Phosphoinositide Signaling: New Tools and Insights

Phosphoinositides constitute only a small fraction of cellular phospholipids, yet their importance in the regulation of cellular functions can hardly be overstated. The rapid metabolic response of phosphoinositides after stimulation of certain cell surface receptors was the first indication that these lipids could serve as regulatory molecules. These early observations opened research areas that ultimately clarified the plasma membrane role of phosphoinositides in Ca$^{2+}$ signaling. However, research of the last 10 years has revealed a much broader range of processes dependent on phosphoinositides. These lipids control organellar biology by regulating vesicular trafficking, and they modulate lipid distribution and metabolism more generally via their close relationship with lipid transfer proteins. Phosphoinositides also regulate ion channels, pumps, and transporters as well as both endocytic and exocytic processes. The significance of phosphoinositides found within the nucleus is still poorly understood, and a whole new research concerns the highly phosphorylated inositols that also appear to control multiple nuclear processes. The expansion of research and interest in phosphoinositides naturally created a demand for new approaches to determine where, within the cell, these lipids exert their effects. Imaging of phosphoinositide dynamics within live cells has become a standard cell biological method. These new tools not only helped us localize phosphoinositides within the cell but also taught us how tightly phosphoinositide control can be linked with distinct effector protein complexes. The recent progress allows us to understand the underlying causes of certain human diseases and design new strategies for therapeutic interventions.

Phospholipids are very important structural elements of all eukaryotic cellular membranes that undergo constant metabolic changes according to the need of the cell to maintain its structural integrity. Each membrane compartment has its unique lipid composition: for example, the plasma membrane (PM) has high phosphatidylinositol (PtdIns) content and can be utilized as a scaffold for regulatory molecules. The PM also has the highest cholesterol content and contains sphingomyelin and complex glycosphingolipids in the outer leaflet of the membrane. Since almost all of the structural lipids or their precursors are synthesized in the endoplasmic reticulum (ER), these lipids have to reach their steady-state destination either with vesicular transport or with the help of lipid transfer proteins. The nuclear processes. The expansion of research and interest in phosphoinositides naturally created a demand for new approaches to determine where, within the cell, these lipids exert their effects. Imaging of phosphoinositide dynamics within live cells has become a standard cell biological method. These new tools not only helped us localize phosphoinositides within the cell but also taught us how tightly phosphoinositide control can be linked with distinct effector protein complexes. The recent progress allows us to understand the underlying causes of certain human diseases and design new strategies for therapeutic interventions.

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Cells also use lipids as signaling molecules. The well known metabolites of arachidonic acid as pro-inflammatory and hemostatic mediators together with the endogenous cannabinoids are good examples of how cells utilize lipid compounds for intercellular communication, although the same lipids can also have signaling roles within the cell. Phosphoinositides are the best examples of how phospholipids, namely phosphatidylinositol (PtdIns), can be utilized as a scaffold to generate by phosphorylation a variety of compounds that control a whole range of cellular functions. It is important to distinguish the small amount of regulatory lipids that show high turnover rates from the structural lipids that have a slower turnover. PtdIns is one of only a few lipids that clearly serves as a structural lipid as well as a precursor of multiple signaling molecules. This dual role often makes it more difficult to analyze the importance of PtdIns in cell regulation. Increased turnover of PtdIns and phosphatidic acid (PtdA) in response to stimulation of some cell surface...
Cellular PtdIns(4,5)P2 and PtdIns(3,4,5)P3 are not regulated by PLC alone. This is clear from those that are not regulated by PLC. For example, the following membrane-associated molecules need to be regulated: the small GTPases, the lipid transfer proteins, and the membrane-associated molecular events (MAME) (8, 39, 103, 147). The role of phosphoinositides in these locations is to recruit exchange factors or GAP proteins. Small GTPases often regulate their nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) to function properly. Although many PLCs are poorly understood, it is evident that any PLC that hydrolyzes PtdIns(4,5)P2 or PtdIns(3,4,5)P3 must be regulated by other mechanisms that are not regulated by PLC. The most common phosphoinositide (PI) PLCs are the G protein-coupled receptors (GPCRs) and the PI PLCs. The role of phosphoinositides in these locations is to recruit adaptor proteins and to work together with the small GTP binding proteins. It is still not clear how phosphoinositides regulate these processes. There are many unanswered questions.
Cellular Processes Regulated by Inositides

It might be easier at this time to list the processes that are not regulated by inositides in a eukaryotic cell than those that are clearly inositol dependent (Figure 1). The following sections will outline some research areas where phosphoinositides have gained high profile and discuss some current questions that the authors feel are important and unresolved. This selection is not exhaustive, and it reflects the authors’ views and interests. The enzymes that recognize phosphoinositides are formed by a large number of kinases and phosphatases and their molecular mechanisms are still not clear. Although these enzyme groups have been extensively researched, and hence could be the basis of any review article covering this field, no attempt will be made in this review to systematically discuss the inositol signaling enzymes. Some summaries can be found in several other recent reviews (5, 8, 39, 103, 147).

Questions still unanswered about the classical canonical signaling roles of phosphoinositides

The general concept of signal transduction utilizing PM phosphoinositides is clearly established for Ca2+-mobilizing hormones when they activate their cell surface receptors. These receptors activate phosphoinositide-specific phosphohydrolysis (PLC) enzymes to hydrolyze phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] (5, 154). In contrast, the latest gene knockout studies have clearly shown that these enzymes primarily hydrolyze PtdIns(4,5)P2 in the PM. However, the limited pool of PtdIns(4,5)P2 needs constant replenishment from a larger PtdIns pool via sequential phosphorylations by PI4-kinase and PIP5-kinase enzymes. As straightforward as it seems, this process is very poorly understood. First, the replenishment of PM PtdIns4P in intact cells appears to be mediated by one of two wormannin-sensitive type II PIP4-kinases, PIP4KIIIα (9, 11). The yeast ortholog of this enzyme, Stt4p, is localized to the PM and is responsible for the synthesis of the PM pool of PtdIns4P and, hence, PtdIns(4,5)P2 (6, 7). However, in mammalian cells, this enzyme is mostly found in the ER and the Golgi (116, 183), and it is not all that clear how it can supply PtdIns4P for the PM since no lipid transfer protein is known to transfer PtdIns4P between membranes. It is possible that a small amount of active PIP4KIIIα enzyme is located at the PM that is below the detection limit of immunocytochemical methods. Alternatively, a fraction of the peripheral ER makes contacts with the PM, and in this functional compartment the enzyme is able to directly phosphorylate PM-localized PtdIns. The existence of such a compartment in yeast is also supported by several observations. In S. cerevisiae, a reciprocal situations exists: the PM-localized Strp is also responsible for the generation of a PtdIns4P pool that is dephosphorylated by the Sac1 phosphatase, a clearly ER-localized enzyme (48). Moreover, Strp is concentrated in specific domains in the PM where it colocalizes with the Ypp1p protein, a molecular chaperone that is required for its activity (7, 188). Ypp1p, presumably a peripherally ER-localized chaperone-like protein that alleviates α-synuclein (a protein that accumulates in familial Parkinson’s disease) toxicity in yeast (46). It is not unreasonable to assume that Strp and Ypp1p are part of the ER–PM contact sites in the yeast where they form a signaling complex (7). These ER–PM contact zones do exist in mammalian cells (170), and they are the sites of the store-operated Ca2+-entry (SOCE) pathway that is activated by the depletion of the ER luminal Ca2+ pools. Here, the contacts are formed by the recently discovered ER-resident Ca2+-sensor molecule STIM1 and the PM Ca2+ channel Orai1 (130). Whether the mammalian PIP4KIIIα is located and functions in this compartment has yet to be demonstrated.
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This suggests that PIP5K I\(\text{human terminology}\)/H9251 is primarily responsible for the synthesis of the agonist-PtdIns(4,5)P2 that is accessed by PLC. While it has been shown that the 87-kDa splice form of PIP5K type I can regulate the restoration of the PtdIns(4,5)P2 pools that regulate the channel (74). This was followed by a large number of studies showing PtdIns(4,5)P2 requirement for proper activity of several ion channels (52, 75). In almost all of these experiments the question was raised whether PtdIns(4,5)P2 is merely a requirement for the optimal functioning of the channels or the channels are actually regulated by PtdIns(4,5)P2 changes that occur during activation of PLC-coupled receptor mechanisms. In some cases, such as the M-current (77, 156) or the cold- and menthol-sensitive TRPM8 channels (33, 135), the channel activity clearly follows very closely the changes in PM PtdIns(4,5)P2. However, in other examples, PtdIns(4,5)P2 has both inhibitory and stimulatory effects on channel activity (108). The TRPV1 channels, for example, are sensitized by PLC-coupled agonists presumably via reduction of PtdIns(4,5)P2 (26), whereas the same lipid is required for their recovery from desensitization (99, 100). More detailed discussion on the inositol regulation of ion channels can be found in excellent recent reviews (55, 134, 155).

The mechanism by which inositol lipids can regulate ion channels remains elusive. Almost all ion channels and transporters contain clusters of basic residues in their membrane-adjacent regions facing the cytosol or within their COOH-terminal tails. Lipid regulation of TRP channels was mapped to the TRP domains of their cytoplasmic tails (118, 135). However, an interesting and common feature of the PtdIns(4,5)P2 regulation of many potassium and TRP channels is that the lipid alters the interaction of the channels with other specific regulators such as calmodulin (87), β-subunits (78), or ligands such as ATP (16). In addition, indirect regulation of channels via inositol-binding channel-interacting proteins is also possible, as shown by the recent identification of Ftr, a molecule that interacts with TRPV1 channels and also binds phosphoinositides (84).

**Phosphoinositides regulate vesicular trafficking**

The presence of phosphoinositide kinases and phosphatases in various membrane fractions of fractionated cells or tissues has been noted by early studies on these enzymes (111). However, the general notion was that these findings either indicated PM contamination of the other membranes or represented intermediate membrane compartments as phosphoinositides were on their way to the PM. Some of the earliest indications that phosphoinositides may be important for membrane fusion or fission events came from studies on dense core vesicles. As early as 1972 it was recognized that these vesicles are crucial for priming the release of their contents from their competent PM when a nerve impulse is delivered to the nerve terminal and the vesicles docked vesicles fuse with the plasma membrane (24). The nerve terminal is a complex membrane domain that contains the machinery for the fusion of vesicles with the PM. This complex machinery is composed of a phosphoinositide-binding element, the SNARE complex, and other molecules that regulate the fusion event. The fusion machinery is composed of a phosphoinositide-binding element, the SNARE complex, and other molecules that regulate the fusion event. The fusion machinery is composed of a phosphoinositide-binding element, the SNARE complex, and other molecules that regulate the fusion event. The fusion machinery is composed of a phosphoinositide-binding element, the SNARE complex, and other molecules that regulate the fusion event. The fusion machinery is composed of a phosphoinositide-binding element, the SNARE complex, and other molecules that regulate the fusion event. The fusion machinery is composed of a phosphoinositide-binding element, the SNARE complex, and other molecules that regulate the fusion event. 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on dense core granule (DCV) exocytosis, where it was recognized that PtdIns(4,5)P_2 synthesis is important for priming the exocytic vesicles (43, 70). DCVs containing their cargo undergo maturation that increases their competence to dock and ultimately fuse with the PM when a rapid rise in Ca^{2+} concentration triggers the fusion process. Some of the mature granules under the membrane will dock to the PM, but these pre-docked vesicles still have to undergo "priming" to become the "readily releasable pool" that is first to be fused upon stimulation (31). Furthermore, the membrane phosphoinositides were important for Rapid depletion of plasma membrane PtdIns(4,5)P_2 and dephosphorylation of the five-position in endosomal membranes. It is recognized that PtdIns(4,5)P_2 synthesis is important to recruit proteins to the site of fusion. Although PtdIns(4,5)P_2 is important to recruit proteins to the site of fusion, it appears that PtdIns(4,5)P_2 has a lipid-dependent component to the PM regardless of the type of endocytosis, it appears that PtdIns(4,5)P_2 has to be dephosphorylated by 5-phosphatase enzymes in order for the exocytic vesicle to find its destination whether recruiting to the PM or sorted via the sorting endosome (106). Thus a high rate of phosphorylation and dephosphorylation of the five-position in PtdIns(4,5)P_2 is crucial for proper membrane cycling at the PM. The presence of the type II PI4Ks in endosomal membranes (10, 113) suggest that PtdIns4P also has an important role in determining the fate of the endocytosed membranes.

A critically important step in recognizing the relevance of phosphoinositides in intracellular vesicular trafficking was the discovery that the yeast Vps4p protein, an essential element of vesicular sorting, was a PtdIns3-kinase (142). This was followed by a whole set of studies that unraveled protein modules capable of recognizing PtdIns4P (22, 149) as well as the enzymes that convert these lipids to PtdIns(3,5)P_2 (56, 148) or back to PtdIns (123, 161) and the signaling complexes that are regulated by them.

Even a superficial summary of the advances made in these respective research fields would exceed the scope of this review. Excellent reviews can be found in all of these topics. We will only discuss some general questions that come to mind when trying to understand the underlying principles common to all of these processes. The current understanding of how inositides contribute to these complex membrane fusion or fission steps is that these lipids interact with protein modules present in the proteins that regulate these processes. Many such inositol binding modules have been identified and characterized. Pleckstrin homology (PH) domains, phox-homology (PX) domains, FYVE-domains, ENTH-domains FERM-domains are the best known ones but others certainly also exist (12, 91). Typical proteins that contain such domains include both tetrameric and monomeric clathrin adaptors, GEF- and GAP proteins of small GTP-binding proteins, belonging to various classes (Arfs, Rabbs, Rho/Rac, and Ras), sorting nexins, protein and lipid kinases, as well as various phosphoplasmas, and probably many others (91). In many instances, the inositol binding to the domain is only a partially effective signal, and it needs an additional input, which in most cases is an active small GTP-binding protein (FIGURE 2A). This mode of operation is often referred to as coincidence detection (23).

Initially, it was believed that the function of the lipid binding domains was to recruit the protein to the site where it needs to function. Although this certainly is the case for many proteins, it is increasingly recognized that membrane localization is often determined by other parts of the molecule, and the inositol binding domain serves as a lipid-dependent regulatory module rather than a localization signal. Another adjustment in our view of the inositol binding domains is concerned with their specificities. Initial examples suggested that these modules show a high degree of specificity in their inositol recognition. And indeed, in many cases, PH domains, PX domains, or FYVE domains show a high degree of inositol binding specificity. However, an increasing number of such modules have proven to be rather promiscuous in their phosphoinositide binding (187) and even showed no lipid binding at all. Often, these modules also have protein binding partners, which is not surprising, since PH domains, for example, have a very similar folding structure to phosphotyrosine binding
monophosphorylated inositides populate the internal membranes whether on endocytic or exocytic routes, and the polyphosphoinositides [PtdIns(4,5)P$_2$ or PtdIns(3,5)P$_2$] are located in the plasma membrane on internal membranes that border a sequestered "outside" compartment such as the dumping organelle, the lysosome). During evolution, phosphoinositide kinases appear together with internal membranes and hence, their functions are likely linked to the identification and fate-determination of the internal membranes. In this context, it is relevant that PtdIns4P is mostly found in the PM (although small amounts it was detected in intracellular membranes in EM studies) (180). The important roles of phosphoinositide 5-phosphatases, such as syntenin-janin and the OCL1-5-phosphatase as well as the PIP 5-kinases at the internalization and recycling of vesicles from and into the PM, indicate that PtdIns(4,5)P$_2$ is a functional identifier of the PM. A delicate balance between PtdIns4P and PtdIns(4,5)P$_2$ in the PM-endo-somal interface is critical both to the fusion (recycling) and the fission (endocytic) processes.

The Golgi contains the largest amount of PtdIns4P, and the various intracellular vesicular compartments contain either PtdIns3P or PtdIns4P and perhaps PtdIns5P, although the distribution of the latter as well as the major route of its production is still not fully understood. Although PtdIns4P has long been viewed only as a precursor of PtdIns(4,5)P$_2$ in the PM, it has become clear in the last decade that PtdIns4P is a major regulatory lipid in the Golgi, the TGN, and some endosomes (35). PtdIns4P working together with Arf1 proteins is important to recruit clathrin adaptors such as AP-1 (179), AP-3 (28), or GGAs (177) to their respective target membranes. It is noteworthy that all of the PH domains that recognize PtdIns4P are part of proteins that transport lipids (see below), revealing a pivotal role of PH kinases as master regulators of structural lipid distribution within the cell. Whether PtdIns4P made in the Golgi contributes to the phosphoinositide supply of PM is still an important open question discussed above.

**Membrane dynamics, actin cytoskeleton, and phosphoinositides**

The plasma membrane of most metazoan cells shows enormous plasticity and undergoes shape changes driven by actin polymerization. This process is very critical for both physiological processes such as chemotaxis, cell adherence, or morphogenesis and to pathological ones such as invasive cancer metastasis or the spreading of pathogens from one cell to another. In all of these instances, a very active membrane deformation is elicited by polymerized actin. We have discussed above the phosphoinositide-binding domains (FERM, ENTH) found in a large number of proteins that interact with the actin cytoskeleton. However, in addition, proteins that regulate actin polymerization are involved. These include gelsolin, profilin, profilin-actin complexes from and into the PM, indicate that PtdIns(4,5)P$_2$ is a functional identifier of the PM. A delicate balance between PtdIns4P and PtdIns(4,5)P$_2$ in the PM-endo-somal interface is critical both to the fusion (recycling) and the fission (endocytic) processes.

**The endless energy metabolism**

Research on PtdIns4P and PtdIns(4,5)P$_2$ has been enriched in the past years, and the understanding of its role in a limited variety of cellular functions has become unconditional. The PI3-kinases are the major enzymes that produce the 3-phosphorylated inositides (PI3P, PI(3,4)P$_2$, PI(3,4,5)P$_3$), and the polyphosphoinositides [PtdIns(4,5)P$_2$ or PtdIns(3,5)P$_2$] are located in the plasma membrane in separate from the endoplasmic reticulum (ER) and the Golgi apparatus. The identification and fate-determination of the internal membranes is a process that is crucial for both physiological processes such as invasion and apoptosis or pathological ones such as invasive cancer metastasis or the spreading of pathogens from one cell to another. In all of these instances, a very active membrane deformation is elicited by polymerized actin. We have discussed above the phosphoinositide-binding domains (FERM, ENTH) found in a large number of proteins that interact with the actin cytoskeleton. However, in addition, proteins that regulate actin polymerization are involved. These include gelsolin, profilin, profilin-actin complexes...
polymers and proteins congregate in the internal cytoskeleton, which is sequestered in the cytoplasm but which is critical for the internalization of actin. The actin polymerization per se also show inositol lipid-binding. These include the actin capping and severing proteins, gelsolin, profilin, and cofilin (185). Actin polymerization requires "nucleation," a process by which filamentous actin free (+) ends are generated. These (+) ends become uncapped by dissociation of gelsolin and ready to accept further actin monomers that are supplied from dissociation from the (-) ends of actin with the help of cofilin (164). PtdIns(4,5)P₂ binding to the capping/severing proteins, hence, determines the rate of actin polymerization. An additional critical component of the actin network is the Arp2/3 complex consisting of seven tightly associated proteins that allow actin polymerization at an angle from an already existing F-actin filament (120). The Arp2/3 complex is regulated by members of the Wscott-Aldrich Syndrome proteins (WASP). Some of these, like N-WASP, have putative phosphoinositide binding motifs (122). Our knowledge on the dynamics of actin polymerization has been greatly enriched by studies on the intracellular movement of the bacterium Listeria monocytogenes with a strong connection to phosphoinositides (89).

The endless complexity of PI3-kinase signaling

Research on PI3-kinases has exploded in the last 20 years, and the progress cannot be summarized in a limited way in this review. The roles of various PI3-kinase isoforms have been clarified using knockout and knock-in mouse models (165). Partly based on such studies, it has become clear that the Class I PI3Ks enzyme with its p85/55 adaptors has major roles in metabolic regulation and cell growth (48, 53, 103) [including cancer (140)], whereas PI3Kδ and PI3Kγ are critical components of immune cell regulation (3, 88, 103). This clear functional separation has generated enormous interest in developing subtype-specific PI3-kinase inhibitors as adjuvant treatment in cancer and as anti-inflammatory or immunosuppressive drugs, respectively (103). Progress in understanding the functions of the Class II and Class III PI3-kinases has been somewhat slower since these enzymes have more complex roles in vesicular trafficking that in many cases might be redundant with other phosphoinositide regulatory enzymes. These developments have been summarized in a number of excellent reviews (e.g., Ref. 47) and will not be further discussed here.

Nuclear processes and phosphoinositides

Several lines of evidence suggest that the nucleus has its own phosphoinositide signaling system (36), although its significance has only recently been emerging (80). Nuclear phosphoinositide changes separate from those detected in the cell membranes have been described in IGF-stimulated Swiss 3T3 cells (37). Moreover, several of the enzymes known to process inositol-cycle intermediates, such as P41Ks (34, 83), PIP3-kinases (20, 106), PLCγ (38, 102), and DAG kinase (124) were found to be present in the nucleus. Phosphoinositides were also detected within the nucleus associated with nuclear speckles, the sites of pre-mRNA splicing (319). This also means that, intriguingly, the lipid does not just associate with the nuclear membranes or the membrane of intranuclear canaliculi but with other nuclear components. This raises important questions concerning the physical state of the lipids, whether they are associated with lipid transfer or other lipid binding proteins.

It is much less clear how phosphoinositides affect nuclear processes. Yeast studies suggested that one of the yeast PI4Ks, Pkh1, which is an essential enzyme and is present both in the Golgi and the nucleus, has to be present in both locations to fully rescue a temperature-sensitive allele (153). Recent evidence also suggest that InsP₃-induced Ca²⁺ changes can be initiated primarily within the nuclei in the case of some cell surface growth factor receptors, such as c-Met (59). The most specific nuclear function so far was linked to a PIP5-kinase iso, which was recently found to associate with and stimulate the activity of a mRNA poly-adenylation enzyme Star-PAP via generation of PtdIns(4,5)P₂ in nuclear speckles (106). By this mechanism, the processing and nuclear export of specific mRNAs can be regulated. What is not clear is what determines which primary RNA transcripts are subject to this regulation. Phosphoinositide binding domains (PHD fingers) have been described in a number of nuclear proteins, such as the tumor suppressor ING2 (64). Another PHD-containing protein, ASH2 from Drosophila, has been found to associate with a PIP kinase (25). The binding of PHD proteins to trimethyl-lysine residues of histones (104) makes these observations even more intriguing.

However, nuclear processes are not only regulated by inositol lipids but also by the multi-soluble multi-phosphorylated inositol, such as InsP₄, InsP₅, or the pyrophosphorylated inositol polyphosphates (143). Although the roles of these compounds as phosphate reservoirs in germinating seeds have been known for a while, the real breakthrough in understanding their significance came from yeast genetic studies that identified the enzymes Ipk1 and Ipk2, which are responsible for the conversion of InsP₄ to InsP₅, (186). Subsequent studies found that this pathway is involved in arginine-specific gene expression responses as well as in chromatin remodelling under the PHOS promoter (152). It is now believed that inositol polyphosphates regulate transcriptional processes, mRNA export, and telomere length (reviewed in Ref. 143 in detail). The importance of the same pathway in mammalian cells is underlined by the embryonic lethality of the knockout of either of the mouse homologs of Ipk2 or Ipk1 (50, 172). However, the exact mechanism of why this happens still awaits identification.
Lipid transport regulated by phosphoinositides

One of the most exciting recent developments in phosphoinositide research was the finding of a link between sphingolipid metabolism and phosphoinositides (66). Earlier analysis of the phosphoinositide-binding specificities of various PH domains revealed that all of the proteins whose PH domains specifically recognized PtdIns4P were lipid transport proteins. These included the oxysterol binding protein (OSBP) [95] and its yeast homologs OSH1 and OSH2 [93, 138, 187], the ceramide-binding protein CERT [67, 94], as well as the FAPP1 and FAPP2 proteins [40]. These findings have already forecasted that lipid transfer function and PH-kinases would be intimately interrelated. This was elegantly demonstrated with the discovery of the CERT protein that transfers ceramide between the site of its synthesis in the ER to the trans side of the Golgi, where ceramide is then flipped and converted to sphingomyelin (SM) in the Golgi lumen. In addition to the ceramide binding module, CERT also contains a PH domain that is important to dock the protein to the Golgi and a mutation within the PH domain that eliminates PtdIns4P renders the CERT protein completely dysfunctional [67]. What is remarkable about the PtdIns4P regulation of CERT transport function is that it appears to require the type III beta PH kinase enzyme [159], even though all of the four PI4-kinase enzymes can produce PtdIns4P in some parts of the Golgi [8]. Based on recent observations, a regulatory loop is emerging in which Golgi DAG levels regulate the recruitment/activity of PKD enzymes that phosphorylate and activate PI4KIIIb [68] but also phospholipase C (PLC), but this phosphorylation decreases CERT/binding to PtdIns4P [52].

Less is known about the inositol regulation of the oxysterol transport function of the OSBP protein or its homologs. However, several clues suggest that phosphoinositides and oxysterol binding proteins are functionally coupled. Deletion of one of the yeast oxysterol binding proteins, Kes1p, bypasses the Sec14 (a yeast PI4-kinase) enzyme and PI4-phosphate and activates PI4KIIIb [68] also phospholipase C (PLC), but this phosphorylation decreases CERT/binding to PtdIns4P [52].

The biology of the FAPP1 and FAPP2 proteins has been more controversial. FAPP1 was initially identified as a protein that contains a PH domain with specific PtdIns4P recognition (four-phosphate adaptor protein) [40]. A highly homologous protein, FAPP2, however, was shown also to contain a glycolipid transfer protein homology (GLTP) domain [174], and it has been proposed that FAPP2 transfers glycosyl ceramide (GlcCer) between membranes in a PtdIns4P-regulated manner [32]. It is not yet clear between which membranes this transfer occurs. GlcCer is synthesized on the outer surface of the cis-Golgi, but its conversion to lactosyl ceramide and the more complex glycosphingolipids (GSLs) occurs in the lumen at the trans-Golgi [64]. One report suggested that FAPP2 is needed to transfer GlcCer from the cis- to the trans-Golgi in a nonvesicular transport step [32], whereas another study proposed that FAPP2 transferred GlcCer from the cis-Golgi back to the ER, where its flipping to the lumen was found most efficient [64]. Regardless of the route(s) of transport of FAPP2 supports, the protein was found critical for the transport of apical cargos to the membrane in polarized cells and for the formation of cilia in the apical membrane, presumably because of the need for organization of glycolipid-rich membrane domains [173]. More studies are expected to clarify this role of the FAPP2 protein as well as to determine which of the PI4-kinases are critical for supporting its PH domain membrane interactions.

The importance of phospholipid transfer proteins in the Golgi to PM secretion process has long been established in S. cerevisiae. Here, the Sec14p protein that encodes a phosphatidylinositol/phosphatidylethanolamine (PP/PC) transfer protein was found to be critical to the yeast secretion process [14]. The function of the Sec14 protein is to supply the Golgi with PtdIns and PtdCho, ultimately to maintain the levels of DAG in this organelle. DAG is an important regulator of a number of Golgi-associated kinases containing cystein-rich Zn+-finger motifs, such as some PKC isoforms [90] and PKD [15]. In addition, PtdIns4P is also a key organizer of the Golgi to PM vesicular secretion process via recruitment of adaptor proteins [179], and Sec14p can also supply PtdIns for the Golgi-localized PI4-kinases [65, 141]. Moreover, there are several Sec14 homologs in yeast that have a role in transferring lipids between the various yeast membranes, and it was recently shown that PtdIns4P synthesis is important in some of these transport functions [137]. One of these Sec14 homologs was also identified as a component of the synthesis of the aminophospholipid phosphatidylethanolamine (PtdEth) via decarboxylation of ER-derived phosphatidylcholine (PtdSer) in Golgi membranes [142]. For this, PtdSer has to be transferred to the Golgi membranes to be decarboxylated, and genetic studies have shown that Stt4p (the yeast homolog of PI4KIIIb) is a regulatory component of this process at the Golgi (but not at the mitochondrial) site [162]. It is not yet understood why the Stt4p kinase is needed for the lipid transfer and whether it acts at the donor or acceptor membrane site. Also, it has yet to be seen whether a similar regulation of aminophospholipid synthesis by PI4-kinases or by other phosphoinositides is present in higher organisms.

Interestingly, mammalian PITPs show only low sequence homology to Sec14, although Sec14 homologs are also found in mammalian cells, and they also transport α-tocopherol and phospholipids (28), and they have been shown to have activities similar to that of ESPTPs [91]. Although mammalian ESPTPs are required cell surface expression of ESPTP.

Methods

Inositol

Most of our current understanding is based on the separation of extracted inositol phosphates using either TLC or high-performance liquid chromatography (HPLC) [145]. The separation of intact inositol phosphates is complicated by the case of labeled inositol phosphates and the labeling of the phosphate. In the case of intact inositol phosphates, intact inositol phosphates are assayed by radioactive methods. In the case of labeled inositol phosphates, intact inositol phosphates are assayed by biochemical methods. As the highly labeled inositol phosphates are not available, intact inositol phosphates are assayed by radioactive methods.

Visualizing dynamics

The first attempts to visualize inositol phosphatase dynamics were based on recombinant PI(4,5)P2 expression in mammalian cells [1].
which membrane syntaxis with the endocytic machinery and glycosphingolipids. A protein such as the larger Nse2 protein (98) or the Golgi-localized PITP (126) is needed to transport lipids from the ER to the TGN, and it is needed to transport lipids from the ER to the TGN, and this process requires the involvement of Sec14p (121) and PtdIns from the ER to the TGN (141) and PtdIns-specific PLCs (128). These studies together outline an important regulatory paradigm in cellular lipid synthesis and transport. Contrary to common beliefs, endogenous membrane lipids are not diffused through the aqueous phase separating the membranes and, in many instances, do not distribute freely with vesicular transport. Their trafficking involves membrane spaces that require specific lipid systems that are not dissimilar to the vesicular transport systems (channels or transporters) that allow the water-soluble products to traverse lipid bilayers. It is intriguing that phosphoinositides appear more and more to be just as important for controlling lipid transport as they are in the regulation of vesicular transport and ion channels.

### Methods to Study the Relevance of Inositol Regulation

Most of our early knowledge on phosphoinositide changes were obtained by metabolic labeling studies using either [3H]-phosphate or myo-[3H]-inositol, followed by separation of the labeled phospholipids by TLC (145). These methods were complemented with separation of the water-soluble inositol phosphates extracted from cells by HPLC (146) and detect the eluted inositol phosphates either by radioimmunoassay in the case of labeled cells, or by metal-dye detection (127) and chemically suppressed conductivity detection (156) to measure absolute amounts of the inositol phosphates. Nineteen inositol phosphates ([1,4,5]P3) mass was also determined by radioimmunoassay (24), but all of these measurements assessed total cellular content without any special information on location or compartmentalization. As the highly localized roles of the lipids became more and more apparent, there was a need to obtain information on the subcellular distribution and rapid dynamics of inositol lipid changes within the cell.

#### Visualizing inositol lipid distribution and dynamics

The first attempts to evaluate the subcellular distribution of inositol lipids without cell fractionation were based on immunocytochemistry using anti-PtdIns(4,5)P2 antibodies (160). This, however, required cell fixation, and many laboratories have expressed frustration over the lack of sensitivity of the methods, to batch-to-batch variability of the antibodies or to the hard-to-standardize subtleties of the fixation procedures. Live cell imaging of lipid distribution has become possible with the availability of protein domains that possess natural phosphoinositide recognition and by using them as GFP-based proteins to visualize the lipids in live cells. These included PH domains to detect PtdIns(4,5)P2 (151, 167), PtdIns(4,5)P3 (85, 144, 168) and PtdIns4P (95), FYVE domains to detect PtdIns(3,4)P2 (57, 86), and C1 domains to follow DAG changes (107). Even though these methods have their limitations (discussed in several reviews; e.g. Ref. 166), they provided enormously important new information on lipid distribution and dynamics in a great number of systems and have become a standard method in cell biology. A special extension of this approach was the use of these domains in fixed cells (just like antibodies) to detect the lipids without disturbing the biology that complicates matters when overexpressing these lipid binding domains (38). This also allowed analysis with EM resolution (180), although the distortion of lipid levels or distribution due to the fixation procedure is a tradeoff in these applications. Detailed description of these methods and their pros and cons of their use has been discussed elsewhere (13, 41, 42) and will not be duplicated here.

#### Manipulating inositol lipid or phosphate levels within the cells

It is difficult to alter inositol lipid levels in whole cells and study the immediate consequences on any signaling or trafficking event. Most of the currently used approaches require prolonged exposure of the cells to the desired lipid change. For example, knockdown of an inositol lipid kinase or phosphatase enzymes, or the overexpression of an active or a dominant negative form takes anywhere between 4 and 7 days to achieve the desired effects. During this period, the primary affected process (such as the release of an adapter protein) initiates a whole sequence of events, leading to changes from which it is hard to deduce what processes have been primarily linked to the lipid changes. The optimal solution to these problems is to use specific inhibitors to evoke acute changes in inositol levels. This is best demonstrated by the enormous boost that discovery of PI3-kinase inhibitors brought to the field of PI3-kinases (4). Unfortunately, there are no good specific inhibitors for many of the inositol converting enzymes. This problem has prompted us (109) and others (156) to design alternative strategies by which inositol levels can be acutely changed in specific membrane compartments. This is based on the regulated recruitment into defined membrane compartments of inositol kinase or phosphatase enzymes that are stripped of their own localization mechanisms. This method relies on the assumption that the enzymes resulting in the cytosol have limited impact.
on the membrane-bound lipids, but this is changed dramatically once the enzyme is recruited to the membrane where its substrate resides. The recruitment is based on the heterodimerization of the FRB domains of mTOR and the FRB12 protein (17) in the presence of rapamycin or an appropriate analog, so fusing an enzyme to one of these domains and targeting the other partner to the desired membrane compartment allows a regulated recruitment process. This method was successfully used to change the PtdIns(4,5)P2 levels in the plasma membrane and assess its consequences on ion channel activity (158, 169) as well as on the membrane binding of clathrin adapters and their roles in the endocytic process (1, 191). This approach can be extended to other enzymes and compartments and will be interesting to study the roles of phosphoinositides in other cellular locations. The recent discovery of voltage-regulated inositol lipid 5-phosphatasases in Ciona intestinalis and other organisms (63, 114, 115) offers an alternative and even more rapid and reversible way of changing PtdIns(4,5)P2 levels within the cells. Use of these enzymes should provide us with lots of interesting data on PtdIns(4,5)P2 regulation of plasma membrane processes.

An alternative method to increase phosphoinositide levels within the cells is to supply synthetic lipids with a delivery system that allows the lipid with its highly polar headgroup to cross the PM. This is achieved by combining the lipid with polyanymes or polybasic proteins (120). The advantages of these “PI-shuttle” systems are their simplicity and that various isomers can be delivered into the cells. Their disadvantage is that they will deplete the cell with the lipid, taking away the local regulatory features of the endogenously produced counterparts. In addition, it is hard to know which way these will metabolize in the cells and what the actually active compound is.

Concluding Remarks

Even from this limited overview, it should be obvious that phosphoinositides have an enormous impact on any membrane-associated signaling processes. Extensive research on these lipids over several decades has led to great advances in our understanding of cell signaling. However, the plethora of information available on these lipids is hard to comprehend. The fact remains that we have fundamental gaps in our understanding of the functions of phosphoinositides and the mechanistic details of how they control membrane dynamics. Improving our research tools and developing more specific inhibitors should help us better answer the open questions. Although this field of research was mostly driven by the curiosity of scientists conducting basic research, the achievements of the field have served the goals of public health very aptly. Ten years ago, PI3-kinase inhibitors were only thought about as research tools, and today they are in clinical trials in targeting diseases such as cancer, autoimmunity, allergy, and metabolic disorders. Right now, “translational research” does not appreciate why we need to develop inhibitors of PI3Ks or PI4-kinases. However, this can change in a heartbeat. As a good example, PI4KIIIα has just emerged as a critical factor in the assembly of the Hepatitis C virus in the liver as reported in three separate recent studies (18, 157, 163). This finding will suddenly make this enzyme a desirable target. There is ample reason to expect that an expanded investment in phosphoinositide research will bring a payback in real medical terms.

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