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# PHOSPHOLIPASE C

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## Abstract

Phospholipase C (PLC) family members constitute a family of diverse enzymes. Thirteen different family members have been cloned. These family members have unique structures that mediate various functions. Although PLC family members all appear to signal through the bi-products of cleaving phospholipids, it is clear that each family member, and at times each isoform, contributes to unique cellular functions. This chapter provides a review of the current literature on PLC. In addition, references have been provided for more in-depth information regarding areas that are not discussed including tyrosine kinase activation of PLC. Understanding the roles of the individual PLC enzymes, and their distinct cellular functions, will lead to a better understanding of the physiological roles of these enzymes in the development of diseases and the maintenance of homeostasis.

### Keywords

Phospholipase C family; G protein-coupled receptors; Phosphatidylinositol 4,5 - bisphosphate; Diacylglycerol; Inositol 1,4,5 - triphosphate; Calcium; Isoform; Structure; Ubiquitous expression; Multiple functions

## Discovery

In 1953, it was reported that the addition of acetylcholine or carbamylcholine to pancreatic cells led to the production of phospholipids [1]. In these studies, $^{32}P$ was used to detect a seven-fold increase in the levels of phospholipids in the samples treated with the drugs, when compared with control slices, which had remained un-stimulated. Although unrecognized at that time, this was the first evidence of the presence of phospholipase C (PLC) function in cells. More than 20 years later, in 1975, it was shown that impure preparations of PLC could be used to cleave phosphatidylinositol [2]. In 1981, the first purified preparation of PLC was isolated [3]. A couple of years later it was found that the inositol 1,4,5 trisphosphate ($IP_3$) generated from the cleavage of phosphatidyl inositol 4,5 bisphosphate (also known as PI (4,5)$P_2$ or $PIP_2$) could induce the release of $Ca^{2+}$ from intracellular stores [4] (Figures 1 and 2). This important observation provided new insight into the function of PLC in living organisms. Eventually, the PLCβ, PLCγ, PLCδ, PLCε, PLCη and PLCζ cDNAs were cloned [5–10]. Although $PIP_2$ is a minor phospholipid in the

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plasma membrane, it plays a central role in regulating a host of cellular processes. PLC is activated following stimulation of cells by either tyrosine kinase receptors, T-cell receptors, B-cell receptors, Fc receptors, integrin adhesion proteins or G protein-coupled receptors via cognate ligands including neurotransmitters, histamine, hormones and growth factors [11–15]. Signaling through PLC family members regulates diverse functions, which will be outlined within this chapter. In addition, we will discuss PLC mediated signaling, common structural domains found in this family of enzymes, current knowledge about the isoforms and areas that have yet to be explored.

## Cleavage of $PIP_2$ and signaling

PLC is a cytoplasmic protein that controls the levels of $PIP_2$ in cells by localizing within or outside of lipid rafts in the plasma membrane and catalyzing the hydrolysis of phosphorylated forms of phosphatidyl inositol in response to cellular stimuli (Figures 1 and 2). These enzymes have been reported to increase the rate of lysis of phosphatidyl inositol $>1000s^{-1}$ at 30°C at low concentrations of substrate, but is likely to reach rates of $>5000s^{-1}$ (as reviewed by [16]). Therefore, targeting of PLC to the plasma membrane plays a critical role in the functioning of this enzyme. The preferred substrate of PLC is $PIP_2$, a relatively uncommon phospholipid in the plasma membrane, followed by phosphatidyl inositol phosphate (PIP), and then phosphatidyl inositol (PI). Cleavage of $PIP_2$ leads to the generation of two products. One product, diacylglycerol (DAG), activates the calcium dependent protein kinase C (PKC), which then phosphorylates downstream effectors such as AKT to activate an array of cellular functions including regulating cell proliferation, cell polarity, learning, memory and spatial distribution of signals [17, 18]. DAG, which remains membrane bound, can then be cleaved to produce another signaling molecule, arachidonic acid. The second product of PLC action on $PIP_2$, $IP_3$ is a small water-soluble molecule, which diffuses away from the membrane, and through the cytosol to bind to $IP_3$ receptors on the endoplasmic reticulum inducing the release of $Ca^{2+}$ from intracellular stores found within the organelle [4]. In turn, the cytoplasmic calcium levels are quickly elevated and cause the characteristic calcium spike that signals cell activation. Once the endoplasmic reticulum stores have been used up, they are replenished through the store-operated calcium channels. $Ca^{2+}$ activates downstream transcription factors resulting in a plethora of gene activation pathways. In this way, signaling through PLC regulates proliferation, differentiation, fertilization, cell division, growth, sensory transduction, modification of gene expression, degranulation, secretion and motility [15, 19–26].

### Structure of PLC

There are thirteen different PLC family members that can be subdivided into six classes, β, γ, δ, ε, η and ζ (Figure 3). Different isoforms have been discovered in a wide range of species including mouse, rat and cattle. PLC-like isozymes have been found in *Drosophila melanogaster, Glycine max* (soybean), *Arabidopsis thaliana, Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [27, 28]. Overall, there is a low level of amino acid conservation between the family members; however, the similarity of the pleckstrin homology domains, the EF hand motifs, the X and Y domains and the C2 domains is greater than 40-50% [15]. Since these domains are common to all organisms they might represent a

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minimum requirement for a functioning PLC [29]. With the exception of the PH domain, which is not expressed on PLCζ, each family member shares all of the core domains. A description of each domain follows:

**Pleckstrin Homology (PH) Domains—**As mentioned, with the exception of PLCζ, all PLC family members have N-terminal pleckstrin homology (PH) domains which consists of approximately 120 amino acids, and is the eleventh most common domain in the human genome. PH domains are found in a large number of distinct protein families involved in signal transduction [30]. PH domains can mediate recruitment of the PLC family members to the plasma membrane via phosphoinositides. Computer simulations and crystal structures of the PH domain found in kindlins, proteins which co-activate integrin adhesion proteins, have revealed that PH domains consist of 7 beta sheets and an alpha helix, and that the beta sheets form the $PIP_2$ binding site [31]. Surface plasmon resonance studies have revealed a 1mM affinity for $PIP_2$ within lipid bilayers.

Notably, membrane binding of PLCδ to $PIP_2$ is blocked by high levels of intracellular $Ca^{2+}$ in hepatocytes due to generation of phosphoinositides [32]. This may also be due to the ability of $Ca^{2+}$ to regulate the conformation of the headgroup of $PIP_2$ [33]. Unlike the PH domain of PLCδ1, which uses the PH domain to bind to the $PIP_2$ in the membrane, the PH domain of PLCβ2, cannot bind to phosphoinositides [34].

PLCγ contains 2 PH domains, one in the N-terminus and a C-terminal split PH domain. This PH domain of PLCγ is unique, since it is split between two tandem Src homology domains [35]. Early on, it was found that the carboxy-terminal region of the PH domains of PLCγ, PLCβ2 and PLCβ3 control the binding of heterotrimeric G protein βγ subunits to PLC following activation of G protein-coupled receptors [36, 37]. Interestingly, the binding of $G_{\beta\gamma}$ to the PH domain, and the binding of $G_{\beta\gamma}$ to Gα are mutually exclusive [36]. Therefore, this competition for binding to $G_{\beta\gamma}$ implicates PLC activation in preventing the regeneration of the $G\alpha/G_{\beta\gamma}$ heterotrimeric G proteins. In this way[34], PLC activation may regulate the signaling of proteins that are turned on in response to stimulation of G protein-coupled receptors. Additionally, downstream of SDF1α (CXCL12) binding to the G protein-coupled receptor CXC chemokine receptor 4 (CXCR4), the PH domains of PLCε1 promote lipase independent activation of Rap1, which leads to β2-integrin-mediated recruitment and adhesion of T-cells to sites of inflammation [38]. Overall, from these observations it can be inferred that PH domains have multiple roles in regulating the signaling via PLC.

In contrast to PLC signaling through heterotrimeric G-protein, it should be noted that Rap1 which belongs to the Rap-family of small GTPases and Ras-family small GTPases are also involved in PLC signaling. Rap and Ras are small, closely related GTP binding proteins. While Rap is an important factor in cell junctions and cell adhesion, Ras is linked to cell proliferation and survival [39]. Both of these small, monomeric G proteins also play critical roles in signaling through PLC as will be discussed below:

**EF-hand motifs:** The EF-hand motifs are helix-loop-helix motifs present in a number of calcium-binding proteins, such as myosin, calmodulin, calreticulin and troponin [40]. EF-hand motifs were first described for PLC when the crystal structure analysis of PLCδ1

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revealed the characteristic helix-loop-helix motifs [41]. Within PLC, the EF-hand is part of the catalytic core that consists of an EF-hand, the X and Y and the C2 domains ([41] and see below). Upon binding to $Ca^{2+}$, the structure of PLC is stabilized as the EF-hand motifs undergo a conformational change to activate calcium-regulated functions, by exposing sites that become ligands for other proteins [42]. For example, in PLCβ, the EF-hands contain sites that mediate association with subunits of heterotrimeric G proteins, while in PLCγ, the EF-hands contain regions that lead to binding of tyrosine kinases [43]. Independent of the $Ca^{2+}$ concentration, deletion of the EF-hands in the enzyme reduces PLC function, [44]; however, binding of $Ca^{2+}$ to the EF-hand motifs can promote binding of PLC to $PIP_2$ via the PH domain. Lacking a PH domain, PLCζ may bind to membrane $PIP_2$ via cationic residues in the EF-hand [45] as well as the X-Y linker (as reviewed [46]).

**X and Y domains:** So far, only PLCδ1 and PLCβ2 have been crystallized and their structures analyzed [34, 41]. The X and Y domains consist of approximately 300 amino acids and lie at the C-terminus of the EF-hand motifs. These domains consist of alternating α-helices and β-sheets that form aβaβaβaβ motif with a triosephosphate isomerase (TIM) barrel-like structure [41]. The X-region, containing all of the catalytic residues, is somewhat conserved across the PLC family members [27, 41]. The X-region forms one half of the TIM-barrel like structure. Within the X-region lies histidine residues that support the generation of the 1,2 cyclic inositol 4,5-bisphosphate [47]. The catalytic activity of this domain increases as the concentration of $Ca^{2+}$ rises from 0.01μM to 10μM. Mutational analysis of rat PLCδ1 revealed that histidine$^{311}$ and histidine$^{356}$ , which are crucial for catalyzing the hydrolysis of $PIP_2$, have an important role within the X domain [47]. These residues are well conserved in PLC family members [47].

Structurally, the Y-domain (residues 489-606) forms the other half of the TIM-barrel-like architecture. This eightfold barrel structure is almost always found within an enzyme that regulates metabolism [48], although the functions of the enzymes are quite diverse. With the exception of an extended loop connection between the β5 and β6 strand, instead of a helix, this domain forms the second half of the TIM-barrel-like structure. This Y-domain is important for substrate recognition and regulates the preference of PLC for $PIP_2$, PIP and PI [49, 50].

PLCγ contains a unique region that splits the X and Y domains. This region contains the split PH domains at the ends and the middle consists of two N-terminal src homology (SH2) domains followed by an SH3 domain. The SH2 domains provide docking sites for tyrosine kinase growth factor receptors such as the platelet derived growth factor receptors (PDGFRs) and the epidermal growth factor receptors (EGFRs) to promote activation of this PLC family member [51–53]. The binding of tyrosine kinase receptors to PLCγ results in phosphorylation and activation of the enzymes [54, 55]. The SH3 domain directs the cellular localization of signaling proteins such as dynamin and the actin cytoskeleton. In addition, the SH3 domains have been found to mediate nerve growth factor-induced cell proliferation through activation of a guanine nucleotide exchange factor for phosphoinositide 3 kinase (PI3K) [56, 57].

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**C2 domains:** C2 domains are formed from about 120 amino acids [58] and can be found in more than 40 different proteins [41]. These motifs have several binding targets and have been implicated in signal transduction and membrane interactions. The C2 domains found within PLC family members are formed by an eight-stranded anti-parallel β-sandwich [41]. There are between three and four C2 domains found within PLCδ family members. In combination with $Ca^{2+}$, the C2 domain mediates the binding of PLCδ1 to anionic phospholipids to mediate signal transduction and membrane trafficking [43]. C2 domains have common structural motifs, which are found in PKCβ, rabphilin 3A [59, 60], and synaptotagmin I [61]. High cooperativity of calcium-dependent phospholipid binding sites implies that there are multiple sites that bind $Ca^{2+}$, which function synergistically [43].

C2 domains belong to the non-continuous $Ca^{2+}$-binding sites in which the $Ca^{2+}$-binding pockets are found far from each other in the amino acid sequence. In contrast EF-hands have binding pockets for $Ca^{2+}$produced by a stretch of continuous amino acids in the primary sequence [62, 63]. Functionally, the EF-hand motif, the most common $Ca^{2+}$ binding motif in proteins, may compete for binding to $Ca^{2+}$ with the C2 domains. The affinity of the EF-hand for $Ca^{2+}$ is within the nanomolar to millimolar range, which overlaps the micromolar to millimolar binding constants of C2 domains [64, 65]. This broad affinity of C2 domains for [$Ca^{2+}$] reflects the diversity of the functions of proteins containing the C2 domains over a wide range of calcium concentrations [66–68].

**PDZ domains:** PDZ (Post synaptic density (PSD)-95, Drosophila disc large tumor suppressor (DlgA), and Zonula occludens-1 protein (zo-1)) regions are separate from C2 domains, and are found in the C-terminal tails of PLCβ and PLCη lipases (Figure 1) [58]. The PDZ domains are formed by 5 of 6 β-strands and 2 or 3 α-helices [69]. This common structural motif is found in many signaling proteins, where it functions as a scaffold for large molecular complexes [70]. In this way, the motif links many proteins to signaling from the cytoskeletal membranes. It has been postulated that each PLCβ form may be used by different G protein-coupled receptors in regulating signaling events [71]. The sequences within the last five amino acids of the C-terminus are thought to regulate the specificity of the interaction of PLC with the Gα or Gβγ subunits [72].

## Roles of each PLC

As mentioned, there are six PLC family members (β, γ, δ, ε, η and ζ) consisting of thirteen different PLCs identified based on structure (Figure 3) and activation mechanism. There is no alpha form of PLC, since the protein that was originally described as the α form turned out to be a protein disulfide isomerase without phospholipase activity [73]. Under most conditions, PLC is a cytoplasmic protein that moves to the plasma membrane. Its role within the membrane lipid rafts is somewhat controversial. For instance, PLC has been shown to accumulate within lipid rafts that consist of cholesterol, sphingomyelin and ceramide, *Xenopus* egg activation, catalyzing the hydrolysis of $PIP_2$ within these frog eggs [16, 74]. In contrast, PLC associates with the tyrosine kinase HER2 within non-raft domains in ovarian cancer cells [72]. In eggs and in ovarian cancer cells, PLC catalyzes hydrolysis of $PIP_2$ to promote classic functions (Figure 3). With the exception of PLCγ2, there have been splice variants reported for each PLC isoform (as reviewed by [44] and [28]). For PLC a different

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gene encodes each isoform. The diversity of the PLC isoforms is created with splice variants. PLC isoforms are quite distinct in regard to tissue distribution, cell localization, expression and regulation. PLCβ and PLCγ are typically activated by extracellular stimuli and are termed first line PLC's, whereas PLCδ, ε, η and ζ are activated by intracellular stimuli and known as secondary PLC's [75]. For the purposes of this chapter, we will focus on the general properties described for each isoform.

## PLCβ$_{1,2,3,4}$

There are four isoforms of PLCβ that range in size from 130kDa for PLCβ4, 140kDa for PLCβ2, 150kDa for PLCβ1 and 152kDa for PLCβ3. In addition, splice variants have been reported for each of these isoforms [76–78]. The PLCβ subfamily consists of a well-conserved core structure with an N-terminal PH domain, four EF-hands, a split X +Y catalytic domain, C2 domain and an extended C-terminal domain (Figure 3). The catalytic domain being the most conserved domain of all PLC's isozymes with a substrate preference for PIP2 over PIP and PI [79]. PLCβ family members show distinct tissue expression and G protein regulation. PLCβ1 and PLCβ3 are ubiquitously expressed, whereas PLCβ2 and PLCβ4 are found only in hematopoietic and neuronal tissues, respectively [80]. These well-characterized isoforms of PLC are classically activated by G protein-coupled receptors and their catalytic activity is entirely dependent upon $Ca^{2+}$. All four PLCβ isoforms are activated by Gα$_q$ subunit. PLCβ2 and PLCβ3 can also be activated by βγ subunits of the Gα$_{i/o}$ family of G proteins and by small GTPases such as Rac and Cdc42 (Figures 1 and 3). In addition, PLCβ's are GTPase-activating proteins (GAPs) for the Gα$_q$ proteins that activate them [80, 81]. While Gα$_q$, Gα$_{11}$, and Gα$_{16}$ can activate PLCβ1, PCLβ2 and PLCβ3 family members [82]. In this case, the G protein-coupled receptor is stimulated by binding to its ligand, undergoing a conformational change to release Gα$_q$ or Gα$_{i/o}$ and Gβ/γ [81, 83, 84]. PLCβ is recruited to the membranes through interactions with Gβγ, but not Gαq [85]. In addition, PLCβ is recruited only through specific Gα subunits and the Gβγ subunits. These studies demonstrate that the PLC family members respond not only to Gα, but to Gβγ as well [37, 86]. Phosphoinositide-specific-phospholipase C β (PLCβ) is the main effector of Gα$_q$ stimulation that is coupled to receptors binding acetylcholine, dopamine, bradykinin, angiotensin II, other hormones and neurotransmitters [87].

The PLCβ family members have an additional 450 amino acid residues in the C-terminus (Figure 3). While all PLCβ family members have been found in the nucleus, PLCβ1 is the major nuclear PLC [88–90]. Within this C-terminal 450 amino acid region, lies the greatest dissimilarity between PLC family members. In this region of the PLCβ1a and 1b splice variants is a nuclear localization signal, which directs localization of PLCβ1 isoforms, mostly to the nucleus while a nuclear export signal allows PLCβ1a to remain in the cytosol [77]. The likely consequence of DAG generation inside the nucleus is activation of nuclear PKC [91, 92]. Nuclear PLCβ1 regulates the cell cycle by modulating cyclin levels with cells overexpressing PLCβ1 producing increased levels of Cyclin D3 and a higher percentage of cells in S phase, in an erythroleukemia cell line [92, 93]. The binding site for Gαq is found within a region that mediates activation of Gαq by regulator of G protein signaling 4 (RGS4) and G alpha interacting protein (GAIP), which are GTPase-activating proteins

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(GAPs)[94]. This binding site blocks activation of PLCβ [95]. PLCβ1 is expressed at high levels in the cerebral cortex, retina, hippocampus and cardiomyocytes [96–98].

As mentioned, the expression of PLCβ2, which shares 48% identity with PLCβ1, appears to be restricted to cells of the hematopoietic lineages [99]. PLCβ2 can be activated by Rac, a member of the Rho-family of kinases [100]. The PH domain of PLCβ2 mediates binding of active forms of Rac (Rac1, Rac2 and Rac3), which leads to activation [101]. In contrast to PLCβ1 and PLCβ2, PLCβ3 lacks 10-20 amino acids within its C-terminus [102], although the significance of this difference is unknown. This PLC isoform is expressed by liver, brain and parotid gland [102].

PLCβ1 and PLCβ4 are expressed within the brain including the cerebral cortex, amygdala, hippocampus, and olfactory bulb and are thought to be involved in brain development and synaptic plasticity [91, 103–105]. Mis-regulation of PLCβ1 and/or PLCβ4 have been linked to several brain conditions such as schizophrenia, epilepsy, depression, Alzheimer's disease, bipolar disease and Huntington's disease [105–107]. In addition, studies of PLCβ1$^{-/-}$ mice revealed roles for PLCβ1 in regulating vision and central nervous system homeostasis and loss of PLCβ1 can lead to seizures and sudden death [108].

PLCβ1 plays important roles in cell differentiation, particularly in osteogenesis, hematopoiesis and myogenesis [79, 80, 109]. At least for myogenic differentiation, PLCβ1 signaling involves inositol polyphosphate multikinase and β -catenin as downstream effectors. By means of c-jun binding to cyclin D3 promoter, the activation of PLC β 1 pathway determines cyclin D3 accumulation and muscle cell differentiation [110]. Also, PLCβ participates in the differentiation and activation of immune cells involved in both the innate and adaptive immune systems including, macrophages, neutrophils, mast cells, T cells and B cells [79]. Consistent with a role of PLCβ3 in neutrophil development, it was reported that PLCβ3$^{-/-}$ mice develop myeloproliferative neoplasm with increased mature neutrophils [80].

A role for PLCβ in several cancers has been proposed. Recently, it has been reported that PLCβ2 acts as a negative regulator of triple negative breast cancer since up-regulation in invasive triple negative breast cancer cells was sufficient to lower the expression of surface antigens required for malignancy and to reduce the number of cells with a stem-like phenotype suggesting that enhancing PLCβ2 expression is a potential therapy for triple negative breast cancer [111]. Similarly, a high expression of PLCβ1 was associated with an enhanced long-term survival of patients with a proneural subtype high grade gliomas [112] and patients affected by myelodysplastic syndromes showed a reduced propensity to develop acute myeloid leukemia when the expression of nuclear PLCβ1 was reduced [91].

## PLCγ$_{1,2}$

There are two isoforms of PLCγ, PLCγ1 and PLCγ2. PLCγ1 is ubiquitously expressed, and operates downstream of tyrosine kinase growth factor receptors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), PDGF and EGF, whereas PLCγ2 is primarily expressed in hematopoietic cell lineages, often functioning downstream of immune cell receptors (Figure 1 and [113, 114]). PLCγ subtypes are primarily activated

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by receptor tyrosine kinases (RTKs). Both PLCγ1 [115] and PLCγ2 can be activated by adhesion receptors, such as integrins [116]. PLCγ1 signaling acts via direct interactions with other signaling molecules via SH domains, as well as its lipase activity [117]. Some PLCγ signaling via nonreceptor tyrosine kinases has been reported [118, 119], including the B-cell receptor and via the Spleen tyrosine kinase (Syk)-activated PLCγ2 signaling in T cells[120] or osteogenic differentiation of bone marrow stromal cells [121]. PLCγ has important roles in differentiation, proliferation, transformation, calcium flux and tumorigenesis [22, 25, 122, 123]. In addition, it has been shown that PLC γ1 is activated by Src tyrosine kinase in Xenopus [124].

PLCγ can regulate proliferation by functions that are independent of its lipase activity. One example is that DNA synthesis does not require phospholipase function, but instead is regulated through the SH3 recruitment of a Ras exchange factor, SOS1 [125]. In addition to the PH domain found in the N-terminus, these PLCγ family members have a second PH domain, which is split into an N-terminal domain of the PH domain that flanks two SH2 domains, followed by an SH3 domain and a C-terminal PH domain (Figure 3). This C-terminal is thought to bind directly to the TRPC3 calcium channel, which then leads to agonist-induced calcium entry into the cell [35]. In addition, Vav1, c-Cbl and Slp76, via interactions with either the SH3 domain or the C-terminal SH2 domain are also required to help stabilize the recruitment of PLCγ1to the plasma membrane [126]. PLCγ2 and PKC are important upstream signals of macrophage-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor (G-CSF) that regulate myelopoiesis through cytokine production. These pathways activate ERK1/2, NFAT and JAK1/STAT-3 pathways [127]. PLCγ isoforms have been reported to be expressed in several innate immune cell types, including natural killer cells, macrophages, neutrophils and mast cells [128–131]. PLCγ activates the innate immune system by regulating respiratory bursts, phagocytosis, cell adhesion, and cell migration. PLCγ also modulates the inflammatory response by controlling Toll-like receptor-mediated signaling [132]. T cells express more PLCγ1 than PLCγ2 and PLCγ1 is activated by ligation of the T cell antigen receptor [126] and recruitment of PLCγ1 by Linker of Activated T cells (LAT) to the plasma membrane [133]. Phosphorylated LAT, in turn, serves as the primary docking site for the amino terminal SH2 domain of PLCγ1 to the membrane [134, 135]. All three SH domains of PLCγ1, however, are required to stabilize association of PLCγ1 with LAT, which is required to activate T cells [126]. Following engagement of the TCR, PLCγ1 production of DAG leads to activation of not only PKC, but also Ras guanyl releasing protein (GRP)-dependent signaling events [136, 137].

PLCγ1 is also activated by certain G protein-coupled receptors. We have shown that PLCγ1 can be activated following stimulation by the G protein-coupled receptor, C-C chemokine Receptor 7, a $G\alpha_{i/o}$ receptor, to mediate activation of β1 integrin, heterodimeric adhesion receptors [138]. In addition, PLCγ1 and PLCγ2 are both activated by the angiotensin and bradykinin G protein coupled receptors.

Homozygous disruption of PLCγ1 in a mouse model revealed that this PLC plays an essential role in growth and development [139]. In the absence of PLCγ1, the mice die at day E9.0, although until that stage of development the embryos appear normal. This mouse

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model revealed that although other PLCγ family members might be available, the role of PLCγ1 is essential and is not compensated by another PLC. In contrast, homozygous deletion of PLCγ2 leads to defects in platelet functions that are stimulated through β1 and β3 integrin adhesion proteins [140, 141]. PLCγ2 plays an essential role in B cell development, and function [20, 26]. Similar to PLCβ2, Rac, a member of the Rho-family of GTPases, can bind to and activate PLCγ2 [100]. This PLC family member can be activated through interactions with growth factor receptors, via phosphorylated tyrosines within their cytoplasmic tails via their intracellular tyrosine activation motifs (ITAMs). PLCγ2 also regulates calcium oscillations induced by the transcription factor, Nuclear Factor of Activated T cells (NFAT). Additionally, the SH2 domains can mediate activation of this receptor.

A role for PLCγ in neural development and certain neurological condition has become increasingly evident. PLCγ1 is highly expressed in the brain and is required for normal neuronal development and activation [114]. Since deregulation of PLCγ1 activation in response to brain derived neuronal factor can alter calcium influx and actin rearrangements that control neuronal migration, this PLC has been linked to diverse neurological disorders, including epilepsy, Huntington's disease and depression [114]. In this case mis-regulation of PLCγ1 function has been observed in animal models of Huntington's disease [142]. Moreover, genomic analysis has revealed a PLCγ2 variant that appears to be protective against Alzheimer's disease, possibly acting via microglia-modulated immune responses [143]. Other physiological roles for PLCγ are provided by recent evidence suggesting that PLCγ1 activates Akt-mediated Notch1 signaling, which is required for intima formation of blood vessels, and also plays a role in influenza viral entry into human epithelial cells [144, 145].

PLCγ1 is often mutated and highly expressed in several cancers being involved in tumorigenic processes including migration, invasion and in some cases, proliferation (as reviewed by [146]). Moderately to poorly differentiated breast tumors showed significantly higher levels of PLCγ1, compared with well differentiated tumors [147, 148]. Also, three distinct mutations in PLCγ2 were described in patients with chronic lymphocytic leukemia that were resistant to Ibrutinib treatment [148]. Indeed, studies have shown that mutated DNA sequences associated with human cancers and autoimmune diseases are well conserved between PLCγ1 und PLCγ2 and these mutations are gain-of-function effectors that destabilize normal regulatory signaling [149].

### PLCδ[1, 3, 4]

There are three identified isoforms of PLCδ with similar amino acid sequences that are highly evolutionary conserved from lower to higher eukaryotes [150]. PLCδ family members are activated by levels of calcium that are normally found in the cytoplasm ($10^{-7}$M to $10^{-5}$M), making them one of the most calcium sensitive PLC isoforms [151, 152]. While PLCδ1 is localized to the cytoplasm in quiescent cells, this PLC isoform shuttles between the nucleus and the cytoplasm in active cells [153]. Human PLCδ4 was found to be primarily nuclear in human adipose derived mesenchymal stem cells [154]. Depletion of PLCδ1 leads to a block in the cell cycle [155]. PLCδ family members are thought to have a

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role in potentiating calcium signaling [151]. This form of PLC is similar to non-mammalian forms of PLC [15, 156] PLCδ1 can be activated by $G_{i/o}$ and $Ga_q$ following stimulation of G protein-coupled receptors [157]. PLCδ is involved in regulating the activation of the actin cytoskeleton. Studies using PLCδ knockout mice have shown that PLCδ1 is required for maintenance of skin homeostasis; a recent study suggested that PLCδ1 is required for epidermal barrier integrity [158], whereas PLCδ3 regulates microvilli genesis within the intestine and the directed migration of neurons in the cerebral cortex of developing brains [159, 160]. Knockout of both PLCδ1 and PLCδ3 resulted in embryonic lethality [161].

Similar to PLCγ1, mis-regulation of PLCδ1 has been linked to Alzheimer's disease [162]. Interestingly, this enzyme function is inhibited by sphingomyelin, a membrane lipid that is found in high concentrations in neurons. PLCδ1 is also mis-regulated in rat models of hypertension [163]. In addition, a decrease in PLCδ1 downregulation in cystic fibrosis cells resulted in dysregulation of Transient Receptor Potential Vanilloid 6 channel activity leading to an increase in the constitutive calcium influx, exacerbating cystic fibrosis effects [164].

PLCδ1 is expressed at high levels in hair follicles. Homozygous deletion of PLCδ1 leads to hair loss [165, 166]. The hair loss was due to an increase in leukocytes, specifically macrophages, neutrophils and T cells within the hair follicle [166]. Homozygous deletion of *Plcδ3* or *Plcδ4* had no apparent affect and the mice appeared normal.

During fertilization, a transient increase in $Ca^{2+}$ precedes egg activation. Like other forms of PLC, this isoform appears to play a role in fertilization. Notably, PLCδ4$^{-/-}$ male mice are sterile [167, 168]. Even when PLCδ4$^{-/-}$ sperm were injected into eggs, few viable embryos developed. These studies implicate this family member in the regulation of fertilization [167]. In the same study, sperm isolated from PLCδ4 knockout mice were found to be inferior to sperm isolated from wild type mice in that the $Ca^{2+}$ oscillations in these mice were delayed or did not occur at all [167].

Similar to several other PLC's, PLCδ's role in carcinogenesis is controversial. In one study, high expression levels of PLCδ significantly correlated with a shorter disease-free survival of patients with poorly-differentiated breast tumors suggesting a possible role as a tumor promoter [147]. In contrast, an unrelated study found that downregulation of PLCδ1 in breast cancers induced cell migration and invasion in an *in vitro* assay by inhibiting the phosphorylation of ERK1/2, suggesting a role as a tumor suppressor [169]. In support of the tumor suppressor effects, another study in colorectal cancer revealed that expression of PLCδ1, as shown by immunohistochemistry, was down-regulated in colorectal cancer samples, which was also linked to suppression of ERK1/2 phosphorylation [170] and increased autophagy of the colorectal cancer cells [171]. These results are in line with the concept that PLCδ1 may function as a tumor promoter or as tumor suppressor [147], and it is clear that further studies are needed to clarify the role of PLCδ in carcinogenesis.

## PLCε

PLCε is the largest of the PLC family members with an apparent molecular weight of ~230 kDa and was originally described in 1998 as a Let-60 Ras binding protein [172]. Two splice variants of PLCε have been reported, termed PLCε1a and PLCε1b that are widely

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expressed, but distinct roles for these variants have not been described [173]. PLCε is expressed at the highest levels in the heart, liver and lung, but can also be found in the skeletal muscle, spleen brain, lungs, kidneys, pancreas, testis, uterus, thymus and intestine [7, 174, 175]. This class of PLC, which was originally identified in *Caenorhabditis elegans*, and was later cloned in humans [7, 172, 174, 175]. The Ras-associated (RA) domains consist of approximately 100 amino acids that interact directly with the Ras-family GTPases, Ras [7, 175] and Rho [176]. A point mutation at a lysine residue in the RA2 domain of PLCε is sufficient to prevent Ras binding of the enzyme in a GTP-dependent manner [7]. Subsequently, it was found that PLCε could also be activated by the $G\alpha_{12}$ and Gβ/γ released by activated G protein-coupled receptors [175, 177]. Later, it was shown that hydrolysis of Golgi-associated phosphatidylinositol 4- phosphate (PI4P) in cardiac myocytes is mediated by Gβγ via the RA2 and N-terminal CDC25 and cysteine-rich domains [178, 179]. G protein-coupled receptors that activate PLCε include the adrenergic and PGE receptors. At the same time Gαs has been shown to stimulate activation of PLCε [180] while $G\alpha_{12}$ and $G\alpha_{13}$ can activate RhoA which can stimulate PLCε [180, 181]. Not only is this PLC family member activated by Ras and RhoA, it can also function as a guanine nucleotide exchange factor (GEF) for the Ras superfamily of GTPases [175]. In a contrasting study, the CDC25 domain of PLCε was found to serve as a GEF for Rap1 but not for other Ras family members [182]. These characteristics of PLCε reveal that this enzyme can be activated not only by subunits of heterotrimeric G proteins, but also by small GTPases.

This ability of PLCε to be regulated by both Ras and Rho suggest that it can contribute to both proliferation and to migration. More interestingly, since PLCβ can be activated by Rho, both PLC family members may work together to regulate signal transduction pathways that are activated following stimulation of cells by Rho to control cell migration. Similarly, since PLCε can be regulated by Ras, a downstream effector of PLCγ signaling following activation of growth factor receptors such as the epidermal growth factor (EGF) receptor, the signaling pathways may work together to promote proliferation. The ability of PLCε to coordinate signaling through these pathways points to regulatory mechanisms that may be more complex than originally thought.

Since PLCε can regulate inflammatory ligands for G protein-coupled receptors, it was suggested that PLCε may protect against ischemia/ reperfusion injuries [183]. In contrast, in a separate study it was shown that PLCε is often upregulated in patients with heart failure [184] and recently it was shown that chronic activation of this isoform leads to cardiac hypertrophy [178]. Additionally, PLCε-null mice have abnormal development of aortic and pulmonary valves [185]. The role of PLCε in carcinogenesis is controversial, although the enzyme is thought to play important roles in the regulation of cancer development and progression, possibly acting as either an oncogene or tumor suppressor depending upon the type of tumor [186, 187]. Inflammatory processes induced by PLCε are thought to be involved in the progression towards cancer [188]. Mutation analysis of the PLCE1 gene landscape via The Cancer Genome Atlas (TCGA) database showed that PLCE1 is an often-mutated gene in several types of cancer, in particular digestive tract cancer such as gastric cancer and esophageal squamous carcinoma, but also including skin cancer, lung cancer and head and neck cancers [187].

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### PLCη1,2

PLCη consists of two members that are the most recently discovered PLC's and are most closely related to PLCδ subtype [189]. The sequence homology between PLCη1 and PLCη2 are ~50% similar. PLCη1 has an apparent molecular weight of 115kDa in mouse and humans, while PLCη2 is larger at 125kDa. PLCη can be activated by G protein-coupled receptors and RTK's [190] with PLC activity amplified by both intracellular $Ca^{2+}$ mobilization and extracellular $Ca^{2+}$ entry [191]. PLCη sequence analysis showed a novel EF-hand domain including a non-canonical EF-loop 2 sequence that is responsible for the enhanced binding of $Ca^{2+}$ and enhanced hydrolysis of $PIP_2$ [189]. The PLCη1 and PLCη2, isoforms are localized to the brain and neurons and are extremely sensitive to changes in calcium levels within the physiological range [8, 9, 192, 193]. Like PLCδ, this form of PLC responds to the 100nM calcium concentrations found inside the cell [194]. However, PLCη is more sensitive than PLCδ [8] and PLCη can modulate a sustained $Ca^{2+}$ release via production of $IP_3$ [189].

PLCη2 is expressed in the infant brain, specifically in the hippocampus, cerebral cortex and olfactory bulb [9], where it may play an important role in calcium mobilization required for axon growth and retraction, growth cone guidance, the generation of synapses and neurological responses [9]. In humans, loss of the human chromosomal region, which encodes PLCη2 has been linked to mental retardation [195] and role for PLCη2 in neurite growth has been postulated [196]. Alzheimer's disease has been linked to altered calcium homeostasis within neurons of the central nervous system with calcium accumulation occurring in disease affected neuronal cells [197]. Since PLCη is expressed in these same regions of the brain, a potential role for PLCη in Alzheimer's disease pathogenesis has been postulated [197].

### PLCζ

PLCζ is the smallest of the mammalian PLC family members with a molecular mass of ~70 kDa in humans and ~74 kDa in mice [10, 198]. Interestingly, studies have shown PLC-like activities in plants with non-specific PLC hydrolyzing membrane phospholipids like phosphatidylcholine (PC) and phosphatidylethanolamine and another PLC with structural similarities to PLCζ [29]. In mammals, PLCζ expression has been confined to sperm heads [10, 198, 199] where it serves to activate eggs during fertilization [10, 200]. Subsequent studies have also identified further mammalian orthologues of PLCζ in human, hamster, monkey, and horse sperm [201, 202]. Although some studies suggested the possibility that a post-acrosomal sheath WW domain-binding protein, termed PAWP, could be responsible for eliciting $Ca^{2+}$ oscillations at egg activation [203–205], more recent studies now convincingly suggest that PAWP is not required to stimulate $Ca^{2+}$ oscillations during egg activation, while strong evidence supports PLCζ as a soluble sperm factor responsible for the $Ca^{2+}$ oscillations [206–210].

In line with its key role as a sperm factor, PLCζ generally localizes to distinct regions of the sperm head in mammals [211–213]. In humans, three distinct populations of PLCζ within the sperm head have been determined in the acrosomal, equatorial and post-acrosomal regions [211, 214–216]. Although this is the only isoform of PLC identified, which lacks the

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N-terminal PH domain, it shares the closest homology with PLCδ1 [217]. The absence of the PH domain demonstrates that presence is not required for membrane localization of PLCζ. It is unclear, however, how PLCζ targets the plasma membrane in the absence of the PH domain. There is some indication that the C2 domain may contribute to targeting PLCζ to membrane-bound $PIP_2$. Following fusion of sperm with the egg, PLCζ is released into an egg, which until that point, is arrested at the second meiotic division. $Ca^{2+}$ oscillations that mediate activation of an egg are due to $IP_3$ mediated $Ca^{2+}$ release. The presence of PLCζ within the cytoplasm leads to $Ca^{2+}$ oscillations, which are classically observed during activation of the egg and release from the meiotic arrest [218]. In addition, immuno-depletion of PLCζ suppresses $Ca^{2+}$ release. After the egg is fertilized the $Ca^{2+}$ oscillations end when the pronuclei merge [219, 220]. Sperm from infertile men who are unable to activate eggs have been reported to exhibit reduced or abolished types of PLCζ [214, 216, 221]. Also, the proportion of sperm expressing PLCζ correlates with fertilization rates following intracytoplasmic sperm injection making PLCζ a diagnostic marker of fertilization [75].

## Methods to inhibit PLC

There are several chemical inhibitors that can be used to block PLC function. A commonly used pan inhibitor, 1-[6-((17β-3-methoxyestra-1,3,5(10)trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione, (U73122), of phospholipase C, is thought to function by blocking translocation of the enzyme to the membrane [222]. For example, using 2μM U73122 in contrast to the control U73343, we found that stimulation of CCR7 through one of its ligands, CCL21 [138], but not CCL19 promoted PLC dependent migration of T cells via β1 integrin adhesion proteins. In the same study were able to determine that the PLCγ1 isoform regulated migration by preventing CCL21 directed migration with targeted siRNA. This data suggests that one G protein-coupled receptor can activate PLCγ1 through two different ligands to control migration in T cells. In this case we speculate that PLCγ1 mediates integrin activation through inside-out signaling leading to activation of β1-integrins.

Recently, it has been shown that U73122 forms covalent associations with human PLCβ3, when the phospholipase is associated with mixed micelles [223]. While U73122 has been used as a pan inhibitor of PLC in numerous studies [21, 138, 224–228], in the study by Klein et al., instead of inhibiting PLC, U73122 activated human PLCγ1, human PLCβ2 and human PLCβ3, which had been incorporated into micelles to differing magnitudes. Since the PLC used in these studies was in a purified form, it is unclear, how U73122 functions to regulate the extent of PLC activation. In a second study, 1μM U73122 was found to directly inhibit G protein activated inwardly rectifying potassium channels. This was in contrast to a second PLC inhibitor, 2-Nitro-4-carboxyphenylN,N -diphenylcarbamate (NCDC), which did not have that effect [229]. NCDC, however, is also thought to have non-specific effects that are not related to PLC functions [230].

It should also be noted that in rabbit parietal cells, use of the U73122 led to a number of unexpected effects including mis-regulation of $Ca^{2+}$ mobilization, and acid secretion induced by an agonist. Of equal concern, the negative control U73343 blocked acid secretion [231]. Therefore, this PLC inhibitor when used, should be used with caution.

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Similarly, there are at least three other known inhibitors and two activators of PLC, yet they are not specific. These inhibitors include O-(Octahydro-4,7-mthano-1H-iden-5-yl)carbonopotassium dithioate, [232], Edelfosine [233] and RHC 80267 (O,O'[1,6-Hexanediyl*bis*(iminocarbonyl)]dioxime cyclohexanone) [234]. The two activators are *m*-3M3FBS (2,4,6-Trimethyl-*N*-[3-(trifluoromethyl)phenyl]benzenesulfonamide) , and the ortho version *o*-3M3FBS [235].

Heterozygous deletion of a specific PLC family member via siRNA, however, can yield targeted results [138]. As mentioned, in these studies, PLCγ1 specific siRNA was used to confirm the role of this PLC isoform in the regulation of β1 integrins during the adhesion of primary T cells. In the future it may be advisable to determine the specific PLC family member involved in a cellular response, by using siRNAs. More recently the discovery of Clustered Regularly Spaced Short Palindromic Repeats-Cas9 (CRISPR Cas9) technology, which was originally described in bacterial systems, allows for long-term targeted disruption or in some cases activation of specific genes[236, 237]. This technology, will likely be used to target specific PLC isoforms in the future.

The highly specific 3-phosphoinositide-dependent protein kinase 1(PDK1) inhibitor 2-O-benzyl-myo-inositol 1,3,4,5,6-pentakisphosphate (2-O-Bn-InsP5) can also block PLCγ1 dependent cell functions such as EGFR-induced phosphorylation of PLCγ1. This interaction takes place through the PH domain of PDK1. The loss of phosphorylation blocks PLCγ1 activity and downstream the cell migration and invasion [238], and has been considered as a lead compound for an anti-metastatic drug.

## Future Directions

### Hierarchy of isozymes

It is unclear how the different isoforms of PLC are activated in cells receiving multiple stimuli from different receptors. With thirteen identified isoforms, expressed in multiple cell types, it will be important to define how the different signaling events that are linked to each isoform are controlled. Since PLC activation leads to release of $IP_3$ and DAG in response to activation, it will be important to determine how cells discriminates between multiple PLC signals to determine the hierarchy, intensity and duration of signaling events. As mentioned, PLCβ2 and PLCγ2 are activated by Rac while PLCε is activated by RhoA. These observations suggest that key regulators of cell motility function through different PLC family members, and may have pivotal roles in defining where and when a cell migrates.

PLC enzymes are found in every cell in the body, where they play critical roles in regulating diverse cellular responses (as reviewed in [28]). As mentioned, some family members serve as scaffolds for other signaling proteins, while others can serve as GAPs or GEFs, for secondary signaling proteins. Other PLCs function to amplify the $Ca^{2+}$oscillations in the cell. Certain PLC family members can travel to the nucleus to control signaling there. With PLC family members playing key roles in numerous cell functions, it will be important to define how each PLC is regulated and how the cellular environment affects the duration and intensity of the response.

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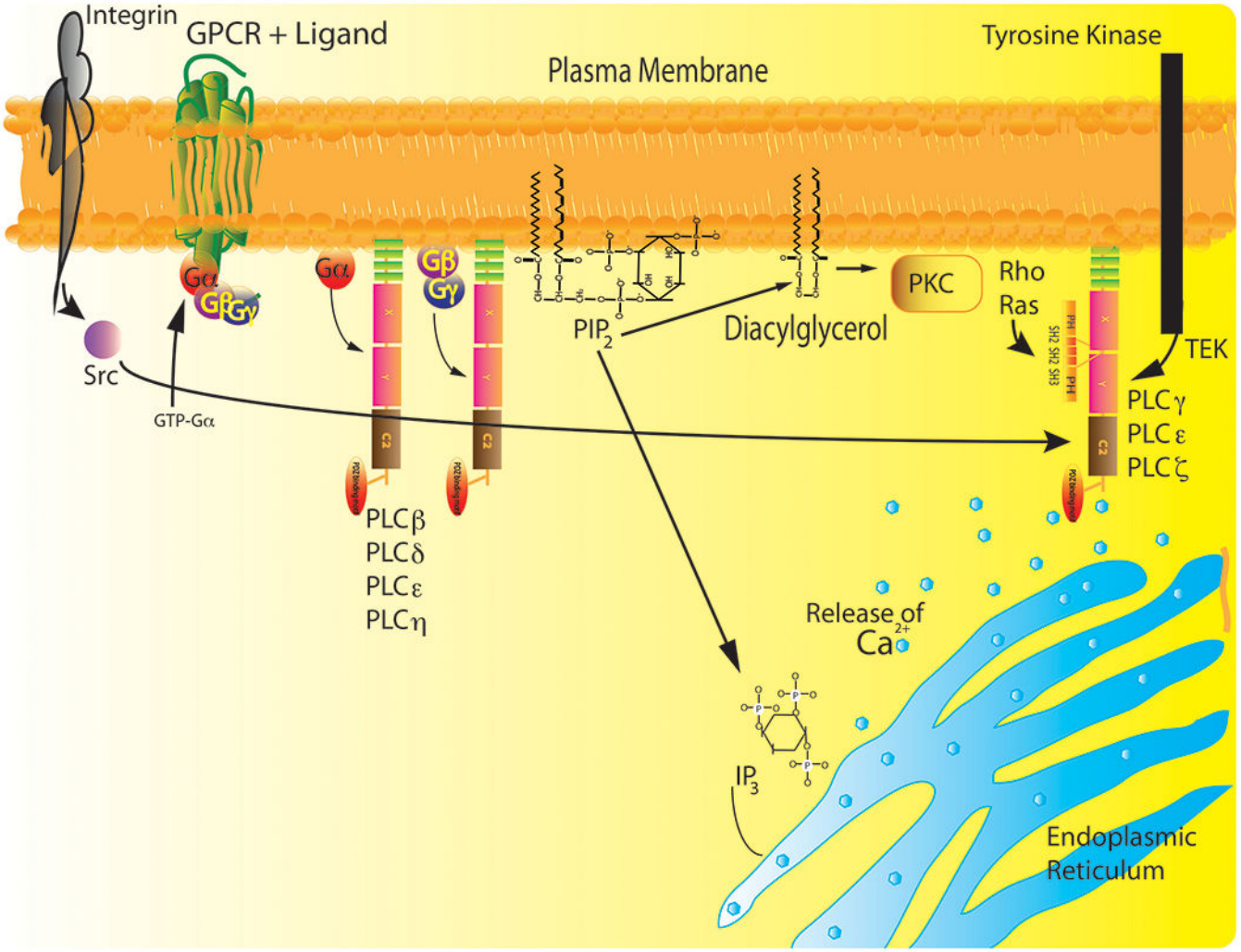


**Figure 1. Three major pathways for activating phospholipase C (PLC).**

PLC can be activated by either tyrosine kinase receptors, T-cell receptors, B-cell receptors, Fc receptors, integrin adhesion proteins or G protein-coupled receptors (GPCRs) via cognate ligands including neurotransmitters, histamine, hormones and growth factors to promote signaling and $Ca^{2+}$ mobilization. For simplicity, this figure shows activation by integrins, GPCRs and tyrosine kinases only.

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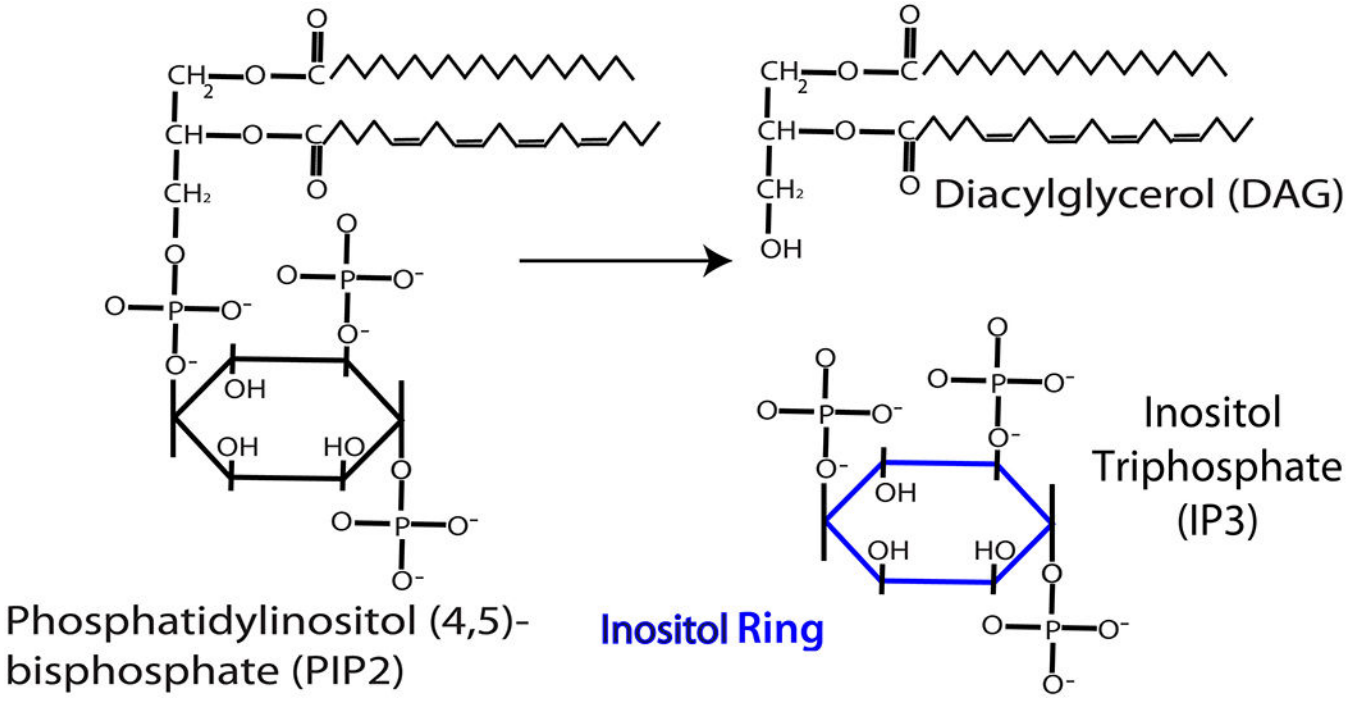


**Figure 2. $PIP_2$ cleaves to produce diacylglycerol and inositol triphosphate.**

Phosphatidylinositol (4,5) – bisphosphate ($PIP_2$) is cleaved by PLC to release diacylglycerol (DAG), which remains membrane bound and Inositol triphosphate ($IP_3$). The inositol ring is outlined in blue.

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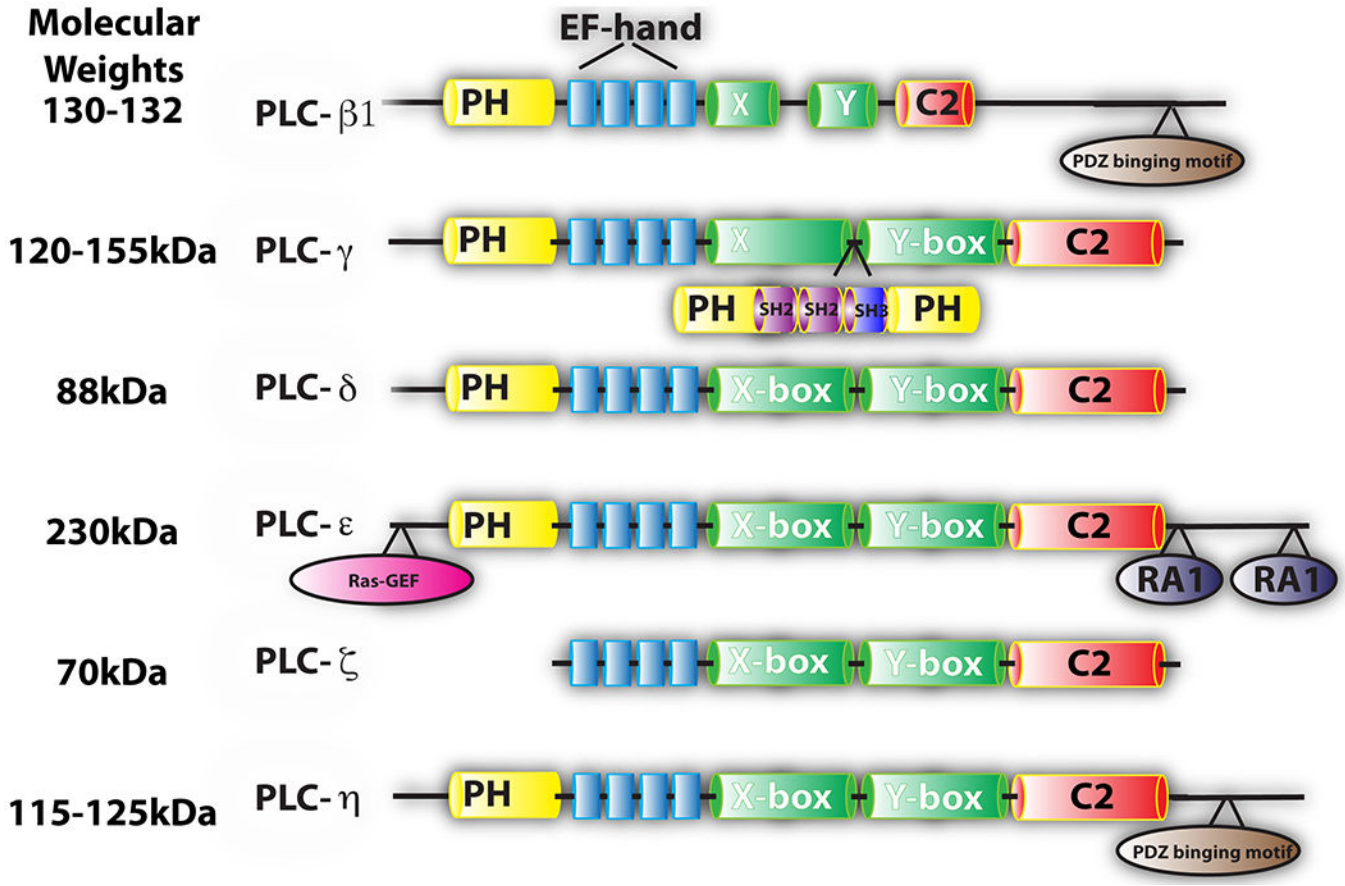


**Figure 3. Structures of the 6 different identified members of the PLC family.**

Relative positions of pleckstrin homology domain (PH), EF-hand, X and Y domains and the C2 domains are shown. Unique domains found in individual family members include the: Post synaptic density (PSD)-95, Drosophila disc large tumor suppressor (DlgA), and Zonula occludens-1 protein (zo-1) (PDZ), src homology 2 (SH2) and src homology 3 (SH3), Ras-GEF binding, and Ras associated (RA) domains. The PDZ domain is only found in one spliced form of PLCη.