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PIP2 as local second messenger: a critical re-evaluation

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PIP₂ as local second messenger: a critical re-evaluation

Jacco van Rheenen

PIP₂ as local second messenger: a critical re-evaluation

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Aan mijn ouders

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Abbreviations

ABP	actin binding protein
CD	methyl- β -cyclodextrin
CCD	charge-coupled-device
CCD	cytochalasin D
CFP	cyan fluorescent protein
DAG	diacylglycerol
EM	electron microscopy
ET	endothelin
ETBr	endothelin B receptor
FLIM	fluorescence lifetime imaging
FLIP	fluorescence loss in photobleaching
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
NKA	neurokinin A
NK2r	neurokinin A receptor
PH	pleckstrin homology
PIP ₂	phosphatidylinositol (4,5) biphosphate
PIP ₃	phosphatidylinositol (3,4,5) triphosphate
PKC	protein kinase C
PLC	phospholipase C
PMT	photomultiplier tubes
PSF	pointsread function
RAP	Reconstructed Axial PSF
RFP	red fluorescent protein
ROI	region of interest
YFP	yellow fluorescent protein

Chapter 1

Introduction

Introduction

Phosphatidylinositol 4,5-bisphosphate (PIP₂) represents less than 1% of membrane phospholipids, but it serves an important function as precursor for the second messengers DAG, IP₃ and PIP₃. Interestingly, PIP₂ has recently been proposed to act as a second messenger itself, with a proposed role in the regulation of ion channels, cell survival, vesicle trafficking and actin cytoskeleton remodeling. If so, then PIP₂ should fulfill two important criteria for second messengers: first PIP₂ levels should vary spatially or temporally under physiological conditions, and secondly, these variations should suffice to influence cellular processes. These criteria are addressed in the first part of this thesis (chapter 2 and 3). We provide evidence that PIP₂ levels may indeed vary dramatically over time and we demonstrate that these variations influence cell survival (chapter 2) and the dynamic behavior of the cortical actin in neuroblastoma cells (chapter 3). Interestingly, these results also suggest that PIP₂ influences multiple physiological processes within the same cell, apparently in a spatially segregated manner. This view also prevails in the literature: it is widely hypothesized that the plasma membrane contains spatially confined PIP₂ pools or domains. In the second part of this thesis (part of chapter 3 and chapter 4) we have investigated this issue by testing to what extent local differences in content or availability of PIP₂ may exist in a cell. Surprisingly, no evidence was found for PIP₂ enrichments, neither at micrometer scale (chapter 3) nor at nanometer scale (chapter 4), presumably due to rapid diffusion. However, at a larger scale, and especially at places where diffusion is limited (e.g. in neurites), PIP₂ gradients could be established. In order to study local variations in PIP₂, we optimized the confocal imaging of Fluorescence Resonance Energy Transfer (FRET) by detecting sensitized emission (chapter 5).

Synthesis and breakdown of PIP₂

Inositol lipids are important signaling mediators in cells. Phosphatidylinositol (PI) is the precursor for all inositol lipids, and phosphorylation of the hydroxyl groups at positions 3, 4 or 5 of the inositol head group results in a number of different phosphoinositides (Hinchliffe et al., 1998). This

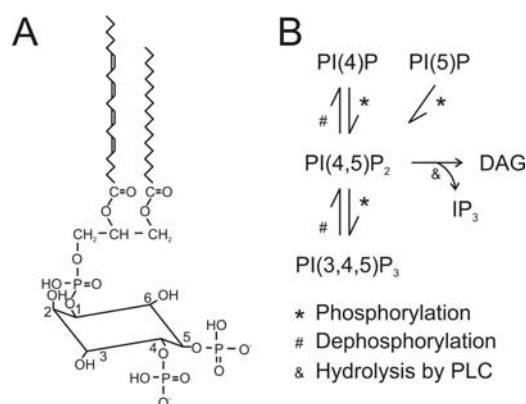


Figure 1, Biosynthesis and removal of PIP₂. (A) The structure of phosphatidylinositol 4,5-bisphosphate (PIP₂). (B) The biosynthesis and removal of PIP₂ are mediated by several phosphorylation (*) and dephosphorylation (#) steps of the inositol headgroup, and by the hydrolysis (&) into DAG and IP₃ by Phospholipase C (PLC).

thesis will focus on the function and the localization of one of these phosphoinositides, PI(4,5)P₂ (hereafter this molecule is referred to as PIP₂, Figure 1A). Several phosphorylation or dephosphorylation steps can generate PIP₂ (Figure 1B). The main route of PIP₂ synthesis is the phosphorylation of PIP (or PI(4)P) at the 5-position by PIP5KI (Anderson et al., 1999). *In vitro*, this kinase also phosphorylates PI(3)P at the 4- and 5-position resulting in the formation of two other forms of PIP₂ (PI(3,4)P₂ and PI(3,5)P₂) and the formation of PI(3,4,5)P₃ (Anderson et al., 1999). Another lipid kinase, PIP4KII, can produce PIP₂ by phosphorylation of PI(5)P at the 4 position (Boronenkov and Anderson, 1995). However, this activity has only been found *in vitro* and thus far *in vivo* data supporting this finding are not available.

Alternatively, PIP₂ is produced via dephosphorylation of PIP₃ by, for example, the 3-phosphatase activity of the tumor suppressor protein PTEN (Maehama et al., 2001). The opposite conversion, the PIP₃ production by phosphorylation of PIP₂ at the 3 position by class I phosphoinositide 3 kinase (PI3K) lowers the PIP₂ level (Rameh and Cantley, 1999; Rameh and Cantley, 1999). However, since levels of PIP₃ are generally much lower than those of PIP₂, these pathways probably play a minor role in determining PIP₂ levels.

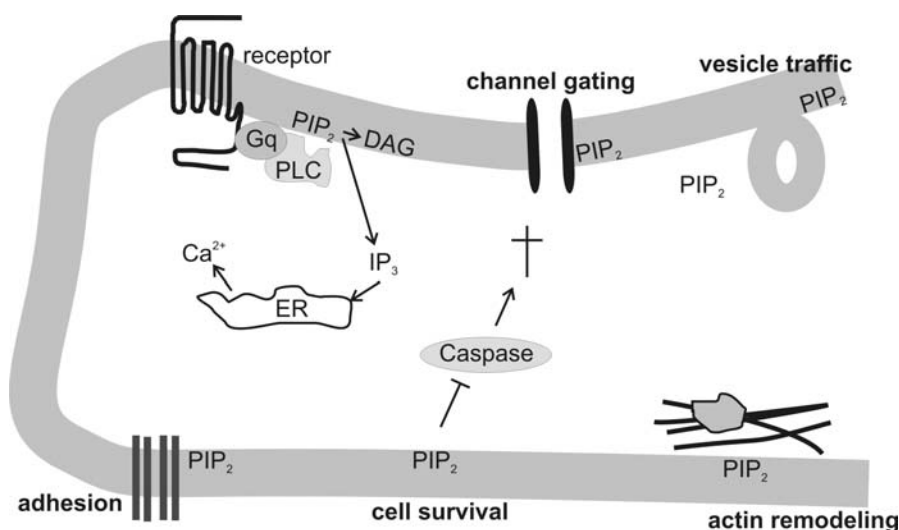


Figure 2, PIP₂ is important in many cellular processes. PIP₂ is the precursor for DAG and IP₃, and it is involved in the gating of ion channels and transporters, cell survival, vesicle trafficking and actin cytoskeleton remodeling.

A major process in PIP₂ turnover is mediated by phospholipase C (PLC). PLC hydrolyzes PIP₂ into two second messengers: DAG, the activator of protein kinase C, and IP₃, which releases Ca²⁺ from intracellular stores. A number of signaling pathways can activate PLC. For example, PLCβ is activated via the heterotrimeric G-protein Gq by G-protein Coupled Receptors (GPCRs), and PLCγ by growth factor receptors that possess intrinsic tyrosine kinase activity (e.g. Meisenhelder et al., 1989) as well as by clustered and activated integrins (e.g. Kanner et al., 1993; Wrenn et al., 1996).

In summary, the levels of PIP₂ are regulated by a variety of signaling pathways constituting a balance between synthesis and breakdown.

PIP₂ as a messenger

As mentioned in the previous section, PIP₂ is an important precursor for the second messengers IP₃, DAG and PIP₃. Apart from being a precursor for second messengers, a growing number of reports have documented a direct signaling role for PIP₂ itself as well. The best-characterized roles will be summarized in this section (see also Figure 2).

Ion channels and transporters are regulated by PIP₂

It is thought that PIP₂ regulates several ion channels and transporters (Hilgemann et al., 2001). On the one hand, PIP₂ is reported to activate plasmalemmal calcium pumps and many types of channels (e.g. potassium, sodium, calcium and some transient receptor potential channels (M5, M7, M8) (Takano and Kuratomi, 2003; Yue et al., 2002). On the other hand, PIP₂ is also reported to inhibit certain channels (e.g. cyclic nucleotide-gated, some transient receptor potential-like (L and V1), and calcium release channels; Womack et al., 2000; Estacion et al., 2001; Chuang et al., 2001). In most cases the underlying mechanism for channel regulation by PIP₂ has not yet been elucidated.

The role of PIP₂ in cell survival

Recent reports suggest that PIP₂ acts as a signaling molecule in cell survival. Prolonged PIP₂ depletion, for example by expression of a constitutively active mutant of the Gq alpha subunit (Althoefer et al., 1997; Adams et al., 1998; Howes et al., 2003), or by expression of a PIP₂ phosphatase such as inositol polyphosphate 5-phosphatase IV (Kisseleva et al., 2002), induces apoptosis. Apoptosis is accompanied by activation of caspases and, interestingly, caspases 3, 8 and 9 are inhibited by PIP₂ (Mejillano et al., 2001; Azuma et al., 2000). This

suggests that PIP₂ depletion may lead to activation of caspases and, as a consequence, to apoptosis. This process may be self-amplifying since caspases can cleave and inactivate the human PIP5KI, thereby inducing further PIP₂ decrease and apoptosis (Mejillano et al., 2001). However, thus far PIP₂ depletion during stress-stimulated apoptosis was never reported. In chapter 2 we show, for the first time, that apoptosis-inducing stress stimuli cause PIP₂ depletion. Moreover, expression of PIP5KI is sufficient to block apoptosis. Thus, in combination with previous studies, our data strongly suggest that PIP₂ depletion is a mediator of apoptosis, and thus that regulation of PIP₂ levels is important for cell survival.

PIP₂ is involved in vesicle trafficking

PIP₂ is thought to be involved in vesicle trafficking (De Camilli et al., 1996). For example, PIP₂ plays a critical role in endo- and phagocytosis. Many adapter and accessory proteins involved in clathrin-mediated endocytosis are recruited to endocytic sites by binding to PIP₂ (AP-2, AP-180, epsin and dynamin; Gaidarov and Keen, 1999; Itoh et al., 2001; Ford et al., 2001; Cremona and De Camilli, 2001). Moreover, inactivation of PLC-mediated PIP₂ hydrolysis impairs the formation of the phagocytic cup, resulting in an inhibition of phagocytosis (Botelho et al., 2000).

PIP₂ is also thought to influence exocytosis. For example, ARF6-regulated membrane trafficking has been suggested to dependent on the generation and removal of PIP₂. Changing the PIP₂ level by expression of a PIP5KI alters exocytosis so that ARF6-containing vesicles cannot recycle back to the plasma membrane (Brown et al., 2001). Moreover, overexpression of this kinase accelerates the actin-based propelling of vesicles (referred as motile actin comets; Rozelle et al., 2000; Martin, 2001).

In conclusion, PIP₂ is involved in vesicle trafficking including endocytosis, phagocytosis, exocytosis and the actin-based propelling of vesicles.

PIP₂ levels influence actin cytoskeleton remodeling

The extensive literature on PIP₂ binding to actin-binding proteins (ABP) suggests that PIP₂ is a messenger during actin cytoskeleton remodeling. However, this is predominantly based on *in vitro* binding studies and lipid biochemistry. Only a limited number of *in vivo*

studies have implicated that changes in the PIP₂ level influence the actin cytoskeleton. For example, elevating PIP₂ levels by overexpression of the PIP5KI results in an increasing number of stress fibers (Yamamoto et al., 2001), and PIP₂ sequestration by membrane-permeable PIP₂-binding peptide disassembles actin filaments (Cunningham et al., 2001). However, these are changes in the PIP₂ level forced by unphysiological methods. In contrast, in the first part of chapter 3 we show, for the first time, that physiological PIP₂ changes influence cortical actin dynamics. The exact underlying mechanisms were not addressed in this study, but a large number of *in vitro* studies suggest that PIP₂ binds to and influences the activity of many actin-binding proteins.

During actin polymerization, globular actin monomers, also called G-actin, polymerize into filamentous (F-) actin. *In vitro* studies suggests that profilin and plectrin influence this process by lowering the free actin monomer concentration by sequestering G-actin in a PIP₂ dependent manner (e.g. Andra et al., 1998; Lassing and Lindberg, 1985; Lassing and Lindberg, 1988). The polymerization of actin occurs mainly at one end of the filament, referred to as the plus end (barbed end). Several PIP₂-dependent mechanisms control the number of barbed ends. For example, the number of barbed ends is increased by breaking (severing) of pre-existing actin filaments. Interestingly, *in vitro* studies showed that the severing activity of proteins such as gelsolin and cofilin is blocked when these proteins bind PIP₂ (Janmey and Stossel, 1989; Chan et al., 2000). This suggests that PIP₂ hydrolysis renders gelsolin and cofilin active. The activity of this proteins leads to increased G-actin concentrations and numbers of free barbed ends, which results in higher numbers of fast-growing actin filaments. An alternative way of raising the number of barbed ends is by adding branches to existing F-actin. This process is mediated by the Arp2/3 complex, which binds to F-actin and nucleates polymerization of new actin filaments. The activity of the Arp2/3 complex appears PIP₂ dependent since PIP₂ synergizes with GTP-bound Cdc-42 *in vitro* to promote the conformational change of N-WASP, which on its turn activates the Arp2/3 complex (Rohatgi et al., 2000; Higgs and Pollard, 2000; Prehoda et al., 2000). The extension of F-actin at new barbed ends, formed by either severing or nucleation, can be prevented when these ends are capped by capping proteins. *In vitro* data suggest that PIP₂

influences this process as well, since it inhibits the activity of the capping proteins CapG (Sun et al., 1997), and protein $\beta 2$ (DiNubile and Huang, 1997).

Apart from regulating actin cytoskeleton remodeling directly, PIP_2 also regulates the interaction between the actin cytoskeleton and integral membrane proteins (e.g. ICAM-ezrin; Heiska et al., 1998) or the extracellular matrix. The latter is mediated by focal adhesions, complexes comprising scaffolding and signaling proteins, such as β -integrins, vinculin, and talin. The conformation of the important focal adhesion component vinculin changes upon PIP_2 binding (Gilmore and Burridge, 1996). Vinculin is in a closed conformation, in which an intramolecular interaction between the head and the tail of the protein masks the binding sites for other focal adhesion proteins such as talin and α -actinin. Upon PIP_2 binding, vinculin changes conformation so that the binding sites for talin and α -actinin become available. Interestingly, talin targets a PIP5KI (γ isoform) to focal adhesions (Ling et al., 2002; Subczynski and Kusumi, 2003). This suggests a (feed-forward) regulatory loop in which PIP5KI elevates PIP_2 levels at focal adhesions, resulting in a vinculin conformational change and the recruitment of talin and the PIP5KI to these focal adhesions. However, PIP_2 enrichments at focal adhesions have not been demonstrated directly. Nevertheless, also increased levels of global PIP_2 enhance the stability of focal adhesions, since the binding of talin to β -integrin is strengthened by PIP_2 (Martel et al., 2001). Thus altogether, the literature suggests that the basis of focal adhesion assembly is regulated by PIP_2 (Martel et al., 2001; Janmey, 1994; Yin and Janmey, 2003).

In summary, the reported interactions of PIP_2 with a host of proteins and its influence on their functions suggest that this lipid is an important messenger in many cellular processes, ranging from cell survival to vesicle trafficking and remodeling of the actin cytoskeleton.

PH domains

As can be concluded from the previous section, the localization and/or activity of many proteins are influenced by binding to PIP_2 in the membrane. These proteins often contain conserved domains with high affinity for PIP_2 .

The pleckstrin homology (PH) domain was the first phosphoinositide-binding domain to be discovered (Bottomley et al., 1998). This domain of 100 to 120 amino acids was identified in pleckstrin, a protein kinase C substrate highly expressed in platelets. Initially, it was thought that PH domains were involved in protein-protein interaction but later it became clear that they mediate phosphoinositide binding. Since then, more than 250 other PH domains have been identified. The affinity and specificity of phospholipid binding varies widely between the different PH domains. Approximately 10% of all PH domains fall into the category of high-affinity binding domains (Lemmon and Ferguson, 2000). For example, $\text{PLC}\delta 1$ contains a PH domain that specifically recognizes PIP_2 with high affinity (Cifuentes et al., 1993). However, most PH domains fall into the category of low-affinity binders. Thus far, the physiological importance of the weak phosphoinositide binding has not been totally clear. Nevertheless, these domains may still be important for membrane targeting of proteins. Two possible mechanisms come in mind. First, increased membrane binding could result from conversion of the PH domain into a high-affinity domain. A second possibility implies the cooperation of multiple low-affinity domains.

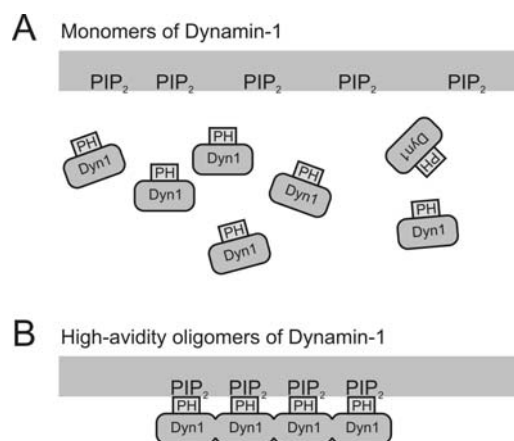


Figure 3, Oligomerization of Dynamin-1 results in a high avidity binding to the membrane. (A) The monomer Dynamin-1 protein will not bind to the plasma membrane since its PH domain has only very low affinity for PIP_2 (K_d in the millimolar range). (B) Dynamin-1 is a tetramer, and the low-affinity PH domains will co-operate resulting in a high avidity membrane binding (by simple addition of binding energies, the effective K_d for the tetramer is in the picomolar range).

Box 1, Fluorescence resonance energy transfer:

FRET, the radiationless transfer of energy from a donor to an acceptor fluorophore occurs when the two fluorophores are in close proximity (< 10 nm) and the emission spectrum of the donor fluorophore overlaps with the excitation spectrum of the acceptor fluorophore (Figure box 1 A and B). Under these conditions, FRET appears as quenching of the donor and as a gain in acceptor fluorescence (Figure box 1 B). The efficiency of this process (E) is defined as the amount of transfer events divided by the amount of photons absorbed by the donor (Figure box 1 C). E is dependent on the distance between the fluorophores (r) and on the Förster radius (R_0). R_0 is the characteristic distance where E is half-maximal for freely rotating fluorophores (Figure box 1 D). The R_0 for the fluorophores used in this thesis are ~ 4 -5 nm. However, in situations where the fluorophores cannot freely rotate, there will not be a random orientation of their dipoles and the R_0 will be different.

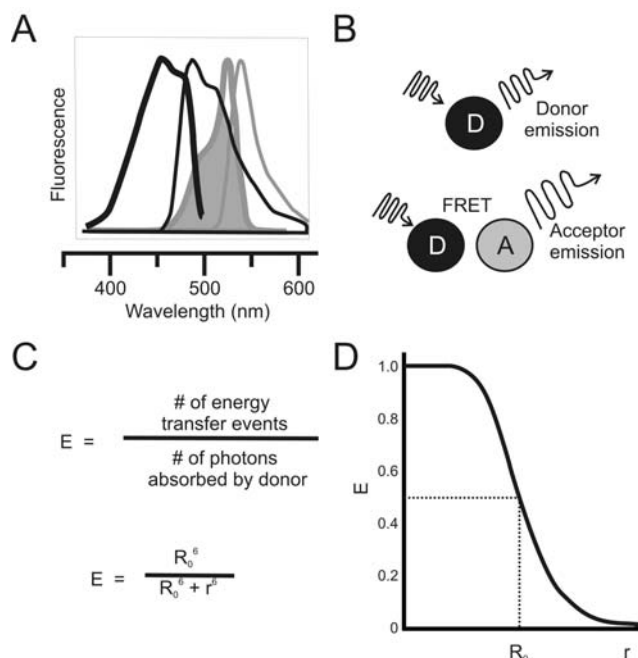


Figure box 1, Fluorescence resonance energy transfer. (A.) The excitation spectra (thick lines) and the emission spectra (thin lines) of donor- (here CFP, black spectra) and acceptor- (here YFP, gray spectra) molecules. The gray area represents the overlap of donor emission and acceptor excitation. (B) An excited donor can relax by emitting a photon, or by transferring energy (FRET) to an acceptor, which can then relax by emitting a photon. (C) The efficiency of FRET (E) is expressed as the amount of energy transfer events divided by the amount of absorbed photons by the donor. E is dependent on the distance between the donor and acceptor molecules (r), and on the Förster radius (R_0). (D) E decreases with the sixth power of distance (r). The R_0 is the distance (r) at half-maximal FRET. R_0 depends on the orientation of the dipoles, on the overlap of the donor emission and acceptor absorption spectra and on the refractive index.

Different approaches exist for measuring FRET. The techniques that are used in this thesis are summarized in table 1. Most of these methods are based on measurement of the intensities of acceptor and donor. The easiest approach, the **ratio of acceptor/donor fluorescence**, is based on the increased acceptor and decreased donor fluorescence upon FRET. This method is easy and it provides high temporal and spatial resolution, but it is not quantitative. **Acceptor photobleaching** is based on the gain in donor fluorescence upon selective photo-destruction of the acceptor. This approach is quantitative, but it is a destructive technique so it is not suited for time-lapse imaging. In **sensitized emission** measurements, the acceptor fluorescence upon donor excitation is calculated quantitatively and can be followed over time. However, this technique is labor-intensive since it requires many corrections (chapter 5). Finally, **fluorescence lifetime imaging (FLIM)** is based on fluorescence decay kinetics. Upon FRET, the fluorescence decay of the donor becomes faster. This approach is quantitative, but it requires dedicated and expensive equipment.

Table 1, different approaches used in this thesis for measuring FRET.

Approach	Principal	Measurements in time	Quantitative
Donor acceptor ratio	gain acceptor, loss donor fluorescence	+	-
Fluorescence lifetime imaging	Decrease Fluorescence lifetime donor	+	+
Sensitized emission	Acceptor fluorescence upon donor excitation	+	+
Acceptor photo bleaching	Gain donor fluorescence after acceptor bleaching	-	+

Data that point to the first possibility stem from studies on the interaction between PLC γ 1 and the plasma membrane channel TRPC3. Upon this interaction, the partial PH domain present in PLC γ 1, together with a complementary partial PH domain in TRPC3, form a full PH domain with high and specific affinity for PIP₂ (van Rossum et al., 2005).

A second possibility is that several low-affinity domains cooperate to increase the avidity for the membrane. For example, by a simple addition of binding energies of three low affinity PH domains ($K_d > \text{mM}$), the effective K_d for this trimer would be in the picomolar range (Lemmon and Ferguson, 2000). The cooperation between membrane-association domains can occur within one protein, or by oligomerization of PH-containing proteins. For example, the plasma membrane binding of Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is mediated by the combination of a covalently attached fatty acid and the electrostatic interactions between a positive stretch and negatively charged lipids (e.g. PIP₂). Deletion of either the fatty acid or the positive stretch results in cytosolic localization (McLaughlin and Aderem, 1995).

As illustrated in Figure 3, oligomerization can be a mechanism to increase the membrane avidity as in the case of dynamin-1 (Klein et al., 1998). While the low affinity of the monomeric dynamin-1 (in the millimolar range) is not sufficient to efficiently bind membranes, the high-avidity tetramer binds

PIP₂-containing vesicles with an effective K_d in the picomolar range (Muhlberg et al., 1997; Klein et al., 1998).

The temporal and spatial regulation of PIP₂ level

The suggestion that PIP₂ can function as a messenger in several cellular processes is predominantly based on the large number of proteins that can interact with PIP₂. If PIP₂ is a specific messenger in such a variety of parallel pathways then one would expect that PIP₂ levels can vary spatially and/or temporally and that these PIP₂ changes influence cellular processes. Here I review the literature on temporal and spatial PIP₂ variations.

Temporal variations in PIP₂ levels

Initially, temporal changes in the PIP₂ level were detected biochemically from populations of [³H]inositol-labeled cells. Most reports showed small and slow PIP₂ decreases upon agonist-induced PLC activation, although agonist- and cell type-dependent variations exist. For example, some studies have shown fast and almost complete PIP₂ hydrolysis (Wijelath et al., 1988; Divecha et al., 1991; Stephens et al., 1993). Since these biochemical analyses have limited time resolution (~1 min) and they rely on cell population measurements (with cells having neither synchronized nor identical responses), it

Box 2, monitoring PIP₂ levels by FRET

Detection of the translocation of GFP-PH from the membrane into the cytosol can be used to follow PIP₂ hydrolysis in living cells (Stauffer et al., 1998; Varnai and Balla, 1998). This process is studied with high temporal resolution by following FRET between color variants of GFP-PH (see box 1). Color variants of GFP-PH (FP-PH) which are used for FRET measurements in this thesis are summarized in table 2. In cells with high PIP₂ levels, the FP-PH proteins are bound to the membrane (Figure box 2 A), where donor and acceptor are in close proximity resulting in high FRET. Upon hydrolysis of PIP₂, the FP-PH proteins translocate to the cytosol. The distance between the donor and acceptor increases and therefore FRET decreases. As a result, the donor emission goes up, while the acceptor emission declines (Figure box 2 B). This method enables detection in flat or motile cells, can be used in single cells as well as in population studies and has high temporal resolution. Several donor-acceptor pairs are well suited for FRET detection, as described in table 1.

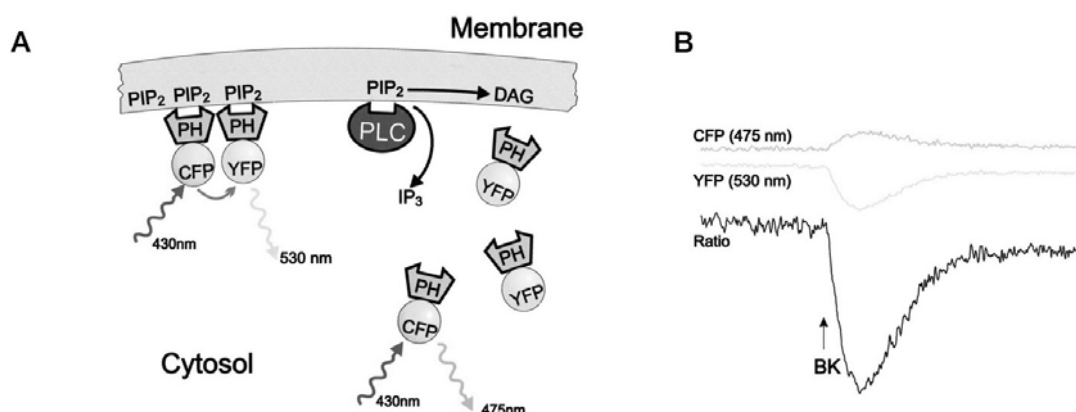


Figure box 2, PIP₂ levels measured by FRET. (A) A schematic representation of FRET between CFP-PH and YFP-PH when these proteins are bound to the membrane. Upon GPCR-mediated PLC activation, these proteins will translocate to the cytosol and FRET will diminish. (B) Donor and acceptor emission signals plus their ratio recorded from a single cell which was stimulated with bradykinin. Figure taken from van der Wal et al, 2001.

Tabel 2, color variants of GFP-PH used in this thesis

Donor	Acceptor	Sensor for
CFP-PH (PLCδ1)	YFP-PH (PLCδ1)	PIP ₂
GFP-PH (PLCδ1)	RFP-PH (PLCδ1)	PIP ₂
GFP-PHPH (PLCδ1)	RFP-PHPH (PLCδ1)	PIP ₂
GFP-PH (GRP1)	RFP-PH (GRP1)	PIP ₃

is difficult to obtain reliable data on PIP₂ kinetics.

Recently, an approach to study PIP₂ kinetics in detail was independently developed in the laboratories of T. Meyer and T. Balla. They visualized PIP₂ levels in single, living cells using a chimera of GFP and the PH domain of PLC δ 1 (GFP-PH) (Stauffer et al., 1998; Varnai and Balla, 1998). GFP-PH binds specifically to PIP₂ in the plasma membrane. Upon PIP₂ hydrolysis, GFP-PH can no longer bind the plasma membrane with a subsequent change in the ratio of membrane and cytosolic fluorescence. Importantly, the affinity of this binding (~ 1.7 μ M; Lemmon et al., 1995) is high enough to localize a substantial part of GFP-PH to the membrane, but not so high that changes in PIP₂ levels would go undetected. Moreover, individual GFP-PH molecules undergo rapid cycles of membrane association and dissociation, as was determined with fast FRAP experiments in our lab (van der Wal et al., 2001). This latter property guarantees that GFP-PH localization rapidly follows the PIP₂ content of the plasma membrane and that all PIP₂ molecules are available for metabolizing steps, such as hydrolysis by PLC. The GFP-PH translocation is usually studied by confocal imaging, but for several reasons we prefer its detection by recording Fluorescence Resonance Energy Transfer (FRET) between color variants of GFP-PH (see box I and II). Studies using the above-mentioned techniques revealed the kinetics of the GPCR induced PIP₂ hydrolysis. In general, decreases of PIP₂ levels after GPCR stimulation recorded with GFP-PH are rapid (a few seconds to 1 minute). The amplitude of hydrolysis and the resynthesis kinetics of plasma membrane PIP₂ vary extensively among several GPCRs (van der Wal et al., 2001).

Although GFP-PH is now generally used to image PIP₂ in living cells, there are two important caveats in the use of this probe. First, as with all life-cell probes, GFP-PH still sequesters (buffers) a part of the PIP₂ pool and theoretically these GFP-PH-bound PIP₂ molecules are not readily available for other interactors (e.g. actin binding proteins). However, GFP-PH rapidly shuttles between membrane and cytosol and therefore, in practice, GFP-PH proteins only temporarily jail PIP₂ molecules. This explains why PLC, when activated, can still hydrolyze the PIP₂ pool nearly completely (except in cases of extreme GFP-PH overexpression; e.g. Raucher et al., 2000).

The second caveat is the specificity of GFP-PH for PIP₂ (Irvine, 2004). Initially, the PH domain of PLC δ 1 was reported to bind only PIP₂ with high affinity as shown by *in vitro* binding assays (Lemmon et al., 1995) and by X-ray crystallography (Ferguson et al., 1995). This affinity is higher than those of the other PIP₂-binding PH domains, such as those of PLC- γ , spectrin, dynamin and pleckstrin. Moreover, a GFP-PH construct with mutations in the lipid-binding pocket of the PH domain (PH*) showed no specific PIP₂ binding (Stauffer et al., 1998) and does not localize to the membrane. These data strongly suggest that GFP-PH binds specifically and with high affinity to PIP₂. However, the product of PIP₂ hydrolysis by PLC, IP₃, has been reported to compete efficiently with PIP₂ for the PH domain of PLC δ 1 (Cifuentes et al., 1994; Lemmon et al., 1995). This suggests that increases in cytosolic IP₃ may also translocate GFP-PH from the membrane. For example, Hirose and co-workers (Hirose et al., 1999) showed that injection of IP₃ causes GFP-PH to translocate from the membrane into the cytosol. Furthermore, they showed that expression of an IP₃ 5-phosphatase (which presumably clamps IP₃ levels down) completely blocked the agonist-induced translocation of GFP-PH. In contrast, work in our laboratory showed via a variety of independent approaches that physiological IP₃ levels cannot translocate GFP-PH from the plasma membrane (van der Wal et al., 2001). Moreover, we recently found that in Rat-1 cells overexpressing the PIP5KI, the GFP-PH probe does not translocate upon agonist-induced PLC-activation (unpublished results), whereas IP₃ rises to at least normal levels, as assayed by a novel (unpublished) FRET probe for IP₃ (T. Balla).

Discrepancies such as in the above-mentioned issue may be due to the different experimental systems (e.g. cell type, *in vitro* versus *in vivo*, used PIP₂ probe). It is known that hydrophobic interactions contribute to the membrane localization of certain PH domains (Lemmon and Ferguson, 2000), and the C-terminal half of the PLC δ 1 PH domain may contain additional features that are required for membrane localization (Varnai and Balla, 1998). Further, dimerization of GFP proteins may contribute to the increased plasma membrane binding of GFP-PH. These factors may influence the avidity of GFP-PH for PIP₂ (see section on PH domains), thereby reducing the relative contribution of IP₃ to GFP-PH translocation during PIP₂ hydrolysis. In summary, although in

some experimental systems IP₃ production may contribute to GFP-PH translocation, in the vast majority of experiments it is predominately caused by PIP₂ hydrolysis.

Domain-delimited PIP₂ hypothesis

As mentioned in the previous section, PIP₂ levels vary in time, but it has also been generally hypothesized that PIP₂ levels vary spatially. In this model, spatially separated pools of PIP₂ molecules specifically regulate separate pathways (e.g., Hinchliffe et al., 1998; Martin, 2001; Simonsen et al., 2001; Janmey and Lindberg, 2004). In this thesis, this model will be referred to the "domain- (or raft-) delimited PIP₂ hypothesis". Several domains with different chemical and/or physical properties have been described in literature. In the next sections three types of such membrane domains will be discussed in more detail.

Confined membrane zones

Single-particle tracking experiments suggested that diffusion of lipids and proteins is restricted to confined zones (300-600 nm) in the plasma membrane (e.g. Sako and Kusumi, 1995; Kusumi et al., 1993). To explain these observations, Fujiwara and co-workers postulated the "membrane-skeleton-fence" model (Fujiwara et al., 2002), in which they propose that the cortical actin cytoskeleton anchored to various transmembrane proteins sterically hinders the free diffusion of lipids and proteins. Occasionally, thermally excited undulations of the plasma membrane may cause lipids and proteins to hop to an adjacent confined zone (Brown, 2003). In this model, long-range diffusion of lipids and proteins can only be explained by successive movements ("hops") to neighboring compartments (Lommerse et al., 2004). It is not very likely that these barriers restrict diffusion of PIP₂ molecules, since long-range diffusion of PIP₂, as measured by Fluorescence Loss In Photobleaching (FLIP), is as fast as one would expect for free diffusion (unpublished results). Thus, although the diffusion of some proteins and lipids might be restricted to confined membrane zones, these zones are not likely to play an essential role in the local function of PIP₂.

Micrometer-sized patches

The light-microscopical distribution of PIP₂ along the plasma membrane has been studied in formaldehyde-fixed cells (e.g. Tran et al., 1999; Aikawa and Martin, 2003). For

example, using PIP₂ specific antibodies, Tran and co-workers showed inhomogeneous PIP₂ labeling in rat hepatocytes (Tran et al., 1999). Similar findings were obtained in formaldehyde-fixed PC12 cells (Aikawa and Martin, 2003). In contrast, these authors noted that in fixed cells the fluorescence of GFP-PH was distributed homogeneously. This inconsistency may be due to the fixation of cells by formaldehyde and by the treatment of cells with detergents for antibody entrance. Formaldehyde binds proteins without any known direct interaction with membrane lipids, whereas lipids are only well fixed by a mixture of formaldehyde, glutaraldehyde and acrolein (Mobius et al., 2002). Thus, non-fixed PIP₂ molecules may be redistributed upon detergent treatment in formaldehyde-fixed cells (see chapter 4). In contrast, the PH-probe is expected to be rapidly fixed in these cells and it will retain its original position at the membrane. Interestingly, this is in line with the interpretation from Laux and co-workers (Laux et al., 2000) who found that the antibody staining pattern of PIP₂ differed with various fixation methods (Laux et al., 2000). In line with the findings of Mobius and co-workers, Laux and co-workers found that fixation with cold methanol resulted in smaller PIP₂ clusters than fixation with formaldehyde, while PIP₂ exhibited nearly homogeneous surface labeling when fixation was carried out with glutaraldehyde (Laux et al., 2000). So, the difficulty to fix lipids and to study PIP₂ with antibodies has led to controversial literature and stresses the importance of studying PIP₂ in live cells.

Several groups have imaged GFP-PH to study the distribution of PIP₂ along the membrane in living cells. A few considerations have to be taken into account. First, one should realize that GFP-PH only reports the free (unbound) pool of PIP₂. PIP₂ molecules sequestered by proteins, such as MARCKS, may represent a second pool, which might be clustered by these interacting proteins. Obviously, lipid-biochemical approaches would detect this second pool. However, in this case proteins cause PIP₂ clustering rather than the other way around, and since these PIP₂ molecules are already occupied, they cannot function as messengers that locally bind or influence proteins. Therefore, this pool of PIP₂ plays a minor role in the domain-delimited PIP₂ hypothesis, although release of these sequestered

Box 3, imaging membrane probes

The resolution of a light microscope is limited by the Point Spread function (PSF), which is the gaussian-shaped intensity profile that is detected when a true point is imaged (Figure box 3 A). Using a standard confocal microscope and an optimal pinhole size, a lateral resolution of 250 nm and an axial resolution of about 1000 nm can be obtained. As studies of the topography of the plasma membrane have shown (van Rheenen et al., 2002A; van Rheenen and Jalink, 2002B), the plasma membrane contains many sub-resolution folds and ruffles (Figure box 3 B). When membranes are fluorescently labeled, these folds and ruffles are observed as fluorescent structures, which cannot be discriminated from real local enrichments (Figure box 3 C). Currently, there are several approaches available to discriminate between lateral enrichments and membrane folds and ruffles, based on co-staining with fluid-phase membrane markers and on FRET imaging.

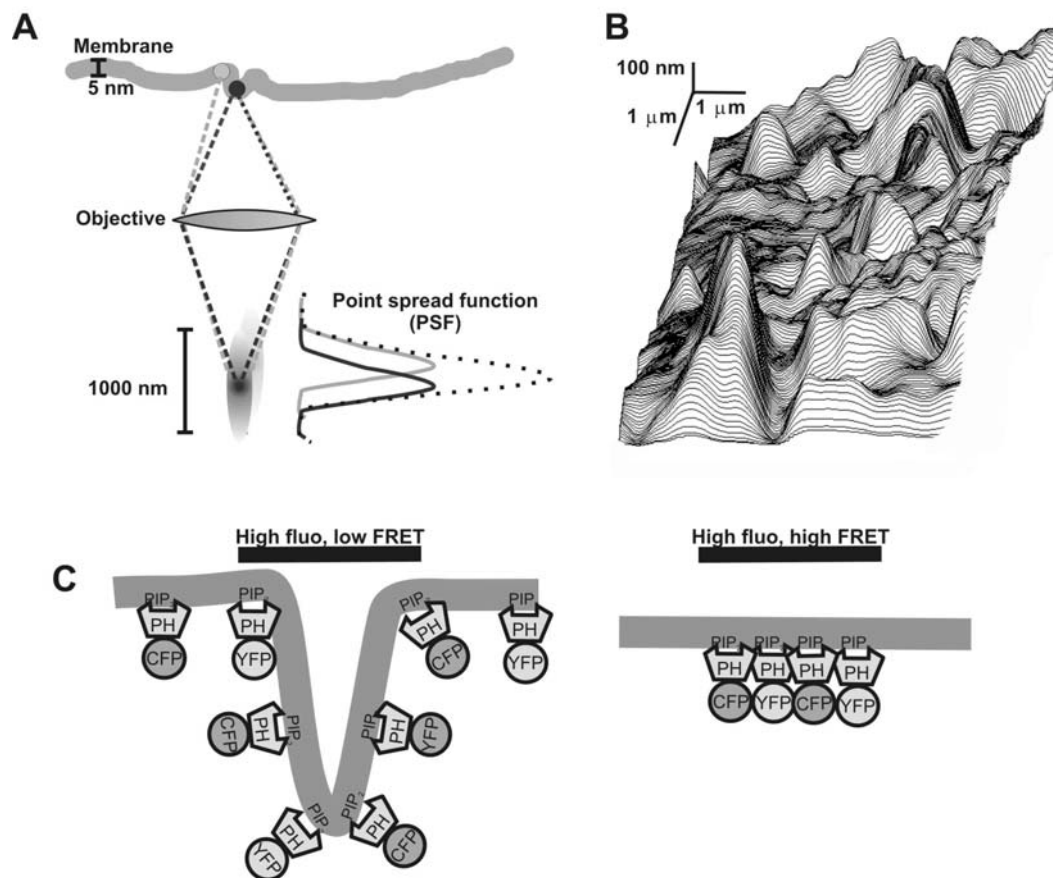


Figure box 3, imaging membranes. (A) When two close point sources in the membrane are imaged with a microscope, the resulting PSFs overlap and will be interpreted as a single PSF derived from a single, bright point source. (B) Apparent flat membranes contain numerous submicroscopic folds, as shown by a novel imaging approach to visualize nm-sized structures (see van Rheenen and Jalink., 2002A and 2002B). (C) Model for PIP₂ labeling in the membrane with CFP-PH and YFP-PH. Structures with increased fluorescence can be a result of PIP₂ clusters (right) or can be a result of a sub-resolution membrane ruffle (left). However, when FRET between CFP-PH and YFP-PH is imaged, only the structures with clustered PIP₂ will yield high FRET efficiencies.

PIP₂ molecules may have an important role in the regulation of PIP₂ levels.

The limitations of a microscope to resolve small structures (laterally ~250 nm; axially, ~1000 nm) complicates the imaging of the PIP₂ distribution using the GFP-PH probe. Sub-resolution folds and ruffles in the plasma membrane appear as structures enriched in GFP-PH and these cannot be discriminated from those caused by PIP₂ enrichments (see Box 3). In the literature, this problem is not always appreciated, and thus structures enriched in GFP-PH were interpreted as PIP₂ domains. In chapter 3, we show that these micrometer-sized domains enriched in GFP-PH are caused by sub-resolution folds and ruffles in the plasma membrane (Box 3 and chapter 3). Despite our detailed study, most studies still lack proper controls for membrane folding and therefore have limited significance (e.g. Golub and Caroni, 2005; Kisseleva et al., 2005).

Several PIP₂ probes based on the PH domain of PLC δ 1 (containing single or tandem PH domains) are currently available, having different membrane (PIP₂) association and dissociation constants. To test whether these constants influence the PIP₂ probe distribution along the membrane, the membrane/PIP₂ binding of these PIP₂ probes was simulated in a computer model (Jalink, unpublished results). This model reveals that the shape of a cell significantly influences the membrane binding of probes containing a single PH domain. For example, in flat cells a larger fraction of GFP-PH is bound to the membrane compared to round cells. This also holds true within a single cell; GFP-PH localizes more to the membrane in slender structures, such as neurites and lamellipodia, than in a cell soma. Thus, the fraction of membrane-bound GFP-PH does not necessarily represent the amount of PIP₂, and this complicates the use of these probes for studying the distribution of PIP₂ along the plasma membrane. For this purpose, the probes containing tandem PH domains should be used. These probes have complete membrane localization (see section on PH domains) independent of cell shape, and therefore cell shape does not influence the lateral distribution of the probe. However, these probes cannot be used to study the turnover of PIP₂, since the membrane binding of these probes is immune to small PIP₂ fluctuations (chapter 4).

With these caveats, imaging PIP₂ probes based on the PH domain of PLC δ 1 revealed that PIP₂ is not enriched in micrometer-sized domains due to fast diffusion of this lipid (Chapter 3). At larger scale, and especially in structures where the diffusion is limited, PIP₂ gradients may be present.

Rafts and caveolae

Rafts are very small (< 250 nm) lipid domains in the plasma membrane and they are thought to function as scaffolds for signal transduction components. Compared to the bulk membrane, rafts are thought to be enriched in cholesterol, sphingolipids and saturated phospholipids. A subset of rafts (caveolae) is also enriched in the protein caveolin. This composition causes the lipids to be in a so-called liquid-ordered state that is thought to limit diffusion significantly.

Biochemically, rafts are characterized as resistant to solubilization in detergents such as Triton X-100 at 4 °C (Chamberlain, 2004) and by their dependence on cholesterol: extraction of cholesterol using methyl- β -cyclodextrin (CD) disrupts the rafts and redistributes the signaling complexes (reviewed by Simons and Ikonen, 1997; Simons and Toomre, 2000). Data obtained by these biochemical assays suggested that PIP₂ is enriched in rafts (Koreh and Monaco, 1986; Hope and Pike, 1996; Pike and Miller, 1998; Pike and Casey, 1996; Hur et al., 2004; Waugh et al., 1998). However, several recent studies showed that artifacts may arise in these assays. For example, detergents such as Triton X-100 may induce non pre-existing clusters (Munro, 2003; Pizzo et al., 2002; Edidin, 2003; Heerklotz, 2002; Mayor et al., 1994; Kenworthy and Edidin, 1998). Furthermore, cholesterol extraction has several additional, raft-independent effects (see chapter 5).

Recently, new insights into the field of rafts have been obtained by several biophysical approaches, including Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Resonance Energy Transfer (FRET)

FRAP was used to study diffusion rates of raft and non-raft markers in the plasma membrane. In model membrane systems, the diffusion rate of lipids in rafts is 2-3 folds lower than in the surrounding membrane (Dietrich et al., 2001; Almeida et al., 1993). However, in live

cells the diffusion of raft markers is similar to non-raft markers, so long-range diffusion of raft markers is dominated by factors other than their association with rafts (Kenworthy et al., 2004 and unpublished results). As expected, similar FRAP results were obtained for fluorescently labeled PIP₂ (chapter 3), high affinity GFP-PH probes and fluid phase lipid markers (chapter 3 and unpublished results). Thus, using FRAP we did not find evidence for PIP₂ enrichment in rafts.

FRET analysis is increasingly used to study rafts. Kenworthy and Edidin analyzed FRET to measure the proximity between glycosylphosphatidylinositol (GPI)-anchored proteins (Kenworthy and Edidin, 1998). GPI-anchored proteins are generally thought to be targeted to and concentrated in rafts. Unexpectedly, based on their FRET analyses, Kenworthy and Edidin concluded that GPI-anchored proteins are randomly distributed (Kenworthy and Edidin, 1998; Kenworthy et al., 2000). However, subsequent FRET studies investigating GPI-anchored proteins and other raft markers yielded contradictory findings and conclusions. For example, Varma and Mayor concluded that GPI-anchored proteins are organized in 70-nm sized rafts (Varma and Mayor, 1998), while Glebov and Nichols found that these proteins are not clustered in resting and activated T-cells (Glebov and Nichols, 2004). Recently, FRET in combination with theoretical modeling suggested that rafts are very small (a few nm) and contain only a few GPI-anchored proteins (Sharma et al., 2004).

To test whether PIP₂ is enriched in rafts, we analyzed FRET between green and red PIP₂ probes (chapter 4). Although several groups used FRET to study rafts, the sensitivity of this assay for different cluster situations was never established. In order to examine this, we used a computer model to simulate, for several cluster conditions, the relationship between density of PIP₂ probes and FRET. From these sensitivity analyses, we concluded that FRET measurements should reveal clustering under a variety of conditions. In our study, we used several independent biophysical approaches and the results showed that PIP₂ is not enriched in rafts (chapter 4). Although the biochemical assays suggest that PIP₂ is enriched in small domains such as rafts, the current knowledge does not unequivocally support this idea.

It is widely suggested that PIP₂ functions as a local second messenger. In this thesis, we addressed this issue. Evidence is provided that PIP₂ can indeed function as a second messenger, since PIP₂ levels were found to vary significantly over time, affecting cell survival (chapter 2) as well as cortical actin motility (chapter 3). Contrary to other studies, however, our work shows that PIP₂ does not act as a very local second messenger, since its fast diffusion limits the establishment and maintenance of small PIP₂ enrichments. In contrast, at larger scale and especially at places where the diffusion is limited, sustained PIP₂ gradients seem to be a realistic possibility.

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Chapter 2

PtdIns(4,5)P₂ depletion is essential for stress-induced apoptosis
submitted

PtdIns(4,5)P₂ depletion is essential for stress-induced apoptosis

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Stress-induced apoptosis is believed to contribute to a number of human diseases however, little is known about the signalling events that regulate this process. Reports demonstrating that the phosphoinositide, phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P₂) inhibits caspase activation and promotes pro-survival signals has led to the hypothesis that PtdIns(4,5)P₂ suppresses apoptosis. However, to date it has not been demonstrated that apoptotic stimuli negatively regulate PtdIns(4,5)P₂ levels. Here, we show that both hydrogen peroxide (H₂O₂) and UV-irradiation, two apoptotic stress stimuli, cause irreversible depletion of PtdIns(4,5)P₂ in a caspase-independent manner. Depletion of PtdIns(4,5)P₂ is essential, as ectopic expression of phosphatidylinositol 4-phosphate 5-kinase (PIP 5-K), a lipid kinase which synthesises PtdIns(4,5)P₂ *in vivo*, rescues cells from H₂O₂-induced apoptosis. We find that H₂O₂ inhibits PIP 5-K activity and simultaneously induces the translocation of PIP 5-K away from its substrate at the plasma membrane. These observations identify PtdIns(4,5)P₂ as an essential regulator of stress-induced apoptosis and establishes PIP 5-K as a target for control by stress stimuli.

Introduction

Stress-induced apoptosis (or programmed cell death) is linked to the aetiology of a number of pathological conditions. For example, both the onset of Alzheimer's disease and cardiac infarction have been linked to apoptosis induced by reactive oxygen species, such as H₂O₂ (Andersen, 2004; Zhao, 2004).

A key survival pathway in cells inhibiting multiple components of the apoptotic machinery is the PKB/Akt signalling cascade. PKB is activated by PtdIns(3,4)P₂

and PtdIns(3,4,5)P₃ which clearly implicates these 3-phosphorylated phosphoinositides in the promotion of cell survival (Alessi, 1996; Stokoe, 1997; Scheid 2003). In contrast, the role of the phosphoinositide, PtdIns(4,5)P₂, in promoting cell survival remains less well defined. PtdIns(4,5)P₂ regulates a wide range of cellular processes, including ion channel activation, actin cytoskeleton remodelling and vesicular trafficking (Yin, 2003; Itoh, 2004; Hilgemann, 2004). PtdIns(4,5)P₂ is also hydrolysed by

phospholipase C (PLC) generating the second messengers, diacylglycerol (DAG) which promotes cellular proliferation (via protein kinase C activity) and IP₃ which regulates intracellular calcium release. PtdIns(4,5)P₂ is synthesised *in vivo* by the phosphorylation of PtdIns4P on the 5 position of the inositol head group by the PIP 5-K family of lipid kinases. The PIP 5-K family is encoded by four genes, which generate a number of different protein products that appear to have non-redundant functions (Loijens, J.C, 1996; Ishihara, 1998). We have shown that in response to H₂O₂, PIP 5-K synthesises PtdIns(3,4,5)P₃ via its PtdIns(3,4)P₂ 5-kinase activity (Halstead, 2001). This data implicates PIP 5-K in cell survival, as activation of the anti-apoptotic protein kinase, PKB/Akt is PtdIns(3,4,5)P₃-dependent. A more direct role for PIP 5-K and PtdIns(4,5)P₂ in the regulation of cell survival has been suggested as PtdIns(4,5)P₂ can inhibit the activation of caspases 8, 9 and 3 (Mejillano, 2001; Azuma, 2000). A possible link between caspase activation and PtdIns(4,5)P₂ levels comes from the observation that human PIP 5-K α (the homologue of murine PIP 5-K β) is cleaved and inactivated by caspases (Mejillano, 2001). In this model of apoptotic control, activated caspases cleave and inhibit PIP 5-K thereby blocking PtdIns(4,5)P₂ synthesis and triggering further caspase activation.

In cardiomyocytes, a role for PtdIns(4,5)P₂ depletion has been suggested in promoting apoptosis. Expression of a constitutively active version of the alpha subunit of Gq causes PtdIns(4,5)P₂ depletion and apoptosis, that is associated with a concomitant decrease in PKB/Akt signalling (Althoefer, 1997; Adams, 1998; Howes, 2003). In line with the concept that depleting PtdIns(4,5)P₂ levels is an apoptotic event, expression of the PtdIns(4,5)P₂ phosphatase, inositol

polyphosphate 5-phosphatase IV, causes apoptosis and inhibits PKB activation in HEK293 cell lines (Kisseleva, 2002). Together, these data strongly suggest that PtdIns(4,5)P₂ is an important survival factor in cells and implicate PIP 5-K as a target for inactivation during apoptosis. If this hypothesis were true, it would be predicted that PtdIns(4,5)P₂ levels would decrease during apoptosis. However, apoptotic stimuli have never been shown to negatively regulate PtdIns(4,5)P₂ levels. Using a combination of biochemical and confocal imaging techniques, this paper addresses the issue of PtdIns(4,5)P₂ in apoptotic regulation. We demonstrate that apoptotic stress stimuli, such as H₂O₂ and UV-irradiation, cause PtdIns(4,5)P₂ depletion and that this event is essential for apoptosis and occurs independently of caspase activation. Moreover, we show that during stress-induced apoptosis, PIP 5-K activity is inhibited by a novel dual mechanism which serves to attenuate PtdIns(4,5)P₂ synthesis at the plasma membrane.

Results

Sustained PtdIns(4,5)P₂ depletion leads to apoptosis.

As a first step towards clarifying the role of PtdIns(4,5)P₂ in apoptosis, we used a constitutively active version of the alpha subunit of Gq (G α q*) ectopically expressed in HeLa cells to deplete PtdIns(4,5)P₂ levels. The GFP fusion of the PH domain of PLC δ 1 (GFP-PH^{PLC}) was used as an *in vivo* PtdIns(4,5)P₂ probe (Varnai, 1998; Stauffer, 1998). GFP-PH^{PLC} is concentrated at the plasma membrane in control cells (not expressing G α q*)(Figure 1A).

In HeLa cells expressing G α q*, GFP-PH^{PLC} was spread diffusely throughout the cell (with an apparent membrane-cytosolic ratio of 1:1) (Figure 1B), indicating that plasma membrane PtdIns(4,5)P₂ levels were

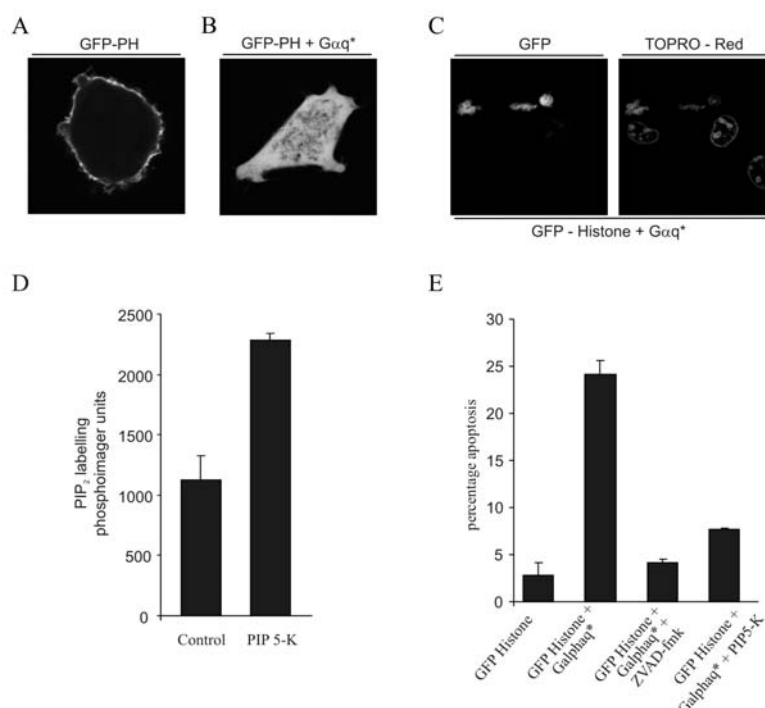


Figure 1, Expression of $G\alpha q^*$ in HeLa cells depletes PtdIns(4,5)P₂. HeLa cells were grown overnight on cover slips and then transfected with GFP-PH^{PLC} alone or together with $G\alpha q^*$. GFP-PH^{PLC} localisation was then examined by confocal microscopy 24 hours after transfection. Shown are representative images of the PtdIns(4,5)P₂ probe transfected alone (A) or co-transfected with $G\alpha q^*$ (B). (C) HeLa cells were grown overnight on glass cover slips and then transfected with GFP-Histone or with both GFP-Histone and $G\alpha q^*$. 36 hrs after transfection cells were fixed, permeabilised and stained with TOPRO-Red to visualise nuclei. Cells were then analysed via confocal imaging. Shown are representative images of GFP and TOPRO-Red channels from cells expressing both GFP-Histone and $G\alpha q^*$. (D) PtdIns(4,5)P₂ labelling is increased in cells expressing murine PIP 5-K α . HeLa cells were transfected with either GFP Histone (designated as control on graph) or GFP-PIP 5-K (designated as PIP 5-K on graph). Cells were then [³²P]-orthophosphate labelled, after which phospholipids were extracted and analysed by TLC. Plotted are the resulting PtdIns(4,5)P₂ levels. Error bars display standard deviation of triplicate samples. This graph is typical of three independent experiments. (E) PIP 5-K α attenuates $G\alpha q^*$ -induced apoptosis. HeLa cells were transfected with the constructs shown. For these experiments a myc-tagged PIP 5-K construct was used. Cells were left for 36 hrs, collected, fixed and stained with Hoechst 33258. Cells were then examined for apoptosis (as defined by nuclear fragmentation). Only transfected cells that were GFP positive were scored. Typically, a total of 500 cells were counted for each sample. Mean values of duplicate samples are plotted. Shown is a graph representative of three independent experiments.

markedly depleted. In line with previous data from cardiomyocytes, expression of $G\alpha q^*$ caused apoptosis in HeLa cells in a caspase-dependent manner (Howes, 2003) (Figure 1C and Figure 1E). A causal link between $G\alpha q^*$ -mediated PtdIns(4,5)P₂ depletion and apoptosis has never been made and to test this point, we sought to inhibit cell death by rescuing PtdIns(4,5)P₂

levels. It has been previously reported by our group and others that the expression of PIP 5-K isoforms elevates PtdIns(4,5)P₂ levels by 1.5 – 2 fold in the majority of adherent cell lines (this figure is based upon 70 % of a cell population ectopically expressing PIP 5-K) and indeed this is also the case in HeLa cells (Figure 1D). Therefore we attempted to rescue

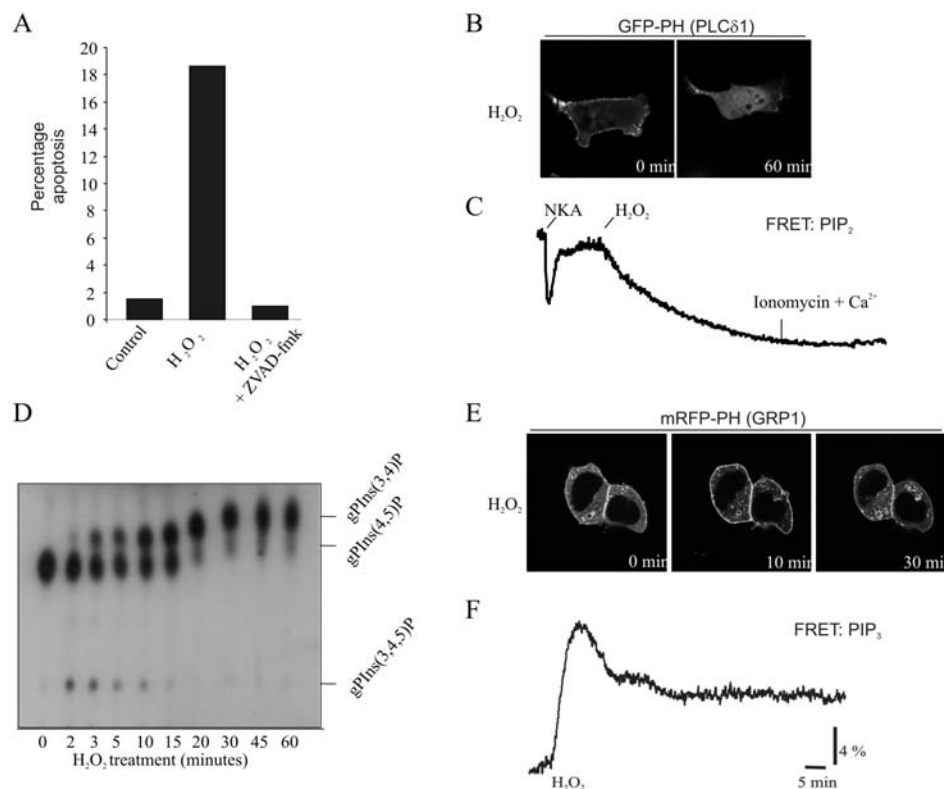


Figure 2, H₂O₂ causes apoptosis in a caspase-dependent manner. (A) HeLa cells were transfected with a GFP Histone. Cells were stimulated with 600 μ M H₂O₂ in the presence or absence of ZVAD-fmk as indicated for 24 hr and fixed. The nuclei of cells were then stained with Hoechst 33258 and examined using fluorescence microscopy to identify transfected cells that displayed fragmented apoptotic nuclei. Plotted graphically are the mean values of duplicate samples. The number of apoptotic cells displaying fragmented nuclei is plotted as a percentage of the total number of transfected cells. Typically, a total of 500 cells were counted for each sample. The data shown are representative of three different experiments. (B) H₂O₂ induces PtdIns(4,5)P₂ depletion in HeLa cells. Cells were grown overnight on glass coverslips and transfected with GFP-PH^{PLC}. Cells were stimulated with 600 μ M H₂O₂ and confocal images taken every minute for 60 min. Shown are representative images of cells prior to stimulation (0 min) with H₂O₂ and 60 min after stimulation with H₂O₂. (C) PtdIns(4,5)P₂ depletion by H₂O₂ can be visualised using FRET. Cells were transfected with YFP and CFP chimeras of the PH domain from PLCδ1. FRET changes were followed on a wide-field microscope by calculating the ratio of the CFP and YFP fluorescence (Van der Wal, 2001). Prior to stimulation the responsiveness of each cell was assessed by NKA addition. Cells were then treated with 600 μ M H₂O₂ and the fluorescence ratio was monitored. 45 minutes post stimulation ionomycin and Ca²⁺ were added. The points of NKA, H₂O₂, ionomycin and Ca²⁺ addition are indicated. Shown is a representative FRET trace of many independent experiments. (D) H₂O₂ induces changes in phosphoinositide metabolism. Cells were [³²P]-orthophosphate labelled for two hours. They were stimulated with H₂O₂ for the time indicated and the phospholipids extracted from the cells. Lipid samples were then deacylated and the resulting glycerophosphoinositides separated on a PEI-cellulose plate. The positions of gPIIns(4,5)P₂, gPIIns(3,4)P₂ and gPIIns(3,4,5)P₃ are indicated. (E) H₂O₂ induces transient synthesis of PtdIns(3,4,5)P₃. HeLa cells expressing mRFP-PH^{Grp1} were treated with 600 μ M H₂O₂ and confocal images taken every minute for 60 min. Shown are representative images of cells prior to stimulation with H₂O₂, 10 min and 30 min after stimulation with H₂O₂. (F) Transient PtdIns(3,4,5)P₃ production by H₂O₂ can be visualised using a FRET based assay. Cells expressing both GFP-PH^{Grp1} and mRFP-PH^{Grp1} were treated with 600 μ M H₂O₂ and FRET changes were followed on a wide-field microscope by calculating the ratio of the GFP and mRFP fluorescence.

PtdIns(4,5)P₂ levels in HeLa cells by co-expressing murine PIP 5-K α . In support of the idea that PtdIns(4,5)P₂ depletion is a critical step during G α q*-induced apoptosis, expression of PIP 5-K α potentially blocked cell death (Figure 1E).

Apoptotic stress stimuli cause sustained PtdIns(4,5)P₂ depletion.

Having demonstrated that PtdIns(4,5)P₂ depletion is essential for G α q*-mediated apoptosis, the study was broadened to test whether other apoptotic stimuli also negatively regulate PtdIns(4,5)P₂ levels.

Therefore, we began by assessing H₂O₂-induced apoptosis in HeLa cells. It is important to note that H₂O₂ influences cell behaviour in a concentration-dependent manner as higher concentrations cause necrosis while lower concentrations cause cell cycle arrest and apoptosis (Finkel, 2003). It was therefore critical to establish a concentration of H₂O₂ that induced apoptosis rather than necrotic cell death. In our cell system, 600 μ M H₂O₂ reproducibly caused apoptosis, which was potentially blocked by the caspase inhibitor, ZVAD-fmk (Figure 2A).

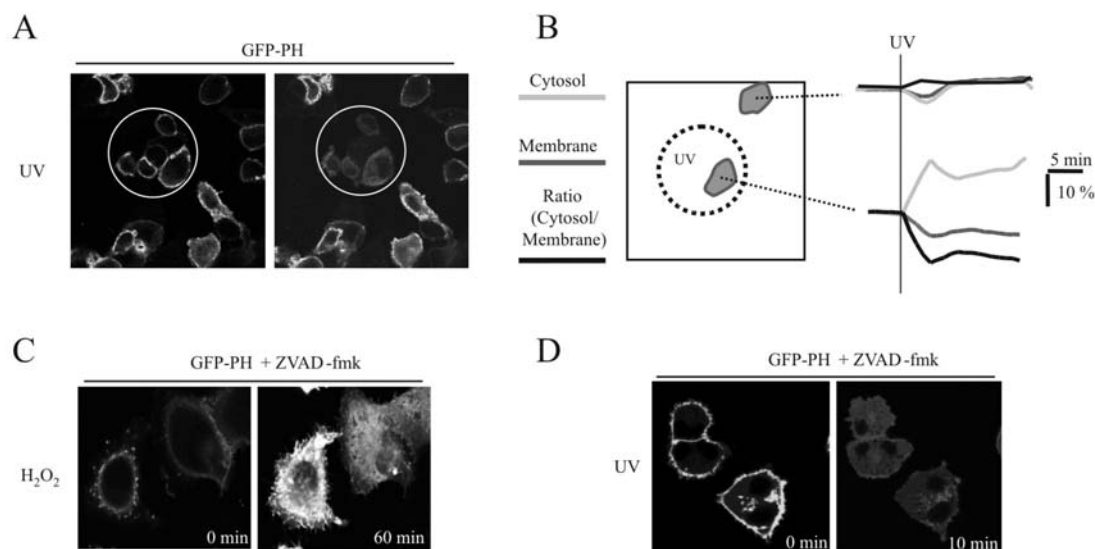


Figure 3, UV-irradiation induces PtdIns(4,5)P₂ depletion. (A) HeLa cells were grown overnight on glass coverslips. They were then transfected with GFP-PH^{PLC}. Using confocal imaging, living cells were recorded for 2-3 min and then stimulated with UV-irradiation for 30 seconds. By closing the field diaphragm only the centre cells (within the white circle) were exposed to UV-irradiation. Cells were then imaged for 10 min with images being recorded every 30 seconds. Shown are representative images of cells prior to stimulation and 10 mins after UV-irradiation. (B) The cartoon highlights two analysed cells for which the intensities of the fluorescence of the membrane (red line), and of the cytosol (blue line), and the membrane/cytosol ratio (black line) were plotted in time. The graphs for each cell is indicated via a dashed line. Note the drop in the membrane/cytosol ratio of the cell within the circle (indicated by the dotted line) after UV-irradiation and that the membrane/cytosol ratio of the cell outside the circle remains constant. Scale bar signal shows percent deviation from baseline ratio value. Time is also indicated, as is the point of UV-irradiation. (C) H₂O₂ induces PtdIns(4,5)P₂ depletion in a caspase-independent manner. HeLa cells were grown overnight on glass coverslips. They were then transfected with a GFP-PH^{PLC} construct. 16 hours post-transfection, cells were incubated with 50 μ M (final concentration) ZVAD-fmk for one hour. Cells were then treated with 600 μ M H₂O₂ and the cells were imaged for 30 minutes with images being taken every minute. (D) Same as in (C) however cells were stimulated with UV-irradiation for 30 seconds. Cells were then imaged for 10 mins with images being recorded every 30 seconds. Shown are representative images prior to treatment and after treatment with H₂O₂ or UV-irradiation (as indicated).

Cells were stimulated with 600 μM H_2O_2 and the localisation of GFP-PH^{PLC} was monitored using live confocal imaging techniques. H_2O_2 caused translocation of GFP-PH^{PLC} from the plasma membrane indicative of sustained PtdIns(4,5)P₂ depletion (Figure 2B). Even after prolonged stimulation with H_2O_2 , PtdIns(4,5)P₂ levels at the plasma membrane did not recover, as GFP-PH^{PLC} remained diffusely spread throughout the cell. To monitor PtdIns(4,5)P₂ depletion with increased temporal resolution, we used a FRET based assay (Van der Wal, 2001). During each experiment, cells were stimulated with neurokinin A to test the responsiveness of individual cells to agonist-induced PtdIns(4,5)P₂ changes. NKA mediated stimulation of PLC activity caused a transient decrease in PtdIns(4,5)P₂ which recovered to near basal levels within 2 minutes. Conversely, H_2O_2 treatment caused a sustained decrease in the FRET ratio, indicating prolonged and sustained PtdIns(4,5)P₂ depletion. Complete activation of PLC by the addition of ionomycin and extracellular Ca^{2+} did not elicit further decreases in the FRET ratio indicating that plasma membrane PtdIns(4,5)P₂ had already been depleted (Figure 2C).

To support our confocal studies, we sought to demonstrate PtdIns(4,5)P₂ depletion by an independent method. Therefore we labelled cells with [³²P]-orthophosphate and monitored phosphoinositide levels during a time course of H_2O_2 treatment. H_2O_2 markedly decreased PtdIns(4,5)P₂ labelling compared to untreated controls (Figure 2D). The labelling data was in good agreement with the FRET and confocal data, indicating that apoptotic concentrations of H_2O_2 negatively regulate PtdIns(4,5)P₂ levels. Moreover,

this was a rapid process as PtdIns(4,5)P₂ labelling was significantly depleted within twenty minutes of H_2O_2 treatment. As previously observed by our group and others, both PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ labelling were increased after H_2O_2 treatment (Van der Kaay, 1999; Halstead, 2001) (Figure 2D). The increase in PtdIns(3,4,5)P₃ labelling however was clearly transient. Intriguingly, the increase in PtdIns(3,4)P₂ labelling was sustained after H_2O_2 treatment suggesting that synthesis of this lipid occurs in the absence of observable PtdIns(3,4,5)P₃ levels. The transient production of PtdIns(3,4,5)P₃ inferred by our labelling studies was confirmed with live cell imaging in which PtdIns(3,4,5)P₃ levels were monitored after H_2O_2 treatment in real-time using a monomeric RFP fusion of the PH domain of Grp1 (mRFP-PH^{Grp1}) as an *in vivo* PtdIns(3,4,5)P₃ probe (Gray, 1999). This probe, which is predominantly cytosolic was transiently recruited to the plasma membrane after H_2O_2 treatment (Figure 2E). The transient synthesis of PtdIns(3,4,5)P₃ production after treatment with H_2O_2 was further confirmed by a FRET based assay in which mRFP-PH^{Grp1} and GFP-PH^{Grp1} were used as a FRET pair to follow PtdIns(3,4,5)P₃ production with improved temporal resolution (Figure 2F).

To examine whether depletion of PtdIns(4,5)P₂ occurred with other apoptotic stress stimuli, HeLa cells expressing GFP-PH^{PLC} were stimulated with UV-irradiation and monitored using confocal microscopy. UV-irradiation also triggered translocation of GFP-PH^{PLC} from the plasma membrane to the cytosol (Figure 3A and Figure 3B). The time-scale of the translocation of GFP-PH^{PLC} after UV treatment was also rapid occurring within 10 min. Together, these data demonstrate that two apoptotic stress stimuli trigger sustained decreases in

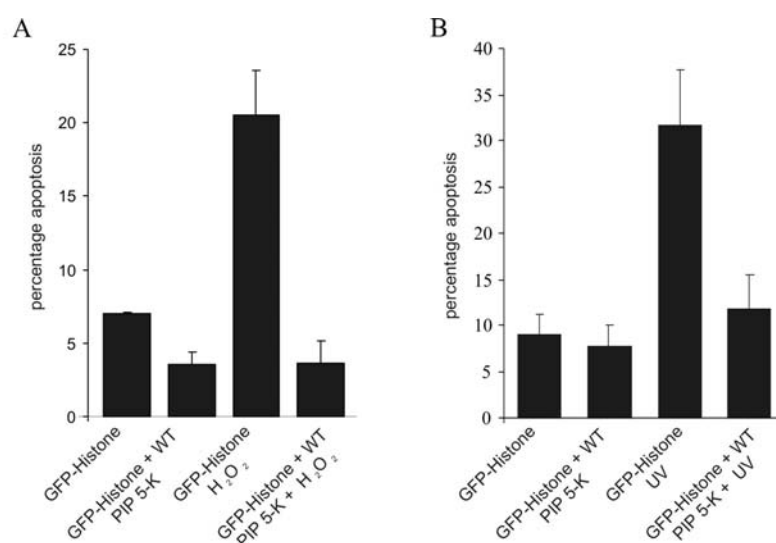


Figure 4, PIP 5-K protects against H₂O₂ induced apoptosis. (A) HeLa cells were transfected with various constructs (as indicated). Cells were stimulated with 600 μ M H₂O₂ for 24 hr and fixed. The nuclei of cells were then stained with Hoechst 33258 and examined using fluorescence microscopy to identify transfected cells that displayed fragmented apoptotic nuclei. Plotted graphically are the mean values of triplicate samples. The number of apoptotic cells displaying fragmented nuclei is plotted as a percentage of the total number of transfected cells. Typically, a total of 500 cells were counted for each sample. The data shown are representative of at least three independent experiments. Error bars display standard deviation of triplicate samples. (B) PIP 5-K protects against UV-irradiation induced apoptosis. Cells were irradiated with UV (18 mJ/cm²) and incubated for a further 24 hours prior to fixation and staining with Hoechst 33258. The data shown are representative of 3 independent experiments. Error bars display standard deviation of triplicate samples.

PtdIns(4,5)P₂ levels.

PtdIns(4,5)P₂ depletion is essential for stress induced apoptosis.

The *in vivo* synthesis of PtdIns(4,5)P₂ is dependent upon the PIP 5-K family of lipid kinases and it is possible that the observed decreases in PtdIns(4,5)P₂ resulted from inactivation of PIP 5-K activity. As apoptosis by H₂O₂ and UV-irradiation are both caspase dependent and one isoform of human PIP 5-K is cleaved and inactivated by caspases, we considered the possibility that PtdIns(4,5)P₂ depletion may occur downstream of caspase activation. While pre-incubation of HeLa cells with the general caspase inhibitor (ZVAD-fmk) attenuated H₂O₂ induced

apoptosis (Figure 2A), ZVAD-fmk, failed to block depletion of PtdIns(4,5)P₂ induced by either H₂O₂ (Figure 3C) or UV-irradiation (Figure 3D). These data demonstrate that apoptotic stress stimuli deplete PtdIns(4,5)P₂ levels in a caspase-independent manner.

Our data indicated that PtdIns(4,5)P₂ depletion was an essential step during Gαq*-dependent apoptosis. To determine if H₂O₂-dependent apoptosis was also reliant upon PtdIns(4,5)P₂ depletion, we sought to block cell death by expressing PIP 5-Kα. Importantly, we found that ectopic expression of PIP 5-Kα completely blocked apoptosis by H₂O₂ in HeLa cells (Fig. 4a). This block in H₂O₂-induced apoptosis was dependent upon the lipid products of PIP 5-Kα, as a ‘kinase dead’ version of PIP 5-K

(that retains 1% residual activity compared to the wild-type enzyme) offered negligible protection against H_2O_2 -induced apoptosis (data not shown). Furthermore, the rescue of cells from apoptosis was not limited to H_2O_2 treatment, as PIP 5-K α also rescued HeLa cells from UV-irradiation-induced apoptosis (Fig. 4b). As PIP 5-K α synthesises PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ *in vivo* (Halstead, 2001), we examined if the protective effect of PIP 5-K was linked to the activation of PKB. Cells were transfected with PIP 5-K α or a constitutively activated PI 3-kinase (CAAX PI 3-kinase) in the absence or presence of PKB and PKB activity was assessed. While the CAAX PI 3-kinase potently activated PKB, no such stimulation was observed with PIP 5-K α (Figure 5A). This result was confirmed by examining endogenous PKB phosphorylation at Serine⁴⁷³ and Threonine³⁰⁸. Insulin, EGF and CAAX PI 3-kinase were potent activators of PKB however PIP 5-K α failed to promote phosphorylation of either amino acid (Figure 5B). As H_2O_2 triggered PtdIns(4,5)P₂ depletion, it was possible that the levels of PtdIns(4,5)P₂ required for PtdIns(3,4,5)P₃ synthesis were maintained by PIP 5-K α expression. H_2O_2 transiently activated PKB, as Ser⁴⁷³ phosphorylation was lost after 60 minutes (Figure 5C). Importantly, ectopic expression of PIP 5-K α failed to prolong PKB Ser⁴⁷³ phosphorylation upon long-term H_2O_2 treatment (Figure 5D). Furthermore, it is unlikely that PIP 5-K α -mediated PtdIns(3,4,5)P₃ synthesis attenuated H_2O_2 -induced apoptosis, as the PI 3-kinase inhibitor LY294002 (Vlahos, 1994), which blocks the ability of PIP 5-K α to generate PtdIns(3,4,5)P₃, had no effect upon the PIP 5-K-dependent rescue of cells after H_2O_2 treatment (data not shown). Together, these data indicated that PIP 5-K-dependent rescue from apoptosis was dependent upon

PtdIns(4,5)P₂ and not PtdIns(3,4,5)P₃; and that PtdIns(4,5)P₂ depletion is an essential step during H_2O_2 and UV-irradiation-induced apoptosis.

Apoptotic stress stimuli attenuate PIP 5-K activity during apoptosis

As there was a close temporal correlation between the labelling decreases in both PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ after H_2O_2 treatment (production of both these lipids is dependent upon PIP 5-K activity), we considered the possibility that PIP 5-K activity was inhibited after prolonged oxidative stress. To test this, we assayed endogenous PIP 5-K activity from HeLa cells after *in vivo* treatment with 600 μ M H_2O_2 . Endogenous PIP 5-K activity was inhibited after 20 minutes incubation with H_2O_2 (Figure 6A). The inhibition was transient as PIP 5-K activity recovered with time, demonstrating that PIP 5-K inhibition by apoptotic concentrations of H_2O_2 was reversible. As H_2O_2 inactivated PIP 5-K in a transient manner, this mechanism was unlikely to fully explain the sustained depletion of PtdIns(4,5)P₂ levels we observed by confocal and labelling studies. Therefore, we examined other potential mechanisms for the down-regulation of PIP 5-K activity. It is known that PIP 5-K activity is abundant in membrane fractions and PIP 5-K localises to membranous structures and is enriched at the plasma membrane (Figure 6B). As the PIP 5-K substrate PtdIns4P is membrane localised and a number of reports have linked *in vivo* PIP 5-K activity to membrane localisation (Kunz, 2000; Kunz, 2002), we tested whether treatment with apoptotic concentrations of H_2O_2 altered PIP 5-K localisation. Indeed, within minutes H_2O_2 treatment, a GFP fusion of PIP5-K α (GFP-PIP 5-K α) translocated from the plasma membrane to the cytoplasm in a manner reminiscent of the H_2O_2 -induced trans-

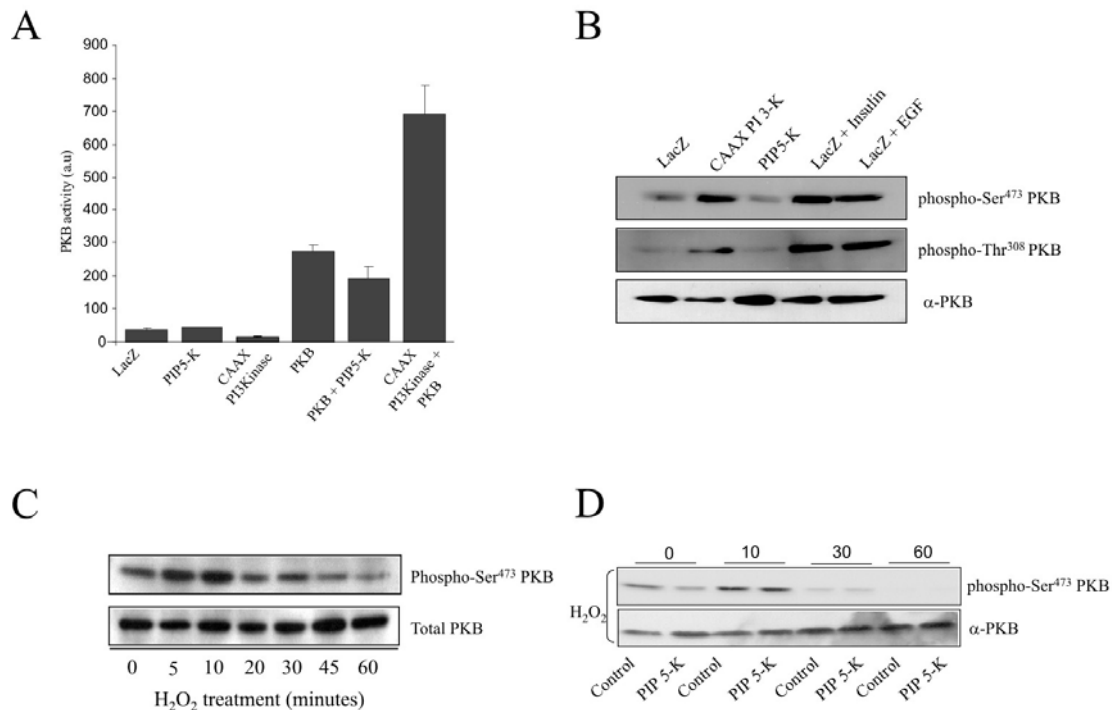


Figure 5, the PIP 5-K rescue from apoptosis is not mediated through PtdIns(3,4,5)P₃ production. (A) PIP 5-K expression has no effect upon PKB activity whereas CAAX PI 3-kinase potently activates PKB *in vivo*. Activity of ectopically expressed PKB was assayed after co-transfection with the various constructs shown. (B) PIP 5-K expression has no effect upon endogenous PKB activity. Activity of endogenous PKB was examined using phospho-specific antibodies raised against the phosphorylated forms of the PKB amino acids Threonine³⁰⁸ and Serine⁴⁷³. Phosphorylation of these residues is indicative of PKB activation. (C) Transient phosphorylation of PKB in cells after treatment with 600 μM H₂O₂. Endogenous PKB activation was examined using an antibody that specifically recognises PKB upon phosphorylation of residue Serine⁴⁷³. (D) PIP 5-K does not prolong activation of endogenous PKB after treatment with 600 μM H₂O₂.

location of GFP-PH^{PLC} (Figure 6B). The translocation of GFP-PIP 5-Kα was also observed after UV-irradiation (Figure 6C). In both cases, we were unable to detect enrichment of GFP PIP 5-Kα at the plasma membrane several hours after treatment. It has been shown previously that PIP 5-K mutants which localise to the cytosol do not efficiently elevate PtdIns(4,5)P₂ levels *in vivo* (Kunz, 2000; Kunz, 2002). The translocation of the enzyme from the plasma membrane could therefore constitute a primary mechanism by which

PtdIns(4,5)P₂ synthesis at the membrane could be attenuated. As GFP PIP 5-Kα translocation after H₂O₂ treatment correlated with a decrease in PtdIns(4,5)P₂, we tested whether sustained PtdIns(4,5)P₂ hydrolysis would alter GFP PIP 5-K localisation. Cells were co-transfected with GFP PIP 5-Kα and a mutated version of the neurokinin A (NKA) receptor, which is not de-sensitised after stimulation, leading to prolonged PLC activation and sustained PtdIns(4,5)P₂ hydrolysis (Alblas, 1995). To observe changes in both GFP-PIP 5-K localisation

and PtdIns(4,5)P₂ levels, we used a monomeric red fluorescent protein fusion of the PH domain of PLC δ 1 (mRFP-PH^{PLC}). After NKA stimulation, mRFP-PH^{PLC} translocated from the plasma membrane to the cytosol within 20 seconds (Figure 6D). GFP PIP 5-K α however remained localised at the plasma membrane indicating that depletion of PtdIns(4,5)P₂ alone, or activation of downstream targets

of the PLC pathway, was insufficient to cause translocation of the lipid kinase. If the translocation of PIP 5-K from the plasma membrane was important for the observed loss of PtdIns(4,5)P₂ levels it would be expected that the translocation of the lipid kinase would precede the translocation of the *in vivo* PtdIns(4,5)P₂ probe. In support of this idea, we observed translocation of GFP PIP 5-K α in HeLa cells prior to

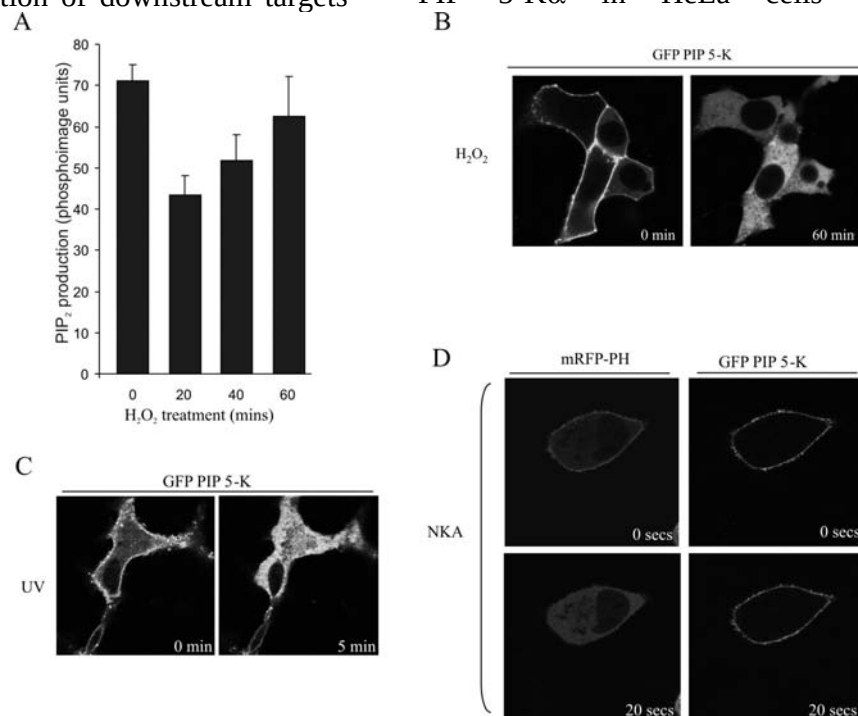


Figure 6, Endogenous PIP 5-K is catalytically inactivated *in-vivo* after treatment with H₂O₂. (A) HeLa cells were stimulated for the time indicated. Cells were lysed and endogenous PIP 5-kinase was immunoprecipitated using a polyclonal antibody. Immunoprecipitates were tested for associated PIP 5-kinase activity using a mixed micelle containing PtdIns(4)P as a substrate (see materials and methods). Samples were extracted and the lipids resolved on using TLC. Plotted graphically are the mean values of triplicate samples. Error bars represent the standard deviation of the mean of triplicate samples. (B) H₂O₂ induces delocalisation of PIP 5-K from the plasma membrane. Shown are confocal images of the mid-section of cells expressing GFP PIP 5-K α . Cells were grown on glass cover slips over night prior to transfection. 16 hours post-transfection, cells were stimulated with H₂O₂. Shown is a representative image of cells prior to stimulation and 60 min post stimulation with H₂O₂. (C) UV-irradiation causes delocalisation of PIP 5-K from the plasma membrane. Identical to Figure 6B however 16 hours post-transfection, cells were stimulated with UV-irradiation and images taken every 20 seconds. Shown is a representative image of cells prior to stimulation and 5 min after treatment with UV. (D) PtdIns(4,5)P₂ depletion does not cause delocalisation of PIP 5-K from the plasma membrane. Shown are the confocal images of the mid-section of HeLa cells transfected with GFP PIP 5-K, mRFP-PH and truncated NKA receptor. Cells were grown on glass cover slips over night prior to transfection. 16 hours post transfection cells were stimulated with NKA and images taken prior to stimulation and for 30 minutes after stimulation. Shown is an image of a representative cell prior to stimulation and 20 seconds after NKA addition. It should be noted that throughout the entire time course of the experiment, GFP PIP 5-K remained localised at the plasma membrane.

translocation of mRFP-PH^{PLC} in cells treated with H₂O₂ (data not shown). These data suggested that distinct signalling events are initiated after H₂O₂ treatment that attenuate PIP 5-K activity at the plasma membrane leading to transient inactivation of the enzyme and displacement of the enzyme away from the plasma membrane. Both these events would serve to deplete PtdIns(4,5)P₂ levels after H₂O₂ treatment, which we have demonstrated are essential for stress-induced apoptosis.

Discussion

We show that apoptotic stress stimuli trigger irreversible PtdIns(4,5)P₂ depletion. PtdIns(4,5)P₂ depletion occurs almost immediately after H₂O₂ or UV-irradiation treatment and is independent of caspase activation. Therefore we propose that H₂O₂ or UV-irradiation mediated PtdIns(4,5)P₂ depletion constitutes an early apoptotic signalling event. A previous report (Mejillano, 2001) has shown that activated caspase-3, cleaves and inactivates human PIP 5-K α (homologue of the murine PIP 5-K β). Primary structure analysis of the other PIP 5-K isoforms (murine PIP 5-K α , γ or H), failed to reveal homologous caspase cleavage sites indicating that these enzymes are unlikely to be directly regulated by caspase cleavage. However, our data and the data from Mejillano *et al.* indicate that apoptotic stimuli down-regulate PIP 5-K activity in both a caspase-dependent and -independent manner. It is possible that caspase-independent mechanisms are required during the early stages of apoptosis and that caspase-dependent mechanisms are employed later on during apoptosis (post-caspase activation) for the inactivation of the

murine β isoform (homologue of human PIP 5-K α). Furthermore, as isoforms of PIP 5-K appear to have non-redundant functions, caspase-dependent and -independent mechanisms may serve to differentially regulate specific pools of PtdIns(4,5)P₂ (nuclear versus cytoplasmic for example).

Expression of PIP 5-K α attenuates both H₂O₂ and UV-irradiation induced apoptosis and moderately inhibits TNF α /cycloheximide-induced apoptosis (JRH, unpublished data), the latter being in good agreement with a previous report (Mejillano, 2001). However, we were unable to observe decreases in PtdIns(4,5)P₂ levels after TNF α /cycloheximide treatment suggesting that certain apoptotic stimuli (stress stimuli) are more dependent on PtdIns(4,5)P₂ depletion than others (death receptor agonists such as TNF α). A role for PIP 5-K in death receptor mediated cell death has been previously suggested as RNAi mediated suppression of PIP 5-K γ sensitises HeLa cells to apoptosis induced by TRAIL (Aza-Blanc, 2003). The anti-apoptotic effect of PIP 5-K is dependent upon kinase activity as a kinase dead version of PIP 5-K offered negligible protection against H₂O₂ and UV-mediated cell death. As LY294002 can block PIP 5-K-induced PtdIns(3,4,5)P₃ formation but not its ability to attenuate apoptosis, it is unlikely that PtdIns(3,4,5)P₃ generation is the mechanism for the inhibition of stress induced-apoptosis. In agreement with this data, expression of PIP 5-K failed to augment or affect PKB activation although it completely rescued cells from H₂O₂-induced apoptosis. We suggest that PtdIns(4,5)P₂ depletion enables an essential event for apoptosis, such as the inhibition of a survival strategy or the activation of a pro-apoptotic signal. With respect to this issue, previous *in vitro* data suggested that PtdIns(4,5)P₂ can inhibit

caspase 8 and 9 activity'. However, so far we were unable to demonstrate that overexpression of PIP 5-K α , which attenuated H₂O₂-induced apoptosis, also blocked caspase activation (unpublished data). Modulation of the cytoskeleton using agents such as cytochalasins potently induces apoptosis (Yamazaki 2000; Rubtsova 1998). As PtdIns(4,5)P₂ is a key regulator of cytoskeletal dynamics, an enticing possibility is that PtdIns(4,5)P₂ depletion induces apoptosis through the modulation of the actin cytoskeleton.

In this paper, we present evidence to support the idea that inactivation of PIP 5-K causes sustained PtdIns(4,5)P₂ depletion during H₂O₂-induced apoptosis. Negative regulation of murine PIP 5-K α (homologue of human PIP 5-K β) occurs through both the inhibition of PIP 5-K catalytic activity and through its relocalisation away from its substrate at the plasma membrane. In our model, inactivation of PIP 5-K by H₂O₂ prevents re-synthesis of PtdIns(4,5)P₂ thereby depleting the cell of this lipid. Interestingly, both H₂O₂ and UV-irradiation can also activate PLC and PtdIns(4,5)P₂ hydrolysis (Scheiven, 1993). Thus, the coordinated activation of PLC and inactivation of PIP 5-K may be the underlying mechanism for stress-induced PtdIns(4,5)P₂ depletion. In *S.cerevisiae*, mutation of the phospholipase C gene, *plc-1* sensitises yeast to UV-irradiation (Andoh, 1998). Furthermore, up-regulation of PKC signalling in mammalian cells can attenuate UV-irradiation induced apoptosis (Matsumura, 2003). Both these data indicate that PLC activity and the resulting activation of PKC may contribute to apoptotic resistance. Thus, maintenance of PtdIns(4,5)P₂, by over-expression of PIP 5-K, may attenuate apoptosis through maintenance of the PLC signalling pathway.

PtdIns(4,5)P₂ is a key intracellular phosphoinositide required for the regulation of many different signalling pathways including the PLC and PtdIns(3,4,5)P₃/PKB pathway. Furthermore PtdIns(4,5)P₂ is essential for vesicle generation and actin dynamics. Dysfunction of these processes is unlikely to be compatible with life. PIP 5-K appears to be a central player in response to oxidative damage. For that reason it is tempting to speculate that PIP 5-K acts as an essential intracellular stress sensor, governing the switch between survival and apoptosis. For example at the point where accumulation of oxidative damage abrogates PIP 5-K activity, the decision is made to embark into a pathway of programmed cell death. The ensuing apoptotic signalling cascade may be triggered by the concomitant decreases in PtdIns(4,5)P₂ levels. It follows therefore that maintenance of PIP 5-K activity and hence PtdIns(4,5)P₂ levels will enable cells to escape apoptosis in response to cellular stress. Clinically, this raises the issue of PIP 5-K in diseases that are characterised by high levels of oxidative stress, such as certain human cancers (Szatrowski, 1991). It is tempting to speculate that these tumour cells may maintain higher than normal levels of PtdIns(4,5)P₂ or alternatively that they have developed a PIP 5-K that is insensitive to H₂O₂. Intriguingly, evidence for aberrant PIP 5-K activity already exists in tumours (Singhal, 1994A; 1994B). In conclusion, PtdIns(4,5)P₂ has an essential role in cellular survival and maintaining levels of this lipid is sufficient to circumvent programmed cell death. Accordingly, certain apoptotic stimuli signal the depletion of PtdIns(4,5)P₂ in the early stages of apoptosis by inactivating PIP 5-K.

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Materials and Methods

Materials, cell culture and Plasmids

Synthetic PtdIns(4)P dipalmitoyl esters were purchased from Echelon (Salt Lake City, UT). Phosphatidylserine, phosphatidic acid, Z-VAD-fmk and hydrogen peroxide were all purchased from Sigma (St. Louis, MO). Topro-Red was purchased from Molecular Probes. ZVAD-fmk was purchased from Sigma. GFP, YFP and CFP-PH^{PLCδ1} constructs are described elsewhere²⁴. GFP-PH^{Grp1} was a kind gift from Professor P. Downes and was subsequently cloned into a mRFP construct. GFP-Histone H2B and activated Gαq* was provided by Prof. W Moolenaar. GFP-PIP 5-Kα and myc-pcDNA PIP 5-Kα were PCR cloned as previously described in our laboratory from a murine brain cDNA library (Divecha, 2000). The CAAX PI 3-kinase construct was a gift from Dr Len Stephens. HeLa cells were routinely cultured in 10 % fetal bovine serum (Gibco) in dulbecco's modified Eagle's medium (DMEM) and transfected using Eugene transfection reagent (Boehringer-Mannheim).

Apoptosis Assays

During our study, two methods were employed to examine cellular apoptosis. The first method was based upon the nuclear morphology of cells visualised using fluorescence microscopy after staining with bisbenzimidazole (Hoescht 33258, Sigma). Cells were collected 24 hours post-apoptotic stimulation including those that had detached from the cell culture plate. Cells that were still attached were washed twice with PBS (washes were saved) and removed by trypsinization. All fractions including PBS wash steps were then pooled and washed a further two times with PBS. The cellular pellet was then fixed in 50 µl of a 3.7 % (v/v) formaldehyde/PBS solution. After 10 min at room temperature, the fixative was removed and the cells were resuspended in 15 µl of PBS containing 16 µg/ml bisbenzimidazole. A 10 µl aliquot was placed on a glass slide, and approximately 500 cells per slide were scored in duplicate or triplicate for the

incidence of apoptotic nuclear changes under a Zeiss Axiovert 135 fluorescence microscope. To be classified as apoptotic we scored the incidence of fragmented nuclei only and did not include the incidence of condensed or partially condensed nuclei as apoptotic. This generated highly reproducible data sets. The second method was also based on nuclear morphology and was used for imaging experiments. Briefly, cells were transfected with GFP Histone2B. After apoptotic stimulation cells were fixed and treated with the nuclear stain Topro-Red and imaged using confocal microscopy (Leica).

PKB assay

Activation of PKB was determined using a phospho-specific antibody that recognises Serine⁴⁷³ of PKB or Threonine³⁰⁸. The PKB activity assays were performed as described previously (Welch, 1998).

FRET and confocal imaging

For confocal imaging, a Leica DM-IRBE inverted microscope fitted with a TCS-SP scanhead (Leica, Mannheim, Germany) was used. Excitation of enhanced GFP was with the 488-nm argon ion laserline, and emission was collected at 500-565 nm. For translocation studies, series of confocal images were taken at 2-10-s intervals and stored on disc. PtdIns(4,5)P₂ FRET studies were performed as described previously (ref) and PtdIns(3,4,5)P₃ FRET studies adapted from these methods. Visualisation and analysis was performed off-line using LCS and Qwin software (Leica). Qwin software was used to automate the assignment of regions of interest.

[³²P]-orthophosphate labelling and PIP 5-K *in vitro* assays

Transfected or non-transfected cells were orthophosphate labelled as previously described¹³. After extraction, phospholipids were treated with monomethylamine and the deacylated products analysed using PEI-cellulose plates (Whatman) as described previously (Halstead, 2001). For the *in vitro* PIP 5-K assays, cells were transfected as described and left for 16 h, after which they were lysed [1 ml lysis buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1% NP-40)], scraped, and nuclei and cellular debris removed by centrifugation (14 000 r.p.m., 4°C Eppendorf centrifuge). Immunoprecipitation of the endogenous PIP 5-K from Hek293T cells was carried out using a polyclonal antibody raised against a peptide present all three isoforms of PIP 5-K. The immunoprecipitates were collected using protein G-Sepharose, and washed three times with IP wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA 0.1% Tween-20), then twice with 1x PIP 5-K buffer. For the PIP 5-K assays, lipid vesicles were

prepared using 1 nmol of PtdIns4P together with 1 nmol PtdSer and 3 nmol of PtdOH. Reactions were carried out at 30°C for 20 min in 1 x PIPkinase buffer (50 mM Tris pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 70 mM KCl) containing cold ATP (20 µM) and 1 µCi of [³²P]ATP in a final volume of 100 µl. Reactions were quenched with 0.5 ml of chloroform

:methanol [1:1 (v:v)] and the phases were split by the addition of 125 µl of 2.4 M HCl. The lower phases were removed to a new tube, dried and separated by TLC either using an alkaline solvent [chloroform:methanol:ammonia (28%):water 45:35:2:8]. Incorporation into PtdIns(4,5)P₂ was quantitated using a phosphoimager.

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Chapter 3

Agonist-induced PIP₂ hydrolysis inhibits cortical actin dynamics: Regulation
at a global but not at a micrometer scale

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Agonist-induced PIP₂ Hydrolysis Inhibits Cortical Actin Dynamics: Regulation at a Global but not at a Micrometer Scale^{[D][V]}

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Phosphatidylinositol 4, 5-bisphosphate (PIP₂) at the inner leaflet of the plasma membrane has been proposed to locally regulate the actin cytoskeleton. Indeed, recent studies that use GFP-tagged pleckstrin homology domains (GFP-PH) as fluorescent PIP₂ sensors suggest that this lipid is enriched in membrane microdomains. Here we report that this concept needs revision. Using three distinct fluorescent GFP-tagged pleckstrin homology domains, we show that highly mobile GFP-PH patches colocalize perfectly with various lipophilic membrane dyes and, hence, represent increased lipid content rather than PIP₂-enriched microdomains. We show that bright patches are caused by submicroscopical folds and ruffles in the membrane that can be directly visualized at ~15 nm axial resolution with a novel numerically enhanced imaging method. F-actin motility is inhibited significantly by agonist-induced PIP₂ breakdown, and it resumes as soon as PIP₂ levels are back to normal. Thus, our data support a role for PIP₂ in the regulation of cortical actin, but they challenge a model in which spatial differences in PIP₂ regulation of the cytoskeleton exist at a micrometer scale.

INTRODUCTION

Phosphatidylinositolbisphosphate (PIP₂) controls many cell processes, ranging from channel gating (Hilgemann and Ball, 1996) to vesicle trafficking (De Camilli *et al.*, 1996) and the actin cytoskeleton. In this latter case, PIP₂ is thought to directly interact with actin binding proteins, influencing the equilibrium of monomeric (G-) to filamentous (F-) actin at the level of G-actin availability (e.g., plectin; Andra *et al.*, 1998, profilin; Lassing and Lindberg, 1985, 1988) and at the level of polymerization into actin fibers. Examples are members of the F-actin-severing and -capping protein family including gelsolin and CapG (Sun *et al.*, 1997), and protein β 2 (DiNubile and Huang, 1997). Cytoskeletal interactions with integral membrane

proteins can also be regulated by PIP₂, as is the case with ezrin binding to ICAM and CD44 (Heiska *et al.*, 1998). Many of these actin binding proteins interact with PIP₂ via pleckstrin homology domains (Kavran *et al.*, 1998; Wang *et al.*, 1999).

The majority of data on the interaction of PIP₂ with the cytoskeleton stem from in vitro binding studies and lipid biochemistry. If membrane lipids are to exert a local signaling function, local differences in content or availability must exist. However, although the phosphoinositide levels at the membrane appear to be tightly regulated by a multitude of specific reactions (Sakisaka *et al.*, 1997; for review see Takenawa *et al.*, 1999), little is known about their distribution along the membrane. Biochemical studies have reported the existence of separate PIP₂ pools within cells (Koreh and Monaco, 1986; Varnai and Balla, 1998), and PIP₂ was reportedly enriched in detergent-insoluble membrane fractions (rafts, caveolae; Hope and Pike, 1996; Laux *et al.*, 2000), although a very recent electron microscopy study challenges these results (Watt *et al.*, 2002). In addition to these biochemical reports, a limited number of in vivo studies have implicated PIP₂ levels in the regulation of the cytoskeleton. For example, increasing [PIP₂] by overexpression of the type I PIP-kinase α lead to an increase of stress fibers in CV1 cells (Yamamoto *et al.*, 2001), and sequestering of this lipid using a membrane-permeable PIP₂-binding peptide blocked motility (Cunningham *et al.*, 2001).

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Abbreviations used: CFP, cyan fluorescent protein; FRAP, fluorescence recovery after photobleaching; FR $\ddot{E}$ T, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; PH, pleckstrin homology; PLC, phospholipase C; PIP₂, phosphatidylinositol(4,5) bisphosphate; PSF, pointspread function; YFP, yellow fluorescent protein.

A detailed understanding of a role for PIP₂ as a local signal requires techniques to study cellular processes with spatial and temporal resolution in single living cells. Recently, an approach to image PIP₂ in living cells was pioneered in the labs of Meyer and Balla (Stauffer *et al.*, 1998; Varnai and Balla, 1998). Both groups used the PIP₂-binding pleckstrin homology domain of PLC δ 1, fused to GFP (GFP-PH), to study PIP₂ in the membrane *in vivo*. In resting cells, GFP-PH is bound to the membrane, and it translocates to the cytosol after agonist-induced PIP₂ hydrolysis. Translocation can be detected at very high sample rate using fluorescence resonance energy transfer (van der Wal *et al.*, 2001). Imaging of GFP-PH bound to the membrane also provides spatial resolution. Interestingly, initial studies reported a rather uniform distribution of GFP-PH along the membrane in unstimulated cells (Stauffer *et al.*, 1998), whereas a recent study (Tall *et al.*, 2000) reported that GFP-PH displays distinct bright patches on a uniformly labeled background. These bright patches are highly dynamic and rich in F-actin content, and they often colocalize with membrane ruffles and microvilli-like structures. Development of new membrane ruffles was reported to start with local concentration of GFP-PH. Based on these observations, and in line with the hypothesized function of PIP₂ as a local regulatory factor in cytoskeletal dynamics, GFP-PH patches were interpreted as local PIP₂ enrichments (Tall *et al.*, 2000).

This study was undertaken to determine to what extent local differences in membrane PIP₂ content influence the cytoskeleton *in vivo*. To this goal, we investigated 1) whether changes in PIP₂ level induced by physiological PLC-activating agonists modulate cortical actin dynamics and 2) whether physiologically relevant differences in membrane [PIP₂] (which, be it relative enrichments or depletions, will here be collectively termed PIP₂ patches) exist locally at the plasma membrane of cultured cells.

MATERIALS AND METHODS

Materials

1-Oleoyl-LPA, endothelin, bradykinin, histamine, phenylarsine oxide, and quercetin were from Sigma Chemical Co. (St. Louis, MO), and ionomycin and neurokinin A were from Calbiochem-Novabiochem Corp. (La Jolla, CA). H₂O₂ was from Riedel-deHaën (Germany), and thrombin receptor activating peptide (SFLLRN) was synthesized in house. Membrane dyes were from Molecular Probes Inc. (Eugene, OR); listed are the dye name, with the structures in parentheses: diphenyl DiI (1,1'-diiododecyl-5,5'-diphenyl-3,3,3',3'-tetramethylindocarbocyanine chloride); SP-DiIC18(3) (1,1'-diiododecyl-6,6'-di(4-sulfophenyl)-3,3,3',3'-tetramethylindocarbocyanine); SP-DiOC18(3) (3,3'-diiododecyl-5,5'-di(4-sulfophenyl) oxacarbocyanine, sodium salt); DiIC18(5) oil (1,1'-diiododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate); TRITC DHPE (N-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt); bis-BODIPY FL C₁₁-PC (1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl)-sn-glycero-3-phosphocholine); BODIPY 564/570 C₁₁ (4,4-difluoro-5-styryl-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid); NBD-PA (7-nitrobenz-2-oxa-1,3-diazol-PA).

Constructs and Transfection

The pcDNA3 expression vectors with inserts eGFP-PH(PLC δ 1), eCFP-PH(PLC δ 1), eYFP-PH(PLC δ 1), and eGFP-CAAX were described elsewhere (van der Wal *et al.*, 2001). pEYFP-Mem was from Clontech (Palo Alto, CA), pcDNA3 with insert GFP-Actin (from the

N-terminus: GFP, flexible linker [GGGLDPRVR] and actin) was obtained from Dr. J. Neefjes, Division of Tumor Biology, and vectors containing the endothelin B receptor, and a human NK2 receptor with C-terminal truncation at position 328 (Alblas *et al.*, 1995) were obtained from Dr. W. Moolenaar, Division of Cellular Biochemistry at our institute. Constructs were transfected using calcium phosphate precipitate, at $\sim 0.8 \mu\text{g DNA/well}$. After transfection for 12 h, cells were washed with fresh medium and incubated for 4–24 h until usage.

Cell Culture and Stimulation

N1E-115 neuroblastoma cells and NIH-3T3 fibroblast cells were seeded in six-well plates at $\sim 25,000$ cells per well on 25-mm glass coverslips and cultured in 3 ml DMEM supplemented with 10% serum and antibiotics. Agonists and inhibitors were added from concentrated stock solutions. It was verified that the PIP kinase inhibitors PAO, quercetin and H₂O₂ did not noticeably affect cell viability over the time course of the experiments.

Determination of PLC-mediated PIP₂ Breakdown by Fluorescence Resonance Energy Transfer

Monitoring of dynamics of PLC activation with FRET was described in detail elsewhere (van der Wal *et al.*, 2001). In brief, cells were transiently transfected with YFP-PH and CFP-PH, at 1:1 ratio. When bound to PIP₂ at the membrane, these constructs are in close proximity and show FRET; upon PIP₂ hydrolysis, CFP-PH and YFP-PH dilute out into the cytosol and FRET ceases. Excitation of CFP-PH was at 425 ± 5 nm, and emission was collected simultaneously at 475 ± 15 (CFP) and 540 ± 20 nm (YFP). FRET was expressed as ratio of CFP to YFP signals, and changes were expressed as percent deviation from the initial value.

Confocal Microscopy

For imaging, coverslips with cells were transferred to a culture chamber and mounted on an inverted microscope. All experiments were performed in bicarbonate-buffered saline (containing in mM: 140 NaCl, 5 KCl, 1 MgCl₂, 10 glucose, and 10 HEPES Ca²⁺), pH 7.2, kept under 5% CO₂, at 37°C. Confocal imaging was with a DM-IRBE inverted microscope fitted with TCS-SP scanhead (Leica, Mannheim, Germany). Excitation of eGFP, eYFP, DiO, NBD-PA, and BODIPY-FL was with the 488-nm laserline, and emission was collected at 500–560 nm. For DiI, Bodipy 564/570, and TRITC, excitation was with 568 nm, and emission was collected at 590–650 nm. DiD was excited at 633 nm, whereas emission was collected at 645–700 nm. Cross-talk between channels was checked and where necessary corrected using Leica Confocal Software.

Image Analysis

Timelapse Analysis. For time lapse studies, series of confocal images were taken at 5–30-s time intervals and stored on harddisc. Visualization and analysis was performed off-line using TCS and Qwin software (Leica) and a suite of analysis routines that were written by one of the authors using the APL+Win development platform (APL2000 Inc., Bethesda, MD), as detailed below.

Motility Assay. To detect motility of actin and GFP-PH patches, pairs of images from a stored timelapse series (8-bit grayscale values) were analyzed essentially for correlation of pixel intensities, using the following steps: (i) Within a series of N images (1.. N), pairwise comparison was carried out for images (1 with $J + 1$), (2 with $J + 2$), ... ($[N - J]$ with N), with J selected to obtain a lapse of 30–120 s between the image pair, which appeared optimal for the detection of changes. (ii) Analysis of movement is best understood by referring to Figure 1A, lower scatterplot. Pixels that are intensely fluorescent in the first but not in the second image appear in the

scatterplot below the blue diagonal line. Slope and abscissa of this line can be set for optimal rejection of unaltered pixels; in addition, a threshold can be included for rejection of background noise. If I_t is the intensity of a given pixel in the first image, and I_s that in the second image, then for these pixels,

$$\{(I_t > B + C \cdot I_s) \text{ AND } (I_t > A)\}, \quad (1)$$

where A is the parameter that determines detection threshold (background; usually <30), B the X-abscissa (range, 5–40) and C the slope (range, 1–1.5). Similarly, for pixels above the red diagonal

$$\{(I_s > B + C \cdot I_t) \text{ AND } (I_s > A)\}, \quad (2)$$

where B is the Y-abscissa. The degree of dissimilarity DD is then calculated as

$$DD = ((\text{nr of pixels that meet } \langle 1 \rangle) + (\text{nr of pixels that meet } \langle 2 \rangle)) / (\text{total nr of pixels}) \quad (3)$$

(iii) Finally, motility traces are constructed by plotting corresponding DD versus image index for $(1 \dots [N - j])$. The reproducibility and sensitivity of this algorithm were checked using simulated and real data. Further details are available upon request from K.J.

Imaging of Reconstructed Axial PSF (RAP Imaging). Stacks of X/Y images of the basal membrane were captured at 40- or 80-nm axial distance, using a 63 $\times$, 1.32 NA oil immersion Planapochromatic objective and a pinhole setting of 1 airy disk. Excitation was at 488 nm, and emission was collected at 525 nm. Because the axial resolution at these conditions is $\sim 1.05 \mu\text{m}$, the objective point spread function (PSF; i.e., the gaussian intensity profile that is detected when a true point source is imaged with an objective) is thus oversampled up to 25 times (see Figure 5). Before processing, individual images were smoothed once. For each pixel (x, y) , plotting its intensity in image 1 to n of the stack of n images ($I_1 \dots I_n$) versus axial position reconstructs the axial PSF, because the thickness of the membrane (~ 5 nm) can be ignored. The “center of intensity” of the axial fluorescence intensity profiles was then determined by a calculation-efficient, simplified fitting algorithm as follows. First, image numbers were sorted and arranged in order of decreasing intensity. Then, these numbers were multiplied by an array of n weighting values ($W_1 \dots W_n$). Values for W were chosen to progressively reduce the influence of out-of-focus (dim) images (see below). The sum P of these products is proportional to the axial position of the center of intensity. For example, be the order of intensity for a given (x, y) pixel:

$$\text{image17} > \text{image16} > \text{image18} > \text{image15} > \dots$$

then the expression for P is:

$$P = W_{1,17} + W_{2,16} + W_{3,18} + W_{4,15} + \dots + W_{n,m}$$

Three-dimensional surface profiles (see Figure 5) were then constructed by plotting P as a function of x and y , using the Surface Plotter plugin of the public domain software ImageJ, version 1.24t (Wayne Rasband, National Institutes of Health, Bethesda, MD).

The array of weighting values W was chosen so that

$$\sum_{i=1}^n w_i = 1.$$

A trivial example is the case where $W_1 = 1$, and $W_2 \dots W_n = 0$. In this case, P will simply be the image number of the highest pixel intensity. Because of the large Poisson-distributed photon noise in the confocal images, this results in extremely noisy surface plots. Good results were obtained with linear weighting arrays that emphasize the images of highest intensity, such as the array $W_i = 0.14, 0.13, 0.12, 0.11, 0.10, 0.09, 0.08, 0.07, 0.06, 0.05, 0.03, 0.02, 0, 0, 0, \dots, 0$. It

should be noted that the described calculation-effective algorithm performed equally well as pixel-by-pixel curve-fitting algorithms that took dramatically longer computer processing. P was calibrated numerically as well as experimentally, using a slightly tilted mirror as the object. It is estimated that a Z-axis resolution up to ~ 15 nm is obtained. Further details are available on request from K.J.

Membrane Staining

Membrane dye stocks were mixed by vigorous pipetting with bicarbonate-buffered saline to a final concentration of $1 \mu\text{M}$. Cells were incubated with the mix for 5–15 min at 37°C . Before imaging, cells were washed three times with bicarbonate-buffered saline.

Fluorescence Recovery after Photobleaching

For fluorescence recovery after photobleaching (FRAP) experiments, cells were imaged using a Leica TCS-SP confocal microscope equipped with 63 $\times$ (NA 1.3) oil immersion objective. Spots were bleached with the 488-nm argon laser line (Bis-Bodipy FL C11-PC; 0.2s) or 568-nm krypton laser line (DiI, 0.2 s), and recovery was sampled at 10 Hz. Data were corrected for slight ($<5\%$) background bleaching and fitted with single exponents using Clampfit software (Axon Instruments, Union City, CA).

RESULTS

Cortical Actin Motility Correlates with Agonist-induced PIP₂ Breakdown

We recently reported the use of a FRET-based assay to monitor the kinetics of receptor-mediated PIP₂ breakdown in single cells (van der Wal *et al.*, 2001). We showed that distinct G protein-coupled receptors (GPCRs) induce PIP₂ hydrolysis with their own characteristic kinetic profiles. Representative examples are depicted in Figure 1, B–F (left traces). To establish the relationship between membrane [PIP₂] and actin dynamics, we set out to study the actin cytoskeleton under identical conditions. N1E-115 cells do not possess actin stress fibers, but the cortical actin cytoskeleton is well developed, and it mediates agonist-induced cell shape changes (Jalink *et al.*, 1993; van Leeuwen *et al.*, 1999). Cortical actin dynamics were studied by *in vivo* time-lapse imaging of cells that express GFP-tagged actin. GFP-actin at the cell cortex displays an inhomogeneous, patchy distribution (Figure 1A, top panel), and it is highly dynamic, with individual structures showing seemingly random as well as directed movements. Strikingly, after addition of PIP₂-hydrolyzing agonists, such as endothelin and neurokinin A, actin movements are inhibited within a minute.

To study these effects in more detail, we set up an assay for the quantification of GFP-actin dynamics. Essentially, in this assay motility is expressed as change (lack of correlation) between successive images in a time-lapse series (see the legend to Figure 1 and MATERIALS AND METHODS). We compared the kinetics of actin dynamics to those of the concomitant PIP₂ decreases after agonist addition. The strong PLC activator endothelin (ET, Figure 1B) causes transient retardation of actin dynamics that correlates well with the decrease in membrane PIP₂ content (representative result of 5 experiments). Agonists that induce weaker PIP₂ hydrolysis, such as histamine, LPA, and thrombin (van der Wal *et al.*, 2001), caused less pronounced or undetectable inhibition of actin motility ($n = 9$; Figure 1C). Bradykinin, which induces a short-lived drop in [PIP₂] caused a minor

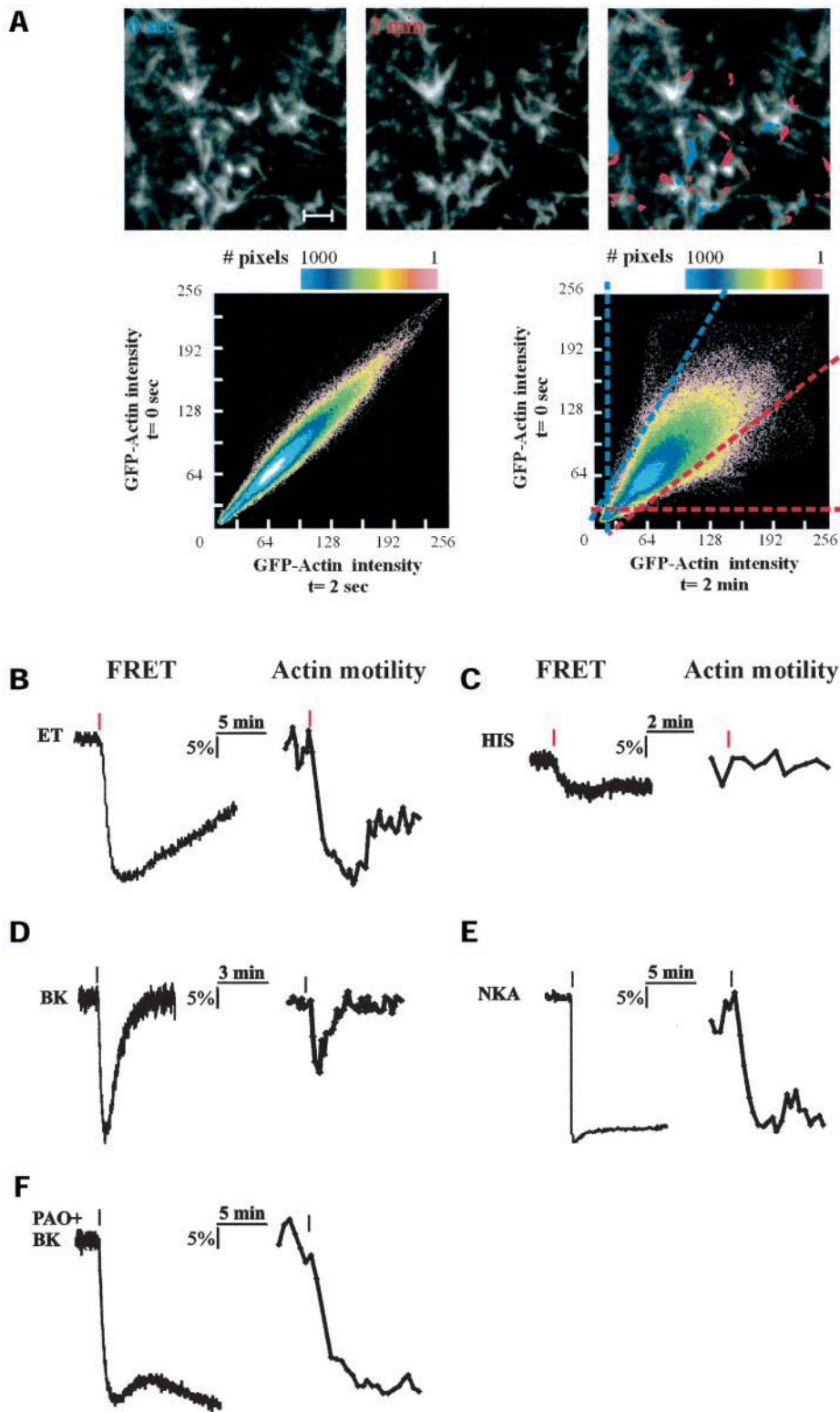


Figure 1. Influence of agonist-induced PLC activation on cortical actin dynamics. (A) Detection of actin motility using intensity scatter plot. Time-lapse series of confocal images of the basal membrane of GFP-actin expressing N1E-115 cells were collected. For each pair of subsequent images X and Y , intensities of each pixel were plotted as dots in a (X, Y) scatterplot (see MATERIALS AND METHODS for further details). Multiple occurrence of identical coordinates is color-coded. For a pair of images collected 2 s apart, the scatterplot shows a distribution along the diagonal (left scatterplot), with some divergence due to the inherent photon noise. For pairs of images taken at longer time intervals (top panel, $t = 0$ and 2 min), bright actin structures that have moved in between collection of the images will be apparent as off-diagonal clusters of dots (right scatterplot). Dots within the blue-dashed area in this plot represent pixels of which the intensity has significantly decreased in 2 min; the red-dashed area contains all pixels whose intensity has increased after 2 min. Taking a fixed time interval (usually 30–120 s), actin motility as a function of time can be quantified extremely sensitively by calculating the fraction of pixels that are off-diagonal for subsequent images in a time-lapse series. In the composite (rightmost) photomicrograph, pixels from the dashed areas are superimposed on the image. Scale bar, 0.5 μm . (B–F) Comparison of agonist-induced PIP_2 hydrolysis (left panels) and actin motility (right panels) in separate N1E-115 cells. Shown are responses from single cells (B) expressing the endothelin B receptor and stimulated with 20 nM endothelin (ET); (C) stimulated with 10 μM histamine (HIS); (D) stimulated with 1 μM bradykinin (BK); and (E) expressing a desensitization-defective neurokinin A receptor and stimulated with 1 μM neurokinin A (NKA) and (F) pretreated with 1 μM phenylarsine oxide (PAO) for 5 min and stimulated with 1 μM bradykinin (BK). Red marks indicate the moment of agonist addition.

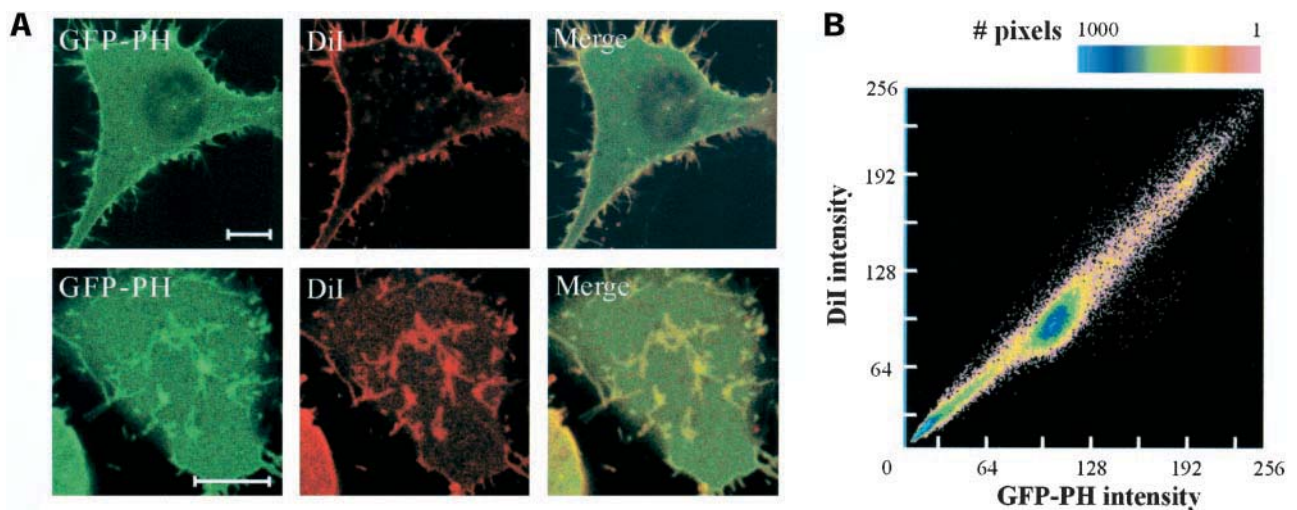


Figure 2. Colocalization of GFP-PH and DiI. Living N1E-115 cells expressing GFP-PH were stained with the lipophilic membrane dye DiI. (A) Images of the fluorescent protein and DiI were collected from both medial (top panels) and basal (bottom panels) sections, using a confocal microscope. Scale bar, 5 μ m. (B) Pixel intensity for DiI was plotted against GFP-PH intensity in a scatter plot. Note the difference from the scatter plots in Figure 1, where one fluorophore is imaged at two points in time.

and transient drop in actin motility ($n = 6$; Figure 1D), whereas the sustained PIP₂ hydrolysis evoked by a desensitization-defective mutant of the NKA receptor (Alblas *et al.*, 1995; van der Wal *et al.*, 2001) correlates with prolonged immobilization of cortical actin (Figure 1E, $n = 6$). These results show that cortical actin dynamics correlate well with membrane PIP₂ content, providing evidence for the causal relationship that was hypothesized in the recent literature. To further address that actin motility changes are secondary to PIP₂ hydrolysis, we inhibited PIP₂ resynthesis by blocking PIP kinases with a low dose (1–4 μ M) of phenylarsine oxide (PAO). As shown in Figure 1F, pretreatment with this drug did not influence basal [PIP₂] or actin motility, but it completely blocked recovery of PIP₂ to basal levels after BK-induced PLC activation (van der Wal *et al.*, 2001). This was paralleled by persistent reduction of actin dynamics. Similar observations were made with the PIP kinase inhibitor quercetin, which blocks ATP binding to the kinase domain, and with H₂O₂ (Mesaali *et al.*, 2000; J. Halstead and N. Divecha, personal communication), which is thought to disrupt PIP-kinase function by modifying critical thiol groups outside the ATP binding domain.

GFP-PH Distribution at the Plasma Membrane Is Not Homogenous

It has been hypothesized (DiNubile and Huang, 1997; Honda *et al.*, 1999; Lanier and Gertler, 2000; Tall *et al.*, 2000) that local differences in membrane PIP₂ content may transmit extracellular signals into local cytoskeletal changes. Do such local enrichments or depletions in PIP₂ content (PIP₂ patches; note that although for brevity we will often mention “PIP₂ enrichments” our analyses were equally focused on PIP₂ enrichments and decreases) at the plasma membrane exist? To study the spatial distribution of PIP₂, cells expressing a GFP-tagged pleckstrin homology domain derived from PLC δ 1 were imaged on the confocal microscope. We noted

that GFP fluorescence along the plasma membranes does not appear to be homogenous. Rather, in several cell types, including N1E-115 mouse neuroblastoma cells (Figure 2A), NIH-3T3 mouse fibroblasts and HEK293 human embryonic kidney cells, GFP fluorescence shows distinct bright patches that are two- to threefold more intense than the rest of the membrane. Bright patches can be observed in medial sections through the cells (top left panel), where they often colocalize with membrane ruffles and lamellae. Patches are also particularly apparent in the basal membranes of cells grown on coverslips, usually displaying a slender, elongated shape (bottom left panel). Similar observations were recently reported by Tall *et al.* (2000).

When imaged in living cells these patches appear highly dynamic: over time, individual patches may disappear, show directed movements, and occasionally branch. These structures further colocalize with F-actin but not with vinculin or other components of focal adhesions. Because many actin-binding proteins can interact with PIP₂ *in vitro*, GFP-PH patches were interpreted to represent local concentrations of PIP₂ (Tall *et al.*, 2000). However, alternative explanations for the local concentration of GFP-PH have not been addressed.

GFP-PH Staining Pattern Reflects Membrane Content

We set out to address the possibility that bright GFP-PH patches reflect local increases in membrane area, due to local membrane folding. Cells expressing GFP-PH were stained with the lipophilic membrane dye diphenyl-DiI and simultaneously imaged for GFP and dye fluorescence on a confocal microscope. Images were collected at the basal membrane and at medial sections through the cell. Strikingly, we observed strong colocalization of GFP and DiI fluorescence in all sections in N1E-115 cells (Figure 2A), NIH-3T3 cells, and several other cell types. A colo-

Table 1. Membrane markers used in colocalization studies

Membrane anchor	Dye	Fluorophore	Colocalization with GFP-PH ^a
Octadecyl (C ₁₈)	Diphenyl DiI	Diphenyl indocarbocyanines	+
	SP-DiI C18(3)	Sulfophenyl indocarbocyanines	+
	SP-DiOC18(3)	Sulfophenyl oxacarbocyanine	+ ^b
	DiI C18(5)	Long λ -ex. Indocarbocyanine	+
	BODIPY 564/570 C ₁₁	Bodipy 564/570	+
Fatty Acid (C ₁₁)	TRITC DHPE	TRITC	+
Lipid, Headgroup labeled	Bis-BODIPY FL C ₁₁ -PC	Bodipy FL	+ ^b
Lipid, Acyl labeled	NBD-PA	NBD	+ ^b
Palmitate	YFP-MEM	Yellow fluorescent protein	+ ^b
Geranyl-geranyl	GFP-CAAX	Green fluorescent protein	+ ^b

Cells were loaded with indicated dyes or transfected with membrane-targeted GFP or YFP as detailed in MATERIALS AND METHODS.

^a Colocalization was assessed directly by simultaneous imaging of the indicated marker and GFP-PH (+) or, for dyes with green emission, indirectly by comparing the pattern of the indicated marker to that of DiI (+^b).

calization analysis was carried out by constructing scatter plots to compare pixel intensities of GFP-PH and DiI (Figure 2B). In these plots, any structures present in the GFP-PH, but not in the DiI image, will be apparent as off-diagonal clusters of dots. No evidence was found for GFP-PH enrichment beyond the level predicted by lipid mass as detected by DiI fluorescence (>35 images analyzed). Conceivably, however, the DiI dye might localize preferentially to places enriched in PIP₂ or F-actin. We therefore used a panel of different membrane markers with widely different physicochemical properties, including lipophilic dyes that intercalate in the lipid double-layer, phospholipids with fluorescently labeled acyl chain or headgroup, and fluorescent proteins, targeted to the membrane with lipid anchors (Table 1). All membrane markers colocalized with GFP-PH, strongly suggesting

that patches represent sites with increased membrane content.

Strong colocalization of GFP-PH with membrane markers was not restricted to the PH domain from PLC δ 1, because similar observations were obtained using the PIP₂-specific PH domain derived from PLC δ 4, and a PIP₂-specific mutant (E41K) derived from the PH domain of Bruton's tyrosine kinase (Btk; Varnai *et al.*, 1999). Furthermore, bradykinin-induced PLC activation caused GFP-PH patches to disappear, whereas the DiI staining pattern remained unaltered. As shown for medial and basal confocal sections in Figure 3, GFP-PH returned to the exact same sites to colocalize again with the DiI patches after resynthesis of PIP₂. Taken together, these data demonstrate that patches enriched in GFP-PH are the consequence of locally increased membrane area (i.e., folds and ruffles), rather than of local PIP₂ enrichment.

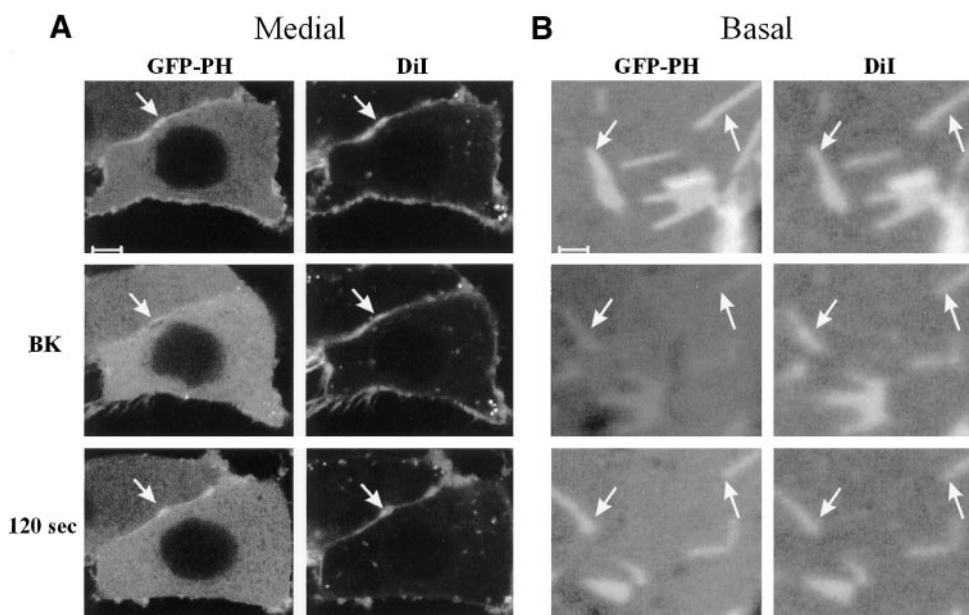


Figure 3. Localization of GFP-PH and DiI during PIP₂ hydrolysis. N1E-115 cells expressing GFP-PH were stained with DiI and imaged on the confocal microscope in medial (A) and basal (B) sections. Bradykinin (BK, 1 μ M) induced PIP₂ breakdown, resulting in translocation of GFP-PH. After resynthesis of PIP₂ (at 120 s), GFP-PH returned to the membrane. In contrast, DiI patches (see arrow) remain at the membrane during PIP₂ hydrolysis. Scale bar: (A) 5 μ m, (B) 1 μ m.

Membrane Folds at GFP-PH Patches Can Be Directly Visualized

Although the above results indicate that GFP-PH patches are sites of membrane folding, detailed confocal imaging studies failed to directly visualize folds in a considerable subset of the patches. This is perhaps not surprising, because the resolution of the confocal microscope is limited by the objective point spread function (PSF), this is the Gaussian intensity profile that is detected when a true point source is imaged with an objective. The PSF of the best objectives are close to two orders of magnitude larger than the thickness (5 nm) of the lipid bilayer. Two types of experiments were performed to investigate whether subresolution membrane folds cause the bright fluorescence in all membrane patches.

First, cells expressing GFP-PH were swollen by a hypotonic shock. The medium was diluted from 350 mOsmol to a final value of 120 mOsmol, while confocal images were continuously collected (Figure 4A). This caused an increase in cell volume of 40–50% that led to straitening out of the membrane with consequent disappearance of bright GFP-PH patches. Disappearance of bright patches was not due to swelling-induced PIP₂ hydrolysis, because [PIP₂] in these cells remained constant (Figure 4B). These experiments strongly argue that bright fluorescent patches are in fact folds in the lipid bilayer.

Second, we set out to directly visualize subresolution membrane folding, using a numerical approach to increase the axial resolution. In these studies, we recorded stacks of images at 40- or 80-nm axial distance, thus oversampling the axial resolution (PSF) of the objective up to 25 times (see Figure 5). The intensity of a small region of interest (ROI) was plotted against the axial position for the images in this stack. Because the thickness of the membrane (~5 nm) can be ignored, the resulting curve basically reconstructs the axial PSF (Figure 5C). When such reconstructed axial PSFs are compared for ROIs inside (blue mask) and just outside (red mask) of the GFP-CAAX patches, the normalized curves consistently show small offsets, indicating differences in Z-position of the fluorescent membrane. It should be noted that by fitting the PSFs, the Z-axis offset can in fact be estimated with a precision considerably higher than the axial step size (see MATERIALS AND METHODS). By applying this technique on a pixel-by-pixel basis to the image stack, the three-dimensional surface profile of the basal membrane could be visualized with ~15-nm axial resolution (Figure 5D). In these images, upward and downward protrusions measuring between 15 and 150 nm in the basal membrane are observed (Figure 5D) that correspond to the bright GFP-CAAX patches. Therefore, these data directly demonstrate that patches in effect represent submicrometer peaks and valleys in the landscape of the basal membrane.

Further analysis of these structures is described in the Supplementary Data section, online. It is demonstrated that GFP-PH patches do not represent sites of cell adhesion, such as focal adhesions. In addition, we show that motility of GFP-PH patches is potently inhibited by cytochalasin D and myosin light chain kinase blockers, indicating that patches are the result, rather than the cause, of local actin-dependent forces.

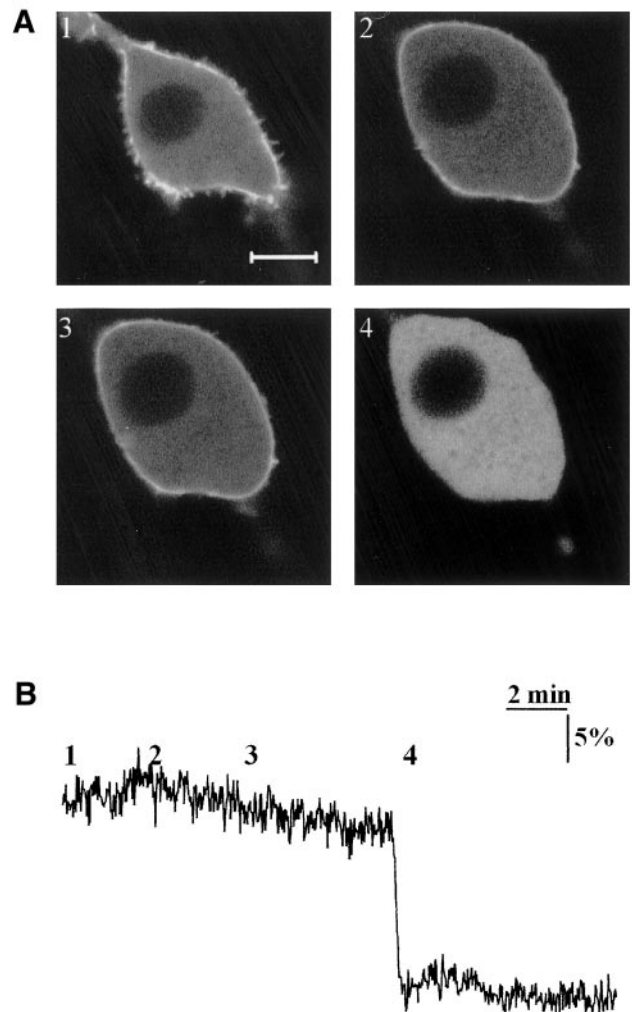


Figure 4. GFP-PH patches disappear after osmotic swelling. (A) Confocal images of NIH-3T3 cells expressing GFP-PH, subjected to hypotonic swelling. Osmolarity of the medium was adjusted from a basal value of 350 mOsmol (1) to 235 (2) and 120 mOsmol (3). Ionomycin, 5 μ M, was added (4) to cause complete translocation of the fluorescent proteins. (B) PIP₂ degradation was assayed by FRET in a single cell, subjected to the same protocol. Note that although swelling eliminates the patches, total PIP₂ in the cell is virtually unaltered. Scale bar, 5 μ m.

Diffusion Limits the Establishment and Maintenance of PIP₂ Gradients

Summarizing the above results, GFP-PH labeling indicates that spatial differences in the concentration of unbound PIP₂, at least at a micrometer scale, do not exist in our cells. However, there might be [PIP₂] differences on a more global scale, e.g., between the leading and trailing edge of polarized cells or in organelles such as lamellipodia. For 3'-phosphorylated phosphatidylinositols, it has been shown that such gradients can exist in chemotactic and phagocytic cells (Haugh *et al.*, 2000; Servant *et al.*, 2000; Marshall *et al.*, 2001). At what scale can spatial

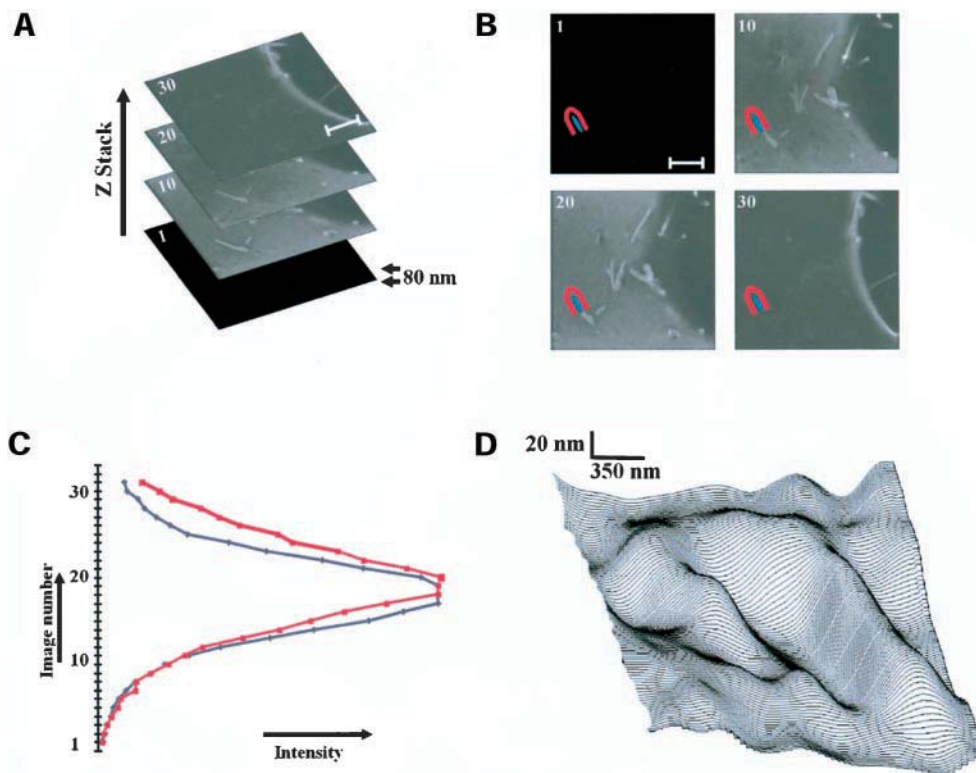


Figure 5. Direct visualization of subresolution membrane folds at the patches. (A) Stack of confocal XY-images, taken 80 nm apart in Z direction, showing the basal membrane of GFP-CAAX expressing N1E-115 cell. (B) Using image analysis software, an area was manually assigned within (blue) and just outside (red) a patch, and applied to the entire image stack. Scale bar, 2 μm . (C) Plotting the measured mean intensity within the blue and red area versus the image number in the stack reconstructs the axial pointspread function of the objective. The PSF (full-width at half-maximum) of the used Leica 63 $\times$ oil 1.32 NA planapochromatic objective is 1.05 μm , at a confocal pinhole setting of 0.58 μm and at emission $\lambda = 525 \text{ nm}$. By analysis of the offset of the normalized curves, the Z-position of the membrane can be estimated with high precision. (D) The three-dimensional surface profile of a patch is visualized with $\sim 15 \text{ nm}$ resolution by applying this analysis on a point-by-point basis to the image stack.

differences in $[\text{PIP}_2]$ be induced in the plasma membrane of living cells?

Gradients in $[\text{PIP}_2]$ are the result of local synthesis and breakdown, combined with lateral diffusion of the lipid in the membrane. We studied the diffusion kinetics of bodipy-labeled PIP_2 in N1E-115 cells using laser-induced photobleaching (see MATERIALS AND METHODS). As can be seen in Figure 6A, the fluorescence in micrometer-sized photobleached spots in the plasma membrane completely recovered within seconds. Thus, it is likely that at this scale, induced gradients in $[\text{PIP}_2]$ rapidly dissipate by diffusion. The observed recovery rate is similar to those of other freely diffusible membrane labels, including DiI and bodipy-labeled phosphatidylcholine (Figure 6A). Published diffusion coefficients (D) for these labels average $\sim 1\text{--}2 \mu\text{m}^2/\text{s}$ (Yechiel and Edidin, 1987; Fulbright *et al.*, 1997), in reasonable agreement with the value reported recently for PIP_3 ($0.5 \mu\text{m}^2/\text{s}$; Haugh *et al.*, 2000). These D values therefore predict that gradients in PIP_2 at a larger scale may occur. To test whether PIP_2 concentration differences can be induced and detected on this scale, we used focal stimulation with Neurokinin A from a micropipette (Figure 6). This caused a rapid initial translocation of GFP-PH at the stimulus site that subsequently spread to the neighboring membrane. In particular in cases where diffusion is restricted, such as in neurites (Figure 6A), sustained local decreases in $[\text{PIP}_2]$ could reliably be evoked (Figure 6B). Taken together, these observations show that agonists can induce PIP_2 differences along the membrane, whereas at a micrometer-scale lateral diffusion limits maintenance of such gradients.

DISCUSSION

In this study, we used GFP-PH as *in vivo* PIP_2 tag to investigate the hypothesis that local pools of free PIP_2 at the plasma membrane (patches) spatially regulate cytoskeletal remodeling. The existence of PIP_2 patches and their involvement in the local regulation of cellular physiology (e.g., actomyosin remodeling, vesicle budding, etc.) has been hypothesized by several groups (DiNubile and Huang, 1997; Raucher *et al.*, 2000; Rozelle *et al.*, 2000; Brown *et al.*, 2001; for review see Caroni, 2001; Gillooly and Stenmark, 2001). In addition, some data have been presented that seems to support this notion (Honda *et al.*, 1999; Botelho *et al.*, 2000; Tall *et al.*, 2000; Micheva *et al.*, 2001). At odds with these reports, the detailed spatial analysis presented here indicates, within the limits of optical microscopy ($\sim 200 \text{ nm}$), that in all cell lines checked the GFP-PH labeling pattern simply reflects the amount of membrane rather than local PIP_2 enrichment. We have previously shown (van der Wal *et al.*, 2001) that the PH domain from PLC $\delta 1$ detects PIP_2 (rather than IP_3) *in vivo* and that membrane-bound GFP-PH is in rapid ($\sim 1 \text{ s}$) equilibrium with a cytosolic pool of approximately equal size. Because in the present article it was shown that gradients in $[\text{PIP}_2]$ could be induced and detected, it is fair to argue that GFP-PH is capable of reporting, at least qualitatively, the local free PIP_2 concentration in single, living cells. From our failure to observe micrometer-sized PIP_2 patches, we conclude that spatial differences in PIP_2 regulation of the cytoskeleton do not exist at this scale.

The above-mentioned contrast between our findings and literature reports deserves further attention. Because this

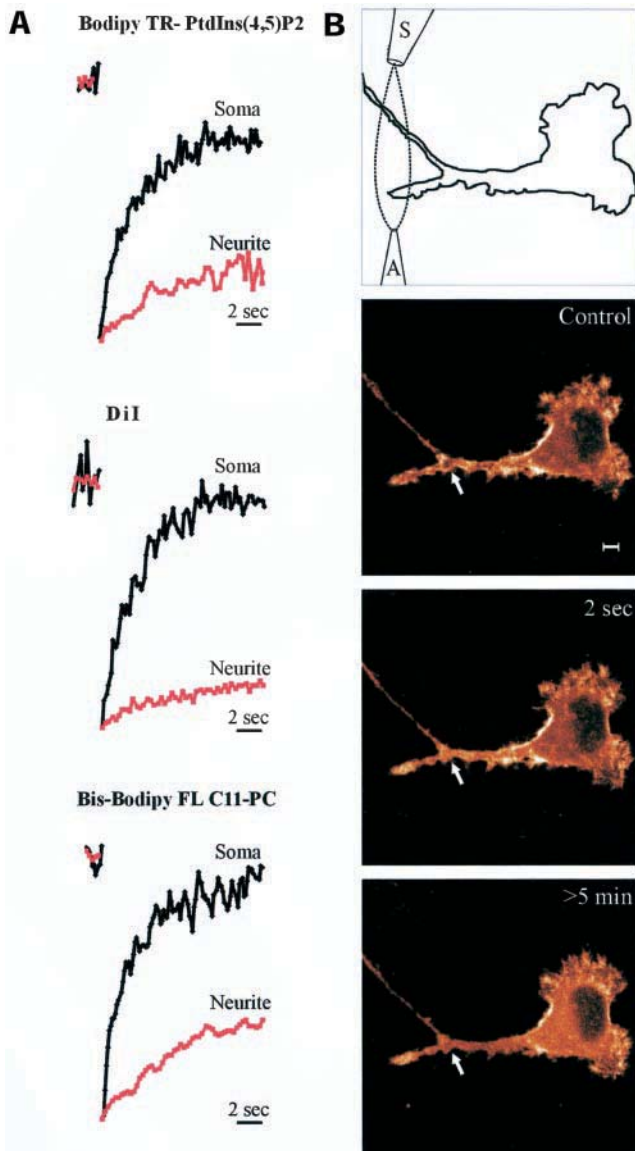


Figure 6. Local PIP₂ gradients are limited by diffusion. (A) Cells were labeled with DiI, bodipy-TR PtdIns(4,5)P₂, or bis-bodipy-FL C11-phosphatidylcholine. Spots were bleached at the plasma membrane in neurites (red trace) or the soma (black trace) with a 0.2-s pulse of 488- or 568-nm laser light, and recovery was quantified from confocal images. Note that recovery upon spot photobleaching in neurites (red lines) was reduced by orders of magnitude. (B) N1E-115 cell expressing GFP-PH and a desensitization-defective NK2 receptor was imaged at the indicated time points using a confocal microscope. PLC was stimulated locally in a developing neurite by a precisely confined stream of neurokinin A (NKA) generated by means of an application (A) and a suction pipette (S). The resulting PIP₂ degradation was monitored as GFP-PH translocation (see arrow). Similar results were obtained with bradykinin. The intensity of the images is color-coded with the “glow” color look-up table of Leica TCS software. Scale bar, 5 μm.

study addressed the hypothesized spatial regulation of the cytoskeleton by PIP₂, we used detection by decoration with GFP-PH to visualize the distribution of the free (unbound)

pool of this lipid along the membrane. PIP₂ that is bound to proteins at the plasma membrane constitutes a second pool that, likely, may display local molar enrichment or gradients due to, respectively, clustering at specific sites or gradients of the PIP₂-interacting proteins. In this case, PIP₂ enrichments are the result, rather than the cause of protein clustering, and because such enrichments are not available for decoration with GFP-PH, our studies will fail to detect them. However, lipid-biochemical approaches, such as those used to study rafts, obviously would. These rafts, which are detergent-insoluble microdomains in the membrane, contain signal-transducing proteins and may have roles in membrane trafficking and signaling (reviewed by Simons and Ikonen, 1997). The resolution of the here used imaging techniques is not sufficient to investigate rafts (<70 nm; Varma and Mayor, 1998; Pralle *et al.*, 2000). Nevertheless, the observed highly homogeneous distribution of GFP-PH along the membrane suggests that hypothetical rafts are either very abundant and evenly distributed along the membrane or they do not contain excess free PIP₂. Reports that rely on labeling of PIP₂ with specific antibodies require fixation and permeabilization of the cells (Laux *et al.*, 2000). However, this procedure may well have caused artifacts (Mayor *et al.*, 1994; Kenworthy and Edidin, 1998; Laux *et al.*, 2000). For example, it was observed by Laux and colleagues that the size of PIP₂ clusters observed in monkey kidney epithelial cells depends on the fixation method. Furthermore, in a recent electron microscopy study (Watt *et al.*, 2002) that used immunogold labeling of PLCδ1-PH-GST to visualize PIP₂ at the membrane, it was shown that lipids are not efficiently fixed with aldehyde at room temperature. Interestingly, these authors showed good labeling results at 0°C but did not observe PIP₂ patches at the membrane.

The published literature generally lacks rigorous colocalization analysis to exclude the possibility of “apparent PIP₂ enrichment” by membrane folding. In our studies, we used a panel of 10 different membrane stains with widely different physicochemical properties, and we have used three different GFP-tagged PIP₂-specific pleckstrin homology domains. Strong colocalization of GFP-PH with lipid dyes was observed in all cell types tested, irrespective of the phase of the cell cycle and of growth conditions (i.e., both when cultured in serum-free and in serum-containing medium). Furthermore, because our data directly contradict the interpretation put forward by Tall *et al.* (2000), we have addressed this issue by additional, independent approaches. These included experiments that involve hypotonic cell swelling, interference reflection microscopy, and the here-introduced technique of reconstructed axial PSF imaging. Together, the experiments show conclusively that GFP-PH patches represent submicroscopic folds and protrusions in the membrane that are caused by actomyosin-based forces, rather than local PIP₂ enrichments. In addition, in vivo FRAP experiments were carried out using fluorescently labeled lipids that incorporated in the plasma membrane after addition to the culture medium. These experiments should be interpreted with some caution, because membrane insertion and possible binding to proteins of the exogenously added PIP₂ has not been characterized extensively. With this caveat, the analysis revealed that diffusion of PIP₂ occurs freely at a rate comparable to that of phosphatidylcholine, showing that micrometer-sized patches will dissipate within

seconds. The recently reported gradients in 3' phosphorylated phosphoinositides (3' PI) in chemotactic and phagocytic cells (Haugh *et al.*, 2000; Servant *et al.*, 2000; Marshall *et al.*, 2001) were analyzed by Haugh and coworkers. In a model of local synthesis and breakdown of 3' PI, the experimentally revealed unhindered diffusion along the membrane ($D = \sim 0.5 \mu\text{m}^2/\text{s}$) and the lifetime of ~ 40 s of 3' PI were consistent with the gradients observed *in vivo*. Our FRAP experiments indicate very similar diffusion rates for PIP_2 . Furthermore, from the observation that agonist-induced PIP_2 breakdown and subsequent resynthesis can take place well within 2 min, we conclude that under these conditions the PIP_2 lifetime may be short enough to enable buildup of gradients. Taken together, our results challenge the view that PIP_2 regulation of the actin cytoskeleton is localized at a micrometer scale. However, on a larger scale and in cases where diffusion is limited, sustained PIP_2 gradients may exist for prolonged periods of time.

We also observed that agonist-induced PIP_2 changes as detected using the FRET assay correlate well with the dynamics of cortical actin as quantified using the assay that was introduced in this article, both with respect to the magnitude and the time course of the response. A possible delay of the actin response was not detected, although it should be noted that the temporal resolution of the assay is limited to ~ 30 s (see MATERIALS AND METHODS). These observations provide experimental evidence for the actin-modulatory role of PIP_2 that has been widely hypothesized (e.g., Lassing and Lindberg, 1988; Sun *et al.*, 1997; Andra *et al.*, 1998; Wang *et al.*, 1999), based on the PIP_2 -binding properties displayed by many actin-binding proteins. Furthermore, this correlation held true when agents were used that circumvent receptor activation to cause PIP_2 decrease (the PIP -kinase inhibitors PAO and quercetin, and H_2O_2 ; Mesaeli *et al.*, 2000; J. Halstead and N. Divecha, personal communication), suggesting that the observed actin changes are not due to signaling events independent from PIP_2 metabolism. In support of this viewpoint, a recent report from Cunningham and coworkers showed that incubation of cells with a membrane permeable peptide with potent PIP_2 -binding activity inhibited cell migration (Cunningham *et al.*, 2001). Indeed, we observed that the transient drop in F-actin dynamics during agonist-induced PIP_2 breakdown was mirrored in loss of motility of the leading edge in fibroblasts. Thus, our data show, for the first time, that the PIP_2 decreases triggered by agonist-induced PLC activation suffice to influence cortical actin motility *in vivo*.

Finally, a notable observation is the existence of highly dynamic, actin-rich structures in the basal membrane of nonmigratory cells. These structures represent places of close membrane-substrate proximity but do not appear to relate to sites of cell adhesion. Whereas it is striking that their dynamic behavior appears PIP_2 dependent, a possible function awaits future investigations.

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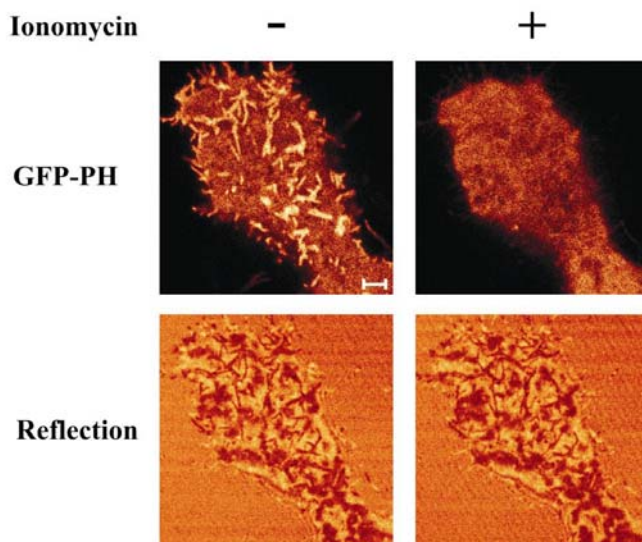
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Supplemental Data

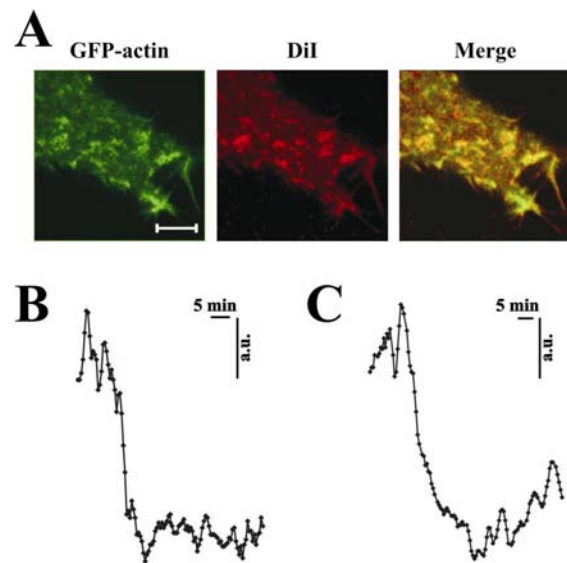
Membrane folds are caused by cortical actomyosin, but do not reflect focal adhesions

The observed patches conceivably reflect sites of interaction with the substratum. In confocal reflection images (Verschuieren, 1985), sites of cell-substratum proximity such as focal adhesions are apparent as marked dark (reflection-free) areas. Indeed, a large fraction of the patches colocalizes with sites of membrane-substratum proximity as observed in reflection images (Supplemental Fig. 1), independently corroborating the results obtained with the reconstructed PSF imaging technique. However, patches do not reflect focal adhesions, since N1E-115 cells do not possess focal adhesions and the staining pattern of antibodies against vinculin and paxillin does not colocalize with the pat-



Supplemental Figure 1. Colocalization of membrane patches with sites of membrane-substratum proximity

Fluorescence and reflection images were taken from the basal membrane of a N1E-115 cell expressing GFP-PH, using the 488 nm Argon ion laser line. To induce complete translocation of GFP-PH to the cytosol, ionomycin (5 μ M) was added. Scale bar: 2 μ m.



Supplemental Figure 2. Actomyosin-based forces drive motility of the patches

(A) N1E-115 cells expressing GFP-Actin were stained with DiI. Time lapse series of confocal images were collected from the basal membrane of these cells, simultaneously registering GFP-Actin and DiI. Actin motility was analyzed after (B) cytochalasin D (CCD, 0.5 μ g/ml) and (C) KT5926 (1 μ M) treatment using the actin motility assay. Scale bar: 2.5 μ m.

ches (data not shown). Furthermore, in a significant subset of cells, basal membrane patches were absent, while adhesion to the cover slip was normal.

Finally, serum-starved N1E-115 cells are quite immobile in cultures on glass or plastic; the highly dynamic nature of the patches therefore suggests a role different from cell adhesion in these cells. Perhaps these structures serve a role in probing the substratum; however, this hypothesis requires further investigation that is beyond the scope of this paper.

How do motile patches arise? Simultaneous imaging of GFP-actin and DiI confirmed the reported colocalization between patches and F-actin (Supplemental Fig. 2) (Tall *et al.*, 2000). Note that while DiI-stained patches strictly

overlap with GFP-actin filaments, the reverse is not always true, since not all actin filaments reach the plasma membrane. Other cytoskeletal components, including tubulin, paxillin and vinculin, do not colocalize with DiI (data not shown). We therefore hypothesized that folds are due to actomyosin contractility. To test this, GFP-actin and DiI images were collected simultaneously and analyzed for colocalization. In time-lapse series, both signals showed strikingly similar dynamics. When cells were treated with cytochalasin D (0.5 µg/ml) to disrupt F-actin (Urbanik and Ware, 1989), GFP-actin structures rapidly shrank with a concomitant drop in motility (Supplemental Fig. 2B), while the DiI patches stopped moving (not shown). Cytochalasin D (Sigma Chemical Co., St. Louis, MO) also blocked motility of GFP-PH patches completely. Similarly, myosin light chain kinase blockers (1 µM KT5926, supplemental Fig. 2C, and 100 nM K252a,

not shown; inhibitors were from Calbiochem-Novabiochem Corp., La Jolla, CA) impeded patch motility. We conclude that actomyosin contractility underlies membrane folding and patch formation.

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Chapter 4

PIP₂ signaling in lipid domains: a critical re-evaluation
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PIP₂ signaling in lipid domains: a critical re-evaluation

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Microdomains such as rafts are considered as scaffolds for phosphatidylinositol (4,5) biphosphate (PIP₂) signaling, enabling PIP₂ to selectively regulate different processes in the cell. Enrichment of PIP₂ in microdomains was based on cholesterol-depletion and detergent-extraction studies. Here we show that two distinct phospholipase C-coupled receptors (those for neurokinin A and endothelin) share the same, homogeneously distributed PIP₂ pool at the plasma membrane, even though the neurokinin A receptor is localized to microdomains and is cholesterol dependent in its PIP₂ signaling whereas the endothelin receptor is not. Our experiments further indicate that detergent treatment causes PIP₂ clustering and that cholesterol depletion interferes with basal, ligand-independent recycling of the neurokinin A receptor, thereby providing alternative explanations for the enrichment of PIP₂ in detergent-insoluble membrane fractions and for the cholesterol dependency of PIP₂ breakdown, respectively.

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Introduction

The phospholipid phosphatidylinositol 4,5-biphosphate (PIP₂) is a minor component in the plasma membrane, but it has important regulatory roles in many cellular processes. Apart from being the precursor for second messengers such as DAG, IP₃ and PIP₃ (Berridge and Irvine, 1984; Rameh and Cantley, 1999), it is also a messenger itself, with reported effects ranging from channel gating to vesicle trafficking and reorganization of the actin cytoskeleton (for reviews, see Czech, 2000; Caroni, 2001; Hilgemann *et al.*, 2001). How can a single lipid species regulate multiple physiological processes in a cell, apparently with spatial resolution? In an attempt to solve this enigma, it has been widely hypothesized that spatially confined PIP₂ pools must exist in the plasma

membrane (e.g., Hinchliffe *et al.*, 1998; Martin, 2001; Simonsen *et al.*, 2001; Janmey and Lindberg, 2004).

The microscopical distribution of PIP₂ along the plasma membrane has been studied in fixed cells with PIP₂-specific antibodies (Laux *et al.*, 2000) as well as in living cells using GFP-tagged pleckstrin homology domains (GFP-PH) as PIP₂ labels (Stauffer *et al.*, 1998; Varnai and Balla, 1998). In a recent detailed study employing this latter technique, we reported that in several cell types tested, GFP-PH labeling of the plasma membrane appeared strictly homogenous. In this study, we also showed that reported local GFP-PH enrichments, which had been interpreted to represent PIP₂ concentrations, appeared due to subresolution folding of the membrane (van Rheenen and Jalink, 2002). In support of this view, fluorescent recovery after photobleaching (FRAP) experiments demonstrated that diffusion of fluorescent PIP₂ is too fast to maintain enrichments in the plasma membrane at a micrometer scale. However, as the resolution of light microscopy does not allow studying structures smaller than ~250 nm, the possible confinement of PIP₂ to smaller structures such as rafts was not addressed in this study.

Rafts are small (<250 nm) lipid domains in the plasma membrane that have recently attracted much attention as (hypothetical) scaffolds for signal transduction components. Rafts differ from the bulk membrane in lipid composition, that is, they are enriched in cholesterol, sphingolipids and saturated phospholipids. This causes the lipids to be in a so-called liquid-ordered state that is thought to limit diffusion significantly. Biochemically, rafts are characterized as resistant to solubilization in detergents such as Triton X-100 at 4°C (Chamberlain, 2004) and by their dependence on cholesterol: extraction of cholesterol using methyl-β-cyclodextrin (CD) disrupts the rafts and redistributes the signaling complexes (reviewed by Simons and Ikonen, 1997; Simons and Toomre, 2000). However, it is important to note that there is no universal support for this hypothesis, and in fact, there is no clear consensus about the size, lifetime and cholesterol dependency of membrane rafts, or even for their very existence (Kenworthy and Edidin, 1998; Kenworthy *et al.*, 2000; Munro, 2003; Glebov and Nichols, 2004). For example, whereas rafts were studied by detergent extraction methods and visualized by clustering them together into micrometer-sized structures with antibodies, it was subsequently shown that these techniques can cluster molecules into non-pre-existing domains (Mayor *et al.*, 1994; Kenworthy and Edidin, 1998; Heerklotz, 2002; Pizzo *et al.*, 2002; Edidin, 2003; Munro, 2003).

Nevertheless, the possible confinement of (a pool of) PIP₂ to rafts would provide a mechanism to prevent spreading of the effects of local PIP₂ breakdown by phospholipase C (PLC). This hypothesis, which we here term 'raft-delimited PIP₂ signaling', is supported by a few biochemical studies (Koreh and Monaco, 1986; Hope and Pike, 1996; Pike and Casey, 1996; Pike and Miller, 1998; Waugh *et al.*, 1998; Hur *et al.*, 2004). For example, Pike and Casey (1996)

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demonstrated enrichment of PIP₂ in the detergent-resistant fraction of A431 cells, which was eliminated by prior stimulation with bradykinin. In the present study, raft-delimited PIP₂ signaling is re-examined. We first establish that PIP₂ breakdown evoked by neurokinin A (NKA) receptors (NK2r) but not endothelin (ET) B receptors (ETBr) shows the hallmarks of raft dependency in HEK293 cells. We then use cell biological, biophysical and electromicroscopical methods to show that PIP₂ is not confined to rafts in these cells.

Results and discussion

Neurokinin A receptor- but not endothelin B receptor-mediated PIP₂ hydrolysis is cholesterol dependent

Because rafts are too small to be resolved with light microscopy, and we wished to avoid detergent extraction methods, we used alternative methods to address possible raft-delimited PIP₂ signaling. One generally employed approach is to study the effects of raft disruption by extraction of cholesterol with CD. To continuously monitor integrity of the receptor-PLC-PIP₂ signaling cascade, we adopted an approach to read out membrane PIP₂ content by fluorescence resonance energy transfer (FRET) (van der Wal *et al*, 2001). In this assay, cells are cotransfected with CFP- and YFP-tagged PIP₂-binding PH domains derived from PLC δ 1 (Figure 1A). At rest, these constructs are concentrated at the membrane by binding to PIP₂, and FRET occurs. Following PIP₂ hydrolysis, the PH domains can no longer bind, and the proteins dilute out into the cytosol, thereby abolishing FRET. Loss of FRET is detected continuously by monitoring the emission ratio of YFP:CFP while exciting CFP at 425 nm. As a control, when non-PIP₂ binding mutant (R40L) PH domains were used, activation of PLC had no effect on FRET (not shown). This technique offers subsecond temporal resolution, while minimizing excitation damage to the cells (for further details, see Materials and methods). We initially focused on the human neurokinin A receptor as a model system, since there is some evidence for its compartmentalization at the plasma membrane (Vollmer *et al*, 1999; Cezanne *et al*, 2004). To assess integrity of the signaling cascade, HEK293 cells expressing NKA receptors were repeatedly stimulated with brief pulses of agonist from a puffer pipette (Figure 1A). Following each puff of NKA, the YFP:CFP emission ratio dropped rapidly, indicative of a decrease in the membrane PIP₂ levels (Figure 1B, left panel). Upon termination of the stimulus, PIP₂ was resynthesized and FRET returned to basal levels. With brief pulses, the receptors do not desensitize, and functional integrity of the signaling cascade can be monitored for hours.

Strikingly, cholesterol extraction compromised the integrity of the signaling cascade in HEK293 cells within minutes, evident as a rapid loss of responsiveness to NKA pulses (Figure 1B, right panel). Maximal suppression of NK2r-induced PIP₂ hydrolysis occurred after 20–30 min of CD treatment. At this time, the cholesterol content of the cells was reduced by >90% (Figure 1C). The effects of CD treatment were reversible, as removal of CD restored cholesterol levels as well as the ability of NKA to induce PIP₂ breakdown (Figure 1C and D, left panel). Similar results were obtained when the membrane localization of GFP-PH (Stauffer *et al*, 1998; Varnai and Balla, 1998) was studied by confocal microscopy (Figure 1E). As a control, α -cyclodextrin, which

does not extract cholesterol, had no effect on PLC signaling (data not shown).

Intriguingly, the effect of CD treatment appeared specific for the NK2r in that PIP₂ breakdown mediated via the ETBr was not affected (Figure 1D, right panel, and Figure 1E, middle panel). Conceivably, this would reflect very strong coupling of the ETBr to PLC. We therefore included a desensitization-defective mutant of the NK2r (Alblas *et al*, 1995) in our experiments. In our hands, this mutant is by far the most potent surface receptor in activating PLC (van der Wal *et al*, 2001); however, it was equally sensitive to CD pretreatment as the wild-type NK2r (Figure 1E, right panel). Thus, we have identified the NKA and ET receptors as, respectively, sensitive and resistant to cholesterol extraction in HEK293 cells, providing a unique opportunity to address raft-delimited PIP₂ signaling.

Neurokinin A and endothelin receptors distribute differently along the plasma membrane

The differential sensitivity to CD treatment of NK2r- and ETBr-mediated PLC signaling suggests that in the former, one or more components of the signaling pathway is/are spatially confined to rafts, whereas in the latter, the entire signaling cascade must be raft-independent. We first sought to assess localization of the G protein-coupled receptors (GPCRs) to rafts. NKA and ET receptors were tagged C-terminally with GFP and expressed in HEK293 cells (Figure 2A and B). The GFP tags do not interfere with receptor signaling, since stimulation with their cognate agonists induced normal PLC-mediated PIP₂ degradation and subsequent internalization of the receptors.

Confocal images of both receptors showed homogeneous labeling at the plasma membrane (Figure 2A and B), which is perhaps not surprising because of the small size of the putative rafts. To obtain higher resolution, cells were fixed and immunogold-labeled with anti-GFP antibodies, followed by analysis of ultrathin slices by electron microscopy (EM). The results revealed striking clustering of the NK2r, but not of ETBr (Figure 2C). To quantitate clustering, we adapted the cluster analysis developed by Ripley (1977) for analysis of the one-dimensional data from ultrathin slices (see Materials and methods). In essence, this statistical method compares the observed distribution of particles with the theoretically expected distribution for random particles, yielding density-independent data on clustering as well as on the mean cluster size and the distance between clusters (Figure 2D, left panel). As expected from the micrographs, analysis of the gold particles showed strong clustering for the NK2r and a random pattern for the ETBr (Figure 2D). Mean cluster size for the NK2r was ~80 nm, and mean cluster distance was ~400 nm. These values correspond quite well to sizes reported for microdomains and, in particular, for diffusion confinement zones recently found for the rat NK2r (Cezanne *et al*, 2004). The observed clustering of the NKA receptor is not noticeably influenced by the GFP tag, since HA-tagged NK2r yielded identical results (Figure 2C, NK2r-HA; cluster analysis not shown). Furthermore, differences in clustering are not due to differences in expression levels of those constructs because ET receptors and NK2r were expressed at comparable levels (Figure 2E), and because cluster analysis of selected cells with low label density gave identical results (not shown). Evidently, the experimentally

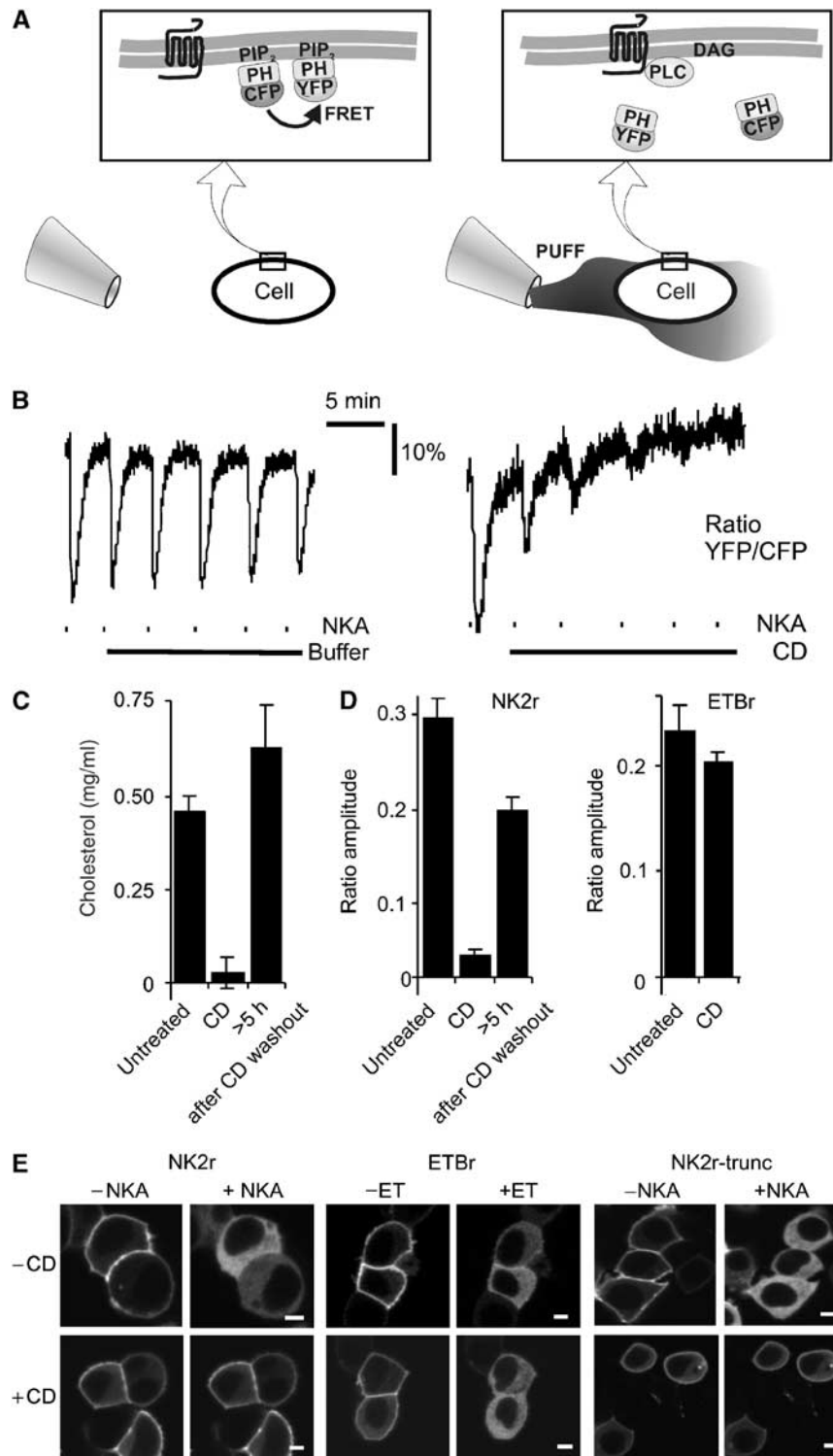


Figure 1 PIP₂ hydrolysis induced by the NK2r, but not the ETB receptor, is cholesterol dependent. (A) Schematic representation of the FRET assay that allows for continuous read out of the integrity of the receptor–PLC–PIP₂ signaling cascade. (Left panel) In a resting cell, CFP-PH and YFP-PH bind to PIP₂ at the membrane and FRET occurs. (Right panel) Using a puffer pipette, the cell is briefly (~10 s) exposed to agonist. This causes rapid degradation of PIP₂, resulting in translocation of CFP-PH and YFP-PH into the cytosol, with consequent loss of FRET. Subsequently, the agonist dilutes out through diffusion, PIP₂ is resynthesized and FRET recovers. For further details, see Materials and methods. (B) HEK293 cells expressing human NK2r were repeatedly stimulated with brief pulses of NKA (dashes, 10-s pulses from a puffer pipette containing 100 μM NKA). (Left panel) Control demonstrating repeated activation of the signaling cascade. (Right panel) Following a test pulse, CD (10 mM) was added (solid line), which rapidly inhibited signaling induced by further NKA pulses. (C) CD treatment causes depletion of cholesterol levels. Upon washout of CD, cholesterol levels recovered. (D) Quantitative analysis of the effects of CD treatment on agonist-induced FRET changes for NKA and ET. (E) HEK293 cells were cotransfected with GFP-PH and either the NK2r, the ETBr or a desensitization-defective truncation mutant of the NK2r as indicated. Confocal images were acquired from untreated (–CD) or CD-treated (+CD) cells before and 30 s after receptor stimulation. Scale bars, 5 μm. Note that in all cases, receptor stimulation induced translocation of GFP-PH to the cytosol (>80% of the cells), except for cells expressing NK2r that were treated with CD (wt NK2r, <30% of cells (partially) responded; desensitization-defective NK2r, <35% of cells responded; *N* > 200; *P* < 0.002).

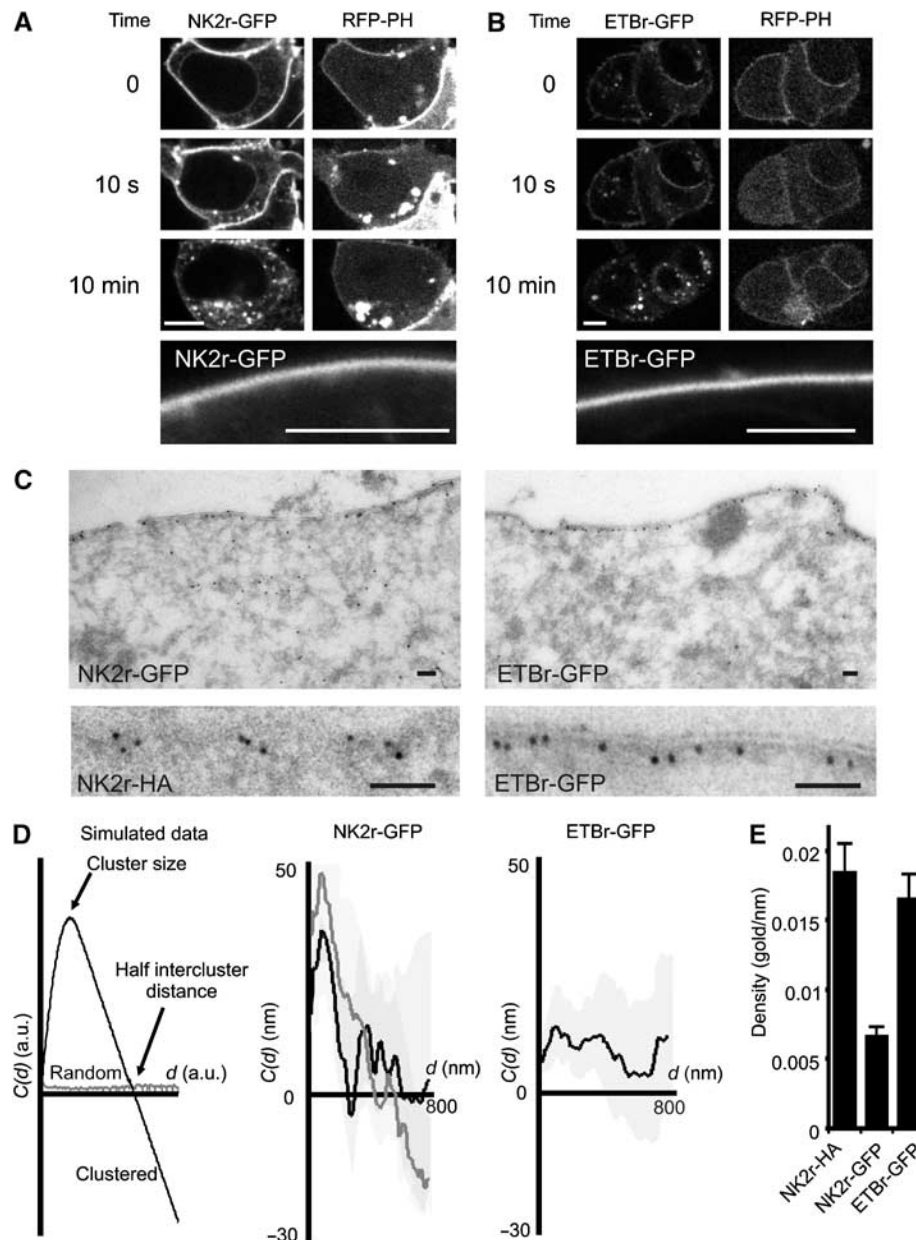


Figure 2 Distribution of NK2r and ETBr at the plasma membrane. HEK293 cells were cotransfected with RFP-PH and with the GFP-tagged NKA (A) or ETB (B) receptor. Confocal images were acquired before, at 10 s, and at 10 min after receptor stimulation. Within 10 s of agonist addition, PLC is fully activated as deduced from the translocation of RFP-PH to the cytosol, whereas receptor internalization takes several minutes to complete. The lower panels show NK2r-GFP and ETBr-GFP at high magnification to illustrate lack of clustering at the light microscopical level in the absence of agonist. Scale bars, 5 μ m. (C) (Left panels) EM micrograph of the localization of GFP-NK2r at the plasma membrane. The lower panel shows a similar result obtained with HA-tagged NK2r, using an antibody against the HA tag. (Right panels) Localization of GFP-ETBr by EM. Scale bars, 100 nm. (D) Cluster analysis of the distribution of gold particles from EM pictures, using a one-dimensional adaptation (see Materials and methods) of Ripley's K analysis. With randomly distributed particles, the cluster parameter $C(d)$ is close to 0 for all cluster diameters d (left panel, gray line). In a clustered data set, for d increasing from 0, $C(d)$ initially becomes positive (indicating over-representation of short distances in the data set), and subsequently crosses the x-axis to become negative. The location of the maximum predicts the average radius of the clusters, and the intersection with the x-axis indicates half of the intercluster distance (left panel, black line). Pooled results from cluster analyses for the NKA receptor (middle panel, black line) confirm strong clustering, whereas the ET receptor (right panel) is not clustered. Treatment of cells expressing NK2r with CD (middle panel, gray line) had little effect on overall clustering and the average cluster size was unaffected. The shaded regions indicate standard errors. (E) Expression levels (mean + s.e.) of HA- or GFP-tagged receptors used for the analysis in (D). Density (gold particles per nanometer membrane) was derived from EM micrographs.

determined size and intercluster distance are in good agreement with the observed homogenous distribution as seen by confocal microscopy.

As CD pretreatment rapidly abolishes NKA-mediated PLC signaling, we next investigated its effects on clustering at the

EM level. Surprisingly, cholesterol depletion had little effect on NK2r clustering (Figure 2D), in that there was only a slight effect on distance between the clusters with no detectable effect on cluster size. This indicates that the loss of signaling is not due to dislodgement of the receptors, and led us to

hypothesize that CD disrupts coclustering of PIP₂ with the receptors.

Ultrastructural analysis reveals homogenous distribution of PIP₂ along the plasma membrane

To address the possibility of PIP₂ confinement to rafts, we first studied clustering of a PIP₂ binding construct by EM. Specific labeling of PIP₂ for immunohistochemistry has been previously carried out using PIP₂-specific antibodies (Laux *et al.*, 2000). In such studies, cells are fixed and permeabilized by detergent to allow entry of the antibodies. However, because it was recently shown that detergent treatment can cluster molecules into non-pre-existing domains (Heerklotz, 2002), we studied the localization of PIP₂ by expressing tagged PH (PLC δ 1) domains in HEK293 cells. Ultrathin frozen sections of these cells were labeled with gold-tagged anti-GFP antibodies, eliminating the need for detergent permeabilization to gain access to the cells (Watt *et al.*, 2002). Because approximately 50% of GFP-PH is cytosolic in resting cells (van der Wal *et al.*, 2001), a tandem-PH construct (GFP-PH-PH; see Materials and methods) with increased membrane affinity was used. Whereas this chimera was absent from the cytosol, its localization at the membrane was identical to that of GFP-PH. High-resolution EM pictures from these preparations showed homogenous distribution of gold particles along the plasma membrane (Figure 3A). Again, a modified Ripley's cluster analysis was performed (Figure 3A, right panel), which confirmed random distribution of GFP-PH-PH in untreated cells ($N = 6$). Thus, by EM, PIP₂ at the membrane is not clustered.

Assessment of PIP₂ clustering *in vivo* by FRET

To confirm this finding *in vivo*, we set out to study the membrane PIP₂ distribution by measuring clustering-dependent FRET, a technique that was pioneered by Kenworthy and Edidin (1998). FRET is proportional to the inverse sixth power of the distance between the donor and the acceptor, and thus a theoretical relationship can be derived for the dependence of resonance on label density. Cells are labeled with suitable donor and acceptor fluorophores, and resonance efficiencies are determined for different concentrations of the fluorophores. To assess clustering, the plot of FRET efficiency versus labeling intensity is then compared to the theoretical relationship. When fluorophores are clustered at the membrane, distances between donors and acceptors are less than in the case of randomly distributed fluorophores, resulting in a left-shifted dose-response curve (Figure 3B). For further details, please refer to Supplementary data (section 1) where the relationship between FRET and clustering is determined by using Monte-Carlo simulations. Results in that section also form a sensitivity analysis delineating the importance of various parameters contributing to clustering-dependent FRET. These calculations demonstrate that at the concentrations of chimeras actually found *in vivo* in our cells, the disruption of clusters containing only a few percent of total PIP₂ leads to significant reduction in FRET, and thus should be readily detectable in our experiments.

We applied this technique to study clustering of membrane PIP₂ by coexpressing chimeras of PIP₂-binding PH domains with (monomeric) green and red fluorescent proteins. Considerable effort was invested into minimizing experimental variability; for details, please refer to 'Optimizing assess-

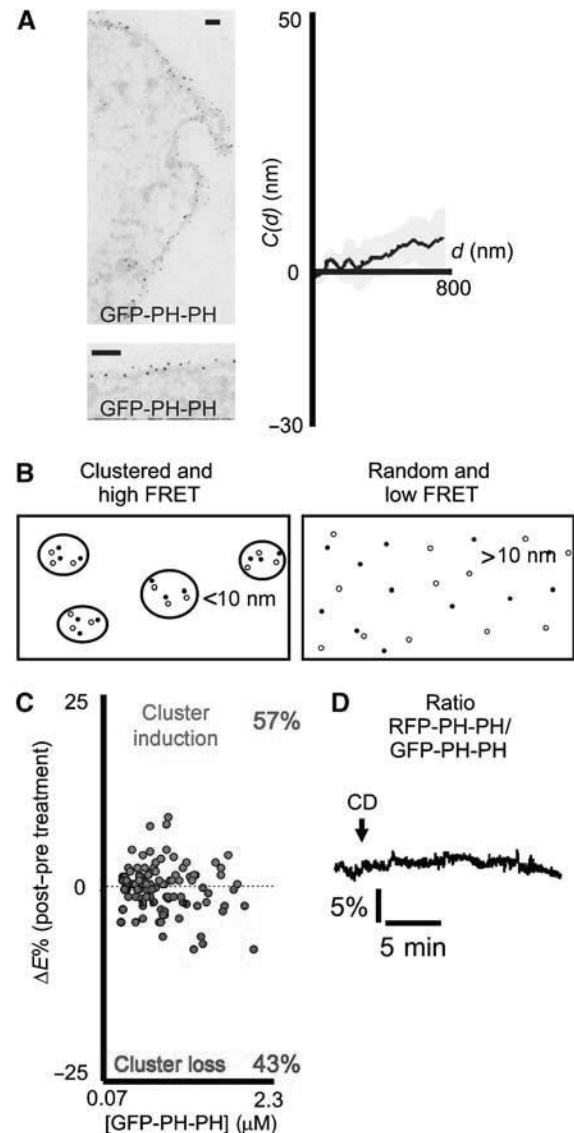


Figure 3 PIP₂ distribution along the plasma membrane is homogeneous. (A) (Left panel) Representative EM images of cells expressing GFP-PH-PH, gold-labeled with antibodies against GFP. The average density of the probe is 0.029 ± 0.003 gold/nm membrane. Scale bar, 100 nm. (Right panel) Cluster analysis of pooled data obtained with GFP-PH-PH indicates randomness of labeling. Results from cells expressing the probe at very low or quite high levels gave similar results. (B) Schematic representation of cluster assay by live cell FRET. When PIP₂ probes are clustered (left panel), the mean probe distance will be smaller, resulting in higher FRET efficiencies in comparison to the random situation (right panel). See Supplementary data for a detailed treatment of the effects of clustering on FRET. (C) For various probe concentrations, the effect of CD treatment on the efficiency of FRET between GFP-PH-PH and RFP-PH-PH was determined using fluorescence lifetime imaging (FLIM). A gain in FRET is depicted in green, and a loss in red. For all concentrations, the FRET changes align along the x-axis, with an average change of $0.024 \pm 0.35\%$, indicating no effect of CD treatment on clustering. Shown is a representative out of five experiments. (D) Representative ratiometric FRET trace from a single HEK293 cell expressing GFP-PH-PH and RFP-PH-PH. Treatment with CD to disrupt clustering did not affect FRET.

ment of clustering by FRET' in Supplementary data (section 2). First, we derived energy transfer from donor fluorescence lifetime measurements (FLIM; see Materials and methods),

which is a quantitative, although rather involved, technique to assess FRET. In five independent experiments, comparison of FRET values before and after disruption of rafts in populations of 80–150 cells failed to show differences (difference: $0.024 \pm 0.35\%$; see Figure 3C). In a second set of experiments, FRET was followed by ratiometry in single living cells during cholesterol extraction. This approach, although not very quantitative, is extremely sensitive to changes from baseline (Figure 3D). Again, disruption of rafts by treatment with CD was without detectable effect ($N=25$). Taken together, our experiments indicate that PIP_2 is not enriched in microdomains at the plasma membrane.

Critical evaluation of the previous results in the context of published literature

Having determined that PIP_2 distributes homogeneously along the plasma membrane, two critical questions remained. First, what might be the reason that the above-mentioned data are at variance with data obtained by biochemical approaches for several cell types (Pike and Casey, 1996; Liu *et al.*, 1998)? In these studies, the enrichment of PIP_2 in low-density Triton X-100-insoluble fractions has been interpreted to reflect its selective partitioning into lipid rafts. Because a more recent study reported that detergent may induce lipid clustering itself (Heerklotz, 2002), we tested the effect of Triton X-100 on PIP_2 clustering in live cells. Figure 4A (left panel) shows that treatment with only 0.0025% Triton X-100 caused a substantial increase in FRET in living cells coexpressing GFP-PH-PH and RFP-PH-PH, as detected ratiometrically. This deviation from baseline does not reflect a direct effect of Triton X-100 on the fluorophores, because the emission ratio of RFP-PH-PH and GFP-PH(R40L), which does not bind to PIP_2 , was not affected (Figure 4A, right panel). To quantitate the magnitude of the effect of Triton X-100, FLIM experiments were performed (Figure 4B and C). In good agreement with the previous result, treatment with Triton X-100 caused an increase ($4.0 \pm 0.52\%$, mean $\pm$ s.e.) in FRET between GFP-PH-PH and RFP-PH-PH in the vast majority of measurements, while it had no effect on control cells expressing only GFP-PH-PH. Statistical analysis by Student's *t*-test for paired observations showed that the difference between post- and

pretreatment values of FRET was extremely significant ($P < 10^{-10}$), contrasting with $P=0.95$ for the effects of CD. Similar results were obtained in other cell types including MDCK and N1E-115 cells. Even larger FRET increases were observed upon addition of 0.5–1% Triton X-100 in ratiometric FRET experiments performed at 4°C. However, as Triton caused rapid lysis of the cells and consequent loss of the labels, quantitation was not experimentally feasible. We next checked whether Triton-induced FRET increases were accompanied by visible clustering of GFP-PH-PH. Confocal images acquired at high magnification (Figure 4D) failed to reveal clusters at 0.0025% Triton X-100 in most cases, but addition

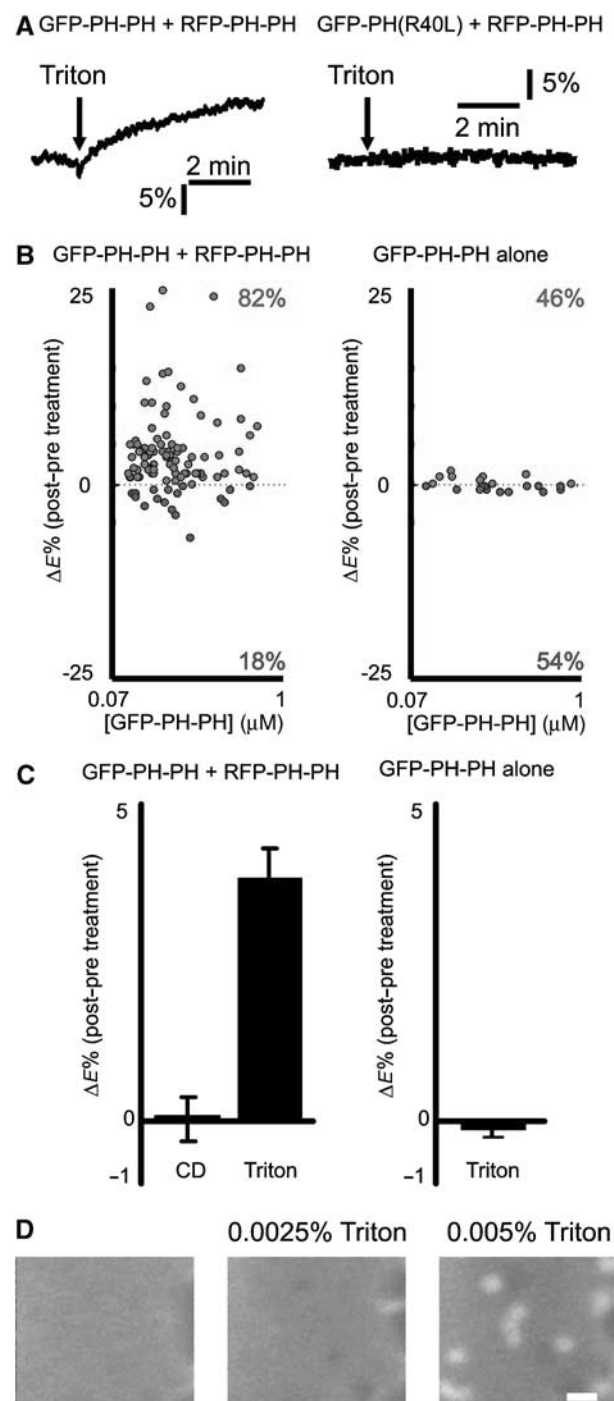


Figure 4 Triton X-100 induces non-pre-existing PIP_2 clusters. **(A)** Ratiometric FRET traces from a cell coexpressing GFP-PH-PH and RFP-PH-PH (left panel) or a control cell with GFP-PH(R40L) and RFP-PH-PH (right panel). Addition of Triton X-100 (0.0025%) to cells expressing GFP-PH-PH and RFP-PH-PH, but not control cells, caused significant increases in FRET. Higher concentrations of Triton X-100 caused larger FRET changes but also increased permeabilization, causing fluorescence to leak out. Representative traces from experiments performed at least 12 times. **(B)** Effect of Triton X-100 treatment on FRET between GFP-PH-PH and RFP-PH-PH (left panel). Cells on a coverslip were marked and FRET was determined by FLIM. After Triton treatment, FRET in the same cells was measured again, and differences are plotted versus the expression level. Triton X-100 treatment did not affect control cells expressing only GFP-PH-PH (right panel). A gain in FRET is depicted in green, and a loss in red. **(C)** Statistical analysis (mean and s.e.) of the data from Figure 3C and panel D of this figure illustrates lack of effect of CD treatment ($P > 0.95$), whereas Triton X-100 induces significant clustering ($P \leq 0.001$) in cells expressing both GFP-PH-PH and RFP-PH-PH. **(D)** Direct demonstration of Triton X-100-induced clustering of GFP-PH-PH in an HEK293 cell. Treatment with just 0.005% Triton X-100 induced visible clusters. Scale bar, 1 μm.

of 0.005% (which caused a larger increase in FRET ratio) induced enrichment of fluorescence in submicrometer-sized domains within a minute (see Supplementary data, Movie 1). This suggests that Triton causes subresolution PIP₂ clusters at 0.0025% whereas at just twice that concentration, domains fuse to become visible clusters. These data indicate, first, that the here employed clustering assays are sensitive enough to report PIP₂ clustering and, second, that Triton X-100 induces non-pre-existing clusters, which likely explains the discrepancy between our findings and previous biochemical reports.

The second critical question concerns the cause of the observed acute disruption of NKA-induced PLC signaling upon cholesterol extraction. Our experiments show that cholesterol extraction does not disrupt NK2r clustering, as determined by EM, or influence PIP₂ distribution, as determined by FRET measurements. How, then, does cholesterol extraction interfere with PIP₂ hydrolysis induced by triggering the NKA receptor but not the ETBr? Apart from its effects on lipid rafts, the cholesterol content of the membrane also critically influences other processes (Munro, 2003), including lateral membrane fluidity (Ohvo-Rekila *et al.*, 2002; Ramstedt and Slotte, 2002) and clathrin-mediated endocytosis (Rodal *et al.*, 1999; Subtil *et al.*, 1999). It was recently reported that certain GPCRs require constitutive, ligand-independent recycling to remain responsive to their cognate agonists (Dale *et al.*, 2001; Kittler *et al.*, 2004; Theriault *et al.*, 2004; Xia *et al.*, 2004). This process, termed tonic recycling, involves clustering by lateral movement in the plasma membrane, followed by clathrin-dependent endocytosis and subsequent exocytosis through lipid vesicles.

To examine whether CD treatment interferes with these processes, we studied the dynamics of GFP-tagged NKA and ET receptors by confocal time-lapse microscopy. First, we studied the effects of monensin, an ionophore that prevents exocytosis of vesicles derived from early endosomes by neutralizing intraluminal pH (Basu *et al.*, 1981). As shown in Figure 5, treatment with monensin caused GFP-NK2r to accumulate in internal structures within minutes (Figure 5A). Concomitantly, NK2r-mediated PIP₂ hydrolysis is blocked (data not shown). Strikingly, monensin treatment did not affect GFP-tagged ETBr (Figure 5B). This suggests that in the absence of exocytosis, tonic recycling causes rapid internalization of NK2r from the membrane. In line with this idea, monensin-induced internalization of NK2r was completely inhibited by CD treatment (Figure 5C). Apart from the effect on receptor endocytosis, cholesterol extraction also strongly interfered with lateral mobility of both GFP-tagged receptors at the plasma membrane, as visualized by FRAP experiments (Figure 5D). Interestingly, the lateral mobility of both GFP-NK2r and GFP-ETBr was blocked by brief CD treatment. The differential effect of CD treatment on NKA- and ET-induced PIP₂ hydrolysis might thus be explained by the dependence of the NK2r, but not the ETBr, on tonic recycling. Whether possible additional effects of cholesterol extraction may play a role (for review, see Munro, 2003) remains to be determined. In any case, our experiments strongly suggest that CD treatment disrupts NKA-mediated signaling by interfering with its tonic recycling, rather than by disrupting rafts.

Concluding remarks

In this study, the hypothesis of raft-delimited PIP₂ signaling was addressed using EM and biophysical techniques. Our

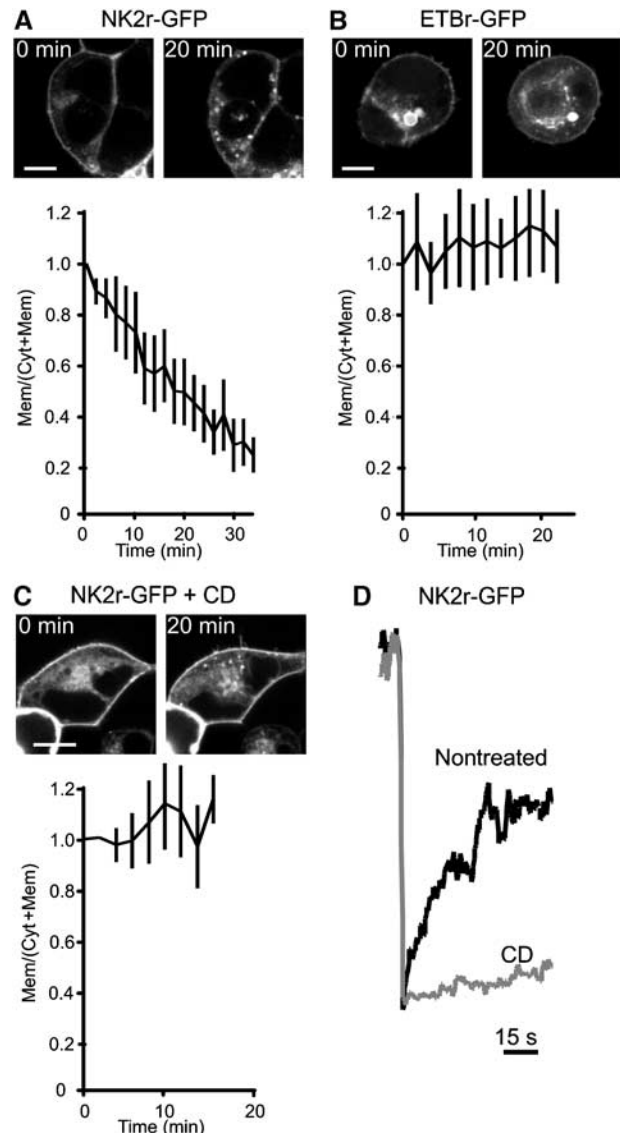


Figure 5 Cholesterol extraction inhibits NKA receptor internalization. HEK293 cells were transfected with GFP-tagged NK2r (A) or ETBr (B) and at $t=0$ s treated with monensin to prevent receptor recycling. Confocal images were acquired every 2–3 min and the ratio of membrane to cytosol fluorescence was determined by image analysis for each time point (see Materials and methods). The initial ratio was normalized to 1.0 to allow averaging of the results from 5 to 10 experiments for each condition. Mean and s.e. are plotted versus time. Scale bars, 5 μ m. Note that receptors have not been exposed to agonists, and that all experiments have been performed in serum-free bicarbonate/HEPES-buffered saline (see Materials and methods). (C) Following treatment of cells with CD for 30 min, at $t=0$ monensin was added and cells were assayed for internalization of fluorescence. Note that tonic recycling of the NK2r is blocked by cholesterol extraction. (D) Diffusion of GFP-tagged NK2r at the plasma membrane was studied with FRAP in nontreated (black line) and CD treated (gray line) HEK293 cells. Cholesterol extraction dramatically lowers NK2r mobility.

experiments showed that the NK2r appears strongly clustered at the membrane, and that its coupling to PIP₂ hydrolysis is completely abolished upon cholesterol extraction. The opposite results were found for the ETBr. Nevertheless, independent lines of evidence presented here reject the possibility of raft-delimited PIP₂ pools, but rather indicate that both

receptors share a single homogenous pool of PIP₂ at the plasma membrane. Our results are at variance with biochemical studies that show that PIP₂ is enriched in detergent-insoluble fractions (Hope and Pike, 1996; Pike and Casey, 1996; Pike and Miller, 1998; Laux *et al.*, 2000; Hilgemann *et al.*, 2001; Klopfenstein *et al.*, 2002; Yin and Janmey, 2003; Hur *et al.*, 2004). It should be emphasized that there is an important distinction between the detection of PIP₂ molecules by lipid extraction assays, on the one hand, and by 'labeling' techniques (such as used in EM and FRET analysis), on the other hand. Labeling with either PIP₂-specific antibodies or PH domains may fail to identify PIP₂ that is already bound to (signaling) proteins, while these lipids are likely detected in extraction assays. Conversely, it is hard to envision how bound PIP₂ molecules could function as messengers since they are not free to interact with the PIP₂-binding domains of signaling proteins. However, the observation that treatment of intact cells with Triton X-100 by itself (even at doses as low as 0.0025%) can induce PIP₂ clustering indicates that, at the least, care should be taken in interpreting the results of detergent extraction experiments.

Our experiments also cast serious doubt on the value of CD-mediated cholesterol extractions to prove involvement of rafts in biological processes. We showed that the CD-induced rapid abrogation of NKA-evoked PIP₂ hydrolysis does not involve disruption of receptor or substrate clustering. Rather, the experiments suggest that CD interferes with agonist-independent tonic recycling by blocking receptor endocytosis, and perhaps also by rigidifying the plasma membrane. In support of this view, in a very recent paper (Cezanne *et al.*, 2004), colocalization of the NK2r with clathrin-coated pre-pits was reported. In any case, the emerging, rather broad, Assortment of reported CD effects does not hamper the interpretation of the data here presented. It remains unchallenged that CD strongly disrupts lipid rafts, and thus its lack of effect on PIP₂ clustering as determined by live cell FRET experiments can only be interpreted to indicate absence of PIP₂ from rafts.

Materials and methods

Materials

Ionomycin and NKA were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA), and ET, CD and α -cyclodextrin were from Sigma Chemical Co. (St Louis, MO). Rhodamine-6G was from Molecular Probes (Eugene, OR) and Triton X-100 was from Merck (Darmstadt, Germany).

Constructs

eGFP-PH(PLC δ 1), eCFP-PH(PLC δ 1) and eYFP-PH(PLC δ 1) in pcDNA3 vectors are as described (van der Wal *et al.*, 2001). Expression vectors encoding the human NK2r (Alblas *et al.*, 1995) and the human ETBr were kind gifts from Dr W Moolenaar, Division of Cellular Biochemistry. To tag the NK2r with GFP, the VSV tag of the NK2r in a pMT2-VSV vector (Alblas *et al.*, 1995) was replaced by the cDNA for GFP using *NotI* and *EcoRI* restriction sites. The ETBr was tagged with GFP by cloning the cDNA for the ETBr (restriction sites *HindIII* and *BamHI*) and the cDNA for GFP (restriction sites *BamHI* and *NotI*) into pcDNA3. The NK2r was cloned N-terminally of the HA tag present in pcDNA3-HA, using *HindIII* and *NotI*. To obtain monomeric GFP-PH, the cDNA for GFP present in eGFP-PH(PLC δ 1) (van der Wal *et al.*, 2001) was exchanged by a cDNA encoding a GFP unable to dimerize (A206K; Zacharias *et al.*, 2002). A second cDNA encoding the PH domain of PLC δ 1 was cloned using *EcoRI* between the regions encoding GFP and the PH domain of monomeric GFP-PH to obtain the GFP-PH-PH. mRFP-PH was a kind

gift from Dr Tamas Balla (National Institutes of Health, Bethesda), where the GFP cDNA of eGFP-PH (Varnai and Balla, 1998) was exchanged for a cDNA encoding the monomeric RFP (Campbell *et al.*, 2002). To obtain mRFP-PH-PH, we cloned a second cDNA encoding the PH domain of PLC δ 1 between the mRFP and PH domain cDNAs using *BamHI*.

Cell culture and transfection

HEK293 cells were seeded on 25-mm glass coverslips in six-well plates (for microscopy) or in a 15-cm Petri dish (for EM or biochemical assays) in DMEM supplemented with 10% FCS and antibiotics. Constructs were transfected using calcium phosphate precipitate, at $\sim 0.8 \mu\text{g}$ DNA per well or $\sim 13 \mu\text{g}$ DNA per Petri dish. After 12 h, the medium was refreshed.

Monitoring of PIP₂ levels by FRET ratiometry

Kinetic analysis of PIP₂ breakdown by FRET was assayed as described (van der Wal *et al.*, 2001). In brief, cells transfected with CFP-PH and YFP-PH at 1:1 ratio were placed on an inverted Zeiss Axiovert 135 microscope equipped with a dry Achromplan $\times 63$ (NA 0.75) objective. Excitation was at $425 \pm 5 \text{ nm}$, and CFP as well as YFP emission was collected simultaneously at 475 ± 15 and $540 \pm 20 \text{ nm}$, respectively, using photomultipliers. For experiments with cells expressing GFP-PH-PH and mRFP-PH-PH, excitation was at $488 \pm 5 \text{ nm}$, and emission was collected at 535 ± 25 and $> 590 \text{ nm}$, respectively. Both photomultiplier signals were filtered at 0.1 Hz and digitized at three samples per second. FRET is expressed as the ratio of acceptor to donor fluorescence. At the onset of the experiment, the ratio was adjusted to 1.0, and FRET changes were expressed as % deviations from base line. It has been previously documented (Stauffer *et al.*, 1998; Varnai and Balla, 1998; van der Wal *et al.*, 2001) that monitoring translocation of the PH domain of PLC δ 1 by either confocal imaging or FRET ratiometry reliably records the activation of the PLC signaling cascade.

Confocal microscopy

Coverslips with cells expressing various constructs were mounted in a culture chamber imaged using an inverted TCS-SP2 confocal microscope (Leica, Mannheim, Germany). Cells were imaged in bicarbonate-buffered saline (containing in mM: 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 23 NaHCO₃, 10 glucose and 10 HEPES at pH 7.2) under 5% CO₂ at 37°C.

Monitoring of receptor internalization

Cells expressing GFP-tagged receptors were imaged every 2–3 min on the confocal, and images were stored for off-line analysis. Membrane localization was expressed as the ratio of the membrane fluorescence to total cell fluorescence. To allow averaging of the results from separate experiments, the ratio was normalized at the onset of the experiment.

Cryoimmunogold electron microscopy

Transfected cells were fixed for 24 h in 4% paraformaldehyde in 0.1 M PHEM buffer (240 mM PIPES, 100 mM HEPES, 8 mM MgCl₂, 40 mM EGTA, pH 6.9) and then processed for ultrathin cryosectioning as previously described (Calafat *et al.*, 1997). Ultrathin frozen sections were incubated at room temperature with mouse monoclonal anti-HA 12CA5 (Boehringer) or rabbit anti-GFP (Clontech), followed by incubation with 10 nm protein A-conjugated colloidal gold (EM Lab., Utrecht University, The Netherlands) as described (Calafat *et al.*, 1997). After immunolabeling, cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM 10 electron microscope (Eindhoven, The Netherlands). For the controls, the primary antibody was replaced by a nonrelevant murine or rabbit antiserum.

Cluster analysis of immunogold-labeled EM images

For analysis of clustering, EM images of immunogold-labeled ultrathin cryosections were digitized and imported in PhotoShop 5.0. Quantitation was by Ripley's K analysis (Ripley, 1977, 1979; Philimonenko *et al.*, 2000), adapted to one-dimensional data as follows. First, positions of the membrane and of individual gold particles were digitized manually. For each photomicrograph, the length of the membrane and total number of particles were determined, and the average density (λ) was calculated from that. Then, for each gold particle, the number (n) of neighbor particles within a given distance d was determined. We varied d between 1

and ~1000 nm; as a boundary condition, when d extended beyond either end of the membrane, data were taken from the opposite end. From these data, we calculated $N(d)$, the mean number of neighbors for each d :

$$N(d) = \text{mean}(n(d)) \quad (1)$$

For randomly distributed particles, the average expected number of neighbors for given d depends on the density:

$$E(d) = 2\lambda d \quad (2)$$

To arrive at a concentration-independent parameter for clustering, the observed frequencies $N(d)$ are first normalized with respect to density:

$$N^*(d) = N(d)/2\lambda \quad (3)$$

Similarly, in the random case, a density-independent estimate is given by

$$E^*(d) = 2\lambda d/2\lambda = d \quad (4)$$

Subsequently, clustering is expressed by subtracting the expected distribution from the observed distribution:

$$C(d) = N^*(d) - E^*(d) = N^*(d) - d \quad (5)$$

The dimension of $C(d)$ is distance (m). Note that for randomly distributed particles, $C(d)$ equals 0 for all d .

Clustering was assessed by plotting $C(d)$ as a function of d . The interpretation of these graphs is analogous to those of the linearly transformed two-dimensional Ripley's K analysis used by Prior *et al* (2003): clusters are apparent as deviations of $C(d)$ from 0, with the position of the first maximum indicating the mean cluster size, and the first intersection of $C(d)$ with the horizontal axis indicating half of the mean distance between clusters.

Fluorescence lifetime imaging

FLIM experiments were performed on an inverted Leica DM-IRE2 microscope equipped with Lambert Instruments (Leutengewolde, the Netherlands) frequency domain lifetime attachment, controlled by the vendors EZFLIM software. GFP was excited with ~4 mW of 430 nm light from a LED modulated at 40 MHz and emission was collected at 490–550 nm using an intensified CCD camera. To calculate the GFP lifetime, the intensities from 12 phase-shifted images (modulation depth ~70%) were fitted with a sinus

function, and lifetimes were derived from the phase shift between excitation and emission. Lifetimes were referenced to a 1 μ M solution of rhodamine-G6 in saline that was set at 4.11 ns lifetime. The measured lifetime of GFP alone was 2.4 ns, and the FRET efficiency E was calculated as

$$E = 1 - \frac{\text{measured lifetime}}{2.4}$$

FRET efficiencies from large populations (80–150 cells per experiment) were measured before and after treatment with CD (10 mM) or Triton X-100. Differences in FRET were plotted against the corresponding concentration of the GFP-PH-PH probe, determined as described (van der Wal *et al*, 2001) from separately acquired images. Very similar results ensued when FRET was plotted against mRFP-PH-PH concentration. All experiments were repeated many times on different days; data from a single representative experiment are shown. The data set was statistically analyzed using MicroCal Origin V5.0.

Cholesterol assay

Cells grown in six-well plates were assayed in triplicate for cholesterol using the Amplex red cholesterol assay kit from Molecular Probes Inc. (Eugene, OR). First, cells were washed once with bicarbonate-buffered saline, followed by incubation with or without 10 mM CD, or with α -cyclodextrin. Subsequently, the saline was refreshed and cells were optionally incubated for different times, or assayed immediately according to the supplier's instructions.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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Supplementary information

Relationship between labeling density and the clustering-dependence of FRET

Because FRET intensity depends to the inverse 6th power of the distance between donor and acceptor fluorophore (here GFP-PH-PH and mRFP-PH-PH), energy transfer between the PIP₂ probes is a function of both label density and clustering. To estimate the concentration range in which FRET is sensitive for clustering, we calculated dependency of FRET efficiency on both labeling density (D ; in molecules per μm^2) and clustering by Monte Carlo simulations using a custom-made Visual Basic program. Briefly, simulations include the following steps.

- [1] First, within a (virtual) flat area A of membrane, N donors and N acceptors are positioned randomly or, alternatively, clustered randomly within small circular subdomains of the membrane area. Number and size of subdomains can be varied. At the onset of each simulation, a fraction (typically $\leq 10\%$) of the donors is excited.
- [2] Then, for each excited donor, the chance of energy transfer to any of the acceptors is calculated as follows:
 - (a) distances of that donor to each of the acceptors are determined and sorted in increasing order $d_1, \dots, d_N$;
 - (b) the theoretical FRET efficiency corresponding to those distances E_d is calculated according to

$$E_d = \frac{R_0^6}{R_0^6 + d^6}$$

Where $R_0 \approx 5$ nm (Patterson et al., 2000).

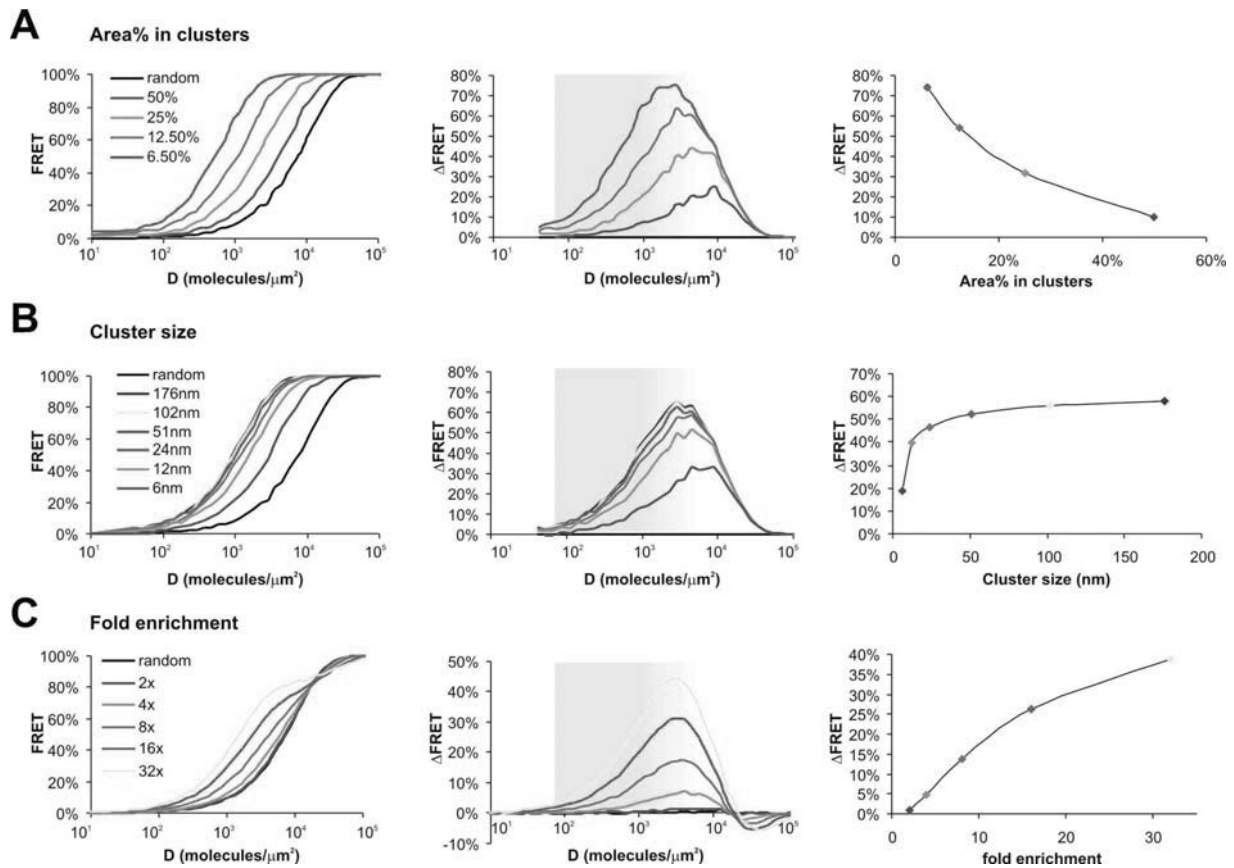
- (c) Energy transfer to the nearest acceptor is defined to occur when a computer-

generated random number r ($0 \leq r \leq 1$) is less than or equal to E_{d1} . When there is no FRET, the procedure is repeated for the next-nearest acceptor (E_{d2}), etcetera, until the chance of transfer is negligible ($< 10^{-4}$), in which case the donor does transfer energy to any acceptor.

- [3] Upon completion of these calculations for all donor-acceptor pairs, the FRET efficiency E^* (percentage of excited donor molecules that transfer their energy) is calculated.

Steps [1]-[3] were carried out for a wide range of densities ($5 \leq D \leq 125,000$) and for various conditions of clustering, including different area% (defined as % of total membrane area that is in clusters), variable cluster sizes, and variations in the fraction of fluorophores that locate to the clusters. For any condition, simulations were repeated for at least 5×10^5 donors, and the results were averaged. The resulting calculated FRET efficiencies were plotted versus probe density at the membrane.

Using confocal imaging and whole-cell patch clamping, we estimated that our cells have an average volume and membrane area of 1.3 pL and 1100 μm^2 , respectively. For GFP constructs containing a single pleckstrin homology domain, which locate for $\sim 50\%$ at the membrane, we previously reported expression levels up to 12 μM (van der Wal et al., 2000) in N1E-115 neuroblastoma cells. Occasionally, cells expressing up to 30 μM were observed. In the HEK293 cells used in this study, expression levels of each of the fluorescently tagged tandem-PH constructs were generally below ~ 2.5 μM . These chimeras localize exclusively to the



Supplementary Figure 1 FRET dependency on fluorophore density and clustering. (A) Fluorophores are present in clusters that constitute the indicated percentage of the total membrane area. (B) Effect of cluster size on FRET for fixed area% = 12.5. Note that down to 12 nm, cluster size has little influence on FRET efficiency. (C) Monte Carlo simulations for area% = 10 with indicated enrichments of labeling in the domains. Grayed regions in the middle panels indicate densities observed in the experiments. For further details, see the text.

plasma membrane, and thus the cellular concentration C is related to the average membrane density D as:

$$D = C \times 6 \times 10^{23} \times 1.3 \times 10^{-12} / 1100$$

$$D = C \times 7.1 \times 10^8 \text{ (molecules / } \mu\text{m}^2\text{)}$$

The results are depicted in **Supplementary Figure 1**. Initially, we determined E^* for the random situation (**A, left panel, black line**). The simulation predicts ~100% energy transfer at 4×10^4 molecules/μm². This corresponds to a concentration of ~56 μM for each of the constructs; expression levels this high have never been observed

in this study. Half-maximal FRET occurs at 8×10^3 molecules/μm², and some transfer should be detectable as low as 3×10^2 molecules/μm² (2% FRET). This graph further depicts the simulation results for localization of the fluorophores in a single domain that covers various percentages of the total area (area%), as indicated. Decreasing the area% causes the curves to progressively shift left by up to 20-fold. The maximal decrease in FRET that can be obtained by complete disruption of clusters is found by subtracting these curves from the random, non-clustered case (**middle panel**). From this graph, it is deduced that significant FRET changes

can be expected even if clusters make up 50% of the membrane area. At a fixed label concentration, the degree of FRET loss is seen to decrease for increasing area% of the clusters (*right panel*).

In **Figure 1B, left panel**, the effects of different cluster sizes are compared for a fixed area% = 12.5%. It is found that deviations from the random situation are maximal at large cluster sizes (> 6 nm). Nevertheless, even at very small cluster sizes, substantial loss of FRET should result from cluster disruption (*middle and right panels*).

Figure 1C depicts the results from mixed-population simulations where labels are present in clusters as well as in the rest of the membrane. In mixed populations, the relationship between expression level and FRET efficiency is distinctly biphasic, with the initial, rapid rise at low levels reflecting FRET in clusters, whereas the second more moderate rise represents the remaining membrane area. In these simulations, the space occupied by clusters was kept constant (area% = 10%) and various enrichments within the clusters were modeled at a fixed total numbers of fluorophores. For increasing enrichment in the clusters, we see a progressive shift to the left within the clusters.

From simulations such as those presented in Figure 1, it is evident that for a wide variety of clustering conditions, micro domain disruption must cause significant loss of FRET at expression levels detected in our cells (0.1-2.5 μ M). For example, from Figure 1C it is deduced that enrichment of only 4-fold in clusters that make up 10% of the membrane will give rise to easily detectable (~ 5%) FRET changes at physiological expression levels. Thus, PIP₂ enrichments of 10- to 20-fold, as proposed by Hope and Pike (Hope and Pike, 1996) based on detergent-dependent methods, should be readily detectable.

We conclude that the lack of effect of CD treatment on FRET efficiency observed in large populations of cells can only be interpreted to indicate that PIP₂ is not clustered in these cells.

Optimizing assessment of clustering by FRET: experimental details

For all clustering experiments, monomeric eGFP (A206K) and monomeric RFP (Campbell et al., 2002) were used as tags to avoid possible problems due to inherent dimerization of the fluorescent proteins (Zacharias et al., 2002).

In line with reported results (Kenworthy et al., 1998; Glebov et al., 2004; Zacharias et al., 2002), our initial experiments showed considerable cell-to-cell variation in detected FRET values. The reproducibility was enhanced by using constructs with tandem-repeats of the PH domain (see M&M), which causes complete membrane localization. This makes the assay immune to small changes in [PIP₂] that may result from CD treatment (Kwik et al., 2003), thus assuring that any observed differences in FRET reflect distribution of the constructs along the plasma membrane rather than a variable degree of membrane localization. It also avoids bias originating from free 'non-fretting' fluorophores in the cytosol. Variability was also considerably reduced by collecting paired observations, i.e. by comparing values pre- and post disruption of rafts from the same cells. Analysis of clustering by FRET requires quantitative measurements, and therefore FRET was previously determined by the acceptor photobleaching method (Kenworthy et al., 1998; Zacharias et al., 2002; Nichols et al., 2003). However, this destructive technique is incompatible with paired observations. Therefore, in this study FRET was detected by donor fluorescence lifetime

measurements (FLIM). Using a very stable FLIM setup with <1% instrumental drift over several hours allowed reliable detection of population FRET changes considerably less than 1%.

To use a second, independent technique, experiments were also repeated using FRET ratiometry (see M&M). This approach, though not very quantitative, is extremely sensitive to changes from baseline and offers high temporal resolution.

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Chapter 5

Correcting confocal acquisition to optimize imaging of fluorescence
resonance energy transfer by sensitized emission
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Correcting Confocal Acquisition to Optimize Imaging of Fluorescence Resonance Energy Transfer by Sensitized Emission

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ABSTRACT Imaging of fluorescence resonance energy transfer (FRET) between suitable fluorophores is increasingly being used to study cellular processes with high spatiotemporal resolution. The genetically encoded Cyan (CFP) and Yellow (YFP) variants of Green Fluorescent Protein have become the most popular donor and acceptor pair in cell biology. FRET between these fluorophores can be imaged by detecting sensitized emission. This technique, for which CFP is excited and transfer is detected as emission of YFP, is sensitive, fast, and straightforward, provided that proper corrections are made. In this study, the detection of sensitized emission between CFP and YFP by confocal microscopy is optimized. It is shown that this FRET pair is best excited at 430 nm. We identify major sources of error and variability in confocal FRET acquisition including chromatic aberrations and instability of the excitation sources. We demonstrate that a novel correction algorithm that employs online corrective measurements yields reliable estimates of FRET efficiency, and it is also shown how the effect of other error sources can be minimized.

INTRODUCTION

Fluorescence resonance energy transfer (FRET), the radiationless transfer of energy from a donor fluorophore to a closeby acceptor fluorophore, is rapidly gaining importance as a means to study molecular interactions in single cells. FRET is apparent as quenching of the donor and increased acceptor emission. Its main applications include the study of interactions between different proteins tagged with either a donor or an acceptor fluorophore (intermolecular FRET), following sterical alterations within a single protein labeled with both a donor and an acceptor (intramolecular FRET), and as the readout signal for biochemical sensors. In the latter case, constructs are engineered to respond to changes in a cellular signal (e.g., cAMP, Ca^{2+} , or protein phosphorylation) by altering FRET. Depending on these different applications, very different design considerations may apply to the detection method. For FRET to occur, the fluorescent dipoles of donor and acceptor must be properly aligned, and there must be overlap between the donor emission spectrum and the acceptor excitation spectrum (Lakowicz, 1999). Furthermore, resonance energy transfer is steeply dependent on the distance between the fluorophores, decreasing with the sixth power of the distance. Characteristic half-maximal distances (Förster radii) for a number of biologically important fluorophores are $\sim 4\text{--}5$ nm, and thus the distance range over which FRET changes ($\sim 2\text{--}10$ nm) is well-matched to the dimensions of individual proteins.

The recent introduction of color mutants of the Green Fluorescent Protein as donor and acceptor labels for FRET has fuelled interest in this technique. Because Green

Fluorescent Proteins are genetically encoded, laborious in vitro conjugation of fluorophores to proteins as well as the introduction into the cell by microinjection or other means are no longer necessary. By far the most popular variants for FRET are the Cyan and Yellow variants, CFP and YFP, respectively (Tsien, 1998). First used to demonstrate a genetically encoded calcium sensor (cameleon; Miyawaki et al., 1997), this FRET pair has been the basis for several interesting sensors developed over the last few years, including those for cAMP, cGMP, PIP_2 , phosphorylation, and protein activation status (Zaccolo and Pozzan, 2002; Honda et al., 2001; van der Wal et al., 2001; Nagai et al., 2000; Mochizuki et al., 2001). Despite their bulkiness, CFP and YFP are also successfully applied to study protein-to-protein interactions and conformational changes. Concomitantly, several approaches to image FRET with this pair from single (living) cells have been exploited (for review, see Wouters et al., 2001). These include acceptor photobleaching, a technique whereby the fluorescent acceptor is destroyed and which therefore is not suited for timelapse imaging, and fluorescence lifetime imaging of the donor. Fluorescence lifetime imaging requires dedicated and expensive equipment, and CFP is not particularly suited for this technique because it intrinsically possesses several fluorescence lifetimes (Pepperkok et al., 1999). The most widely employed approach therefore is to calculate sensitized emission (i.e., the acceptor fluorescence resulting from energy transfer from excited donor molecules) from separately acquired donor and acceptor images. Because the spectra of CFP and YFP show considerable overlap, the detected sensitized acceptor emission must be corrected for leakthrough of the donor emission into the acceptor emission channel and for direct excitation of the acceptor during donor excitation. The latter correction requires that an additional image is captured from the acceptor, directly excited at its own wavelength. Several correction schemes were worked out for images that were acquired with wide-field fluores-

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cence microscopes equipped with charge-coupled-device (CCD) cameras (Gordon et al., 1998; Nagy et al., 1998; Hoppe et al., 2002).

In this study, we focus on CFP/YFP FRET imaging by confocal microscopy. Confocal imaging has a number of advantages over wide-field imaging, the most important of which is that it produces crisp optical sections of the preparation. However, detecting sensitized emission by multiexcitation confocal acquisition raises a number of complications in the correction scheme. Unlike FRET imaging with digital camera systems, which have a single detector and a fixed ratio of excitation intensities that is determined by the filter sets, during (conventional) confocal imaging at least two individual detectors (photomultiplier tubes, PMT) are used, as well as two independent excitation laser lines. Dependent on the design of the deployed confocal instrument the spectral response of the detectors may even be tuned individually. Both donor and acceptor excitation intensities and PMT gain provide additional degrees of freedom and can be independently controlled by the user. Therefore, relative sensitivity for given fluorophores and leakthrough coefficients are not necessarily constant and need in all cases to be determined for each set of experimental conditions. Furthermore, errors stem from temporal variations in the relative intensities of the excitation lines for CFP and YFP, from slight misalignment between laser lines, and from the axial chromatic aberrations of the optical system. We quantified these effects and describe methods to correct for them. We also show that CFP is optimally excited at 430 nm to detect FRET, and we demonstrate suitability of a frequency-doubled diode laser for this application. These improvements result in a significant increase in quality of confocal sensitized emission images.

MATERIALS AND METHODS

Materials

Ionomycin was from Calbiochem-Novabiochem (La Jolla, CA), BAPTA was from Sigma Chemical (St. Louis, MO), and 0.17- μ m, yellow-green fluorescent beads (490/515, component B from the PS-Speck Microscope Point Source Kit P-7220) were from Molecular Probes (Eugene, OR).

Constructs and transfection

The yellowameleon (2.0 and 3.1) in pcDNA3 were a kind gift of Drs. R. Tsien and A. Miyawaki (Miyawaki et al., 1997). The GST-tagged yellowameleon 2 proteins were purified from an *Escherichia coli* culture expressing the pGEX261 vector inserted with the yellowameleon 2.0 into the *Hind*III and *Not*I sites. eYFP-PH(PLC δ 1) and eCFP-PH(PLC δ 1) in pcDNA3 expression vector were described elsewhere (van der Wal et al., 2001). Transfections were performed using calcium phosphate precipitate, at $\sim$ 0.8 μ g DNA/well. After overnight transfection, cells were washed with fresh medium and incubated until usage.

Fluorometry

For fluorometry, a dual-emission channel Quantamaster fluorometer (Photon Technology International, Lawrenceville, NJ) was used. Purified fluorescent proteins were dissolved at a final concentration of $\sim$ 1 μ M in HEPES-buffered intracellular solution. Free $[Ca^{2+}]$ of the solution was set to 50 nM using BAPTA. Fluorescence was detected from 2-ml aliquots of solution, kept at 37°C in a stirred cuvette.

Confocal microscopy

For registration of images, coverslips with transfected cells were transferred to a culture chamber and mounted on the inverted microscope. The cells were kept in bicarbonate-buffered saline (containing in mM: 140 NaCl, 5 KCl, 1 MgCl₂, 10 glucose, 1 CaCl₂, 23 NaHCO₃, and 10 HEPES, pH 7.2), under 5% CO₂ at 37°C. Imaging was with a DM-IRE2 inverted microscope fitted with TCS-SP2 scanhead (Leica, Mannheim, Germany). CFP was excited at 430 nm and detected from 460 to 490 nm, and YFP was excited at 514 nm, and detected from 528 to 603 nm. Excitation power was $\sim$ 100–400 μ W.

Image processing

Image acquisition and specimen refocusing were automated from within a custom-made Visual Basic (v6.0) program by calling commands from the Leica macro tool package. To obtain FRET images, the following post-acquisition image processing steps were carried out. First the imported images were shading-corrected, and optionally smoothed. Then regions of interest (ROIs) were designed corresponding with cells expressing only CFP or YFP. From these ROIs, correction factors were measured and calculated. With these factors, sensitized emission was calculated as outlined in Results and Discussion. The sensitized emission image was ratioed to the excitation intensity-corrected $M_{\text{DirectAcceptor}}$ or F_{Donor} (see Appendix) image to obtain the apparent FRET efficiency picture. Images were scaled appropriately for onscreen visualization. To suppress excessive noise in dim parts of the images, a mask was applied as follows. First, the FRET efficiency image was smoothed with a spatial filter to distinguish noise from signal. Then, a mask was created by setting a threshold equal to the background from this image. Subsequently, unwanted noise in dim areas was rejected by applying this mask to the original, unfiltered FRET image.

RESULTS AND DISCUSSION

Experimental setup and corrective terms for sensitized emission

In the most general case, proteins with CFP and YFP labels are independently expressed in living cells. Relative fluorescence levels are thus not fixed, and pixel-to-pixel intensities may differ widely for each fluorophore. To image sensitized emission, acceptor fluorescence is to be detected while exciting the donor. However, due to spectral overlap the recorded image in the acceptor emission channel contains components of leakthrough of donor emission into the acceptor channel and of direct excitation of the acceptor at the donor excitation wavelength (Gordon et al., 1998; Nagy et al., 1998; Hoppe et al., 2002). Estimation of the latter term requires information on the acceptor distribution, which is gained by taking an additional image at acceptor excitation and emission wavelength. In the following treatment, it is assumed that detector gain and

offset are correctly adjusted, and that autofluorescence of the cells is either negligible, or properly subtracted (for an excellent correction method, see Nagy et al., 1998). In the more extensive treatment given in the Appendix the individual factors that influence brightness of the images (such as PMT gain, laser intensities, the CFP and YFP quantum yield, etc.) are factored out to allow clear assessment of the influence of these factors.

Thus, provided that independent estimates of cross-talk magnitude are present, straightforward corrections can be carried out from three acquired images (denoted M for Measured): donor excitation with donor emission, M_{Donor} ; donor excitation with acceptor emission, $M_{\text{IndirectAcceptor}}$; and acceptor excitation with acceptor emission, $M_{\text{DirectAcceptor}}$.

The measured images are composite images consisting of multiple terms as follows: M_{Donor} is the sum of fluorescence of the donor diminished by donor fluorescence lost to energy transfer ($F_{\text{Donor}} - F_{\text{Sen}}$), and of leakthrough components consisting of fractions of $F_{\text{DirectAcceptor}}$ (the actual acceptor fluorescence) and of F_{Sen} , as

$$M_{\text{Donor}} = F_{\text{Donor}} - F_{\text{Sen}} + \alpha F_{\text{DirectAcceptor}} + \delta F_{\text{Sen}}, \quad (1)$$

where α is the correction factor for acceptor fluorescence excited and detected at donor wavelength, and δ that for leakthrough of sensitized emission back into the donor filters.

$M_{\text{IndirectAcceptor}}$ represents the sum of fluorescence of energy transfer (F_{Sen}), leakthrough of the donor minus the component lost to energy transfer ($F_{\text{Donor}} - F_{\text{Sen}}$), and of the directly excited acceptor (F_{Acceptor}),

$$M_{\text{IndirectAcceptor}} = F_{\text{Sen}} + \beta(F_{\text{Donor}} - F_{\text{Sen}}) + \gamma F_{\text{DirectAcceptor}}, \quad (2)$$

where β is the leakthrough factor of the fluorescence of donor into acceptor filters, and γ is the excitation efficiency of the acceptor upon excitation at donor wavelength.

Finally, $M_{\text{DirectAcceptor}}$ represents the acceptor fluorescence. Formally, a component consisting of donor fluorescence, excited and emitting at acceptor wavelengths, is present. However, using the 514-nm argon ion laser line and the CFP/YFP pair, the magnitude of this component is essentially zero. Thus,

$$M_{\text{DirectAcceptor}} = F_{\text{DirectAcceptor}}. \quad (3)$$

To derive the sensitized emission, Eqs. 1 and 3 are combined as

$$F_{\text{Donor}} - F_{\text{Sen}} = M_{\text{Donor}} - \alpha M_{\text{DirectAcceptor}} - \delta F_{\text{Sen}}, \quad (4)$$

and Eqs. 3 and 4 are substituted into Eq. 2, yielding

$$F_{\text{Sen}} = (M_{\text{IndirectAcceptor}} - M_{\text{Donor}}\beta - M_{\text{DirectAcceptor}}(\gamma - \alpha\beta)) / (1 - \beta\delta). \quad (5)$$

For detailed derivation, see the Appendix (corresponding equation is Eq. A11). In Eq. 5, the parameters α , β , γ , and δ are effectively used as correction factors that must be determined independently. Estimates for α , γ , and δ can be obtained by imaging a sample with only acceptor molecules, and can then be calculated as

$$\alpha = M_{\text{Donor}} / M_{\text{DirectAcceptor}} \quad (6)$$

$$\gamma = M_{\text{IndirectAcceptor}} / M_{\text{DirectAcceptor}} \quad (7)$$

$$\delta = M_{\text{Donor}} / M_{\text{IndirectAcceptor}}. \quad (8)$$

Similarly, β is estimated from a sample with only donor molecules, as

$$\beta = M_{\text{IndirectAcceptor}} / M_{\text{Donor}}. \quad (9)$$

To obtain an indication for apparent FRET efficiency, the derived expression for F_{Sen} (Eq. 5) can be related to the total acceptor levels as

$$E_A = F_{\text{Sen}} / M_{\text{DirectAcceptor}}, \quad (10)$$

or it can be related to the donor levels, which makes the calculated efficiency over time independent of laser fluctuations (see Appendix for further detail),

$$E_D = F_{\text{Sen}} / F_{\text{Donor}}. \quad (11)$$

It is evident that any changes in cell morphology (e.g., locomotion) that occur in between acquisition of the images will severely compromise the accuracy. Therefore, the images should be acquired in rapid succession by simultaneously detecting M_{Donor} and $M_{\text{IndirectAcceptor}}$, immediately followed by $M_{\text{DirectAcceptor}}$ at its own excitation line. When acquisition parameters are chosen with some care, the Leica TCS SP2 confocal system used here, controlled by our in-house developed macro program, is capable of grabbing a full-sized (512×512 pixels) set of images in two seconds.

It should be stressed that whereas the derived expressions for FRET efficiency allow direct comparison of FRET between different preparations and for different laser intensity and PMT settings, information on either the fraction of acceptor in complex with donor or the characteristic maximum FRET efficiency between donor and acceptor in complex is lacking. Since we anticipate this to be the reality in the vast majority of experiments, estimates

of the actual fraction of donors and acceptors that engage in FRET (such as presented in FRET stoichiometry; Hoppe et al., 2002) cannot be derived from our data.

The confocal acquisition parameters

Although corrective factors for emission leakthrough and indirect excitation are analogous to those described for wide-field CCD imaging of FRET (Gordon et al., 1998), confocal acquisition introduces a major complication in that relative sensitivities for donor and acceptor emission of the detector channels are no longer fixed. With CCD acquisition, weaker fluorescent cells are imaged with increased integration time, causing both direct signals from the fluorophores as well as leakthrough terms to increase proportionally. Thus, leakthrough factors are fixed for a particular combination of fluorophores and filters, and alterations in integration time can be easily compensated for. In contrast, during confocal imaging, sensitivity is adjusted by finetuning the individual excitation line intensities and by controlling PMT gain (high voltage) and offset settings for each channel separately. In addition, since excitation sources and PMT channels are physically separate, drift will have differential effects on sensitivity for donor and acceptor fluorophores. Taken together, these factors necessitate that new estimates for α , β , γ , and δ be determined for each experiment, even if identical filter and pinhole settings are used from experiment to experiment. The advantage—on the other hand—is that the added flexibility allows simultaneous optimized acquisition of the often weak FRET signals without compromising acquisition time.

In the Appendix, dependence of the parameters α , β , γ , and δ on instrument settings is derived (Eqs. A7–A10). Note that parameters β and δ depend on signal amplifications in the utilized detector (PMT), which normally operate non-linearly, and elements in the optical path (optical filter, spectral detection bands) only, whereas α and γ are additionally influenced by relative laser line intensities. Furthermore, from Eqs. A7–A10, it is seen that $\delta = \alpha/\gamma$. This relationship, as well as the dependencies of correction factors on PMT and/or laser intensity settings were verified experimentally by imaging cells expressing CFP or YFP under a variety of settings (data not shown, but available on request).

For experiments with cells expressing CFP- and YFP-tagged constructs at ~1:1 stoichiometry, spectral detection bandwidth of the SP2 channels were set up to balance minimal cross talk with optimal collection efficiency of CFP and YFP (460–490 nm and 528–603 nm, respectively). Under these conditions, typical ranges for the parameter values were $0.00001 < \alpha < 0.0005$; $0.2 < \beta < 1.5$; $0.02 < \gamma < 0.5$; and $0.0003 < \delta < 0.003$. However, at different expression stoichiometry, or when spatial distribution is very inhomogeneous for one of the fluorophores, selection of widely different instrument settings may be favorable, with consequent large changes in α , β , γ , and δ . Thus, optimized

instrument settings for cells expressing low CFP and high YFP levels caused large δ - and α -values, whereas cells expressing high CFP and low YFP resulted in δ and α being negligibly small. In the latter case, Eq. 5 may be simplified to the numerator.

As outlined above, parameters α , β , γ , and δ are determined by imaging cells expressing either CFP or YFP alone. Stochastic errors in the calculated values for either of these parameters systematically bias the FRET efficiency results over the entire image, and should therefore be minimized. Thus, it is important to obtain the parameter values from extended image regions, averaging out statistic fluctuations over many pixels. The correction factor δ is particularly sensitive to noise because it is calculated by dividing M_{Donor} by $M_{\text{IndirectAcceptor}}$ (Eq. 8) from a cell expressing only YFP. Both of these images are very dim, because they stem from acceptor molecules excited at donor wavelength (430 nm). Since δ depends on filter and PMT settings, but not on relative laser line intensities (see Eq. A9), δ may be acquired using increased laser power or with 514-nm excitation. In practical experiments, errors in calculated FRET efficiencies for each pixel are dominated by the rather large noise in the M_{Donor} , $M_{\text{DirectAcceptor}}$, and $M_{\text{IndirectAcceptor}}$ images, with stochastic variations in the parameter values contributing <1%, on average.

CFP excitation for sensitized emission is optimal at 430 nm

The 458-nm and 514-nm Argon ion laser lines have been used (He et al., 2003; Karpova et al., 2003) to excite CFP and YFP in FRET experiments. However, as deduced from the excitation spectra of these fluorophores (see Appendix Fig. 1), the 458-nm line overlaps considerably with the YFP excitation spectrum, resulting in direct acceptor excitation and poor discrimination. To determine the optimal wavelength for CFP excitation in sensitized emission experiments, we expressed and purified the CFP/YFP-based Ca^{2+} sensor yellowameleon (Miyawaki et al., 1997) from bacteria. When dissolved at ~1 $\mu\text{g}/\text{ml}$ in a Ca^{2+} -free intracellular buffer solution, this construct shows little FRET in the fluorometer. Upon addition of 1 mM Ca^{2+} , a robust and reliable increase in FRET is detected. In a series of experiments, the excitation wavelength was varied in the range of 340–452 nm, and both the magnitude of the CFP emission, as well as the magnitude of the Ca^{2+} -induced FRET change (defined as percent change in the ratio YFP/CFP induced by Ca^{2+}) were recorded (Fig. 1, A and B). It is apparent from Fig. 1 A that FRET changes are most efficiently detected at excitation wavelength below 432 nm, whereas direct YFP excitation caused the Ca^{2+} -induced change in ratio to drop dramatically at higher wavelength. Conversely, decreasing wavelength below ~425 nm had little effect on Ca^{2+} -induced ratio changes but significantly

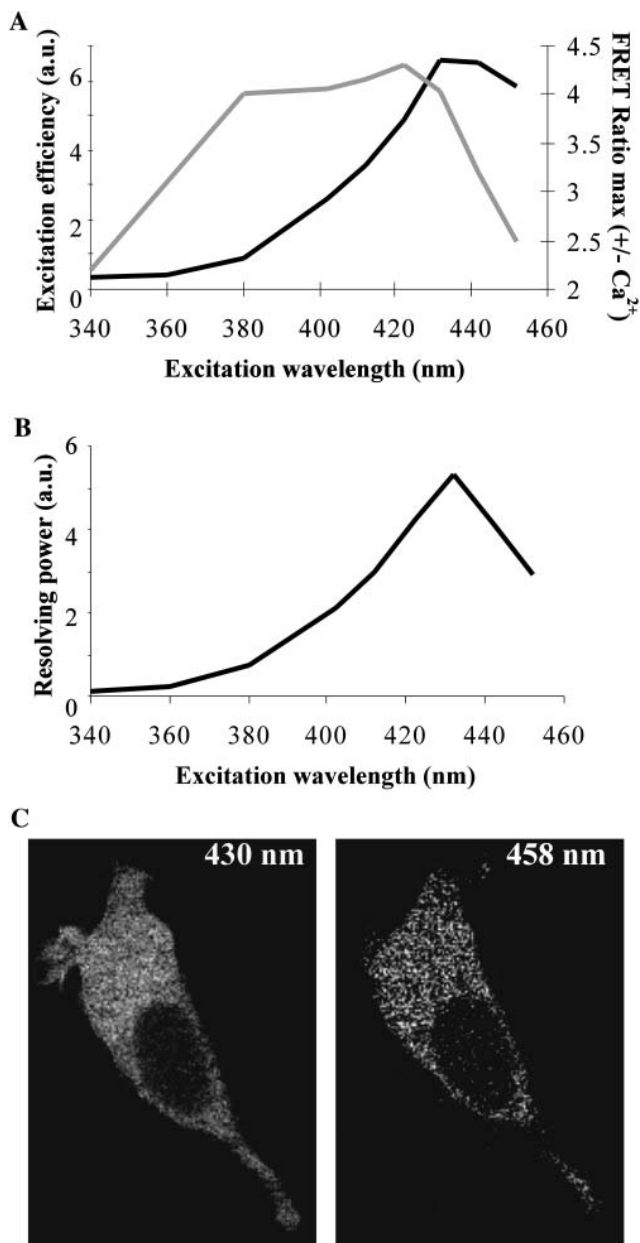


FIGURE 1 Optimization of CFP excitation wavelength to resolve FRET from CFP/YFP. Yellowameleon 2.0 was expressed and purified from bacteria, and introduced at $\sim 1 \mu\text{M}$ in a 2-ml cuvette in a spectrofluorometer. FRET changes were measured upon increasing the Ca²⁺ concentration from 50 nM to 1 mM. (A) Excitation efficiency of CFP (black line) and Ca²⁺-induced change in YFP/CFP emission intensity (shaded line) are plotted as a function of wavelength. (B) The efficacy of various excitation wavelengths in resolving FRET changes was approximated by multiplying the excitation efficiency with the efficiency to resolve Ca²⁺-induced ratio changes. Note the considerable decline at wavelengths longer than 432 nm. (C) Sensitized emission of yellowameleon was imaged using either 430-nm (left panel) or 458-nm (right panel) CFP excitation, and 514-nm YFP excitation. The average sensitized emission in the cytosol was 111 ± 40 with 430-nm excitation and 48 ± 41 with 458-nm excitation (8-bits grayscale).

reduced CFP excitation. From Fig. 1, it can be concluded that optimal excitation to resolve FRET changes is at 432 nm.

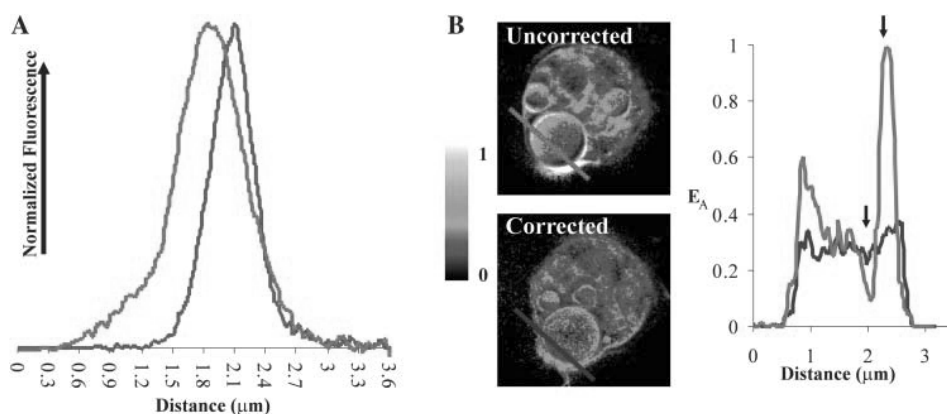
We employ a 10-mW Melles Griot (Irvine, CA) type 58-BTL-008 frequency-doubled diode laser to excite CFP at 430 nm on our Leica TCS-SP2 confocal microscope. YFP excitation is by the 514-nm Argon laser line. The use of 430-nm CFP excitation, rather than the more commonly used 458-nm excitation, also allows collection of a larger part of the CFP emission spectrum, resulting in brighter CFP images. Together with the aforementioned optimal discrimination between CFP and YFP, this significantly increases the signal/noise ratio. Fig. 1 C shows FRET images of a cell that expresses yellowameleon using either 430-nm or 458-nm excitation.

Correcting misfocusing deviations

As the calculation of F_{Sen} involves mathematical operations based on three raw images, it is of the utmost importance that these channels spatially overlap tightly, both in lateral and in axial direction. Compared with wide-field microscopy, the focusing deviations—i.e., deviations that occur if donor and acceptor images are offset in the axial direction—are emphasized by the confocals' inherent optical sectioning. The CFP and YFP images are effectively taken from slightly different planes in the cell (Fig. 2 A), causing erroneous results during calculation of the sensitized emission, resulting in pixels with extreme high or low FRET efficiencies (Fig. 2 B). Two main sources for this type of deviation exist: chromatic aberrations within the objective and other optics, and slight differences in the collimation of the laser beams. Chromatic aberrations are due to the wavelength dependency of the refractive index of optical glasses, which causes axial misregistration of images taken at different wavelengths (Cogswell and Larkin, 1995). Depending on the objective used, chromatic aberrations may be several micrometers (worst case). Chromatically corrected objectives are available, but it should be stressed that these are optimized only for a limited spectral range, typically in the midvisible range. Therefore, significant chromatic aberration may still be present at 430 and 458 nm.

Using a good, standard corrected $63\times$ magnification, 1.32-NA oil immersion objective (HCX PL APO CS, #506180, Leica), we noticed focusing deviations of ~ 400 nm (Fig. 2 A). Use of a UV-corrected $63\times$ magnification objective (HCX PL APO lbd.BL, #506192, Leica) significantly, but not completely, remedied this chromatic aberration. Chromatic focusing deviations are not limited to violet wavelengths because significant deviations exist for dye pairs excited throughout the visible spectrum (Table 1).

Slight collimation differences between the laser beams are the second source of focusing inaccuracies, in particular if donor and acceptor excitation wavelength are derived from separate lasers. Lasers which are coupled via separate collimation lenses are normally optimized for three-di-



upper photomicrograph, respectively). The intensity profiles plotted along the indicated line (red, uncorrected routine; blue, refocusing routine) show the extreme FRET values in the profile from the uncorrected FRET image (arrows).

mensional resolution. This causes the focal plane of excitation to vary depending on excitation wavelength, resulting in an offset between the images and also in inefficient excitation with consequent unnecessary specimen bleaching. In principle, slight adjustments in collimation of one beam could be used to correct the objective chromatic aberration, at least partially. The lower-wavelength beam can be adjusted to be a little bit more divergent, which compromises three-dimensional resolution, but brings the different focus planes nearer to each other. However, this is not a practical solution, as chromatic aberrations vary with lens types, and even for different objectives of the same type (L. Oomen and K. Jalink, unpublished; Zucker and Price, 2001).

To provide a more generic approach to overcome focusing deviations, we used the fine focusing capacity of the Z-galvanometer of the microscope stage. First, M_{Donor} and $M_{\text{IndirectAcceptor}}$ images were recorded using 430-nm excitation. Then, before taking the $M_{\text{DirectAcceptor}}$ image with 514-nm excitation, the preparation is refocused to minimize chromatic aberration. Because for a given combination of objective and excitation lines the focus deviation is constant, the correction distance needs to be determined only once. We used x/z -scanning of fixed cells or fluorescent beads for this goal (Fig. 2 A). Applying this focus correction in an automated acquisition routine (macro), M_{Donor} , $M_{\text{IndirectAcceptor}}$, and $M_{\text{DirectAcceptor}}$ images are collected from the same focal plane in the biological sample. Thus, the FRET efficiency

calculated from images acquired in this manner is effectively corrected for misfocusing as shown in Fig. 2 B.

Lateral image errors

Lateral image errors occur when raw images do not overlap precisely in the image plane (x/y direction). Both geometric and intensity errors may occur. Geometric errors are most apparent at the borders of the image, and errors of this type can best be avoided by zooming in slightly. Lateral intensity errors may be present over the entire image and occur on CCD and confocal systems alike. For CCD systems, a standard correction algorithm exists: corrections are carried out by normalizing pixel intensities using a reference image, a procedure called shading correction (Tomazevic et al., 2002). On the confocal system with independent excitation lines, these corrections are slightly more complex because spatial excitation intensities may vary independently (L. Oomen, L. Brocks, and K. Jalink, unpublished; Zucker and Price, 2001) and similar effects also occur in the detection path. This necessitates that both channels be normalized by shading correction.

For 430- and 514-nm lines, excitation inhomogeneities were measured by registration of reference images of a solution of the FRET calcium sensor yellow cameleon (Miyawaki et al., 1997). We observed significant deviations from unity flatness: 430-nm excitation intensity dropped by as much as 50% at the image corners, whereas 514-nm deviated by $\sim 15\%$ (Fig. 3 A, left panels). Importantly, significant differences (up to 20%) may also occur over the center of the images. Deviations of this magnitude are not uncommon in confocal systems (Zucker and Price, 2001), although they can be diminished by increasing the zoom factor. Therefore, shading correction was routinely applied to M_{Donor} and $M_{\text{IndirectAcceptor}}$ by normalizing to the 430-nm reference, and to $M_{\text{DirectAcceptor}}$ by normalizing to the 514-nm reference image. This completely corrects for lateral

TABLE 1 Differences in focus distance between commonly used laser line pairs using a standard 63 $\times$, 1.32 NA oil immersion objective

Laser line pair (nm)	Distance (μm)
458/514	0.3
488/568	0.17
514/633	0.04

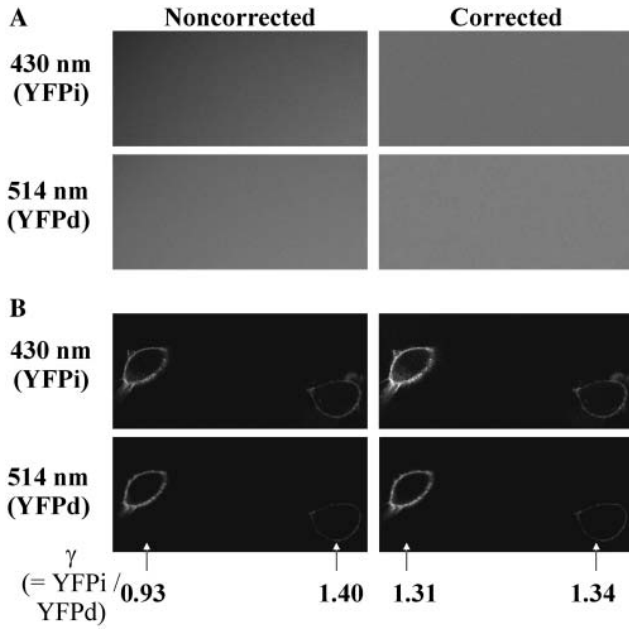


FIGURE 3 Lateral image errors. (A) Shown are parts of reference images that were acquired by averaging eight confocal images of a solution of yellow cameleon at 430-nm excitation (*upper left panel*) and at 514-nm excitation (*lower left panel*). The right panels demonstrate shade correction of (single-pass) confocal images through division by the respective normalized reference images. (B) Two cells expressing YFP-PH were registered with 430- and 514-nm excitation (*upper and lower left panels*, respectively). Using the shade correction reference image, fluorescence inhomogeneities were corrected (*right panels*). The γ -values, calculated according to Eq. 7, are indicated for the two cells. Note that the differences in γ -values in the uncorrected images are remedied in the right panel.

fluorescence inhomogeneities (Fig. 3, *A and B, right panels*). To illustrate the impact of shading correction on practical experiments, the γ -values calculated for two cells expressing YFP-PH are also indicated in Fig. 3 *B*. Whereas in the uncorrected images (*left panels*) these values differ by as much as 50%, shading correction (*right panels*) effectively canceled out the differences. Consequently, we used shading-corrected images to determine F_{Sen} as well as the correction factors throughout this study.

Temporal errors: laser intensity fluctuation

Unstable excitation sources generate temporal intensity variations. Excitation stability is extremely important because the correction factors α and γ depend on relative laser line intensities. We observed considerable drift and slow oscillations (at a timescale of one to several minutes) in excitation line intensity on several different confocal systems (Fig. 4 *A*). Changes of several percent are common, whereas worst-case variations of up to 20% are detected in poorly aligned systems. Importantly, individual laser line intensity variations are independent, even for different lines from the

same laser. Although intensity variations may also occur in arc lamps from wide-field fluorescence microscopes, these changes are often much smaller (compare Fig. 4, *A and B*). Furthermore, slow arc lamp intensity variations affect the three raw images to the same degree if images are gathered in rapid succession, and thus have no effect on the apparent FRET image (Eqs. 10 and 11).

The independent variations in laser line intensity on confocal systems pose a major problem for timelapse FRET measurements. This is illustrated in Fig. 4 *C*, where the FRET efficiency (E_D) of Yellow cameleon was followed over time (*red line*). Although in these unstimulated cells the FRET efficiency remains constant over time, the independent intensity variation of the 430 and 514-nm laser line cause fluctuations in E_D . A supplier-installed stabilization system improved the excitation stability considerably, but not completely. In particular when expected FRET signals are a small fraction of the total fluorescence, the realized stability of $\sim 3\%$ hampers acquisition of meaningful results. We therefore implemented an online correction scheme by recalculating the leakthrough factors for each image. To this goal, the cells under study were plated together with a mix of cells expressing either CFP or YFP on the same coverslip. In an image taken at low zoom factor, regions of interest (ROIs) were assigned to single CFP- or YFP-transfected cells (Fig. 4 *D*). From these ROIs, correction factors were determined as detailed in Eqs. 6–9. Sensitized emission was then calculated using these correction factors from cells expressing both CFP and YFP within the same image, or from a separate image collected at higher zoom factor. Provided that proper shading correction is carried out (see Lateral Image Errors), this procedure completely removed the effect of laser fluctuations, resulting in superior registration of FRET during acquisition of timelapse series (Fig. 4 *C, black line*).

Post-acquisition analysis

Having compensated for the most important sources of confocal acquisition deviations, significant improvements in image quality may still be obtained by post-acquisition procedures. The prime consideration is noise present in the images. Since photon noise is Poisson-distributed (with the standard error being the square root of the number of photons), its effects will be most evident in low intensity regions of the image (see Fig. 5). In these regions, noise will be emphasized by image arithmetic, because it leads to extreme values in ratios as well as subzero intensity values in subtractions. This causes pixels with “false” high FRET values to appear in dim image regions (Fig. 5, *upper right panel*). Therefore, care must be taken to acquire M_{Donor} , $M_{\text{IndirectAcceptor}}$, and $M_{\text{DirectAcceptor}}$ images with a good signal/noise ratio. This can be accomplished in a number of ways on the confocal, including increasing the laser power which

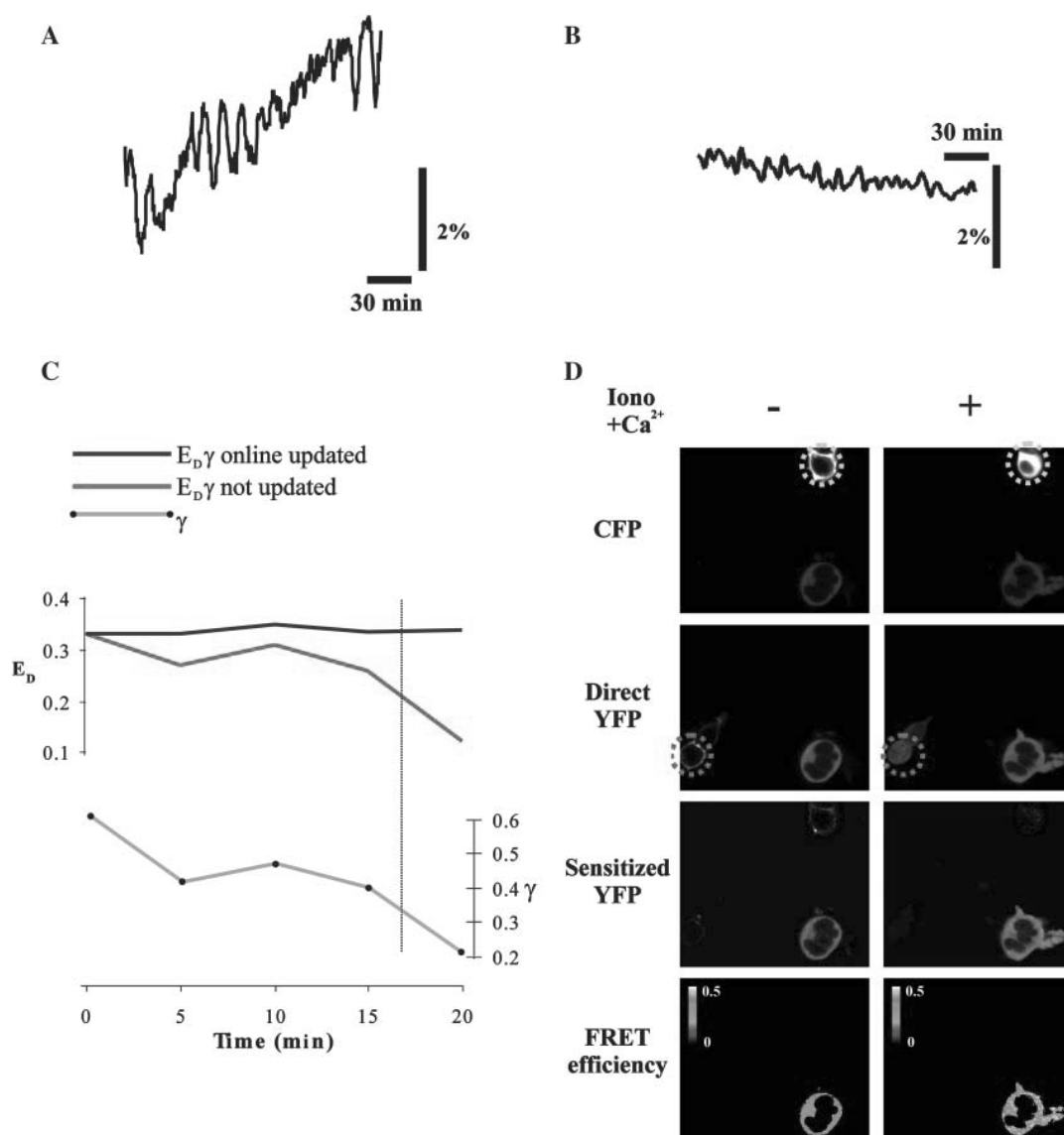


FIGURE 4 Temporal intensity variations in excitation sources. The intensity of a 514-nm argon ion laser line (A) and a mercury arc lamp (B) were measured every 20 s for a 3-h time period and plotted after normalization. (C) A mixed population of cells expressing yellow cameleon, YFP-PH, or CFP-PH was imaged and analyzed for sensitized emission. The FRET efficiency (E_D) and the correction factor γ (shaded line) are plotted versus time. FRET efficiency was calculated using a single fixed (red line) γ -factor and the online-updated (black line) γ -factor. After 15 min a large intensity fluctuation in the 514-nm laser line was simulated by manually diminishing laser power with $\sim 60\%$. (D) To correct for variations in excitation intensity, the leakthrough factors were determined in every pair of images from regions of interest (ROIs) corresponding to cells expressing either CFP-PH (blue ROI) or YFP-PH (red ROI), as detailed in the text. The calcium ionophore ionomycin and 2 mM extra Ca^{2+} were added to the medium to increase the FRET signal, and to translocate the PH-chimeras to the cytosol. Factors α , β , γ , and δ in the first acquired images were 0.00005, 0.47, 0.04, and 0.0013, respectively.

allows using lower PMT voltage, averaging of acquired images, and opening the pinhole. However, these measures come at the expense of increased fluorophore bleaching, prolonged imaging time, and degraded resolution.

Post-acquisition spatial filtering (smoothing) can also be applied to reduce noise, but this will equally degrade the resolution and blur fine details. To abolish the incidence of false high FRET values in dim image regions, while simultaneously circumventing image blurring in the other

regions, a masking technique was applied (Fig. 5, *middle right panel*). In the apparent FRET image, resonance can be distinguished from noise by smoothing the image with a spatial filter. Isolated noise pixels are averaged out, whereas consecutive adjacent pixels with positive FRET remain visible. Setting a threshold to just above background intensity, a mask is then generated from this image that contains only regions of true FRET. This mask is subsequently applied to the original, unfiltered FRET image.

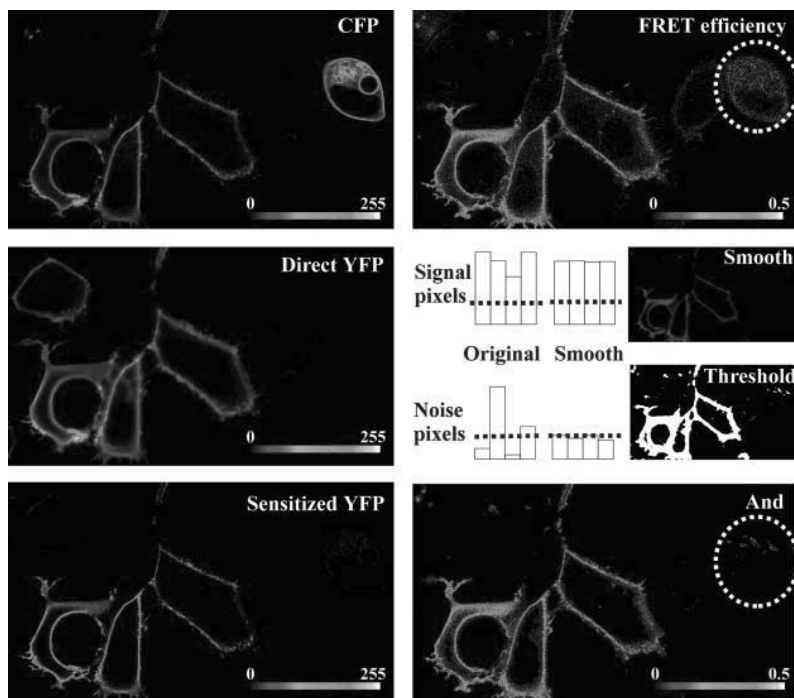


FIGURE 5 Sensitized emission calculated from noisy confocal images. Confocal images of cells expressing CFP-PH, YFP-PH, or both were registered as in Fig. 4. (*Upper left panel*, CFP image; *middle left panel*, YFP image; and *lower left panel*, corrected sensitized emission.) Apparent FRET efficiency (*upper right panel*) was calculated as detailed in the text. Note the appearance of excessive noise in dim areas of the FRET image. The calculated FRET efficiency image was smoothed with a spatial filter to distinguish noise from signal and a threshold equal to background intensity (*dotted line*) was applied to this image to reject pixels without FRET signal (*middle right panel*). Applying this mask to the original nonsmoothed FRET image effectively rejects the noise (*lower right panel*; compare to the *upper right panel* to assess the noise rejection introduced by this step). Factors α , β , γ , and δ were 0.0004, 0.65, 0.46, and 0.001, respectively.

Fig. 5 (*middle and lower right panels*) illustrates that this approach results in near-complete rejection of noise pixels.

Final remarks

Confocal microscopy has a number of advantages over wide-field fluorescence microscopy, the most important of which is that it records thin optical sections from the preparation. We therefore aimed to optimize confocal imaging of FRET between CFP and YFP by detecting sensitized emission. Earlier confocal studies focused on acceptor photobleaching (Karpova et al., 2003), and main confocal suppliers now support this application with dedicated software. However, acceptor photobleaching is a destructive technique that cannot be used for timelapse studies, and we therefore focused on detecting sensitized emission. In this study, it was demonstrated that a 430-/514-nm excitation line pair outperforms the more commonly used 458-/514-nm lines (Karpova et al., 2003) by discriminating better between CFP and YFP, resulting in a marked decrease in noise of the FRET image. We also identified a number of confocal-specific error sources that complicate the leakthrough correction schemes commonly applied in wide-field fluorescence microscopy.

In this study, an approach was introduced to compensate for individual laser line intensity fluctuations, leakthrough, and detector gain by simultaneous imaging of cells expressing either CFP or YFP alone, present within the same image field. This complicates the experimental design because it requires the user to establish co-cultures with CFP- and YFP-

expressing cell lines, unless in the cell under study reliable regions can be identified that contain either CFP or YFP fluorescence only. However, the advantages are numerous, because the online calibration procedure not only compensates for excitation intensity fluctuations, but also allows semiquantitative assessment of FRET efficiency, independent of system settings such as PMT gain. Importantly, this enables direct comparison of FRET values from experiment to experiment, even when detector gain and laser intensities have been adjusted by the user.

Careful consideration of the practical implementation of automated acquisition and analysis steps in the macro is necessary. For example, within a timelapse series, the correction factor δ , that is updated along with the other parameters, is deduced from division of M_{Donor} by $M_{\text{IndirectAcceptor}}$ from a cell expressing YFP only. Both images are very dim, because they stem from acceptor molecules excited at donor wavelength, and this may result in some noise in consecutive determinations of δ . The independence of this parameter on relative laser line intensities (see Appendix) allows δ to be determined just once, e.g., at the onset, for the whole timelapse series, if needed at increased laser power or using the 514-nm laser line, as long as no further adjustments are made to the instrument during the experiment.

In summary, online corrected confocal imaging is a fast, sensitive, and straightforward approach to detect sensitized emission from the CFP/YFP pair. The speed is particularly important for live cell imaging, since it minimizes artifacts due to movement of organelles during acquisition. Further-

more, the introduced corrective approaches can be readily adapted to other FRET pairs.

APPENDIX: IMAGING FRET BY SENSITIZED EMISSION

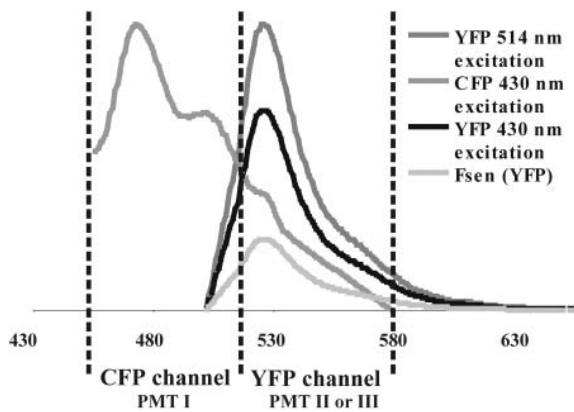
In this Appendix, we will assume 430-nm donor and 514-nm acceptor excitation to image FRET from the CFP/YFP pair. As detailed in the text, three images are collected that allow independent estimates of cross-talk magnitude to perform correction of leakthrough: 430-nm excitation with CFP emission, M_{Donor} ; 430-nm excitation with YFP emission, $M_{\text{IndirectAcceptor}}$; and 514-nm excitation with YFP emission, $M_{\text{DirectAcceptor}}$.

The acquired images are composite images that consist of multiple terms (see Appendix Fig. 1; for symbols, see Appendix Table 1) as described in the following equations.

M_{Donor} is the output grayscale value after amplification by the PMT I detector (g_1) of the sum of the fraction (A) of CFP fluorescence in the CFP channel and the fraction (B) of YFP fluorescence in the CFP channel. The fluorescence of CFP depends on the number of CFP molecules (N_{CFP}), the 430-nm laser excitation ($\epsilon^{430\text{CFP}}$), the quantum yield (Q^{CFP}), diminished by the number of CFP molecules that lose their excited state energy due to FRET (N_{Sen}). Note that $\epsilon^{430\text{CFP}}$ models both the laser intensity and the excitation efficiency of CFP at 430 nm. The fluorescence of YFP depends on its quantum yield (Q^{YFP}), and the sum of the number of YFP molecules (N_{YFP}) excited with 430-nm laser ($\epsilon^{430\text{YFP}}$) and those excited by FRET (N_{Sen}). Because the relaxation of excited CFP molecules by FRET results in equal amounts of excited YFP molecules, both pools are denoted by N_{Sen} . Since resonance is due to excited CFP, N_{Sen} is also dependent on the excitation efficiency of CFP at 430 nm ($\epsilon^{430\text{CFP}}$), as

$$M_{\text{Donor}} = (N_{\text{CFP}} - N_{\text{Sen}})\epsilon^{430\text{CFP}}Q^{\text{CFP}}Ag_1 + N_{\text{YFP}}\epsilon^{430\text{YFP}}Q^{\text{YFP}}Bg_1 + N_{\text{Sen}}\epsilon^{430\text{CFP}}Q^{\text{YFP}}Bg_1. \quad (\text{A1})$$

$M_{\text{IndirectAcceptor}}$ is the output grayscale value after the PMT II detector scaling (g_2) of the sum of the fractions of CFP fluorescence in the YFP channel (C) and of YFP fluorescence in the YFP channel (D). The CFP fluorescence depends on Q^{CFP} , the 430-nm laser excitation efficiency ($\epsilon^{430\text{CFP}}$), the number of CFP molecules (N_{CFP}), and the CFP molecules that lose their energy by FRET (N_{Sen}). The fluorescence of YFP depends on Q^{YFP} , the



APPENDIX FIGURE 1 Spectral overlap of CFP and YFP. Emission spectra of CFP and YFP were recorded on a spectrofluorometer from bacterially expressed purified protein. Note that the two fluorophores have considerable spectral overlap. The graph also illustrates the difference in excitation efficiency of YFP depending on the method of excitation; 514 nm (dark shaded line), 430 nm (black line), and FRET (light shaded line); not to-scale.

APPENDIX TABLE 1 Glossary of used symbols

Factor	Name	Description
Laser	$\epsilon^{430\text{CFP}}$	Excitation efficiency of CFP with 430 nm
	$\epsilon^{430\text{YFP}}$	Excitation efficiency of YFP with 430 nm
	$\epsilon^{514\text{YFP}}$	Excitation efficiency of YFP with 514 nm
Fraction (Spectral)	A	Fraction of CFP spectrum in the CFP channel
	B	Fraction of YFP spectrum in the CFP channel
	C	Fraction of CFP spectrum in the YFP channel
	D	Fraction of YFP spectrum in the YFP channel
PMT detector	g_1	Scaling factor relating fluorescence to donor channel grayscale value
	g_2	Scaling factor relating fluorescence to indirect acceptor channel grayscale value
	g_3	Scaling factor relating fluorescence direct acceptor channel grayscale value
Quantum yield	Q^{CFP}	The quantum yield of CFP is 0.40 (Tsien, 1998)
	Q^{YFP}	The quantum yield of YFP (Citrine) is 0.76 (Griesbeck et al., 2001)

amount of YFP molecules (N_{YFP}) excited with 430-nm laser ($\epsilon^{430\text{YFP}}$), and on the amount of YFP molecules excited by FRET (N_{Sen} , which is linear to $\epsilon^{430\text{CFP}}$), as

$$M_{\text{IndirectAcceptor}} = N_{\text{Sen}}\epsilon^{430\text{CFP}}Q^{\text{YFP}}Dg_2 + (N_{\text{CFP}} - N_{\text{Sen}})\epsilon^{430\text{CFP}}Q^{\text{CFP}}Cg_2 + N_{\text{YFP}}\epsilon^{430\text{YFP}}Q^{\text{YFP}}Dg_2. \quad (\text{A2})$$

Finally, $M_{\text{DirectAcceptor}}$ is the output grayscale value after the PMT III detector scaling (g_3) of the YFP fluorescence in the YFP channel (D), which depends on the quantum yield of YFP (Q^{YFP}) and the amount of YFP molecules (N_{YFP}) excited with 514 nm ($\epsilon^{514\text{YFP}}$). Note that PMT III generally will be the same physical detector as PMT II, but operated at a different gain setting. Formally, donor fluorescence, excited with 514 nm is also present. However, using the 514-nm argon laserline, the magnitude of this component is essentially zero. Thus,

$$M_{\text{DirectAcceptor}} = N_{\text{YFP}}\epsilon^{514\text{YFP}}Q^{\text{YFP}}Dg_3. \quad (\text{A3})$$

To derive the sensitized emission, Eqs. A1 and A3 are combined as

$$N_{\text{CFP}} - N_{\text{Sen}} = \frac{M_{\text{Donor}}}{\epsilon^{430\text{CFP}}Q^{\text{CFP}}Ag_1} - \frac{M_{\text{DirectAcceptor}}\epsilon^{430\text{YFP}}Q^{\text{YFP}}Bg_1}{\epsilon^{514\text{YFP}}Q^{\text{YFP}}Dg_3\epsilon^{430\text{CFP}}Q^{\text{CFP}}Ag_1} - \frac{N_{\text{Sen}}\epsilon^{430\text{CFP}}Q^{\text{YFP}}Bg_1}{\epsilon^{430\text{CFP}}Q^{\text{CFP}}Ag_1}, \quad (\text{A4})$$

and Eqs. A3 and A4 are substituted into Eq. A2, yielding

$$N_{\text{Sen}} = \frac{M_{\text{IndirectAcceptor}} - M_{\text{Donor}} \frac{C_{g2}}{A_{g1}} - M_{\text{DirectAcceptor}} \left(\frac{\epsilon^{430\text{YFP}} D_{g2}}{\epsilon^{514\text{YFP}} D_{g3}} - \frac{\epsilon^{430\text{YFP}} B_{g1}}{\epsilon^{514\text{YFP}} D_{g3}} \frac{\epsilon^{430\text{CFP}} C_{g2}}{\epsilon^{430\text{CFP}} A_{g1}} \right)}{\epsilon^{430\text{CFP}} Q^{\text{YFP}} D_{g2} - \frac{\epsilon^{430\text{CFP}} C_{g2} \epsilon^{430\text{CFP}} Q^{\text{YFP}} B_{g1}}{\epsilon^{430\text{CFP}} A_{g1}}}. \quad (\text{A5})$$

Relating back to Eq. 5 from Results and Discussion, the sensitized emission grayscale image F_{Sen} is composed of the emission from N_{Sen} , which depends on the quantum yield of YFP (Q^{YFP}), scaled by factors for PMT II gain (g_2), fraction of YFP fluorescence in the YFP channel (D), and CFP excitation efficiency $\epsilon^{430\text{CFP}}$, as

To obtain an indication for FRET efficiency, the derived expression for F_{Sen} (Eq. A11) can be related to the total acceptor level, or to the total donor level. Depending on the biological application, either way may have specific advantages. Relating F_{Sen} to $F_{\text{DirectAcceptor}}$, the expression for efficiency becomes

$$N_{\text{Sen}} \epsilon^{430\text{CFP}} Q^{\text{YFP}} D_{g2} = \frac{M_{\text{IndirectAcceptor}} - M_{\text{Donor}} \frac{C_{g2}}{A_{g1}} - M_{\text{DirectAcceptor}} \left(\frac{\epsilon^{430\text{YFP}} D_{g2}}{\epsilon^{514\text{YFP}} D_{g3}} - \frac{\epsilon^{430\text{YFP}} B_{g1}}{\epsilon^{514\text{YFP}} D_{g3}} \frac{\epsilon^{430\text{CFP}} C_{g2}}{\epsilon^{430\text{CFP}} A_{g1}} \right)}{\frac{\epsilon^{430\text{CFP}} D_{g2}}{\epsilon^{430\text{CFP}} D_{g2}} - \frac{\epsilon^{430\text{CFP}} C_{g2} \epsilon^{430\text{CFP}} B_{g1}}{\epsilon^{430\text{CFP}} A_{g1} \epsilon^{430\text{CFP}} D_{g2}}}. \quad (\text{A6})$$

In Eq. A6, the constants α , β , γ , and δ (see Results and Discussion) are identified as detailed in Eqs. A7–A10. Values for α , γ , and δ can be deduced from imaging of a sample with only acceptor molecules,

$$E_A = \frac{N_{\text{Sen}} \epsilon^{430\text{CFP}} Q^{\text{YFP}} D_{g2}}{N_{\text{YFP}} \epsilon^{514\text{YFP}} Q^{\text{YFP}} D_{g3}} = \frac{N_{\text{Sen}} \epsilon^{430\text{CFP}} g_2}{N_{\text{YFP}} \epsilon^{514\text{YFP}} g_3}. \quad (\text{A12})$$

This corresponds to Eq. 10 from Results and Discussion. It is evident that E_A depends on the excitation of YFP at both laser lines and on PMT II and III settings. Therefore, E_A is useful to compare FRET efficiencies within a cell, or between different cells in the same image, but not to compare efficiencies when excitation intensities or PMT settings may have changed unless an additional correction is introduced to compensate for such changes. The magnitude of this corrective term is

$$\frac{Y_{\text{FP}} M_{\text{Donor}}}{Y_{\text{FP}} M_{\text{DirectAcceptor}}} = \frac{N_{\text{YFP}} \epsilon^{430\text{YFP}} Q^{\text{YFP}} B_{g1}}{N_{\text{YFP}} \epsilon^{514\text{YFP}} Q^{\text{YFP}} D_{g3}} = \frac{\epsilon^{430\text{YFP}} B_{g1}}{\epsilon^{514\text{YFP}} D_{g3}} = \alpha, \quad (\text{A7})$$

$$\frac{Y_{\text{FP}} M_{\text{IndirectAcceptor}}}{Y_{\text{FP}} M_{\text{DirectAcceptor}}} = \frac{N_{\text{YFP}} \epsilon^{430\text{YFP}} Q^{\text{YFP}} D_{g2}}{N_{\text{YFP}} \epsilon^{514\text{YFP}} Q^{\text{YFP}} D_{g3}} = \frac{\epsilon^{430\text{YFP}} g_2}{\epsilon^{514\text{YFP}} g_3} = \gamma, \quad (\text{A8})$$

$$\frac{Y_{\text{FP}} M_{\text{Donor}}}{Y_{\text{FP}} M_{\text{IndirectAcceptor}}} = \frac{N_{\text{YFP}} \epsilon^{430\text{YFP}} Q^{\text{YFP}} B_{g1}}{N_{\text{YFP}} \epsilon^{430\text{YFP}} Q^{\text{YFP}} D_{g2}} = \frac{B_{g1}}{D_{g2}} = \delta. \quad (\text{A9})$$

$$\frac{\epsilon^{430\text{CFP}} g_2}{\epsilon^{514\text{YFP}} g_3} = \frac{\epsilon^{430\text{CFP}}}{\epsilon^{430\text{YFP}}} \gamma = \kappa \gamma, \quad (\text{A13})$$

where κ is a constant relating the efficiency of CFP excitation by the 430-nm laser line to that of YFP (using our settings, $\kappa \sim 15$).

Alternatively, when FRET efficiency is expressed by relating F_{Sen} to F_{Donor} , the results become directly independent from excitation intensity and PMT settings. To arrive at an expression for E_D , the loss of signal due to FRET from the grayscale image M_{Donor} is related to the total (i.e., when no FRET occurs) donor grayscale image,

Similarly, β is calculated from a sample with only donor molecules, as

$$\frac{C_{\text{FP}} M_{\text{IndirectAcceptor}}}{C_{\text{FP}} M_{\text{Donor}}} = \frac{N_{\text{CFP}} \epsilon^{430\text{CFP}} Q^{\text{CFP}} C_{g2}}{N_{\text{CFP}} \epsilon^{430\text{CFP}} Q^{\text{CFP}} A_{g1}} = \frac{C_{g2}}{A_{g1}} = \beta. \quad (\text{A10})$$

Note that in contrast to β and δ , α and γ depend on the relative laser line intensities.

Analogous to Eq. 5, we can thus rewrite Eq. A6 as

$$E_D = \frac{N_{\text{Sen}} \epsilon^{430\text{CFP}} Q^{\text{CFP}} A_{g1}}{N_{\text{CFP}} \epsilon^{430\text{CFP}} Q^{\text{CFP}} A_{g1}} = \frac{N_{\text{Sen}}}{N_{\text{CFP}}}, \quad (\text{A14})$$

$$N_{\text{Sen}} \epsilon^{430\text{CFP}} Q^{\text{YFP}} D_{g2} = \frac{M_{\text{IndirectAcceptor}} - M_{\text{Donor}} \beta - M_{\text{DirectAcceptor}} (\gamma - \alpha \beta)}{1 - \beta \delta}. \quad (\text{A11})$$

where in the numerator, the emission lost (the CFP quantum yield times N_{Sen} , see Eq. A5) is scaled by factors for PMT I gain (g_1), fraction of CFP fluorescence in the CFP channel (A), and CFP excitation efficiency $\epsilon^{430\text{CFP}}$. Analogous to Eq. 6,

$$N_{\text{Sen}} \epsilon^{430\text{CFP}} Q^{\text{CFP}} A_{g1} = \frac{Q^{\text{CFP}}}{Q^{\text{YFP}}} \frac{M_{\text{IndirectAcceptor}} - M_{\text{Donor}} \frac{C_{g2}}{A_{g1}} - M_{\text{DirectAcceptor}} \left(\frac{\epsilon^{430\text{YFP}} D_{g2}}{\epsilon^{514\text{YFP}} D_{g3}} - \frac{\epsilon^{430\text{YFP}} B_{g1}}{\epsilon^{514\text{YFP}} D_{g3}} \frac{\epsilon^{430\text{CFP}} C_{g2}}{\epsilon^{430\text{CFP}} A_{g1}} \right)}{\frac{C_{g2}}{A_{g1}} \frac{D}{C} \left(1 - \frac{C_{g2}}{A_{g1}} \frac{B_{g1}}{D_{g2}} \right)}, \quad (\text{A15})$$

which, using Eqs. A7–A10, can be rewritten to

$$N_{\text{Sen}} \varepsilon^{430\text{CFP}} Q^{\text{CFP}} A g_1 = \frac{Q^{\text{CFP}} M_{\text{IndirectAcceptor}} - M_{\text{Donor}} \beta - M_{\text{DirectAcceptor}} (\gamma - \alpha \beta)}{Q^{\text{YFP}} \frac{\beta D}{C} (1 - \beta \delta)}. \quad (\text{A16})$$

In the divisor of Eq. A14, the expression for the grayscale image of the total donor fluorescence is

$$N_{\text{CFP}} \varepsilon^{430\text{CFP}} Q^{\text{CFP}} A g_1 = M_{\text{Donor}} (1 + \zeta) - M_{\text{IndirectAcceptor}} \left(\frac{\zeta}{\beta} \right) - M_{\text{DirectAcceptor}} \left(\alpha - \frac{(\gamma - \alpha \beta) \zeta}{\beta} \right), \quad (\text{A17})$$

where

$$\zeta = \left(\frac{\delta \beta - \frac{C Q^{\text{CFP}}}{D Q^{\text{YFP}}}}{1 - \beta \delta} \right).$$

Note that ζ does not depend on photomultiplier gain settings or laser intensity fluctuations, because $\beta \delta = BC/AD$. For a given combination of confocal filter settings and fluorophores ζ is therefore a constant (for our settings, $\zeta = -0.248$). It can be reliably determined by acquiring the M_{Donor} , $M_{\text{IndirectAcceptor}}$, and $M_{\text{DirectAcceptor}}$ images before and after complete acceptor photobleaching. Since postbleach M_{Donor} is equal to $N_{\text{CFP}} \varepsilon^{430\text{CFP}} Q^{\text{CFP}} A g_1$ (see Eq. A1), ζ can be determined by rewriting Eq. A17 as

$$\zeta = \frac{\text{postbleach } M_{\text{Donor}} - \text{prebleach } M_{\text{Donor}} + \frac{\text{prebleach } M_{\text{DirectAcceptor}} \alpha}{\beta}}{\frac{\text{prebleach } M_{\text{IndirectAcceptor}}}{\beta} + \frac{\text{prebleach } M_{\text{DirectAcceptor}} (\gamma - \alpha \beta)}{\beta}}. \quad (\text{A18})$$

Equation A17 is derived by combining Eqs. A1, A3, and A11,

The constant ζ is brought outside the parentheses,

$$N_{\text{CFP}} \varepsilon^{430\text{CFP}} Q^{\text{CFP}} A g_1 = M_{\text{Donor}} \left(1 + \left(\frac{\beta \delta - \frac{Q^{\text{CFP}} C}{Q^{\text{YFP}} D}}{(1 - \beta \delta)} \right) - M_{\text{IndirectAcceptor}} \left(\frac{1}{\beta} \left(\frac{\beta \delta - \frac{Q^{\text{CFP}} C}{Q^{\text{YFP}} D}}{(1 - \beta \delta)} \right) - M_{\text{DirectAcceptor}} \left(\alpha - \frac{(\gamma - \alpha \beta)}{\beta} \left(\frac{\beta \delta - \frac{Q^{\text{CFP}} C}{Q^{\text{YFP}} D}}{(1 - \beta \delta)} \right) \right) \right), \quad (\text{A21})$$

and Eq. A21 is used as the template to arrive at Eq. A17.

$$M_{\text{Donor}} = N_{\text{CFP}} \varepsilon^{430\text{CFP}} Q^{\text{CFP}} A g_1 - \left(\frac{(M_{\text{IndirectAcceptor}} - M_{\text{Donor}} \beta - M_{\text{DirectAcceptor}} (\gamma - \alpha \beta)) \varepsilon^{430\text{CFP}} Q^{\text{CFP}} A g_1}{(1 - \beta \delta) \varepsilon^{430\text{CFP}} Q^{\text{YFP}} D g_2} \right) + M_{\text{DirectAcceptor}} \frac{\varepsilon^{430\text{YFP}} Q^{\text{YFP}} B g_1}{\varepsilon^{514\text{YFP}} Q^{\text{YFP}} D g_3} + \left(\frac{(M_{\text{IndirectAcceptor}} - M_{\text{Donor}} \beta - M_{\text{DirectAcceptor}} (\gamma - \alpha \beta)) \varepsilon^{430\text{CFP}} Q^{\text{YFP}} B g_1}{(1 - \beta \delta) \varepsilon^{430\text{CFP}} Q^{\text{YFP}} D g_2} \right). \quad (\text{A19})$$

Equation A19 can be rewritten as

$$N_{\text{CFP}} \varepsilon^{430\text{CFP}} Q^{\text{CFP}} A g_1 = M_{\text{Donor}} \left(1 - \frac{Q^{\text{CFP}} C}{(1 - \beta \delta) Q^{\text{YFP}} D} + \frac{\beta \delta}{(1 - \beta \delta)} \right) + M_{\text{IndirectAcceptor}} \left(\frac{Q^{\text{CFP}} C}{(1 - \beta \delta) Q^{\text{YFP}} D \beta} - \frac{\delta}{(1 - \beta \delta)} \right) + M_{\text{DirectAcceptor}} \left(\frac{(\gamma - \alpha \beta) \delta}{(1 - \beta \delta)} - \frac{(\gamma - \alpha \beta) Q^{\text{CFP}} C}{(1 - \beta \delta) Q^{\text{YFP}} D \beta} - \alpha \right). \quad (\text{A20})$$

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Summary

Phosphatidylinositol 4,5-bisphosphate (PIP₂) at the inner leaflet of the plasma membrane has been proposed to act as a second messenger in the regulation of many cell processes, ranging from channel gating to vesicle trafficking. If so, then PIP₂ should fulfill two important criteria's for second messengers: first PIP₂ levels should vary spatially or temporally under physiological conditions, and secondly, these variations should suffice to influence cellular processes. In this thesis we have addressed this issue and provide data that support the idea that PIP₂ can function as a second messenger, since PIP₂ levels were found to vary significantly over time affecting cell survival, as well as cortical actin dynamics.

Interestingly, these results also suggest that PIP₂ influences multiple physiological processes within the same cell, apparently with spatial resolution. This view also prevails in the literature: it is widely hypothesized that the plasma membrane contains spatially confined PIP₂ pools or domains. Indeed, recent studies that used GFP-tagged pleckstrin homology domains (GFP-PH) as fluorescent PIP₂ sensors suggest that this lipid is enriched in membrane patches. However, we report here that this concept needs revision.

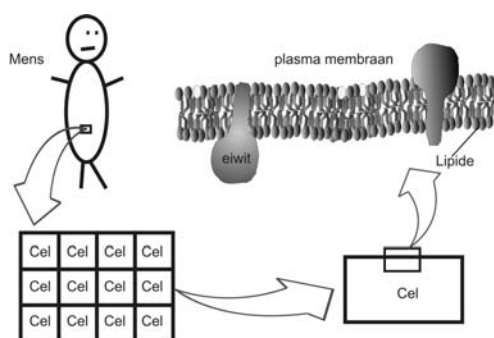
Using three distinct fluorescent GFP-tagged pleckstrin homology domains, we show that highly mobile GFP-PH patches colocalize perfectly with various lipophilic membrane dyes and, hence, represent increased lipid content rather than PIP₂-enriched microdomains. We show that bright patches are caused by submicroscopical folds and ruffles in the membrane that can be directly visualized at 15 nm axial resolution with a novel numerically enhanced imaging method. Moreover, to test sub-resolutional clustering, we analyzed clustering of PIP₂-binding pleckstrin homology domains by electron microscopy as well as by FRET in live cells. These assays suggest that PIP₂ is neither clustered in micrometer nor in sub-resolution domains. This can be explained by the fast diffusion of PIP₂ which limits the formation of small domains, as established by FRAP experiments. However, at larger scale, and especially at structures where diffusion is limiting, PIP₂ gradients can be induced.

Thus, our data support a model in which PIP₂ has a second messenger role in the regulation of cell survival and the cortical actin cytoskeleton, but they challenge a model in which this regulation exist at a small local scale.

Nederlandse samenvatting

Cell communicatie

Het lichaam bestaat uit miljarden cellen die continu met elkaar communiceren (Fig. 1). Deze communicatie is zeer belangrijk bij bijvoorbeeld de heling van een wond. Daarvoor moeten veel nieuwe cellen worden aangemaakt die zich vervolgens verplaatsen (**migreren**) in de wond. Cellen gaan pas delen en migreren, wanneer ze signalen hebben gekregen dat er een wond is. Het afgeven en ontvangen van deze signalen gaat bijna altijd goed. Hierdoor zullen wonden genezen. Helaas gaat het soms ergens in het lichaam mis en “denken” cellen dat ze moeten delen en migreren terwijl hun omgeving dit niet aangeeft. Wanneer cellen zich ongecontroleerd gaan delen en niet opgeruimd worden, ontstaat een tumor. Er is sprake van kanker, als deze zieke cellen naar andere plekken migreren (uitzaaien). Het is dus heel belangrijk om te bestuderen hoe een cel omgaat met signalen van haar omgeving.



Figuur 1, van mens tot plasma membraan. De mens bestaat uit miljarden cellen. Eén zo’n cel is opgebouwd uit veel verschillende bouwstenen (moleculen). Lipiden en eiwitten zijn belangrijke bouwstenen/moleculen voor de buitenste laag van de cel. Dit gedeelte van de cel noemen we de plasma membraan.

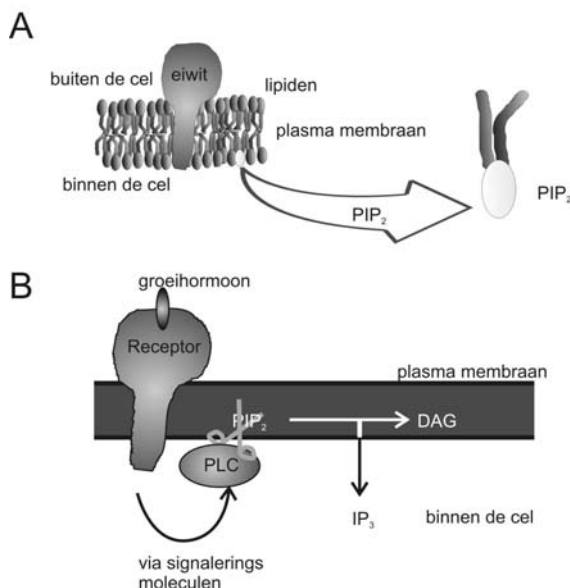
De cel en de plasma membraan

Een cel is opgebouwd uit veel hele kleine bouwstenen (moleculen). Het buitenste gedeelte van de cel, “**de plasma membraan**” (Fig. 1), is opgebouwd uit lipide bouwstenen (die zeep-achtige structuur hebben), en eiwit bouwstenen. De plasma membraan werkt zoals een grens van een land. Het bestuur van het land beslist wat wel en niet de grens over mag. Als de cel “beslist” iets uit de omgeving nodig te hebben, of juist iets kwijt te willen raken, dan laat de plasma membraan specifiek dat molecuul door.

Vroeger werd gedacht dat de lipide bouwstenen alleen maar belangrijk waren voor het opbouwen van de membraan. Tegenwoordig weten we dat sommige lipiden ook een rol spelen bij het doorgeven van signalen tussen de binnen- en buitenkant van de cel. Ze zijn bijvoorbeeld van belang bij de overdracht van wondsignalen, waarbij cellen moeten gaan delen en migreren.

PIP₂

In dit proefschrift is gekeken naar de lipide met de chemische naam Phosphatidyl-Inositol(4,5)bisphosphate. Om het makkelijk te houden wordt dit lipide ook wel **PIP₂** genoemd. PIP₂ heeft twee belangrijke functies. Ten eerste vormt het met andere lipiden en



Figuur 2, PIP₂. (A) Een schema van een stukje plasma membraan. Het plasma membraan bestaat uit eiwitten en een dubbele laag lipiden. PIP₂ (witte kop) is één van de vele soorten lipiden. (B). Bovendien is PIP₂ belangrijk bij het doorgeven van signalen. Wanneer een groei hormoon aan een receptor eiwit bindt wordt het eiwit PLC geactiveerd (aangezet). Dit eiwit knipt PIP₂ kapot waardoor DAG en IP₃ worden gevormd die op hun beurt weer vele processen in de cel activeren.

eiwitten de plasma membraan (Fig. 2A). Daarnaast is PIP₂ belangrijk voor het doorgeven van signalen. Wanneer bijvoorbeeld een wondsignaal herkend wordt door een **receptor** (een eiwit in de plasma membraan dat als een antenne “zoekt” naar signalen) in de plasma membraan, wordt vervolgens het eiwit Phospholipase C (**PLC**) geactiveerd (Fig. 2B). Dit PLC eiwit werkt als een soort schaar die PIP₂ kapot knipt, waardoor twee nieuwe signaalmoleculen ontstaan, DAG en IP₃. Deze activeren op hun beurt weer diverse processen in de cel. PIP₂ is dus een belangrijke voorloper van signaalmoleculen binnen de cel.

Er wordt echter door steeds meer wetenschappers gesuggereerd dat PIP₂ niet alleen maar een voorloper is van signaalmoleculen, maar dat PIP₂ misschien zelf ook kan functioneren als een signaalmolecuul. Maar hoe bewijs je dat?

Als PIP₂ inderdaad een signaalmolecuul is dan moet het voldoen aan de volgende signaal criteria;

1. het niveau van het PIP₂ moet kunnen veranderen.
2. deze verandering moet een reactie teweegbrengen.

Een goed voorbeeld van een signaal uit het dagelijks leven is het fluitsignaal van een voetbalscheidsrechter. Het niveau van het signaal kan veranderen: het is namelijk stil, totdat de scheidsrechter een beslissing neemt en fluit. Hierop wordt, als het goed is, gereageerd; het spel wordt bijvoorbeeld tijdelijk gestopt. Echter, als de spelers zich misdragen en ondanks het fluitsignaal door blijven gaan, kan een wedstrijd behoorlijk uit de hand lopen. Dit geldt ook voor cellen. Als cellen niet op sommige signalen reageren, of juist gaan reageren zonder een signaal kan dit leiden tot kanker.

Nu terug naar PIP₂. De plasma membraan bevat veel PIP₂ moleculen. Door een signaal van buiten de cel wordt het eiwit PLC geactiveerd wat resulteert in PIP₂ afbraak (Fig.

2B). Daardoor neemt het niveau van PIP_2 af. Het niveau varieert afhankelijk van de activiteit van PLC. PIP_2 voldoet dus aan bovengenoemd criterium 1.

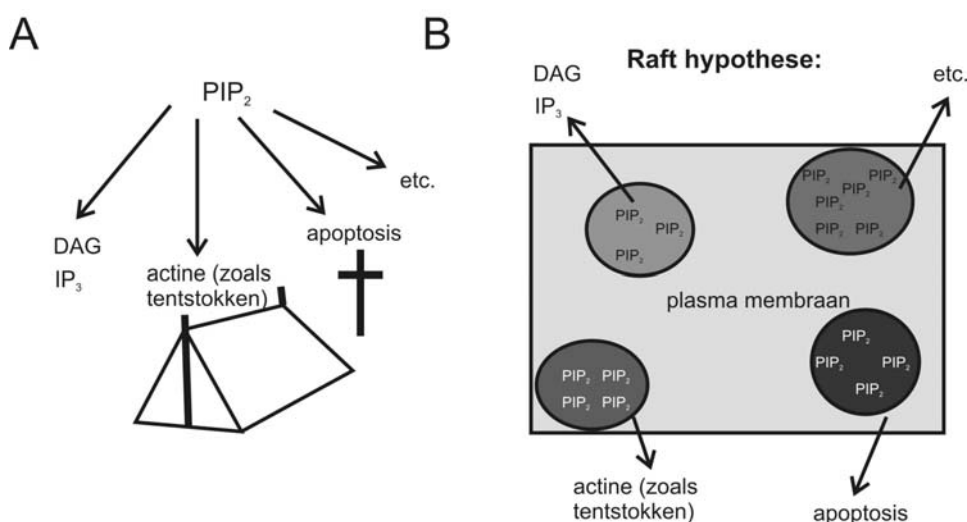
Maar voldoet PIP_2 ook aan het tweede criterium? Brengt de verandering van het PIP_2 niveau ook een reactie teweeg.

PIP_2 is een signaalmolecuul

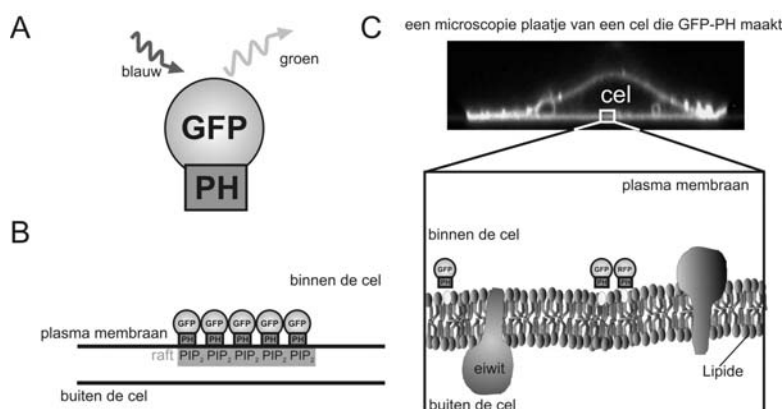
In het eerste gedeelte van mijn proefschrift bestuderen we PIP_2 als signaalmolecuul. In hoofdstuk 2 laten we zien dat de aanmaak van PIP_2 stopt, wanneer de cel te veel beschadigd is door fysische of chemische stress vanuit de omgeving om “gezond” te kunnen doorleven. Dit treedt bijvoorbeeld op in huidcellen na langdurige blootstelling aan UV straling tijdens het zonnebaden. Het afgenomen PIP_2 niveau in de plasma membraan is voor de cel een signaal om dood te gaan. Dit proces heet geprogrammeerde celdood, met een mooi woord: **apoptose**. Dit is belangrijk, want als een beschadigde cel niet dood gaat, zou deze zich tot een kankercel kunnen ontwikkelen!

In hoofdstuk 3 laten we zien dat verschillende signalen van buiten de cel het eiwit PLC (binnen in de cel) activeren. Het geactiveerde PLC breekt vervolgens PIP_2 af. De verlaging van PIP_2 remt de opbouw en afbraak van structuren (**actine**) die voor de vorm van de cel zorgen, net zoals de tentstokken van een tent.

Op basis van dit onderzoek onderschrijven we de hypothese dat PIP_2 inderdaad als een signaalmolecuul functioneert, omdat het voldoet aan beide genoemde criteria. Ten eerste kan het niveau van PIP_2 veranderen en ten tweede wordt op deze verandering gereageerd (cel gaat dood of de dynamiek van de actine structuren verandert).



Figuur 3, PIP_2 en rafts (A) PIP_2 is een belangrijk signaal molecuul bij verschillende processen in de cel. In dit proefschrift hebben we beschreven dat PIP_2 betrokken is bij de formatie van DAG en IP_3 , bij de dynamiek van actine, en bij apoptose (B). Om te kunnen begrijpen hoe PIP_2 betrokken kan zijn bij verschillende processen in één cel, hebben wetenschappers een hypothese gemaakt die ze de vlotten (raft) hypothese noemen. In deze hypothese wordt gesuggereerd dat de plasma membraan in verschillende domeinen of vlotten (rafts) verdeeld is. In deze rafts kan PIP_2 mogelijk clusteren. Wanneer dit inderdaad gebeurt dan kunnen verschillende PIP_2 rafts diverse functies hebben.



Figuur 4, het zichtbaar maken van PIP_2 . (A) Om PIP_2 zichtbaar te maken, hebben we in dit proefschrift gebruik gemaakt van het eiwit GFP-PH. GFP kan blauw licht opnemen en groen licht uitstralen. PH bindt heel sterk aan PIP_2 . (B) Hierdoor bindt GFP-PH aan het PIP_2 in de plasma membraan. (C) Een zij aanzicht foto gemaakt met een confocale microscoop van een cel die GFP-PH maakt. Omdat GFP-PH aan PIP_2 in de plasma membraan bindt, is de plasma membraan van de cel zichtbaar.

PIP_2 in vlotten (rafts)

In hoofdstuk 2 en 3 is duidelijk geworden dat PIP_2 een belangrijke voorloper is van signaalmoleculen (namelijk IP_3 en DAG) en dat PIP_2 ook zelf een signaal is tijdens processen zoals apoptose en de dynamiek van actine structuren. Ook andere wetenschappers hebben bewezen dat PIP_2 in veel processen binnen de cel een belangrijke rol speelt. Het lijkt dus wel of PIP_2 meerdere functies kan hebben in een cel (Fig. 3A). Maar hoe kan PIP_2 afbraak bijvoorbeeld specifiek de dynamiek van actine structuren beïnvloeden zonder apoptose te induceren? Om dit te verklaren is in de literatuur de volgende hypothese gepostuleerd (Fig. 3B):

“De plasma membraan is mogelijk verdeeld in verschillende domeinen. Deze domeinen worden ook wel vlotten genoemd (in dit proefschrift gebruiken we het engelse woord *rafts*). Deze domeinen heten zo omdat gedacht wordt dat ze als een vlot drijven in een zee van lipiden. In deze vlotten kunnen specifieke eiwitten en lipiden clusteren. De eiwitten en lipiden binnen een raft hebben een andere functie dan dezelfde eiwitten en lipiden buiten de raft.”

Zitten PIP_2 moleculen ook specifiek in dergelijke rafts? En hebben ze daar verschillende functies? Om deze interessante onderzoeksvragen te kunnen beantwoorden, moesten we PIP_2 zichtbaar kunnen maken.

PIP_2 zichtbaar maken in de cel

Sommige kwallen in de zee lichten s’ nachts op. Dit komt omdat ze eiwitten bevatten dat blauwe licht kunnen absorberen (opnemen) en dan zelf groen licht kunnen uitzenden. Het eiwit dat daar verantwoordelijk voor is heet het “groene fluorescentie eiwit” (GFP). Dit GFP eiwit is “geplakt” aan een gedeelte van een PLC eiwit dat het PH domein wordt

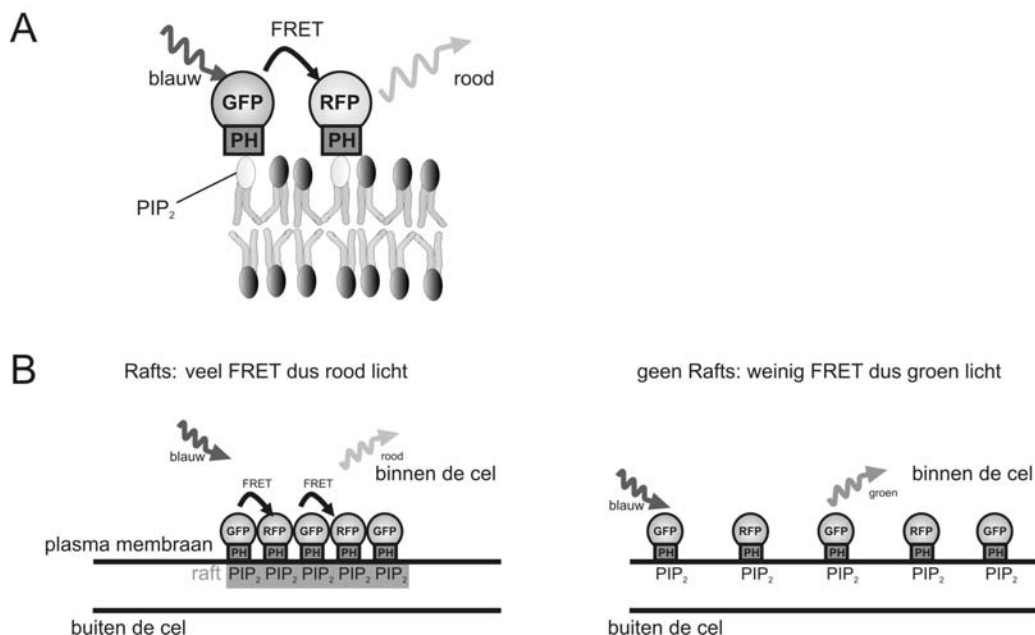
genoemd (Fig. 4A). In dit proefschrift noemen we dit aan elkaar geplakt eiwit GFP-PH. Omdat het PH domein heel specifiek aan PIP₂ kan binden, doet het GFP-PH eiwit dat ook. Dus in cellen die GFP-PH maken, binden de GFP-PH eiwitten aan de PIP₂ in de plasma membraan (Fig. 4B). Door deze cellen te bestralen met blauw licht, is onder de microscoop het groene licht van GFP-PH zichtbaar en kunnen we de positie van GFP-PH en dus ook van PIP₂ bepalen (Fig. 4C).

In hoofdstuk 3 hebben we met behulp van GFP-PH gekeken of PIP₂ geclusterd is in de plasma membraan. In het tweede gedeelte van dit hoofdstuk laten we zien dat GFP-PH homogeen verdeeld is over de plasma membraan, hoewel er kleine domeinen zijn. In deze domeinen/rafts lijkt GFP-PH geclusterd te zijn. Andere wetenschappers zagen dat ook en zij concludeerden dat GFP-PH en dus ook PIP₂ geclusterd is in de plasma membraan. Deze materie is echter veel complexer, hetgeen we in het hoofdstuk 3 laten zien. In deze GFP-PH rijke domeinen is de plasma membraan namelijk heel erg geplooid en gerimpeld. Deze plooien en rimpels zijn zo klein dat je ze niet ziet met de microscoop. Hierdoor ontstaat ten onrechte de indruk dat in deze gebieden GFP-PH en dus ook PIP₂ geclusterd is (voor verdere uitleg zie Box 3, hoofdstuk 1).

FRET

Om toch PIP₂ clustering in kleine domeinen te kunnen bestuderen, hebben we de afstand tussen GFP-PH eiwitten gemeten. Als bijvoorbeeld 5 GFP-PH eiwitten bijengepakt zijn in een kleine raft dan is de onderlinge afstand klein, maar wanneer diezelfde 5 GFP-PH eiwitten over de hele plasma membraan verdeelt zijn, dan is de onderlinge afstand erg groot (zie Fig. 5B, linker plaatje, PIP₂ is geclusterd in raft. In het rechter plaatje is PIP₂ niet geclusterd.) De afstand tussen GFP-PH eiwitten en dus ook de clustering van PIP₂ in raften kunnen we meten met een natuurkundige truc.

Ze recentelijk zijn er naast groene, ook cyane, gele en rode fluorescentie eiwitten beschikbaar (resp. CFP, YFP, RFP). Met behulp van deze eiwitten hebben wij cyane, gele en rode PIP₂ sensoren gemaakt (respectievelijk CFP-PH, YFP-PH en RFP-PH) (Fig. 5A). Bij belichting met blauw licht (488 nm) van cellen die GFP-PH en RFP-PH maken, zal GFP-PH het licht absorberen en groen licht uitstralen. Echter, wanneer er een RFP-PH heel dicht bij de GFP in de buurt zit (minder dan 10 nm, een miljoenste van 1 cm) dan kan GFP-PH de energie van het geabsorbeerde licht geven aan RFP-PH (Fig. 5A). Hierdoor komt er rood licht uit de cel in plaats van groen licht. Dit proces heet fluorescentie resonantie energie overdracht (FRET). FRET is erg afhankelijk van de afstand tussen GFP-PH en RFP-PH. In een cel die erg rood is, is de gemiddelde afstand tussen GFP-PH en RFP-PH zeer klein. Dus als PIP₂ moleculen en daardoor ook GFP-PH en RFP-PH in vlotten zitten, dan zullen GFP-PH en RFP-PH dicht bij elkaar zitten. Hierdoor zal er veel FRET zijn en is de cel dus rood-achtig (Fig. 5B). Als er geen vlotten in de plasma membraan aanwezig zijn, dan is de gemiddelde afstand tussen GFP-PH en RFP-PH groot wat resulteert in weinig FRET en een groene cel (Fig. 5B). Met behulp van FRET hebben we gevonden dat PIP₂ niet verrijkt is in vlotten of domeinen. Dit is een belangrijke conclusie omdat de regulatie van verschillende processen door PIP₂ niet gebeurt door verschillende PIP₂ clusters (zoals raften) hetgeen de hypothese van Fig. 3B stelde. Om deze studie te kunnen volbrengen, moesten we diverse nieuwe meet methodes



Figuur 5, met behulp van FRET is onderzocht of PIP₂ geclusterd is in rafts. (A) GFP-PH en RFP-PH eiwitten binden aan PIP₂ in de plasma membraan. Door de cel met blauw te bekijken zal GFP-PH soms het licht absorberen. Als RFP-PH heel dicht in de buurt zit van GFP-PH (minder dan 10 nm), dan kan GFP-PH de geabsorbeerde energie van het licht overgeven aan RFP-PH. RFP-PH zal dan een rood licht uitstralen, in plaats dat GFP-PH het groene licht. Dit proces heet FRET. (B) Omdat FRET afhankelijk is van de afstand tussen GFP-PH en RFP-PH, kunnen we door FRET te meten een uitsluitsel geven of GFP-PH en RFP-PH (en dus ook PIP₂ moleculen) dicht bij elkaar zitten in een vlot of domein. Als PIP₂ moleculen clusteren in rafts (linker plaatje), dan is de afstand tussen GFP-PH en RFP-PH klein en is er veel FRET waardoor de cel rood-achtig is. Echter, als PIP₂ moleculen niet geclusterd zijn in rafts, dan is de afstand tussen GFP-PH en RFP-PH erg groot en er is geen FRET. Dus bij deze grote afstand (rechter plaatje) zal voornamelijk groen licht te zien zijn.

ontwikkelen. De belangrijkste is een kwantitatieve methode om FRET in detail te kunnen meten op een confocale microscoop (hoofdstuk 5).

Conclusie

In dit proefschrift is bekeken of PIP₂ een lokaal signaal kan zijn voor processen binnen de cel. Op basis van ons onderzoek onderschrijven we de hypothese dat PIP₂ een signaalmolecuul is, echter we hebben geen bewijs kunnen vinden voor lokale PIP₂ signalen.

Curriculum Vitae

Jacobus Emiel van Rheenen geboren op 12 januari 1978 te Utrecht, behaalde in 1995 zijn HAVO diploma aan de Breul in Zeist. Vervolgens behaalde hij zijn propedeuse aan de Hogere Laboratorium School te Utrecht. In 1996 stroomde hij door naar de Universiteit van Amsterdam waar hij aan zijn studie Biologie begon. Tijdens zijn specialisatiefase liep hij stage in de groep van Dr. Frans Klis onder begeleiding van Dr. Edwin Vink aan het Swammerdam Institute of Life Sciences (Universiteit van Amsterdam). Daar bestudeerde hij de functie van het kleine G-eiwit Rho1p in de biosynthese van de celwand van gist. Tijdens deze stage raakte hij geïnteresseerd in het bestuderen van processen in levende cellen. Vervolgens liep hij stage in de groep van Dr. Charles Brearley en Prof. Robin Irvine van de Department of Pharmacology aan de University of Cambridge. Met behulp van small silencing RNAs probeerde hij de functie van PIP 5-Kinase te bestuderen in planten. Tijdens deze stage bekwaamde hij zich in microscopie en moleculaire biologie. September 2000 studeerde hij af als biotechnoloog en begon direct daarna aan zijn promotie onderzoek. Dit deed hij bij de vakgroep Celbiologie van het Nederlands Kanker Instituut onder begeleiding van Dr. Kees Jalink. Daar bestudeerde hij met behulp van diverse microscopische technieken, fosfolipiden signalering in levende cellen. Met de microscopische ervaringen op zak, wilde hij vervolgens nog een stap verder gaan door met behulp van microscopie de processen die betrokken zijn bij tumor progressie te bestuderen in levende organismen. Om dit mogelijk te maken vroeg hij een beurs aan bij het Koningin Wilhelmina Fonds, die hij toegekend kreeg in Oktober 2005. Dit stelt hem in staat om vanaf Januari 2006 onderzoek te gaan doen aan de Albert Einstein College of Medicine in New York onder begeleiding van Prof. John Condeelis.

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