Complex roles of PIP$_2$ in the regulation of ion channels and transporters

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Huang C-L. Complex roles of PIP$_2$ in the regulation of ion channels and transporters. Am J Physiol Renal Physiol 293: F1761–F1765, 2007. First published October 10, 2007; doi:10.1152/ajprenal.00400.2007.—The regulation of ion channels and transporters by phosphoinositides has received much attention over the past 10 years. There are multiple potential mechanisms for regulation of ion channels and transporters by PIP$_2$, including a direct binding of PIP$_2$ to the target proteins, alterations of membrane insertion, and retrieval. Added to the complexities of multiple potential mechanisms is how cells use PIP$_2$ to regulate so many different processes. Here, I briefly review several past and recent studies to illustrate the complexities and raise outstanding questions for future studies.

endocytosis; exocytosis; phosphoinositide; PIP$_5$K; Arf; Rho

PHOSPHOINOSITIDES ARE MINOR phospholipids that regulate diverse eukaryotic cellular processes including vesicular transport, growth factor and calcium signaling, organization of the cytoskeleton, and ion channels and transporters (15, 18, 20, 26, 28, 32, 37). The precursor of all phosphoinositides, phosphatidylinositol (PI), is the most abundant. Among the D-4 isoforms, phosphatidylinositol 4,5-bisphosphate (PIP$_2$), the precursor of inositol-1,4,5-trisphosphate (IP$_3$) and diacylglycerol, comprises ~1% of the total phospholipids in the plasma membrane. Phosphatidylinositol-4-phosphate (PI$_4$P) is equal to of the PIP$_2$. The D-3 isoforms, phosphatidylinositol-3,4-bisphosphate [PI(3,4)P$_2$] and phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P$_3$], are considerably less abundant.

The role of phosphoinositides in the regulation of ion transport proteins was first reported by Choquette et al. (5) as early as 1984. By studying brain Ca$^{2+}$-ATPases reconstituted into liposomes consisting of phosphatidylcholine and with or without PIP$_2$, they found that PIP$_2$ stimulates activity of the pump in the liposomes. Subsequently, Kobayashi et al. (21) in 1989 and Chu and Stefani (6) in 1991 reported that PIP$_2$ is a potent activator of Ca$^{2+}$ release channels from skeletal muscle when isolated in vesicles or incorporated into lipid bilayers. Furukawa et al. (9) reported that spontaneous or Ca$^{2+}$-induced rundown of the ATP-sensitive K$^+$ channels in excised inside-out patches can be inhibited by the application of actin filament stabilizer or PIP$_2$. In 1996, Hilgemann and Ball (14) reported in Science their findings that PIP$_2$ regulates the Na$^+$/Ca$^{2+}$ exchanger and ATP-sensitive K$^+$ (K$_{ATP}$) channels, which led to explosive interests in the role of phosphoinositides in the regulation of ion channels and transporters. Since then, more than 300 original research papers or reviewers have been published reporting on the regulation of a wide range of ion channels and transporters by PIP$_2$. For interested readers, a list of PIP$_2$-regulated ion channels and transporters published up to the year 2005 can be found in a review by Suh and Hille (43).

Mechanisms of Regulation by PIP$_2$

Direct binding with PIP$_2$. The earlier studies using reconstituted lipid bilayers suggest a direct regulation of ion transporters by PIP$_2$ (5, 6, 21). The effects of PIP$_2$ on ion channels and transporters in excised patches are consistent with this mechanism of regulation (8, 19, 38). Huang et al. (19) provided the first experimental evidence to support that PIP$_2$ directly binds to ion channels in vitro. They showed that fusion proteins containing the COOH terminus of inward rectifier K$^+$ channels bind to liposomes composed of phosphatidylincholine (PC) and radioactive PIP$_2$ and identified several basic amino acids in the proximal COOH terminus of channels critical for the interaction (19). Since then, direct binding with PIP$_2$ has also been shown for other channels (25, 48). More recently, Enkvetchakul et al. (7) reconstituted purified bacterial K$^+$ channel KirBac1.1 into liposomes and demonstrated that the activity of KirBac1.1 is inhibited by PIP$_2$ in the liposome. The inhibition of KirBac1.1 by PIP$_2$ is interesting in light of the fact that bacterial membrane does not contain PIP$_2$ and that KriBac1.1 is normally constituatively open. Evidence for direct regulation of purified ion channel proteins by the physiological membrane concentration of PIP$_2$ in reconstituted lipid bilayers remains lacking. Nevertheless, the totality of evidence is compelling for the case of direct regulation to be made.

With respect to PIP$_2$-binding sites on ion channels and the mechanism of regulation, mutagenesis studies have identified clusters of basic, hydrophobic, and hydrophilic amino acids important for interaction with PIP$_2$ (43). These amino acids interact with the phosphate head groups of PIP$_2$ via electrostatic, hydrogen-bonding, and/or hydrophobic forces. Mechanistically, these interactions stabilize channel proteins in a certain conformational state, as reflected by the effects of PIP$_2$ on the open probability of ion channels. For example, the interaction with PIP$_2$ stabilizes the inward rectifier K$^+$ channel, ROMK, IRK1, and G protein-gated inward rectifying K$^+$ (GIRK) channels in the open state (8, 19, 38). Conversely, it stabilizes the capsaicin receptor (as known as TRPV1) in the closed state (36).
Promoting membrane insertion of ion channels by PIP2. PIP2 plays essential roles in the exocytotic insertion of vesicles into the plasma membrane (26). More abundant in the plasma membrane than in the intracellular membrane, PIP2 is a plasma membrane recognition signal for docking of vesicles (26). In addition, many proteins involved in the exocytosis of vesicles are recruited and localized to the plasma membrane by binding to PIP2 (12, 26). Phosphoinositide 5-kinase (PIP5K) catalyzes the formation of PIP2 from phosphatidylinositol-4-phosphate (33). The activity of PIP5K is tightly regulated by phosphatidic acid (PA) (30, 33). Phospholipase D (PLD) is a phosphodiesterase that specifically cleaves phosphatidylcholine to release PA and choline (34). Interestingly, PIP2 is a critical cofactor for PLD, which affects the activity and localization to the plasma membrane (34).

After budding from the trans-Golgi network, exocytotic vesicles recruit PLD and PIP5K en route to the plasma membrane (34). PLD releases PA, which simulates the activity of PIP5K to synthesize PIP2. Formation of PIP2 by PIP5K further recruits and stimulates PLD. This reciprocal feed-forward stimulation of PLD and PIP5K leads to an explosive production of PA and PIP2, driving the insertion of vesicles into the plasma membrane. Thus increased insertion may underlie the stimulation of ion channels or transporters by PIP2 in cells.

The activity of both PLD and PIP5K is under the control of small GTPases of the ADP-ribosylation factor and Rho families (33, 34). Rho GTPases are downstream of growth factors. Bezzerides et al. (1) reported that epidermal growth factor (EGF) increases the activity of TRPC5 channels at the cell surface by promoting plasma membrane insertion. This effect requires the stimulation of the Rho GTPase Rac1 by EGF and production of PIP2 via an isoform of PIP5K, PIP5K\(\alpha\). Pochynyuk et al. (35) showed that activation of RhoA signaling stimulates the epithelial Na\(^{+}\) channels (ENaC) by promoting channel insertion. The upstream pathway leading to RhoA activation in their system was not examined.

Stimulation of endocytosis of ion channels by PIP2. The process of endocytosis requires concerted actions of many proteins. Many of these proteins, including AP2 adaptors protein, epsin, AP180, dynamin, and synaptojanin, are recruited to the plasma membrane via binding to PIP2 produced by PIP5K at the sites of endocytosis (12, 26). Recruitment of AP2, epsin, and AP180 by PIP2 is the priming step for formation of a clathrin coat. Recruitment of dynamin is critical for fission of coated vesicles, whereas synaptojanin (which is an inositol polyphosphate 5-phosphatase that dephosphorylates PIP2) is crucial for uncoating of vesicles after endocytosis (12). Depletion of PIP2 in the plasma membrane by forced expression of PIP2 5-phosphatase leads to loss of clathrin-coated pits (50). Conversely, production of PIP2 by PIP5K locally would be expected to enhance endocytosis. An increase in PIP2 synthesis by PIP5K at endocytosis hot spots may be mediated by Rho GTPases (for regulation by growth factors) or by ADP-ribosylation factor, which localized in the plasma membrane and endosomal structures (2). In addition, a recent paper reports that the AP2 complex directly interacts with PIP5K\(\gamma661\), a major PIP5K isoform in the brain (31). The direct interaction between the AP2 complex and PIP5K provides an additional mechanism for enhanced production of PIP2 at the endocytosis sites.

Despite the unequivocal role of PIP2 in the endocytosis of membrane receptors and ligands, evidence for PIP2 regulation of ion channel and transporters by increasing their endocytosis is relatively scarce. Yaradanakul et al. (47) recently reported that PIP2 exerts a biphasic effect on the cardiac Na\(^{+}/\text{Ca}^{2+}\) exchanger NCX1, the first phase presumably by direct regulation and the second phase by causing internalization of the exchanger. Multiple lines of evidence support the conclusion of internalization of NCX1 by PIP2 in the second phase. Depletion of PIP2 by stimulation of PLC via M1 muscarinic receptors increases the activity of NCX1 in parallel with an increase in membrane area measured by cell capacitance. Increasing membrane PIP2 by direct perfusion of PIP2 into cells or transient overexpression of PIP5K\(\beta\) causes parallel decreases in NCX1 and cell membrane area. Finally, transgenic mice with cardiac-specific overexpression of a PI4-kinase have reduced expression of the exchanger at the cell surface.

Indirect regulation by PIP2 via the cytoskeleton. The role of PIP2 in the regulation of the actin cytoskeleton is also well known (21). The polymerization of actin monomers into filaments is regulated by the actin-related protein (Arp) complex Arp2/3 (29). The Arp2/3 complex is the key regulator of actin polymerization in vivo by promoting filament assembly through enhanced nucleation of actin subunits (29). Spontaneous depolymerization of actin filaments is slow. Actin depolymerization factors such as gelsolin and cofolin enhance disassembly of actin filaments, thus facilitating remodeling of the actin cytoskeleton in dynamic cellular processes (44). Activation of Arp2/3 by Wiskott-Aldrich syndrome protein (N-WASP) is critically dependent on PIP2 in the plasma membrane (29). PIP2 is inhibitory to gelsolin and cofolin (44). Thus the overall function of PIP2 is to favor actin filament polymerization.

The observation that both actin filament stabilizer and PIP2 prevent rundown of the K\(_{\text{ATP}}\) channels led Furukawa et al. (9) to conclude that actin is the regulator of the channel. Despite more recent evidence of direct regulation of the K\(_{\text{ATP}}\) channel by PIP2, the possibility of dual regulation by direct binding and indirect effect through the actin cytoskeleton remains. Reorganization of the actin cytoskeleton is crucial for membrane trafficking (26, 37). The effects of PIP2 on endo- and exocytosis of ion channels and transporters may conceivably be in part from its effects on the actin cytoskeleton. Yaradanakul et al. (47) considered this possibility in their interpretation of the effect of PIP2 on the internalization of NCX1.

Local PIP2 Signaling

Does it exist? Thus PIP2 can regulate ion channels by direct binding, increasing membrane insertion, increasing endocytosis, and/or via the actin cytoskeleton. For example, ENaC is regulated by clathrin-mediated endocytosis (46), which would be stimulated by membrane PIP2. In addition, PIP2 promotes insertion of ENaC and directly gates the channel (35, 48). How can cells use PIP2 to regulate so many different processes without creating chaos? It would seem obvious that there must be localized PIP2 signaling with temporal and spatial resolution (16). However, experimental results addressing this question are not straightforward.

Using fluorescent-attached PH domain to visualize PIP2, some observe that the distribution of PIP2 appears clustered or
localization, whereas others report a homogenous distribution (37). However, it should be cautioned that PH domain binds free PIP2 only, not an indicator of protein-bound PIP2. The existence of “lipid rafts” with enrichment of PIP2 is also unsettling (10, 45). With respect to PIP2 regulation of ion channel, Cho et al. (4) reported that cardiac GIRK channels are regulated by specific G protein-coupled receptors. Cho et al. further reported that the low mobility of PIP2 underlies the receptor-specificity for GIRK regulation (3). They found that, in cell-attached recording, inclusion of acetycholine in the pipette causes a localized depletion of PIP2 in the area of plasma membrane within the pipette but not beyond the pipette (3). Conversely, bath application of ACh does not deplete PIP2 within the pipette, nor does it activate GIRK. To examine the mobility of PIP2, they applied fluorescent PIP2 (BODIPY-PIPP2) from the pipette and found that little PIP2 diffuses beyond the membrane area isolated by the pipette. Application of an actin depolymerizer allows PIPP2 diffusion to the membrane area outside the cell-attached pipette (3). Thus, low mobility of PIP2 in the membrane, at least in part due to restriction by the actin cytoskeleton, contributes to local PIP2 signaling of GIRK channels.

These interesting findings by Cho et al. (4), however, are in direct contrast to those by Horowitz et al. (17) and by Yaradanakul et al. (47). In the latter two studies, the authors reported that the lipid signal induced by hydrolysis via PLC (i.e., depletion of PIP2) propagates freely from the extrapipette membrane area to the intrapipette by direct measurement of PIP2 content in the membrane. Horowitz et al. (17) showed that application of an agonist depletes the plasma membrane PIP2 and the total cellular PIP2 content membrane by >80% in less than 1 min. The reasons behind differences between studies by Cho et al. (4) and those by Horowitz et al. (17) and Yaradanakul et al. (47) are not clear. Differences in cell types have been suggested (41). Irrespective of whether PLC activation causes a global or localized depletion of PIP2, the notion of local PIP2 signaling seems inescapable. Without it, every time, agonist activation of PLC would impact membrane trafficking, actin organization, as well as multiple ion channels and transporters all at once.

**Mechanisms of local PIP2 signaling.** One mechanism for local PIP2 signaling is the low diffusion mobility of PIP2 in the plasma membrane relative to its density and space constant (13). As discussed above, the experimental results on this subject remain discrepant. Another mechanism is a marked increase in PIP2 production locally. This mechanism occurs during exo- and endocytosis when the reciprocal feed-forward stimulation of PLD and PIP5K leads to explosive production of PIP2 (33, 34). Subcellular compartmentalization of PIP2 metabolically machinery and ion channels will conceivably underlie many instances of local PIP2 signaling (11, 16).

The other mechanism is due to local positive electrostatic potential conferred by proteins. This mechanism can cause local PIP2 signaling independently of PIP2 content in the bulk membrane. McLoughlin and Murray (27) discussed this in detail in a recent review, to which interested readers are referred. In essence, proteins with a cluster of four or more basic amino acids located at the membrane-cytosol interface serve as a basin to laterally sequester PIP2. Laux et al. (23) suggested that myristoylated alanine-rich C-kinase substrate and growth-associated protein 43, both with long stretch of basic clusters, reversibly associate with membrane PIP2 through these protein electrostatics to control the level of free PIP2 in the plasma membrane and to regulate cortical actin dynamics locally. Although PIP2-regulated ion channels may not contain as many basic residues as myristoylated alanine-rich C-kinase substrate and growth-associated protein 43 per subunit, the multimeric structure of ion channels would help to provide concentrated local charges for sequestering the multivalent PIP2.

Sequestration of PIP2 by ion channels may involve interaction beyond protein electrostatics. Liou et al. (24) showed that, in inside-out patches after rundown of ROMK via depletion of PIP2 by Mg2+, direct application of PIP2 or application of Mg-ATP to generate PIP2 via lipid kinase reactivates the channel. Application of an anti-PIP2 antibody inhibits ROMK, which, however, cannot be reversed by subsequent application of PIP2 or Mg-ATP. The only maneuver that reactivates the channel is by application of a reducing agent to inactivate the antibody. These results support the idea that PIP2 is tightly bound to ROMK. Binding of an antibody to PIP2 uncouples its regulation of ROMK. The complex formed by PIP2 and the antibody, however, remains associated with ROMK in situ, preventing subsequent activation by additional application of PIP2 but allowing channel reactivation by PIP2 released from the antibody that is inactivated by the reducing agent.

The tight association between PIP2 and ROMK may involve acyl chains of PIP2 besides protein electrostatics. Using lipids with different acyl chains, Rohacs et al. (38) showed that natural PIP2 with arachidonoyl stearyl chains activates GIRK1/4 channels more than synthetic dipalmitoyl or shorter chain dioctanoyl PIP2. Activation of IRK1 is not different among these lipids. Zeng et al. (49) also found that the maximal activation of ROMK by dioctanoyl PIP2 is only ~30% of that by natural PIP2. As much as 10 mM of each lipid was applied, suggesting that the difference in the maximal activation is not due to differences in incorporation into the plasma membrane.

**Concluding Remarks**

PIP2 is predominantly in the plasma membrane. One advantage for cells to use PIP2 to regulate activity as well as membrane trafficking of channels and transporters is that ion transport proteins would be active only at the cell surface but not during export to and retrieval from the cell surface. To orchestrate the multiple roles of PIP2, local signaling is essential. The feed-forward stimulation of PLD and PIP5K produces massive PIP2 locally to drive exocytosis and endocytosis. Local signaling for ion channels and transporter may depend on subcellular compartmentalization of signaling complexes with ion channels. In addition, sequestration of PIP2 by ion channels through protein electrostatics and through interaction with acyl chains of the lipid may also contribute to local signaling of ion channels. These local PIP2 signaling processes may occur on a scale of 20 nm (e.g., neck of budding vesicles) or smaller (e.g., PIP2 bound to a single molecule of channel). Technology with resolution beyond conventional light microscopy is required to study these processes.

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