Regulation of PLCβ1a membrane anchoring by its substrate phosphatidylinositol (4,5)-bisphosphate

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Summary

Basic knowledge as to the subcellular location and dynamics of PLCβ isozymes is scant. Here, we report on the subcellular location of GFP-PLCβ1a and the use of total internal reflection fluorescence (TIRF) microscopy to examine the dynamics of GFP-PLCβ1a at the plasma membrane upon stimulation of Gq-coupled receptors. Using this technique, we observed PLCβ1a dissociation from the plasma membrane upon addition of agonist. An increase in intracellular calcium and a decrease in PtdIns(4,5)P2 both coincided with a translocation of PLCβ1a from the plasma membrane into the cytosol. In order to differentiate between calcium and PtdIns(4,5)P2, rapamycin-induced heterodimerization of FRB and FKBP12 fused to 5-phosphatase IV was used to instantaneously convert PtdIns(4,5)P2 into PtdIns(4)P. Addition of rapamycin caused PLCβ1a to dissociate from the plasma membrane, indicating that removal of PtdIns(4,5)P2 is sufficient to cause translocation of PLCβ1a from the plasma membrane. In conclusion, PLCβ1a localization is regulated by its own substrate.

Key words: PLCβ, PtdIns(4,5)P2, TIRF

Introduction

The family of phosphatidylinositol-specific phospholipases (PI-PLCs) consists of six subfamilies: β, γ, ε, η and ζ (Citro et al., 2007; Hwang et al., 2005; Katan, 2005; Rebecchi and Pentyala, 2000; Rhee, 2001; Saunders et al., 2002). Upon activation, they all preferably hydrolyze the phospholipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2], which is present in the inner leaflet (1-3%) of the plasma membrane (McLaughlin et al., 2002). Hydrolysis of PtdIns(4,5)P2 leads to the formation of diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate [Ins(1,4,5)P3], of which the latter opens calcium channels in the endoplasmic reticulum (Streb et al., 1983).

The PLCβ subfamily in mammals consists of four genes that encode PLCβ1, PLCβ2, PLCβ3 and PLCβ4. PLCβ enzymes can be activated by members of the Gq class or by Gβγ subunits, or by both, depending on the isozyme (Rebecchi and Pentyala, 2000). PLCβ1 is nearly ubiquitous, with high expression levels detected in brain tissue (Caricasole et al., 2000). This protein exists in two splice variants that differ in their C-terminal (CT) domain: PLCβ1a and PLCβ1b (Park et al., 1993). In COS-7 cells and in rat brain, 70% of PLCβ1a is present in the particulate fraction (Jhon et al., 1993; Wu et al., 1993). It was proposed that the remaining 30% moves to the plasma membrane upon activation of Gq-coupled G-protein-coupled receptors (GPCRs).

The mechanism by which PLCβ binds to membranes remains elusive, but is probably mediated by electrostatic interactions because treatment with high salt (1-2 M KCl) leads to depletion of PLCβ1 from the particulate fraction (Kim et al., 1996). The CT domain is unique to PLCβ isozymes and makes up about one-third of the protein (Singer et al., 1997). Localization of PLCβ1 to the particulate fraction is dependent upon this domain (Kim et al., 1996). CT domains contain a high number of charged amino acids. In addition, the crystal structure of the CT domain of turkey PLCβ shows a clear charge separation, which led to the assumption that the side of the domain with a high positive charge orients towards the plasma membrane, whereas the negatively charged side faces the cytosol (Singer et al., 2002).

Activation of PLCβ1 by Gq is contingent upon the presence of the CT domain. In addition, this domain displays GTPase-accelerating activity (GAP activity) for Gq (Paulssen et al., 1996). Experiments in reconstituted vesicles indicate that Goq forms a complex with both the GPCR and PLCβ in the presence of agonist and GTP. This phenomenon is termed kinetic scaffolding and is caused by the GAP activity of PLCβ1 that keeps the supply of Goq-GDP high enough for the receptor not to lose its affinity for the Gα subunit. Since the complex does not dissociate, several GDP/GTP exchange cycles can occur consecutively (Biddulcrome et al., 1996). Wu et al. (Wu et al., 1993) delineated the Goq-interaction site in PLCβ1 and the amino acids involved in particulate fraction binding, and dubbed them the G-box (Gln1030-Leu1142) and the amino acids involved in Goq interaction.

Here, we report on the hitherto poorly defined subcellular location of PLCβ1a and its regulation upon activation by Gq in living cells. Confocal and total internal reflection fluorescence (TIRF) microscopy were used to detect the localization of Gqβγ-tagged PLCβ1a and follow its dynamics. Dissociation of PLCβ1a from the plasma membrane upon activation of Gq was found to be triggered by conversion of PtdIns(4,5)P2.

Results

Subcellular location of PLCβ1a

In order to investigate the subcellular location and dynamics of PLCβ1a, the protein was equipped with a GFP tag fused to its N-terminus and transiently transfected into HeLa cells. YFP-PLCβ1
has been reported to show wild-type basal activity and activation by Gqα, indicating that it retains functionality upon GFP fusion (Dowal et al., 2006). By confocal microscopy, the GFP-PLCβ1a protein was found to reside in the cytosol, as well as at the plasma membrane (Fig. 1A). We did not detect GFP-PLCβ1a in the nucleus, similar to what has been observed in HEK293 and PC12 cells (Dowal et al., 2006). In other cell types, PLCβ1 is detected in the nucleus, depending on the differentiation state of the cell type (for a review, see Cocco et al., 2006).

Plasma membrane anchoring does not require Gq because PLCβ1a localizes to the plasma membrane in cells deficient in both Gqα and Gq11 (Fig. 1B). In these cells, PLCβ1a is found at the plasma membrane, albeit to a lower extent than in HeLa cells. However, the distribution is similar to that observed in wild-type MEF cells, suggesting that the distribution of PLCβ1a is dependent on the cell type but not on the presence of Gq proteins (Fig. 1C).

Removal of the CT domain abolished enrichment at the plasma membrane (Fig. 1D).

TIRF microscopy selectively excites a thin layer above the coverslip, allowing visualization of processes specifically at the plasma membrane and with high contrast (Axelrod, 2001). The highly fluorescent patches observed in the TIRF image of GFP-PLCβ1a (Fig. 1E) are sites with increased membrane content, consisting of submicroscopic folds and ruffles (van Rheenen and Jalink, 2002). Upon activation of a Gq-coupled receptor, PLCβ isoforms become active and start hydrolyzing PtdIns(4,5)P2. Surprisingly, the location of PLCβ1a was influenced by the addition of the H1R-specific agonist histamine, which resulted in dissociation from the membrane, leading to a loss of fluorescence as observed with the TIRF technique. This fluorescence was rapidly re-established and, at variable time spans, consecutive transients were observed in ~67% of the cells (n=30) (Fig. 1F).

PLCβ1a and intracellular Ca2+

In order to determine the cause of the oscillatory membrane binding of PLCβ1a, the protein was studied in the context of several probes. Using confocal fluorescence microscopy, RFP-PLCβ1a and the Ca2+-sensor YFP-C2γ were co-imaged. The C2 domain of PKCγ is a typical CalB domain that binds the phospholipid phosphatidylinositol serine (PS) present in the plasma membrane upon an increase in intracellular Ca2+ (Teruel and Meyer, 2002). Upon addition of histamine, the cytosolic C2γ domain moved to the plasma membrane (Fig. 2A-C,G) and, in ~10-20% of the cells, it oscillated between the cytosol and the plasma membrane (Fig. 2H,I, green traces). The translocation of the C2γ domain to the plasma membrane coincided with the translocation of PLCβ1a from the plasma membrane to the cytosol (Fig. 2D-I, red traces).

The duration of the C2γ translocations was equal to that of the PLCβ1a translocations. Moreover, the oscillations were phase-locked. The coincident translocation of RFP-PLCβ1a and YFP-C2γ suggests a role of Ca2+ in the regulation of PLCβ1a binding to the plasma membrane. However, (Ca2+-mediated) phosphorylation by PKC could also decrease PLCβ1a membrane binding (Litiosch, 2003). Such a phenomenon has been described for MARCKS, a protein that loses its affinity for negatively charged phospholipids upon phosphorylation by PKC (Allen and Aderem, 1995). To investigate a possible role of PKC in the translocation of PLCβ1a, we examined the translocation dynamics of PLCβ1a in cells in which PKC is downregulated. PKC downregulation can be achieved by incubating cells overnight with PMA (1 μM), as described (Harootonian et al., 1991; Liu and Heckman, 1998; Lu et al., 1998). Addition of histamine led to a transient increase in Ca2+ as measured using the calcium-indicator dye Fura Red (Fig. 3A, black trace). Similarly, in all cells, the translocation of PLCβ1a still occurred, although in both Ca2+- and PLCβ1a oscillations were no longer observed (Fig. 3A, red trace). Our data suggest that PKC activity is required for both PLCβ1a and Ca2+ oscillatory behavior, but is not required for the initial translocation. In support of this, Violin et al. (Violin et al., 2003) showed that PKC is required for Ca2+ oscillations, suggesting that the Ca2+ oscillations are required for the PLCβ1a oscillations.

PLCβ1a and PtdIns(4,5)P2

Another explanation for the observed coincidence of Ca2+ spiking and PLCβ release from the membrane is an interaction between PLCβ and its substrate, PtdIns(4,5)P2. Upon activation, PtdIns(4,5)P2 is cleaved and its product PtdIns(1,4,5)P3 releases Ca2+ from the ER. To examine whether plasma membrane PtdIns(4,5)P2 levels are profoundly changed during PLCβ stimulation, a PtdIns(4,5)P2-binding biosensor, consisting of the PH domain of

Fig. 1. Subcellular location of PLCβ1a. (A-D) Confocal images of (A) HeLa cells expressing GFP-PLCβ1a, (B) MEF cells deficient in Gqα11 expressing GFP-PLCβ1a, (C) wild-type MEF cells expressing GFP-PLCβ1a and (D) HeLa cells expressing YFP-PLCβ1a without its CT domain. (E) TIRF image of a HeLa cell expressing GFP-PLCβ1a. (F) Fluorescence of GFP-PLCβ1a detected in TIRF mode upon stimulation with histamine (100 μM, arrow). Scale bars: 10 μm.
YFP-C2 from cells 1, 2 and 3 (as depicted in A), respectively. The green lines indicate the intensity of three different cells. Graphs G, H and I show the normalized fluorescence intensity quantified μ(100 timepoints as indicated by the vertical lines in the graphs beneath (G-I). Addition of histamine μbar: 10 βRFP-PLC1fused to GFP, was expressed. In resting cells, this sensor is located at the plasma membrane (Fig. 3B). We proceeded by co-imaging PLCβ1a and the PHδ1 domain in the presence of the overexpressed bradykinin B2 receptor. At first, histamine was added to stimulate endogenous receptors and after 100 seconds bradykinin was added to obtain a maximized PLCβ1a dissociation. Oscillations were observed for both PLCβ1a and PHδ1 upon addition of histamine. After adding bradykinin, a complete dissociation from the plasma membrane of both proteins occurred (Fig. 3C). This indicates that PtdIns(4,5)P2 levels indeed oscillate and change profoundly. However, this experiment cannot discriminate between PtdIns(4,5)P2 breakdown and an increase in intracellular Ca2+ levels as the mechanism that induces PLCβ1a translocation into the cytosol.

In order to discriminate between the role of Ca2+ and PtdIns(4,5)P2 in mediating the membrane dissociation of PLCβ, we co-transfected RFP-PLCβ1a and murine GFP-PtdIns(4)P 5-kinase type Ix (GFP-PIP5K), which is also located at the plasma membrane (Fig. 4A,B). PIP5K uses PtdIns(4)P as a substrate to produce PtdIns(4,5)P2. If PtdIns(4,5)P2 breakdown alone mediates the translocation of PLCβ1a, then we would expect it to be abrogated in the presence of this kinase because PIP5K counteracts PtdIns(4,5)P2 depletion upon GPCR activation (van Zeijl et al., 2007).

Upon addition of histamine, a transient dissociation of the GFP-PLCβ1a protein was still observed in a number of cells. However, a two-tailed Student’s t-test analysis of the data indicates that those cells in which PLCβ1a does not move to the cytosol express significantly more PIP5K than those in which PLCβ1a diffuses into the cytosol (Fig. 4C). This suggests that in the presence of a larger amount of PLCβ1a, PtdIns(4,5)P2 hydrolysis cannot be effectively counteracted by PIP5K. Throughout the duration of the experiment, PIP5K did not dissociate from the plasma membrane. In addition, we calculated the ratio between plasma membrane fluorescence and cytosolic fluorescence of YFP-PLCβ1a for cells expressing PLCβ1a alone or in the presence of PIP5K. Statistical analysis indicates that significantly more PLCβ1a localizes at the plasma membrane in the presence of PIP5K (Fig. 4F).

In a complementary approach, we examined the effect of overexpression of 5-phosphatase, an enzyme that converts PtdIns(4,5)P2 into PtdIns(4)P. If PtdIns(4,5)P2 specifically mediates plasma membrane binding, this conversion would be expected to decrease the amount of PLCβ1a found at the plasma membrane. Others have shown that upon expression of 5-phosphatase, the PH domain of PLCδ1 loses its affinity for the plasma membrane (Kisseleva et al., 2002). Since we used untagged 5-phosphatase,

**Fig. 2.** PLCβ1a and intracellular Ca2+. HeLa cells coexpressing YFP-C2γ (A-C, green) and RFP-PLCβ1a (D-F, white, for higher contrast). Confocal images are shown at three different timepoints as indicated by the vertical lines in the graphs beneath (G-I). Addition of histamine (100 μM) is indicated by the arrow in G. Regions of interest were selected in the cytosol of three different cells. Graphs G, H and I show the normalized fluorescence intensity quantified from cells 1, 2 and 3 (as depicted in A), respectively. The green lines indicate the intensity of YFP-C2γ in the regions of interest; the red lines indicate the intensity of RFP-PLCβ1a. Scale bar: 10 μm.

**Fig. 3.** PLCβ1a and PtdIns(4,5)P2. (A) HeLa cells were stimulated with histamine (100 μM, arrow) after overnight incubation with 1 μM PMA. The red trace depicts the mean normalized fluorescence intensity of GFP-PLCβ1a; the black trace depicts the mean normalized fluorescence intensity of the Fura Red dye in a region of interest in the cytosol (n=7; the error bars depict standard error). The Fura Red dye was excited at 488 nm and detected with a long-pass 650-nm filter. Therefore, an increase in intracellular Ca2+ correlates with a decrease in fluorescence intensity. For the exact settings used, see Materials and Methods. (B) HeLa cells expressing RFP-PHδ1. Scale bar: 10 μm. (C) HeLa cells coexpressing PLCβ1a (red trace), PHδ1 (black trace) and bradykinin type 2 receptor (B2R) were first stimulated with histamine (100 μM; at t=15 seconds, first arrow) and then with bradykinin (1 μM; at t=115 seconds, second arrow).
the PHδ1 domain was used as an indicator for the presence and enzymatic activity of the enzyme. YFP-PLCβ1a, CFP-PHδ1 and untagged 5-phosphatase were coexpressed in HeLa cells. In several cells, both PHδ1 and PLCβ1a were found in the cytosol, suggesting 5-phosphatase activity (Fig. 4D,E). However, in the remaining cells, the PHδ1 domain was found in the cytosol, whereas PLCβ1a was still partly localized at the plasma membrane (Fig. 4G,H). We calculated the ratio between plasma membrane fluorescence and cytosolic fluorescence of YFP-PLCβ1a for cells expressing PLCβ1a alone or in the presence of 5-phosphatase. Statistical analysis indicates that significantly less PLCβ1a localizes at the plasma membrane in the presence of 5-phosphatase. Error bars depict standard error.

Fig. 4. The effect of PIP5K and 5-phosphatase on the location of PLCβ1a. (A,B) HeLa cells coexpressing GFP-PIP5K (imaged separately in A) and RFP-PLCβ1a (imaged separately in B). (C) The fluorescence intensity ratio of PLC to PIP5K was calculated for cells in which RFP-PLCβ1a does (TL (translocation)) and does not (no TL) dissociate from the plasma membrane upon stimulation with 100 μM histamine. A two-tailed Student’s t-test (confidence level of 95%) indicates that the cells in which PLCβ1a does not translocate to the cytosol express significantly more PIP5K than those in which PLCβ1a diffuses into the cytosol. Error bars depict standard error. (D,E,G,H) HeLa cells coexpressing untagged 5-phosphatase IV, YFP-PLCβ1a (D,G) and CFP-PHδ1 (E,H). (F) The ratio between plasma membrane fluorescence and cytosolic fluorescence of YFP-PLCβ1a was calculated for cells expressing PLCβ1a alone (PLC), in the presence of PIP5K (PLC+PIP5K) and in the presence of 5-phosphatase (PLC+5ptase). A two-tailed Student’s t-test (confidence level of 95%) indicates that significantly more PLCβ1a localizes at the plasma membrane in the presence of PIP5K, and that significantly less localizes at the plasma membrane in the presence of 5-phosphatase. Error bars depict standard error.

PLCβ1a partly dissociates from the plasma membrane upon translocation of RFP-5-phosphatase to the plasma membrane, indicating that PLCβ1a detects PtdIns(4,5)P2 and that conversion leads to dissociation from the plasma membrane. This process is schematically depicted in Fig. 5E,F. For these experiments, we selected cells with a low expression level of RFP-5-phosphatase, as YFP-PLCβ1a is found in the cytosol upon high-level expression of this protein. This can be explained by the fact that we used a truncated version of 5-phosphatase that lacks the domains required for negative regulation (Varnai et al., 2006). Through diffusion, the truncated 5-phosphatase is therefore expected to convert PtdIns(4,5)P2 into PtdIns(4)P even in the absence of rapamycin.

Complete dissociation of PLCβ1a from the plasma membrane was observed upon addition of bradykinin (1 μM) in cells overexpressing the bradykinin B2 receptor (Fig. 5A). In addition, we monitored the effect of 5-phosphatase recruitment on the localization of the PH domain of PLCβ1. This domain also partly dissociates from the plasma membrane upon addition of rapamycin, albeit to a greater extent than does PLCβ1a. Complete dissociation was obtained by stimulation of the overexpressed B2 receptor (Fig. 5B). In order to exclude a role for Ca2+ in this process, we co-transfected cells with YFP-C2γ. No change in the location of this probe was detected, indicating that the intracellular Ca2+ concentration did not increase in the presence of rapamycin (Fig. 5C). Ionomycin was subsequently added to check the integrity of the calcium sensor. In the absence of the RFP-5-phosphatase-FKBP12 protein fusion, YFP-PLCβ1a did not dissociate from the plasma membrane upon addition of rapamycin (Fig. 5D).

Discussion

Phospholipases have to reside at membranes in order to encounter their substrates. Some of these proteins are thought to be present at the membrane in the resting state, whereas others are thought to be targeted to this locale in response to receptor activation. This is the first report on the localization and dynamics of PLCβ1 in living cells, and shows that PLCβ1 moves from the plasma membrane to the cytosol upon activation of the GPCR-Gq pathway and does so in a transient manner. Importantly, plasma membrane binding is dependent on the CT domain and on PtdIns(4,5)P2, but not on the presence of members of the Gq class of Gα subunits.

PtdIns(4,5)P2 regulates the subcellular location of PLCβ1a

Negatively charged phospholipids have been proposed to mediate the interaction between the positively charged face of the CT domain of PLCβ and the plasma membrane (Singer et al., 2002). Rapamycin-induced dimerization of an FRB domain and a FKBP domain coupled to 5-phosphatase (Varnai et al., 2006) enabled us to show that PLCβ1a has affinity for PtdIns(4,5)P2 in living cells and that its localization to the plasma membrane is regulated by this lipid.

Many proteins have affinity for PtdIns(4,5)P2 and the domains that dictate their binding vary extensively (Ballal, 2005). Even short
Importantly, it should be noted that the affinity of PLCβ2 relative-charge densities (McLaughlin and Murray, 2005). The clusters of basic residues in the CT domain of PLCβ1 are likely to determine its affinity for PtdIns(4,5)P2 because addition of rapamycin did not trigger a complete dissociation of PLCβ1 from the plasma membrane. Another explanation might be that the PtdIns(4,5)P2 pool is not entirely converted, as suggested by PHδ1 (Fig. 5B), which also did not completely dissociate upon addition of rapamycin. Alternatively, a concurrent increase in intracellular Ca2+ might have an additive effect on the amplitude of the translocation. Moreover, data in the literature suggest that PLCβ1 has affinity for phosphatidic acid (PA) (Litochs, 2003). Therefore, PLCβ1 localization might also be influenced upon activation of phospholipase D (PLD) through PA and receptor tyrosine kinase signaling through PtdIns(3,4,5)P3, enabling cross-talk between diverse pathways. However, with respect to Gq-mediated signaling, the effect of PtdIns(4,5)P2 is of immediate significance, as it is this lipid that is specifically hydrolyzed.

The other PLCβ isozymes might also function in a similar way. However, their CT domains await structural resolution and it might very well be that additional or differently positioned charges negatively influence their membrane anchoring. For instance, PLCβ2 is not enriched at the plasma membrane in a manner comparable to PLCβ1a. However, by virtue of its CT domain, PLCβ2 does not display a pure cytosolic localization, as concluded from FRAP data (Illenberger et al., 2003).
In vitro, James et al. (James et al., 1995) observed PtdIns(4,5)P₂-dependent attachment of PLCβ to mixed PtdIns(4,5)P₂/detergent micelles, whereas such a dependence was not found using phospholipid vesicles (Jenco et al., 1997; Runnels et al., 1996). Boguslavsky et al. found PS to facilitate PLCβ anchoring to lipid vesicles (Boguslavsky et al., 1994). Similarly, PIP₂ strip overlay assays have shown that both PLCβ1 and PLCβ3 have affinity for phospholipids (McCullar et al., 2007). It is not clear why PtdIns(4,5)P₂ did not have a clear effect on the binding of PLCβ to phospholipid vesicles. The different physical properties of synthetic phospholipid vesicles and dot blots from the native cellular plasma membrane might explain the inconsistency between the data obtained in vitro and our data obtained in living cells.

**Processive catalysis**

PLCβ1 binds non-catalytically to a PtdIns(4,5)P₂ molecule at the plasma membrane by virtue of its PH domain. This initial binding event is followed by binding of a second PtdIns(4,5)P₂ molecule at the catalytic site, which results in hydrolysis of the substrate. The PH domain of PLCβ1 mediates stable attachment of the enzyme at the plasma membrane, which permits multiple hydrolytic cycles to occur before the enzyme detaches from the interface. This process is termed dual substrate/scooting behavior and leads to processive catalysis. Several other lipid-metabolizing enzymes have been suggested to work in a similar way, including PLA₂, PI(4)-K and PLCγ. Successive lipid-binding events have also been discerned for turkey erythrocyte PLC and mammalian PLCβ1 and PLCβ2 (James et al., 1995). Interestingly, we report here a non-catalytic PtdIns(4,5)P₂-binding site in PLCβ1, and the findings described here imply the existence of a non-catalytic PtdIns(4,5)P₂-binding site in PLCβ1a that can explain its dual substrate behavior. If the CT domain is able to sequester PtdIns(4,5)P₂ molecules in the plasma membrane, it might also be able to increase the local PtdIns(4,5)P₂ concentration sufficiently to enhance the catalytic activity of the enzyme. Recently, a similar phenomenon has been described for PLCζ, which contains a cluster of basic residues, close to the catalytic site, that increases both membrane binding of the enzyme and the concentration of PtdIns(4,5)P₂ adjacent to the catalytic domain (Nomikos et al., 2007).

The relevance of a transient response

Receptor-mediated increases in intracellular Ca²⁺ often oscillate and are decoded by frequency-modulated proteins, such as PKC and calcmodulin. Whereas prolonged increases in intracellular Ca²⁺ trigger cell death, oscillations allow the cell to use Ca²⁺ as a messenger. PtdIns(4,5)P₂ is implicated in the activation of ion channels, the attachment of the cytoskeleton to the plasma membrane, exo- and endocytosis, phagocytosis, chemotaxis and polarization (Di Paolo and De Camilli, 2006; McLaughlin et al., 2002). Constitutive depletion of PtdIns(4,5)P₂ promotes apoptosis (Halstead et al., 2005). Obviously, a cell must strictly regulate the levels of this important lipid. Therefore, the transient redistribution of PLCβ1a to the cytosol upon activation of Gq-coupled receptors is expected to enable the cell to replenish PtdIns(4,5)P₂ in the plasma membrane. Thus, PLCβ1a is regulated by PtdIns(4,5)P₂, which functions both as its substrate and its plasma membrane targeting signal.

Two models have been described to explain oscillations similar to the ones observed for Ca²⁺, PtdIns(4,5)P₂ and PLCβ1a in this paper. The first model is based on calcium-induced calcium release, whereas the other is triggered by dynamic phosphorylation of the GPCR, thereby uncoupling the PLCβ pathway (Hirose et al., 1999; Nash et al., 2001; Sauve et al., 1991). Both models depend on PKC activity. The phase-locked oscillations observed for Ca²⁺ and PLCβ1a are fine-tuned by PKC, as they are absent after a 24-hour treatment with PMA. PKC is involved in desensitizing both the Ca²⁺ and the PLCβ1a response, as they show a delayed recovery in its absence. Importantly, the initial translocation of PLCβ1a is normal after downregulation of PKC by means of PMA, indicating that phosphorylation by PKC is not mandatory for the first dissociation of PLCβ1a from the plasma membrane to occur. Apparently, the mechanism of PLCβ1a translocation is not comparable to that of MARCKS (Allen and Aderem, 1995), the membrane affinity of which is reduced by phosphorylation.

**Implications for downstream signaling**

Other domains of the full-length PLCβ protein might enhance its affinity upon reaching the plasma membrane. However, except for the CT domain, the individual domains show no significant affinity for the plasma membrane (data not shown).

Dissociation of PLCβ from the plasma membrane constitutes yet another way to downregulate phosphoinositide signaling, in addition to the GAP activity that PLCβ displays. In fact, translocation of PLCβ is likely to abrogate kinase-mediated behavior. This phenomenon is thought to lead to spatial focusing of signaling by decreasing the amount of active G proteins capable of activating more-distant effector molecules (Zhong et al., 2003). Dissociation of PLCβ from the complex might therefore lead to a ‘reshuffling’ of signalosomes by enabling Gq to engage other partners, such as p63RhoGEF (Lutz et al., 2005). Alternatively, the translocation might increase the interaction of PLCβ with cytosolic proteins.

Importantly, PLCβ1a interacts with the PDZ-domain-containing protein NHERF1 via its C-terminal four amino acids. NHERF1, which also interacts with certain GPCRs and channels, connects signaling complexes to the actin cytoskeleton via ezrin, radixin and moesin proteins (Suh et al., 2001). Such PDZ-domain-containing scaffolds might inhibit PLCβ1a dissociation from the plasma membrane by physical scaffolding. For instance, Dowal et al. did not observe dissociation of eYFP-PLCβ1a from the plasma membrane in PC12 cells upon stimulation with acetylcholine (Dowal et al., 2006). Such a mechanism would enable the cell to modulate the extent of PLCβ1a dissociation by regulating the expression levels of its scaffold proteins.

**Materials and Methods**

Constructs and materials

pcDNA5/V5-HisB containing rat PLCβ1a was a kind gift of L. Runnels (University of Medicine and Dentistry of New Jersey, Jersey City, NJ). Full-length PLCβ1a was fused to the C-terminus of monomeric spectral variants of GFP in pClontech-c1 vectors (Kremers et al., 2007). RFP (mStrawberry) was a kind gift of R. Y. Tsien (Howard Hughes Medical Institute, San Diego, CA). GFP-C2γ (PKCγ) and GFP-PHγ1 were kind gifts of T. Meyer (Stanford University Medical Center, Stanford, CA). Experiments were performed with the C2γ domain fused to a monomeric YFP. The cDNA encoding 5-phosphatase IV was donated by P. Majerus (Washington University School of Medicine, St Louis, MO). The GFP-pm-FRB and RFP-5-phosphatase (truncated)-FKBP12 were obtained from T. Balla (National Institutes of Health, Bethesda, MD). Mouse PIP5K type Ia cDNA was provided by Y. Oka (Tohoku University Graduate School of Medicine, Miyagi, Japan) and fused to the C-terminus of GFP in a pEGFP-c2 vector (Clontech). The pcDNA3 vector containing human bradykinin type 2 receptor (B2R) was a gift from C. Liebmann (Friedrich-Schiller-University, Jena, Germany). All constructs were verified by sequencing.

Rapamycin, histamine, bradykinin, phorbol 12-myristate 13-acetate (PMA) and ionomycin were obtained from Sigma. The Fura Red AM compound was obtained from Molecular Probes.
Mammalian cells were imaged at room temperature using a Zeiss LSM 510 confocal laser-scanning microscope. A Zeiss Plan-Apochromat 63×/1.4 oil immersion objective was used. GFP-PLC-β1 was excited with a 488-nm argon laser line controlled by an acousto-optical tunable filter. For GFP-Fura Red, the following settings were used: HFT 488 as primary dichroic mirror, NFT 570 as secondary dichroic mirror, thereby splitting the Fura Red fluorescence into the GFP and Fura Red image. For GFP/RRF, the following settings were used: samples were excited with a 488-nm argon laser and a 568-nm argon/krypton laser line. The primary dichroic mirror was HFT 488/568, the secondary NFT 570. The 505-550 band-pass filter was used to yield the GFP image. The long-pass 650 filter was used to obtain the Fura Red image. In order to abolish cross-talk, the images were acquired in the multi-tracking mode, in which the 488-nm laser line was coupled to the GFP-detection channel and the 568-nm laser line to the RFP-detection channel.

TIRF microscopy

Cells were imaged at room temperature using a Zeiss Axiovert 200M inverted microscope equipped with the TIRF Zeiss microscope. A Zeiss Plan-75×/1.4 oil-immersion objective was used. GFP-PLCβ3 was excited with a 488-nm argon laser line and GFP emission was selected using a 510-530 band-pass emission filter (Chroma Technology). Images were acquired with a cooled CCD camera (CoolSnap HQ, Roper Scientific). Software for image control, acquisition and analysis was written in C++ using Matlab 6.1 (The Mathworks).

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