Thus, even if we add it to the outside of the cell, we expect most of the DiD to be located on the inner leaflet: both the negative transmembrane potential and the negative electrostatic surface potential of the inner leaflet (produced by acidic phospholipids) favor this distribution. As expected, D for DiD was the same, within experimental error, whether the lipid was added to the inside (3 ± 1 $\mu m^2/s$, average ± SD; n = 11) or outside (3 ± 1 $\mu m^2/s$; n = 12) of cells. These values of D agree with those reported for FRAP measurements of DiD diffusion (1–4 $\mu m^2/s$) in cell membranes (Fulbright *et al*., 1997; Weisswange *et al*., 2005). The observation that DiD diffuses more rapidly than PIP_2 support the interpretation that the D of PIP_2 on the inner leaflet is lower because approximately two thirds of the polyphosphoinositide is bound reversibly. As discussed in the Supplemental Material, however, the measurements are difficult, particularly when DiD is added to the bathing solution. Thus, we repeated the experiments on the plasma membrane inner leaflets with rhodamine-labeled phosphoethanolamine (PE).

Rhodamine-PE Diffuses 1.9-fold Faster than PIP_2 on the Inner Leaflet

Rhodamine-PE is a more typical fluorescent lipid: it has a net charge of −1 (vs. −4 for PIP_2, and it should not flip-flop rapidly across the plasma membrane bilayer (Melikyan *et al*., 1996)). Our FCS measurements produced an average ± SD D value for rhodamine-PE on the inner leaflet of HEK293, Cos1, HeLa, or Rat1 cells of 1.5 ± 0.9 $\mu m^2/s$ (n = 107), 1.9-fold higher than the average D of PIP_2, 0.8 ± 0.2 $\mu m^2/s$. This value agrees with previous measurements of lipid diffusion in cell membranes (Jacobson, 1983). In contrast to PIP_2, rhodamine-PE did not diffuse significantly faster in the blebs (D = 1.5 ± 0.4; n = 5) than in the unperturbed plasma membranes of Rat1 cells. The rhodamine-PE results argue against the possibility that lipids in general have tortuous diffusion pathways on the plasma membrane inner leaflet, lending support to the hypothesis that reversible association with proteins is responsible for the reduced value of D for PIP_2 we observe.

Potential Effects of Phosphoinositide-specific Phospholipase Cs and Lipid Phosphatases on our Measurements

The fluorescent PIP_2 we injected into the cell was >90% pure, based on TLC (data not shown). Methods to analyze phosphoinositides have been discussed previously (Rusten and Stenmark, 2006). Microinjected Bodipy TMR-PIP_2 could, however, be degraded by phosphoinositide-specific PLCs (Rebecchi and Pentyala, 2000; Rhee, 2001) and by lipid phosphatases. PLCs, for example, could hydrolyze a fraction of fluorescent PIP_2 to produce fluorescent diacylglycerol (DAG), which should diffuse more rapidly than PIP_2 because it will not be electrostatically sequestered by basic clusters on proteins. If this occurs to a significant degree, our estimate that two thirds of the PIP_2 is bound reversibly could be an underestimate. Thus, we did three important control experiments. First, we measured the D of Bodipy TMR-phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2] in the inner leaflet; PLCs prefer PI(4,5)P_2 and they do not hydrolyze this lipid rapidly (Serunian *et al*., 1989). Nevertheless, PI(3,4)P_2 binds to unstructured basic clusters with the same affinity as PI(4,5)P_2, as expected for nonspecific electrostatic interactions (Wang *et al*., 2002). Second, we measured D in cells treated with a lipid phosphatase inhibitor. Third, we reduced the time between microinjection and FCS measurements.

FCS measurements of Bodipy TMR-PI(3,4)P_2 microinjected into Cos1 and Rat1 cells produced a D = 0.9 ± 0.2 $\mu m^2/s$ (average ± SD; n = 47), which agrees within experimental error with the value obtained with Bodipy TMR-PI(4,5)P_2 (0.8 ± 0.2 $\mu m^2/s$). This suggests PLCs did not hydrolyze a major fraction of fluorescent PI(4,5)P_2 into DAG in our experiments. We examined the effects of phosphatases by exposing NIH3T3 and HEK293 cells to 0.5 μM of the lipid phosphatase inhibitor RV001 (Rosivatz *et al*., 2006) ≥10 min before the FCS measurements (RV001 was a generous gift of Drs. Rudiger Woscholski [Imperial College, London] and Banafshe Larijani [Cancer Research UK, London]). The average D of Bodipy TMR-PI(3,4)P_2 in cells exposed to phosphatase inhibitor, 0.7 ± 0.3 $\mu m^2/s$ (n = 12), did not differ significantly from the value obtained without inhibitor.

For most FCS measurements we first microinjected about a dozen cells at a time using a fluorescence microscope with a long working distance objective and attached microinjector, then moved the dish to the adjacent FCS microscope for the diffusion measurements. The time from microinjection to measurement of D was 10–30 min, during which lipid phosphatases (or PLCs) could convert a significant fraction of the fluorescent PIP_2 to phosphatidylinositol monophosphate, phosphatidylinositol, or DAG. We did not expect this to be an important problem because we starved the cells for 1–2 h, and we maintained them at 25°C (rather than 37°C) for the injection and diffusion measurements. Nevertheless, we tested our assumption by mounting the microinjector on the FCS confocal microscope and making FCS measurements of D of Bodipy TMR-PIP_2 as rapidly as possible (<2 min) after microinjection of a single Rat1 cell. These measurements yielded D = 0.7 ± 0.3 $\mu m^2/s$ (average ± SD; n = 6), a value similar to that obtained using our standard protocol (0.8 ± 0.2 $\mu m^2/s$; n = 218). We also measured D in cells starved for 4–6 h before microinjection: again, the results, D = 0.9 ± 0.2 $\mu m^2/s$ (n = 34), did not differ significantly from those reported above.

Our final experiment was a positive control: we tested the effect of maintaining cells at 37°C for 45 min after microinjection. Specifically, we microinjected the cells with Bodipy-

TMR PIP_2, returned them to a 37°C incubator for 45 min., and then performed FCS measurements at 25°C. This protocol increased D significantly, to 1.2 ± 0.3 $\mu m^2/s$ (average ± SD; n = 50). This result suggests phosphatases or lipases may hydrolyze a significant fraction of the PIP_2 at 37°C.

DISCUSSION

Summary and Comparison with Other Results in the Literature

Our main result (Figure 3) is that the diffusion coefficient, D, of fluorescent PIP_2 on the inner leaflet of fibroblast plasma membranes is significantly (2- to 3-fold) lower than the D of PIP_2 in blebs or in the outer leaflet, and the D value of other lipids (rhodamine-PE and DiD) on the inner leaflet. The simplest interpretation is that approximately two thirds of the PIP_2 on the inner leaflet is bound reversibly to cytoskeletal or other membrane proteins. (Note that PIP_2 must be bound reversibly on the time scale of the FCS experiment, ~10 ms. The Supplemental Material includes a discussion of why this is reasonable for electrostatic interactions with unstructured basic clusters.)

Our measurements and conclusion agree well with the other reported experiment we know of that addresses this question directly. Hagelberg and Allan (1990) measured the phospholipid composition of microvesicles from erythrocytes and noted the only phospholipids depleted in the microvesicles were "polyphosphoinositides: the microvesicles only contained about half as much of the polyphosphoinositides as the original cells (compared on the basis of total phospholipids content), suggesting that only half of these lipids are free to diffuse." They suggested PIP_2 could be bound to proteins such as glycophorin, which contains a cluster of four basic residues in its juxtamembrane cytoplasmic domain and binds PIP_2 (Anderson and Marchesi, 1985). Glycophorin and Band III (and presumably other cytoskeletal proteins) are depleted in the microvesicles from erythrocytes.

Our FCS experiments measure the average ± SD D value of PIP_2 in the plasma membranes of fibroblasts and epithelial cells. Our estimate of D on the inner leaflet (0.8 $\mu m^2/s$) from FCS measurements agrees well with an estimate (0.7 $\mu m^2/s$) from measurements made with an indirect technique over longer distances (Yaradanakul and Hilgemann, 2007). Both our estimates disagree with the observation of Cho *et al*. (2005) that the D of PIP_2 in atrial myocytes is orders of magnitude lower; Hilgemann (2007) discusses the Cho *et al*. (2005) measurements.

As we discuss in the Supplemental Material, our conclusion that two thirds of the PIP_2 on the plasma membrane inner leaflet is bound reversibly is consistent with many partitioning measurements of green fluorescent protein (GFP)-pleckstrin homology (PH) domains (from both phospholipase C-δ1 and pleckstrin) between the plasma membrane and the cytoplasm. We conclude the free concentration of PIP_2 is C = 10 μM, and the sequestered concentration is S = 20 μM (concentrations calculated by assuming PIP_2 is dissolved in the cytoplasm of a hypothetical 10-μm-diameter cell).

Possible Location of the Sequestered PIP_2

If the plasma membrane contains 20 μM PIP_2 that is reversibly bound, where is it? One possibility is that it is sequestered through nonspecific electrostatic interactions with clusters of basic residues on proteins (McLaughlin and Murray, 2005). The many membrane proteins capable of sequestering PIP_2 include ~40 members of the Ras, Rab, Arf, and Rho families of GTPases that have basic clusters and use them to interact with PIP_2 (Heo *et al*., 2006). Moreover, the major protein kinase C (PKC) substrate MARCKS has a basic cluster that binds to the membrane and sequesters three PIP_2 (Gambhir *et al*., 2004). MARCKS has been estimated to be present at a concentration ~10 μM in neuronal tissue and at somewhat lower concentrations in other cells; so, MARCKS by itself could sequester a significant fraction of the PIP_2 (McLaughlin and Murray, 2005). Many integral proteins (e.g., EGFR, pIgR, glycophorin, and syntaxin) have basic clusters on their cytoplasmic juxtamembrane domains, as predicted by the "positive inside" rule formulated by von Heinje and colleagues (Nilsson *et al*., 2005). These basic clusters produce a local positive potential at the surface of the membrane that acts as a basin of attraction for the tetravalent PIP_2, as discussed in detail previously (McLaughlin *et al*., 2002; Gambhir *et al*., 2004; Wang *et al*., 2004; McLaughlin and Murray, 2005). Experiments confirm (Gambhir *et al*., 2004) PIP_2 is concentrated ~100-fold in the neighborhood of the basic cluster (i.e., PIP_2 interacts with a basic cluster with an electrostatic energy of ~3 kcal/mol). Engelman (2005) made the strong point that we should expect a highly nonuniform distribution of a lipid in the plasma membrane except when its interaction energy with proteins is less than thermal energy (~0.6 kcal/mol). The interaction energy of PIP_2 with a basic cluster (>4 basic residues) is much greater than thermal energy. Thus, a tentative picture of PIP_2 distribution in the plasma membrane emerges: approximately two thirds should be concentrated 100-fold in local neighborhoods (thickness ~1 nm = Debye length; see McLaughlin and Murray, 2005) adjacent to basic clusters on proteins such as K-Ras, EGFR, MARCKS, and syntaxin, whereas the remaining one third is free to diffuse and distributes randomly over the bulk of the membrane (~100-fold larger area). As discussed in the Supplemental Material, many cells have regions where proteins with clusters of basic residues are concentrated, e.g., MARCKS in ruffles and nascent phagosomes; syntaxin in regions where exocytosis occurs.

Possible Release of Sequestered PIP_2

If a significant fraction of plasma membrane PIP_2 is sequestered, could physiological factors, such as a local increase in Ca^{2+} or activation of PKC, release it? Experiments on MARCKS and peptides corresponding to the basic domains from e.g., MARCKS, EGFR, and gravin show these domains laterally sequester PIP_2. Additional experiments show Ca/CaM can bind with high affinity to the basic clusters on MARCKS (K_d = 30 pM) and EGFR (K_d = 10 nM), and rapidly rip them from phospholipid vesicles, releasing the sequestered PIP_2 (McLaughlin and Murray, 2005). There is ample evidence that cells experience local increases in Ca^{2+}—and consequently Ca/CaM—in response to many stimuli (Clapham, 1995; Chandler *et al*., 2003; Hilgemann, 2007; Muller *et al*., 2007); can local increases in Ca/CaM produce a local increase in the level of free PIP_2? The Supplemental Material includes data from experiments attempted to release some of the sequestered PIP_2. For example, we reversed the net negative charge on the cytoplasmic leaflet with amphipathic weak bases. This should cause dissociation of some basic clusters from the membrane: as we hoped, this procedure significantly increased the D value of PIP_2.

PKC phosphorylation of MARCKS can also cause desorption of the protein from plasma membrane to cytoplasm and release of sequestered PIP_2 in a model system (Murray *et al*., 2002). Importantly, J. Sable, M. Sheetz, and colleagues have

evidence that PKC activation increases the level of free PIP_2 in fibroblasts (personal communication). Specifically, PKC-induced desorption of MARCKS from the plasma membrane resulted in a twofold decrease in the level of cytoplasmic GFP-PH and a concomitant rise in the membrane bound GFP-PH. The simplest interpretation of this result is that MARCKS binds a significant fraction of the PIP_2 in the plasma membrane and that translocation of this protein from membrane to cytoplasm increases the level of free PIP_2 in the plasma membrane. This experiment provides the strongest evidence to date that the level of free PIP_2 in the plasma membrane of living cells can be modified by protein translocation.

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