Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P_2 concentration monitored in living cells

Thomas P. Stauffer, Seungkril Ahn and Tobias Meyer

Although phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is a well-characterized precursor for the second messengers inositol 1,4,5-trisphosphate, diacylglycerol [1] and phosphatidylinositol 3,4,5-trisphosphate [2], it also interacts with the actin-binding proteins profilin and gelsolin [3], as well as with many signaling molecules that contain pleckstrin homology (PH) domains [4]. It is conceivable that stimuli received by receptors in the plasma membrane could be sufficiently strong to decrease the PtdIns(4,5)P_2 concentration; this decrease could alter the structure of the cortical cytoskeleton and modulate the activity of signaling molecules that have PH domains. Here, we tested this hypothesis by using an *in vivo* fluorescent indicator for PtdIns(4,5)P_2, by tagging the PH domain of phospholipase C δ1 (PLC-δ1) with the green fluorescent protein (GFP–PH). When expressed in cells, GFP–PH was found to be enriched at the plasma membrane. Binding studies *in vitro* and mutant analysis suggested that GFP–PH bound PtdIns(4,5)P_2 selectively over other phosphatidylinositol lipids. Strikingly, receptor stimulation induced a transient dissociation of GFP–PH from the plasma membrane, suggesting that the concentration of PtdIns(4,5)P_2 in the plasma membrane was effectively lowered. This transient dissociation was blocked by the PLC inhibitor U73122 but was not affected by the phosphoinositide (PI) 3-kinase inhibitor wortmannin, suggesting that it is mostly mediated by PLC and not by PI 3-kinase activation. Overall, our studies show that PtdIns(4,5)P_2 can have second messenger functions of its own, by mediating a transient dissociation of proteins anchored in the plasma membrane.

Address: Department of Cell Biology, Duke University Medical Center, Nanaline Duke Building, Room 346, Box 3709, Durham, North Carolina 27710, USA.
E-mail: tobias@cellbio.duke.edu

Received: 19 November 1997
Revised: 19 January 1998
Accepted: 22 January 1998

Published: 2 March 1998

Current Biology 1998, 8:343–346
http://biomednet.com/elecref/0960982200800343

 ISSN 0960-9822

Results and discussion

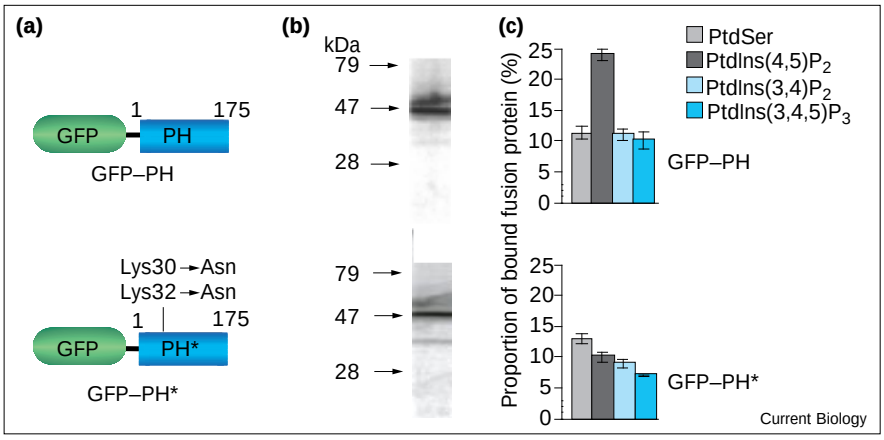
PH domains are protein modules of about 100 amino acids found in a variety of enzymes [5]. They are thought to serve as localization modules for signaling molecules by binding to phosphatidylinositol lipids in membranes [6]. Recent *in vitro* studies have shown that distinct PH domains have a selectivity for different phosphatidylinositols (i.e. PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3) [7–9]. We used this selectivity to create a fluorescent single-cell *in vivo* indicator which recognizes specifically PtdIns(4,5)P_2 but not other phosphatidylinositols.

To achieve this goal, we made fusion constructs between GFP [10] and the PH domain of PLC-δ1 (GFP–PH). This PH domain has a high selectivity for PtdIns(4,5)P_2 as shown in *in vitro* binding assays [8] and by X-ray crystallography [11]. Furthermore, it binds to PtdIns(4,5)P_2 with higher affinity than do the other PtdIns(4,5)P_2-binding PH domains that have been tested thus far — PLC-γ, spectrin, dynamin, pleckstrin and the Son of sevenless protein [12]. Because we wanted to express GFP–PH by RNA transfection, we cloned a cassette containing sequences encoding GFP followed 3′ by sequences encoding the PLC-δ1 PH domain into an RNA expression vector [13] (Figure 1a). As a control, we also made a construct (GFP–PH*) in which two point mutations were introduced into sequences encoding the phosphatidylinositol-binding pocket (Lys30→Asn and Lys32→Asn) (Figure 1a).

The RNA used for transfection was transcribed and polyadenylated by sequential *in vitro* steps [13]. The integrity of the expressed protein was tested by SDS–PAGE following *in vitro* translation in the presence of [^{35}S]methionine. Both *in vitro* translated proteins appeared as major bands at about 49 kDa (Figure 1b) which correspond to the calculated molecular weight of 48.2 kDa for the GFP–PH fusion protein. We then used phosphatidylserine liposomes with either no phosphatidylinositol or 5% PtdIns(4,5)P_2, PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 to test whether the GFP tag changed the phosphatidylinositol specificity of the PLC-δ1 PH domain. As expected, GFP–PH showed a much higher binding to liposomes that contain PtdIns(4,5)P_2 (Figure 1c). The binding to liposomes containing PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 was indistinguishable from the binding to liposomes that did not contain phosphoinositols. This suggests that the specificity of the PLC-δ1 PH domain was not altered by the addition of the GFP tag. As an additional control, we confirmed that the GFP–PH* fusion construct with the mutations in the lipid-binding pocket showed no specific PtdIns(4,5)P_2 binding (Figure 1c).

The GFP-based fluorescent indicator for PtdIns(4,5)P_2 was expressed in rat basophilic leukemia (RBL) cells by

Figure 1



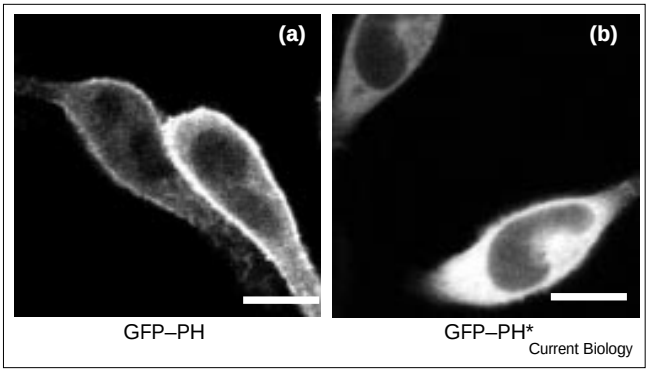
The PH domain of PLC-δ1 was fused with GFP and expressed in rat basophilic leukemia (RBL) cells. **(a)** The GFP–PH and GFP–PH* fusion proteins. The PLC-δ1 PH domain, or a mutant version (PH*) carrying mutations in the phosphatidylinositol-interacting residues (Lys30→Asn and Lys32→Asn), was cloned 3′ to a cycle 3 mutant of GFP [13]. **(b)** The integrity of the radiolabeled GFP–PH fusion proteins was determined by SDS–PAGE following translation *in vitro* in the presence of [^{35}S]methionine. **(c)** Specific binding of GFP–PH to PtdIns(4,5)P_2. ^{35}S-labeled GFP–PH or GFP–PH* was incubated with liposomes containing phosphatidylserine (PtdSer) and 5% (w/w) PtdIns(4,5)P_2, PtdIns(3,4)P_2, or PtdIns(3,4,5)P_3. For each fusion protein, the amount of bound fusion protein was determined by scintillation counting and standardized against the total amount of the fusion protein used. No selective PtdIns(4,5)P_2 binding was observed with the GFP–PH* mutant. The results shown are the average of three independent experiments with the bars indicating the standard error.

microporation of the RNA encoding the GFP–PH [14] and visualized, typically three hours after microporation, by confocal laser microscopy. Interestingly, GFP–PH was highly enriched at the plasma membrane and nearly uniformly distributed along the membrane (Figure 2a). The plasma membrane localization is likely to be due to the binding interaction between the PH domain and PtdIns(4,5)P_2 in the plasma membrane as shown by the failure of the GFP–PH* fusion protein to localize to the plasma membrane (Figure 2b). These observations strongly suggest that the PH domain of PLC-δ1 binds to PdtIns(4,5)P_2 in living cells and can therefore be used a fluorescent indicator for PtdIns(4,5)P_2.

Because the plasma membrane localization of GFP–PH is likely to provide a direct measure of the plasma membrane concentration of PtdIns(4,5)P_2, we tested whether a receptor stimulus that induces the hydrolysis of PtdIns(4,5)P_2 could be sufficiently strong to lower the PtdIns(4,5)P_2 concentration and thereby induce a dissociation of the GFP–PH probe from the plasma membrane. An RBL cell line stably transfected with the receptor for the platelet activation factor (PAF) was used for these studies [15]. Strikingly, within less than 60 seconds of PAF addition, the GFP–PH probe almost completely dissociated from the plasma membrane and became uniformly distributed within the cytosol (Figure 3a, upper panels). Nevertheless, the GFP–PH probe dissociated from the plasma membrane only transiently and relocalized to the plasma membrane within 3–8 minutes after stimulation (a movie of the dissociation and reassociation of GFP–PH after receptor stimulation is available as Supplementary material published with this paper on the internet). As expected, the cytosolic localization of the GFP–PH* mutant did not change during stimulation (Figure 3a, lower panels).

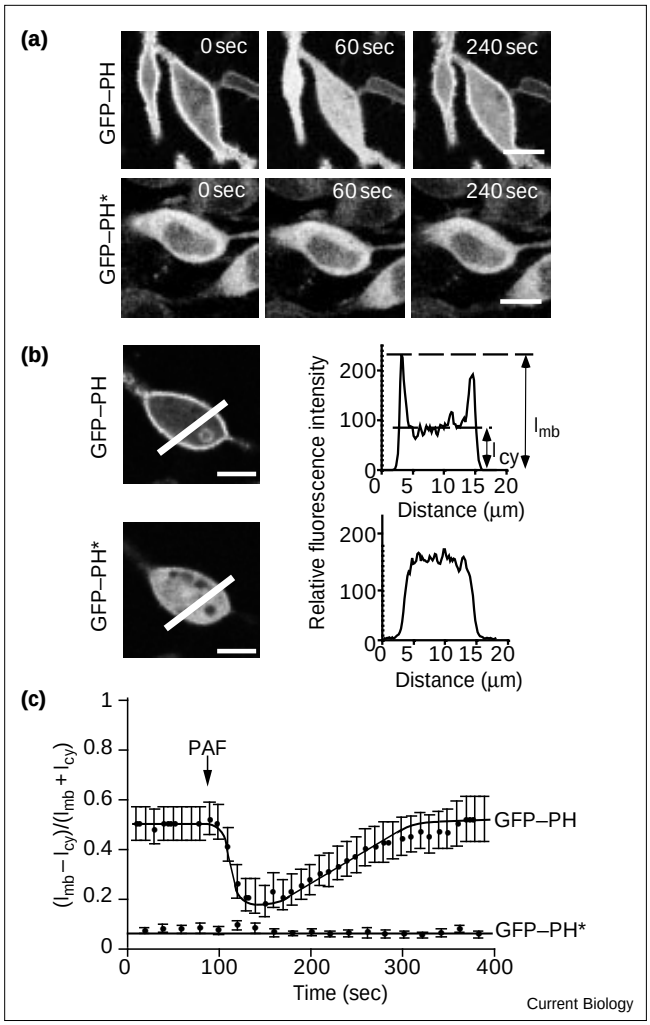
To determine the time course of the transient dissociation of the GFP–PH probe from the plasma membrane more quantitatively, a timed series of confocal fluorescence images of cells expressing the GFP–PH probe were recorded before and after receptor stimulation. The relative change in the plasma membrane fluorescence intensity was determined in each image using line intensity profiles across each of the cells (Figure 3b). The relative decrease in the fluorescence intensity of the plasma

Figure 2



Localization of GFP–PH or GFP–PH* in RBL cells. The fusion proteins were expressed in adherent RBL cells by microporation of *in vitro* transcribed and polyadenylated RNA, and visualized by confocal laser scanning microscopy. **(a)** GFP–PH is predominantly localized to the plasma membrane. **(b)** The GFP–PH* mutant is uniformly distributed. Each scale bar is 10 μm.

Figure 3



Receptor-induced transient dissociation of GFP–PH from the plasma membrane. (a) Sequential images of RBL cells expressing GFP–PH (upper panels) or GFP–PH* (lower panels) taken immediately before, or 60 sec and 240 sec after receptor stimulation with PAF. (b) Schematic representation of the method used to calculate the relative decrease in plasma membrane staining. For each cell in a given image, a line intensity profile across the cell was obtained. Typical intensity profiles before and after receptor stimulation are shown. The relative decrease in plasma membrane localization was calculated from the plasma membrane fluorescence intensity [I_{mb}] and the average cytosolic fluorescence intensity [I_{cy}]. The scale bar in (a) is 10 μm and applies to all images; the scale bar in (b) is 5 μm. (c) Plasma membrane translocation was reflected as a relative increase in plasma membrane localization when plotted as a function of time. Each resulting curve shows the time course of the transient plasma membrane dissociation of GFP–PH in response to PAF receptor activation. An average of 12 curves is shown in the plot; the bars indicate the standard error.

membrane to that of the cytosol was calculated by measuring the amplitude of the fluorescence signal at the plasma membrane and dividing it by an average intracellular fluorescence intensity $[(I_{mb} - I_{cy})/(I_{mb} + I_{cy})]$. Figure 3c shows the intensity ratio for each time point as a function of time.

Although the dissociation of GFP–PH from the plasma membrane could at least in theory be the result of a displacement by other plasma membrane binding proteins, it is more likely to be caused by the receptor-induced degradation of PtdIns(4,5)P_2. Several enzymes including PLC, PI 3-kinase, which is activated after PAF receptor stimulation (T.P.S., unpublished observations), or PtdIns(4,5)P_2 phosphatase might contribute to the removal of PtdIns(4,5)P_2 after receptor stimulation. To test the role of PLC and PI 3-kinase in the reduction of PtdIns(4,5)P_2 concentration, we incubated GFP–PH-transfected RBL cells with the relatively specific PLC inhibitor U73122 [16], or the PI 3-kinase inhibitor wortmannin [17]. Before stimulation, the localization of the GFP–PH probe was not altered by the two drugs (Figure 4a,b). Cells incubated with U73122 failed to show the transient plasma membrane dissociation of GFP–PH (Figure 4a), whereas cells incubated with wortmannin showed a normal response (Figure 4b), which indicates that PI 3-kinase is not a major contributor in the reduction of PtdIns(4,5)P_2 concentration after receptor activation. These results strongly suggest that G-protein activation can induce a transient and cell-wide hydrolysis of plasma membrane PtdIns(4,5)P_2 that is mediated by PLC. This model is further supported by the finding that the time course of the PAF-induced Ca^{2+} transient directly corresponds to the dissociation of GFP–PH from the plasma membrane (Figure 4c).

These results are of particular interest since a number of signaling proteins as well as cortical cytoskeletal structures are attached to the plasma membrane by binding to PtdIns(4,5)P_2. The transient reduction in plasma membrane PtdIns(4,5)P_2 concentration may therefore represent a negative feedback mechanism which can downregulate signaling processes by releasing the signaling complex from the plasma membrane. In addition, the parallel dissociation of the actin-binding proteins gelsolin and profilin from the plasma membrane might activate their severing activity and might trigger a restructuring of the cortical cytoskeleton.

Materials and methods

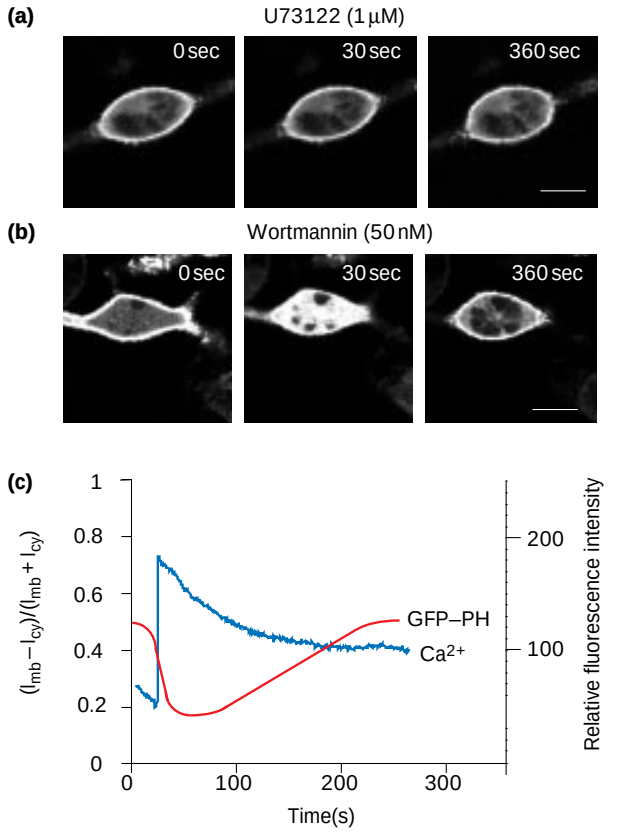
cDNA constructs and in vitro *RNA synthesis*

The PH domain of human phospholipase C δ1 (amino acids Met1–Ile175) as well as the corresponding lipid-binding mutant PLC-δ1 Lys30→Asn, Lys32→Asn were subcloned 3′ to cycle3 GFP [18] into the eukaryotic RNA expression vector Hiro3 [13]. An additional Ser65→Thr mutation was added to cycle3 GFP to increase its brightness [19]. The RNA was transcribed and polyadenylated by sequential *in vitro* steps as described in [13].

In vitro *translation and binding assay*

The PH domains were transcribed, translated and labeled with [^{35}S]methionine *in vitro* using the TNT coupled reticulocyte lysate

Figure 4



Receptor-induced dissociation of GFP–PH is mediated by PLC. (a) RBL cells were incubated for 10 min with 1 μM U73122 (PLC inhibitor). Sequential images of RBL cells expressing GFP–PH were taken immediately before, and 30 sec and 360 sec after receptor stimulation with 100 nM PAF. (b) The PI 3-kinase inhibitor wortmannin (50 nM) was added to RBL cells for 30 min prior to activation. Sequential images of RBL cells expressing GFP–PH were taken immediately before, and 30 sec and 360 sec after receptor stimulation with 100 nM PAF. In both (a) and (b), the size bar is 10 μm. (c) The PAF-dependent Ca^{2+} release from internal stores was measured in single cells using the calcium indicator Fluo-3. The average change in fluorescence with time is shown for eight cells, together with the time course of the transient plasma membrane dissociation of GFP–PH in response to PAF receptor activation.

system (Promega). The specificity of the PH domains was determined as described [7].

Cell preparation and microporation of RNA

The RNA was microporated into rat basophilic leukemia cells (2H3 type) expressing PAF receptors [15] as described [14,20].

Supplementary material

A movie of the dissociation and reassociation of GFP–PH after receptor stimulation is published with this paper on the internet

Acknowledgements

We thank M. Rebecchi for providing the PLC-δ1 cDNA constructs, R. Richardson and R. Snyderman (Duke University) for RBL cells expressing the PAF receptor, and M. Teruel and E. Oancea for assistance. T.P.S is a recipient of a fellowship from the Swiss National Science Foundation (Grant 823A-050457). T.M. was supported by a fellowship from the David and Lucile Packard Foundation. This work was supported by Proctor & Gamble grant SRA 1617 and the National Institute of Health grant GM-51457.

References

1. Lee SB, Rhee SG: Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. *Curr Opin Cell Biol* 1995, 7:183-189.
2. Kapeller R, Cantley LC: Phosphatidylinositol 3-kinase. *Bioessays* 1994, 16:565-576.
3. Jammey PA: Protein regulation by phosphoinositol lipids. *Chem Biol* 1995, 2:61-65.
4. Shaw G: The pleckstrin homology domain: an intriguing multifunctional protein module. *Bioessays* 1996, 18:35-46.
5. Gibson TJ, Hyvonen M, Musacchio A, Saraste M, Birney E: PH domain: the first anniversary. *Trends Biochem Sci* 1994, 19:349-353.
6. Lemmon MA, Ferguson KM, Schlessinger J: PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell* 1996, 85:621-624.
7. Salim K, Bottomley MJ, QuerfurthE, Zvelebil M J, Gout I, Scaife R, *et al.*: Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J* 1996, 15:6241-6250.
8. Lemmon MA, Ferguson KM, O'Brien R, Sigler PB, Schlessinger J: Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. *Proc Natl Acad Sci USA* 1995, 92:10472-10476.
9. Franke TF, Kaplan DR, Cantley LC, Toker A: Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* 1997, 275:665-668.
10. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC: Green fluorescent protein as a marker for gene expression. *Science* 1994, 263:802-805.
11. Ferguson KM, Lemmon MA, Schlessinger J, Sigler PB: Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* 1995, 83:1037-1046.
12. Lemmon MA, Falasca M, Ferguson KM, Schlessinger J: Regulatory recruitment of signalling molecules to the cell membrane by pleckstrin-homology domains. *Trends Cell Biol* 1997, 7:237-242.
13. Yokoe H, Meyer T: Spatial dynamics of GFP-tagged proteins investigated by local fluorescence enhancement. *Nat Biotechnol* 1996, 14:1252-1256.
14. Teruel MN, Meyer T: Electropopration induced formation of individual calcium entry sites in cell body and processes of adherent cells. *Biophys J* 1997, 73:1785-1796.
15. Richardson RM, HaribabuB, Ali H, Snyderman R: Cross-desensitization among receptors for platelet activating factor and peptide chemoattractants. Evidence for independent regulatory pathways. *J Biol Chem* 1996, 271:28717-28724.
16. Bleasdale JE, Thakur NR, Gremban RS, Bundy GL, Fitzpatrick FA, Smith RJ, Bunting S: Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J Pharm Exp Therap* 1990, 255:756-768.
17. Ui M, Okada T, Hazeki K, Hazeki O: Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem Sci* 1995, 20:303-307.
18. Crameri A, Whitehorn EA, Tate E, Stemmer PC: Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat Biotechnol* 1996, 14:315-319.
19. Heim R, Tsien R: Improved green fluorescence. *Nature* 1995, 373:663-664.
20. Stauffer TP, Meyer T: Compartmentalized IgE-receptor-mediated signal transduction in living cells. *J Cell Biol* 1997, 137:1447-1454.