Topological organisation of the phosphatidylinositol (4,5) bisphosphate-phospholipase C resynthesis cycle: PITPs bridge the ER-PM GAP

Shamshad Cockcroft and Padinjat Raghu*

Dept. of Neuroscience, Physiology and Pharmacology, Division of Biosciences, University College London, London WC1E 6JJ, UK;

*National Centre for Biological Sciences, TIFR-GKVK Campus, Bellary Road, Bangalore 560065, India

Address correspondence to: Shamshad Cockcroft, University College London UK; Phone: 0044-20-7679-6259; Email: s.cockcroft@ucl.ac.uk

Abstract
Phospholipase C (PLC) is a receptor-regulated enzyme that hydrolyses phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) at the plasma membrane (PM) triggering three biochemical consequences, the generation of soluble inositol 1,4,5-trisphosphate (IP_3), membrane–associated diacylglycerol (DG) and the consumption of plasma membrane $PI(4,5)P_2$. Each of these three signals triggers multiple molecular processes impacting key cellular properties. The activation of PLC also triggers a sequence of biochemical reactions, collectively referred to as the $PI(4,5)P_2$ cycle that culminates in the resynthesis of this lipid. The biochemical intermediates of this cycle and the enzymes that mediate these reactions are topologically distributed across two membrane compartments, the PM and the endoplasmic reticulum (ER). At the plasma membrane, the DG formed during PLC activation is rapidly converted to phosphatidic acid (PA) that needs to be transported to the ER where the machinery for its conversion into PI is localised. Conversely, PI from the ER needs to be rapidly transferred to the plasma membrane where it can be phosphorylated by lipid kinases to regenerate $PI(4,5)P_2$. Thus two lipid transport steps between membrane compartments through the cytosol are required for the replenishment of $PI(4,5)P_2$ at the PM. Here, we review the topological constraints in the $PI(4,5)P_2$ cycle and current understanding how these constraints are overcome during PLC signalling. In particular, we discuss the role of lipid transfer proteins in this process. Recent findings on the biochemical properties of a membrane-associated lipid transfer protein of the PITP family, PITPNM proteins (alt. name RdgBα/Nir proteins) that localize to membrane contact sites is discussed. Studies in both *Drosophila* and mammalian cells converge to provide a resolution to the conundrum of reciprocal transfer of PA and PI during PLC signalling.

Short Title:
Lipid transfer by PITPNM during PLC signalling

Key Words:
Phosphatidylinositol; PITP; RdgB; Phospholipase C; $PI(4,5)P_2$; phototransduction; phosphatidic acid; PA transport proteins: ER-PM membrane contact sites;

Abbreviations:
Phosphatidylinositol(4,5)bisphosphate, PI(4,5)P_2,
Plasma membrane, PM;
Endoplasmic reticulum, ER;
VAMP associated proteins, VAP;
Vesicle-associated membrane protein, VAMP;
Diacylglycerol, DG;
Phospholipase C, PLC;
Phosphoinositide 3-kinase, PI3K;
Inositol(1,4,5)P_3, IP_3;
Phosphatidic acid, PA;
Phosphatidylinositol, PI;
Phosphatidylcholine, PC;
Phospholipase D, PLD;
Membrane contact sites, MCS;
G-protein-coupled receptor, GPCR;
Sub-microvillar cisternae, SMC;

Introduction

Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) is the most versatile inositol-containing phospholipid enriched at the inner leaflet of the plasma membrane (PM). $PI(4,5)P_2$ levels govern many cellular functions including endocytosis, ion channels and transporter activity and cytoskeleton-PM interactions [1]. Additionally, $PI(4,5)P_2$ is a substrate for two signalling pathways, phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K). Both are ubiquitous cell signalling pathways that can be activated by agonists acting on appropriate cell surface receptors. Both G-protein-coupled receptors and receptor tyrosine kinases can regulate PLC activity. In addition, both pathways can also be activated by small GTPases and some PLCs by a rise in cytosolic Ca^{2+}. Hydrolysis of $PI(4,5)P_2$ by PLC generates two second messengers, diacylglycerol (DG) and inositol(1,4,5)trisphosphate (IP_3). A secondary consequence of PLC activation is a transient reduction in $PI(4,5)P_2$ levels and this can have a profound impact on cellular functions which depend on the intact lipid. Thus mechanisms have to be in place to replenish $PI(4,5)P_2$ levels during cell signalling. This review focuses on these potential mechanisms.

Phospholipase C-mediated signalling has a long history and the first papers were published in the 1950s when it was noted that activation of cells with appropriate agonists caused an increase in the labelling of phosphatidylinositol (PI) and phosphatidic acid (PA) when cells had been pre-incubated with $^{32}P_i$ to label the ATP pool. This stimulated increase in PA and PI labelling was a ubiquitous phenomenon observed in a wide range of cell types (see Fig. 1). The time-course of PA and PI labelling indicated a product precursor relationship whereby PA was converted into PI and by 1964, the metabolic interconversion between PA and PI was established and became known as the 'PI cycle' (Fig. 1A) [2]. In 1975, it was proposed that the 'PI cycle' stimulated by agonist receptor interactions was responsible for causing the increase in cytosol Ca^{2+} [3]. Although much of the data fitted into this paradigm, there were clearly situations where Ca^{2+} was able to activate the 'PI cycle' [4]. The issue about Ca^{2+} and PLC activation was ultimately resolved when it became clear that there are multiple PLC isozymes with different characteristics. The 'PI cycle' was later modified to include $PI(4,5)P_2$ as the cellular substrate for the PLC rather than PI and is referred here as the '$PI(4,5)P_2$ cycle'. PI became an intermediate in the pathway and was converted into $PI(4,5)P_2$ by two lipid kinases, PI 4-kinase and PIP 5-kinase (Fig. 1B). The discovery that IP_3 was capable of mobilising Ca^{2+} from intracellular stores secured the link between the $PI(4,5)P_2$ cycle and changes in cytosol Ca^{2+} [5]. The subsequent discovery of the IP_3 receptor at the endoplasmic reticulum finally established the '$PI(4,5P_2$ cycle' as an important signal transduction system. In addition to generating IP_3, the other product of PLC activation is DG. This lipid was also identified as a second messenger; it activates several proteins including protein kinase C by interactions with C1 domains. Thus PLC activation became established as a signal transduction system at par with adenylyl cyclase-cAMP second messenger system. It has subsequently become clear that the receptor activated $PI(4,5)P_2$ cycle is a conserved feature of most metazoan cells.

An outstanding question that was highlighted in a review in 1973 and again in 1975 was the issue of lipid transfer between the plasma membrane and the endoplasmic reticulum. PLC-mediated $PI(4,5)P_2$ hydrolysis was confined to the PM but the machinery to resynthesise PI was present at the endoplasmic reticulum (Fig. 1B) [3;6]. In 1974, a cytosolic activity had been identified that was able to transport PI between membrane compartments and the concept arose that lipids could be moved by 'lipid transfer proteins' (LTPs) [7]. The identification of soluble PITPα as an exchange protein that could bind and transfer PI and phosphatidylcholine (PC) and subsequently shown to support IP_3 production in permeabilised

cells established the principle that PITPs participated in lipid transfer during PLC signalling [8;9]. However, the outstanding issue for PA transport from the PM (where it is produced by the sequential action of PLC and DGK) to the ER (where it is channelled into PI resynthesis) remained unclear until recently. In 2012, we identified that some members of the PITP family, namely the RdgB proteins were able to bind and transfer PA in addition to PI [10]. This was the first example of a PA transfer protein. Previous experiments conducted in permeabilised cells where the PI resynthesis cycle was reconstituted indicated that PA transfer did not require cytosolic proteins or vesicular transport and it was suggested that PA transfer occurs at membrane contact sites [11]. Here we highlight the journey that has led to the identification of a class of PITPs (PITPNM/RdgB proteins) that can exchange PA for PI between the ER and the PM to complete the '$PI(4,5)P_2$ cycle'. This journey has taken 40 years.

To a large extent, studies of the $PI(4,5)P_2$ cycle have been performed in cell culture models. Although studies conducted in cell-lines where the individual components can be over-expressed or knocked out have provided fundamental information about PLC signalling, there are limitations. Studies in cultured cell-lines have relied on over-expression of multiple proteins simultaneously including receptors to enhance PLC signalling, PITPNM proteins and probes targeted to specific membranes to monitor changes in lipids. In these studies, the PLC signalling pathway has been hyper-stimulated and moreover, do not take into consideration the expression levels of endogenous PITP proteins or receptors. Nonetheless, these studies provide a valuable insight into the PLC signalling pathway. The human body contains approximately 200 different cell-types that are specialised to perform specific functions. More importantly, the protein composition as well as protein abundance varies depending on cell type. Thus it is critical to examine PLC signalling in the context of a specific cell function. The study by Kruse et al [12] highlights the importance of studying PLC signalling under physiological conditions.

The *Drosophila* phototransduction cascade remains one of the best characterized systems where the application of genetics coupled with measurements of the physiological output i.e. light response provides context to PLC signalling [13-16]. In these cells photon absorption by rhodopsin is transduced into opening of two classes of Ca^{2+}-permeable ion channels, TRP and TRPL [13]. This transduction pathway requires Gq-mediated PLC activation leading to $PI(4,5)P_2$ hydrolysis. Several generic elements of the '$PI(4,5)P_2$ cycle' including the G-protein coupled receptor (rhodopsin encoded by *ninaE*), heterotrimeric G-protein (Gq), phospholipase Cβ4 (PLCβ4 encoded by *norpA*), DG kinase (DGK encoded by *rdgA*), CDS (*cds*), PI synthase (*pis*) and PITPNM (encoded by *rdgB*) and PIP5K [reviewed in [13]] have been identified and characterized in fly photoreceptors. The ability to combine genetics with measurements of the physiological outcome has made *Drosophila* photoreceptors a valuable tool to dissect the questions related to the $PI(4,5)P_2$ cycle. Thus we will draw upon this system as an exemplar in our review and in many instances, provide a comparative analysis between studies in mammalian cell lines and *Drosophila* photoreceptors.

Biochemistry of the PI cycle

PI constitutes about 5-7 % of the total cellular phospholipids in mammalian cells. *De novo* synthesis of PI occurs at the ER from PA (see Fig. 1B). PA is synthesised by the acylation of glycerol-3-P (G3P) to lysophosphatidic acid (LPA) and a second acylation reaction to generate PA. The PA is converted to cytidine diphosphate diacylglycerol (CDP-DG) by the enzyme, CDP-DG synthase (CDS) in the presence of CTP. The final step is the condensation

of CDP-DG and *myo*-inositol into PI by PI synthase (PIS). An intriguing feature of PI in mammalian cells is its distinctive fatty acid composition; it is enriched in stearic acid (18:0) at the *sn*-1 position and arachidonic acid (20:4) at the *sn*-2 position of its glycerol backbone [17]. In mammalian cells, PI acquires its characteristic fatty acid composition by fatty acid remodelling after *de novo* synthesis of PI through sequential deacylation and reacylation reactions [18;19]. The mammalian enzyme responsible for the enrichment of arachidonic acid into PI is LPIAT (lyso-PI acyltransferase) (also known as MBOAT7) [17;20;21]. The importance of this fatty acid configuration is underscored by the observation that disruption of this gene in mice leads to severe defects in brain development including a substantial reduction in the levels of PI [17;21].

PI can be sequentially phosphorylated by PI 4-kinase (PI4K) to PI4P and finally to PI(4,5)P_2 by PIP 5-kinase (PIP5K). However, of the total cellular pool of PI, only 10-12 % is present in the phosphorylated forms with the PI4P and PI(4,5)P_2 being the most abundant. The family of PI 3-kinases can also phosphorylate PI, PI4P and PI(4,5)P_2 to make PI3P, PI(3,4)P_2 and PI(3,4,5)P_3 respectively but these species are present at much lower abundance [22]. PI4P and PI(4,5)P_2 are both enriched at the PM although PI4P is also found in other compartments including the Golgi, endosomes and lysosomes [23;24]. The seven phosphorylated derivatives of PI are enriched in specific membrane compartments due to the presence of unique lipid kinases and phosphatases that respectively add and remove phosphates from unique positions on the inositol ring. In many internal compartments, these phosphoinositides are interconverted by lipid kinases and phosphatases but in this case the basic backbone of PI is not destroyed. It is only the PLC signalling system that destroys the lipid requiring the resynthesis of PI.

Biochemical constraints in the PI(4,5)P_2 cycle

One of the central assumptions of the 'PI(4,5)P_2 cycle' is that the PA produced from PLC activity does not 'mix' with other cellular pools of PA and is maintained separately for recycling back to PI [3;25;26]. This assumption is partly based on observations that increased labelling of PI with [^{32}P]P_i is not normally accompanied with enhanced labelling with radio-labelled glycerol or fatty acids. Additionally, measurements of the fatty acid composition of newly-formed 'stimulated PA' reflects the fatty acid composition of PI i.e. PA formed is enriched in stearate and arachidonate. In principle, there are at least two other major pathways that can generate PA which can then enter the PI(4,5)P_2 cycle. One of these is the *de novo* synthesis from glycerol and fatty acids. In the second pathway, PA can also be produced by the hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD). In mammalian cells there are two PLDs, PLD1 and PLD2 that are also activated when cell surface receptors bind their agonist [27]. It is not uncommon for both PLC and PLD to be activated by the same receptors. Since the fatty acid composition of PA derived from PLD activity will reflect the composition of PC, which mainly contains mono-saturated fatty acids, it is possible to distinguish the source of the PA [28] although recent studies with PLD2 knockout mice have failed to verify this model [29]. There is some evidence to suggest that the backbone of PI(4,5)P_2 is recycled back into PI and the PA from the PLD pathway is kept separate [11;26]. This question needs to be revisited in intact cells where selective inhibition of the PLD pathway could be used to address this question.

Although the PI(4,5)P_2 cycle is ubiquitous in distribution across cell types, it acquires complexity because the mammalian genome contains multiple genes that produce variants of a specific enzyme activity that can catalyse a specific step of the cycle. For example there are

thirteen PLCs and ten DG kinases (excluding splice variants) in mammalian cells. As a further complexity, other lipid metabolising enzymes that are not core steps of the $PI(4,5)P_2$ cycle can also generate lipid intermediates of the cycle. For example, PA can be produced by phospholipase D (PLD1 and PLD2 and NAPE-PLD) [30;31] and DG can exit the cycle by being metabolised to 2-arachidonylglycerol (2-AG), an activator of the endocannabinoid receptor, by DG lipaseα [32]. This is particularly important in the brain as postsynaptic synthesis and release of endocannabinoid lipids activate presynaptic cannabinoid1 receptor to mediate short and long term depression in many brain regions. In platelets, DG can exit the cycle by being metabolised by DG lipase to form arachidonic acid for thromboxane synthesis [33]. Thus the assumption that the intermediates in the 'PI(4,5)P_2 cycle' might be conserved [3] may not be valid. In platelets, mass measurements of phosphoinositides, PA and DG following stimulation with thrombin revealed that the loss of phosphoinositide was not completely recovered as either DG or PA [33]. What is clear is that hormone-stimulated PI resynthesis always occurs when PI(4,5)P_2 hydrolysis by PLC takes place to replace the pool of degraded PI(4,5)P_2 [3;34]. Below we describe the key enzymes that participate in the cycle and their sub-cellular locations.

Phospholipase C

Thirteen PLC enzymes have been identified and grouped into six classes, PLCβ1-4, PLCγ1,2, PLCδ1,3,4, PLCε, PLCζ and PLCη1,2 on the basis of domain structure and regulatory mechanisms (Table 2). The presence of a catalytic X and Y domain, a PH domain, a C2 domain and 4 EF hands are the common features shared by these PLCs. The presence of additional subtype-specific domains contributes to specific regulatory mechanisms. The enzymes of the PLCβ family can be stimulated by G-protein-coupled receptors and the Gq family of G-proteins are responsible for their activation. PLCε is regulated by Ras, $G_{\beta\gamma}$ and by G_{12}. Receptor tyrosine kinases, on the other hand, activate PLCγ1 and 2 whilst PLCδ family, PLCζ and PLCη are regulated by a rise in cytosol Ca^{2+} from the nanomolar to the micromolar range. *In vitro*, PLCs can hydrolyse PI, PI4P and PI(4,5)P_2 but it is thought that in cells, PI(4,5)P_2 is their main substrate. However, there may be exceptions; for example, PLCε is present at the vicinity of the nuclear envelope where it hydrolyses Golgi PI4P [35]. The majority of the PLCs have been deleted in the mouse individually and the animals exhibit a range of phenotypes including epilepsy (PLCβ1), embryonic lethality (PLCγ1) and B cell development defects (PLCγ2) [36].

Although the PLC signalling pathway was first characterised as a means to generate the second messengers, IP_3 and DG, it is now clear that a decrease in PI(4,5)P_2 also has significant consequences. The intact lipid, PI(4,5)P_2 is a key regulator of many ion channels [37;38]. The best example is the regulation of the M-current carried by KCNQ2/3 (Kv7.2/7.3) potassium channels [39;40]. The M-current is activated by muscarinic receptors and depletion of PI(4,5)P_2 causes the closure of these channels; IP_3 and DG do not play a significant role. In a recent study, detailed analysis of two GPCRs present in the same cell, concluded that the number of receptors activated determined how much PI(4,5)P_2 depletion occurred [41]. Interestingly, this concept has a longer history. Early studies had indicated that the 'PI cycle' tracked receptor occupancy but not changes in cytosol Ca^{2+} [42]. The recent studies used the M-current as a surrogate for PI(4,5)P_2 hydrolysis, and substantial PI(4,5)P_2 hydrolysis only occurred when receptors were present at a high density. Thus only activation of the high abundance muscarinic receptor led to closure of the KCNQ2/3 potassium channels but not by the activation of the low abundance P2Y2 receptors for ATP [12;38;41]. Nonetheless

activation of either receptor by saturating concentrations of agonist provoked a full amplitude Ca^{2+} response. This study confirms earlier work that only a small amount of $PI(4,5)P_2$ needs to be hydrolysed to obtain a maximal rise in cytosol Ca^{2+}. Secondly, a high receptor occupancy is required to obtain a substantial decrease of $PI(4,5)P_2$. This is an important conclusion as it clarifies how different GPCRs in the same cell can produce different functional outputs. Further it implies that receptor density with maximal receptor occupancy will determine how much $PI(4,5)P_2$ will get depleted. Under these circumstances, the PI resynthesis pathway must be tuned to cope with the extra demand when $PI(4,5)P_2$ depletion occurs following high rates of receptor-triggered PLC activity. In this setting, another important factor that needs consideration is the speed of $PI(4,5)P_2$ replenishment. Is the rate of $PI(4,5)P_2$ synthesis variable between different cell types? Comparing the dynamics of $PI(4,5)P_2$ resynthesis following PLC signalling in sympathetic neurons and in a non-excitable cell line reveals that synthesis of PI4P is restored much faster in sympathetic neurons than in a non-excitable cell-line [12]. In neurons, $PI(4,5)P_2$ has many essential functions including exocytosis, endocytosis, actin assembly and ion channel regulation and may be more vulnerable to $PI(4,5)P_2$ depletion. This may require additional mechanisms to cope with the PI resynthesis pathway. The resynthesis of $PI(4,5)P_2$ at the plasma membrane requires a supply of PI and it is here that lipid transporters may play an important role.

In the *Drosophila* genome, there are only three PLCs; two are highly related to the PLCβ family, NORPA and Plcβ21C and a single PLCγ (Table 2) [43-47]. Unlike in mammals, loss of PLCγ does not lead to lethality but causes small wings [45]. Plcβ21C is implicated in olfaction transduction whilst NORPA is required for phototransduction. NORPA expression is enriched in photoreceptors and in these cells there are high levels of basal PLC activity [48]. Following illumination, PLC activity increases dramatically and under bright light condition ca. $1x10^6$ PLC molecules are active [49]. Thus *Drosophila* photoreceptors are an example of cells in which very high rates of PLC activity are achieved. Despite these high levels of PLC activation, $PI(4,5)P_2$ levels show only a 50% reduction under daylight equivalent illumination in vivo [50]. This was however not the case when recordings were done in the absence of extracellular Ca^{2+} or in the *trp* mutant which reduced Ca^{2+} influx into photoreceptors [50;51]. Thus the end point of PLC activation, namely Ca^{2+} influx may offer a mechanism by which PLC activity can be inhibited and tuned to prevent $PI(4,5)P_2$ depletion. Interestingly a biochemical study of PLC activation in *Drosophila* heads has reported a bell shaped dependence of PLC activity on Ca^{2+} concentration [52].

A further question that arises is the mechanism by which specificity of $PI(4,5)P_2$ signalling arises during PLC activation. $PI(4,5)P_2$ has multiple functions at the plasma membrane and therefore during PLC signalling it is essential to have mechanisms that prevents the non-PLC dependent functions of $PI(4,5)P_2$ being affected. There are multiple mechanisms by which the specificity of $PI(4,5)P_2$ functions can be preserved; these have recently been reviewed [53]. In many neuronal and also some non-neuronal cells, many of the phospholipases form macro-molecular complexes with other proteins during cell activation which means that PLC activation at the plasma membrane is likely to be restricted to microdomains. If $PI(4,5)P_2$ hydrolysis at these domains is tightly coupled to the local delivery of PI for replenishment, this would lessen the impact of $PI(4,5)P_2$ depletion on other $PI(4,5)P_2$ dependent systems.

In *Drosophila* photoreceptors, NORPA is localized at the plasma membrane along with the GPCR, rhodopsin and Gq. In this location it is found as part of a macromolecular protein complex assembled on the multi-PDZ domain protein INAD. In addition to NORPA, this complex includes the TRP channel, PKCα and NINAC, an unconventional myosin [reviewed

in [16;54]]. The 90 a.a. PDZ domain was originally identified as a conserved element in three structurally unrelated proteins, **P**SD-95, **D**LG and **Z**O-1. The PDZ domain binds to short carboxy-terminal peptide sequences of target proteins. Multiple PDZ domains are often present in a single protein promoting the scaffolding of several target proteins [55]. All PLCβ subtypes have PDZ binding motifs and the PDZ-containing NHERF (Na^+/H^+ exchanger regulatory factor) family and SHANK proteins form signalling complexes bringing together the PLCβ isozymes with the different receptors [56]. Despite considerable interest in the idea that the presence of NORPA in the INAD complex of *Drosophila* photoreceptors provides speed and specificity to PLC signalling [57], a number of studies have failed to support the idea that the presence of an intact INAD complex is required for normal activation through PLC signalling [58;59]. However the notion that confining PLC activation to signalling platforms would avoid effects on the cytoskeleton and vesicular transport and allow channelling of substrate to these regions remains attractive. As discussed below, these signalling platforms could reside at membrane contact sites allowing for efficient and localised signalling to occur.

Diacylglycerol Kinases

The product of PLC activity is DG that is rapidly phosphorylated to PA by DGK. The first DG kinase was cloned in 1990 and now there are ten DG kinases in the mammalian genome and five in the *Drosophila* genome (Table 2). DGK enzymes are subdivided into five subtypes based on the domain structures [60]. The expression of DGKs is cell-type specific [60]; for example, in T cells the predominant isoforms expressed are DGKα and DGKζ. Since $PI(4,5)P_2$ in mammalian cells is enriched in C18:0/C20:4, the DG formed will also have the same fatty acid composition. DGKε has been shown to specifically utilise stearoyl arachidonyl glycerol [61]. However, this specificity is not absolute as all the other DGKs can utilise C18:0/C20:4 species.

PA made following the activation of PLC activity is found at the plasma membrane as demonstrated using subcellular fractionation [62]. However DGK may not be constitutively localized at the plasma membrane. For example, in T cells the predominant isoforms expressed, DGKα and DGKζ are both recruited to the plasma membrane following T-cell receptor activation where they contribute to the termination of DG-mediated signalling downstream following PLCγ activation by T cell receptor-ligand complexes [63;64]. An important consequence of this observation is that PA, the product of DGK activity being a lipid, will be spatially restricted to the PM while the next enzyme in the $PI(4,5)P_2$ cycle, CDS that utilizes PA as a substrate, is localized to the ER. Thus a mechanism is required to transport PA from the PM to the ER.

In *Drosophila* photoreceptors, the DGK that is utilised in the removal of DG after PLC activation is encoded by *rdgA* [65]. The mammalian equivalent of the RDGA protein is the type IV family that includes DGKζ and DGKι. Loss of *rdgA* results in enhanced electrical responses to light, a defect in response termination [66;67] and severe retinal degeneration [68;69]. All of these phenotypes are dependent on ongoing PLC activity [reviewed in [13]] and underscore the importance of DGK in regulating light-induced $PI(4,5)P_2$ turnover. The biochemical basis of phenotypes resulting from reduced *rdgA* function remains to be resolved. Reduction in *rdgA* function results in lower levels of PA; surprisingly elevated DG levels have not been demonstrated [70;71]. It has been proposed that the DG may be metabolised via liberation of polyunsaturated fatty acids (PUFA) that may then activate TRP channels [reviewed in [72]]. An interesting feature of RDGA is that in photoreceptors, the

protein is localized to the sub-microvillar cisternae (SMC) [73], a specialization of the ER that forms a membrane contact site with the microvillar plasma membrane [reviewed in [74]] This localization of RDGA to an ER sub-compartment resembles, conceptually, the recruitment of DGKα and DGKζ from the ER to the PM of mammalian cells following receptor-mediated PLC activation. In the case of *Drosophila* photoreceptors, RDGA could act in trans across the small 10 nm gap at this MCS to access DG generated at the microvillar plasma membrane by NORPA.

Type II PA phosphatase

Biochemical experiments have identified two phosphatidic acid phosphatase activities in mammalian cells. One of these, Type I PA phosphatase (also called Lipins), a Mg^{2+} dependent and N-ethylmaleimide-sensitive activity is associated with the cytosol and microsomal fraction and involved in phospholipid biosynthesis. The other, a Type II phosphatidic acid phosphatase (also called lipid phosphate phosphohydrolase, is Mg^{2+} independent and N-ethylmaleimide-insensitive and is associated with the plasma membrane (reviewed in [75]). This latter class of PA phosphatases have been suggested to be involved in regulating signalling reactions and may in principle regulate the $PI(4,5)P_2$ cycle where PA is generated as an intermediate by DGK activity. However, although a role for Type II PA phosphatase in regulating PA levels in *Drosophila* is shown, its role in regulating $PI(4,5)P_2$ levels in mammalian cells remains to be established.

A *Drosophila* ortholog of Type II PA phosphatase has been implicated in the regulation of the $PI(4,5)P_2$ cycle in *Drosophila* photoreceptors [71;76]. The *Drosophila* genome encodes seven Type II PA phosphatase-like genes. One of these *lazaro (laza)*, in enriched in the eye. Loss of function mutants in *laza* show increased accumulation of PA during illumination that triggers G-protein coupled hydrolysis of $PI(4,5)P_2$. *rdgA* (DG kinase) mutants show reduced levels of PA and these are further reduced by overexpression of *laza*. Importantly, in addition to PA, the levels of PI are also reduced in *rdgA* mutants and these are further reduced by overexpression of *laza*. Thus a Type II PA phosphatase activity regulates the levels of PA and PI, two key intermediates during G-protein coupled PLC activation in photoreceptors. The retinal degeneration and electrophysiological defect in *rdgA* can be enhanced by overexpression of *laza* and suppressed in *rdgA;laza* double mutants. Together these findings suggest that Type II PA phosphatase regulates PA and PI turnover during PLC activation. The sub-cellular localization of endogenous LAZA remains to be established.

Cytidine diphosphate diacylglycerol synthase

The next enzyme in the $PI(4,5)P_2$ cycle is CDP-DG synthase (CDS) which catalyses the conversion of PA to CDP-DG, an essential intermediate step in the biosynthesis of PI. In mammals, two homologous genes of *CDS (CDS1* and *CDS2*) have been cloned that are 73% identical and 92% similar [77-79]. Genes homologous to mammalian Cds are found in *E. coli*, *S. cerevisiae*, *Drosophila*, plants and cyanobacteria [79-81]. In *S. cerevisiae*, the single CDS1 is an essential gene and appears to account for the majority of the synthase activity [82] (but see later). In *Drosophila*, complete loss of CDS results in lethality. However an eye-enriched isoform of CDS is expressed and mutant alleles that result in its downregulation result in defects in phototransduction and retinal degeneration [83]. In *Drosophila* photoreceptors, CDS is distributed throughout the cell body; it is likely localized to the ER as has been reported for mammalian cells [84;85].

The two isoforms of mammalian CDS show different substrate specificities. Mammalian CDS2 appears to be selective for the acyl chains of PA, the most preferred species being 1-stearoyl, 2-arachidonoyl-*sn*-PA [84]. The fatty acid composition of PI and its phosphorylated derivative, PI(4,5)P_2 is mainly 1-stearoyl, 2-arachidonyl, and during PLC signalling, the PA generated would retain this composition. This is also supported by studies in zebrafish where CDS2 controls the availability of PI(4,5)P_2 for VEGF signalling, and when mutated, there is a failure of angiogenesis [86]. CDS1, on the other hand, shows no particular substrate specificity [84]. Expression studies indicate that CDS1 and CDS2 exhibit quite different tissue specificity. In the mouse, Cds2 appears to be ubiquitously expressed whilst CDS1 has a restricted pattern of expression.

Early biochemical studies in yeast and mammalian cells showed that CDS activity was present in both microsomal (mainly ER) and mitochondrial fractions [87-89] hence CDS1 was thought to be present in both ER and mitochondria. However, recent studies in yeast have identified a new enzyme, Tam41, which possesses CDP-DG synthase activity. Tam41 is a peripheral membrane protein localised in the inner mitochondrial membrane where it is involved in cardiolipin synthesis [90]. Tam41 and the yeast CDS1 have different evolutionary origins and are not related by sequence although both possess CDS activity [90]. It is of interest that the substrate, PA, is transported from the outer mitochondrial membrane to the inner mitochondrial membrane-localised Tam41 by the yeast PA transporter, Ups1/Mdm35 complex (TRIAP1/PRELI complex in mammals) [91-95]. Interestingly, this complex has structural homology to mammalian PITPα [96;97].

PI synthase

Phosphatidylinositol synthase (PIS) is involved in the final step of PI synthesis, converting CDP-DG and *myo*-inositol into PI. In addition to *de novo* biosynthesis, PIS has exchange activity where the inositol of the PI can be replaced for free inositol in the presence of Mn^{2+}, in the absence of CDP-DG [98]. PIS activity localises mainly to the ER and molecular identification was first achieved in yeast and ultimately made it possible to clone the mammalian enzyme [79;99]. There is a single gene for PIS in yeast and in mammalian cells and share sequence identity throughout their primary sequence. The human *PIS* gene is predicted to encode for an integral protein of 213 a.a. (23 kDa) and studies in yeast indicate that the active site faces the cytosol [100]. In *Drosophila*, there is a single *PIS* gene (*dPIS*); loss of function mutants in *dPIS* result in both organismal and cell lethality underscoring the central role of PI signalling in eukaryotic cell biology. Hypomorphic alleles of *dPIS* show defects in phototransduction, a process that requires PI(4,5)P_2 regeneration after its hydrolysis by PLC [101].

The ER is a heterogeneous compartment and studies conducted in COS 7 cells indicate that that over-expressed PIS can co-localise with Rab10 to an ER-derived sub-compartment [85;102]. This sub-compartment makes contacts with other membranes implying that PI can be delivered to other membranes presumably by a lipid transfer mechanism. These dynamic structures required the GTPase Sar1, indicating that they were generated from the ER. However, CDS1 or 2 did not co-localise in these dynamic structures [85]. The dependence on Sar1 implies that the vesicles are initially made as COPII-coated vesicles. One possibility is that PIS, normally an ER-resident enzyme, when over-expressed at high levels results in being captured into COPII vesicles. The fundamental issue would be access to CDP-DG for the synthesis into PI in these mobile structures. Further study on these dynamic compartments is required preferably in other cell types.

Phosphatidylinositol 4-kinase

Of the four PI 4-kinases encoded in the mammalian genome (PI4KIIIα (*Stt4* in yeast), PI4KIIIβ (*Pik1* in yeast), PI4KIIα and PI4KIIβ (*Lsb6* in yeast), PI4KIIIα has recently been shown to be the isoform localized at the plasma membrane [103-105]. The role of PI4KIIIα in regulating PI4P synthesis during PLC signalling was first studied using pharmacological inhibitors and siRNA [103]. In this setting, the role of PI4KIIIα in regulating PI4P synthesis during PLC signalling has been studied in the context of overexpressed GPCRs; the role of this enzyme in the context of an endogenous G-protein coupled PLC pathway remains to be demonstrated. Also, the identity of the PI4K that regulates PI4P synthesis during *Drosophila* phototransduction remains to be established.

An interesting feature of PI4KIIIα that has emerged from recent studies is that in multiple systems the enzyme exists as part of a protein complex. In yeast, the PI4KIIIα ortholog, Stt4 exists as part of a complex consisting of three adaptor proteins, the peripheral membrane protein, EFR3, the scaffolding protein, Ypp1 (TTC7 in mammals) and an integral membrane protein Sfk1 (TMEM150A in mammals); this has also been observed in mammalian cells [105;106]. Efr3 is palmitoylated and is targeted to the plasma membrane due to a basic surface patch and by interactions with TMEM150A and recruits the PI4KIIIα/TTC7 complex by binding to the C-terminus of Efr3 [107]. Collectively, these studies also strongly suggest that PI4KIIIα is localized at the PM and the synthesis of PI4P occurs at this location. More recently, the leukodystrophy protein FAM126A (also known as hyccin) has also been shown to regulate PI4P synthesis and binds to TTC7 and possibly PI4KIIIα as well [108]. These findings suggest that the generation of PI4P by PI4KIIIα is a highly regulated process controlled by multiple proteins; this remains to be understood. Recent studies also indicate that in mammalian cells, protein kinase C can stimulate PI4K activity and so increase PI4P levels [109;110].

PIP 5-kinase

Phosphorylation of PI4P to $PI(4,5)P_2$ is catalysed by PIP 5-kinase (PIP5K). In the mammalian genome, there are three genes (PIP5Kα, -β, and –γ) encoding PIP5K enzymes that are recruited at the plasma membrane as homodimers or heterodimers [111]. One of the striking features of the PIP5Ks is that *in vitro*, their activity can be enhanced by PA [112]. This positive feedback by PA may in part ensure that $PI(4,5)P_2$ levels are restored promptly since $PI(4,5)P_2$ is important for other functions including maintenance of the actin cytoskeleton. PIP5Kγ has a number of splice variants and one specific splice variant, PIP5Kγ87 was found to be responsible for the generation of $PI(4,5)P_2$ utilised to make IP_3 in histamine-stimulated HeLa cells [113]. In a separate study, PIP5Kα was found to be responsible for thrombin-stimulated IP_3 generation in platelets [114]. Mice deleted of PIP5Kα or β are born and develop normally indicating that PIP5Kγ suffices to maintain $PI(4,5)P_2$ levels. Mice deleted of PIP5Kγ, on the other hand, die shortly after birth due to defects in neuronal function. In the *Drosophila* genome, there are two PIP5K genes, *sktl* and *dPIP5K*. *dPIP5K* appears to be equivalent to mammalian PIP5Kγ; it is enriched in the adult head and appears to regulate $PI(4,5)P_2$ resynthesis during phototransduction [115]. By contrast, loss of *sktl* is lethal during development and is also cell-lethal in multiple tissues where this has been tested [[116] and P.Raghu lab, unpublished]. Although some isoforms of PIP5K have been reported at intracellular membranes, the vast majority of isoforms across

multiple species are localized to the plasma membrane. Thus the synthesis of $PI(4,5)P_2$ from PI4P occurs primarily at the PM.

Topological constraints in the $PI(4,5)P_2$ cycle

Activation of both G-protein-coupled receptors and receptor tyrosine kinases stimulates the PLC signalling pathway to hydrolyze $PI(4,5)P_2$ and generate the two ubiquitous second messengers, IP_3 and DG in almost all metazoans. The activity of PLC is auto-inhibited and external cues lead to its activation [117;118]. The substrate, $PI(4,5)P_2$ is mainly localised at the PM and when cleaved by PLC, generates a series of lipid-soluble intermediates that are spatially restricted to and cannot diffuse away from the membrane at which they are generated. The immediate lipid product of PLC activity is DG that is rapidly converted into PA by DGK; these two reactions are thought to occur at the PM in mammalian cells (Fig. 1B). PA is converted to PI by the action of two sequentially acting enzymes, CDS and PIS which are localized to the ER. This separation of PA generation at the PM from the enzyme that metabolizes it, CDS at the ER membrane constitutes a topological problem necessitating the need to transfer PA from the PM to the ER (Fig. 1B).

The final steps in the $PI(4,5)P_2$ cycle are the sequential phosphorylation of PI at the 4, and 5 position to regenerate $PI(4,5)P_2$ (Fig. 1B). The kinases that mediate the phosphorylation of PI to PI4P are not present in the ER but thought to reside at the PM, once again generating a spatial separation of non-diffusible substrate from the enzymes. To maintain $PI(4,5)P_2$ homeostasis following PLC signalling, the first question to be addressed is the subcellular distribution of PI and how changes in PI levels reflect changes in $PI(4,5)P_2$ levels. PI distribution in cells has been monitored by subcellular fractionation and the majority of PI is found intracellularly, notably at the ER [119;120]. Unlike probes available for detecting the spatial distribution of phosphorylated PIs, no probes for monitoring the spatial distribution of PI has been identified so far. When PIS, an ER localized enzyme was knocked down, a 40% loss of total PI was noted and this was accompanied by a corresponding decrease in both PI4P and $PI(4,5)P_2$ indicating that PI, PI4P and $PI(4,5)P_2$ were in equilibrium and changes reflected in PI manifested itself in the total pool of phosphoinositides [85]. Thus in PIS knockdown cells, changes in PI synthesis at the ER are reflected in changes in plasma membrane $PI(4,5)P_2$ reflecting the dependence of plasma membrane $PI(4,5)P_2$ synthesis on the availability of PI from the ER. Since it is generally the case that hydrolysis of $PI(4,5)P_2$ manifests itself as a reduction in the PI pool [34;121-123], this would imply that the plasma membrane depends on PI being made available from the ER. Indeed, in permeabilised cells when PLC activation is intensely-driven, the amount of IP_3 produced far exceeds the resting levels of $PI(4,5)P_2$ and more importantly, a reduction in PI and not $PI(4,5)P_2$ is mainly observed [121]. Essentially, hydrolysis of $PI(4,5)P_2$ is dependent on on-going conversion of $PI(4,5)P_2$ from PI.

To demonstrate that PI at the ER contributes to $PI(4,5)P_2$ synthesis at the plasma membrane in intact cells, a bacterial phospholipase C was targeted to the ER to deplete PI in this compartment and noted that recovery of $PI(4,5)P_2$ at the plasma membrane was delayed following PLC activation [85;124]. Studies by Dickson et al have recently suggested that PI4P at the Golgi participates in the maintenance of the plasma membrane $PI(4,5)P_2$ [125]. Targeting of a PI4P phosphatase, Sac1, to the Golgi or the PM led to a decrease in PM $PI(4,5)P_2$. They suggested that 30% of the PM $PI(4,5)P_2$ is derived from a Golgi PI(4)P pool under steady state. In these studies, the KCNQ2/3 current was monitored as an indirect measure of $PI(4,5)P_2$ content. They suggest that Golgi-derived vesicles are responsible for

this transport. However, a recent study examined this question and concluded otherwise. The Golgi was disrupted using brefeldin A and under these conditions $PI(4,5)P_2$ synthesis at the PM (as monitored by the translocation of the PH domain of PLCδ) was enhanced [124] and not reduced as expected. Thus from this data it is clear that PI originating from the ER is the precursor lipid for $PI(4,5)P_2$ synthesis at the PM. Since the on-going production of IP_3 in permeabilised cells was dependent on the presence of a PI transport protein, this would suggest a supply of PI from the ER to the plasma membrane. In this experimental condition, any protein that has PI transfer activity was capable of supporting PLC signalling. Thus PITPα, PITPβ and even yeast Sec14p which has no structural or sequence identity to mammalian PITPs, were capable of supplying PI in these artificial conditions [126;127]. What these experiments establish is that the endoplasmic reticulum is the main source of PI and moreover, it has to be transported to the plasma membrane.

Bridging the topological constraints in the $PI(4,5)P_2$ cycle

Conceptually, one of the ways of bridging the topological gap in the $PI(4,5)P_2$ cycle would be the use of lipid transport proteins that would move lipid molecules between the ER and PM across the aqueous cytosol. This process could also be further optimized if one were to use sites where the ER and PM come in close contact with each other, namely the ER-PM MCS (membrane contact sites). A brief introduction to the current state of ER-PM contact sites is given below and we then discuss lipid transfer proteins in the context of the $PI(4,5)P_2$ cycle.

ER-PM contact sites

The components of the $PI(4,5)P_2$-PLC cycle are distributed in two membrane compartments, the ER and PM (Fig. 1B). Recent studies indicate that these two compartments can come into close proximity allowing lipid exchange to occur. The endoplasmic reticulum (ER) exhibits a complex network of tubules and cisternae continuous with the nuclear membranes and is involved in vesicular transport, protein synthesis, Ca^{2+} storage and lipid metabolism [128-130]. It is also the largest organelle of the cell representing nearly 50% of the total cellular membranes [131]. The ER can be closely opposed to other organelle membranes including endosomes, peroxisomes, mitochondria in addition to the plasma membranes. These ER-PM membrane contact sites (MCS) have been by observed by electron microscopy (EM) in both mammalian cells e.g. in muscle cells, neurons and in fly photoreceptors [74;132;133].

To maintain regions of close membrane contact tethering proteins that bring the membranes together are necessary. The distance between the ER and the PM at ER-PM contact sites is estimated to be in the range of 10 to 20 nm, sufficiently close to allow proteins in one compartment to directly interact with proteins or even lipids in the other compartment. Several tethering proteins have been identified in the formation of ER-PM contact sites. These include the junctophilins, Ist2 in yeast (related to TMEM16 proteins in mammals), VAMP-Associated Proteins (VAPs) (Scs2 and 22 in yeast) and most recently the Extended synaptotagmins (E-Syts; Tricalbins in yeast). These proteins are all anchored in the ER and many have domains allowing them to bind lipids on the PM in trans [130;134-136]. The junctophilins are necessary for sarcoplasmic reticulum (SR)-PM junctions in muscle cells [137], whereas VAPs are involved in numerous interactions at ER-PM contact sites, notably with oxysterol-binding (OSBP)-related protein (ORP) and PITPNM family members. VAPs bind to a FFAT motif on LTPs such as ORPs and PITPNMs. E-Syts are transmembrane proteins with multiple C2 domains and are tethered at the ER whereas the C2 domains interact with plasma membrane $PI(4,5)P_2$. In yeast approximately 40% of the PM contacts the ER whilst in mammalian cells, ER-PM contacts are less prominent; in many non-excitable

cells less than 1% of the PM makes contact with the ER. In yeast, combined deletion of the six potential yeast ER-PM tethering proteins, Ist2, the VAP homologs Scs2/22, and all three Tricalbins was necessary to eliminate ER-PM junctions [138].

In mammalian cells, membrane contact sites can be induced during Ca^{2+} signalling e.g. as it occurs with Stim and Orai [139;140]. Depletion of ER Ca^{2+} stores by IP_3 binding to the IP_3 receptor on the ER leads to the process whereby store-operated calcium entry (SOCE) can occur. In brief, the Ca^{2+} sensors, STIM1 and STIM2 detect the decrease in Ca^{2+} in the ER stores and this causes a conformational change in STIM proteins such that they expose the SOAR domain, resulting in STIM redistribution to ER-PM contact sites where they assemble with the plasma membrane-localised Orai1, which is the store-operated Ca^{2+} channel [141]. This transition is aided by an ancillary protein, STIMATE (TMEM110) which stabilises the ER-PM contact sites [142;143].

In mammalian cells, ER-localised E-Syts have recently been identified as tether proteins that bind to the plasma membrane upon elevation of Ca^{2+} [144]. E-Syts are a family of ER integral membrane proteins with multiple C2 domains that are conserved from yeast to mammals. E-Syts contain an amino- terminal ER-membrane anchor, an SMP (synaptotagmin-like mitochondrial-lipid binding protein) domain and multiple C2 domains. The E-Syt family has three members; E-Syt1 has five C2 domains and E-Syt2 and E-Syt3 each have three C2 domains (Table 2). C2 domains are independently folded domains that can mediate Ca^{2+}/phospholipid interactions. The SMP domains belong to the TULIP superfamily of lipid/hydrophobic ligand binding domains [145;146] and the SMP domain of E-Syt2 has a β-barrel structure and dimerizes to form an approximately 90 Angstrom long cylinder traversed by a channel lined entirely with hydrophobic residues. The channel can accommodate lipids with the lipid fatty acid tails embedded in the hydrophobic channel whereas the polar headgroup protrudes in the aqueous phase. The SMP channel lacks specificity and binds any glycerolipids without any discrimination but does not bind cholesterol, sphingomyelin or ceramide [147;148]. The C2 domains of E-Syt2 /3 interacts with plasma membrane $PI(4,5)P_2$ thus tethering the ER to the plasma membrane. Tethering of E-Syt1 to the plasma membrane is more complex; it occurs when Ca^{2+} is elevated and is dependent on the C2C domain of E-Syt1 [149]. In the presence of Ca^{2+} this domain binds to $PI(4,5)P_2$. Since hydrolysis of $PI(4,5)P_2$ by PLC causes a rise in Ca^{2+}, E-Syt1 localises to the PM, but only transiently due to hydrolysis of $PI(4,5)P_2$ [144;150].

E-Syt1 can apparently use their SMP domain to transfer lipids between two membrane compartments *in vitro* provided that the C2C domain is intact. Mutations in the Ca^{2+} binding sites abrogate tethering as well as lipid transfer. Thus E-Syt1 is a Ca^{2+}-dependent lipid transfer protein that functions at membrane contact sites [150;151]. Unlike other LTPs described later, SMP domains do not bind a single lipid like LTPs but forms a channel and can transfer any glycerolipid.

PI transfer proteins

Given the distribution of lipid intermediates of the $PI(4,5)P_2$ cycle between the PM and the ER, it is essential for cells to have in place a mechanism that allows the exchange of lipid intermediates between these two compartments (Fig. 1B). Given the rapid rates that PLC signalling can achieve *in vivo*, transfer of lipid intermediates via vesicular transport is likely to be a slow and unwieldy solution. The use of lipid transport proteins (LTPs) is a potential solution for the exchange of such lipid intermediates. Multiple families of lipid transport

proteins (LTPs) have been identified in eukaryotic cells that can engage in non-vesicular lipid transport. LTPs fall into two categories: they can be either single domain cytosolic proteins or they can be multi-domain proteins with an LTP domain. To date, intracellular families of LTPs include START, OSBP, CRAL-TRIO, GLTP and PITP. The hallmark of all LTPs is their ability to accommodate a lipid within a hydrophobic cavity and shield it from the aqueous environment. Structures are available for many of the LTPs showing the lipid-protein interactions that take place to hold the lipid in position during transport between membranes [152]. These structures reveal that the entrance to the cavity is blocked by a flexible 'lid'. In mammalian cells, the PITP family has been identified as the main LTP that can bind and transfer PI and therefore are the pertinent LTPs in ensuring a supply of PI for $PI(4,5)P_2$ synthesis during PLC signalling [153;154].

PITP overview

Since the original discovery of PITPs as a biochemical activity that can mediate PI transfer between membranes *in vitro* [7] and support PLC signalling *in vivo*, a large number of genes encoding PITPs have been discovered in metazoa through both molecular cloning and genome sequencing efforts. In the mammalian genome, there are five genes that encode proteins with a PITP domain; these are subdivided into two classes based on sequence homology as well as their lipid binding characteristics [155;156] (see Table 1 and Fig. 2)) Class I PITPs, PITPα and PITPβ are single domain PITPs and can bind and transfer PI or PC whilst Class II PITPs are proteins that can bind and transfer PI and PA, both of which are lipid intermediates that need to be exchanged during the $PI(4,5)P_2$-PLC cycle. Class II PITPs comprise three members; PITPNC1, PITPNM1 and PITPNM2. The founding member of the Class II PITPs is *Drosophila* retinal degeneration B (RDGB), a multi-domain protein with a N-terminal PITP domain [157]. The N-terminal PITP domain of RDGB is followed by a region of low complexity, a FFAT motif, a DDHD domain and a LNS2 domain (Fig. 2). The FFAT motif can bind to the ER-resident integral protein, VAP. A third member of the PITPNM family is PITPNM3 that lacks the PITP domain. PITPNM3 also contains the FFAT motif and could potentially be a negative regulator by competing to bind with VAP. The structural arrangements of the different domains in PITPNM1 and 2 are not known but one of the key questions that need to be addressed is whether the PI transfer activity of the PITP domain is kept inhibited by protein interactions and only made accessible after PLC activation. An acidic region has been shown to bind Ca^{2+} and therefore changes in cytosol Ca^{2+} could potentially influence the structure [157;158]. In addition to PITPNM, Class II also includes the short Class IIB PITPs (RdgBβ, PITPNC1).

Table 1 provides a summary of the PITP proteins and the various nomenclatures in use. We propose that the HUGO nomenclature (PITPNA, PITPNB, PITPNM and PITPNC1) for the gene names should be adopted for the corresponding proteins and will be used throughout this review. Recent studies in both mammalian cells and in *Drosophila* converge to suggest that, the PITPNM proteins are the long sought for proteins that can facilitate the transfer of PI and PA between the PM and the ER at membrane contact sites (MCS) during the PLC-$PI(4,5)P_2$ cycle.

Biochemical properties of the PITP domain

(a) PI transfer function: The PITP family of proteins are well-characterised with respect to their lipid binding specificities. PITPs have dual specificity. The first PITP protein to be identified, PITPα, was shown to be present in two complexes when purified from brain; PITPα bound to either PI or to PC [7;8]. The PITPα-PI (or PC) complex is stoichiometric,

involving a single lipid molecule [159;160]. The binding pocket can accommodate either a PI or a PC molecule; whilst the phosphate moiety and the two lipid tails occupy identical positions, the headgroup makes contact with different amino acids. The inositol headgroup makes contact with four amino acid residues (T59, K61, E86 and N90) and these residues are conserved in all PITPs. Mutation of any of these amino acids leads to loss of PI binding and transfer but has little effect on PC binding and transfer. The PI and PC bound forms are readily convertible by incubating with excess lipid and can be separated based on charge; the PI-PITPα complex is more negative that the PC-PITPNA complex [161]. The lipid-bound forms of PITPs have a 'closed' conformation whilst the apo-form has an 'open' conformation. The apo-form is only transiently observed during lipid exchange at the membrane [152]. A recent study has shown that these key determinants of PI binding and transfer activity are also conserved in PITPNM1 when tested both i*n vitro* [127] and *in vivo* [127;149].

(b) PA transfer function: In contrast to PITPα, the PITP domain of *Drosophila* PITPNM1 does not transfer PC; rather it binds and transfers PA [127]. The PITP domains of PITPNM proteins bind PI at much higher levels compared to Class I PITPs and can also bind PA but not to the same extent as PITPNC1. PITPNC1 is a cytosolic protein with a PITP domain followed by an unstructured 80 amino acid C-terminal tail. PITPNC1 bound similar amounts of PI and PA despite the fact that PA constitutes less than 1% of the total lipids suggesting a very affinity for PA binding [10]. Recent studies have shown that the isolated PITP domain of human PITPNM1 or *Drosophila* PITPNM was able to transfer PA between membrane compartments *in vitro* very efficiently [156].

An independent study also analyzed the ability of PITPNM1 to remove PA from the plasma membrane to the ER, in HEK cells expressing the Ang II receptor [162]. Using a PA reporter, these authors followed the decrease in cytoplasmic fluorescence as the PA probe translocated to the plasma membrane upon Ang II stimulation. In cells that co-express PITPNM1 the PA probe was removed from the plasma membrane faster than in the surrounding cells not expressing the probe. From these experiments, the authors concluded that over-expression of PITPNM1 facilitates the removal of PA from the plasma membrane. The PITP domain was required for PA removal. Mutation of T59 to A or E of the PITP domain of PITPNM1 prevented PA clearance following stimulation. Unfortunately no analysis of the PA transfer and binding activity of these mutants *in vitro* were provided. The structure of the PITP domain of PITPα coupled with mutational analysis has identified that T59 is in the binding pocket and is important for PI binding and transfer. In PITPα, T59 when mutated to E is unable to transfer PI but when mutated to A retains partial transfer activity [159;161]. This is also the case for the PITP domain of *Drosophila* PITPNM1. Thus it is surprising that the T59A mutant completely prevented PA clearance following stimulation. Analysis of PA transfer *in vitro* of the two mutants shows that it was unaffected [127]. This result is not surprising because T59 only makes contact with the inositol ring and does not contribute to the binding of the phosphate moiety in the binding pocket. A detailed analysis of the PITP domain of the human PITPNM protein is warranted to clarify these discrepancies. Identification of mutants that are defective in PA but not PI transfer would assist in this analysis. One of the key questions is how the PITP domain accommodates PA in the lipid binding cavity. It is interesting to note that PITPNC1 which can bind PA extremely well but transfer PA less efficiently was without effect in clearing PA from the plasma membrane [162]. In contrast, the PITP domain of PITPNM1 alone was still partly functional indicating that membrane contact sites make the transfer more efficient.

Spatial organization of PITPNM in relation to function

(a) PITPNM proteins are membrane-associated proteins

In *Drosophila* PITPNM, the founding member of the PITPNM family, early work had identified multiple hydrophobic segments downstream of the PITP domain and it was speculated that they could act as trans-membrane spanning segments [157]. Subsequently, PITPNM proteins were also identified in mammals where two separate genes encoding for PITPNM1 and PITPNM2 were identified [163;164]. A study by Lu et al, investigated whether PITPNM2 was an integral membrane protein [164]. They performed subcellular fractionation studies and found that although PITPNM2 was associated with the membrane pellet, conditions that disrupt protein–protein interactions, such as 1 M NaCl, high pH, or strong denaturants such as urea or guanidine, solubilized PITPNM2 partially or completely. In contrast, the detergent Triton X-100 failed to solubilize any PITPNM2. Thus PITPNM2 did not behave as an integral membrane protein. Similar studies conducted with *Drosophila* PITPNM confirmed that the protein is membrane-associated and can be solubilised under conditions that disrupt protein-protein interactions confirming that endogenous PITPNM proteins are not integral or cytosolic proteins but are membrane-associated [127].

In 2000, a zebrafish PITPNM homolog (alt names: PITP-less RdgB, pl-RdgB, PITPNM3) that lacks the amino-terminal PITP domain, was identified [165]. PITPNM3 is also present in mammals but not in *Drosophila*. The mouse PITPNM3 shares over 45% amino acid identity with the two mouse PITPNM proteins (PITPNM1 and 2) and was also found to be membrane-associated; it could be released when membranes were treated with the protein denaturant, guanidine. In contrast, Triton X-100 which solubilises the membranes did not solubilise the protein but was found in the pellet likely associated with the cytoskeleton. Similar experiments have also been conducted for PITPNM1 and it was concluded that PITPNM proteins are not integral membrane proteins, but may associate with the membrane through protein–protein interactions [166]. Thus three independent studies on different PITPNM proteins concluded that they are membrane-associated proteins [164-166] and not integral membrane proteins as originally suggested [157]. Thus it comes as a surprise that PITPNM3 has been recently identified as a transmembrane receptor for the chemokine, CCL18 [167;168]. It was proposed that extracellular CCL18 binds to PITPNM3 and can activate signal transduction pathways including PI3K [169]. This finding has been recently challenged however [170] and we suggest that PITPNM proteins are membrane-associated proteins rather than integral proteins and it is unlikely that PITPNM3 is a transmembrane receptor for the chemokine, CCL18. Determinants that mediate membrane association of PITPNM proteins include interactions between the FFAT motif with the integral VAP proteins as well as the LNS2 that can interact with PA as reported recently.

(b) Localization of PITPNM proteins to membrane contact sites

Recent studies indicate that the transfer of PI from the ER to the PM takes place at membrane contact sites by PITPNM1. PITPNM1 was found to translocate to ER-PM contact sites following PLC activation by histamine [149;162]. Secondly, knockdown of PITPNM1 resulted in decreased recovery of $PI(4,5)P_2$ at the PM whilst over-expression enhanced recovery. The PI transfer activity was essential for $PI(4,5)P_2$ replenishment as mutation of key residues that abolished PI transfer were not functional although they still translocated to the ER-PM contact sites. It is interesting to note that knockdown of PITPNM1 did not inhibit

the initial loss of $PI(4,5)P_2$ following histamine stimulation indicating that basal levels of $PI(4,5)P_2$ were not affected by loss of PITPNM1. This is in contrast to what is observed in *Drosophila* photoreceptors, where deletion of PITPNM led to a pronounced decrease in resting $PI(4,5)P_2$ levels [127].

In mammalian cells there are two PITPNM proteins and it is suggested that these two proteins have subtle differences in $PI(4,5)P_2$ replenishment at the plasma membrane. PITPNM2 is more sensitive and functions at low levels of $PI(4,5)P_2$ hydrolysis whilst PITPNM1 restores $PI(4,5)P_2$ homeostasis at intense levels of stimulation [124]. This was demonstrated by the use of two inhibitors, U73122 (PLC inhibitor) and R59022 (DGK inhibitor). Both drugs inhibited the translocation of PITPNM2 to the PM. Although U73122 has been used in the literature as a PLC inhibitor, it is clear that it is NOT a direct inhibitor of phospholipase C enzymes. U73122 is a cationic amphiphilic drug and very likely decreases the availability of the $PI(4,5)P_2$ substrate [171]. This would affect $PI(4,5)P_2$ interactions with the cytoskeleton as well. Likewise R59022 only inhibits the Type I DGKα and γ and moderately inhibits type II δ and κ [172]. In mammalian cells there are 10 DGK subdivided into 5 classes (see Table 2 and [60]) and Type III DGKε which is specific for arachidonyl stearoyl DG is not inhibited by this inhibitor. Identification of the DGK responsible for conversion of PLC-derived DG to PA will assist in clarifying the situation.

What drives the translocation of PITPNM1 to ER-PM contact sites? Translocation to ER-PM contact sites requires PITPNM protein to be associated with the ER and the plasma membrane simultaneously. Several studies have shown that PITPNM1 is localised to the ER and translocated to ER-PM contact sites following PLC activation [124;149;162]. Moreover, co-expression of VAP-B increased the ER association of PITPNM1 and upon stimulation both proteins clustered and co-localised at membrane contact sites. It is interesting to note that CDS2 was also found in the same clusters [162].

Association with the ER is dependent on the FFAT motif that binds to ER-localised VAP proteins. This is supported by studies in HeLa cells where PITPNM1 mutated at FFAT binding motif failed to get recruited at ER-PM contacts [149;162]. Recruitment of PITPNM1 was dependent on prior recruitment of E-Syt1 that decreased the distance between the ER-PM contacts [149]. Thus these results suggested that the increase in cytosol Ca^{2+} following PLC activation results in E-Syt1 recruitment which reduces the distance between the two membranes allowing PITPNM1 to bridge the GAP with the FFAT motif facilitating ER association. In this scenario, it is suggested that PITPNM1 can promote the transfer of PI for $PI(4,5)P_2$ replenishment after receptor-induced hydrolysis via its PITP domain [149]. The main evidence to support this can be summarised [149]: (1) Downregulation of E-Syt1 results in a delay in the kinetics of $PI(4,5)P_2$ resynthesis measured using fluorescent reporters for this lipid (2) knockdown of PITPNM1 leads to a decrease in $PI(4,5)P_2$ replenishment as monitored by measuring the dynamics of $PI(4,5)P_2$ reporters (iii) over-expression of PITPNM1 enhances $PI(4,5)P_2$ replenishment via PI binding to the PITP domain. These findings suggest that ER-PM MCS may support the $PI(4,5)P_2$ cycle. The effect of reducing MCS function on clearing PA from the PM is not known.

Saheki *et al* have examined the recruitment of endogenous E-Syt1 to ER-PM contacts and report that whilst ionomycin or thapsigargin rapidly translocated E-Syt1, the translocation observed with oxotremorine as a stimulus was transient. Since E-Syt1 binds to $PI(4,5)P_2$ at the membrane in a Ca^{2+} dependent manner, the pronounced decrease in $PI(4,5)P_2$ by oxotremorine is likely responsible for the transient nature of E-Syt1 recruitment [150].

In one study, a synthetic construct, MAPPER (membrane-attached peripheral ER) was used that selectively monitors ER-PM contact sites. The MAPPER construct comprises of a signal peptide, GFP, transmembrane segment, a linker, FRB (FKBP12-rapamycin binding) domain, a linker followed by phosphoinositide binding (PB) domain. Essentially the TM resides in the ER whilst the PB domain can bind to $PI(4,5)P_2$ at the PM. It was found that the increase in cytosol Ca^{2+} increased ER-PM junctions and this was dependent on the translocation of E-Syt to the junctions [149]. Ca^{2+}-dependent translocation of E-Syt1 to ER-PM junctions has also been demonstrated by another study [144].

To examine how PITPNM proteins sense $PI(4,5)P_2$ hydrolysis, and translocate to the ER-PM contact sites, it was shown that the LNS2 domain of PITPNM1 is recruited by increases in PA [173]. During cell stimulation of cell–surface receptors, many agonists stimulate both phospholipase C and phospholipase D. Both phospholipases produce PA; PLD produces PA directly whilst PLC produces DG that is rapidly phosphorylated to PA by DG kinase. A commonly-used PLC inhibitor, U73122 was found to inhibit translocation of PITPNM1 to the ER-PM contact sites as was a DG Kinase inhibitor, R59022 suggesting that phospholipase C was the main source of PA [162].

However in HeLa cells, endogenous PITPNM1 was found to be localised to the Golgi in interphase cells and translocated from the Golgi to the PM upon stimulation with EGF [173]. Localisation to the Golgi required the PITP domain whilst translocation to the plasma membrane required the C-terminal LNS2 domain (a.a. 911-1244). The LNS2 domain has been identified in lipins (PA phosphatase) and is the catalytic domain; however, in PITPNM proteins, a critical residue is substituted by a serine meaning that PITPNM would lack intrinsic PA phosphatase activity but could bind PA. Both exogenous addition of PA or stimulation of phospholipase D activity led to the translocation of PITPNM1 to the plasma membrane. The isolated LNS2 domain was found to bind liposomes when PA was present. Over-expression of PITPNM1 was found to enhance EGF-stimulated Akt phosphorylation whilst knockdown reduced EGF-stimulated Akt phosphorylation [173]. The effects of PITPNM1 on Akt required both the PITP domain as well as the LNS2 domain. Additional studies showed that PITPNM1 regulated both $PI(4,5)P_2$ and $PI(3,4,5)P_3$ levels and this required both the PITP domain intact for PI transfer and also the LNS2 domain that could bind PA. The main conclusion from this study was that PITPNM1 could translocate to the plasma membrane upon increases in PA levels and that the PITP domain transferred PI to the plasma membrane [173].

In contrast to mammalian tissue culture cells, in *Drosophila* photoreceptors, the single PITPNM is constitutively localized at the SMC a MCS between the ER and the PM [74]. There is no detectable change in this localization on receptor stimulation. However it is possible that there are subtle changes or rearrangements in 3D conformation between the various domains of PITPNM thus regulating lipid transfer function. These remain to be discovered.

Concluding remarks

In this review, we put the PITPNM proteins as major players in moving PI and PA during the resynthesis of $PI(4,5)P_2$ after PLC signalling (Fig. 2). We answer the major outstanding question that was highlighted in the review by Michell in 1975 [3]. A neat solution is found by identifying a single protein that can accomplish reciprocal transfer of PA for PI to

complete the ‘PI cycle’. An important feature that emerges is that lipid transfer takes place at privileged sites where the ER and the plasma membrane come into close proximity such that lipid exchange occurs over very short distances. One outstanding question that remains is the structures of the different domains of the PITPNM proteins. This will shed light on whether the PITP domain is held in an inactive state and only becomes operational during PLC signalling. A second question is to identify key residues in the PITP domain of the PITPNM proteins that are important for PA binding as these mutants will facilitate in further testing of the model.

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Gene name	Full name	Protein name	Synonyms
PITPNM1	phosphatidylinositol transfer protein membrane-associated 1	PITPNM1	Nir2, RdgBα1,
PITPNM2	phosphatidylinositol transfer protein membrane-associated 2	PITPNM2	Nir3, RdgBα2,
PITPNM3	PITPNM family member 3 (lacks a PITP domain	PITPNM3	Nir1, RdgBα3, pI-RdgB,
PITPNC1	phosphatidylinositol transfer protein, cytoplasmic 1	PITPNC1	RdgBβ,
PITPNA	phosphatidylinositol transfer protein alpha	PITPα	PITPNA VIB1A
PITPNB	phosphatidylinositol transfer protein beta	PITPβ	PITPNB VIB1B

Table 1: Nomenclature of PITP proteins in common use

Enzymes of the PI(4,5)P_2 cycle	**Mammals**	***D.melanogaster***
Phospholipase Cs PLCβ PLCγ PLCδ PLCε PLCζ PLCη	**13** PLCβ1; β2; β3; β4 PLCγ1; PLCγ2 PLCδ1; δ3; δ4; PLCε PLCζ PLCη1;η2;	**3** *norpA* (CG3620); PLC21C (CG4574) PLCγ (CG4200)
Diacylglycerol kinase Type I Type II Type III Type IV Type V	**10** DGKα, DGKβ, DGKγ, DGKδ, DGKη,DGKκ, DGKε, DGKζ, DGKι, DGKθ	**5** *Dgk* (CG34361), CG31187, DGKε (CG8657) *rdgA*(CG42667), CG31140,
CDP-diacylglycerol Synthase	**3** Cds1; Cds2; Tam41	**2** *Cds* (CG7962) Tam41 (CG33331)
PI Synthase	**1** PIS	**1** PIS (CG9245)
Class I PITPs	**2** PITPα; PITPβ	**1** PITP (*vib*) (CG5269)
Class II PITPs (RdgB) IIA IIB	**3** PITPNM1; PITPNM2; PITPNC1	**2** *RdgB* (CG11111); *RdgBβ* (CG17818)
Phosphatidylinositol 4-kinase Type II PI4K TypeIII PI4K	**4** PI4KIIA, PI4KIIB PI4KIIIA, PI4KIIIB,	**3** PI4KIIA (CG2929); PI4KIIIA (CG10260); *fwd*, (PI4K3B; CG7004)
PIP 5-kinase	**3** PIP5K1A; PIP5K1B, PIP5K1C	**2** *Sktl* (CG9985); *dPIP5K (CG3682)*
E-Synaptotagmin	**3** E-Syt1; E-Syt2; E-Syt3	**1** E-Syt2 (CG6643),
VAMP associated Protein (VAP)	**2** VAPA, VAPB	**3** dVAP-A (CG5014), dVAP-B (CG33523), *fan* (CG7919)

Table 2 Diversity in the enzymes of the PI(4,5)P_2 cycle required for phospholipase C signalling: Comparison between mammals and *Drosophila*.

Figure Legends

Fig. 1 The 'PI(4,5)P_2 cycle' : metabolic interconversions involved in resynthesis of phosphoinositides after hydrolysis by phospholipase C

[A] The 'PI cycle' as proposed by Mabel and Lowell Hokin in 1964 [2].

[B] Topological organization of the 'PI(4,5)P_2 cycle': The 'PI(4,5)P_2 cycle' operates between two membrane compartments, the ER and the PM requiring the transfer of PA and PI in opposite directions. The 'PI(4,5)P_2 cycle' was routinely observed by monitoring the incorporation of $^{32}P_i$ into PA and PI (phosphate labelled in blue).

Fig. 2 Restoration of PI(4,5)P_2 levels following phospholipase C signalling is accomplished by PITPNM proteins that can reciprocally transfer PA and PI at membrane contact sites.

Model illustrating how PITPNM participate in PA and PI transfer at membrane contact sites for the restoration of PI(4,5)P_2 levels. The 'PITP domain' is modelled on the structure of PITPα. The 'FFAT' motif that binds to VAP is indicated. Participation of E-Syt in tethering the two membranes is not included in the diagram for clarity. Abbreviations are: MSP, major sperm protein homology domain; FFAT motif sequence, EFFDAxE; TM, transmembrane domain.

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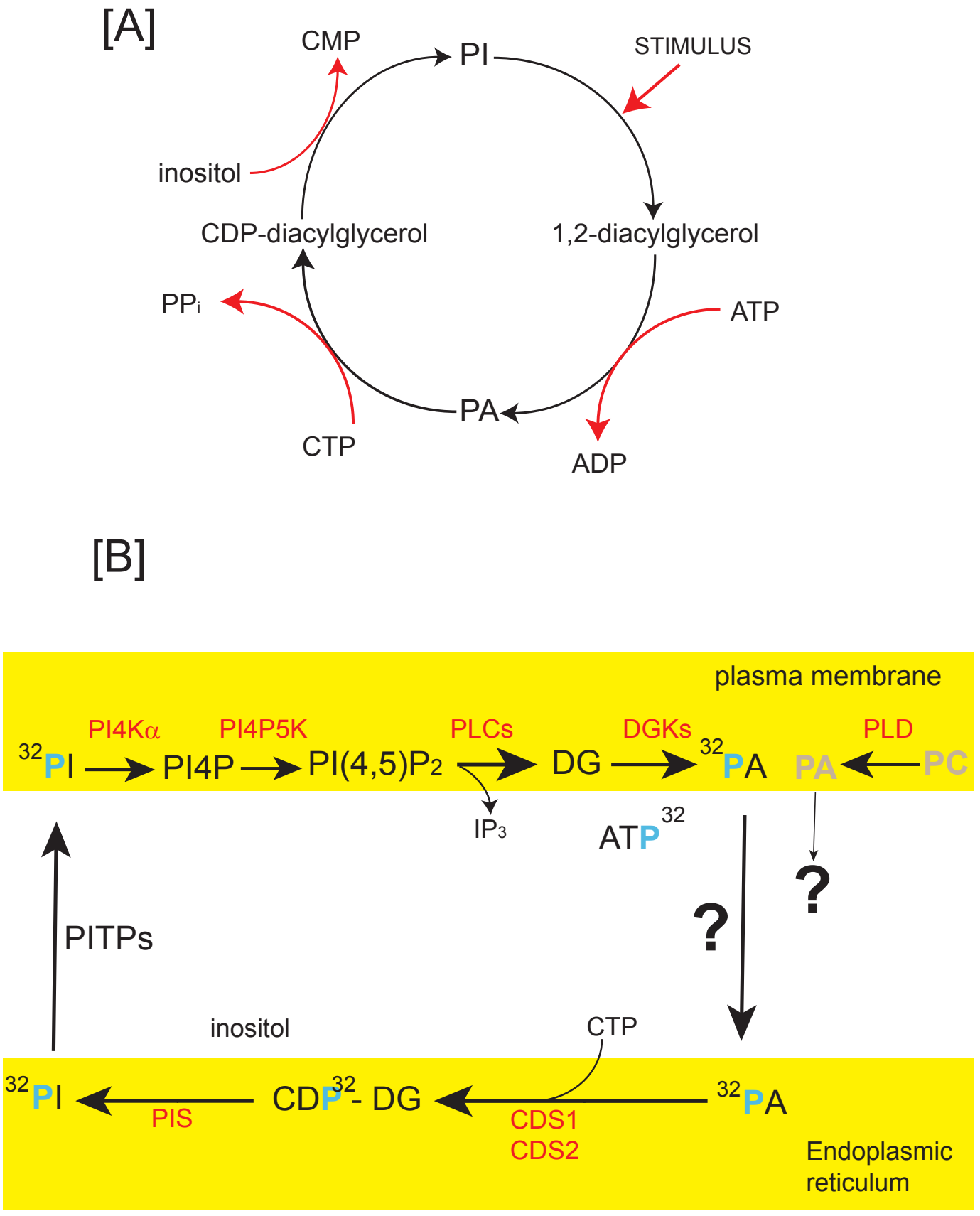
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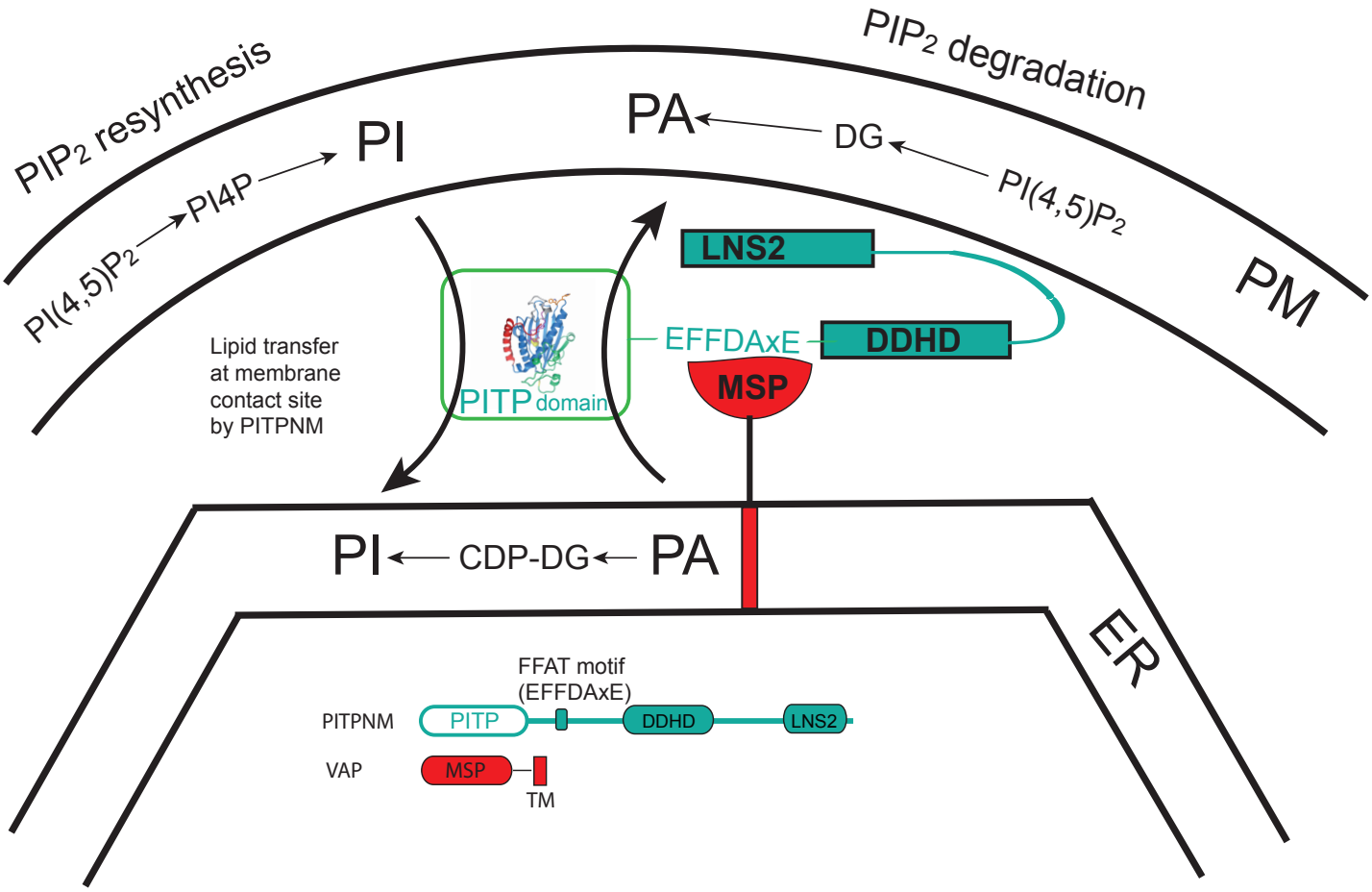
Fig. 1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DDHD
MSP
Lipid transfer
at membrane
contact site
by PITPNM
PITP domain
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CDP-DG
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VAP
MSP
TM

Fig. 2