Protein regulation by phosphatidylinositol lipids

Phosphatidylinositol lipids are best known as a source of the second messengers inositol trisphosphate and diacylglycerol. Some phosphatidylinositol lipids may directly regulate cytoskeletal events, however. To understand the role of lipids in signaling, help from both synthetic chemists and physical chemists is needed.

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Phosphatidylinositol lipids, or polyphosphoinositides (PPIs), constitute only a small fraction (<1 %) of total cell membrane lipid. They are disproportionately important in biology, however; changes in the synthesis or distribution of PPIs appear to be involved in the signaling pathways that trigger a wide range of cellular processes, including cell division, secretion, motility, and cytoskeletal reorganization. Most attention has focused on phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$; Fig. 1) as a substrate for the phosphodiesterase phospholipase C (PLC). Cleavage of $PI(4,5)P_2$ by PLC (Fig. 2) yields two second messengers, inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 enters the cytosol, where it opens channels that elevate cytoplasmic Ca^{2+} levels, while DAG remains in the cell membrane, stimulating the activity of protein kinase C (PKC) and possibly other proteins that function in signal transduction or cytoskeletal changes [1]. In this series of events, $PI(4,5)P_2$ is only a precursor of the second messenger molecules, IP_3 and DAG. More recent work, however, indicates that $PI(4,5)P_2$ itself can act as a messenger, directly activating PKC and other enzymes. These observations are consistent with some of the earliest work on PPIs (originally called di- or tri-phosphoinositides [2]), which suggested that these membrane lipids themselves bind to proteins [3].

Direct effects of phosphatidylinositol lipids on protein function

One clear example of a direct effect of phosphatidylinositol lipids on protein function is their ability to modulate the activities of components of the cytoskeleton. PI(4)P and $PI(4,5)P_2$ specifically interact with several actin-binding proteins, altering their affinity for actin. The first interaction of this kind to be identified was that of $PI(4,5)P_2$ with profilin, a soluble protein that sequesters actin monomers [4]. $PI(4,5)P_2$ decreases the affinity of profilin for actin and may thereby promote actin polymerization, a process that is essential for cell motility. It was later found that gelsolin, which severs and caps actin filaments and thus breaks up actin networks, is inactivated by $PI(4,5)P_2$. Gelsolin binds extremely tightly to actin (K_d<nM), and phosphatidylinositol lipids are the only physiological agents known to dissociate these complexes. It has been nearly ten years since these first reports that gelsolin and profilin, both of which are presumably cytosolic proteins, might be regulated by these rare plasma membrane lipids. Since then, several lines of evidence have accumulated which support this notion. First, despite much experimentation, no agent has been found that is better than PI(4)P or $PI(4,5)P_2$ at inhibiting actin binding by either gelsolin or profilin. Second, stimulated polymerization of actin *in vivo* appears to take place at the interface of the cytoplasm with the plasma membrane, not mainly in the cytosol, making the notion that the

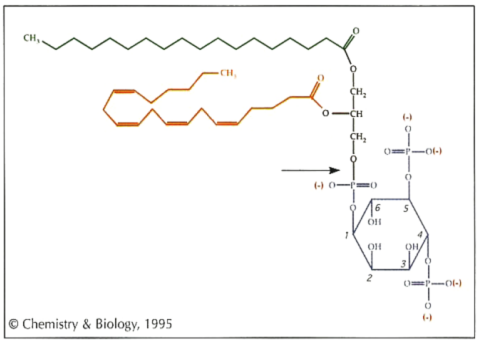

Fig. 1. Structure of the phosphatidylinositol lipid $PI(4,5)P_2$. The typical glycerol backbone of a phospholipid (shown in black) is linked by ester bonds to a saturated fatty acid (typically stearate, shown in green) at the 1 position and an unsaturated fatty acid (typically arachidonate, shown in orange) at the 2 position. The 3 position is linked by a phosphodiester bond to inositol (shown in blue). The parent phosphatidylinositol (PI) is phosphorylated by a specific PI 4 kinase and a specific PI(4)P 5-kinase to obtain the $PI(4,5)P_2$ species shown. PI and its derivatives are substrates for phospholipase A2, releasing arachidonic acid, which is further processed to make a number of signaling molecules, including prostaglandins, leukotrienes and hydroxyeicosatetraenoic acid derivatives (HETEs) by cellular lipoxygenases, cyclooxygenases, and epoxygenases. $PI(4,5)P_2$ is also a substrate for several types of PI-specific phospholipase C isoforms which respond to different stimuli to hydrolyze the phosphodiester bond adjacent to glycerol (arrow), releasing IP_3 to the cytosol and leaving diacylglycerol in the cell membrane. A different class of enzymes, the PI-3 kinases, which are implicated in cell growth and division, phosphorylate the inositol ring at the 3' position and can use PI, PI(4)P and $PI(4,5)P_2$ as substrates. The resulting products appear not to be substrates for phospholipase C.

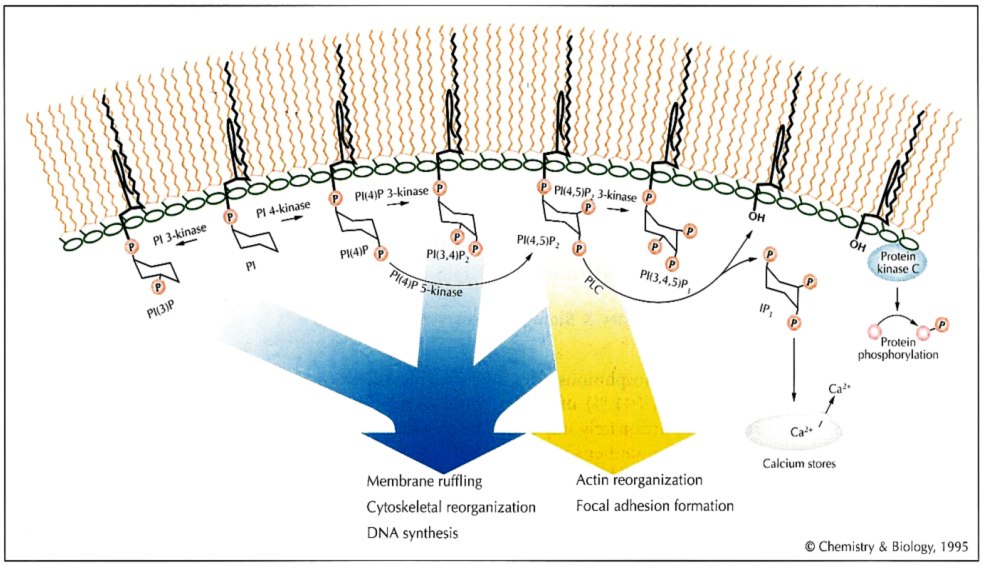

Fig. 2. Summary of phosphatidylinositol lipid turnover. Phosphatidylinositol can be phosphorylated at the 4' position, leading to PI(4)P and subsequently to $PI(4,5)P_2$, which is a substrate for phospholipase C (PLC). Hydrolysis of $PI(4,5)P_2$ by phospholipase C produces IP_3, which triggers the release of Ca^{2+} from intracellular stores, and DAG, which activates protein kinase C to phosphorylate a number of proteins. PI, PI(4)P and $PI(4,5)P_2$ can also be phosphorylated at the 3' position, giving rise to PI(3)P and higher phosphorylated derivatives, which are not substrates for PLC but instead appear to have direct effects on cytoskeletal organization, secretion, and DNA synthesis. Not shown are the functions of specific and non-specific phosphatases. In resting cells, the opposing actions of the phosphatases and kinases can produce a large flux within the pathway, without altering steady-state concentrations.

proteins responsible might interact with lipids more reasonable. And finally, more than a dozen actin-binding proteins have now been found to bind to PPIs, notably filament-severing proteins and proteins that are involved in forming adhesion sites, where actin filaments are attached to the cell membrane. Binding to PPIs sometimes enhances and sometimes inhibits the interaction between actin and the protein in question (reviewed in [5]). Although both PI(4)P and $PI(4,5)P_2$ interact with actin-binding proteins, the fact that PI(4)P 5-kinase is a target of Rho, a small G-protein whose activation lies upstream of signals leading to actin polymerization and formation of protein complexes in adhesion sites [6], suggests that $PI(4,5)P_2$ is the more important signal for actin polymerization.

At least some types of phosphatidylinositol lipids, notably phosphatidylinositol-3-phosphate (PI(3)P) and its higher phosphorylated derivatives $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Fig. 2), appear not to be substrates for PLCs and thus cannot be precursors of IP_3 or DAG. The enzyme that makes PI(3)P, phosphatidylinositol (PI) 3-kinase, is required for DNA synthesis and cell growth [7], suggesting that PI(3)P itself also has a target, as yet unknown, in the signaling pathway leading to cell division. In the discussion below, PIP_2 will be used to designate PI derivatives whose phosphorylation structure is either not known, or not important for the biological effect discussed.

PPI-binding sites on proteins

Three structures of PPI-binding domains have been determined by X-ray or NMR analysis (Fig. 3), and they appear to bind PPIs in different ways. Two sites in gelsolin (one in segment 1, the other in segment 2) bind PIP_2 micelles as well as PIP_2 incorporated into phosphatidylcholine vesicles, and this binding dissociates gelsolin–actin complexes. One of these sites appears to be at or near residues 135–142. A recombinant gelsolin segment 1 mutant lacking residues 135–142 (FKSGLKYKK) binds actin but, unlike its normal counterpart, is not regulated by PIP_2. Furthermore, a synthetic peptide based on this sequence competes with intact gelsolin for PPIs [8]. In the crystal structure of gelsolin segment 1, this linear sequence spans a coil and β-strand exposed near the gelsolin–actin interface [9] (Fig. 3a). A peptide based on the second PPI-binding site of gelsolin (residues 150–169) undergoes a coil–helix transition *in vitro* upon binding $PI(4,5)P_2$ [10].

The pleckstrin homology domain is a motif found in several proteins and is thought to anchor them to the cell membrane. Pleckstrin also binds $PI(4,5)P_2$, diluted in small detergent micelles, but the structural motif responsible for binding is fundamentally different from that of gelsolin [11]. NMR studies suggest that the amino acids that contact the lipid lie at the edges of turns forming a β-barrel in the pleckstrin structure.

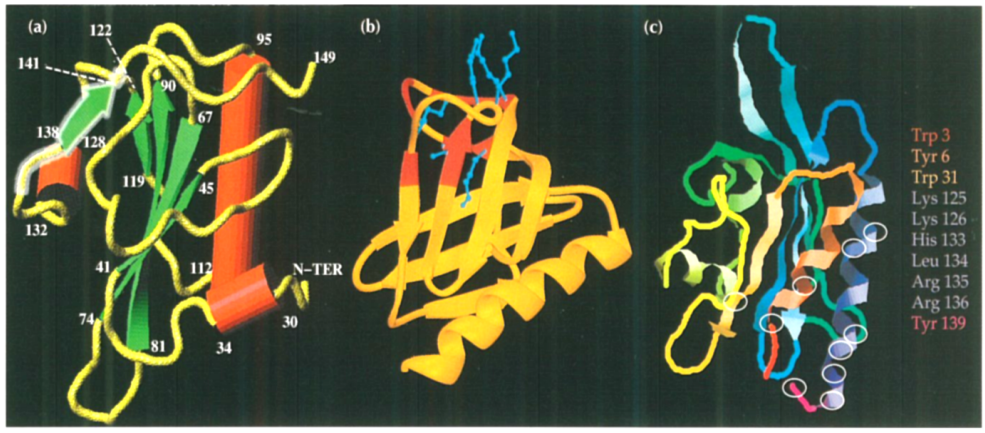

Fig. 3. Structures of $PI(4,5)P_2$-binding protein domains. **(a)** Gelsolin segment 1. Two $PI(4,5)P_2$ sites are found in gelsolin, one at residues 135–142 (outlined in white), the other at residues 150–169 (found in gelsolin segment 2, whose structure is unknown, but probably homologous to that of segment 1). **(b)** Pleckstrin. The $PI(4,5)P_2$-binding site is formed by amino acids at the edges of a series of turns forming a β-barrel in the pleckstrin structure. The side chains of the six Lys residues at the $PI(4,5)P_2$ binding site are highlighted in blue and the residues that show chemical shift changes upon binding $PI(4,5)P_2$ are shown in red. **(c)** Profilin. The white circles indicate the proposed $PI(4,5)P_2$-binding site, which comprises a number of basic residues in the carboxy-terminal helix that forms part of profilin's actin-binding site (listed on the right in mauve) and a cluster of hydrophobic residues that are found close by at the base of the protein (color-coded to their position in the structure).

These residues, which lie close together in space, are far apart in the amino acid sequence (Fig. 3b).

The third PPI-binding protein whose structure is known, profilin [12], is not quite so informative. The PPI binding site on this protein is not known with certainty, but there are several plausible candidates. One is a linear sequence with some similarity to gelsolin residues 135–142, which lies on an α-helix that constitutes part of the actin binding site (Fig. 3c). Other surfaces with positive charge have also been proposed to be the PPI-binding site [13].

Structures of PPI lipids bound to proteins

PPI binding to profilin and gelsolin is not exclusively electrostatic. The proteins have little affinity for water-soluble derivatives lacking acyl chains, and binding to PIP_2 is strong over a pH range where at least one of the phosphomonoesters of PIP_2 becomes protonated, reducing the charge. There is also only a modest difference in the binding of PPIs to profilin isoforms that differ radically in isoelectric point. Instead, binding of these proteins is very sensitive to the physical state of the lipid (Fig. 4). A variety of studies suggest that clusters of PIP or PIP_2 containing between 5 and 10 PPI-headgroups must form to stabilize the protein–lipid complex. For example, gelsolin is not affected by PIP_2 incorporated at below 10 mol % in either triton micelles or phosphatidylcholine vesicles [14]. Therefore, the distribution of lipids in the biological membrane may be at least as important as their bulk concentration for biological activity. The importance of packing geometry for PIP_2 function *in vivo* is illustrated by what happens when a polar derivative of arachidonic acid (15-HETE) is substituted in the pool of cellular PIP_2. In neutrophils, 15-HETE selectively enters the PPI pool and replaces arachidonate at the 2 position of the glycerol backbone. In cells carrying these modified PPIs, stimulation by inflammatory agents no longer leads to hydrolysis of $PI(4,5)P_2$ by PLC, and the neutrophils no longer reorganize their cytoskeleton or migrate across endothelial cell boundaries when stimulated to do so [15]. Since the phosphodiester bond from glycerol to inositol cleaved by PLC is still abundant, the inability to generate IP_3 appears to be due to an effect of the 15-HETE on the way in which the PIP_2 is presented to the enzyme on the cytoplasmic surface of the membrane.

Next steps: synthetic chemistry

Progress in this area has been made difficult by the lack of sufficient amounts of lipids with defined structure and stereochemistry to allow biochemical tests. For example, although it seems clear that PI(3)P functions differently from PI(4)P *in vivo*, it has not been practical to determine the effects of PI(3)P on cytoskeletal protein function *in vitro* because insufficient amounts of the lipid can be made. The obvious synthetic route for a biologist is to use purified or recombinant PI 3-kinase; however, the enzyme stops working once it has phosphorylated 10 % of its substrate, even though it retains activity when presented with a fresh supply of PI (C.L. Carpenter and L.C. Cantley, personal communication). This result probably has important implications for the role of surface charge or curvature in controlling PI-3 kinase activity, but is also a strong barrier to making pure PI(3)P. A more promising route is the complete synthesis of phosphorylated inositol lipids.

Synthetic phosphatidylinositol and some derivatives were first reported in 1970 [16]. One example of the use of synthetic PPIs to shed light on lipid–protein interactions

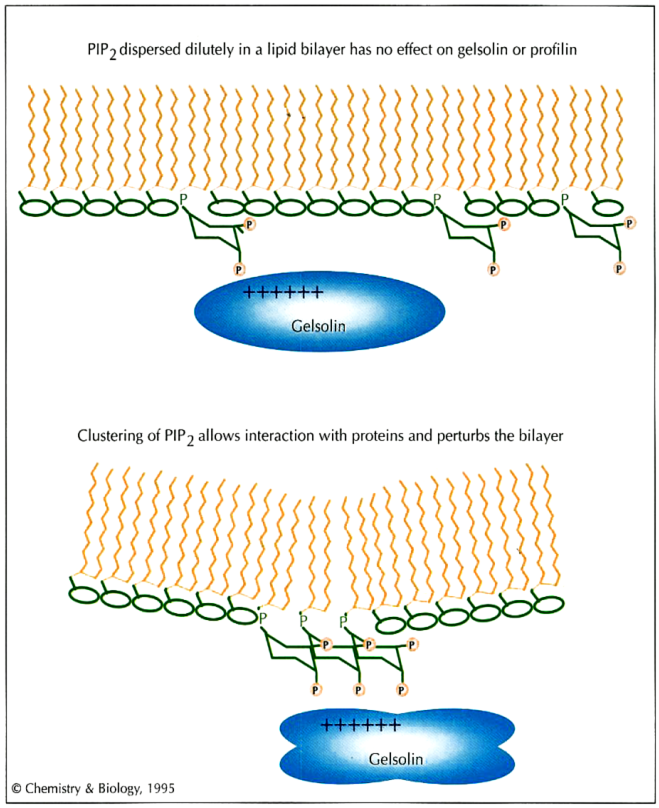

Fig. 4. Hypothetical role of membrane curvature or PPI clustering in binding to proteins. Using gelsolin as an example, a membrane with PI(4,5)P_2 widely dispersed has little or no affinity for the positively charged PPI-binding site of the protein (top). A rearrangement that concentrates the phosphorylated inositol rings stabilizes the lipid–protein interaction and induces a structural rearrangement within the protein (bottom). Aggregation of PIP_2 headgroups which are too large to form stable flat bilayers would tend to deform the membrane locally, possibly contributing to binding.

is a study of four stereoisomers of dihexadecanoyl PI. This study showed that the chirality of the inositol ring is important for the activity of PI 4-kinase, but that this enzyme can tolerate changes in the stereochemistry of the glycerol backbone and the nature of the acyl chains [17]. In light of the biological importance of isomers of PI-monophosphates, it is interesting to note that synthetic PI(2)P was reported in 1970, and PI(5)P was produced by alkaline phosphatase treatment of PI(4,5)P_2 in 1968 [18]. Neither of these isomers has been studied *in vivo* or with purified proteins.

Physical chemistry

It seems likely that the interesting biological functions of PPIs are connected to their unusual physical properties. PIP_2 is unique among common phospholipids with two acyl chains in that it forms small micelles instead of bilayer vesicles in water, because of the large electrostatic charge (−4 to −5) of the headgroup. PIP_3, whose synthesis *in vivo* correlates with cytoskeletal changes, would presumably be even more water-soluble, but this has not yet been tested with the native lipid. For PIP_2 or PIP_3 to be stable in a cell membrane, their large charged headgroups must presumably be compensated for by other phospholipid types. Divalent cations also bind avidly to these phosphorylated headgroups. Such binding would be strongly enhanced by the electrostatic potential of a large cluster of like-charged lipids in the inner leaflet of the cell membrane [19,20]. Understanding how charged lipids like PPIs distribute in domains in the cell membrane and how to relate such macroscopic surface features to protein–lipid interactions remains a challenge to cell biology and chemistry.

The roles of PI phosphorylation in cell signaling might be difficult to define using only a single purified lipid, since the context in which the lipid is found may also be extremely important. A naive analogy might be made with protein tyrosine phosphorylation, which places a single phosphomonoester on a hydroxyl group of a six-membered carbon ring similar in size to inositol. Tyrosine phosphorylation can be the 'switch' that makes a protein into a target for the next protein in the signaling pathway, typically a protein that contains a Src homology 2 (SH2) domain. The binding specificity of SH2 domains for phosphotyrosines is dictated by the topography of the surrounding polypeptide chain, however, and, although the SH2 domain will not bind to its ligand in the absence of tyrosine phosphorylation, the presence of phosphotyrosine is not enough to lead to binding. The binding of individual proteins to PI(3)P or PI(4,5)P_2 might similarly depend on the nature of the surface on which the phosphorylated PI ring is found.

The radical changes that occur in stimulated PI turnover *in vivo*, in which 50% of the cell's PIP_2 is hydrolyzed and reformed within seconds, could cause large changes in membrane potential and curvature, which might be important for signaling to intracellular proteins. Modeling such effects theoretically or experimentally by constructing synthetic surfaces and linear polymers bearing multiple inositol phosphates would begin to address these issues. In addition, the thermodynamics of hydration and divalent cation binding to PPI-rich surfaces are complex [21], and the steric and electrostatic effects of placing multiple phosphates on the small inositol ring cannot be neglected. For example, direct measurements suggest that the free energy change of hydrolysis of the 5' phosphate from $PI(4,5)P_2$ is more negative than that for hydrolysis of ATP [22]. Whether this large free energy change is exploited by the cell is unknown.

Practical uses of synthetic PPI analogs and PPI ligands

PPI derivatives have potential use as antiproliferative agents and other pharmaceuticals based on modulating cell signaling by PPIs. For example, thiophosphate [23,24] and deoxy [25] analogs of PI have been synthesized and have biological activity, and phosphonate substrate analogs can inhibit PPI-specific PLC [26]. Sulfated analogs might also be useful. Such agents, as well as peptides that bind to PPIs, might be able to modulate PPI turnover *in vivo*. Some attempts in this direction have already been made, with surprising results; although PPI-binding peptides would be expected to inhibit the access of enzymes to PPIs, in one case a peptide based on a sequence of phospholipase C-β, with similarity to one of the PPI-binding sites of gelsolin, strongly accelerated rather than inhibited hydrolysis of $PI(4,5)P_2$ by this enzyme [27]. These results again emphasize the probable relevance of the physical packing of PPIs to their biological function. A collaboration between synthetic organic chemists, physical chemists, and cell biologists will probably be required to understand the function of PPIs completely.

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