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Review

Regulation and cellular roles of phosphoinositide 5-kinases

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Abstract

The membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), plays a critical role in various, apparently very different cellular processes. As precursor for second messengers generated by phospholipase C isoforms and class I phosphoinositide 3-kinases, PIP_2 is indispensable for cellular signaling by membrane receptors. In addition, PIP_2 directly affects the localization and activity of many cellular proteins via specific interaction with unique phosphoinositide-binding domains and thereby regulates actin cytoskeletal dynamics, vesicle trafficking, ion channel activity, gene expression and cell survival. The activity and subcellular localization of phosphatidylinositol 4-phosphate 5-kinase (PIP5K) isoforms, which catalyze the formation of PIP_2, are actively regulated by membrane receptors, by phosphorylation and by small GTPases of the Rho and ARF families. Spatially and temporally organized regulation of PIP_2 synthesis by PIP5K enables dynamic and versatile PIP_2 signaling and represents an important link in the execution of cellular tasks by Rho and ARF GTPases.

Keywords: PIP5K; PIP_2; Phosphoinositide metabolism; Rho; ARF

Contents

1. Introduction

Two breakthrough discoveries in the 1980s accelerated the progress in our understanding of the role of phosphoinositides in cellular signaling by membrane receptors. The demonstration by Streb et al. (1983) that inositol 1,4,5-

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trisphosphate (IP_3) is a second messenger that mobilizes Ca^{2+} from stores in the endoplasmic reticulum directed the attention towards the receptor-regulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC). Five years later, the identification of the phosphoinositide kinase activity associated with the Src oncoprotein and the platelet-derived growth factor (PDGF) receptor as a phosphoinositide 3-kinase (PI3K) by Whitman et al. (1988) initiated a strong research interest in the role of PI3K and its lipid products in cell proliferation and transformation. The appreciation that PIP_2 not only serves as a substrate for PLC and PI3K, but can directly interact with intracellular proteins and is involved in an amazing amount of various and fundamental cellular processes, opened a further exciting area in phosphoinositide signaling and resulted in a renewed interest in the regulation of PIP_2 synthesis in the past years. The finding that the activity of phosphatidylinositol 4-phosphate 5-kinase (PIP5K), the enzyme that catalyzes the last step in PIP_2 synthesis, is regulated by monomeric GTPases of the Rho and the ARF families places the classical phosphoinositide cycle in a new context and provides a regulatory mechanism that is likely to direct the novel roles for the phosphoinositides. This review will focus on the regulation of PIP_2 synthesis by PIP5K and on the involvement of the enzyme in cellular processes.

2. Synthesis of PIP_2 by PIP5K

PIP_2 contains a *myo*-inositol headgroup connected to an 1,2-diacylglycerol backbone via a phosphodiester bond (Fig. 1A). In the canonical phosphoinositide pathway, the synthesis of PIP_2 is assumed to result from the sequential phosphorylation of phosphatidylinositol (PI) on the *D*-4 and *D*-5 position of the inositol ring by phosphatidylinositol 4-kinase (PI4K) and PIP5K, respectively. Originally, two distinct PIP5K isoforms (type I and II) were isolated from erythrocytes by chromatographic procedures and found to have different immunological and biochemical properties (Bazenet et al., 1990). PIP5K type I was characterized by an ~20-fold lower K_m for PI4P and could be stimulated by phosphatidic acid (PA), heparin and spermine (Loijens et al., 1996). Further examination of the substrate specificities showed that the type II enzymes actually phosphorylated PI5P at the *D*-4 position and unmasked the type II kinase as a phosphatidylinositol 5-phosphate 4-kinase (PIP4K) (Rameh et al., 1997). Thus, type II PIP4K enzymes phosphorylate PI5P, but also PI3P at the *D*-4 position, whereas the type I enzymes preferentially phosphorylate PI4P, but also PI3P, $PI(3,4)P_2$ and, to a lesser extent, PI at the *D*-5 position in vitro (Zhang et al., 1997; Tolias et al., 1998).

The discovery of further phosphoinositide species, the appreciation of broader substrate specificities of the PIPKs and the existence of various specific phosphatases have remodeled the phosphoinositide metabolism from the original linear cascade to a highly dynamic network system (Fig. 1B). Thus, PIP_2 can theoretically be generated in two phosphorylation reactions, from PI4P by (type I) PIP5K, and from PI5P by (type II) PIP4K. Pulse-labeling with [^{32}P]orthophosphate and determination of the relative labeling rate of the *D*-4 and *D*-5 positions suggested that in intact cells the phosphorylation of PI4P by PIP5K represents the major route for PIP_2 synthesis (Stephens et al., 1991; Whiteford et al., 1997). Also the very low amount of PI5P within cells (~2% of total PI monophosphates) makes PI5P a most unlikely supplier for large quantities of PIP_2 (Rameh et al., 1997). Alternatively, PIP_2 arises by the specific dephosphorylation of the *D*-3 position of $PI(3,4,5)P_3$ by the lipid phosphatase PTEN (Maehama and Dixon, 1998), but whether this reaction contributes to PIP_2-regulated processes or merely has the purpose to terminate PIP_3 signaling is not yet clarified. In analogy, PIP_2 is not only consumed by PLC-mediated hydrolysis and *D*-3 phosphorylation by PI3K, dephosphorylation of PIP_2 by class II

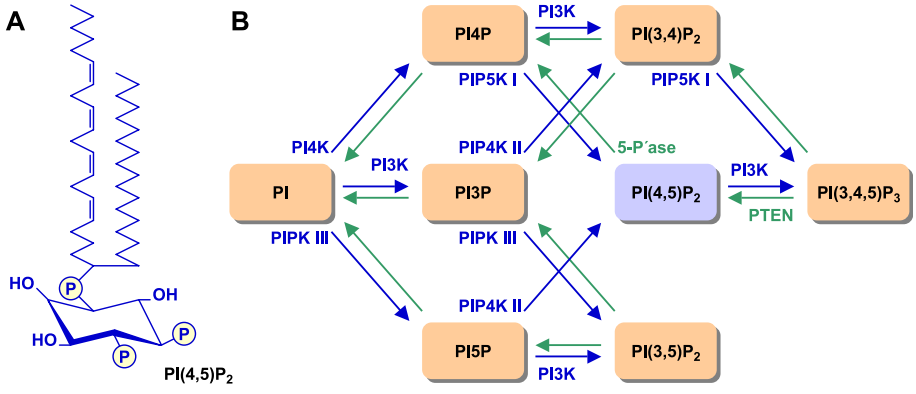

Fig. 1. PIP_2 and phosphoinositide metabolism. (A) Chemical structure of PIP_2. The acronym $PI(4,5)P_2$ considers the positions *D*-4 and *D*-5 of the inositol ring that are phosphorylated. (B) The phosphoinositide network. The inositol ring of PI can be reversibly phosphorylated at one or any combination of the *D*-3, *D*-4 and *D*-5 positions, resulting in the seven identified phosphoinositides that are interconnected by kinase (blue) and phosphatase (green) reactions. Note that $PI(4,5)P_2$ can be generated by the phosphorylation of PI4P and PI5P as well as by the dephosphorylation of $PI(3,4,5)P_3$, and that PIP5K type I catalyzes the synthesis of $PI(4,5)P_2$ as well as $PI(3,4,5)P_3$ in vivo. For the sake of clarity, only the dephosphorylation reactions that directly affect $PI(4,5)P_2$ are labeled.

inositol polyphosphate 5-phosphatases, such as synaptojanin, is involved in the regulation of cellular PIP_2 pools and is indispensable for PIP_2 recycling during vesicle trafficking (Majerus et al., 1999).

Three isoforms of type I PIP5K (designated Iα, Iβ and Iγ; unfortunately, the nomenclature for human and murine Iα and Iβ isoforms is reversed) with alternative splice variants (Ishihara et al., 1996, 1998; Loijens and Anderson, 1996) and also three type II PIP4K isoforms (IIα, IIβ and IIγ) (Boronenkov and Anderson, 1995; Castellino et al., 1997; Itoh et al., 1998) have been cloned and characterized. The PIP5K isoforms have molecular masses of 68 kDa (Iα and Iβ) and 90 kDa (Iγ), respectively. Sequence analysis has shown that the PIP4K and PIP5K enzymes are related, but that they share no identity to most other lipid (PI3K and PI4K) or protein kinases. Remarkable homologs to the PIPKs are the yeast gene products, Mss4p and Fab1p. The mammalian ortholog of the yeast Fab1p lipid kinase, p235 PIKfyve, was found to synthesize *D*-5-phosphoinositides (Sbrissa et al., 1999) and to support PI5P and $PI(3,5)P_2$ synthesis in vivo (McEwen et al., 1999; Sbrissa et al., 2002). On account of the homology and activity of Fab1p/PIKfyve, these enzymes are now proposed to be designated as PIPK type III. The sequence similarity between the PIP4Ks and PIP5Ks is clustered in the catalytic core of the kinases, and the little sequence homology outside this region suggests that the individual isoforms may be distinctly regulated (Hinchliffe et al., 1998; Anderson et al., 1999). Elegant studies by Kunz et al. (2000, 2002) demonstrated that an activation loop spanning the catalytic domain determines both substrate specificity and subcellular targeting of type I and type II PIPKs, which can be swapped by substitution of a single amino acid in the activation loop. When type II PIPK was provided with the type I activation loop, by constructing a IIβbackbone-Iβloop-chimera, the type II enzyme now localized to the plasma membrane and produced PIP_2. However, the chimera was less effective in inducing actin remodeling, suggesting that other regulatory features of PIP5K isoforms may be required. In murine PIP5K-Iβ, two dimerization domains were identified, which may contribute to the proper subcellular localization and functioning of the enzyme (Galiano et al., 2002).

3. Cellular roles of PIP_2 and PIP5K

Hydrolysis of PIP_2 by PLC isoforms into the second messengers, IP_3 and 1,2-diacylglycerol, is a general and well-defined answer of cells in response to stimulation of a variety of membrane receptors (Rhee, 2001), and the resulting rise in intracellular Ca^{2+} and the activation of protein kinase C (PKC) isoforms are early components in many signaling pathways. Phosphorylation of PIP_2 by class I PI3K results in the rapid accumulation of PIP_3, which recruits and activates mediators involved in actin remodeling, mitogenesis and survival (Vanhaesebroeck et al., 2001). Its position as precursor of three second messengers in two essential signaling pathways would have sufficed to denote PIP_2 a crucial component in cellular signaling. But PIP_2 is also a messenger by itself and affects by direct binding via its phosphorylated headgroup to phosphoinositide-binding motifs in a variety of effector molecules the localization, the catalytic activity or the functionality of these proteins. Several highly conserved phosphoinositide-binding sequences are now identified, including the pleckstrin homology (PH); phox homology (PX); epsin N-terminal homology (ENTH); four-point-one, ezrin, radixin, moesin (FERM); Fab1p, YOTB, Vps27p, EEA1 (FYVE); and tubby domains (Santagata et al., 2001; Itoh and Takenawa, 2002; Cozier et al., 2004).

The perception that PIP_2 is involved in the regulation of the actin cytoskeleton started with the observation by Lassing and Lindberg (1985) that PIP_2 specifically interacts with profilin. Since this initial report, the association of PIP_2 with a plethora of actin-binding proteins that regulate the structure and dynamics of the actin cytoskeleton has been described (Yin and Janmey, 2003; Hilpela et al., 2004). Binding of PIP_2 to actin regulatory proteins can lead to the displacement of these proteins from actin monomers (profilin) or from barbed ends of actin filaments (gelsolin), thereby driving rapid and local actin polymerization. Binding of PIP_2 to vinculin promotes its oligomerization with talin, actin and vasodilator-stimulated phosphoprotein (VASP), and leads to the bundling of the distal ends of actin filaments in focal adhesion contacts (Gilmore and Burridge, 1996; Hüttelmaier et al., 1998). In addition, the binding of PIP_2 to cytoskeletal proteins may control the adhesion between actin-based cortical cytoskeleton and the plasma membrane (Raucher et al., 2000). Consistent with a function for PIP_2 in the control of actin assembly, overexpression of type I PIP5K isoforms was shown to dramatically affect actin cytoskeletal dynamics and to induce, dependent on the cell type used, stress fibers (Shibasaki et al., 1997; Yamamoto et al., 2001), membrane ruffles (Honda et al., 1999), microvilli (Matsui et al., 1999) and motile actin comets (Rozelle et al., 2000). On the other hand, reduction of cellular PIP_2, through forced hydrolysis by synaptojanin or the *Salmonella* phosphatase SigD, or through sequestration of PIP_2 by the PH domain of PLC-δ1 or a gelsolin-derived peptide, resulted in a decreased number of actin stress fibers (Sakisaka et al., 1997), reduced membrane-cytoskeletal adhesion energy (Raucher et al., 2000; Terebiznik et al., 2002) and blockade of actin assembly and cell motility (Cunningham et al., 2001). Using green fluorescent protein-tagged PH domains or antibodies to visualize PIP_2, the lipid was found to concentrate in highly dynamic, actin-rich regions (Tall et al., 2000) and lipid rafts (Laux et al., 2000; Parmryd et al., 2003) in the plasma membrane, feeding the idea that spatially organized PIP_2 synthesis regulates actin polymer-

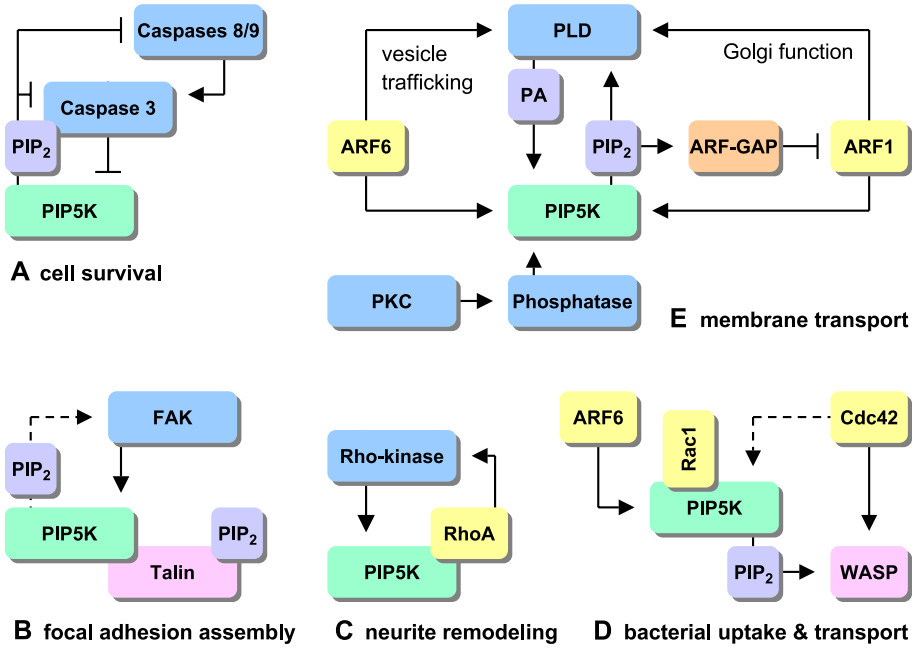

Fig. 2. Role of PIP5K in cellular signaling processes. (A) PIP_2 protects against apoptosis by inhibiting the initiator caspases 8 and 9 as well as their common effector caspase 3 by direct binding, whereas PIP5K-Iα is cleaved and inactivated by caspase 3 during apoptosis. (B) PIP5K is targeted to focal adhesions by talin, and phosphorylation of PIP5K by FAK, which is sensitive to PIP_2, increases lipid kinase activity and binding to talin. (C) RhoA binds to PIP5K, and activation of PIP5K by RhoA is mediated by Rho-kinase. Activation of PIP5K by RhoA and Rho-kinase induces neurite retraction, and this process may involve modulation of cell–matrix interactions. (D) Rac1 binds to and activates PIP5K. Bacterial uptake involves recruitment of PIP5K by Rac1 to the phagosome, and activation of PIP5K by ARF6. Actin-based motility of pathogens requires coordinate activation of WASP by PIP_2 and Cdc42, but Cdc42 has also been shown to stimulate PIP_2 synthesis. (E) PIP5K is activated by ARF1 and ARF6, which are involved in the regulation of Golgi structure and function, and vesicle trafficking, respectively. Also, PLD, which regulates membrane traffic, is activated by ARF GTPases, and the activities of PLD and PIP5K can be amplified by reciprocal stimulation via their products PA and PIP_2. PKC-induced dephosphorylation of PIP5K, apparently by protein phosphatase 1, results in enhanced lipid kinase activity, and phosphatase-liberated PIP5K can restore exocytosis. PIP_2 can affect the function of exchange factors for ARF1 (not shown), as well as terminate ARF1 activation by stimulating ARF-GAP activity. Arrows and blunted lines represent stimulatory and inhibitory interactions, respectively. Dotted interactions have been observed, but are not necessarily involved in the concerned processes.

ization and other cellular processes. The localization of PIP_2 in rafts is supported by biochemical data (Pike and Casey, 1996); however, specific PIP_2 clustering has been disputed by others (van Rheenen and Jalink, 2002; Watt et al., 2002).

PIP_2 is also an intrinsic membrane recognition signal involved in membrane trafficking processes, such as endocytosis, exocytosis and vesicle motility (Martin, 2001; Simonsen et al., 2001). Recruitment of the endocytic proteins, AP2, epsin and AP180, by PIP_2, the latter via their ENTH domains (Itoh et al., 2001; Ford et al., 2001), is the priming step for clathrin-coat formation preceding endocytosis. Expression of catalytically active murine PIP5K-Iβ strikingly increased epidermal growth factor (EGF) receptor endocytosis (Barbieri et al., 2001), whereas a truncated, inactive mutant interfered with colony-stimulating factor-1 receptor internalization and, hence, increased receptor signaling (Davis et al., 1997). The PIP_2 pool involved in regulated exocytosis is also localized in the plasma membrane (Holz et al., 2000; Micheva et al., 2001), and PIP_2 synthesis by PIP5K is required for dense-core vesicle secretion (Hay et al., 1995), and perhaps synaptic vesicle secretion as well (Zheng et al., 2004), and likely depends on the direct interaction of PIP_2 with the vesicle protein synaptotagmin-1 (Bai et al., 2004).

Application of phosphoinositides, in particular PIP_2, to inside–out membrane patches led to the activation of K_{ATP} channels and antagonized ATP inhibition of the channels (Hilgemann and Ball, 1996; Fan and Makielski, 1997). In intact cells, manipulation of membrane PIP_2 levels by expression of wild-type or inactive PIP5K significantly modulated the nucleotide sensitivity of K_{ATP} channels (Shyng et al., 2000). PIP_2 is now appreciated to regulate a variety of ion transporters and channels, including all inward rectifier K^+ channels, cardiac Na^+/Ca^{2+} exchangers (NCX1), Na^+/H^+ exchangers (NHE1-4), epithelial Na^+ channels (EnaC), mammalian rod cyclic nucleotide-gated (CNG) channels, *Drosophila* transient receptor potential-like (TRPL) channels, the IP_3 receptor, and capsaicin-activated (TRPV1) and melastatin-related (TRPM7) transient receptor potential channels (Hilgemann et al., 2001).

In agreement with a role for dynamic PIP_2 synthesis in various processes, PIP5Ks are found in several cellular compartments, including the plasma membrane, Golgi and vesicles, but also in the internal nuclear matrix (Payrastre et

al., 1992), and phosphoinositides may regulate nuclear processes such as gene transcription, mRNA export, cell cycle progression and DNA repair (Irvine, 2003). Indeed, the retinoblastoma susceptibility gene product, pRB, which regulates progression of cells from G1 through S phase, has been shown to interact with and stimulate PIP5K isoforms, leading to increased nuclear PIP_2 levels (Divecha et al., 2002).

Activation of phosphoinositide-dependent kinase-1 and Akt/protein kinase B, initiated by *D*-3-phosphorylated phosphoinositides as PIP_3, is a well-established mechanism in cell survival. The availability of PIP_2 as substrate for PI3K might indirectly influence this protective pathway. Indeed, expression of constitutively active $G\alpha_q$ attenuated Akt phosphorylation and induced apoptosis, most probably by inducing continuous PLC signaling and depletion of PIP_2 (Howes et al., 2003). In addition, PIP_2 was found to protect against apoptosis, alone or in complex with gelsolin, by directly inhibiting the initiator caspases 8 and 9, as well as by binding and inhibiting their common effector, caspase 3 (Azuma et al., 2000; Mejillano et al., 2001). During apoptosis, PIP5K-Iα was cleaved and inactivated by caspase 3 (Mejillano et al., 2001), suggesting a feedback mechanism between the apoptotic machinery and phosphoinositide synthesis (Fig. 2A). In human cancer cells, PI4K and PIP5K activities were markedly increased (Singhal et al., 1994), and PIP_2 was found to mediate actin cytoskeleton rearrangement during malignant transformation by oncogenic Ras mutants (He et al., 1998).

We must realize that receptor-induced hydrolysis of PIP_2 by PLC, and in isolated membrane areas perhaps phosphorylation by PI3K, leads, in addition to the generation of second messengers, to the consumption of PIP_2, which is likely to affect cellular processes (Stauffer et al., 1998). Stimulation of the muscarinic M_1 receptor or the α_1-adrenoceptor, which couples to PLC, resulted in the inactivation of K_{ATP} (Xie et al., 1999; Haruna et al., 2002), G-protein-gated inwardly rectifying K^+ (GIRK) (Kobrinsky et al., 2000) and TRPM7 channels (Runnels et al., 2002), not through PKC activation, but most probably through a decrease in PIP_2 levels. Agonist-induced PIP_2 hydrolysis and subsequent release of gelsolin in leading lamellipodia have been suggested to drive cytoskeletal rearrangement and protrusion during cell motility (Chou et al., 2002). Reversely, sequestering of PIP_2 by profilin and tubulin at high concentrations was shown to inhibit PLC signaling (Goldschmidt-Clermont et al., 1990; Popova et al., 2002). Furthermore, not all effects of PIP5Ks necessarily depend on the exclusive production of PIP_2, as PIP5K can also phosphorylate $PI(3,4)P_2$ at the *D*-5 position and is likely responsible for PIP_3 synthesis during oxidative stress in intact cells (Halstead et al., 2001).

The tremendous variety of functions of PIP_2 demands the synthesis and turnover of the lipid in particular subcellular compartments to be detailed and distinctly regulated. Decisive for this is the organized regulation of the enzymes that modulate PIP_2 levels: *D*-5-phosphatases, PLC, PI3K and PIP5K isoforms.

4. Regulation of PIP5K isoforms

4.1. Regulation of PIP5K by membrane receptors

Upon receptor activation, the formation of IP_3 by PLC can be accompanied by reduction of the cellular PIP_2 level by ~80%, and obviously, resynthesis of PIP_2 is required to allow full early and sustained phases of receptor signaling (Willars et al., 1998). Unlike original conceptions, synthesis of PIP_2 does not simply depend on mass requirement of the lipid. Labeling of cells with [^{32}P]orthophosphate or *myo*-[^{3}H]inositol has revealed that the synthesis of PIP_2 can be stimulated by G-protein-coupled and tyrosine kinase receptors. Treatment of A431 cells with EGF resulted in rapid stimulation of PI4K and PIP5K activities (Pike and Eakes, 1987), and both enzymes were found to be present in anti-phosphotyrosine immunoprecipitates (Payrastre et al., 1990), to interact with the activated EGF receptor (Cochet et al., 1991) and to associate with the actin cytoskeleton (Payrastre et al., 1991). Modulation of PIP_2 synthesis by G-protein-coupled receptors has been mainly studied in platelets, where stimulation with thrombin resulted in enhanced PIP_2 formation (Nolan and Lapetina, 1990) and in increased association of PIP5K with the cytoskeleton (Grondin et al., 1991). The receptor-induced stimulation and cytoskeletal association of PIP5K may be directly involved in actin cytoskeletal regulation and to represent the assembly and retention of enzymes into signaling complexes, whose formation is likely to guarantee efficient signaling, for instance, by PLC. We found that pulse stimulation of various G-protein-coupled receptors in human embryonic kidney (HEK)-293 cells induced a strong and long-lasting potentiation of repeated PLC activation by G-protein-coupled and tyrosine kinase receptors (Schmidt et al., 1996b, 2000). The sensitization of the PLC response was apparently dependent on pertussis toxin-sensitive G_i proteins and activation of a PKC isoenzyme, and could be partly explained by an increase in the cellular level of PIP_2. In agreement, stimulation of PIP_2 synthesis in permeabilized neutrophils by *N*-formylmethionyl-leucyl-phenylalanine and platelet-activating factor was found to be blocked by pertussis toxin (Stephens et al., 1993), and stimulation of PKC isoforms by phorbol esters has been shown to greatly enhance PIP_2 synthesis in platelets (de Chaffoy de Courcelles et al., 1984; Halenda and Feinstein, 1984) and lymphocytes (Boon et al., 1985).

In lymphocytes, sustained calcium signaling requires appropriate recruitment of nonreceptor tyrosine kinases and PLC-γ into signaling complexes at the plasma membrane (Kurosaki, 2002). Upon ligand binding, the activated B cell receptor translocates to lipid rafts, and its phosphorylation by the Src family kinase Lyn generates binding sites for the

tyrosine kinase Syk. Activated Syk and Lyn now recruit the adaptor proteins BLNK (B cell linker protein) and BCAP (B cell adaptor for PI3K), which serve as docking stations for PLC-γ2, the Tec family tyrosine kinase Btk, and PI3K. PIP_3, produced by coincident activation of PI3K, anchors both Btk and PLC-γ2 in the complex, and supports phosphorylation and activation of PLC-γ2 by a concerted action of Syk and Btk. Very recently, PIP5K was found to associate with Btk and to be shuttled to the plasma membrane upon B cell receptor activation as well (Saito et al., 2003). Obviously, the presence of PIP5K will guarantee the local availability of PIP_2 as common substrate for PI3K and PLC-γ2. It will be interesting to follow whether PIP_2 modulates further the downstream effects of Btk, especially as Btk has been shown to induce cytoskeletal rearrangement involving Rac (Nore et al., 2000). An attractive feedback mechanism may be induced as well, as the Rac exchange factor Vav, which is activated by *D*-3-phosphoinositides, is inhibited by PIP_2 (Han et al., 1998).

4.2. Regulation of PIP5K by phosphorylation

Class I PI3Ks possess intrinsic protein kinase activity that is inseparable from their lipid kinase activity, and phosphorylation of serine residues within the catalytic subunit itself or the associated regulatory subunit down-regulates the enzyme activity (Fruman et al., 1998). Also, all three PIP5K isoforms were shown to have protein kinase activity, and the absence of protein kinase activity in a lipid kinase-negative PIP5K-Iα mutant suggested the same catalytic mechanisms for the lipid and the protein kinase activities (Itoh et al., 2000). Autophosphorylation of PIP5K was markedly and specifically enhanced by PI and resulted in strong suppression of the lipid kinase activity. Phosphorylation of *Schizosaccharomyces pombe* PIP5K by casein kinase I (Vancurova et al., 1999) and of mouse PIP5K-Iα at serine residue 214 by cyclic AMP-dependent protein kinase (PKA) (Park et al., 2001) also resulted in inactivation of the enzymes. In contrast, lysophosphatidic acid-induced PKC activation increased PIP5K activity, not by direct phosphorylation, but most probably by stimulating PIP5K dephosphorylation by the okadaic acid-sensitive protein phosphatase 1 (Park et al., 2001). Interestingly, depolarization of synaptosomes resulted in Ca^{2+}-dependent dephosphorylation of PIP5K-Iγ (Wenk et al., 2001), and restoration of exocytosis in permeabilized PC12 cells by this isoform was PKC-dependent and required Ca^{2+}-induced dephosphorylation of PIP5K-Iγ at serine 264 (Aikawa and Martin, 2003). PI4K and PIP5K were also found to exclusively associate with catalytically active PKCμ, an unusual member of the PKC family which has a putative transmembrane domain, but it is not clear whether this interaction affects PIP5K activity or serves the positioning of the lipid kinases at specific membrane locations (Nishikawa et al., 1998).

In platelets, stimulation of phosphoinositide phosphorylation by the thromboxane A_2 mimetic U46619 (9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$) was strongly inhibited by the tyrosine kinase inhibitor genistein (4′,5,7-trihydroxyisoflavone) (Gaudette and Holub, 1990), suggesting that PIP5K may be regulated by tyrosine phosphorylation. Indeed, treatment of cells with the tyrosine phosphatase inhibitor pervanadate was shown to increase cellular PIP_2 levels (Rozelle et al., 2000), and this mechanism may involve Rho proteins (Rümenapp et al., 1998). Recently, the predominant brain splice variant of PIP5K-Iγ was found to be targeted to focal adhesions by association with the FERM domain of talin (Di Paolo et al., 2003) and to become tyrosine phosphorylated by focal adhesion kinase (FAK), leading to both increased lipid kinase activity and binding of PIP5K to talin (Ling et al., 2003). PIP_2 also interacts with talin, is necessary to retain talin in focal adhesions (Martel et al., 2001) and may additionally be required for FAK activation (Linseman et al., 1999). The recruitment and activation of PIP5K by talin appear critical for focal adhesion assembly, but additional factors such as small GTPases probably cooperate in this process (Fig. 2B).

4.3. Regulation of PIP5K by Rho family GTPases

Small GTPases of the Rho family (Rho, Rac and Cdc42) control a wide variety of signal transduction pathways and have profound effects on the actin cytoskeleton (Etienne-Manneville and Hall, 2002). Since both Rho family members and PIP_2 are involved in the regulation of actin dynamics, it was hypothesized that Rho proteins might partly exert their effect by modulating phosphoinositide metabolism. Chong et al. (1994) found that activated RhoA stimulates PIP_2 synthesis in vitro and that specific inactivation of Rho proteins with *Clostridium botulinum* C3 exoenzyme prevents the stimulation of PIP5K by GTP. In agreement, inactivation of Rho GTPases by *Clostridium difficile* toxin B reduced cellular PIP_2 levels, resulting in inhibition of receptor-mediated inositol phosphate formation by PLC (Schmidt et al., 1996a) as well as diminished stimulation of PIP_2-sensitive phospholipase D (PLD) (Schmidt et al., 1996c). A dominant-negative RhoA mutant, which induced the disassembly of actin stress fibers, did not affect stress fibers induced by PIP5K-Iα, suggesting that PIP5K functions downstream of RhoA in actin organization (Shibasaki et al., 1997). We have recently shown that the RhoA effector, Rho-kinase, which mediates signals from RhoA to the actin cytoskeleton (Amano et al., 1997), is involved in RhoA-dependent regulation of PIP5K activity (Oude Weernink et al., 2000b) (Fig. 2C). In agreement, in intact CV1 cells (Yamamoto et al., 2001) and platelets (Gratacap et al., 2001), PIP_2 accumulation was found to be reduced by the Rho-kinase inhibitor, Y-27632 ((+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)-cyclohexanecarboxamide). PIP5K was recently shown to play an essential role

as downstream effector of RhoA and Rho-kinase in neurite remodeling (van Horck et al., 2002; Yamazaki et al., 2002). Thus, overexpression of wild-type PIP5K-Iα, but not of PIP4K-IIα, led to cell rounding that could not be prevented by Y-27632, whereas neurite retraction induced by RhoA, Rho-kinase and receptor agonists was completely blocked by a kinase-deficient PIP5K mutant. Interestingly, PIP5K-induced cell rounding was not sensitive to inhibition of myosin light chain kinase, suggesting that the effect of PIP_2 is not mediated by enhanced actomyosin contraction, but, alternatively, may depend on reduced integrin-extracellular matrix interaction (van Horck et al., 2002). Ezrin, radixin, and moesin (ERM) proteins crosslink actin filaments to plasma membranes, and the activation and recruitment of ERM proteins by activated RhoA result in the formation of microvilli (Bretscher et al., 2002). PIP_2 can activate ERM proteins in vitro (Hirao et al., 1996), and overexpression of PIP5K mimicked the effects of RhoA on ERM activation and microvilli formation (Matsui et al., 1999), whereas sequestration of polyphosphoinositides by the antibiotic neomycin resulted in the loss of microvilli (Yonemura et al., 2002).

Also, Rac has been shown to stimulate PIP_2 synthesis, and PIP5K isoforms may be critical mediators of Rac-dependent actin remodeling. In permeabilized platelets, addition of active Rac1 induced rapid PIP_2 synthesis, leading to uncapping and polymerization of actin filaments (Hartwig et al., 1995). PIP5K-Iα also induced actin filament assembly, whereas a kinase-deficient PIP5K-Iα mutant blocked actin assembly induced by thrombin or Rac1 (Tolias et al., 2000). In fibroblasts, membrane ruffle formation by PDGF required coordination of PIP5K-Iα and Rac signaling, and Rac-induced ruffles were blocked by kinase-deficient PIP5K-Iα (Doughman et al., 2003). In pollen tube cells, Rac and PIP_2 colocalize to the tip plasma membrane, and PIP_2 functions downstream of Rac in pollen tube elongation (Kost et al., 1999). Thus, Rac1 can activate PIP5K to produce PIP_2, but also inhibits endocytosis by direct interaction with the inositol 5-phosphatase, synaptojanin 2 (Malecz et al., 2000).

Rho GTPases were also found to physically associate with PIP5K. Ren et al. (1996) showed the binding of RhoA to a 68-kDa PIP5K isoform in Swiss 3T3 cell lysates, while Tolias et al. (1995) found that PIP5K activity from rat liver cytosol exclusively associated with recombinant Rac1, but not with RhoA or Cdc42, and that PIP5K activity was present in Rac immunoprecipitates. We have recently shown that actually all three type I PIP5K isoforms do associate with both RhoA and, to a lesser extent, with Rac1, but not with Cdc42, both in vitro and in vivo (Oude Weernink et al., 2004). In support of our observation, C-terminal peptides of RhoA and Rac1, but not of Rac2 or Cdc42, were found to precipitate PIP5K activity from HL60 cell lysates (van Hennik et al., 2003). All studies do agree in the observation that the binding of PIP5K to the Rho GTPases is independent of the nucleotide-binding state (GDP or GTP) of the GTPases. Most other effectors of Rho GTPases exclusively bind to the active, GTP-loaded GTPases. Although the constitutive interaction of PIP5K with RhoA and Rac1 is apparently specific and direct and likely exists in intact cells, binding of the PIP5K isoforms to the Rho GTPases appears not to be a trigger or prerequisite for kinase activation, but may serve another function, e.g. recruitment of the kinase to specific cellular compartments. Indeed, disruption of the PIP5K binding by a point mutation in the C-terminus of Rac1 inhibited thrombin- or Rac-induced actin polymerization in platelets (Tolias et al., 2000).

Although Cdc42 did not interact with PIP5K, Cdc42 was found to markedly stimulate PIP5K activity and to elevate cellular PIP_2 levels (Oude Weernink et al., 2004). Whereas the stimulation of PIP5K isoforms by RhoA was entirely mediated by Rho-kinase, the stimulatory effects of Cdc42, and of Rac1, were specific and largely independent of RhoA and Rho-kinase. Cdc42 has been shown to be required for phosphoinositide-induced actin assembly in *Xenopus* egg extracts (Ma et al., 1998), and synergistic activation of neuronal Wiskott–Aldrich syndrome protein (N-WASP) by Cdc42 and PIP_2 promotes potent actin filament nucleation by the actin-related protein 2/3 complex (Prehoda and Lim, 2002) (Fig. 2D).

The existence of three PIP5K isoforms obviously might enable cells to coordinate their PIP_2 production for specific processes, either by differential regulation or/and by selective subcellular localization of the individual isoforms. Indeed, Rac1 was shown to associate with both murine PIP5K-Iα and PIP5K-Iβ in vitro, but only PIP5K-Iα induced actin filament uncapping downstream of Rac1 in permeabilized platelets (Tolias et al., 2000). Human PIP5K-Iα was found to specifically localize to Rac1-induced membrane ruffles, whereas PIPK-Iβ was detected primarily in cytosolic vesicular structures, but not in membrane ruffles (Doughman et al., 2003). In agreement, small interfering RNA specific for human PIP5K-Iβ, but not for Iα or Iγ, affected the PIP_2 pool involved in constitutive endocytosis (Padrón et al., 2003). PIP5K-Iγ is highly expressed in brain and targets to focal adhesion sites (Di Paolo et al., 2003; Ling et al., 2003) and nerve terminals (Wenk et al., 2001). Three splice variants of Iγ are now found, and the newly identified Iγc appears to be selectively expressed in brain, and to be specifically involved in the maintenance of neuronal processes (Giudici et al., 2004).

Regulation of PIP_2 synthesis by Rho GTPases may be under control of membrane receptors and adhesion signals. Integrin-mediated adhesion of fibroblasts to fibronectin resulted in a rapid and strong increase in PIP_2 synthesis (McNamee et al., 1993). Likewise, in suspended cells, PIP_2 levels were reduced and PLC signaling was attenuated. After inactivation of Rho proteins with clostridial toxins, PIP_2 levels were not further affected by cell detachment (Oude Weernink et al., 2000a), and PIP_2 levels and PLC signaling in detached cells could be restored by micro-

injection of activated Rho or transfection with the Rho exchange factors, Lbc or Dbl (Schwartz et al., 1996). In platelets, stimulation of the thromboxane A_2 receptor, which couples to G_{12}/G_{13} and G_q proteins, resulted in the rapid activation of both RhoA and Rac1, and in a transient increase in PIP_2 content, which could be partially reduced by Y-27632 (Gratacap et al., 2001). In $G\alpha_q$-deficient platelets, only Rho activation occurred, and the remaining PIP_2 formation could be completely blocked by Y-27632. These data show that Rac1 and RhoA/Rho-kinase mediate receptor-dependent formation of PIP_2 by heterotrimeric G_q and G_{12}/G_{13} proteins, respectively, in two independent pathways. In COS-7 cells, stimulation of the thrombin receptor, PAR1, or expression of a constitutively active $G\alpha_q$ mutant induced the activation and translocation of PIP5K-Iα to the plasma membrane (Chatah and Abrams, 2001). $G\alpha_q$-induced translocation of PIP5K was dependent on both RhoA and Rac1, but not on ARF, and dominant-negative RhoA blocked translocation induced by active Rac1, suggesting that receptor-mediated PIP5K translocation may involve the sequential activation of $G\alpha_q$, Rac1 and RhoA. Interestingly, also PI4K-IIβ, the enzyme that produces PI4P was recently shown to be activated by Rac and to translocate to the plasma membrane (Wei et al., 2002), suggesting that Rac may promote the synthesis of PIP_2 at the plasma membrane by the recruitment and activation of both PI4K and PIP5K enzymes.

4.4. Regulation of PIP5K by ARF family GTPases

ADP-ribosylation factor (ARF) GTPases regulate intracellular vesicle trafficking. ARF1 is localized to the Golgi complex and is required for Golgi structure and function, whereas ARF6 regulates membrane traffic between the plasma membrane and endosomal compartments and organizes cortical actin. ARF1 was suggested to restore secretion in cytosol-depleted cells by promoting PIP_2 synthesis (Fensome et al., 1996). Honda et al. (1999) showed that PIP5K-Iα is directly activated by ARF proteins in the presence of PA, and that ARF6 colocalizes with PIP5K in ruffling membranes. PIP5K is also a direct effector of ARF1, and ARF1 mediates PIP5K activation (Jones et al., 2000a) and recruitment to the Golgi complex (Godi et al., 1999). ARF6 and PIP_2 colocalize on the plasma membrane and endosomal structures (Brown et al., 2001), and the ARF6-regulated PIP_2 pool at the plasma membrane is apparently involved in regulated secretion. In MIN6 pancreatic β cells, dominant-negative ARF6 reduced PIP_2 levels and impaired the slow phase of insulin secretion (Lawrence and Birnbaum, 2003). Expression of constitutively active ARF6 induced the trapping of PIP5K and PIP_2 at endosomal membranes, and corresponding depletion of PIP_2 from the plasma membrane interrupted exocytosis from dense-core vesicles in PC12 cells (Brown et al., 2001; Aikawa and Martin, 2003). Direct activation of PIP5K-Iγ by ARF6 was also shown to stimulate clathrin-coat recruitment to synaptic membranes, thereby allowing synaptic vesicle recycling (Krauss et al., 2003). Together, these data show that regulation of PIP5K activity and organized PIP_2 turnover is critical for ARF6-induced membrane trafficking. In cultured rat hippocampal neurons, ARF6 was recently shown to affect neurite extension and branching, and this process apparently involved PIP5K (Hernández-Deviez et al., 2004). Focal and transient accumulation of PIP_2 by PIP5K is required for phagocytosis as well (Botelho et al., 2000; Coppolino et al., 2002), and examination of integrin-mediated bacterial uptake into mammalian cells revealed efficient collaboration of Rac1 and ARF6 to attain localized PIP_2 synthesis. The results indicate that Rac1 regulates the recruitment of PIP5K to the phagosomal cup at early stages of the ingestion process, whereas ARF6 controls the activity of the membrane-recruited kinase (Wong and Isberg, 2003) (Fig. 2D).

The relationship between ARF and PIP_2 is more complicated, as phosphoinositides can regulate ARF activity by binding and activating both ARF exchange factors (Paris et al., 1997; Klarlund et al., 1998) and ARF-GTPase-activating proteins (ARF-GAPs) (Kam et al., 2000; Nie et al., 2002) via their PH domains. The high and specific affinity of the ARF-GAPs for PIP_2 may represent an attractive feedback mechanism for terminating ARF activation after a cycle of ARF-induced PIP_2 synthesis.

4.5. Interaction of PIP5K with PLD

The activity of many enzymes involved in phospholipid metabolism is modulated by lipids. PIP5K purified from bovine and rat brain was shown to be specifically and potently stimulated by PA (Moritz et al., 1992; Jenkins et al., 1994). All three PIP5K isoforms respond to PA (Ishihara et al., 1998), and this stimulation may be essential for resynthesis of PIP_2 in response to PIP_2 hydrolysis by PLC and subsequent conversion of 1,2-diacylglycerol to PA by diacylglycerol kinase (DGK). Alternatively, PA arises from phosphatidylcholine hydrolysis by PLD, which is involved in various cellular processes, including vesicle trafficking. Indeed, DGK- as well as PLD-derived PA was able to stimulate PIP5K activity in vivo (Jones et al., 2000b). Stimulation of PIP5K by PA is not restricted to mammalian cells. Also, the membrane-associated PIP5K homolog Mss4p in *Saccharomyces cerevisiae* (Homma et al., 1998) and *Candida albicans* (Hairfield et al., 2002) is stimulated by PA, and activation of PIP5K activity may be involved in the establishment of cell morphology and during temperature-induced hyphal growth, respectively. Both mammalian PLD isoforms, PLD1 and PLD2, interacted with PIP5K-Iα, and PLD2 recruited PIP5K-Iα to a submembraneous vesicular compartment (Divecha et al., 2000). PLD1 and PLD2 have an almost absolute requirement for PIP_2, and PLD1 is also directly regulated by ARF and Rho GTPases (Exton, 2002; Powner and Wakelam, 2002). The reciprocal stimulation of PIP5K and PLD, and the regulation of these enzymes by the

same GTPases, points to concerted mechanisms in cellular actions, involving acute, localized PIP_2 synthesis supported by the feed-forward regulatory loop. Indeed, quenching of PA production with primary alcohols inhibited PIP_2 synthesis and led to disruption of Golgi membranes (Sweeney et al., 2002), blockade of clathrin-coat assembly (Arneson et al., 1999) and inhibition of ARF1-reconstituted secretion (Way et al., 2000), demonstrating that PLD-induced PIP_2 synthesis is essential for maintaining the function of the Golgi apparatus and the endo- and exocytotic machinery. Using an ARF1 point mutant that could selectively activate PIP5K, but not PLD activity, it was concluded that both PLD-derived PA and direct activation of PIP5K by ARF1 contribute to the regulation of PIP_2 synthesis in intact cells (Skippen et al., 2002) (Fig. 2E).

5. Conclusion

The synthesis of PIP_2 by PIP5K regulates a large number of cellular processes (Fig. 2). The identification of the three type I PIP5K isoforms raised the expectation of a differential regulation by cellular signal transduction components, but up to now, the regulatory properties of PIP5K-Iα, Iβ and Iγ appear to be remarkably similar. All three isoforms are stimulated by PA (Ishihara et al., 1998), are negatively regulated by PI-dependent autophosphorylation (Itoh et al., 2000) and PKA (Park et al., 2001), and specifically interact with Rho GTPases (Oude Weernink et al., 2004), pRB (Divecha et al., 2002) and PIP4K-IIα (Hinchliffe et al., 2002). Nevertheless, evidence has been provided that specific PIP5K isoforms are involved in particular processes in different cell types, for instance, actin reorganization downstream of Rac1 in platelets, which specifically involves PIP5K-Iα. The undisturbed completion of the various PIP_2-modulated processes is very likely achieved by the strict subcellular concentration of appropriate signaling partners within discrete membrane microdomains.

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