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Features of the Phosphatidylinositol Cycle and its Role in Signal Transduction

Richard M. Epand[1]

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Abstract The phosphatidylinositol cycle (PI-cycle) has a central role in cell signaling. It is the major pathway for the synthesis of phosphatidylinositol and its phosphorylated forms. In addition, some lipid intermediates of the PI-cycle, including diacylglycerol and phosphatidic acid, are also important lipid signaling agents. The PI-cycle has some features that are important for the understanding of its role in the cell. As a cycle, the intermediates will be regenerated. The PI-cycle requires a large amount of metabolic energy. There are different steps of the cycle that occur in two different membranes, the plasma membrane and the endoplasmic reticulum. In order to complete the PI-cycle lipid must be transferred between the two membranes. The role of the Nir proteins in the process has recently been elucidated. The lipid intermediates of the PI-cycle are normally highly enriched with 1-stearoyl-2-arachidonoyl molecular species in mammals. This enrichment will be retained as long as the intermediates are segregated from other lipids of the cell. However, there is a significant fraction (>15 %) of lipids in the PI-cycle of normal cells that have other acyl chains. Phosphatidylinositol largely devoid of arachidonoyl chains are found in cancer cells. Phosphatidylinositol species with less unsaturation will not be as readily converted to phosphatidylinositol-3,4,5-trisphosphate, the lipid required for the activation of Akt with resulting effects on cell proliferation. Thus, the cyclical nature of the PI-cycle, its dependence on acyl chain composition and its requirement for lipid transfer between two membranes, explain many of the biological properties of this cycle.

Keywords Phosphatidylinositol · Phospholipid acyl chains · Phosphatidylinositol cycle · Nir proteins · Diacylglycerol kinase · CDP-diacylglycerol synthase

Abbreviations

CDP-DAG	Cytidine diphosphate-diacylglycerol
CDS	CDP-DAG synthase (Phosphatidate cytidylyltransferase)
DAG	Diacylglycerol
DGK	Diacylglycerol kinase
LPIAT1	Lysophosphatidylinositol acyltransferase 1 (*O*-acyltransferase containing domain 7) (MBOAT7)
PA	Phosphatidic acid
PI	Phosphatidylinositol
PI-cycle	Phosphatidylinositol cycle
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI4K	Phosphatidylinositol-4-kinase
PI4P	Phosphatidylinositol-4-phosphate
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PIP_n	Phosphorylated forms of PI
PI4P5K	Phosphatidylinositol-4-phosphate 5-kinase
PIS	PI synthase (CDP-diacylglycerol-inositol 3-phosphatidyltransferase)
PKC	Protein kinase C
PLC	Phospholipase C (1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase)
PLD	Phospholipase D

✉ Richard M. Epand
epand@mcmaster.ca

[1] Department of Biochemistry and Biomedical Sciences, McMaster University, 1280 Main Street West, Hamilton, ON L8S 4K1, Canada

PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (Phosphatase and tensin homolog)
TrpC channel	Transient receptor cation channel

Lipid Synthesis and Signaling Lipids

Lipids serve many roles in biology. The synthesis of lipids to form cell membranes is often the rate-limiting step in cell proliferation. Phospholipids are major constituents of cell membranes and they have roles in the formation of membrane domains and in the modulation of the activity of membrane proteins. Triglycerides form fat bodies that can be later oxidized as a source of metabolic energy. There are many pathways for lipid synthesis and many species of signaling lipids.

In the present review we will focus on the phosphatidylinositol cycle (PI-cycle) that is the major pathway for the synthesis of phosphatidylinositol (PI). This cycle also plays a major role in lipid signaling. We will discuss some of the properties of the PI-cycle that are important for signal transduction. These features include the fact that this is a metabolic cycle with the consequence that intermediates of the cycle will perform a catalytic role and will tend to remain at a constant concentration. Thermodynamics requires that the cycle function in only one direction since it consumes a large amount of energy. As a metabolic cycle it may be unique in requiring two different membranes. Hence the transfer of lipids between these two membranes becomes an important step in the cycle. In normal cells the cycle enriches lipid intermediates with 1-stearoyl-2-arachidonoyl acyl chains, but it can also produce other molecular species of PI. In order to maintain a particular acyl chain composition, the lipid intermediates of the PI-cycle must be isolated from other lipids of the same type that are present in the cell.

The Phosphatidylinositol Cycle

The PI-cycle is a series of enzyme-catalyzed biochemical reactions that form a cyclical process, such that all of the intermediates of the cycle are regenerated each time the cycle goes around once. There are many known biochemical metabolic cycles and as cycles they all have the property that each intermediate in the cycle has a catalytic role in accelerating the cycle, while the intermediate itself is never depleted or increased in amount. The particular example of the PI-cycle is shown in Fig. 1.

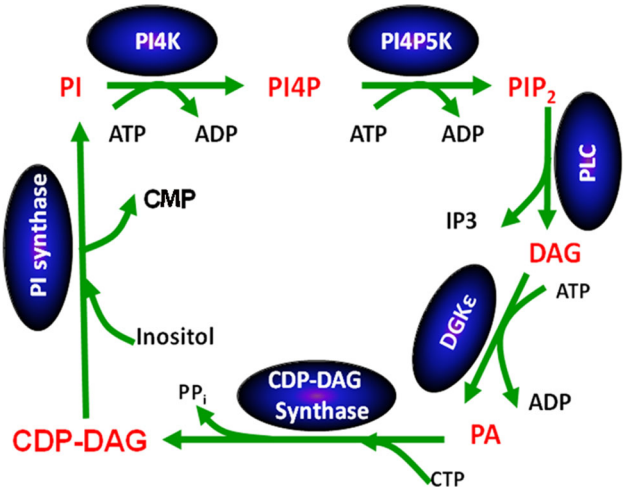

Fig. 1 The phosphatidylinositol cycle. Enzymes involved in the catalysis of each step in the cycle are written in a *blue oval* above the *arrow* for the reaction, using abbreviations. Each lipid intermediate in the cycle is written in *red* (Color figure online)

The PI-cycle is unique among biochemical cycles in that it is not located in a single organelle or membrane within the cell. Phospholipase C (PLC) (1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase (EC:3.1.4.11)) and phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) (EC:2.7.1.68) that are involved in the PI-cycle are located in the plasma membrane, while the enzymes CDP-DAG synthase (CDS) [Phosphatidate cytidylyltransferase (EC:2.7.7.41)] and PI synthase (PIS) [CDP-diacylglycerol-inositol 3-phosphatidyltransferase (EC:2.7.8.11)] are in the endoplasmic reticulum. This means that lipids have to be transferred between the endoplasmic reticulum and the plasma membrane in order to complete the cycle. Portions of the endoplasmic reticulum are juxtaposed closely to the plasma membrane (Fig. 2). The routes of lipid transfer will be discussed below.

Each cycle of the PI-cycle converts 3 ATP + CTP + inositol to 3 ADP + CMP + pyrophosphate + inositol triphosphate. Thus the PI-cycle consumes a fair amount of energy and is required to "turn" in the clockwise direction, according to the way it is drawn in Fig. 1. Furthermore, one of the products of the PI-cycle, pyrophosphate, would be hydrolyzed by endogenous pyrophosphatases to inorganic phosphate, further making the cycle uni-directional.

The cycle also produces inositol triphosphate that is an important ligand in opening calcium channels in the endoplasmic reticulum. Hence, indirectly the PI-cycle contributes, in part, to the regulation of intracellular calcium levels. In addition to inositol triphosphate increasing cellular calcium levels by opening calcium channels in the endoplasmic reticulum, the loss of Ca^{2+} from the endoplasmic reticulum activates a Ca^{2+}-sensor protein, Stim1

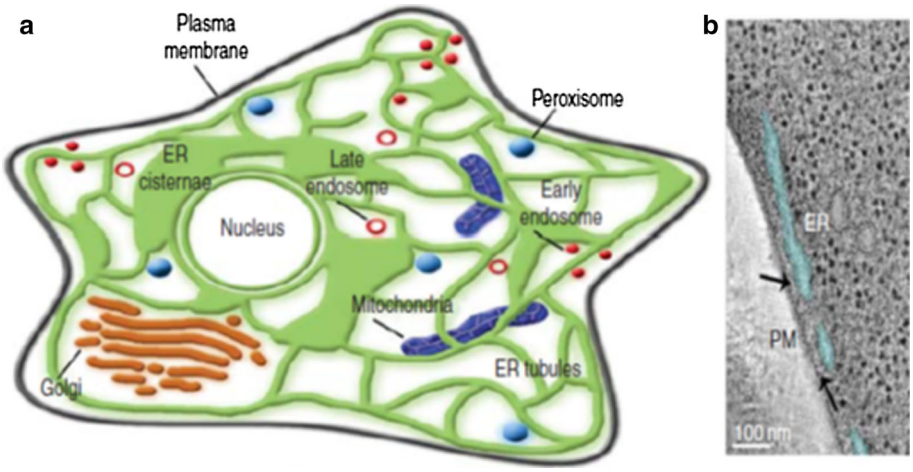

Fig. 2 Close apposition between the endoplasmic reticulum and plasma membrane. **a** Schematic model showing the observed membrane contact sites between the endoplasmic reticulum and plasma membrane, as well as other organelles. **b** An electron microscope tomography of a yeast cell illustrating the close contact between the peripheral endoplasmic reticulum (shown in *blue*, labeled ER) and the plasma membrane (the *dark edge*, labeled as PM). Taken from English and Voeltz (2013) (Color figure online)

that in turn activates a store Orai1 Ca^{2+} channel in the plasma membrane, resulting in a further increase in the intracellular calcium through influx into the cell (Putney and Tomita 2012). PLC, an enzyme of the PI-cycle, can also activate certain transient receptor potential cation (TrpC) channels in the plasma membrane, likely as a result of changes in the PI-cycle intermediates PIP_2 and DAG that are the substrate and product, respectively, of this enzyme. With some TrpC channels, DAG can act indirectly by activating PKC that in turn inhibits TrpC channels (Putney and Tomita 2012).

Lipids that are Derived from or form Intermediates of the PI-Cycle

Although there is no net synthesis or consumption of any of the lipid intermediates in the cycle, the lipid intermediates do have connections with other metabolic pathways that can consume or synthesize intermediates of the cycle (Fig. 3). For example, there are many sources of DAG including the cleavage of phospholipids by different isoforms of phospholipase C (PLC), the hydrolysis of PA by PA phosphohydrolases and lipid phosphate phosphatases, as a product in the synthesis of sphingomyelin from ceramide by sphingomyelin synthase and by the hydrolysis of triglycerides with lipases (Carrasco and Merida 2007). In addition, PA, which is an another intermediate in the PI-cycle, is the precursor for the formation of most phospholipids, including those not made through the PI-cycle. PA can also be hydrolyzed back to DAG, which is not the reverse of the step in the PI-cycle, since the hydrolysis of PA produces inorganic phosphate together with DAG and

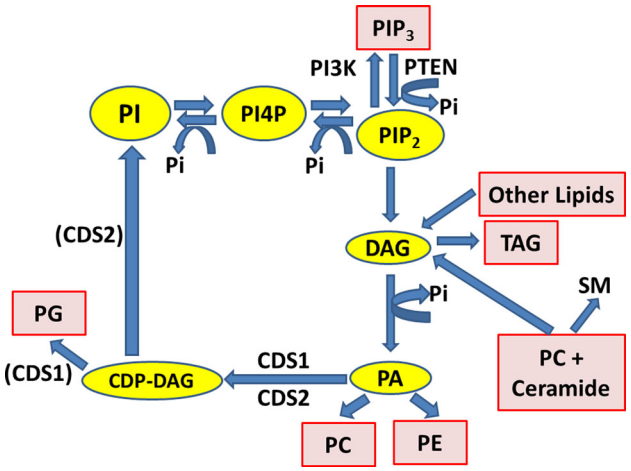

Fig. 3 Some of the metabolic processes outside the PI-cycle in which lipid intermediates of the cycle participate. Lipids that are intermediates in the PI-cycle are in *yellow ovals*, lipids made from intermediates in the PI-cycle or lipids forming these intermediates are shown in pink rectangles. *TAG* triacylglycerol, *SM* sphingomyelin. CDP-DAG is synthesized by both CDS1 and CDS2. We suggest that the CDP-DAG formed by CDS1 results in the formation of phosphatidylglycerol, while that formed by CDS2 leads to the synthesis of PI. CDS does not catalyze the formation of these lipids, but is shown in *brackets* to distinguish which CDS isoform catalyzes the previous step (Color figure online)

does not regenerate ATP. The lipid intermediate in the PI-cycle, CDP-DAG, is a precursor not only for the formation of PI but also for the formation of phosphatidylglycerol. In yeast CDP-DAG is also converted to phosphatidylserine. Many phosphorylated species of PI exist in the cell as lipid signaling agents. Only two of them, PI4P and PIP_2, are intermediates in the PI-cycle. There can be interconversion among PI and its phosphorylated forms through the action

of specific lipid kinases and phosphatases. Thus, in addition to the energy required to drive the whole PI-cycle, there can also be combinations of kinases and phosphatases that can catalyze the interconversion of PI or phosphorylated forms of PI with other more highly phosphorylated forms of PI. There is the possibility of futile cycling between any pair of phosphorylating and dephosphorylating reactions that will utilize additional ATP.

An important connection with the PI-cycle for cell proliferation and cancer is the phosphorylation of PIP_2 to PIP_3 by the enzyme phosphatidylinositol 3-kinase (PI3K) (Phosphatidylinositol-4,5-bisphosphate 3-kinase, EC:2.7.11.1) and the hydrolysis of PIP_3 to PIP_2 by the enzyme Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Phosphatidylinositol-4,5-bisphosphate 3-kinase, EC 3.1.3.16, EC:3.1.3.48, EC:3.1.3.67). The interconversion among PI and phosphorylated forms of PI both within the PI-cycle and with reactions connected to the cycle is an important and complex aspect of lipid signaling and will be discussed in the next section in relation to the PI-cycle.

Phosphorylation of PI

The entire group of phosphorylated PI, having varying numbers and positions of phosphorylation on the inositol ring are referred to as PIP_n. The concentration of PIP_n species outside the PI-cycle is very low and does not remove a large fraction of lipid from the cycle (Table 1). Many species of PIP_n play important roles in lipid signaling. Many forms of PIP_n bind to specific sites on proteins (Hammond and Balla 2015). The parent molecule, PI, represents 10–20 mol% of the total cellular phospholipid. It is synthesized in the endoplasmic reticulum via the CDS/PIS pathway, but its distribution among cellular membranes is not yet well established. In the PI-cycle, PI is first converted to PI4P. At steady state in a typical mammalian cell, PI4P constitutes only 2–5 % of the PI. PI4P is then converted in the PI-cycle to $PI(4,5)P_2$. $PI(4,5)P_2$ is known to be present almost exclusively in the plasma membrane. However, the enzyme PI4K that catalyzes the conversion of PI to PI4P is located mainly in the Golgi and endosomal compartments (Balla and Balla 2006). There are two classes of PI4K, Type II and Type III, with each of these having two different isofomrs, α and β (Delage et al. 2013). Types II and III PI4K differ from one another in their size, Km values, and sensitivity to inhibitors. The major fraction of the product of this reaction, PI4P, has recently been found in the plasma membrane (Hammond et al. 2009, 2014; Sarkes and Rameh 2010). An explanation for different subcellular location of PI4K and the products of its catalysis came when it was found that different isoforms of PI4K are responsible for making PI4P in the two organelles. PI4KA is present in the plasma membrane where it generates PI4P, while PI4KB is present in the Golgi (Godi et al. 1999). PI4P is not detected in the endoplasmic reticulum but the enzyme PI4KA is present in that organelle. It is possible that PI4P is located in the endoplasmic reticulum exit sites (Blumental-Perry et al. 2006). The other PIP_n in the PI-cycle is $PI(4,5)P_2$. It represents 2–5 % of the total PI. The level of $PI(4,5)P_2$ is maintained in spite of changes in the concentration of its precursor, PI4P (Bojjireddy et al. 2014; Hammond et al. 2012). This may be a result of increased movement of PI from the endoplasmic reticulum to the plasma membrane as a result of lipid exchange when the PI-cycle is activated (see discussion of lipid transfer, below). In addition to being hydrolyzed by PLC to form the secondary messengers DAG and inositol triphosphate, $PI(4,5)P_2$ also has other important roles in signal transduction as well as in the regulation of enzymatic activities, membrane transport, the actin cytoskeleton, and nuclear signaling (Delage et al. 2013). $PI(4,5)P_2$ affects the actin cytoskeleton by binding to specific actin-binding proteins (Moseley and Goode 2006; Saarikangas et al. 2010) as well as affecting membrane properties including membrane bending, fusion, fission, and affecting membrane trafficking and signaling (Ischebeck et al. 2010; Vicinanza et al. 2008). $PI(4,5)P_2$ regulates a number of ion channels and transporters which are essential for many signal transduction pathways (Rohacs 2009; Suh and Hille 2008; Suh et al. 2010; Yaradanakul et al. 2007).

There are also several other PIP_n that are present in very low amounts, generally only a few percent of the concentration of PI4P. Many of these PIP_n have roles in signal transduction (Balla 2013). We will not review all of the

Table 1 Cell content of PI and PIP_n

Lipid	% Relative to PI	Major cell location	PI-cycle intermediate
PI	100[a]	Not known	Yes
PI4P	2–5 %	Plasma membrane and Golgi apparatus	Yes
$PI(4,5)P_2$	2–5 %	Plasma membrane	Yes
$PI(3,4,5)P_3$	0.0002–0.0025 %	Plasma membrane	No

[a] By definition it is 100 %. Relative to other phospholipids, PI is 10–20 % of cell lipids

species of PIP_n, but will consider one of them that is in higher concentration. This lipid is $PI(3,4,5)P_3$, whose concentration in the cell is 1–5 % that of $PI(4,5)P_2$.

Because of their important roles in cell signaling, several mechanisms exist for the modulation of the activities of PI4K as well as PI4P5K. The activity of these enzymes is modulated by small G-proteins in response to extracellular signals (Balla and Balla 2006; Krauss and Haucke 2007; Santarius et al. 2006). There is also evidence for multimolecular complexes channeling substrate into particular pathways for end product formation (Balla et al. 2009 125/id; Lee et al. 2004 126/id). In addition to the kinases, lipid phosphatases exist that can reverse the direction of the metabolic flow. Both the kinases and the phosphatases also have their enzymatic activities regulated by post-translational protein phosphorylation and dephosphorylation. In addition, PI4P5K is activated by PA (Moritz et al. 1992; Jenkins and Frohman 2005). The acyl chain composition of the PA has a large effect on the extent of activation. There is a growing realization of the importance of the whole lipid molecule, including the acyl chains in the biological activity of certain lipids (Kimura et al. 2016). The presence of a saturated acyl chain at the sn-1 position of PA markedly lowers the extent of activation (Shulga et al. 2012). For example, dioleoyl phosphatidic acid is a good activator of PI4P5K, but 1-stearoyl-2-oleoyl phosphatidic acid hardly activates at all (Shulga et al. 2012), even though these two forms of PA differ by only one double bond.

Both PI4P and PA are intermediates in the PI-cycle. The major acyl chain composition of these lipids as intermediates in the PI-cycle in normal cells is 1-stearoyl-2-arachidonoyl. This provides the possibility that there could be positive feedback within the cycle, resulting in an increased rate of interconversions within the cycle (Oude Weernink et al. 2007). However, 1-stearoyl-2-arachidonoyl phosphatidic acid is not among the best activators of PI4P5K; diarachidonoyl phosphatidic acid is a much better activator (Shulga et al. 2012). Another indication that there is not a feedback regulation of the PI-cycle is that although the PA formed by DGKζ (Luo et al. 2004), DGKα (Jones et al. 2000) as well as by phospholipase D (PLD) (Pettitt et al. 2001) can all increase the activity of PI4P5K. The PA formed within the PI-cycle by DGKε catalysis, i.e., 1-stearoyl-2-arachidonoyl phosphatidic acid, does not produce a product that can activate PI4P5K (Jones et al. 2000). There may however be a positive augmentation in the rate of the PI-cycle by coupling with PLD. A product of the PI-cycle, $PI(4,5)P_2$, activates PLD (Hammond et al. 1997; Liscovitch et al. 1994) that generates PA. This PA product of PLD can then activate PI4P5K resulting in the feedback acceleration of the PI-cycle.

Increased PIP_3 in Cancer Cells

It has recently been shown that transformed mammalian cells contain little 1-stearoyl-2-arachidonoyl PI, but rather have species of PI with shorter and less unsaturated acyl chains compared with normal cells (Naguib et al. 2015). These transformed cells still have a normal content of PI but the specific species of PI are different. This demonstrates that the synthesis of PI can be altered so that other acyl chains besides 1-stearoyl-2-arachidonoyl can be incorporated into this lipid. There is also recent evidence that the positions of double bonds in acyl chains of the same molecular mass (isobaric) differ in cancer cells (Ma et al. 2016).

Cancer cells have more PIP_3 than normal cells. This is often a consequence of the mutation of PI3K, resulting in increased activity, as well as the inactivation or gene deletion of PTEN, the enzyme that catalyzes the hydrolysis of $PI(3,4,5)P_3$ to form $PI(4,5)P_2$. In addition, there is another mechanism, through the mutation of the transcription factor p53, that can result in higher levels of $PI(3,4,5)P_3$. Mutation of p53, which occurs in many cancer cells, results in an alteration of the acyl chain composition of several phospholipids. P53 is a repressor of the expression of stearoyl-CoA desaturase (Rueda-Rincon et al. 2015). Many cancer cells have inactivating mutations in p53 leading to an increased expression of stearoyl-CoA desaturase and an increased amount of monounsaturated phospholipid species (Igal 2011; Minville-Walz et al. 2010; Naguib et al. 2015; Rueda-Rincon et al. 2015). The rate determining step in the synthesis of PIP_2 is the phosphorylation of PI4P to $PI(4,5)P_2$ catalyzed by PI4P5K. There are three isoforms of PI4P5K, α, β, and γ. There is evidence that these isoforms may have different biological functions. PI4P5Kβ is involved in regulating the pool of $PI(4,5)P_2$ that controls store-operated Ca^{2+} channels, while PI4P5Kγ catalyzes the formation of inositol triphosphate (Calloway et al. 2011; Vasudevan et al. 2009). For all three isoforms of this enzyme, PI4P with no unsaturation is a very poor substrate compared with PI4P containing one or more double bonds (Shulga et al. 2012). Increased expression of p53 causes a shift of phospholipid acyl chains in the cell from those composed of two monounsaturated acyl chains to those with one or no unsaturation in the two acyl chains (Rueda-Rincon et al. 2015). The PI4P with fully saturated acyl chains will block the conversion of PI4P to $PI(4,5)P_2$ (Shulga et al. 2012). Thus, mutation of p53 in cancer cells causes an increase of stearoyl-CoA desaturase, in turn increasing the amount of phospholipids with two monounsaturated acyl chains, allowing more efficient conversion of PI4P to PIP_2 and thus higher substrate concentration for PI3K to catalyze the

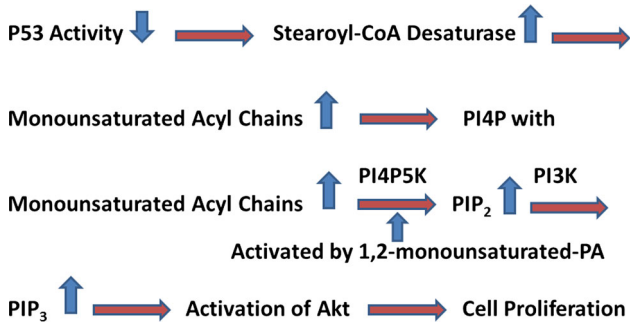

Fig. 4 Mechanism connecting reduced p53 activity to increased cell proliferation as a consequence of changes in the acyl chain composition of lipid intermediates of the PI-cycle. Progression of steps indicated by horizontal *red arrows*. *Vertical blue arrows* indicate that as a result of a loss of p53 activity, there is a resultant increase in a series of enzyme activities or lipid products (Color figure online)

formation of PIP_3. The resulting increase in PIP_3 affects oncogenesis through modulation of the activity of Akt. Akt is a protein kinase that is recruited to the cell membrane by interaction with PIP_3, resulting in the activation of Akt by phosphorylation. Increased activation of Akt mediates downstream responses that are typical of cancer cells including cell survival, growth, proliferation, and cell migration (Fig. 4). The increased synthesis of PIP_3 in cancer cells is indirectly a result of an increased level of the substrate PIP_2 caused by changes in the acyl chain composition of PI4P (Rueda-Rincon et al. 2015). In support of this relationship are the findings that inhibition of stearoyl-CoA desaturase decreases the activity of Akt (Scaglia and Igal 2008) and that the addition of monounsaturated oleic acid to cells reverses the p53-induced repression of Akt activation by phosphorylation (Rueda-Rincon et al. 2015).

Acyl Chain Composition of Other Lipid Intermediates of the Phosphatidylinositol Cycle

The above discussion demonstrates the importance of the acyl chain composition of PIP_n in determining the rate of formation of PIP_3. The acyl chain composition of all of the lipid intermediates of the PI-cycle in non-transformed mammalian cells is predominantly 1-stearoyl-2-arachidonoyl. Some of the steps contributing to the enrichment of these lipids with 1-stearoyl-2-arachidonoyl chains in normal mammalian cells have been identified. It is at least in part, the result of the acyl chain dependence of substrate specificity of two enzymes of the PI-cycle. One of these enzymes is the epsilon isoform of DGK (DGKε). There are 10 isoforms of DGK, in addition to gene splicing variants in mammals, each having its own function, subcellular localization and organ distribution (Shulga et al. 2011). Among all of these variants of DGK, only one form exhibits specificity for DAG substrates having an arachidonoyl chain at the sn-2 position (Rodriguez de Turco et al. 2001; Milne et al. 2008; Tang et al. 1996) as well as a stearoyl chain at the sn-1 position (Lung et al. 2009). Other isoforms of DGK have similar activities against most DAG species, independent of their acyl chains. Another enzyme that exhibits specificity for a 1-stearoyl-2-arachidonoyl lipid substrate is CDS2. CDS has only two isoforms in mammals; CDS1 and CDS2. There is a marked contrast between the acyl chain specificities of these two isoforms. Only CDS2 is highly specific for 1-stearoyl-2-arachidonoyl phosphatidic acid, while CDS1 shows almost no effect of the acyl chain composition of the substrate on enzymatic activity (D'Souza et al. 2014).

The PI-cycle intermediate, CDP-DAG is a precursor for the synthesis of phosphatidylglycerol as well as PI. Unlike PI, phosphatidylglycerol is not enriched in 1-stearoyl-2-arachidonoyl, suggesting that CDS1 may be responsible for the synthesis of that lipid and CDS2 would pass the CDP-DAG product to PIS for the synthesis of PI. In the brain, an organ having a relatively high level of expression of DGKε, the arachidonoyl content in the sn-2 position of CDP-DAG, is 44.6 % while that for PI in this organ is 62.6 % (Thompson and MacDonald 1976). This increase in arachidonoyl content is not a result of the enzyme specificity in the last step of PI synthesis, catalyzed by PIS, since that enzyme shows no acyl chain specificity (D'Souza and Epand 2015). Remodeling of PI by the Lands cycle (see below) could contribute to the additional acyl chain enrichment. It is also possible that there is a direct transfer of 1-stearoyl-2-arachidonoyl-CDP-DAG from CDS2 to PIS, while other species of CDP-DAG are used for the synthesis of phosphatidylglycerol (Weeks et al. 1997) through the action of CDS1 (Fig. 3).

CDP-DAG is also a precursor for cardiolipin that is synthesized and located in the mitochondria. It is not known if a mitochondrial CDS1 or a mammalian homolog of the yeast enzyme Tam41 (Tamura et al. 2013) or a combination of these paths is involved in cardiolipin synthesis.

Although the major fraction of PI is the 1-stearoyl-2-arachidonoyl species, there is a minor fraction of PI (~25 %) that has other acyl chains. Transformed cells contain little of 1-stearoyl-2-arachidonoyl PI (Naguib et al. 2015). Thus there must also be pathways to synthesize PI with other acyl chain compositions. The simplest explanation for the incorporation of other acyl chains into PI is that other isoforms of DGK, besides DGKε and CDS1 in addition to CDS2 contributes to the respective steps in the PI-cycle. These other isoforms exhibit little or no acyl chain specificity for their substrates. The molecular mechanism controlling the relative contributions of these other isoforms to the synthesis of PI is yet to be determined.

Compartmentalization

There is a requirement for substrate channeling or compartmentalization in order to explain the observation that although there are many molecular species of PA in the cell, predominantly one of these species, the 1-stearoyl-2-arachidonoyl-PA, is normally converted into PI. Most cells express both CDS1 and CDS2. Why does so little of other molecular species of PA normally end up as PI with different acyl chains? Similarly, if CDS2 contributed to the synthesis of phosphatidylglycerol, why does this phospholipid not contain more of the 1-stearoyl-2-arachidonoyl-PA species?

Neither total cellular DAG nor PA is highly enriched with arachidonoyl-containing forms (Milne et al. 2008). This is because these lipids are not confined to the PI-cycle, but are involved in other pathways of lipid metabolism. Another question is how is the substrate selectivity of DGKε for acyl chains is maintained in the next step of the PI-cycle, catalyzed by CDS? If the 1-stearoyl-2-arachidonoyl phosphatidic acid synthesized by DGKε mixed with the large excess of PA from other sources in the cell, the enrichment with particular acyl chains through catalysis by DGKε would be lost. Hence there must be some segregation of the lipid intermediates of the PI-cycle from other lipids in the cell, either by physical isolation in different cellular structures or in membrane domains or by the formation of multiprotein complexes containing different enzymes of the PI-cycle. There has to be some mechanism to have the product of one step in the PI-cycle passed on directly to the enzyme catalyzing the next step, without allowing mixing with other species of the PI-cycle. There is also the feature that this is a cycle, so that each time the PI-cycle goes around, the lipid intermediates can become progressively enriched with specific acyl chains as a result of the substrate specificities of some of the enzymes in the cycle, provided that the cycle is largely isolated from other lipids of the cell. Additional factors contributing to the extent of acyl chain enrichment in PI are the levels of expression of DGKε and CDS2. DGKε is found mostly in the brain (Kohyama-Koganeya et al. 1997) but CDS2 is very widely expressed. In addition, there is remodeling of the PI that is formed (see below). In mammalian organs other than brain, in which the expression levels of DGKε are low, PI is still found to be highly enriched with the 1-stearoyl-2-arachidonoyl species. A contributing factor to determine which acyl chains are incorporated into PI may be compartmentalization. This is supported by the findings that using special resolution imaging mass spectrometry, both breast cancer tissue (Kawashima et al. 2013) as well as tissue from prostate cancer patients (Goto et al. 2014) have PI species with altered acyl chain compositions that are spatially clustered.

CDP-DAG is synthesized within the PI-cycle. In normal mammalian cells this lipid is already significantly enriched in the 1-stearoyl-2-arachidonoyl species (Thompson and MacDonald 1976), even though remodeling does not occur until PI is synthesized. This suggests that most of the CDP-DAG is synthesized via the reaction catalyzed by CDS2. If any CDP-DAG is made using CDS1, this CDP-DAG, without acyl chain enrichment, must be rapidly converted to other phospholipids so that it does not form a major fraction of the CDP-DAG intermediate. The species of CDP-DAG enriched in 1-stearoyl-2-arachidonoyl chains would be converted to PI by PIS, maintaining the high enrichment of 1-stearoyl-2-arachidonoyl acyl chains.

Non-Vesicular Lipid Transfer

As discussed above, the PI-cycle can only be completed if there is transfer of lipid intermediates of the cycle between the endoplasmic reticulum and the plasma membrane. The protein Nir2 has been shown to transfer PI between membranes in vitro (Garner et al. 2012). In addition to being required to complete the PI-cycle, it is possible that some of the lipid transfer processes preferentially transfer lipids with specific acyl chain compositions. The acyl chain specificity of this exchange has not yet been evaluated.

It has recently been found that Nir2 facilitates the exchange of PI in the endoplasmic reticulum for PA in the plasma membrane (Chang and Liou 2015; Kim et al. 2013, 2015). This exchange can account for the rapid replenishment of PIP_2 subsequent to its hydrolysis on the stimulation of PLC (Chang and Liou 2015; Kim et al. 2015). Another reason that the concentration of PIP_2 remains constant is because it is part of a metabolic cycle, the PI-cycle. Intermediates of a metabolic cycle are maintained at a constant level and are neither increased nor decreased in amount. This is particularly true for PIP_2 that is found exclusively in the plasma membrane where it is likely to be a part of the PI-cycle. However, PI4P is found in many subcellular compartments, not all of which are directly associated with the PI-cycle. This could explain the observation that the concentration of PI4P varies to a greater extent than that of PIP_2. For lipids that are part of the PI-cycle, each intermediate is buffered by other intermediates of the cycle which can all be interconverted through the cycle. When the cycle is stimulated, as for example by the activation of PLC, there can be a brief rise in the concentration of certain intermediates of the PI-cycle, but levels promptly return to the steady-state levels. However, when the cycle is broken, as for example by eliminating exchange of PA and PI between the endoplasmic reticulum and the plasma membrane, the

concentration of intermediates of the cycle markedly fall for a more prolonged period of time.

Binding of Nir2 to the endoplasmic reticulum is also associated with another protein of the endoplasmic reticulum, VAP-B (Kim et al. 2015). VAP-B expression increased the association of Nir2 to the endoplasmic reticulum. Furthermore after stimulation, Nir2 and VAP-B showed colocalization in contact sites between the plasma membrane and endoplasmic reticulum. In addition, Nir2, that is difficult to detect by fluorescence microscopy in resting cells, rapidly forms puncta that localize between the endoplasmic reticulum and the plasma membrane, colocalizing with CDS2 after stimulation (Kim et al. 2015). These results provide evidence that both Nir2 and CDS2 are closely associated with the PI-cycle. There is also another isoform, Nir3, which is a homolog of Nir2. These two proteins appear to be related, but somewhat play different roles. While Nir2 functions to transfer PI so as to maintain PIP_2 signaling capacity in the plasma membrane, Nir3 maintains the basal level of PIP_2 in the plasma membrane in the resting state (Chang and Liou 2015). Both Nir2 and Nir3 are sensors for PA formed from the hydrolysis of PIP_2 and the phosphorylation of the resulting DAG by DGK. Of the two Nir proteins, Nir_3 has a stronger binding affinity for PA and may serve to maintain the basal level of PIP_2. By contrast, Nir_2 has a weaker translocation to the endoplasmic reticulum–plasma membrane junction following receptor stimulation and appears to have a higher capacity to transfer PI, while at the same time avoiding the formation of excess PIP_2 at the plasma membrane. The importance of PI transfer from the endoplasmic reticulum membrane to the plasma membrane to enable continued PIP_2 signaling is shown by the observation that depletion of PI at the plasma membrane results in defective PIP_2 replenishment at the plasma membrane. However, initial signaling from PIP_2 at the plasma membrane can occur even after acute depletion of PI from the endoplasmic reticulum. It is suggested that PIP_2 can be regenerated from a small pool of precursors in the plasma membrane but this regeneration of PIP_2 cannot be sustained without the presence of the Nir proteins. These studies also suggest that both Nir2 and Nir3 function by binding PA and translocating to the endoplasmic reticulum–plasma membrane contact sites, where they can facilitate the exchange of PA and PI. Thus receptor activation of PLC to hydrolyze PIP_2 will produce PA that can then activate the movement of the Nir proteins to be non-vesicular lipid exchange proteins. It is possible that one of the Nir proteins functions to transfer lipids with 1-stearoyl-2-arachidonoyl chains, while the other is not specific for the acyl chain, as we have seen with other steps of the PI-cycle catalyzed by more than one isoform of the enzyme involved. Since Nir2 maintains the basal level of PIP_2 in the plasma membrane, we suggest that it is the isoform specific for binding 1-stearoyl-2-arachidonoyl-PA. Its lower binding affinity may be a consequence of non-optimal species of PA being used for the binding studies.

It has been shown that the molecular species of PI and PA are similar in the endoplasmic reticulum and in the plasma membrane. However, the PI:PA ratio is >1 for 34:2, 36:1, 38:3, and 38:4, while for most other species it is <1 (Shulga et al. 2010). This demonstrates a selective enrichment of PI with 1-stearoyl-2-arachidonoyl (38:4) and related acyl chains from its precursor PA. There are also short chain PA species 30:1 and 30:0 making up 21 % of the PA in the endoplasmic reticulum, but apparently they do not get converted to PI since no PI with this acyl chain composition is found (Shulga et al. 2010). Furthermore, these species of PA are twice as abundant in the endoplasmic reticulum as in the plasma membrane, suggesting that not only they are not metabolized to PI, but they also are not as likely to be exchanged between the endoplasmic reticulum and the plasma membrane. It is possible that they are required to stabilize regions of high curvature in the endoplasmic reticulum. There are also species of PI for which there is no corresponding PA (Shulga et al. 2010). These PI species are found equally prevalent in the endoplasmic reticulum and in the plasma membrane. The most abundant of these species of PI is 38:5 PI that comprises about 17 % of the PI of both the endoplasmic reticulum and the plasma membrane. 38:5 PI is quite similar to the most abundant 1-stearoyl-2-arachidonoyl (38:4) species, but apparently is not acted on by PLC. These results also suggest that these species of PI for which there is no corresponding PA are readily exchanged between the two membranes. The endoplasmic reticulum is the site of synthesis of PI, so that lipid would be present in the endoplasmic reticulum for exchange with PA in the plasma membrane. In order to form PA in the plasma membrane as part of the PI-cycle, to complete the lipid exchange, would require the presence of DGK in the plasma membrane. There are several isoforms of DGK that partition to the plasma membrane, in some cases after stimulation (Shulga et al. 2011). However, for the acyl chain-specific formation of 1-stearoyl-2-arachidonoyl phosphatidic acid, the DGK isoform has to be DGKε. Most of the DGKε is found in the endoplasmic reticulum (Kobayashi et al. 2007; Matsui et al. 2014), although there is evidence for some of this isoform to be in the plasma membrane (Decaffmeyer et al. 2008). These findings would suggest that DGKε in the endoplasmic reticulum might function as a reservoir form to be transferred to the plasma membrane upon stimulation of PLC. An observation linking DGKε with the fraction of PA that participates in the in the PI-cycle is the finding that the content of PA in the plasma membrane is decreased by 3-fold in mouse embryo fibroblasts knocked out for DGKε

(Shulga et al. 2010). This could be explained by the removal of DGKε disrupting the PI-cycle and the generation of PA in the plasma membrane. This change in lipid composition in the plasma membrane occurs despite the fact that the total cellular PA is unaffected in these cells (Milne et al. 2008).

Alternatively, in addition to the PA-PI transfer process there may also be some DAG that is transferred from the plasma membrane to the endoplasmic reticulum where it is phosphorylated by DGKε. In addition to the lipid transfer from the plasma membrane to the endoplasmic reticulum, there also has to be lipid transfer in the opposite direction to complete the PI-cycle. With the Nir proteins the reverse lipid movements are coupled by the lipid exchange process. PI synthesized in the endoplasmic reticulum is exchanged for PA in the plasma membrane. There may also be other ways to deliver PI to the plasma membrane. There are PI-specific lipid transfer proteins that could perform this function (Cockcroft and Garner 2011; Routt and Bankaitis 2004). It has also been shown that in cells that overexpress PIS, highly mobile membrane compartments form at the endoplasmic reticulum that can deliver PI to various membranes by vesicular transport (Kim et al. 2011). There are also contact sites between the endoplasmic reticulum and plasma membrane through which lipids can be transferred (Henne et al. 2015). At these sites, the movement of PI from the endoplasmic reticulum to the plasma membrane can be balanced by movement of DAG and/or PA in the opposite direction.

It has been shown that the lipid transfer protein Nir2 enhances the epithelial–mesenchymal transition and facilitates breast cancer metastasis mediated through the PI3K/Akt pathway (Keinan et al. 2014). We discussed above how the acyl chain composition of PI could affect cancer progression by affecting the phosphorylation of Akt. Nir2 may have a role in this process by completing the PI-cycle and allowing more rapid formation of PIP_2, the substrate of PI3K to form PIP_3 and activate Akt (final steps in Fig. 4).

Lipid Remodeling

All phospholipids undergo acyl chain remodeling through the actions of acyl transferases and phospholipases. This process is collectively known as the Land's cycle (Lands 1958, 1960; Lands and Merkl 1963; Lands et al. 1982). Remodeling allows certain lipids to attain a specific acyl chain composition, which is important for signaling functions. Several classes of enzymes that are needed for remodeling have been characterized. Lysophospholipid acyltransferases are enzymes that transfer an acyl group from acyl-CoA to a lysophospholipid (Matsuda et al. 2008). Other enzymes required for remodeling include the phospholipase A1 and A2 families, which cleave acyl chains off the phospholipid to produce the lysophospholipid that can accept an acyl chain from an acyl-CoA (Aoki et al. 2002; Puttmann et al. 1993). The Land's cycle could result in acyl chain enrichment through the selective incorporation and/or removal of acyl chains.

There is a specific mechanism for enriching PI with arachidonoyl chains. Lysophosphatidylinositol acyltransferase 1 (LPIAT1), also known as membrane bound *O*-acyltransferase containing domain 7 (also referred to as MBOAT7), catalyzes the transfer of an acyl group from acyl-CoA to lysoPI (Gijon et al. 2008). LPIAT1 has a high specificity for arachidonoyl-CoA (Gijon et al. 2008). Thus, LPIAT1's arachidonoyl specificity contributes to the enrichment of PI with an arachidonoyl chain (Fig. 5).

Knocking out LPIAT1in mice with the same genetic background (C57/B16) was found independently by two groups to significantly decrease arachidonoyl-containing PI, and $PI(4,5)P_2$ (Anderson et al. 2013; Lee et al. 2012). LPIAT1 is critical for neural development of mice; $LPIAT^{-/-}$ mice were viable up to 30 days after birth, but exhibited a smaller, atrophied cerebral cortex, and hippocampus. The laminal structure of the neocortex was also disordered due to delayed neural migration, which indicated a role for LPIAT1 in cortical lamination (Lee et al. 2012).

Other lipid products can form arachidonic acid that is released from the sn-2 position of lipid intermediates in the PI-cycle by the action of phospholipase A2. The arachidonic acid released can be further metabolized to form signaling eicosanoids. Another lipid signaling product could be formed by the hydrolysis of the stearoyl group of 1-stearoyl-2-arachidonoyl glycerol by phospholipase A1.

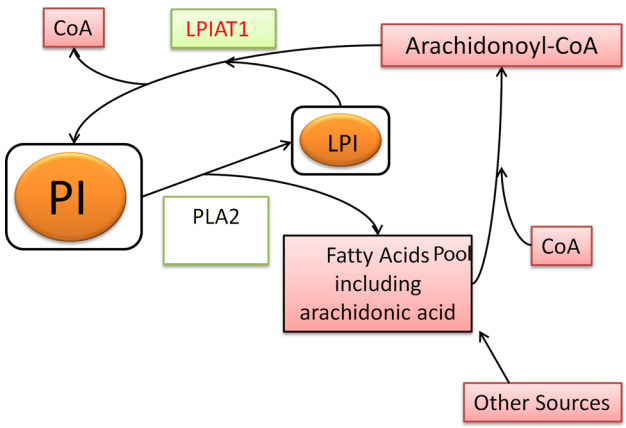

Fig. 5 The Land's cycle of acyl chain remodeling of PI. The acyl chains of PI can be remodeled through phospholipases (PLA2) and acyltransferases (LPIAT1). PLA2 exhibits little acyl chain specificity, while LPIAT1 is highly specific for transferring an acyl chain from arachidonoyl-CoA to lysophosphatidylinositol (LPI). The combination of these two steps results in the enrichment of PI with arachidonoyl chains at the sn-2 position

The resulting product of this reaction is 2-arachidonoyl glycerol that is an endogenous endocannabinoid ligand. The resulting 2-arachidonoyl glycerol can no longer efficiently enter the PI-cycle as a lysophosphatidic acid (Gantayet et al. 2011). In addition to remodeling with an arachidonoyl group at the sn-2 position, the sn-1 position is also remodeled with a stearoyl group, catalyzed by the enzyme that was originally named lysocardiolipin acyl transferase (Imae et al. 2012).

Roles of the Acyl Chains of PI-Cycle Intermediates and Lipid Signaling

The predominant molecular species of $PI(4,5)P_2$ has 1-stearoyl-2-arachidonoyl acyl chains in normal mammalian cells and tissues. $PI(4,5)P_2$ has many functions, making it complex to assess the role of acyl chains in the signaling by $PI(4,5)P_2$. One role of $PI(4,5)P_2$ is as a precursor of $PI(3,4,5)P_3$ that is important for the activation of Akt. The rate of formation of $PI(4,5)P_2$ and $PI(3,4,5)P_3$ is acyl chain dependent as described above. $PI(4,5)P_2$ plays a major role in endocytosis in the synapse (Haucke 2005). To end this process, synaptojanin-1 dephosphorylates $PI(4,5)P_2$ (Wenk and De Camilli 2004). In vitro studies have shown that natural $PI(4,5)P_2$ (largely the 1-stearoyl-2-arachidonoyl form) is hydrolyzed more rapidly than the dipalmitoyl form of $PI(4,5)P_2$ (Schmid et al. 2004), demonstrating a role for the acyl chains in modulating this process.

In addition to $PI(4,5)P_2$, the PI-cycle also includes other lipid signaling intermediates such as DAG and PA. There are two major signaling routes for the formation of DAG and PA that are activated by the stimulation of cells with certain agonists. One is the hydrolysis of PI by PLC to produce DAG, as part of the PI-cycle (Fig. 1). The DAG formed by PLC hydrolysis uses predominantly inositol phospholipids as substrate and produces DAG enriched with 18:0/20:4 and 18:0/20:3 species (Pettitt and Wakelam 1993). This DAG can be converted to PA by most mammalian DGK isoforms, including DGKε, the isoform that has specificity for 1-stearoyl-2-arachidonoyl glycerol (D'Souza and Epand 2014). The other pathway for the formation of PA is by PLD hydrolysis of phospholipids, mostly phosphatidylcholine. There may be a crosstalk between the two pathways since $PI(4,5)P_2$ from the PI-cycle activates PLD and PA from the PLD pathway activates PI4P5K, an enzyme of the PI-cycle, to form more PIP_2. The PA can also be hydrolyzed by phosphatidate phosphohydrolase to produce DAG. The DAG species produced from PC through this pathway has distinct acyl chain compositions compared with the DAG produced from $PI(4,5)P_2$ (Madani et al. 2001; Pettitt et al. 1997). It has been shown that in endothelial cells, activation of PLD by lysophosphatidic acid produces DAG that does not activate protein kinase C (PKC) (Pettitt et al. 1997).

In vitro studies of PKC activation with DAG confirms that the nature of the acyl chains on DAG determines its potency in stimulating PKC (Marignani et al. 1996; Madani et al. 2001). Sustained PKC activation has been shown to be oncogenic and contributes to malignant phenotypes seen in cancers (Koivunen et al. 2006; Rajotte et al. 1992). PKC-βII is maximally activated by 1-stearoyl-2-arachidonoyl glycerol (Deacon et al. 2002) and expression of this PKC isoform is required for the proliferation of human leukemic cells (Thompson and Fields 1996). PKC-βII is targeted to the nucleus during the G2/M phase of the cell cycle (Deacon et al. 2002). The translocation of PKC-βII to the nucleus during the G2/M is triggered in part by the buildup of 1-stearoyl-2-arachidonoyl glycerol in the nucleus (Deacon et al. 2002). This DAG species arises from the hydrolysis of $PI(4,5)P_2$ in the nucleus (Cocco et al. 1987; Irvine and Divecha 1992) as part of the PI-cycle that is also known to be present in the nucleus, independently of the cycle occurring in the endoplasmic reticulum/plasma membrane (Cocco et al. 1989).

Other evidence of acyl chain specificity of DAG comes from recent studies of the activation of caged DAGs with different acyl chains (Nadler et al. 2013). It was demonstrated that the photoactivation to liberate 1-stearoyl-2-arachidonoyl glycerol, and not other DAGs, resulted in a massive increase of intracellular Ca^{2+} levels. This can be understood in terms of the intermediates of the PI-cycle having a catalytic role as well as the fact that a product of the cycle is inositol triphosphate that is a ligand capable of opening Ca^{2+} channels in the endoplasmic reticulum. Thus, these experiments tie in specific acyl chains of DAG with signaling resulting from the PI-cycle.

Another signaling lipid intermediate of the PI-cycle is PA. PA plays an important role in activating PI4P5K with some dependence on its acyl chain composition (Shulga et al. 2012). PA also activates a protein kinase in platelets (Khan et al. 1994); PA has modulatory effects on membrane fusion (Rogasevskaia and Coorssen 2015); PA regulates the formation of actin stress fibers (Cross et al. 1996). Less is known about the influence of the acyl chains of PA on its various functions. Recently it has been shown that PA binds to the transcriptional repressor Opi1 in a manner that distinguishes between PA with a C16 versus a C18 acyl chain (Hofbauer et al. 2014). In addition, the activation of DNA synthesis by lysophosphatidic acid is dependent on the acyl chain composition of this lipid (van Corven et al. 1992).

Conclusions

The PI-cycle has particular properties as a consequence of the fact that it is a cycle so that all of the lipid intermediates of the cycle are regenerated and the intermediates can act

as catalysts. The PI-cycle consumes a large amount of energy and can thus proceed only in one direction. The PI-cycle is the major pathway for the synthesis of PI from which the signaling PIP_n lipids are derived. In addition, some lipid intermediates of the cycle are also important in signaling, including DAG and PA. The PI-cycle requires transfer of lipid to and from the endoplasmic reticulum and the plasma membrane. This is facilitated by the Nir proteins that exchange PI formed in the endoplasmic reticulum with PA formed in the plasma membrane. The substrate specificity of two enzymes in the PI-cycle contributes to the enrichment of PI with 1-stearoyl-2-arachidonoyl acyl chains in normal mammalian cells. Some of the intermediates in the PI-cycle are common to other lipid metabolic pathways. In order for the acyl chain composition to be maintained by the intermediates of the cycle, they must be isolated from the same lipids having different acyl chains. This suggests that the PI-cycle is largely isolated from the lipid metabolic processes occurring outside the cycle. However, at least 15 % of PI in these cells has other acyl chains and with cancer cells, as a consequence of alterations in lipid metabolism, PI is even less enriched with the 1-stearoyl-2-arachidonoyl acyl species. The arachidonoyl content of PI in transformed cells is very low, yet the same amount of PI is still synthesized. This suggests that there can be alternative isoforms of DGK and CDS that allow the completion of the PI-cycle.

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