Evidence for a fence that impedes the diffusion of phosphatidylinositol 4,5-bisphosphate out of the forming phagosomes of macrophages

Urszula Golebiewska¹,², Jason G. Kay³, Thomas Masters⁴, Sergio Grinstein⁵, Wonpil Im⁶, Richard W. Pastor⁷, Suzanne Scarlata⁸, and Stuart McLaughlin⁹,¹⁰

¹Department of Physiology and Biophysics, Stony Brook University, Stony Brook, NY 11794; ²Department of Biological Sciences and Geology, Queensborough Community College, Bayside, NY 11364; ³Program in Cell Biology, Hospital for Sick Children, Toronto, ON MSG 1X8 Canada; ⁴Mechanobiology Institute, National University of Singapore, 117411 Singapore; ⁵Department of Molecular Biosciences and Center for Bioinformatics, University of Kansas, Lawrence, KS 66047; ⁶Laboratory of Computational Biology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892

ABSTRACT To account for the many functions of phosphatidylinositol 4,5-bisphosphate (PIP₂), several investigators have proposed that there are separate pools of PIP₂ in the plasma membrane. Recent experiments show the surface concentration of PIP₂ is indeed enhanced in regions where phagocytosis, exocytosis, and cell division occurs. Kinases that produce PIP₂ are also concentrated in these regions. However, how is the PIP₂ produced by these kinases prevented from diffusing rapidly away? First, proteins could act as “fences” around the perimeter of these regions. Second, some factor could markedly decrease the diffusion coefficient, D, of PIP₂ within these regions. We used fluorescence correlation spectroscopy (FCS) and fluorescence recovery after photobleaching (FRAP) to investigate these two possibilities in the forming phagosomes of macrophages injected with fluorescent PIP₂. FCS measurements show that PIP₂ diffuses rapidly (D ∼ 1 μm²/s) in both the forming phagosomes and unengaged plasma membrane. FRAP measurements show that the fluorescence from PIP₂ does not recover (>100 s) after photobleaching the entire forming phagosome but recovers rapidly (<10 s) in a comparable area of membrane outside the cup. These results (and similar data for a plasma membrane–anchored green fluorescent protein) support the hypothesis that a fence impedes the diffusion of PIP₂ into and out of forming phagosomes.

INTRODUCTION

The lipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂ or PIP₂) plays many roles in the plasma membrane of mammalian cells. For example, it is the source of three different second messengers. It also acts as a regulator or second messenger itself when it activates scores of ion channels, mediates both endocytosis and exocytosis, facilitates phagocytosis, and contributes to attaching the cytoskeleton to the membrane (Di Paolo and De Camilli, 2006). How does one lipid do so much? A number of investigators have proposed there must be separate pools of PIP₂ in the plasma membrane. For example, Hinchliffe et al. (1998) stated that if PIP₂ “does function as a second messenger in its own right … the existence of discrete, functionally distinct, independently regulated PtdIns(4,5)P₂ pools seems inescapable.” There is some evidence for “functionally distinct pools” of PIP₂ in the plasma membrane of mast cells (Vasudevan et al., 2009) and in caveolae (Fujita et al., 2009). There is good evidence that spatially, as well as functionally, distinct pools of PIP₂ exist in three different mammalian cell types: specifically, the surface concentration of PIP₂ is enhanced significantly (approximately fivefold) in the vicinity of syntaxin clusters, where exocytosis occurs in neuronal cells (Aoyagi et al., 2005; Milosevic et al., 2005; James et al., 2008); in the forming phagosomes of macrophages...
(Botelho et al., 2000; Scott et al., 2005); and in the furrows of dividing fibroblasts (Emoto et al., 2005; Field et al., 2005). The kinases that produce PIP$_2$, mainly the phosphatidylinositol 4-phosphate 5-kinases (PIPK5s)—which exist as $\alpha$, $\beta$, and $\gamma$ isoforms—are also concentrated in these three regions [for reviews of PIP5Ks see Weemink et al. (2004), Santarius et al. (2006), and Mao and Yin (2007)]. For example, Milosevic et al. (2005) observed colocalization of PIP5K$\gamma$ and green fluorescent protein (GFP)-PH-PLC-81, a probe for PIP$_2$, in 300-nm clusters localized to the region where exocytosis occurs in PC12 cells, extending the work of Wenk et al. (2001). Emoto et al. (2005) showed that PIP5K$\beta$ accumulates at the cleavage furrow during cytokinesis. Doughman et al. (2003) discussed the evidence that PIP5K$\alpha$ is recruited to the phagosomal cup upon stimulation with opsonized beads in macrophages; a recent study (Mao et al., 2009) documented the unique roles of PIP5K$\gamma$ and PIP5K$\alpha$ in receptor-mediated phagocytosis. Furthermore, the enhanced local concentration of PIP$_2$ in forming phagosomes is critical for phagocytosis; a kinase-dead PIP5K$\alpha$ blocks both local accumulation of PIP$_2$ and phagocytosis (Coppolino et al., 2002). Similarly, an inactive PIP5K$\beta$ blocks both accumulation of PIP$_2$ at the furrow and cytokinesis (Emoto et al., 2005).

This different isoforms of PIP5Ks are concentrated, presumably by specific protein–protein interactions, in forming phagosomes, furrows, and syntaxin clusters. These PIP5Ks enhance the local surface concentration of PIP$_2$ in these regions. However, how is the PIP$_2$ produced by these PIP5Ks prevented from diffusing rapidly away from the source? Diffusion of a lipid over short subcellular distances in a fluid membrane is an extremely rapid process: the diffusion time is proportional to the square of the distance. Thus different isoforms of PIP5Ks exist as $\alpha$, $\beta$, and $\gamma$ isoforms that produce PIP$_2$ in forming phagosomes is critical for phagocytosis; a kinase-dead PIP5K$\alpha$ blocks both local accumulation of PIP$_2$ and phagocytosis (Coppolino et al., 2002). Similarly, an inactive PIP5K$\beta$ blocks both accumulation of PIP$_2$ at the furrow and cytokinesis (Emoto et al., 2005).

Specifically, the measured diffusion coefficients of PIP$_2$ in the inner leaflet of the plasma membrane of different cell types are all of order $D \sim 1 \text{μm}^2/$s (Golebiewska et al., 2008). If the diffusion coefficient of PIP$_2$ in the forming phagosome has a similar value, the Einstein relation ($x^2 = 4Dt$) predicts that in $t = 1$ s the extra surface PIP$_2$ produced in the forming phagosome would diffuse a distance $x = 2 \text{μm}$, comparable to the radius of a moderately large phagosome. However, the forming phagosomes maintain an enhanced level of PIP$_2$, a process mediated by PIP5Ks, for $\sim 100$ s under some conditions (Botelho et al., 2000; Scott et al., 2005). Then, prior to the final fission, the level of PIP$_2$ is significantly depressed, a process mediated by PLC-$\gamma$ and PI3K (Swanson, 2008). The depressed level of PIP$_2$ and enhanced level of PIP$_3$ prior to scission are also maintained for $\sim 100$ s under some circumstances. How?

We envision two simple explanations for why the PIP$_2$ produced by the localized PIP5Ks does not diffuse away more rapidly than it can be produced. First, proteins could act as “fences” or “corrals” at the perimeter of the forming phagosomes. The fences would act as a barrier to impede the diffusion of PIP$_2$ out of the forming phagosomes, allowing the surface concentration of PIP$_2$ to rise upon activation of PIP5Ks within the fence. We refer to this as the “fence hypothesis.” In yeast, septin filaments act as a fence/corral to limit the diffusion of membrane proteins (Faty et al., 2002; Finger, 2005; McMurray and Thorner, 2009) and possibly PIP$_2$ (Garrenton et al., 2010). The fence constituents in mammalian cells such as macrophages may include septin filaments and/or actin-associated proteins, as the actin cytoskeleton is swept away from the central base region of the forming phagosome and concentrates in a band at the perimeter (e.g., Swanson, 2008). Second, some factor(s) could decrease significantly (e.g., >10-fold) the diffusion coefficient of PIP$_2$ in the forming phagosomes or regions where syntaxin is clustered. For example, proteins with clusters of basic residues, such as syntaxin (Lam et al., 2008; Williams et al., 2009) and myristoylated alanine-rich protein kinase C substrate (MARCKS; Gambhir et al., 2004), can bind PIP$_2$ rapidly and reversibly by a simple electrostatic mechanism (McLaughlin and Murray, 2005). Thus these proteins can act as buffers of PIP$_2$. Syntaxin is concentrated at regions where exocytosis occurs, and MARCKS is concentrated in forming phagosomes: if they were present at a sufficiently high local surface concentration to bind 90% (or 99%) of the PIP$_2$, a simple analysis shows that they would decrease the effective value of the diffusion coefficient 10-fold (or 100-fold; Golebiewska et al., 2008). Alternatively, there could be a latticework of proteins throughout the forming phagosomes that force PIP$_2$ to move by a relatively slow “hop diffusion” mechanism, as postulated for the diffusion of proteins in the plasma membrane (Kusumi et al., 2005, 2010). Alternatively, lipid domains such as cholesterol-enriched rafts (Lingwood and Simons, 2010) might impede diffusion of PIP$_2$. We refer collectively to these buffer/raft mechanisms that might decrease the D of PIP$_2$ within the phagosomes as the “reduced–diffusion coefficient hypothesis.”

To distinguish between the fence and the reduced–diffusion coefficient hypotheses, we measured directly the diffusion coefficient of fluorescent PIP$_2$ in the forming phagosomes of macrophages and in the bulk plasma membrane outside the phagosomal cup. The process of phagocytosis in macrophages is reviewed elsewhere (Cougoule et al., 2004; Stuart and Ezekowitz, 2005; Yeung et al., 2006; Swanson, 2008): a major advantage of using this system to test whether PIP$_2$ fences exist is that macrophages will engulf large-diameter (8 μm) beads, which facilitates diffusion measurements in the forming phagosome. Specifically, we microinjected micelles containing fluorescent PIP$_2$ into macrophages and measured the diffusion coefficient of individual PIP$_2$ molecules that incorporated into the plasma membrane by using fluorescence correlation spectroscopy (FCS). To test the fence hypothesis more directly, we photobleached the PIP$_2$ within the forming phagosome. We reasoned that if a PIP$_2$ fence surrounds the forming phagosome, the fluorescence should not recover. We also reasoned that if a fence prevents the diffusion of PIP$_2$ out of the forming phagosome, it should also prevent the diffusion of a larger peripheral protein. Thus we obtained FCS and fluorescence recovery after photobleaching (FRAP) data on a GFP construct anchored to the plasma membrane through two acyl chains (GFP-tagged N-terminal region of Lyn kinase [PM-GFP]).

**RESULTS**

The level of PIP$_2$ in the forming phagosomes of J774a.1 macrophages first increases and then decreases

We used murine macrophages of the J774 line to study PIP$_2$ diffusion during phagocytosis. Although a biphasic change in PIP$_2$ during phagocytosis was demonstrated earlier in another line of murine macrophages, RAW 264.7 (Botelho et al., 2000; Scott et al., 2005), it was important to validate the occurrence of similar changes in J774 cells. As before, GFP-tagged PH domain from PLC-81 was used to monitor PIP$_2$ in live cells. As shown in Supplemental Figure S1, the fluorescence of the probe first increases and then
Figure 1: Methods used to study the diffusion of fluorescent PIP2 in the forming phagosomes of macrophages. (A) Cartoon showing a J774a.1 macrophage and adjacent microinjector needle loaded with micelles containing Bodipy-TMR-PIP2. (B) After microinjection, monomers of fluorescent PIP2 incorporate rapidly into the inner leaflet of the plasma membrane, which is now colored red. The cell is then exposed to 8-μm-diameter latex beads coated with human IgG. One bead, colored gray, is shown in the process of landing on top of the cell. (C) The cell begins to ingest the bead by the process of Fc receptor–mediated phagocytosis. The laser focus (green hourglass) is positioned on the top membrane in the middle of the forming receptor–mediated phagocytosis. The laser focus (green hourglass) is positioned on the top membrane in the middle of the forming phagosome to obtain the FCS data from fluorescent PIP2 molecules diffusing into and out of this area.

As predicted by the fence hypothesis, the signal from fluorescent PIP2 diffusing in the inner leaflet of the plasma membrane of a phagosomal cup does not recover after photobleaching of the entire cup.

To test more directly the hypothesis that there is a fence/corral that limits the diffusion of PIP2 across the perimeter of the forming phagosome, we carried out FRAP measurements on 1-oleoyl-2-[6-(4-(dipyrrometheneboron difluoride)butanoyl)amin]hexanoyl-sn-glycero-3-phosphoinositol-4,5-bisphosphate (TopFluor-PIP2) in the phagosomal cups of J774 macrophages. TopFluor-PIP2 bleaches more easily than Bodipy-TMR-PIP2 and is thus more suitable for FRAP measurements. We microinjected fluorescent PIP2 into the cytoplasm of cells in the form of mixed micelles (see Materials and Methods) and observed, as expected, that TopFluor-PIP2 rapidly incorporated into the plasma membrane of cells; a fraction of this lipid also remained in cytosolic compartments (Figure 3).
We then added 8-μm-diameter latex beads coated with human IgG to the solution bathing the cells. The microinjected cells initiated the process of phagocytosis. For FRAP measurements, we selected cells in early stages of phagocytosis (plasma membrane envelops ∼10–20% of the bead) using transmitted light and the LSM module. Figure 3 shows an example of one of these FRAP measurements. We recorded three images prior to the bleach (Figure 3A), bleached the TopFluor-PIP2 in the phagosomal cup within 1.5 s (Figure 3B), and monitored fluorescence for ∼100 s after the bleach (Figure 3C). Fluorescence was recorded in the region indicated by the dashed white lines. As shown in Figure 3D, the level of fluorescence in this cell did not recover significantly. The simplest interpretation of this result is that a fence/corrail, presumably of protein origin, impedes diffusion of fluorescent PIP2 into and out of the phagosomal cup during its formation.

As a control, we made FRAP measurements on TopFluor-PIP2 in the plasma membrane of quiescent macrophages. As shown in Figure 3E, the fluorescence signal recovered rapidly (recovery half-time was ∼10 s for a region of radius ∼3 μm) and fully (to 95% of corrected fluorescence in unbleached portion of membrane), consonant with a relatively unconstrained diffusion of fluorescent PIP2 into the bleached area. The data in Figure 3E represent one typical example of these control measurements; see Supplemental Section 2 for more details.

A similar FRAP experiment with the fluorescent lipid 1,1′-didodecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate (with 12 C chains [DiIC12]) shows that the lack of recovery in Figure 3D with PIP2 is not due to technical problems in monitoring fluorescence recovery in the vicinity of a bead during phagocytosis. When we bleached the fluorescence due to DiI in a forming phagosome, the fluorescence recovered fully (to >80% of the initial value) and rapidly (with a time constant of ∼10 s), as shown in Supplemental Figure S2. This result was expected: DiI can rapidly flip-flop across the membrane (Melikyan et al., 1996). The evidence suggests that there is no barrier to the diffusion of DiI on the outer leaflet (or GPI-GFP; see Supplemental Section 3).

**FCS and FRAP measurements on PM-GFP suggest that its diffusion into the forming phagosome is, like that of PIP2, limited by a fence**

We made FCS measurements on PM-GFP expressed in macrophages to measure how rapidly this model peripheral protein diffuses in both the cup of the forming phagosome in RAW macrophages and in the membrane outside the cup. The construct tags the N-terminal 10 residues of the Src family kinase Lyn with GFP. Thus the construct should be myristoylated as well as palmitoylated in the RAW macrophages and should be targeted to the plasma membrane by the same mechanism as Lyn. We observed that PM-GFP, which is found mainly on the plasma membrane, diffuses rapidly both inside and outside of the phagosomal cup. Specifically, the measured correlation times (average values of 32 ms inside the cup and 27 ms outside the cup) correspond to a D ∼ 0.7 ± 0.4 μm²/s (n = 28 ± SD) within the cup and 0.9 ± 0.5 μm²/s (n = 25 ± SD) outside the cup. Hammond et al. (2009) reported a very similar value for the D of PM-YFP in HEK cells (0.79 μm²/s) using FRAP. (We note in passing that PM-GFP diffuses almost as rapidly as the lipid PIP2 in both cases D is ∼1 μm²/s. The simplest interpretation is that the predominant component of the diffusional drag on PM-GFP is exerted by the two acyl chains, which insert into the hydrophobic—and relatively high viscosity—interior of the plasma membrane. Although GFP is larger (a cylinder ~3 nm in diameter by 4 nm in height) than the head group of PIP2 (~1 nm diameter), the GFP moiety presumably exerts a minimal effect on D because it is diffusing in a medium of relatively low viscosity.)

If a protein fence impedes the diffusion of PIP2 into and out of the forming phagosome, we expect that it will also impede the movement of the larger PM-GFP. Figure 4 shows that when we photobleached the PM-GFP within a phagocytic cup the
fluorescence recovered (with a time constant of 15 s) to a maximum value of only 20% within 100 s (Figure 4, A, Cup; and B, black squares). However, when we subsequently bleached a comparable area of membrane (radius ~2 μm) in the same cell in a region outside the cup, the fluorescence recovered more fully to ~70% of the initial value (Figure 4, A, bottom, PM; and B, open circles). Thus results with this membrane-anchored probe recapitulate the results we obtained with PIP2: the fluorescence recovers only partially within the cup but more fully outside the cup. This result provides additional support for the existence of a corral/fence around the perimeter of the forming phagosome. (The recovery of ~70% illustrated in Figure 4B is typical of 20 measurements for the PM outside the cup. In two cases we prebleached the area: this resulted in a recovery of ~100% for the second photobleach, as shown in Supplemental Figure S3.)

We interpret the FCS and FRAP measurements reported here differently than the similar FRAP measurements on PM-GFP reported previously for macrophages by Corbett-Nelson et al. (2006), as discussed in Supplemental Section 3. The results shown in Figures 4 and Supplemental Figure S3 are in good accord with those obtained by Schmidt and Nichols (2004), who reported a barrier for diffusion of PM-GFP (aka Lyn-GFP) at the cleavage furrow of dividing mammalian cells.

Diffusion of molecules is reduced, but only slightly, in actin-rich areas at the periphery of the forming phagosome

As discussed in reviews (e.g., Swanson, 2008), actin accumulates at sites of phagocytosis and is particularly abundant at the tips of advancing pseudopods (Figure 5A). We therefore considered the possibility that actin-rich structures constitute a diffusional barrier that confines lipids and proteins within the forming phagosomal cup. For these experiments the mobility of fluorescent membrane components was assessed by FRAP. To stabilize the focal plane during the course of the measurements, we used a model of frustrated phagocytosis in which the ligand, IgG, is attached to the surface of a coverslip. The cells are allowed to settle by gravity onto the coated surface, whereupon they extend an aborted phagocytic cup. The development of this process is illustrated in Figure 5B, where the fluorescence of cells transfected with mCherry-actin is monitored at the plane of contact. Actin forms a distinct rim around the periphery of the extending cup, replicating the behavior observed in Figure 5A. To assess whether the actin rim serves as a diffusional barrier, we cotransfected cells with mCherry-actin and with GFP-tagged membrane-associated probes. These included either lipid-anchored GFP (the diacylated PM-GFP or the farnesylated and palmitoylated GFP-tH) and a GFP-tagged protein that spans the membrane (GT46-GFP). The mobility of these probes was assessed during the course of frustrated phagocytosis in two areas: near the center, where actin is largely depleted, and near the edge, where the actin rim is most prominent (Figure 5C). For comparison, the diffusion of the probes was also measured in the ventral membrane of cells that were grown on coverslips not coated with IgG (designated as “control”). Figure 5D summarizes the results of these experiments. The two probes attached to the inner leaflet of the plasma membrane (PM-GFP and GFP-tH) behaved in a similar manner: their diffusion coefficient in the center of the frustrated phagosome was not retarded compared with that in control membranes, whereas diffusion was modestly retarded in the actin-rich region of the membrane. In contrast, the mobility of an exofacial probe—glycosylphosphatidylinositol-linked GFP (GPI-GFP)—was not different in the different regions of the forming phagosome.

Whereas diffusion of the transmembrane or inner leaflet-associated probes was reduced by actin and/or actin-associated components, the observed changes in mobility are comparatively modest (less than twofold; Figure 5D). Thus actin filaments are unlikely to account for the lack of recovery after photobleaching noted in the cup region for PM-GFP (Figure 4).

Actin filaments by themselves are unlikely to constitute the PIP2 fence

Although most of the actin filaments in the cortical cytoskeleton are probably located some distance (>2 nm) from the bilayer leaflet, electron tomography measurements suggest that a fraction of the actin filaments may be located in close proximity (<1 nm) to the plasma membrane (Morone et al., 2006). Actin has a net negative charge, and the electrostatic potential adjacent to most of the filament is negative (Supplemental Figure S4). Given that PIP2 is also negatively charged (valence of ~4 at pH 7 [McLaughlin et al., 2002]),
DISCUSSION

FCS and FRAP measurements on fluorescent PIP<sub>2</sub> and PM-GFP provide evidence for a fence that limits diffusion

The surface concentration of PIP<sub>2</sub> first increases and then decreases in the phagosomes of J774 macrophages (Supplemental Figure S1), as previously observed in RAW macrophages (Scott et al., 2005). Our FCS measurements show directly that the diffusion coefficient of PIP<sub>2</sub> D, has similar high values both inside and outside of the phagosome cup (Figure 2, D ~ 1 μm<sup>2</sup>/s). This result falsifies the low–diffusion coefficient hypothesis. Instead, the data are consistent with the alternative fence hypothesis: molecules located at the perimeter of the phagosomal cup impede the diffusion of PIP<sub>2</sub> into and out of the cup.

The fluorescence associated with PIP<sub>2</sub> does not fully recover within 100 s when we bleach the area of the cup but does rapidly recover when we bleach an area outside of the cup (Figure 3): this FRAP result supports the hypothesis there is a fence at the perimeter or lip of the cup. Similar FCS and FRAP measurements on PM-GFP show the fence also limits the diffusion of this construct into and out of the forming phagosome (Figure 4 and Supplemental Figure S3).

The collar of cortical actin filaments apparent at the leading edge of the forming phagosome (Figure 5A) is associated with only a moderate decrease in the diffusion coefficients of model peripheral proteins such as PM-GFP (Figure 5D); the decrease (less than twofold) is not sufficient to account for the lack of recovery after photobleaching observed with PM-GFP in Figure 4 and Supplemental Figure S3. Furthermore, our theoretical calculations suggest that actin filaments alone are unlikely to exert a sufficient barrier to account for the limited diffusion of PIP<sub>2</sub> out of a forming phagosome (Supplemental Figure S4).

The relatively sharp boundary between the enhanced level of PIP<sub>2</sub> and PIP<sub>3</sub> in the forming phagosome and the lower levels of PIP<sub>2</sub> and PIP<sub>3</sub> in the contiguous extraphagosomal membrane (e.g., Supplemental Figure S1) also support the fence hypothesis. What proteins might act as fences?

Nature of the fence

Septin filaments, which are described in detail elsewhere (Hall et al., 2008; McMurray and Thorner, 2009; Oh and Bi, 2011; Estey et al., 2011), can act as diffusional barriers in yeast. Their structure is known: specifically, six human (or eight yeast) septin monomers join together to form hetero-oligomeric rods (Sirajuddin et al., 2007; see also Supplemental Figure S5). The rods join together to form filaments: in yeast the filaments separate mother cell from daughter bud. FRAP measurements show that fluorescently tagged proteins diffusing freely in the daughter buds do not cross the barrier to the mother cell until the temperature is raised and the septin structure is disrupted (Barral et al., 2000; Takizawa et al., 2000), as reviewed elsewhere in detail (Faty et al., 2002; Finger, 2005). Less direct evidence suggests that the split septin rings at the neck of budding yeast may allow PIP<sub>2</sub> to accumulate in this membrane microdomain (Yoshida et al., 2009; Garrenton et al., 2010). Garrenton et al. (2010) also showed that PIP<sub>2</sub> accumulates in the protrusions of yeast responding to pheromones (shmoo formation); of importance, they observed a septin lattice network at the boundary of the PIP<sub>2</sub>-rich protrusion. This region of enhanced PIP<sub>2</sub> concentration lasts ~100 min, which, as they note, requires a diffusion barrier. In Supplemental Section 5 we consider how mammalian septin filaments could bind to the inner leaflet of the plasma membrane.

Septins appear in the right place at the right time to be implicated in the formation of PIP<sub>2</sub> fences in macrophages; they appear...
in the forming phagosomes when the IP2 level in the forming phagosome starts to increase, and procedures that move septins off the membrane also inhibit the final step of phagocytosis (Huang et al., 2008). Septin filaments are also present near syntaxin clusters in neuronal cells (Froese and Trimble, 2008) and in the furrows of dividing mammalian cells (Schmidt and Nicholls, 2004): thus septin filaments are candidates to act as IP2 fences at these locations.

**Importance of the fence for phagocytosis**

Several lines of evidence suggest that local changes in the level of the phosphoinositides IP2 and IP3 are important for phagocytosis (Araki et al., 1996; Coppolino et al., 2002; Swanson, 2008). However, it is not fully understood why IP2 is important (Swanson, 2008). A reasonable speculation is that an increase in the local level of IP2 may be required to enhance attachment of the actin-rich cytoskeleton to the membrane; a decrease in the local level of IP2 may facilitate cytoskeletal dissociation from the membrane and scission of the phagosome (Scott et al., 2005). A similar dependence of membrane-cytoskeleton interactions on the global level of IP2 has been observed in a number of cells (Raucher et al., 2000). Whatever the mechanism(s) by which IP2 acts, it is clear that because IP2 diffuses rapidly in the plasma membrane, a fence is required to maintain an enhanced level of IP2 synthesized by local PI3K.

In conclusion, we note that there is much indirect evidence for separate pools of IP2 in the plasma membrane. Our FCS (Figure 2) and FRAP (Figure 3) measurements on fluorescent IP2 in the plasma membrane of macrophages provide direct support for the hypothesis that there is an IP2 fence around the forming phagosomes in macrophages: the fence impedes the diffusion of IP2 into and out of the cup. This fence allows the surface concentration of IP2 in the cup to first increase (due to the action of PI3Ks) and then decrease (due to the action of PLC, PI3K) during phagocytosis. Previous work shows these changes in IP2 in the forming phagosome are functionally important for phagocytosis. The nature of the fence is unknown, but septin filaments are a viable candidate.

**MATERIALS AND METHODS**

**Materials**

Bodipy-TMR-IP2 was purchased from Echelon Bioscience (Salt Lake City, UT). The chemical structure of this lipid is illustrated in Gambhir et al. (2004). TopFluor-Pi(4,5)P2 was purchased from Avanti Polar Lipids (Alabaster, AL). DiIC2 was purchased from Invitrogen (Carlsbad, CA). Arachidoyl Lyso-PC was purchased from Avanti Polar Lipids. Rhodamine B and human IgG were from Sigma-Aldrich (St. Louis, MO). The 8-μm latex beads were from Bangs Labs (Fishers, IN). Glass capillaries, thin walled with filament inner diameter 1.0 mm, were purchased from World Precision Instruments (Sarasota, FL). Needles were pulled on a Flaming Brown micropipette puller, model P80/PC, Sutter
Instrument (Novato, CA). The 35-mm glass-bottom dishes were purchased from MatTek Corporation (Ashland, MA).

Cell culture
J774A.1 macrophages were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were maintained at 37°C with 5% CO₂ in DMEM supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin sulfate, and sodium pyruvate. J774A.1 cells used in the examination of PIP₂ and PIP₃ levels with GFP-tagged probes were maintained at 37°C with 5% CO₂ in DMEM with 1.5 g/l sodium bicarbonate from Wisent (St. Bruno, QC, Canada) supplemented with 10% FBS. RAW 264.7 cells were used for the actin localization and frustrated phagocytosis experiments were from ATCC and were maintained at 37°C with 5% CO₂ in RPMI-1640 from Wisent supplemented with 5% FBS. RAW 264.7 cells were also used for the PM-GFP FCS and FRAP experiments. They were cultured at 37°C in DMEM (Life Technologies, Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Life Technologies). 50 U/ml penicillin, 50 μg/ml streptomycin sulfate, L-glutamine, and 110 mg/ml sodium pyruvate. As recommended by ATCC, CO₂ was maintained at 10% due to the high sodium bicarbonate level of the Life Technologies media used.

Sample preparation
Bodipy-TMR-PIP₂ and TopFluor-PIP₂ were injected into J774a.1 cells in the form of arachidoyl Lyso-PC/fluorescent-PIP₂ micelles as described previously (Golebiewska et al., 2008). The composition of micelles was 82% arachidoyl Lyso-PC and 18% fluorescent-PIP₂. Lipids were suspended in 1 mM EDTA solutions, warmed to 37–42°C, mixed, and diluted with 1 mM EDTA to a concentration of −500 μM, and then sonicated in a bath sonicator for 3–5 s to form the mixed micelles. We used mixed micelles rather than PIP₂ micelles because PIP₂ micelles aggregate in the presence of the 1 mM Ca²⁺ present in the bathing solution, causing the pipette tips to clog. DiC₁₂ was dissolved in ethanol and added to cells by mock microinjections (with the Pi and Pc set to 0). The final concentration of ethanol in the cell was <0.01%.

Latex beads were coated with human IgG as previously described (Steinberg and Grinstein, 2009). Briefly, beads were washed three times with phosphate-buffered saline (PBS), incubated with human IgG for 1 h at room temperature, and again washed three times with PBS.

For frustrated phagocytosis measurements, the coverslips were incubated with 1% BSA at PBS at room temperature for 1 h, extensively washed in PBS, and then incubated with PBS containing 10 μg/ml mouse anti-BSA antibody for ≥3 h at room temperature. After three washes with PBS the coverslips were left in PBS (to prevent drying) until they were moved to the microscope.

Microinjection
Two days before the microinjection experiments, cells were plated in glass bottom MatTek dishes that were marked with a diamond knife (to aid in locating microinjected cells). Needles were pulled on the Flaming Brown instrument using the following settings: heat 780, pull 15, velocity 13, time 20. These needles had slightly larger tips than the commercially available needles from Eppendorf to minimize clogging of the tips. For microinjections we used InjectMan NI2 with FemtoJet pump from Eppendorf (Hauppauge, NY). PIP₂ micelles were microinjected into the cytoplasm. For FCS measurements we set the injection pressure Pi at 17–25 hPa and kept the compensation pressure Pc at 0 (to avoid leakage of PIP₂ micelles from the needle into the bathing solution). For FRAP measurements Pi was set to 35–55 hPa. The injection time was set to 0.4 s for FCS measurements and to 0.6 s for FRAP measurements. Prior to the microinjections we replaced the medium bathing the cells with phenol-free Liebovitz's 15 (L15) medium.

We typically injected ~10 cells in a 10-min period. Microinjections were performed on a Zeiss (Jena, Germany) Axiovert 200M microscope equipped with a 40x long-distance phase 2 objective. We examined the microinjected cells using the phase 2 and epifluorescence to select cells that were both viable and contained a suitable level of fluorescent PIP₂.

We then transferred the cells to the FCS microscope. We measured the diffusion of fluorescent molecules in the plasma membrane of macrophages 10–30 min after microinjection. For the measurements in the phagosomal cup we exposed cells to 8-μm-diameter latex beads coated with human IgG. We examined the cells using both transmitted light and laser scanning function. We placed the FCS focus on the top portion of the membrane enveloping a bead that was at the early stage of phagocytosis.

FCS measurements
As reviewed in detail elsewhere, FCS allows one to measure the fluorescence as a function of time and determine the correlation time, which may be considered as the average time for a single PIP₂ to diffuse out of the confocal area on the plasma membrane illuminated by laser (Elson and Rigler, 2001; Haustein and Schwille, 2007). As the width of the illuminated spot can be determined experimentally, the diffusion coefficient, D, can be determined from the Einstein relation.

Confocal imaging, FCS, and FRAP measurements were performed on a Zeiss LSM 510 Meta/Confocor 2 apparatus and a Zeiss Confocor 3 using standard configurations. Minimal laser powers were chosen to avoid photobleaching of the fluorescent probes. We used a 40x numerical aperture–1.2 C-Apochromat water immersion objective and adjusted the pinholes of the Confocor 2 daily. For FCS measurements of PIP₂ diffusion, we excited Bodipy-TMR-PIP₂ with the 543-nm HeNe laser and collected emission spectra through a 560 LP filter.

To calibrate the effective radius of the detection volume of the Confocor 2 we measured the correlation time of rhodamine (D = 420 μm²/s) in L15 medium and used the Einstein relation, Eq. 2. The effective radius of the detection volume for the 543-nm line was ω₂ = 0.22 ± 0.01 μm. In our previous work we assumed that rhodamine diffuses with D = 300 μm²/s. To compare our previous measurements with our current results we recalculated the D of PIP₂ in the plasma membrane (0.8 μm²/s; Golebiewska et al., 2008) and obtained the slightly larger value of D = 1.2 μm²/s.

To calculate the effective radius of the confocal volume of the Confocor 3, we measured the correlation time τ₂ of several fluorescent dyes with known diffusion coefficients (D) (Ruttinger et al., 2008). We used fluorescein with D = 436 μm²/s (at 23°C) to calibrate the 488-nm laser line and TMR with D = 420 μm²/s (at 23°C) to calibrate the 543-nm laser line (Petrasek and Schwille, 2008). The FCS measurements yield τ₂ = 37 ± 1 μs for fluorescein and 38 ± 1 μs for TMR with laser powers of 10 μW and pinhole sizes of 1 airy unit. The effective radius (ω₂) under these conditions is thus 0.25 μm for both the 488-nm and 543-nm laser lines, as calculated from Eq. 2.

We monitored the count rate during data acquisition and rejected measurements with either a substantial increase or decrease in light intensity to avoid artifacts due to either membrane movements or bleaching.
Autocorrelation curves were fitted to the model equation for free Brownian diffusion in two dimensions:

\[ G(r) = \frac{1}{N} \frac{1}{1 + (r / \tau_d)} \]

where \( N \) is the average number of particles in the confocal volume and \( \tau_d \) is the average residence time in the confocal volume. We used software from both SigmaPlot (Systat, San Jose, CA) and Zeiss, employing a least squares algorithm to fit Eq. 1 to the data.

We then obtained the diffusion coefficient, \( D \), from

\[ D = \frac{\omega_r^2}{4\tau_d} \]

where \( \omega_r \) is the radius of the detection volume and \( \tau_d \) is the correlation time. For cases with populations of molecules diffusing with different diffusion coefficients but with the same fluorescence quantum yields the equation becomes

\[ G(r) = \frac{1}{N} \sum Y_i \frac{1}{1 + (r / \tau_{d_i})} \]

where \( N \) is the number of molecules and \( Y_i \) is the fraction of molecules diffusing with a diffusion coefficient \( D_i \) and a correlation time \( \tau_{d_i} \). To minimize hydrolysis of PIP\(_2\) after microinjection, all measurements using the Confocor 2 were performed at room temperature, 25 \( \pm \) 1°C, which was monitored throughout the experiment using a thermocouple. The FRAP measurements on PIP\(_2\) with the Confocor 3 were conducted at room temperature, -23°C. The FRAP measurements of PM-GFP made on the Confocor 3 were conducted at 37°C.

FRAP measurements

TopFluor-PIP\(_2\). To monitor the fluorescence produced by TopFluor-PIP\(_2\), we excited using 1% intensity of the 488-nm line from the argon laser; we recorded images using a 505 LP filter. The scan speed was set to 1.6 μs/pixel, resulting in ~900 ms/frame. The desired area (4–10 μm\(^2\)) around a bead that was engulfed 10–50% or around a bead that was engulfed 10–50% was bleached with 4–10 scans with 100% intensity of the 488-nm line from the argon-ion laser. Typically, as shown in Figure 3, A–D, we recorded three images prior to bleaching and 97 images postbleach with 1-s intervals. The success rate of these experiments was very low. For example, the cells were often damaged upon microinjection of the high concentration of PIP\(_2\); we did not succeed in rapidly bleaching most of the TopFluor-PIP\(_2\) in the cup; and the bead moved during the 100 s we followed the recovery. TopFluor-PIP\(_2\) was superior for the FRAP experiments because it bleached more readily; Bodipy-TMR-PIP\(_2\) was superior for the FCS experiments because it did not bleach as easily and for other reasons discussed elsewhere (Golebiewska et al., 2008).

We excited DiIC\(_{12}\) with the 546-nm line of the HeNe laser and recorded images using a 560 LP emission filter. To bleach DiIC\(_{12}\) we used full power of the 488-nm line of the argon-ion laser.

We analyzed the FRAP images using ImageJ or Fiji software and fitted the intensity curves using SigmaPlot.

PM-GFP. RAW macrophages were transfected with either palmitoylated/myristoylated PM-GFP (derived from the N-terminal region of Lyn kinase, see Corbett-Nelson et al., 2006) or PMT-GFP (derived from a different protein [Liu et al., 2007], a kind gift of Thorsten Wohland). We used the Neon electroporation system (Invitrogen). The 8-μm-diameter latex beads (Bangs Labs) were opsonized with IgG (Sigma-Aldrich) and introduced to the cells bathed in phenol-free L15 medium and incubated at 37°C on the microscope stage. For FRAP, measurements were taken using an LSM 710 confocal microscope (Zeiss), employing the argon-ion 488-nm line to excite fluorescence, and emission longer than 500 nm was collected. Bleaching was performed with 100% power (700 μW), with up to 10 images acquired before and 90 after.

FRAP of RAW macrophages undergoing frustrated phagocytosis was performed on a WaveFX spinning disk microscopy system (Quorum Technologies, Guelph, ON, Canada) equipped with a Mosaic digital diaphragm for photobleaching and operated with the software Volocity, version 4.4 (PerkinElmer, Waltham, MA). Macrophages for frustrated phagocytosis were lightly scraped to lift them from the growth flask, resuspended in RPMI with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and added to coverslips coated with IgG in a heated chamber on the microscope. Cells were allowed to settle by gravity onto the coverslip; when mCherry-actin was observed to form a ring at the beginning of frustrated phagocytosis, FRAP of the coexpressed GFP-tagged protein was performed.

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