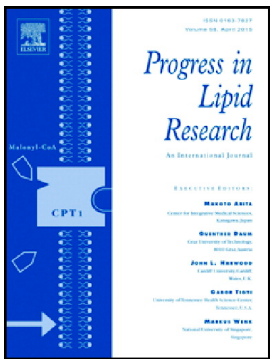

Phospholipase C families: Common themes and versatility in physiology and pathology

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Phospholipase C families: common themes and versatility in physiology and pathology

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Contents

List of abbreviations

AKT, serine/threonine kinase (also known as PKB);
APLAID, Autoinflammation, antibody deficiency, and immune dysregulation;
CDC25, cell division cycle 25;
cIP, cyclic inositol-1,2-phosphate;
Cryo-EM, Single particle cryo electron microscopy;
CTD, C-terminal domain;
DAG, diacylglycerol;
DCC, deleted in colorectal cancer;
ER, endoplasmic reticulum;
FMLP, FMetLeuPhe;
GADS, Grb2-related adaptor downstream of Shc;
G_h, transglutaminase II;
GH, Growth Hormone;
GPCR, G-protein-coupled receptor;
GPI, Glycosylphosphatidylinositol;
HSC, haematopoietic stem cells;
HDX-MS, Hydrogen Deuterium exChange-mass spectrometry;
$I(1,4,5)P_3$, inositol(1,4,5)trisphosphate;
ICSI, intracytoplasmic sperm injection;
IL-8, interleukin 8;
INAD, inactivation no after potential;
ITAM, Immunoreceptor tyrosine-based activation motif;
ITK, IL-2-inducible T cell kinase;
Jak2, Janus kinase 2
LAT, linker for activation of T cells;
LPS, Lipopolysaccharide;
LSC, leukemia stem cells;
Munc, mammalian uncoordinated,
ORPs, oxysterol binding protein-related proteins;
PH, pleckstrin homology;
PLAID, PLCγ2-associated antibody deficiency and immune dysregulation;
PLC, phospholipase C;
$PI(3,4,5)P_3$, phosphatidylinositol(3,4,5)trisphosphate;
$PI(4,5)P_2$, phosphatidylinositol(4,5)bisphosphate;
PI(4)P, phosphatidylinositol(4)phosphate;
PKC, protein kinase C;
PKD, protein kinase D;
PM, plasma membrane;
PS, phosphatidylserine;
PTP-1B, protein tyrosine phosphatase-1B;
RA, Ras association;
RTK, receptor tyrosine kinase;
SHP-1, SH2-containing phosphatase-1;
SLP-76, SH2 domain-containing leukocyte protein of 76kDa;
Stat5, Signal transducer and activator of transcription 5;
TAK1, TGFβ-activated kinase 1;
TRPM5, Transient Receptor Potential melastatin 5;
TRPV1, Transient Receptor Potential Vanilloid 1;

Abstract

Phosphoinositide-specific phospholipase Cs (PLCs) are expressed in all mammalian cells and play critical roles in signal transduction. To obtain a comprehensive understanding of these enzymes in physiology and pathology, a detailed structural, biochemical, cell biological and genetic information is required. In this review, we cover all these aspects to summarize current knowledge of the entire superfamily. The families of PLCs have expanded from 13 enzymes to 16 with the identification of the atypical PLCs in the human genome. Recent structural insights highlight the common themes that cover not only the substrate catalysis but also the mechanisms of activation. This involves the release of autoinhibitory interactions that, in the absence of stimulation, maintain classical PLC enzymes in their inactive forms. Studies of individual PLCs provide a rich repertoire of PLC function in different physiologies. Furthermore, the genetic studies discovered numerous mutated and rare variants of PLC enzymes and their link to human disease development, greatly expanding our understanding of their roles in diverse pathologies. Notably, substantial evidence now supports involvement of different PLC isoforms in the development of specific cancer types, immune disorders and neurodegeneration. These advances will stimulate the generation of new drugs that target PLC enzymes, and will therefore open new possibilities for treatment of a number of diseases where current therapies remain ineffective.

Keywords: Phospholipase C families; enzyme activity; mechanism of activation; Phosphatidylinositol(4,5)bisphosphate; Disease development; Lipid signaling;

1. Introduction

The control of cellular processes by a plethora of extracellular stimuli involves inositol lipid signaling, triggered by their specific cognate receptors. Upon activation of different types of eukaryotic cells, phospholipase C (PLC) selectively catalyses the hydrolysis of the minor membrane lipid, phosphatidylinositol(4,5)bisphosphate [PI(4,5)P_2] resulting in its decrease, and an increase in water-soluble inositol(1,4,5)trisphosphate [I(1,4,5)P_3] and membrane-bound diacylglycerol (DAG) **(Figure 1)**. The main current concepts for PLC signalling are based on the ground-breaking work between 1950s and 1980s that established the importance of inositol lipids in cellular responses to external stimulation (for insights by the key researchers see [1-5]). Both products of PLC hydrolysis, I(1,4,5)P_3 and DAG, are second messengers regulating a range of functions by engaging ever-increasing number of protein targets and also through their further conversion by metabolic enzymes. I(1,4,5)P_3 binds to IP_3 receptors present at the ER to release Ca^{2+} into the cytosol from the ER stores whilst hydrophobic DAG binds to C1 domains of proteins for membrane recruitment and activation. I(1,4,5)P_3 is also a substrate for the synthesis of inositol polyphosphates including pyro-phosphates such as IP_7 and IP_8 which are recognised as signaling molecules, including metabolic messengers or energy sensors [6]. Members of the protein kinase C (PKC) and Munc13 family as well as RasGRP4 are prime examples of proteins that are regulated by transient changes in DAG [7-9]. Conversion of DAG to PA also generates a bioactive metabolite with multiple functions [10-12]. Together, the products of PI(4,5)P_2 hydrolysis regulate many aspects of cellular function, such as transcription, cytoskeleton remodelling, membrane trafficking, neurosecretion and metabolism.

In addition to the generation of second messengers upon PI(4,5)P_2 hydrolysis by PLC, the decrease in the levels of PI(4,5)P_2 can impact on a number of processes mainly by affecting recruitment of peripheral membrane proteins and by regulation of integral membrane proteins. Miscellaneous proteins depend on PI(4,5)P_2 as a membrane anchor and PI(4,5)P_2 is a known regulator of membrane dynamics, actin cytoskeleton dynamics and activity of different ion channels and receptors (reviewed in [13-19]). Furthermore, PI(4,5)P_2 is also a substrate for a signaling pathway where phosphorylation by PI 3-kinases converts PI(4,5)P_2 to PI(3,4,5)P_3. PI(3,4,5)P_3 recruits PH domain-containing proteins such as AKT that play major roles in many signaling events including glucose uptake and cell growth (reviewed in [20]). Thus PI(4,5)P_2 hydrolysis by PLCs could have multiple downstream effects **(Figure 1)**. Because cellular PI(4,5)P_2 levels are controlled by many enzymes involved in inositol lipid synthesis and degradation, the activation of PLCs is one of the routes that contributes to the overall regulation, through stimulation by agonists acting through cell surface receptors.

Strict regulation of PLC activity is thus of crucial importance due to the sheer number of functions attributed to the simple reaction, PI(4,5)P_2 hydrolysis by PLC. Phospholipase Cs, as enzymatic and molecular entities, have a long history. Two independent reports described PLCs in 1959 [21] and 1961 [22] as phosphatidylinositol [not PI(4,5)P_2] hydrolysing enzymes. Many attempts at enzyme purification and identification were undertaken in the ensuing years but it was in the late eighties that the first PLC enzymes were purified and later cloned. It should be noted that PLC was also known as a phosphoinositidase C [22] or polyphosphoinositide phosphodiesterase (PPI-PDE) [23]. The identification of the first regulator of PLC was a G-protein and this was prior to the identification of the PLC enzymes [24]. The purification of multiple PLC enzymes to homogeneity only occurred after the

second messenger function of $I(1,4,5)P_3$ [25] and DAG [26] was discovered (reviewed in [27, 28]). Purification of the first PLCs occurred in the late 1980's when three mammalian PLC subtypes (β, γ and δ), were first isolated and their corresponding cDNA determined [29-32]. Subsequent studies led to the identification of multiple PLCs, which share conserved core domains, and these have been grouped into 6 families (β, γ, δ, ε, ζ, and η). In mammals, within each family are multiple members: four PLCβ (1-4), 2 PLCγ (1 and 2), 3 PLCδ (1, 3, 4), 1 PLCε, 1 PLCζ and 2 PLCη (1 and 2) making 13 PLCs in total (reviewed in [33-39]). In 2012, a seventh family of PLCs was discovered across different eukaryotic species, including 3 isoforms in humans, and named PLC-XD [40]. Thus, the PLC superfamily has expanded from 13 members to 16 members **(Figure 2)**.

Building on the insights about these important signaling enzymes covered in earlier reviews [33-39, 41], we here emphasize new, significant developments in the PLC field. These important advances include the identification of a new family (PLC-XD) of PLCs. Furthermore, discoveries of disease-linked mutations by comprehensive genetic investigations, most notably in the PLCγ family, provide direct evidence for their involvement in disease development. Combined with recent insights from cellular and structural studies, these findings highlight the key roles of PLCs and the underpinning mechanisms both, in physiology and diverse pathologies.

2. Domain organization of PLC families and main signaling connectivity

The well-established PLC families (β, γ, δ, ε, ζ, and η) share a conserved core structure in addition to a variety of other domains specific for each family **(Figure 2A)** [33-39]. The PLC-core comprises of an N-terminal pleckstrin homology (PH) domain, followed by four EF hand domains, a catalytic domain (with two conserved halves, X and Y) and a C2 domain. Only PLCζ enzymes lack the PH domain within the PLC-core **(Figure 2A)**. The catalytic domain is conserved with respect to both, structure and function. However, other domains in the common PLC-core incorporate a number of distinct features in different PLCs, mainly implicated in regulation (see Section 3). Some of the examples are highlighted in **Figure 2A**, including diverse interactions mediated by PH domains in PLCδ and PLCβ isoforms and a unique function of EF hands in PLCβ.

Each of these PLC families, except PLCδ and PLCζ, has additional regulatory domains **(Figure 2A)**. PLCβ and PLCη isoforms have C-terminal extensions. In PLCβ, this region has been implicated in interactions with Gαq and with the membrane. PLCγ isoforms contain a split PH domain (spPH), two Src homology 2 domains (nSH2 and cSH2), and a Src homology 3 (SH3) domain. The region encompassing these domains is known as the regulatory region or the γ-specific array (γSA); the γSA is inserted between the two halves of the catalytic domain. The well-defined contacts with some members of the receptor tyrosine kinases (RTKs) and a small GTPase Rac, are examples of many regulatory interactions mediated by the γSA. PLCε contains a Cdc25 domain and two Ras association (RA) domains, both related to the regulatory interplay with small GTPases.

Together, the regulatory interactions embedded in the PLC-core and contained within the additional domains, provide links with numerous and diverse cell-surface receptors; many of these links have been previously summarized [33-39] and are discussed further in Sections covering each PLC family **(Sections 5.1-5.6)**. Overall, the signaling connectivity remains

best defined for the G-protein-coupled receptors (GPCRs) and PLCβ isoforms, mediated by the α and βγ subunits of G-proteins, and for the receptor tyrosine kinases (RTKs) and tyrosine kinases linked to ITAM associated receptors, that activate PLCγ enzymes by direct phosphorylation. The regulation that involves small GTPases, activated by a range of different receptors, is also documented for several classical PLC families but the understanding of signaling links within relevant physiological contexts requires further studies. The importance of changes in cytosol Ca^{2+}, in particular for the regulation PLCδ and PLCη isoforms, has also been suggested; however, precise binding sites on these PLCs are not clearly determined.

As highlighted in the introduction, a recent discovery of the PLC-XD family not only increases the total number of PLC enzymes that hydrolyze inositol lipids but also reveals their unexpected diversity **(Figure 2)**. Unlike all other known eukaryotic PI-PLCs, the PLC-XD family is more similar to bacterial PI-PLCs and is characterized by a single catalytic domain structure. Initial studies of PLC-XDs suggest their varied and important functions, contributing, alongside other PLCs, to processes controlled by inositol lipid signaling (see **Section 5.7**). Taking into the account these new discoveries, we now propose that the eukaryotic enzymes from six families (PLCβ, γ, δ, ε, ξ and η) are collectively designated as classical PI-PLCs and PLC-XDs as atypical PI-PLCs **(Figure 2)**. Together with bacterial enzymes, PLC-XD enzymes are also described as "minimalist PI-PLCs" [42].

In contrast to the classical PLCs, the PLC-XD family contains the catalytic domain with the sequence similarity only within the X-region **(Figure 2B)**; this characteristic is shared with most bacterial PI-PLCs [42].

The general outline illustrated in **Figure 2** is supported by numerous studies covering structure determination, biochemical and biophysical characterization, as well as cellular and genetic dissection of biological functions regulated by individual enzymes. In the following Sections we provide extensive and detailed coverage of these different aspects and discuss how the main current concepts have been extended and consolidated by recent findings. We highlight some common themes, many revealed by structural studies, as well as great functional diversity that became particularly apparent when comparing isoforms from each of the PLC families.

3. Structural insights into PLC function

A substantial number of 3D structures are currently available for PLC enzymes with the specificity for inositol lipids **(Table 1)**. They provide a valuable basis for the understanding of various functional properties, including their PLC activity and regulatory mechanisms. Although the key insights for mammalian PLCs have been obtained from studies of mammalian species, identification of PLC-XD with similarity to bacterial enzymes highlights the importance for a wider comparison, in particular when discussing the structural basis for the enzyme activity. Comparison of different families of the classical PLCs defines common structural features associated with the PLC-core, as well as features related to their specific regulatory mechanisms. Notably, recent studies highlighted that despite the diversity of their interacting proteins, the general mechanism for regulation of classical PLCs is centred on intramolecular interactions that maintain PLCs in their inactive form, also referred to as autoinhibition, that becomes released in the process of activation.

3.1. Structural basis for the enzyme activity

As peripheral membrane enzymes, PLCs have a complex task. They must first recognize and bind the membrane, access and selectively bind their substrate, catalyse the reaction, and then proceed to the next substrate molecule within the membrane surface. While the substrate recognition and catalytic reaction takes place within the active site of the catalytic domain, various structural features across different domains, including the catalytic domain as well as the PH and C2 domain, can contribute to direct interactions with cellular membranes. In addition to this established and well-documented model, the possibility that additional proteins could be involved in extracting and directly presenting the substrate to PLCs, has also been considered [43, 44]. However, these potentially exciting concepts require further experimental support.

Based on extensive structural studies, the enzyme activity in all PI-PLCs is encapsulated in the βα-barrel structures, the most common fold present in about 10% of all enzymes [45, 46] **(Table 1, Figure 3A)**. A catalytic $(\beta\alpha)_8$ or TIM-barrel domain in classical PLCs, first defined in PLCδ1, has high sequence similarity in two regions, X and Y, corresponding to the two halves of the barrel [47, 48]. The linkers between the two halves (X/Y linkers) vary in length and in sequence and incorporate elements that can regulate enzyme activity [49]. In contrast, some minimalist PI-PLCs, including the eukaryotic enzymes, share a certain degree of similarity with classical PI-PLCs in the first half of the barrel (X region or domain, "XD") while the second half is variable; they also lack the X/Y linker [40, 42] **(Figure 2A)**. The overall structure of the βα-barrel determined for a number of bacterial PI-PLCs is distinctly characterized by a distorted barrel with only six alpha helices ($\beta_8\alpha_6$) **(Table 1)**.

A number of studies have provided a detailed catalytic mechanism for the eukaryotic classical PLCs and minimalist PI-PLCs from Gram-positive bacteria. Over the years, the main conclusions from the initial studies focused on PLCδ1 and PI-PLC from *Bacillus cereus* [47, 48, 50-53] have been reinforced and extended [42]. As a key point, these PLCs share the same catalytic mechanism despite the structurally defined differences in their substrate recognition and a requirement for Ca^{2+}. Specifically, classical enzymes have a preference for $PI(4,5)P_2$/PI(4)P and are calcium-dependent while bacterial enzymes are selective for PI and GPI (Glycosyl-PI) and, in most cases, calcium-independent. Both types of PI-PLCs cleave their inositol lipid substrates in a two-step reaction by a general acid/general base mechanism **(Figure 3B)**. The first step is a phosphotransferase reaction. The substrate is cleaved to yield DAG and stable catalytic intermediates, cyclic inositol-1,2-phosphates (cIP). For the second step, a cyclic phosphodiesterase reaction, the enzymes prime water to be the nucleophile that can then attack the bound cIP to yield linear inositol-1-phosphates (I-1-P).

The key residues involved in substrate recognition and catalysis are highly conserved among families of classical PLCs; this has been further reiterated by new structural insights into PLCβ and PLCγ enzymes, showing similar position and orientation of these residues within the TIM-barrel structure as in PLCδ1 **(Table 1)**. A comparison of PI-PLCs from Gram-positive bacteria also revealed conservation of several key residues involved in these functions [42]. However, the overall conservation between these two groups is limited to some of the residues directly involved in a general acid/general base catalysis (for example, His356 in PLCδ1 and His82 in *B. cereus* PI-PLC) and the recognition of the common inositol ring in their different substrates (for example, Tyr551 in PLCδ1 and Tyr200 in *B. cereus* PI-PLC).

In contrast to the above comparison, partial insights for PI-PLCs from Gram-negative bacteria suggest that there can be variations in the basic enzymatic mechanism; similarly, it has been suggested that the catalytic mechanism of at least some PLC-XD enzymes (for example plant DNF2) is likely to be different from the mechanism described in **Figure 3B** [42]. Furthermore, a range of substrates and substrate preferences remain to be defined for PLC-XDs. Nevertheless, despite sequence divergence, the structures of PLC-XDs can be homology modeled based on PI-PLC structures from Gram-positive bacteria, providing new insights and highlighting unresolved questions. A homology model for hPLC-XD3, for example, shows that the two histidine residues critical for the catalysis are structurally conserved **(Figure 3A).** However, residues that coordinate calcium and those that contribute to the preference for $PI(4,5)P_2$ and PI(4)P in classical PLCs do not appear to be conserved. This does not preclude that other structural features, unique for these proteins, could contribute to their ability to recognize $PI(4,5)P_2$/PI(4)P, as suggested in one of the recent studies [54]. Further enzymatic and structural characterization of these PLCs is clearly required to define their properties related to substrate recognition and detailed mechanism of catalysis. In turn, such characterization could provide valuable insights into cellular function of these enzymes.

The membrane interaction surfaces on the catalytic domain include the loops, containing a number of hydrophobic residues exposed to the surface, that together form a ridge region in the vicinity of the active site opening. The position of this ridge and additional experimental evidence for some classical PLCs, have suggested a model where this region needs to penetrate the membrane bilayer to allow PLCs to access the substrate [47, 48, 55, 56]. Based on general membrane association mechanisms for peripheral proteins, this penetration is preceded by interactions involved in membrane recognition and binding [57, 58]. Such interactions can range from nonspecific electrostatic forces to binding of specific phospholipids; they can involve surfaces on the catalytic domain or other domains in the PLC-core of classical PLCs. The importance and role of these membrane interactions is further discussed in the context of specific regulatory mechanisms covered in the following Sections.

3.2. Common organization of the core domains in classical PLCs

As already described in **Section 2**, the domains comprising the PLC-core in most PLC families include, in addition to the catalytic domain, the PH, EF-hand and C2 domains **(Figure 2)**. Comparison of several PLC-cores from PLCδ, PLCβ and PLCγ enzymes (PDB IDs in **Table 1**) reveals common relative orientation between the catalytic, EF-hand and C2 domains. As originally described for PLCδ1, the main interactions involve two extensive interfaces formed by the C2 domain with the catalytic domain and with the EF-hand domain; the EF-hands are not in contact with the catalytic domain. Interestingly, the PH domain can be flexibly linked to the rest of the core, as suggested for PLCδ1, or make extensive contacts with the catalytic domain and EF-hands, as shown for PLCβ and PLCγ enzymes. One important consequence of the closer integration of all four domains is that the binding of regulatory proteins or lipids to one of the domains can govern overall positioning of the PLC-core, for example, with respect to the cellular membrane.

Despite this conservation of the overall architecture, domains in the PLC-core can have specific binding properties for various regulatory proteins or other ligands **(Figure 2)**, distinct from those commonly recognized by these modular domains in other proteins [59, 60]. For example, calcium binding by the EF-hands or the C2 domain (originally observed in PLCδ1 structures) has not been generally documented for PLC enzymes. Instead, the EF-

hands in PLCβ enzymes underpin the GAP function that accelerates GTP hydrolysis by Gαq [61-63]. Similarly, binding of the inositol lipid headgroups by the PH domains has been clearly shown only for PLCδ1 (PDB ID 1MAI) [64-66]. Based on recent structural insights (PDB ID 6PBC), it is not certain that it is the property of the PH domain from PLCγ1, as originally proposed [67]. Instead, other interactions have been shown, notably the binding of small GTPase Rac by the PH domain from PLCβ2 (PDB ID 2FJU).

3.3. Autoinhibition as a general regulatory mechanism in classical PLCs

In the absence of extracellular stimuli, most classical PLCs exhibit very low intrinsic substrate hydrolysis. They are robustly activated upon direct interactions with their specific regulatory proteins or other ligands. Following extensive structural and functional studies, we are now able to define some of the restraints that maintain this low-activity status. Because PLCs interact with phospholipid bilayers to hydrolyze $PI(4,5)P_2$, the control of membrane localization provides one mechanistic route for regulation of basal enzymatic activity. However, several lines of experimental evidence revealed another important aspect of regulation, different from the control of subcellular localization. For example, it has been demonstrated for many classical PI-PLCs that removal of specific segments results in an enhancement of basal PLC activity, implicating these regions in direct occlusion of the active site or, more subtly, restriction of productive orientation of the catalytic domain towards the substrate-containing membrane. These auto-inhibitory elements and their implications for the activation mechanisms are best defined for PLCβ enzymes and very recently also for PLCγ (**Figure 4).**

3.3.1 Insights from PLCβ variants and their complexes with regulatory proteins

The main regulatory proteins for this family include Gα and Gβγ subunits and also the small GTPase, Rac. As outlined in **Figure 2A** and **Figure 4A**, PLCβ enzymes comprise the PLC-core and an ~400-amino-acid extension, the C-terminal domain (CTD), which is unique to this family. The proximal CTD and distal CTD, that forms an extended coiled-coil structure, are connected by a variable linker. Extensive structural insights, summarized in **Table 1**, mainly cover partial structures such as C-terminal truncations or the isolated distal CTD obtained by X-ray crystallography. Structures of complexes of the C-terminal truncations, that include the PLC-core and in most cases also the proximal CTD, with regulatory proteins Rac1 or Gαq, have also been solved [68, 69]. Importantly, one full-length structure, the structure of PLCβ3 in complex with Gαq, combined X-ray crystallography and cryo-EM, provided more comprehensive and in-depth understanding of molecular aspects of PLCβ function [70]. Together, these structural insights and related functional studies define the key regions involved in the autoinhibition and interactions with specific regulatory proteins; they also provide a framework to propose models for regulation of PLCβ enzymes by these upstream signalling components and membrane surfaces.

Based on extensive supporting evidence, two distinct regions contribute to the autoinhibition of PLCβ: the X-Y linker of the catalytic domain and the proximal CTD [49, 68-72]. Additionally, the distal CTD coil-coil structure, that contains the key membrane interaction sites, also participates in autoinhibition [70, 73].

The X-Y linker varies in length among PLCβ isoforms, nevertheless, all have two conserved elements, namely, an acidic stretch and a C-terminal region that forms the active site lid helix blocking access of the phosphoinositide head group to the active site [49, 68-70]. It is likely that the acidic stretch acts as a clasp to stabilize the lid helix by interacting with highly

conserved basic residues in close proximity to the active site [72]. Together, these elements block substrate binding.

Within the proximal CTD, ~25 amino acids form a well-ordered helical hairpin. The Hα2' helix of the hairpin binds to a cleft on the PLC-core formed at the interface of the catalytic barrel and C2 domains. The cleft contains residues that are uniquely conserved in the PLCβ family and places the helical hairpin in close proximity to the active site and the X–Y linker [69-71]. However, the Hα2' helix inhibits PLC activity independently of the X-Y linker [72]. It is hypothesized that the Hα2' helix/PLC-core interaction stabilizes the PLC-core in a catalytically quiescent state and/or somehow prevents the active site from achieving optimal interactions with lipid bilayers.

The role for the distal CTD in autoinhibition has been suggested by findings that in the structure of the full-length PLCβ3 this region interacts with the ridge of the catalytic domain [70]. As a consequence, the basic surface of the distal CTD implicated in interactions with the membrane, could be sequestered and the ridge region of the catalytic domain prevented from the membrane binding or insertions. Additional mapping of the interaction sites and conformational changes by Hydrogen Deuterium eXchange (HDX-MS), performed for the full-length and truncated PLCβ2 variants in a set up containing a membrane mimetic, has similarly suggested that the distal CTD is inhibitory to PLC activity *via* direct binding to the PLC-core [73]. Interestingly, this approach also revealed that the distal CTD rearrangements could destabilize the proximal CTD, linking these two structural features in the context of autoinhibition [73].

Structural studies have also suggested how interactions with regulatory proteins could result in release of the autoinhibition. In particular, PLCβ3-Gαq structures and associated biochemical data, defined interaction sites with Gαq in the proximal and distal CTD as well as several sites on the PLC-core [69, 70, 73]. The interaction with the canonical effector binding site on Gαq involves a helix-turn-helix (Hα1/Hα2) in the first 25 residues of the proximal CTD that precede the autoinhibitory Hα2' helix. Taking into account the insights into the PLCβ autoinhibition and the data related to interactions with Gαq, a following molecular mechanism for PLCβ3 activation by Gαq has emerged **(Figure 4A)**. In the autoinhibited state, the Hα2' helix of the proximal CTD is bound to the PLC-core, suppressing basal activity, and the preceding Hα1/Hα2 element and distal CTD sites are freely accessible to Gαq. The distal CTD and the X–Y linker are also positioned to suppress basal activity. Upon activation, Gαq binds to Hα1/Hα2 and other sites, including the distal CTD that itself contains autoinhibitory elements and is linked to the autoinhibitory segment (Hα2' helix) in the proximal CTD. These interactions displace the Hα2' helix element from the PLC-core and disrupt the interactions between the distal CTD and the PLC-core, leading to allosteric activation of PLCβ. The interactions between the membrane, the palmitoylated N-terminus of Gαq, and the distal CTD help bring the PLC-core into close proximity with the membrane. In such proximity, the negatively-charged membrane surface could displace the acidic stretch in the X-Y linker by electrostatic repulsion to release the clasp on the lid-helix and unmask the active site. Alternatively, this unmasking could be achieved by the competition between the acidic stretch and the membrane for the basic patch on the catalytic core. In both scenarios, it is hypothesized that Gαq utilizes the membrane as a conduit to allosterically enhance the phospholipase activity.

Regulation of PLCβ enzymes also involve deactivation of Gαq by the GAP activity underpinned by the EF-hand structures in the PLCβ-core and, together with unresolved mechanistic detail, provides additional layers of complexity for the proposed mechanistic models [62, 63, 73, 74].

Molecular mechanisms for the release of autoinhibition by Rac or Gβγ proteins, that appear to bind different sites on the PLC-core, have also been addressed [68, 72, 75-78]. Notably, the sites distinct from the binding of Gαq are consistent with the well documented synergistic activation by Gαq and Gβγ [79, 80]. The site of interaction on the PLC-core has been structurally defined only for Rac1and resides on the PH domain [68]. These studies using a truncated PLCβ2, where the complex formation with Rac1 has no marked consequences for the overall conformation, lead to a suggestion that the interaction with the membrane-bound Rac1 could result in local and limited rearrangements in the X-Y linker in the membrane proximity, contributing to the unmasking of the active site. Similar considerations of a membrane-mediated allosteric impact on the X-Y linker have been applied to PLCβ3 activation by Gβγ [72, 78]. However, recent studies of the Gβγ binding to the full-length PLCβ2 reported extensive conformational changes in the distal CTD structure that also contains autoinhibitory elements [73]. Thus, unlike Gαq that directly binds the distal CTD, Gβγ appears to indirectly impact on conformation of this region contributing to the release of autoinhibition and PLC activation.

Overall, the structural studies of PLCβ family highlighted the elements of autoinhibition within and outside the X-Y linker and implicated their involvement in the activation process by different regulatory proteins. Interestingly, the allosteric impact resulting from the binding of these regulatory proteins seems to be in part mediated by the membrane surfaces. To further build on these important advances, more comprehensive studies including the full-length enzymes as well as membrane structures are clearly required.

3.3.2 Structural insights and complexity of PLCγ regulation

As outlined in **Figure 2**, PLCγ enzymes are characterized by an array of domains within the X-Y linker, referred to as "γ-specific array (γ-SA)" or as "regulatory region". Over the years, a number of studies described partial structures of domains from the regulatory region and several regulatory complexes, providing useful but limited information as well as some conflicting data **(Table 1)**. Partial insights into PLCγ autoinhibition and phosphorylation-mediated activation have been also obtained in some of these earlier studies [81, 82]. Although it has been assumed that the core domains are organized as determined for PLCδ and PLCβ enzymes **(Table 1)**, a direct structural determination of the PLCγ-core lagged behind these two families. Furthermore, the overall organisation of an intact PLCγ, that would fully inform molecular aspects of their regulation, have also been lacking. Only recently, new insights have been reported that addressed these limitations. Specifically, the structure of a near-intact PLCγ1 has been solved by X-ray crystallography [83] and the architecture of the complex, including an intact PLCγ1 and an intracellular part of FGFR1, determined using cryo-EM [84]. In both studies PLCγ1 adopts an inactive, autoinhibited conformation. Nevertheless, these studies not only fully defined regions involved in the autoinhibitory interactions but also suggested mechanisms of activations *via* physiologically relevant phosphorylation of PLCγ and also by gain-of-function mutations discovered across diverse pathologies.

The autoinhibitory interfaces lock the regulatory array on top of the PLC-core [83, 84] **(Figure 4B).** The first interface is formed by interaction of the spPH with the ridge region, positioned close to the active site opening, of the TIM barrel. A second interface is formed between loops of the cSH2 domain and the loops of the C2 domain. These two interfaces do not overlap. In the crystal structure where the large portion of the cSH2/SH3 linker is deleted, the SH3 domain lies between the cSH2 and spPH domain, away from the autoinhibitory surfaces [83]. The SH3 domain is not visible in the cryo-EM map presumably because of the flexibility of relative positions of this domain towards the rest of the molecule caused by the long linker present in the intact protein used in this study [84]. The main consequence of the relative position of domains in the autoinhibited form is that the membrane interaction surfaces on the PLC-core are occluded, preventing access of the membrane-bound substrate to the active site.

Previous studies of complexes of isolated regulatory domains with regulatory proteins described interactions between the intracellular portion of FGFR with the PLCγ1 nSH2 or cSH2 domain, interaction of small GTPase Rac with the spPH domain from PLCγ2 and some other interactions including binding of a motif from the adapter protein SLP76 to the PLCγ1 SH3 domain [85-88]. Recent studies with the intact PLCγ1 in the complex with FGFR confirmed the interaction with the nSH2 domain and excluded binding to the cSH2 as a physiologically relevant interaction [84]. Importantly, all of the defined, relevant interactions with regulatory proteins are at the sites far from the autoinhibitory surface [83, 84]. This suggests that an impact on the autoinhibition requires propagation of an input from these distant sites *via* allosteric networks. Another implication of relative arrangements of different regulatory proteins from several separate studies [84-86, 88] is that they all could be accommodated simultaneously by a full-length PLCγ1 without clashes. In turn, this could allow integration of different signals in overall control of the PLC activity.

The best documented activation route of PLCγ enzymes is by phosphorylation and the recent structural insights for PLCγ1 suggest a detailed mechanistic model for this process triggered by FGFR **(Figure 4B)**. In order that the key phosphorylated tyrosine (pY783) in the cSH2/SH3 linker can intramolecularly bind to the site on the cSH2 domain, that is inaccessible in the autoinhibited form, a conformational change has to take place. It is suggested that such change could be propagated from the site of FGFR binding on the nSH2 domain to the autoinhibitory interface *via* an allosteric network that involves residues from both SH2 domains [83]. Binding of the phosphorylated segment to the cSH2 domain competes out its autoinhibitory interactions with the C2 domain, leading to activation.

Available data show that PLCγ enzymes activated by phosphorylation maintain high PLC activity as free proteins, independently of complexes with their regulatory proteins [82, 83]. Furthermore, it has been shown that phosphorylation of PLCγ1 by FGFR weakens their interaction, potentially allowing for the activation of many PLCγ1 molecules by a single FGFR kinase [82, 89]. This model of signal amplification also implies that the main event leading to inactivation could be the dephosphorylation of the enzyme. However, different considerations apply to phosphorylation-independent activation, such as activation of PLCγ2 by Rac.

Overall, based on our current understanding, the autoinhibition that keeps PLCγ enzymes in an inactive form and general requirements for the release of this autoinhibition are well defined. However, a direct and detailed characterization of an active form is required to

support various models for the overall activation mechanisms of the PLCγ family. In particular, it will be important to confirm and define the predicted, substantially rearranged conformation of the active PLCγ, its precise orientation towards the membrane and the role of individual interacting proteins and the membrane surfaces in the activation process and stabilisation of the active form.

3.3.3 Other PLC enzymes

In addition to structural evidence presented for the role for a typical (in PLCβ) or a multidomain (in PLCγ) X-Y linker, other studies support the role of the X/Y-linker in autoinhibition of most classical PLCs. Notably, removal of this region enhances activity of PLCδ and PLCε, without affecting activation of PLCε by the Ras or Rho GTPases [49, 90]. Despite the sequence diversity, the X-Y linkers, similarly, as illustrated for PLCβ, have clusters of negatively charged residues. Consequently, it has been hypothesized that a common activation mechanism could involve an interfacial repulsion between the negatively charged X-Y linker, that occludes the catalytic domain, and the proximal membranes; this would prevent the access to the membrane-bound substrate. However, further, direct experimental evidence is needed to confirm this model and identify other elements involved in activation that are not dependent on the X-Y linker.

With respect to the above considerations, some of the PLCζ enzymes, resulting from alternative splicing, seem to differ [91-93]. These PLCζ splice variants have a highly basic X-Y linker rather than a negative charge. It has been speculated that these segments serve as a membrane targeting signal, to facilitate interaction with phospholipids, or as a nuclear localization signal.

The main limitation in our further, detailed understanding of regulation of different families is the lack of 3D-structures; the structures that include almost an entire protein are available for PLCδ, PLCβ and PLCγ enzymes, while for all other families they only cover isolated regulatory domains from PLCε (**Table 1).** Considering that in the proposed mechanism of autoinhibition for PLCβ, the X-Y linker is one of several regulatory elements **(Figure 3A),** it is expected that in other PLC families additional structural features are also involved. Overall, it is likely that a general concept for autoinhibition includes some common elements as well as elements unique for specific families and individual enzymes.

3.4. Localized activation of PLCs: role of scaffold proteins

An additional layer of regulatory complexity arises from the observation that PLC enzymes interact with numerous adapter and scaffolding proteins to form higher-order signaling complexes. Although such complexes represent the next frontier for structural studies of PLC enzymes, some notable, recent insights in scaffold proteins are beginning to address this level of complexity. Scaffold proteins provide platforms for segregating PLC signaling events and the best-characterized system is the INAD scaffold-organized signaling complex in *Drosophila* photoreceptors. INAD comprises of 5 PDZ domains arranged in tandem and organizes the core components of the phototransduction pathway with NORPA (PLCβ4), Ca^{2+}-permeable transient receptor potential (TRP) channel and eye protein kinase C (PKC) by binding to PDZ motifs present in each of the molecules [94]. INAD PDZ2 binds to eye PKC, PDZ3 binds to the TRP channel and PDZ4/5 tandem binds to PLCβ4. Both the PDZ domains of the PDZ45 tandem bind not only the PDZ binding motif but also to the adjacent coiled-coil domain which is separated by 6 a.a. residues. The PLCβ coiled-coil domain is

important for the membrane targeting of the enzyme, which is necessary for the full activity of the PLC. The tight complex between PDZ45 domain of INAD and the NORPA coiled-coil domain however, does not interfere with membrane binding. Thus, during *Drosophila* phototransduction, INAD allows the assembly of a complex where Gαq-PLCβ4 is simultaneously bound to the membrane and to INAD which brings together PKC and the TRP channel in one multi-protein complex. During PLC activation, a hydrogen ion is released and decrease in pH allows dissociation of the INAD-NORPA complex allowing for signal termination.

Studies in mammalian cells have implicated PDZ-containing proteins in PLC signaling (reviewed in [95]). In mammals, INADL (also known as PATJ) was identified as the counterpart to INAD. INADL comprises of 10 PDZ domains and binds to PLCβ4. The tandem PDZ89 was found to interact with the C-terminal coiled-coil-PDZ binding motif of PLCβ4. PLCβ4 is the vertebrate homologue of NORPA and is the key regulator of melanopsin-mediated signaling in ipRGC (intrinsically photosensitive retinal ganglion cells). Melanopsin is also activated by light and interacts with Gαq, which in turn activates PLCβ4. The interaction between INADL and PLCβ4 is highly specific as MUPP1, a close homologue of INADL has no detectable binding to PLCβ4 [94].

Other PDZ-containing proteins that are implicated in PLC signaling are members of the NHERF (Na^+/H^+ exchanger regulatory factor) family, SHANK2 and Par3. NHERF1 and NHERF2 comprise of two PDZ domains with the N-terminus containing an ERM-binding domain capable of linking to the cytoskeletal proteins, ezrin, radixin and moesin (ERM). NHERF3 and NHERF4 comprise of four PDZ domains [96]. Like INADL, NHERFs also interact with PLCβ enzymes. For example, NHERF3 (also known as PDZK1) forms a ternary complex between the somatostatin receptor 5 and PLCβ3 and enhances PLC signaling [97]. Of the four PDZ domains of NHERF2, PLCβ3 interacts with the first N-terminal PDZ domain whilst the somatostatin receptor 5 interacts with the third PDZ domain. NHERF2 contains two PDZ domains in tandem and the interaction between the second PDZ domain of NHERF2 and the PDZ motif of PLCβ3 results in the potentiation of carbachol-stimulated PLCβ3 activity when co-expressed in COS-7 or HeLa cells [98]. In further studies, the LPA_2 receptor which also contains a PDZ motif was also found to interact with the second PDZ domain of NHERF2 and it was found that LPA_2 receptor, PLCβ3 and NHERF2 are present in a complex; where the first PDZ domain interacts with a second molecule of NHERF2 to allow the association between these three proteins [99]. NHERF2 also interacts with the mGluR5 and regulates Ca^{2+} signaling [100]. Par3 which contains 3 PDZ domains, uses its first PDZ domain to bind to PLCβ1 and its third PDZ domain to bind to the bradykinin receptor B2 [101]. Shank2 contains a PDZ domain in addition to a SH3 domain, a SAM domain and a proline-rich domain. PLCβ3 associates with the PDZ domain allowing for the formation of a multi-molecular complex with metabotropic glutamate receptor [102].

In addition to PDZ domains binding to the PDZ motif of PLCβ, complex formation of PLCβ3 with CD3, ORP4L and Gαq has been reported in T cell acute lymphoblastic leukemia cells [103]. Whilst no structural information is presently available, it would appear that different molecular assemblies are possible functioning in a cell-type specific manner. PLCβ2 which is activated by βγ subunits also associates with WDR26 [104, 105]. WDR26 is a WD40 repeat-containing protein and is required for optimal signaling in leukocytes. It is also a βγ-interacting protein and exists in a higher order oligomer and simultaneously binds both Gβγ

and PLCβ2. It promotes PLCβ2 membrane translocation and functions as a scaffolding protein to bring PLCβ2 in close proximity to Gβγ for activation. WDR26 promotes signaling through βγ such as Ca^{2+} signaling and Akt thus leading to leukocyte migration.

Higher-order regulatory complexes that incorporate PLC enzymes from other families have also been identified. For example, PLCγ enzymes interact with several adapter proteins in different types of immune cells **(*see* Section 5.2.)**. However, structures of these complexes have not yet been solved.

PLCγ1 has also been shown to interact with Jak2 and the protein tyrosine phosphatase 1B resulting in negatively regulating Growth Hormone (GH) and IL-6 signalling [106]. Binding of GH to its receptor initiates the recruitment of the tyrosine kinase, Jak2 (Janus kinase 2) leading to its activation by autophosphorylation. Downstream of Jak2 is the activation of STATS (signal transducer and activator of transcription) resulting in transcription of multiple genes. PLCγ1 reduces GH-induced Jak2 activation by binding to both, Jak2 and to the protein tyrosine phosphatase 1B (PTP-1B). Jak2 binds to the N-terminal SH2 domain whilst PTPB1 binds to the SH3 domain of PLCγ1. Activation of PLCγ1 through phosphorylation by GH is required and this occurs through Jak2. Using PLCγ1$^{-/-}$ MEFs (murine embryonic fibroblasts), growth hormone-dependent c-Fos was upregulated and proliferation was potentiated. It would appear that PLCγ1 functions here as a scaffold protein to recruit PTP-1B and Jak2, that can then restrain Jak2 activity during growth hormone signalling. During erythropoiesis, erythropoietin also activates PLCγ1 via Jak2, and here PLCγ1 is essential for erythroid maturation. In the absence of PLCγ1, erythroid differentiation does not occur [107]. Whether, PTP-1B has a restraining influence on erythropoiesis is not known. The interactions and stability of the PLCγ1/ PTP-1B /Jak2 complex, as well as the structural features, have not been explored.

4. Phospholipase C enzymes – why so many?

The mammalian genome encodes 16 PLC enzymes and the number increases many fold when their splice variants are taken into consideration [108, 109]. They all catalyse the same reaction (with some exceptions) raising the question of the purpose of this diversity. Firstly, the presence of multiple PLCs provides differential means of regulation of PLC activity. As described in **Section 2**, PLC activation occurs not only following activation of cell surface receptors by appropriate agonists, but also occurs downstream of small GTPases and changes in cytosol Ca^{2+}. Secondly, expression patterns of specific isoforms in different cell types allows for spatial and temporal complexity in cell signaling. Thirdly, the extent of $PI(4,5)P_2$ hydrolysis can be regulated with greater precision. Fourthly, signal amplification can be achieved as different PLCs have different Ca^{2+} thresholds for activation.

Our initial understanding of PLC signaling was the generation of second messengers, $I(1,4,5)P_3$ and DAG [110], but now it has become increasingly clear that $PI(4,5)P_2$ levels regulate many cellular functions **(Figure 1)**. Depending on the mode of PLC activation (e.g. RTK, GPCR, monomeric G-proteins or increase in cytosol Ca^{2+}), $PI(4,5)P_2$ hydrolysis could be either minimal, transient that recover within minutes or sustained leading to a pronounced loss of $PI(4,5)P_2$ [111]. Maximal calcium responses saturate with very low amounts of $I(1,4,5)P_3$ and therefore require minimal $PI(4,5)P_2$ hydrolysis [112, 113]. Moreover, the

catalytic activity of the different PLCs varies [29] and can be modulated by other factors such as phosphorylation [114]. Thus depending on the PLC activated, the duration and the strength of its activity will determine how much PI(4,5)P_2 levels will decrease. Another consideration is the number of receptors that are present for a particular agonist. Maximal activation with diverse agonists can lead to different outcomes dependent on the GPCR that is stimulated. For example maximal stimulation with UTP causes a maximal rise in cytosol Ca^{2+} and PKC activation but no decrease in PI(4,5)P_2. In contrast, maximal stimulation with a muscarinic cholinergic agonist causes the same level of increase in cytosol Ca^{2+} and PKC activation but is now accompanied by a substantial decrease in PI(4,5)P_2 [112, 113]. Another consideration is the temporal difference in PLC activation dependent on the isoform stimulated. As discussed in **Section 5.4**, activation of PLCε enzymes mediates sustained signaling compared to activation by PLCβ enzymes [115, 116]. There are several PLC families whose activity is directly regulated by changes in cytosol Ca^{2+} in the μmolar range. Cells generally maintain Ca^{2+} at 100nM and many mechanisms exist that can increase intracellular Ca^{2+} including opening of Ca^{2+} channels, as well as I(1,4,5)P_3 [41]. Thus, PLC activation can be amplified when GPCR/RTK cause a rise in cytosol Ca^{2+} or Ca^{2+} entry could directly trigger PLC activation.

PI(4,5)P_2 is mainly localized at the plasma membrane (PM) but not necessarily localized homogenously throughout the membrane [117-119]. Here, PI(4,5)P_2 is synthesised from PI by sequential phosphorylation. The first product, PI(4)P is mainly generated by PI4KIIIα, which is found in an evolutionary conserved complex containing TTC7B, FAM126A and EFR3 [120]. PI(4)P is subsequently phosphorylated by PIP 5-kinases to PI(4,5)P_2. At the PM, not all the PI(4)P is converted to PI(4,5)P_2, meaning that there is a significant pool of PI(4)P at the PM [121]. What regulates the balance between PI(4)P and PI(4,5)P_2 is complex as cholesterol, PS and PA all regulate PIP 5-kinase activity [12, 122]. There are a significant number of processes at the PM that depend on PI(4)P and PI(4,5)P_2. PI(4)P (and possibly PI(4,5)P_2) is used as a counter transport lipid for oxysterol-related proteins (OSBP) and related proteins (ORPs) to deliver cholesterol or PS from intracellular compartments (e.g. ER or lysosomes) to the PM [123-126] and hence decreases in PI(4)P/PI(4,5)P_2 due to PLC activity would affect cholesterol and PS homeostasis at the PM. This in turn would affect PI(4,5)P_2 homeostasis. Moreover, decreases in PI(4,5)P_2 affects the actin cytoskeleton [18], endocytosis [127], channel function [128] and exocytosis [117]. Another question that remains controversial is whether PLCs also degrade PI(4)P in cells. This possibility cannot be excluded as *in vitro* both PI(4,5)P_2 and PI(4)P are substrates for PLC as is PI [48]. PLC enzymes preferentially hydrolyze PI(4,5)P_2, but also hydrolyze PI(4)P and to a much lesser extent PI [29]. Interestingly, in dorsal rat ganglion (DRG) neurons, capsaicin-activated TRPV1 cation channels increase cytosol Ca^{2+} that stimulate PLCδ4 and this causes a substantial decrease in both PI(4)P and PI(4,5)P_2 whilst stimulation with bradykinin only leads to depletion of PI(4,5)P_2 [129]. Is the decrease in PI(4)P due to its utilisation by PIP 5-kinase or is it hydrolyzed by PLC? This point was not addressed in the study.

PLC signaling can also result in the generation of bioactive metabolites downstream of DAG. The substrate, PI(4,5)P_2 is highly enriched in stearic acid at the *sn*-1 position and arachidonic acid at the *sn*-2 position particularly in the brain (reviewed in [130, 131]. Thus, the DAG produced will retain this specific composition. In the brain, the DAG-lipases (α and β) can specifically remove the stearic acid, generating the endocannabinoid, 2-arachidonoyl glycerol

(2-AG), an agonist for endocannabinoid receptors used for retrograde signaling in neurons [132, 133]. Another aspect of PLC is the activation of PI 3-kinases and MAPK signaling through the production of DAG. In a limited number of cell-types that express the Ras exchange factor, RasGRP4, DAG can activate the small GTPase, Ras. (RasGRP4 contains a C1 domain that binds to DAG.) Ras proteins can directly regulate both Class I PI3K and the Raf/p42/p44 MAP kinase pathway. For example, in human neutrophils, the GPCR for FMetLeuPhe activates PI3Kγ and Raf/p42/p44 MAPK signaling downstream of PLCβ2/β3 activation [7].

Below we present the recent advances for each of the PLCs with respect to their regulatory input, their cellular and biological functions and dysfunctions in disease; these are summarized in **Tables 2 and 3**. In particular, the new findings resulting from genetic studies (Table 3) not only extend our understandings of their roles in control of physiological processes but also reveal their contribution to development of specific pathologies.

5. Phospholipase families and their specific characteristics

Phospholipase C enzymes were initially purified over several decades from a variety of tissues; a number of PLCs of different molecular masses, isoelectric points and calcium dependency were identified [134-139]. In the late 80's, three PLC isozymes, namely PLC-γ, -β, and -δ, were isolated from bovine brain cytosol and their cDNA sequences were obtained [29, 140, 141]. Subsequently, multiple sub-types were shown to exist in each of these three classes from various tissues using protein purification, RT-PCR using specifically-designed primers or a screening method using low stringency hybridization with probes made from the conserved X or Y regions [30, 142-150]. Thus 4 PLCβ (1-4) and 2 PLCγ (1, 2) enzymes were found to exist. It was initially thought that there were 4 PLCδ enzymes but PLCδ2 and PLCδ4 are the same; bovine PLCδ2 was found to be a homologue of mouse/human PLCδ4 [151]. There is no PLCα because a protein originally designated as PLCα [152], was found to be a luminal ER protein [153]. Additional PLC isozymes (ε, ζ, η) were subsequently discovered and cloned. PLCε was identified in mammalian cells in 2001 and contains several domains not present in other PLC isoforms [154-156]. (PLCε is a homolog of PLC 210, which was initially identified from a *Caenorhabditis elegans* cDNA library in 1998 [157].) In 2002, PLCζ was discovered that was specifically present in sperm [158]. Two PLCη enzymes were identified in 2005 [159-162] whilst the atypical PLCs, PLC-XD, were found in 2012; this brings the current total number of PLCs to 16 in mammalian cells. Other eukaryotes such as yeast and slime moulds contain only δ-type suggesting that the mammalian forms acquired additional domains to facilitate regulatory input.

5.1. PLCβ family

There are 4 PLCβ genes in the mammalian genome with diversity created by multiple splice variants for some genes [108]. The four isozymes of PLCβ (β1, β2, β3, β4) shows a distinct tissue distribution with most cells expressing at least one or two isoforms. PLCβ1 and β3 are widely expressed whilst PLCβ2 and PLCβ4 have limited tissue expression. PLCβ2 is highly enriched in haematopoietic cells including neutrophils whilst PLCβ4 is highly enriched in the

cerebellum and retina. PLCβ isozymes are well-established effectors downstream of GPCRs with the signal transduced from receptor to PLCβ by either Gαq family or Gβγ subunits of heterotrimeric G-proteins. Gαq family comprises of Gα11, Gα14 and Gα16 and do not appear to differ in their ability to activate PLCβs. PLCβ1, PLCβ3 and PLCβ4 are activated by Gαq whilst PLCβ2 and PLCβ3 can be activated by Gβγ subunits. PLCβ1 also functions as a GTPase Activation Protein (GAP) for Gαq. PLCβs can also directly bind and be activated by members of Rho family of GTPases such as Rac isoforms. This input links PLCβ activity to receptors (e.g. LPA and SIP receptors) that couple to Gα12 that activate exchange factors for small GTP binding proteins. As discussed in **Section 3.3.2**, the structural basis for PLCβ activation by Gαq, βγ and Rac proteins is beginning to be understood. Another feature of PLCβ enzymes is the presence of a PDZ binding motif at the C-terminal end allowing for binding to scaffold proteins containing PDZ domains as discussed in **Section 3.4**.

Using both whole animal and conditional knockouts of mice for the individual PLCβ isoforms, the specific physiological functions of the individual enzymes have been identified **(Table 2).** Also, genome analysis of patients provides an insight into the function of these enzymes in humans **(Table 3).** It is clear from comparing the two Tables that there are some differences in phenotypes between mice and humans. Below we discuss the individual isoforms and their roles in specific physiological functions. It is known that some members of the PLCβ family (and some other PLCs) localize and function in the nucleus; we do not cover this aspect in this review and interested readers are guided to the following reviews, where it has been excellently covered [163-165].

5.1.1 PLCβ1

PLCβ1 enzyme is ubiquitously expressed and tissue-specific requirement for this PLC has been identified in diverse cell-types including cardiomyocytes, neurons and β-cells of the islet of Langerhans. Below we summarise some studies that highlight the specific involvement of PLCβ1 enzymes in the physiology or pathology of different organs.

Two splice variants, PLCβ1a and PLCβ1b, that differ only in the extreme C-terminal sequence are present. PLCβ1b uses an alternate exon at the 3' end resulting in a shorter protein with a distinct C-terminus. PLCβ1a is longer by 43 a.a. and has a C-terminal PDZ-interacting sequence. Studies in cardiomyocytes indicate that PLCβ1b is responsible for cardiac dysfunction [166-168]. Activation of several GPCRs including angiotensin II receptors, Arg-vasopressin (V1) receptors and α-adrenergic receptors in cardiomyocytes leads to hypertrophy and decreased cardiac contractility both *in vivo* and in tissue culture models [169, 170]. PLCβ1b localizes to the PM through association with the scaffolding protein, Shank3 [168]. Downstream to PLCβ1b activation, it is the increased activity of PKCα that is associated with contractile failure.

PLCβ1 is widely expressed in the brain and activation of PLCβ1 isozymes have prominent roles in neuronal signaling as observed from the knockout mice **(Table 2)**. PLCβ1 is highly expressed in the cortex, the hippocampus and dentate gyrus [171]. The muscarinic cholinergic (M1) receptor is highly expressed in the brain and couples to PLCβ1 [172, 173]. PLCβ1$^{-/-}$ mice suffer from epileptic seizures due to defects in the inhibitory neuronal circuitry. Inhibitory interneurons are stimulated by cholinergic input and loss of PLCβ1 results in disruption of PKC activity leading to a deficit in GABAergic inhibition [174]. In

human patients, loss of PLCβ1 also results in infantile epileptic encephalopathy [175-177] (Table 2). This is a rare event and to date, only seven cases have been identified.

In pancreatic β-cells, some GPCRs that activate PLCβ enzymes enhance glucose-stimulated insulin secretion. Using islets prepared from conditional knockout mice for each of the 4 PLCβ isozymes, deletion of PLCβ1was found to reduce glucose-stimulated insulin release and intracellular levels of Ca^{2+} compared to islets prepared from control mice [178]. Additionally, the conditional knockout mice lacking PLCβ1 had a lower ability to clear glucose from the blood when challenged with high glucose. Several GPCRs on the islet cells including arg-vasopressin, 5-hydroxytryptamine and kisspeptin enhance glucose-stimulated insulin release and β-cells prepared from mice lacking islet-expressed PLCβ1 exhibited a marked defect in glucose-stimulated insulin release, leading to glucose intolerance. Furthermore, the conditional knockout mice when maintained on a high fat diet developed an even more severe glucose intolerance [178, 179]. Although islets expressed at least three of the four PLCβs isozymes, only conditional knockout of PLCβ1 resulted in defects in enhanced glucose-stimulated insulin secretion. Thus, these GPCR appear to specifically use PLCβ1 for signaling purposes.

5.1.2 PLCβ2

PLCβ2 has restricted expression mainly to cells of the haematopoietic origin including neutrophils, platelets and macrophages. In addition, PLCβ2 is part of the taste receptors transduction system which includes the G-protein, gustducin and TRPM5 [180-182]. In platelets, two splice variants of PLCβ2 (a and b) are present that differ in the carboxyl-terminal region. Both splice variants are expressed at the protein level and are similarly activated by Gαq. The only difference is their localization; PLCβ2a which is longer by 15 amino acid residues, is more enriched in the nucleus compared to PLCβ2b which is more cytosolic [109]. PLCβ2 is often expressed with PLCβ3 in these cell-types. Mice knockouts of PLCβ2 are fertile and viable but show defects in neutrophil function. Chemoattractants including FMLP (derived for bacteria) and IL-8 use PLCβ2 and PLCβ3 as their sole PLC isoforms and are required for superoxide production and increases in intracellular Ca^{2+} levels in neutrophils [183, 184]. Interestingly, chemotaxis is enhanced in mouse neutrophils lacking PLCβ2.

PLCβ2 is central to taste receptor signaling and is present in taste receptor cells where it is activated by βγ subunits released by multiple GPCRs that respond to bitter, sweet and umami tastes [185]. Additionally, the major taste transduction signaling elements including gustducin, PLCβ2 and TRPM5 are also found is several other epitheliums including the olfactory epithelium in the nasal passage and also in the gingival junctional epithelium in the oral cavity. In these epithelia, the signaling elements are present in specific solitary chemosensory cells. In the gingival junctional epithelium, the bitter taste receptors respond to pathogenic bacterial metabolites triggering host defense to control bacterial infection [181].

PLCβ2 knockout mice are more resistant to infections by viruses. Viral infections cause uncontrolled release of proinflammatory cytokines through activation of the MAPKKK, TAK1 (TGFβ-activated kinase 1) [186]. TAK1 interacts with PLCβ2 as well as with $PI(4,5)P_2$. Activation of TAK1 requires $PI(4,5)P_2$ binding and hydrolysis of $PI(4,5)P_2$ by PLCβ2 suppresses TAKI activation. Thus, PLCβ2 is a negative regulator and viral infection was found to upregulate PLCβ2 levels. In contrast, LPS (lipopolysaccharide), a component of the cell walls of gram-negative bacteria, is a major activator of innate immune responses, and

a potent inducer of the expression of cytokines and inflammatory mediators from macrophages. LPS is an agonist for the TLR4 receptor and has been shown to suppress PLCβ2 levels in macrophages and this plays a role in switching M1 (inflammatory) macrophages into an M2-like state (angiogenic) [187]. LPS also down-regulates PLCβ2 in B-lymphocytes.

5.1.3 PLCβ3

Like PLCβ1, PLCβ3 is widely expressed and is involved in many physiological/pathological responses. PLCβ3 can be activated by both Gβγ and Gαq in a synergistic manner. Additionally, curated phospho-proteome databases indicate that PLCβ3 can be phosphorylated at Ser537 and Ser1105. Phosphorylation at Ser1105 is inhibitory [188, 189], whilst Ser537 phosphorylation does not appear to impact on PLC activity [190]. Ser537 is phosphorylated in the basal state and increases upon stimulation causing its localization to the PM [103, 191]. PLCβ3 appears to function not only as an enzyme but in some cases, it also functions as an adaptor protein as discussed below.

Mice lacking PLCβ3 mice have increased behavioural and cellular responses to opioid agonists. The opioid receptor couples to PLCβ3 via Gβγ and modulates opiate-mediated analgesia. The hypersensitivity to morphine observed in the knockout mice implies that PLCβ3 inhibits some aspect of the opioid-mediated responses [192]. In DRG neurons, μ-opioids inhibit voltage-dependent calcium channels, and in neurons lacking PLCβ3, the inhibition is more pronounced. The increased sensitivity of the PLCβ3-deficient cells to μ-opioid-mediated regulation of voltage-dependent Ca^{2+} channels is also seen with PMA treatment indicating that PKC modulates some aspect of μ-opioid signaling.

Further analysis of the PLCβ3 knockout mice reveal that mice also develop myeloproliferative disease, lymphoma and other tumours. PLCβ3-deficient mice exhibit increased numbers of HSC (haematopoietic stem cells) and myeloid progenitors as well as preferential granulocytic differentiation. The transcription factor, Stat5 (Signal Transducer and Activator of Transcription 5) is widely expressed throughout the hematopoietic system, both in stem and progenitor cells as well as in committed erythroid, myeloid and lymphoid cells. PLCβ3 normally restrains proliferation of HSC and myeloid progenitors by interacting simultaneously with Stat5 and SHP-1 (SH2-containing phosphatase I), a phosphatase that deactivates Stat5. The activity of PLCβ3 is not required; instead the C-terminal extension of PLCβ3 recruits both Stat5 and SHP-1 [193]. A similar mechanism also occurs in mast cells. Here, the C-terminal extension of PLCβ3 physically interacts with the FcεR1, Lyn and SHP-1 to regulate cytokine release from mast cells. In mast cells, PLCγ2 is absolutely required for FcεRI-mediated calcium mobilisation, degranulation, and cytokine production [194]. In addition to PLCγ2, studies *in vivo* indicate that PLCβ3 is also required particularly for the late phase; both migration and secretion of TNFα, IL6 and IL13 is reduced in antigen-stimulated PLCβ3$^{-/-}$ mast cells.

PLCβ3 is also involved in atherosclerosis [195]. Macrophages from PLCβ3-knockout mice are hypersensitive to apoptotic induction and the atherosclerotic lesion size is reduced in these mice [195]. In macrophages, PLCβ3 is found in a complex with Gαq and ORP4L at the PM. ORP4L comprises of a N-terminal PH domain, a FFAT motif that binds to VAP proteins and an ORD domain that can bind and exchange cholesterol for PI(4)P/PI(4,5)P_2. The binding site for PLCβ3 has been mapped to a region which comprises the FFAT domain and

extending to the beginning of the ORD domain (amino acid residues 445-513). The binding site on PLCβ3 has not been identified. Like PLCβ3 knockout mice, ORP4L$^{-/-}$ mice also display a reduction in atherosclerosis [196]. ORP4L is highly expressed in macrophages and is required for PLCβ3 activation by chemokines such as C5a. The increase in I(1,4,5)P_3/Ca^{2+} activates the calmodulin/CaMKII/CREB pathway to upregulate Bcl-XL expression promoting survival. In the absence of ORP4L or PLCβ3, macrophages in the atherosclerotic lesions die by apoptosis displaying a reduction in atherosclerosis. In the presence of oxysterols, the complex of ORP4L/Gαq/PLCβ3 dissociates and also results in increased apoptosis as PLCβ3/Ca^{2+} signaling is reduced.

In contrast to macrophages, ORP4L is absent in T lymphocytes but is aberrantly expressed in T cell acute lymphoblastic leukemia cells (T-ALL cells) and in leukemia stem cells (LSCs), a rare subpopulation of abnormal hematopoietic stem cells (HSCs) that propagates leukemia. In T-ALL cells, ORP4L acts as a scaffold bringing together CD3ε (cluster of differentiation), Gαq and PLCβ3 in a complex allowing for PLCβ3 activation. (Normally CD3 activation leads to activation of PLCγ1 but in the T-ALL cells, activation of PLC is switched from the PLCγ1 isozyme to PLCβ3.) ORP4L is essential for cell survival via increased oxidative phosphorylation and robust ATP production due to metabolic reprogramming [103]. In the Jurkat cell-line, a human leukemic T-cell lymphoblast, ORP4L is also highly expressed and is present in a complex with CD3ε, PLCβ3, and Gαq in anti-CD3-stimulated cells.

ORP4L is normally not expressed in HSCs but is aberrantly expressed in LSCs. Aberrant expression of ORP4L has been noted in other immortalised and transformed cell-lines where it is required for proliferation and survival [197]. Similar to T-ALL cells and Jurkat cells, ORP4L forms a complex with PLCβ3 and Gαq and is required for LSC survival [43]. An inhibitor of ORP4L, LYZ-81, inhibits Ca^{2+} signaling. ORP4L can bind PI(4,5)P_2 in its binding pocket and it is suggested that ORP4L extracts PI(4,5)P_2 from the PM for PLCβ3 to hydrolyze it [43]. However, based on the structures of other ORPs, the phosphodiester bond of PI(4,5)P_2 would not be accessible to the PLC for hydrolysis. Moreover, as discussed in Section 3.1, most PLCs dock onto the membrane to hydrolyze the membrane-resident PI(4,5)P_2 as the phosphodiester bond is accessible. In the case of PLCβ enzymes. the distal C-terminal domain is a coiled-coil domain with highly conserved clusters of lysine and arginine residues arrayed along one face that function as a major membrane binding determinant. Indeed, the membrane acts as an allosteric activator of PLCs [198, 199]. Thus conceptually, the model that PLCβ3 only hydrolyzes ORP4L-extracted lipid is at odds with our general understanding of how PLCβ enzymes work in general.

Studies from human patients have identified mutations in PLCβ3. Mutations either have a protective effect in cystic fibrosis patients [200, 201] or cause autosomal recessive spondylometaphyseal dysplasia [202]. The lungs of patients with cystic fibrosis are highly inflamed due to secretion of IL-8 from the bronchial epithelial cells. IL-8 attracts neutrophils and exacerbates the inflammatory response. PLCβ3 is abundantly expressed in bronchial epithelial cells; in humans a genetic variant (S845L) is protective and confers a milder phenotype in cystic fibrosis patients [201]. When the bronchial epithelial cells are exposed to *P.areugunosa*, the cells secrete ATP which activates the G-protein-coupled P2Y2 receptor in an autocrine fashion. The increase in cytosolic Ca^{2+}/DAG results in PKC activation and subsequently NFκB which contributes to IL-8 expression and secretion. PLCβ3 (S845L) variant is a loss of function mutant, resulting in a decrease in IL-8 release. and thus slows the

progression of the pulmonary disease [200]. Ser845 is localized in the C-terminal tail in close proximity to the C2 domain of the PLCβ3 and its conversion to leucine will result in a loss of function mutant. At the C-terminal part of PLCβ3, the helix–turn–helix Hα1/Hα2 forms the centre of the binding interface by making extensive contacts with multiple residues of Gαq. Ser845 in PLCβ3 is found at the base of the Hα1/Hα2 segment in a region that could affect Gαq-dependent PLCβ3 activation [200].

A rare mutation in PLCβ3 (A878S) has also been identified in two cousins from a consanguineous family [202]. The two patients have spondylometaphyseal dysplasia (SMD). SMD is a rare form of chondrodysplasia characterised by severe abnormalities in vertebrae and metaphyses of tubular bones. Fibroblasts from the patients were found to have increased $PI(4,5)P_2$ levels and a disorganized actin cytoskeleton. The mutation localises to the proximal CTD and appears to destabilise the protein as its expression was greatly reduced when expressed in COS-7 cells compared to the wild type. It is interesting to note that the mutation A878S and S845L both localise to the proximal CTD that is important for Gαq interactions but result in very distinct phenotypes.

5.1.4 PLCβ4

Many physiological functions of PLCβ4 have been identified based on mice knockouts and patient studies **(Table 2 and Table 3)**. It is clear that PLCβ4 is activated by a number of G-protein-coupled receptors. PLCβ4 is enriched in the cerebellum and the retina and mice knockouts show a range of defects including in cerebellar development leading to locomotor ataxia [172], long term depression through the Type 1 metabotropic glutamate receptor in the Purkinje cells in the rostral cerebellum [203], synapse elimination, endocannabinoid signaling [204], impairment in their visual processing abilities [205] and impairment of sleep-dependent memory consolidation [206]. In the cerebellar Purkinje cells, MGluR1/PLCβ4 is crucial for the release of the endocannabinoid, 2AG, for retrograde suppression of transmitter release. PLCβ4 is also required for melanopsin signaling in the iris for the pupillary light reflex and in the intrinsically-photosensitive retinal-ganglion cells (ipRGCs) [207]. PLCβ4 is also highly expressed in the thalamocortical (TC) neurons where it functions downstream of Type 1 metabotropic glutamate receptors to tune the firing mode of the TC neurons via the simultaneously regulation of T and L-type Ca^{2+} currents. In the absence of PLCβ4, mice experience absence seizures (a generalised non-convulsive seizure, characterised by a brief and sudden impairment of consciousness) [208].

Studies in patients indicate other receptors that couple to PLCβ4. Cysteinyl leukotriene receptor 2 (CysLT2R) couples to Gα11/Gαq and activates PLCβ4. Mutations in the receptor, the G-proteins or PLCβ4 have all been identified resulting in uveal melanomas that arise from melanocytes of the uveal tract and are the most prevalent tumors of the eye [209]. The most recurring mutation in PLCβ4 occurs at Asp630 to Tyr, Phe or Asn and results in a hypermorph [210]. Asp630 localises to the Y-domain.

PLCβ4 loss of function mutations or mutations in the catalytic X and Y domains have also been described and results in Auriculocondylar syndrome (ACS) [211-213]. In this case, PLCβ4 is activated by endothelin -1 binding to the endothelin receptor type A. ACS is a rare craniofacial disorder with a specific malformation of the external ear, known as “question mark ears”.

5.2. PLCγ family

As described in **Sections 2 and 3**, there are two PLCγ isoforms that in addition to the core domains are characterised by a large multidomain insert in the X-Y linker that is central to their regulation **(Figure 2)**. PLCγ1 and PLCγ2 are similar in structure and regulation but varies in their expression. PLCγ1 is widely and uniformly expressed, whereas PLCγ2 is highly expressed in the immune and haematopoietic cells. As described in **Section 2**, PLCγ isozymes are regulated by RTKs or by non-receptor tyrosine kinases. The nSH2 domain of PLCγ is recruited to tyrosine-phosphorylated residues on either receptor tyrosine kinases or to non-receptor tyrosine kinases or their adapter proteins. Activation of PLCγ is through phosphorylation of a single conserved tyrosine residue (Tyr783 in PLCγ1, Tyr759 in PLCγ2) [214].

Although PLCγ enzymes have long been recognized as key components in the intracellular signal transmission, it is only during the last 5 years that their roles in disease development have become apparent **(Tables 2 and 3).** Crucially, a number of genetic studies revealed a PLCγ subnetwork as an important regulator of cell functions that can be subverted in various diseases that ultimately require new treatment options. Variants of PLCγ1 and PLCγ2 have been linked to cancer, complex immune disorders, inflammation (further implicated in other diseases such as cancer and steroid-sensitive nephrotic syndrome) as well as Alzheimer's and related neurodegenerative diseases. The major, comprehensive discoveries are described in a number of publications [215-224].

Interestingly, in a number of cases where functional characterization of the effect of genetic changes on PLC activity has been performed, these alterations (predominantly single amino-acid substitutions) result in an increase of PLC activity under basal and/or stimulated conditions [83, 84, 215, 217, 219, 225-228]. Consequently, terms "gain-of-function mutation" (a mutation that confers new or enhanced activity on a protein) and "hypermorphic mutation" (mutation in which the altered gene product possesses an increased level of activity) have been used when describing these PLCγ variants. Superimposing position of mutated amino-acids on the structure of PLCγ1 reveals that the majority of mutations, including most of the hot spot alterations, are likely to directly release an autoinhibition **(Figure 5A)**. However, there are other PLCγ variants where different mechanisms appear to lead to dysfunction. For example, some of the variants could attain higher PLC activity by adopting a more stable active form or by enhanced interactions with regulatory proteins or membrane. Some of the specific mutations are further described in the sections below.

5.2.1 PLCγ1

In many different cell types PLCγ1 is activated *via* receptors for growth factors, such as EGF, FGF, PDGF, BDNF, VEGF and NGF, that are RTKs, or by integrins. The function of PLCγ1 has been analysed by targeted disruption in mice. Whole animal knockout of PLCγ1 is embryonic lethal (after E9) due to defects in vital developmental processes, vasculogenesis and erythropoiesis, both dependent on VEGF signaling [229, 230].

PLCγ1 is also an important signaling component downstream of T-cell antigen receptor (TCR). Conditional PLCγ1-deficient mice, in which PLCγ1 deficiency is restricted to the T-cell lineage, revealed an essential role of this PLC in several aspects of T-cell biology [231]. Notably, PLCγ1 depletion severely impairs TCR-induced activation of multiple signaling molecules and transcription factors and affects T-cell development, activation and tolerance.

In contrast to activation *via* growth factor receptors that provide both, the scaffold and the kinase activity, in T-cells several interconnected adapter proteins (LAT, Gads and SLP76) are involved in positioning of PLCγ1 for subsequent phosphorylation by a non- receptor tyrosine kinase, ITK, and for access to the membrane-bound substrate. Mechanistically, this more intricate activation process is poorly understood compared to the activation by growth factor receptors **(**see **Section 3.3.2)**. Nevertheless, the quaternary PLCγ1/LAT/Gads/SLP76 complex can be reconstructed *in vitro*, where the multiple, cooperative protein–protein interactions generate a circular arrangement of binding interfaces [232]. It has been hypothesized that the relative instability of this quaternary complex can be the target of regulation, enabling the dynamic control of transient assembly and disassembly in a cellular setting.

Both signaling contexts described above, direct activation by growth factor receptors and activation by non-receptors tyrosine kinases in T-cells, are relevant for PLCγ1 dysregulation, most notably in cancer. A number of early and follow up studies implicated PLCγ1 in diverse cancers based on links with well-established oncogenes, mainly growth factor receptors, and overexpression of PLCγ1; these studies also highlighted the tumour progression and development of metastases as the main processes facilitated by these aberrations [233-239]. However, it was only relatively recently that recurrent somatic mutations in *PLCG1* gene were discovered in specific, non-common cancer types. Such mutations were first reported in angiosarcoma, a rare, highly aggressive cancer of endothelial cells of blood vessels [216]. Functions of PLCγ1 that are affected in angiosarcoma include links to angiogenesis, notably to VEGF signaling, and invasiveness [216, 240-242]. The PLCγ1 link with VEGFR2 is further supported by the finding that mutations in these two proteins are mutually exclusive [240].

Subsequently, *PLCG1* mutations were discovered in another type of rare malignancies, T-cell lymphomas, including cutaneous T-cell lymphoma (CTCL) and adult T-cell leukaemia/lymphoma (ATLL or ATL; linked to a human T-lymphotropic virus type 1/HTLV-1) [222, 228, 243-260]. In ATL, *PLCG1* is the most frequently mutated gene (36%, based on the largest study [222]) and, as in other T-cell lymphomas, have been linked to the aberrant TCR signal transduction pathway. Further studies of PLCγ1 variants established that several hot-spot mutations (S345F, E1163K and D1165/H/G/V) and a number of less frequent mutations (e.g. D342G, P867R, Q1016L, F1167I, M1166R and p.VYEEDM1161V indel), resulting in higher PLC activity, are all suitably positioned to disrupt auto-inhibition [83, 84]. However, one of the hotspot substitutions in T-cell lymphomas (R48W in the nPH domain) represents a different mechanistic class that needs further characterization [83, 84]. Another hotspot mutation unique to angiosarcoma, R707Q, appears to have an indirect effect on autoinhibition by affecting stability of the cSH2 domain [84].

Several analyses of clinical data for T-cell lymphomas show that the patients with mutations occurring in components of TCR signaling and in *PLCG1* specifically, have reduced overall or progression-free survival [247, 251, 257]. It has also been shown that identical *PLCG1* mutations are present in multiple tumour compartments of individual patients and persist several years after diagnosis, suggesting that these are driver gene mutations, which are positively selected [228]. Collectively, these new discoveries of mutations and further characterization showing gain-of-function and clinical impact, support the inclusion of

PLCG1 in the current list of oncogenes. Future studies are, however, needed to establish how PLCγ1 regulates cancer-associated cellular processes and in particular the nature and relative impact on cell motility and cell proliferation in specific cancer types [261].

In addition to well-recognized roles in physiology and pathology illustrated above, there is also supporting evidence for the role of PLCγ1 in regulation of various functions of the brain. By specifically knocking out PLCγ1 in neuronal precursors in mice, its role in axon guidance has been revealed [262-264]. The mice are outwardly normal but a deficit in midbrain axon guidance is observed due to defects in netrin/DCC/Src/PLCγ signaling. Netrin interacts with DCC (deleted in colorectal cancer) receptor, functioning as a guidance cue for migrating neuronal progenitors and axons in nervous system development. DCC does not contain an intracellular catalytic domain but contains three highly conserved protein-binding domains termed P1, P2, and P3. These domains mediate the assembly of various combinations of multiple signaling components such as the non-catalytic region of tyrosine kinase adaptor protein 1 (NCK1), Src family kinases and PITPα, which are necessary for the integration of axon guidance cues. PLCγ1 deficiency causes a structural change in the mesencephalon dopaminergic system where the axons do not project to the appropriate locations.

The requirement of PLCγ1 is widespread in the brain and selective knockout of PLCγ1 in the forebrain (e.g. hippocampus, cortex, striatum) results in mice that appear outwardly normal but exhibit several behavioural defects [265]. These include hyperactivity due to deficits in BDNF-stimulated TrkB receptors that mediate PLCγ1 signaling. This signaling pathway is required for the formation and function of GABAergic inhibitory synapses. The hyperactivity phenotype is rescued by treating the animals with lithium chloride, frequently used to treat bipolar disorder in humans [265]. Previous studies had already hinted that *PLCG1* was a susceptibility locus for bipolar disorder [266]. These results support the notion that benefits of lithium salts treatment are at least partially due to an impact on PLC signaling. Lithium chloride inhibit inositol monophosphatase thus resulting in lower inositol levels which impacts on the resynthesis of $PI(4,5)P_2$, the substrate for PLC [267].

Work from many laboratories suggest that enhanced activation of the brain-derived neurotrophic factor (BDNF) receptor, TrkB, promotes development of temporal lobe epilepsy in both human and animal models [268]. BDNF binding to TrkB receptors enhances the TrkB tyrosine kinase activity, resulting in autophosphorylation of Y816, creating a docking site for PLCγ1 recruitment [269]. Using either pilocarpine or kindling to evoke seizures in mice, increased activation of PLCγ1 was observed. (In kindling, repeated electrical stimulations, most often delivered in the amygdala or hippocampus, result in progressive intensification of epileptiform responses, culminating in a generalized seizure.) PLCγ1 activation was required for LTP (long term potentiation) of the mossy fibre -CA3 pyramid synapse of the hippocampus. Importantly, disruption of PLCγ1 recruitment to the TrkB receptor by mutation of Y816 resulted in inhibition of limbic epileptogenesis [269]. Similar results were obtained when PLCγ1 heterozygous mice were used. Kindling development was impaired [270]. Indeed, a peptide that selectively inhibits TrkB-mediated activation of PLCγ1 was found to prevent temporal lobe epilepsy [271].

The above studies have focused on the role of PLCγ1 for epileptogenesis in excitatory neurons. In a study where PLCγ1 was specifically deleted from inhibitory GABAergic neurons genetically, mice developed handling-induced seizures in aged mice [272]. In

contrast, in the temporal lobe epilepsy model, hyperexcitation of excitatory neurons causes the activation of cellular signaling pathways, including the elevated phosphorylation of PLCγ1 mediated by BDNF-TrkB pathway. In this case, uncoupling the BDNF receptor TrkB from PLCγ1 prevents epilepsy [271]. It is clear that the effects PLCγ1 on epilepsy appears to be dependent on the specific neuronal population.

5.2.2 PLCγ2

PLCγ2 is highly expressed in hematopoietic cells and has the key role in signaling downstream of B-cell antigen receptor (BCR) and Fc receptors (FcRs) that bind immunoglobulins and immune complexes. Mice genetically deficient in PLCγ2 are viable but have a distinct phenotype that includes depressed B cell numbers and impaired mast cell, natural killer (NK) cell and platelet function [194, 273]. Defects in B-cells development and responses to stimulation have been extensively characterized and linked to BCR signaling. The ability of platelets to aggregate in response to collagen is absent in the PLCγ2$^{-/-}$ mice. Mast cells fail to respond to antigen that stimulates the IgE-primed Fc receptor, FcεR1. Collagen stimulation of platelets, likewise, depends on the Fc receptor, FcγR. Signaling from other Fc receptors, FcRIIγ/III and 2B4 receptors on NK cells, is similarly impaired. Antibody-induced FcR aggregation, similarly to activation of TCR that is linked to PLCγ1, stimulates protein tyrosine kinases, including Src, Syk and Tec family kinases. Phosphorylation of PLCγ2 by the tyrosine kinases (mostly from Tec family) is mediated by binding to adapter proteins. In addition to BCR and Fc receptors, the PLCγ2/adapters/kinases signaling hub could be linked to other types of receptors (such as TREM receptors) that belong to the same, ITAM-associated receptor family [274]. Data obtained from the analyses of Rac deficient platelets and B-cells [275, 276] support the contribution of this small GTPase in activation of PLCγ2, as observed *in vitro* [277, 278].

Consistent with the insights from the knockout mice, a number of disease-linked mutations and rare variants of PLCγ2 highlight its important role in regulation of B-cell function as well as function of different cell types involved in innate immunity, including mast cells and NK-cells. Notably, the first clear link between a human disease and genetic aberrations in PLC enzymes came from studies of families with dominantly inherited complex and, in many cases, severe immune disorder characterized by cold urticaria (cold-induced swelling) [215]. The cold urticaria in these patients likely results from the fact that mast cells expressing the mutant PLCγ2 spontaneously activate when exposed to lower temperatures. In frame deletions in *PLCG2*, removing/altering the cSH2 autoinhibitory domain in the protein, have been linked to this disorder, subsequently designated as PLCγ2-associated antibody deficiency and immune dysregulation or PLAID [215]. Follow up studies have shown that a gain-of-function in a temperature-dependent manner in PLCγ2 PLAID variants is mechanistically more complex compared to direct disruption of the auto-inhibition [279].

A related dominantly inherited complex immune disorder, designated as autoinflammation, antibody deficiency, and immune dysregulation or APLAID, has been described in several families [217, 219, 280-282]. The associated mutations in PLCγ2 include S707Y, A708P, L848P, L845-L848del and M1141K, and are characterized by gain-of-function resulting from the disruption of intramolecular autoinhibition [84, 217, 219, 280-282]. It is possible that immunodeficiency results from the disruption of B-cell development owing to upregulation

of normal BCR signaling pathway. Studies of other immune cell types from the patients have shown a link with the NLRP3 inflammasome *via* enhanced intracellular Ca^{2+} signaling or, possibly, other mechanisms [282, 283]. This suggests that the inflammation in patients with APLAID is at least in part driven by the activation of the inflammasome, a multiprotein intracellular complex that activates the pro-inflammatory cytokines. The severity for this rare disease ranges from a combination of cutaneous inflammatory manifestations with serious immunodeficiency to a mild phenotype similar to the common variable immune deficiency (CVID) [217, 219, 280-282]. Considering that this range has been observed for the same mutation (M1141K) [281], other intrinsic or extrinsic factors, impacting on the immune system of an individual, seem to contribute to manifestations of the disease. In addition to material from patients, the mouse strains with similar indications, expressing PLCγ2 gain-of-function mutations (D993G in *Ali5* [284] and Y495C in *Ali14* [285] mice), provide useful models to further characterize this type of immune disorders.

In addition to causing monogenic autoinflammatory diseases such as APLAID, variants of *PLCG2* influence susceptibility to inflammatory bowel disease (IBD), a polygenic inflammatory disease. A genome-wide association study of IBD patients, characterized by a chronic disorder of the gastrointestinal tract in genetically susceptible individuals, identified two rare variants of PLCγ2 (H244R and R286W) that have > 50% probability of being causal [218]. These PLCγ2 variants, however, require further characterisation. Similarly, recent genetic studies of steroid-sensitive nephrotic syndrome (SSNS) identified rare variants of *PLCG2* as candidate risk loci for this disease; a compound-heterozygous variant (R268W and P522R) linked to familial SSNS, appears to result in gain-of-function [223, 286]. These studies also suggest a major role for adaptive and autoimmunity in the pathogenesis of SSNS.

The function and dysregulation of PLCγ2 in B-cells is also well documented in the context of cancer drug resistance. Chronic lymphocytic leukemia (CLL) is one of the common haematological malignancies that requires the BCR-signaling pathway for proliferation and survival of CLL cells. Components downstream of BCR include BTK, a Tec family kinase, and PLCγ2 that is directly phosphorylated and, consequently, stimulated by BTK. Although activating mutations in BTK have not been found in CLL, inhibitors of the kinase proved to be potent drugs [287, 288]. Ibrutinib, a covalent, irreversible inhibitor of BTK, has an overall response rate of 70-90%. Despite this success, resistance to ibrutinib does occur and in about 80% of the cases the development of resistance is associated with mutations in BTK itself as well as in PLCγ2 [221, 289-297]. While the BTK mutations affect the residue to which ibrutinib covalently binds (C481), mutually exclusive mutations occurring in PLCγ2 result in gain-of-function that sustains signaling through the BCR pathway even in the presence of ibrutinib. Position and functional impact of a number of PLCγ2 mutations (for example, mutations affecting residues D334, D993, S707, A708, L845, M1141 and D1140) are consistent with an impact on autoinhibition, resulting in gain-of-function [83, 84, 226]. Interestingly, several of these acquired somatic mutations in CLL (S707Y, A708P, M1141K and D993G) are identical to those causing APLAID or related dysfunctions in mice (*Ali5* strain) as germ-line mutations. Together with the similarities in PLCγ1 features affected by mutations, this further highlights the common mechanistic basis for gain-of-function in PLCγ enzymes. One of the frequent mutations in CLL (R665W), however, is likely to stabilize the active form rather than disrupt autoinhibition [83, 84].

Recently, one mutation in PLCγ2 (Q548R; co-occurring with mutations in two other proteins) has been observed in patients that do not respond or lose responsiveness to common therapies for another haematological malignancy, namely, myelodysplastic syndrome (MDS) [298]. However, the functional impact of the mutation on PLC activity and the link of this variant with the changes in the responsiveness to treatments, remain unclear.

Finally, recent observations highlight the importance of PLCγ2 in the context of regulation of macrophages and, specifically, the brain-resident macrophages or microglia. In an extensive genetic study, one rare *PLCG2* variant (P522R) has been reported to strongly associate with the protection from the development of Alzheimer's disease (AD) [220]; this observation has been supported by other, follow up studies [224, 299-302]. Together with identification of other immune and microglia-related genes associated with AD, this *PLCG2* variant has been implicated in immune modulation that plays an important role in neurodegeneration. Genetic studies have shown that this variant also reduces the risk of other dementia (dementia with Lewy bodies and frontotemporal dementia) and, consistent with its protective role, increases the likelihood of longevity [224]. Functional assessment of the P522R PLCγ2 variant has demonstrated a relatively small increase in PLC activity [225] and the position of the substitution (within a linker away from the autoinhibitory surface) differs from the majority of mutations observed in other PLCγ2-linked diseases [34]. With respect to its role in the context of neurodegeneration, further studies are required to better define links of PLCγ2 P522R with ITAM receptors in microglia and with amyloid-β accumulation and/or TAU pathologies that characterize dementia; recent generation of mouse strains expressing PLCγ2 P522R variant, together with other approaches, will facilitate progress in this area [303-305]. Recently, two other different haplotypes around *PLCG2* have been associated with AD and reinforce the role of this genomic region in the AD susceptibility [302].

5.3. PLCδ family

There are three members of the PLCδ family, PLCδ1, δ3 and δ4 [151]. (PLCδ2 was found to be the bovine homologue of PLCδ4). An inactive protein with the same domain structure to PLCδ, (PRIP, phospholipase C-related but catalytically inactive protein) has been identified and will not be covered in this review and interested readers can refer to the following papers [306-308].

5.3.1 PLCδ1

PLCδ1 was the first mammalian PLC whose structure was solved [47]. Unlike PLCβ and PLCγ enzymes which are regulated by activation of appropriate cell surface receptors, the activity of PLCδ can be stimulated by μmolar levels of Ca^{2+}. These levels are in the physiological range that are achieved in cells by activation of other PLCs or entry of Ca^{2+} through calcium channels [309-312]. PLCδ1 localises to the PM due to its PH domain; the PH domain of PLCδ1 binds to $PI(4,5)P_2$ and with $I(1,4,5)P_3$ with very high affinity [64, 313]. Thus increases in $I(1,4,5)P_3$ levels competes with plasma-membrane-anchored PLCδ1 [314]. Thus the activity of PLCδ1 in cells will be influenced by intracellular Ca^{2+} levels, the amount of $PI(4,5)P_2$ at the PM and by $I(1,4,5)P_3$ levels. The mechanism of Ca^{2+} activation of PLCδ enzymes remains unclear.

Two other putative positive regulators of PLCδ1 are a high molecular weight GTP binding protein known as G_h (transglutaminase II) and Ral, a member of the Ras family of small GTPases [315, 316]. Several receptors have been identified that couple to G_h including the FSH receptor on Sertoli cells [317], oxytocin receptor in the myometrium [318] and the thromboxane receptor [319]. G_h binds PLCδ1 via the residues 720-736 of the C2 domain. In the triple-negative breast cancer cells, G_h-PLCδ1 interaction drives metastatic progression [320]. G_h is a bifunctional enzyme with two distinct activities which are mutually exclusive, transamidation and GTP binding and hydrolysis. When bound to GTP, G_h has a closed conformation whilst transamidation occurs in the open conformation. The GTP-bound protein has a high affinity for PLCδ1 and stimulates PLCδ1 activity. Whether these *in vitro* observations have any physiological or pathological relevance remains obscure and requires further study.

Studies from mouse models indicate that PLCδ1 plays a role in keratinocytes. Mice knockouts of PLCδ1 show progressive hair loss, epidermal hyperplasia and epidermoid cyst formation and inflammation of the skin [321-324]. In these knockout mice, calcium mobilization and activation of calcineurin and NFAT is disrupted in the keratinocytes.

A number of genetic studies of patients with hereditary leukonychia support an association with mutations in the *PLCD1* gene [325-329]. Hereditary leukonychia is a nail disorder characterised by nail plates whitening on all finger and toe nails referred to as porcelain nails. Inheritance is either autosomal-recessive or -dominant depending on the mutation [327]. Protein-truncating mutations result in autosomal-recessive inheritance whilst autosomal-dominant mutations display amino acid substitutions. The mutations (including C209R, A285G, E464K, A574T and frameshift deletions/premature stop codons at positions 473 and 740) are scattered over the entire protein **(Figure 5B)**. Functional assessments suggest that some of these aberrations reduce protein expression, stability or PLC activity [327, 330]. PLCδ1 is highly expressed in the nail matrix and bed, and hair follicles and matrix but no hair or skin abnormalities are observed in these patients. Interestingly, a synonymous and missense variant seen in tandem constitutes a high-risk allele (P301P and S460L) for hereditary trichilemmal cyst formation. (Trichilemmal cysts are benign tumours that mostly occur on the scalp.) When this "high risk allele" is further mutated in the C2 domain (S745L), cyst formation on the scalp occurs [331, 332]. Analysis of the PLCδ1 activity of the "high risk allele" indicates no impact but when combined with the cyst-specific mutation, activity is dramatically reduced.

A number of studies also indicate that PLCδ1 may play a role in several other cancers. For example, *PLCD1* plays an important role in different tumors as a tumor-suppressor gene. It is epigenetically silenced through hypermethylation in oesophageal squamous cell carcinoma, breast cancer, gastric cancer, and chronic myeloid leukaemia [333-337].

5.3.2 PLCδ3

PLCδ3 knockout mice do not show any obvious abnormality [338, 339]. PLCδ1 and PLCδ3 share high sequence homology and similar tissue distribution and simultaneous loss causes embryonic lethality at embryonic day 11.5 to 13.5 due to apoptosis of placental trophoblasts and defects in vascularisation of the placenta [338]. Mice where the embryonic lethality of the double knockout is by-passed by expressing PLCδ1 in extra-embryonic tissues, are found

to have cardiac abnormalities. The earliest defect is apoptosis of the cardiomyocytes, a result that could be recapitulated in differentiated H9c2 cardiomyoblasts [322].

5.3.3 PLCδ4

The function of PLCδ4 is enigmatic; mice knockouts of PLCδ4 are male infertile due to defects in the acrosome reaction in sperm, an exocytic event required for fertilisation [340]. Recent studies have identified functions of PLCδ4 that are independent of their PLC activity. PLCδ4 has at least four splice variants that are protein-coding [341-344]. Annotations for human PLCδ4 in UniProt, encodes for two different isoforms of PLC, the shorter isoform PLCδ4b (1-272 a.a.) and PLCδ4a (1-762 a.a). PLCδ4a, the canonical isoform of PLCδ4, contains all the domains characteristic of this family (in order from the N- to C-terminus): the PH domain that can bind phosphoinositides, EF-hands, catalytic TIM barrel (with X- and Y-boxes) responsible for the catalytic activity, and the C-terminal C2 domain. PLCδ4b comprises only of the PH domain and EF-hands. Residing in the EF hands is a conserved sequence of 13 a.a. called the Gα-binding and activating (GBA) motif. The GBA motif binds to $G\alpha_{i3}$ with high affinity docking into a cleft. It has guanine nucleotide exchange activity and can activate G-proteins [342]. The short splice variant, PLCδ4b, is highly active and when expressed in cells as a non-receptor activator of G-proteins. The splice variant is expressed at similar levels to the full-length protein and was shown to function as an activator of heterotrimeric G-proteins in cells [345].

Other studies have identified a role for PLCδ4 in non-canonical Wnt/Ca^{2+} signaling [343]. Activation of the GPCR, Frizzled by Wnt ligand, stimulates an unidentified PLC [346]. Sec14L2/3 are lipid transfer proteins that comprise a CRAL-TRIO domain that can bind lipids and a GOLD domain. In addition, the protein also harbours GTPase activity [347]. It forms a complex with Frizzled and Dishevelled upon activation by Wnt. Sec14L2/3 then binds GTP and activates PLCδ4a. PLCδ4a also acts as a GAP to inactivate GTP-bound Sec14L2/3. These results suggest that PLCδ4 is the unidentified PLC in Wnt signaling.

5.4 PLCε family

PLCε was first discovered in *C.elegans* in 1998 [157]. It is the largest of the PLCs and in addition to the core domains found in most PLCs, it contains a CDC25 domain at its N-terminal and two C-terminal RA domains. PLCε can be activated by both GPCRs as well as RTKs [348, 349]. Activation occurs via the small GTPases of the Ras, Rap and Rho family as well as by βγ subunits [350, 351] **(Figure. 2)**. Ras and Rap binds to the RA2 domain for recruitment to the membrane and for activation [154, 156, 348, 352] whilst Rho activation occurs through a 65 amino acid insert within the catalytic core of the Y domain which is absent in other PLCs [353]. The insert maps to a small surface-exposed loop within the catalytic core that is accessible to interact with Rho proteins. Rho activation occurs downstream to Gα12 and Gα13 stimulated by receptors for LPA and thrombin [348, 354] whilst Ras activation occurs downstream to EGF receptors [355]. Rap2B activation occurs via the β2-adrenergic receptor and by the M3 muscarinic cholinergic receptor; both stimulate cAMP production that regulates the Rap guanine nucleotide exchange factor, EPAC [356, 357]. The CDC25 domain is functional as a RasGEF and the isolated domain is sufficient to activate the MAP kinase pathway through activation of Ras [155]. Yet another activator of PLCε is βγ subunits. Activation by βγ subunits requires the RA2 domain as well as the N-

terminal CDC25 and PH domain. A truncated PLCε comprising of the EF hands, the catalytic domain, C2 domain and RA1 cannot be activated by βγ subunits but can still be activated by Rho [351]. Thus, multiple inputs have been identified for PLCε activation mainly based on studies in cultured cell lines using co-expression studies. Moreover, the CDC25 domain also interacts with Rap1, providing active Rap1 locally for activation through the RA2 domain [358].

Recent studies suggest that PLCε activation shows a more sustained pattern of signaling compared to PLCβ. In rat-1 fibroblasts, GPCR agonists, endothelin 1, LPA and thrombin stimulate both endogenous PLCβ3 and PLCε. PLCβ3 only contributes to the acute activity seen in the first 1-3 minutes; sustained activity (10-60 min) is dependent on PLCε activation [115]. Studies in astrocytes also show that LPA, SIP and thrombin also stimulate sustained signaling through PLCε. LPA and SIP receptors generally couple to $G\alpha_{12/13}$ which couple to exchange factors to activate RhoA [116]. Upon binding to Ras and Rap1, PLCε translocates to spatially distinct sites, the plasma membrane and the perinuclear region respectively. For the sustained stimulation by PLCε, recruitment to the perinuclear region by Rap1 and further activation of Rap1 by the CDC25 domain allows signaling to be maintained for longer periods [358, 359].

Recent studies in mice highlight the function of PLCε both at the PM and at the Golgi. Studies in cardiomyocytes where PLCε is expressed at high levels show the importance of sustained signaling due to the intracellular-localized PLCε. Chronic stimulation by β1-adrenergic receptors in the heart leads to cardiac hypertrophy and subsequently heart failure. PLCε and EPAC is scaffolded at the nuclear envelope by muscle-specific AKAPβ (A-kinase anchoring protein) in close proximity to Golgi [155, 360, 361]. Golgi-localized β1-adrenergic receptors have been identified that generate a specific pool of cAMP with privileged access to EPAC/AKAPβ/PLCε. PLCε is able to access PI(4)P at the Golgi for hydrolysis [360]. The internal pool of receptors is activated by noradrenaline which is transported into the cells via the membrane cation transporter, OCT3 [362]. Diacylglycerol produced at the Golgi activates nuclear PKD [363]. Endothelin-1 can also regulate PI(4)P hydrolysis at the Golgi via release of Gβγ subunits [364, 365].

In a separate study, PLCε was required for vesicle budding from the TGN. PLCε activation at the TGN can also occur via RhoA. GEF-H1/ARFGEF2 is an exchange factor for RhoA associated with microtubules. Release of the exchange factor by nocadazole activates RhoA leading to PLCε activation using PI(4)P as substrate. The RhoGAP, DLC3 is a negative regulator that provides a restraint. DAG activates nPKC which in turn phosphorylates PKD. Active PKD recruits Rab8 to TGN membranes and induces the fission of Rab6-positive vesicles that travel along microtubules. One cargo identified was TNFα which was delivered at focal adhesions [366]. This pathway was also stimulated by LPA and thrombin.

PLCE1 is mutated in the X domain in humans causing nephrotic syndrome [367]. Nephrotic syndrome is characterised by proteinuria due to disruption of the glomerular filtration barrier executed by podocytes. Podocytes exhibit a unique cytoskeletal structure that maintains the kidney filtration barrier and mutations in the podocyte cytoskeletal proteins including advillin (a member of the gelsolin/villin family) also results in proteinuria. PLCε binds to tyrosine-phosphorylated advillin and knockdown of advillin inhibits EGF-stimulated PLCε activation and subsequently lamellipodia formation [368]. Thus, PLCε is downstream to advillin and its

recruitment to advillin is essential for podocyte function in humans. Advillin is a homologue of villin and tyrosine-phosphorylated villin also recruits PLCγ1 and regulates its catalytic activity [369]. PLCε does not possess SH2 domains for recruitment to phospho-tyrosine residues and thus mapping the sites on PLCε that are important for this interaction is required. Interestingly, mouse models of PLCε do not have a nephrotic phenotype yet again an example where mouse and human protein function seems to diverge **(**compare **Table 2 with Table 3)**.

Direct interaction between the Ras oncoprotein and PLCε and subsequent stimulation described above, prompted further research to assess the possibility that this PLC functions as an effector of Ras in cancer. Despite generation of two mouse strains deficient in PLCε and analyses in the context of Ras-driven cancers, there is no clear answer to this question [370, 371] **(**see **Table 2).**

5.5. PLCζ family

First reported in 2002, PLCζ is the smallest mammalian PLC and consists of four domains; EF-hands, the X and Y catalytic domains and a C2 domain [158, 372, 373]. The PH domain found in all the classical PLCs is absent **(Figure 2)**. This PLC is sperm-specific and is the physiological trigger responsible for generating $I(1,4,5)P_3$-mediated Ca^{2+} oscillations that induces oocyte activation during mammalian fertilisation. Spermatozoa from PLCζ knockout mice fail to induce Ca^{2+} oscillations when microinjected into mouse oocytes. Nonetheless, males are not completely infertile; some offspring were produced. However, *in vitro* fertilisation with such sperm produce fewer Ca^{2+} oscillations which were delayed, cause polyspermy accompanied by failure to activate the oocytes [374, 375].

PLCζ is unique in its ability to activate oocytes compared to other PLCs. PLCζ is the most Ca^{2+}-sensitive PLC requiring nanomolar Ca^{2+} found in resting oocytes. This feature implies that PLCζ must be inhibited in some way when present in sperm. Interestingly, PLCζ does not hydrolyze PM $PI(4,5)P_2$ but hydrolyzes substrate localized to distinct vesicular structures inside the egg cortex.

Consistent with the important role of the sperm PLCζ in fertilization, many studies from male patients with fertility problems have identified mutant variants in this enzyme; the inheritance patterns underlying male infertility, however, vary [376-380]. Amino acid substitutions in the EF-hands (I120M), catalytic domain (R197H, L224P, H233L, H398P and P420L) and C2 domain (I489F and S500L) as well as frameshift deletions /premature stop codons (in the X-Y linker and catalytic domain), have all been identified in these patients **(Figure 5B)**. The EF hands and the X-Y linker region are essential for the interaction of PLCζ with the $PI(4,5)P_2$ containing membranes whilst the C2 domain is essential for PLCζ activity *in vivo* but not *in vitro* [381, 382]. Functional studies support loss-of-function of many PLCζ variants found in patients and highlight their link to the failure of oocyte activation regulated by calcium oscillations [376-380].

5.6. PLCη family

The PLCη family (PLCη1 and PLCη2) was identified in 2005 [159-162]. Like the other mammalian PLCs, the domain organisation of PLCη consists of PH, 4 EF-hands, catalytic X and Y domains, separated by a linker region, C2 domain and a long C-terminal domain (Fig. 2). The linker region in PLCη enzymes is at least 100 amino acids longer than that of PLCδ enzymes. Although this domain organisation is similar to the PLCβ family, amino acid comparison show that PLCη enzymes are more similar to PLCδ enzymes [162]. Three splice variants of PLCη1 and five splice variants of PLCη2 have been identified, all of which differ in the length of the C-terminus [161, 383]. Both PLCη enzymes also contain a PDZ binding motif similarly to PLCβ. However, three of the PLCη2 splice variants have lost the PDZ domain.

PLCη1 is expressed in a range of mice tissues with the highest expression in the brain and kidney with lower expression in lung, spleen, intestine, thymus and pancreas [159]. However, western blot analysis indicates the presence of protein only in several brain regions including the cerebellum, cerebrum and the spinal cord but not in other tissues including the kidney, most likely due to lower expression. On the other hand, PLCη2 appears to be exclusively present in specific regions of the brain where it is developmentally regulated [162]. Expression only increases after birth. It is particularly enriched in the pyramidal cells of the hippocampus, cerebral cortex, the olfactory bulb, habenula, retina, pituitary and neuroendocrine cells [383, 384]. PLCη2 localises to the PM via its PH domain, similar to PLCδ1 [161].

All mammalian PLCs with the exception of the newly identified PLC-XD family (see below) need Ca^{2+} for their catalytic activity. Ca^{2+} sensitivity varies amongst the different PLCs with PLCη2, PLCδ1 and PLCζ being the most Ca^{2+} sensitive. Compared to PLCδ1, PLCη2 is even more sensitive; *in vitro* maximal activation of PLCη2 occurs at 1μM whilst PLCδ1 is maximally activated at 10μM [162]. The EF hand of PLCη2 senses Ca^{2+} as mutation of the residue D256A in the EF-loop1 results in a 10-fold loss in Ca^{2+} sensitivity in transfected COS-7 cells [385]. In addition to regulation by Ca^{2+}, PLCη2 but not PLCη1 can be specifically activated by $\beta_1\gamma_2$ subunits when co-expressed in COS-7 cells, an indication of regulation by GPCRs [161, 386].

Mice knockouts of PLCη2 show no obvious phenotypes [384]; however, no analysis of neurological function was conducted. It was suggested that the function of PLCη2 is modulatory and is probably restricted to specific neuronal populations. Arguably, the most intriguing feature of PLCη2 is their apparent ability to respond to large changes in intracellular Ca^{2+} as are often observed locally in neurons. Neurons can increase their Ca^{2+} concentrations through either voltage-gated Ca^{2+}- or through receptor-activated Ca^{2+}-channels. Activation of PLCη2 could locally regulate $PI(4,5)P_2$ levels. Because $PI(4,5)P_2$ has multiple functions including regulation of ion channels and actin cytoskeleton and is required for exocytosis, PLCη2 could be an important modulator of neuronal function. The function of PLCη2 has been explored in neuronal cultured cell-lines and results suggest that it may be involved in neuronal differentiation and vesicle exocytosis in neuroendocrine cells [387, 388]. Using the NeuroA cells as a model for neuronal differentiation by retinoic acid, it was found that PLCη2 activity was required for differentiation. A mutant devoid of PLC activity inhibited differentiation as did knockdown by siRNA [387]. LIMK-1 (LIM domain kinase 1) was found to interact with PLCη2 [387, 389]. LIMK-1 phosphorylates cofilin1 and 2

suggesting a link between PLCη2 and the cytoskeleton. Furthermore, studies in PC12 cells showed that activation of PLCη2 can modulate exocytosis when Ca^{2+} levels are significantly elevated. Hydrolysis of $PI(4,5)P_2$ results in F-actin disassembly, thereby removing the physical barrier for dense core granules to be recruited to the plasma membrane [388, 390].

5.7. PLC-XD family

The most recent family of PLCs to be discovered is the PLC-XD family in 2012 bringing the total number to seven distinct classes of PLCs [40]. The family was named as phospholipase C X-domain containing protein (PLC-XD) due to the presence of only the conserved X-domain in the catalytic βα-barrel. We classify this family as atypical PLCs whilst the rest of the PLCs as classical PLCs (**Figure 2**). Three tissue-specific PLC-XD isoforms exist in humans, comprising hPLC-XD1, -XD2 and -XD3, with hPLC-XD2 exhibiting three C-terminal splice-forms. Specific histidines essential for the catalytic function of PI-PLCs are conserved in all three human PLC-XDs and overexpression of all three PLC-XD proteins in the HeLa cell-line increased basal PLC activity compared to non-transfected cells. PLC-XD3 showed the highest activity. Addition of the calcium ionophore, A23187 only marginally increased PLC-XD1 and -2 activity whilst no increase was observed for PLC-XD3. Human PLC-XD isoforms exhibit tissue-specific expression profiles in mice with all three PLC-XDs highly expressed in brain. In humans, PLC-XD3 is highly enriched in heart with lesser amounts in lung and kidney [40]. Over-expression of tagged enzymes in HeLa cells indicated that PLC-XD1 and PLC-XD3 are intracellular in an uncharacterised compartment which could be ER (PLC-XD1) or Golgi (PLC-XD3). PLC-XD2 localized to the nucleus.

The sex chromosomes, X and Y, differ in their gene content but share a small region of sequence homology known as the pseudo autosomal region (PAR). The PLC-XD1 gene is the most terminal protein-coding gene of the PAR in humans, dogs and horses (but not in ruminants including sheep, goat or cattle, where it is X-specific). The PAR region shows a high identity of sequence similarity and identical gene content between the X- and Y-chromosomes and is needed for sex chromosome segregation in male meiosis [391].

Recent studies have identified the PLC-XD3 gene might confer vulnerabilities to early onset bipolar disorder [392]. A mutation (R93H) was found in one individual and a deletion of the PLC-XD3 locus in another. Bipolar disorder has been linked with phosphoinositide signaling and drugs used to treat bipolar disorder such as lithium chloride are known to target the phosphoinositide signaling. It is notable that PLC-XD3 is highly enriched in the brain.

PLC-XD3 has also been identified as a potential regulator of insulin secretion from islets of Langerhans [393, 394]. PLC-XD3 is highly expressed in human islets and in the INS-1 cell-line and islets from diabetic patients have a significant reduction in PLC-XD3 expression [393]. In islets obtained from human tissue, expression of PLC-XD3 correlated positively with insulin secretion and negatively with increased glycated haemoglobin A1c (HbA1c), a marker for Type II diabetes [394]. Down regulation of PLC-XD3 from β-cells, INS-1 cell line, resulted in decreased insulin secretion when stimulated with high glucose [393]. It would appear that insulin secretion can be modulated by different PLCs as some GPCRs use PLCβ1 to modulate glucose-stimulated insulin secretion as discussed above [178, 179].

Drosophila has a single *PLCXD* gene and is involved in regulating endosomal $PI(4,5)P_2$ [54]. Endosomal $PI(4,5)P_2$ is generally maintained at low levels by the $PI(4,5)P_2$ phosphatase, OCRL1 in mammals and dOCRL in flies; depletion of OCRL results in abnormal accumulation of $PI(4,5)P_2$, disorganisation of the endocytic compartments and cytokinetic defects. Recent studies identified PTEN together with dPLC-XD could compensate for depletion of dOCRL in *Drosophila*. Although PTEN is a $PI(3,4,5)P_3$ phosphatase, phosphatase activity was not required. PTEN comprises of five conserved domains, an N-terminal $PI(4,5)P_2$ binding domain (PBD), a catalytic domain, a C2 domain, an auto-inhibitory C-tail domain and a C-terminal PDZ binding motif. The PBD and the C2 domain were sufficient to rescue the dOCRL phenotype and importantly reduced the level of $PI(4,5)P_2$ on endosomes. PTEN functions by activating PLCXD on endosomes although a physical interaction between PTEN and dPLC-XD was not identified.

In the legume, *Medicago truncatula*, DNF2 (Deletion in nitogen fixation 2) is identified as the plant PLC-XD and DNF2 mutants cannot fix nitrogen [395]. Nitrogen fixation is dependent on rhizobia bacteria present in the nitrogen-fixing nodules.

6. PLCs and their complex interplay in physiology and pathology

PLCs play a role in many aspects of physiology/pathology that preclude inclusion of all the available possibilities in this review. PLCs are expressed in virtually all cell-types and in most cases multiple PLC family members are co-expressed. Below, we have selected three examples where we examine how PLCs are used to regulate cellular functions. The first example is the integration of ion channel regulation by activation of PLCβ isozymes to regulate blood flow in the brain, the second example is the use of multiple PLCs in cardiovascular function and finally, PLC activation in Alzheimer's disease (AD). These examples are supported by a number of recent findings.

6.1. Ion channel regulation by PLCβ to regulate blood flow in the brain

The excitable behaviour of neurons is determined by the activity of their endogenous membrane ion channels. Several ion channels are indirectly regulated by GPCRs that signal through PLCβ families. The first ion channel that was described to be regulated by activation of muscarinic (M1) cholinergic receptors was the M channel composed of Kv7.2 and 7.3 subunits (KCNQ2 and KCNQ3 gene products) (reviewed in [396]). The M1 receptor activation closes the channels by activating Gq-PLCβ to reduce membrane $PI(4,5)P_2$ to levels below those needed to keep the channels open. Since the original observations on the M1 muscarinic receptors, ion channel regulation by $PI(4,5)P_2$ has been identified as a widespread process in many cell-types including the brain. Interestingly, reduction in $PI(4,5)P_2$ in a single cell can result in closure of some channels and opening of others allowing for signal integration [397, 398].

Below we will review recent studies where a decrease in $PI(4,5)P_2$ due to PLCβ activity modulates blood flow in the brain by closure of K^+ channels and opening of TRPV4 (transient receptor potential vanilloid 4) channels [397, 399, 400]. Capillaries in close proximity to neurons respond to the metabolic demands of neurons, by enhancing blood flow by dilating upstream arterioles, a process known as neurovascular coupling. The endothelial cells of the capillaries are connected by gap junctions and thus can transmit information from

the capillaries to the arterioles. The potassium channel, Kir.2.1 present in endothelial cells allows the inward flow of K^+ when extracellular K^+ increases during neuronal activity. This causes a vasodilatory hyperpolarizing signal. Like many Kir2 channels, channel activity of Kir2.1 is regulated by $PI(4,5)P_2$. $PI(4,5)P_2$ binds to specific positively-charged residues of the channel to keep the channel in the open state [16, 17]. Release of agonists from astrocytes or neurons that can act on GPCRs that regulate PLCβ activity modulate the channel activity by causing decreases in $PI(4,5)P_2$ levels. Thus, PGE_2, carbachol and ATP inhibit the Kir2.1 current due to inhibition of the capillary to arteriole signaling and thus modulating blood flow [399].

In addition to Kir2.1, endothelial cells of the capillaries also express depolarizing channels that intersect with Kir2.1 mediated signaling. Ca^{2+}/Na^+-permeable TRPV4 channels are expressed and are tonically inhibited by $PI(4,5)P_2$, the opposite to Kir2.1 channels. Thus depletion of $PI(4,5)P_2$ by agonists, that signal through GPCRs causes simultaneous disinhibition of TRPV4 channels and suppression of Kir2.1 channels [397]. Thus, GPCR activation functions as a molecular switch to favour capillary TRPV4 activity over Kir2.1 signaling.

6.2. Phospholipase Cβ and PLCε in cardiac hypertrophy

The mammalian heart is a dynamic organ that can adapt in response to physiological stimuli or pathological insults. The heart responds by hypertrophic enlargement, which is characterized by an increase in the size of individual cardiac myocytes being postmitotic cells (reviewed in [401]). Several GPCRs are involved in triggering pathologic cardiac hypertrophy and both PLCβ1 and PLCε play specific roles in mediating these responses. Transgenic expression of active Gαq in animal models have long established that sustained stimulation of PLCβ results in cardiac hypertrophy mediated by DAG-mediated PKC activation [402]. Activation by Angiotensin II, vasopressin, adrenaline acting via α1-adrenergic causes cardiomyocyte hypertrophy in isolated cardiomyocytes, cardiomyocyte cell-lines and in *in vivo* models [169, 170, 403, 404]. These receptors all couple to Gαq and activate PLCβ family and are thought to induce myocyte hypertrophy through a mechanism termed excitation–transcription coupling. The splice variant PLCβ1b appears to be specifically responsible for the hypertrophy responses [166]. PLCβ1b lacks the PDZ domain but contains a proline rich domain at its C-terminus which can bind to the SH3 domain of Shank3 at the sarcolemma [166-168, 405]. PLCβ1b expression and activity is also upregulated in diseased myocardium from mice and humans [167].

According to a recent study, Gαq signaling in cardiac myocytes is compartmentalised in different subcellular locations resulting in unique hypertrophy phenotypes dependent on the GPCR activated [406]. Angiotensin II and adrenaline through α1-receptors both activate gene transcription but adrenaline activates a more robust response compared to Ang II. PLCβ1 was found to localise not only at the sarcolemma and T tubules but also at the nuclear envelope. (The specific splice variant was not identified in this study). α1-adrenergic receptors co-localized with PLCβ1and $PI(4,5)P_2$ at the nuclear membrane whilst Ang II receptors localized to the sarcolemma with PLCβ1. Both stimuli induce transcription but the response stimulated by Ang II is mostly a subset of the α1-adrenergic-induced transcription. In summary, these results link Gq-receptor compartmentalization in cardiac myocytes to unique hypertrophic transcription.

Hypertrophic responses can also be stimulated through β-adrenergic receptors. Chronic stimulation of β-adrenergic receptors by noradrenaline stimulates PLCε through Rap1. In this case, cAMP activates EPAC, the Rap1 exchange factor [407]. PLCε is scaffolded to a muscle-specific A kinase anchoring protein (mAKAP) together with protein kinase A and EPAC at the nuclear envelope [361]. The substrate for the intracellular-localized PLCε is not $PI(4,5)P_2$ but Golgi-localized PI(4)P [360]. DAG formed at the Golgi activates PKD which is thought to be responsible for the hypertrophic responses [360, 362].

Thus, hypertrophic response in cardiomyocytes can occur via activation of different PLCs. Moreover, PLC activation is not confined to the plasma membrane but can occur intracellularly. The substrate for PLC is generally accepted to be $PI(4,5)P_2$ which is mainly localized at the plasma membrane. PI(4)P is highly enriched at the Golgi and it appear that PLCε uses this as its substrate. Whether other PLCs also function intracellularly is a distinct possibility with the identification of PLC-XD family; the possibility that not only PI(4)P can be used as a substrate, but also PI may be subject to hydrolysis by PLCs.

6.3. Phospholipase C in Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia and patients experience a progressive decline in cognition and memory loss. The pathological hallmarks of AD include aggregation of amyloid-β peptides (Aβ) as extracellular senile plaques and the formation of neurofibrillary tangles of hyperphosphorylated tau proteins (reviewed in [408-410]). Extracellular deposits of abnormally-folded amyloid β with 40 or 42 amino acids (Aβ40 and Aβ42) are two by-products of the cleavage of the amyloid precursor protein (APP) by γ-secretase. Autosomal dominant mutations in either presenilin-1, -2 or APP is the cause of 50-70% of familial AD and these highly penetrant mutations alter APP cleavage to produce an increased ratio of Aβ42 compared to Aβ40, the predominant product found in non-pathogenic aged brains. Aβ42 is the most toxic form of the peptide as it appears to be more prone to the formation of amyloid fibrils, one of the early events in AD pathogenesis.

$PI(4,5)P_2$ is highly enriched in neurons and in the synapse, two enzymes regulate $PI(4,5)P_2$ degradation, PLCβ and synaptojanin 1 (Synj1), a $PI(4,5)P_2$ 5-phosphatase; both enzymes are implicated in Alzheimer's disease [411-416]. Early studies indicated that familial AD-associated pathogenic presenilin mutations affected the transient receptor potential melastatin 7 (TRPM7)-associated Mg^{2+}-inhibited cation (MIC) channel, through a $PI(4,5)P_2$-dependent mechanism. Mutations in presenilin was found to suppress TRPM7 currents that could be rescued by exogenous addition of $PI(4,5)P_2$. Moreover, $PI(4,5)P_2$ levels modulated the amount of Aβ42; inhibition of PLC activity decreased Aβ42 secretion whilst activation of PLC increased Aβ42 secretion. To confirm that $PI(4,5)P_2$ was the major contributor, the $PI(4,5)P_2$ phosphatase, Synj1, also increased Aβ42 secretion [411].

Subsequent studies show that Aβ42 can reduce $PI(4,5)P_2$ levels through PLC activity when incubated with primary cortical neurons or with hippocampal neurons [412, 416]. Addition of oligomeric Aβ42 *in vitro* to cortical neuronal cultures caused a decrease in $PI(4,5)P_2$. Not only did synthetic Aβ42 oligomers, but also cell-derived Aβ from APP-mutated mice caused a decrease in $PI(4,5)P_2$ levels when added to primary cortical neurons, hippocampal neurons or to neuroblastoma or PC12 cells [412]. The decrease in $PI(4,5)P_2$ required extracellular Ca^{2+} and was inhibited by putative PLC inhibitors. Addition of Aβ42 oligomers reduced $PI(4,5)P_2$ acutely and was sustained over a period of 7 days. Removal of Aβ42 from the cells

reversed the decrease in $PI(4,5)P_2$. It was suggested that Ca^{2+} entry through NMDA (glutaminergic N-methyl-D-aspartate) receptors at the post synapse was required for PLC activity [412]. Blockade of NMDA with the selective inhibitor D(–)-2-amino-5-phosphonovaleric acid (AP5) caused a partial rescue of the $PI(4,5)P_2$ deficiency, suggesting that oligomeric Aβ42 in part requires functional NMDA receptors to exert its effects on $PI(4,5)P_2$ levels. Using PC12 cells, they demonstrated that Aβ42 stimulated the loss of $PI(4,5)P_2$ from the plasma membrane with a concurrent increase in DAG. Putative inhibitors of PLC activity suggested that addition of Aβ42 led to activation of PLC. The PLC activated was not identified but the potential candidate PLCs that are activated by cytosol Ca^{2+} at the postsynapse would include the PLCδ and PLCη enzymes which are specifically enriched in the brain.

Another study indicates that there is also a pre-synaptic deficit in excitatory hippocampal synapses in AD patients and is also regulated by altered $PI(4,5)P_2$ metabolism. In this study, it was noted that Aβ42 significantly depleted total, axonal and dendritic $PI(4,5)P_2$ in cultured hippocampal neurons [416]. In an AD mouse model, pharmacological mGluR5 inhibition prevents cognitive impairment and a reduction in disease pathology implying a role for the metabotropic GluR5 [417]. Aβ42 was found to directly activate the mGluR5 at the pre-synapse in the hippocampus decreasing the release probability at the synapse between the Schaffer collateral (CA3) and CA1 pyramidal neurons in mouse models of AD with elevated Aβ production. Aβ42 also suppressed the release probability at the Schaffer-collateral-CA1 synapse in wild-type mice. The metabotropic GluR5 works through Gαq PLCβ pathway resulting in depletion of $PI(4,5)P_2$ in axons. Selectively inhibiting Aβ-induced $PI(4,5)P_2$ hydrolysis in the CA3 region of the hippocampus prevented oligomeric Aβ-induced suppression of release probability at the Schaffer collateral-CA1 synapse and rescued synaptic and spatial learning and memory deficits in APP/Presenilin 1 mutant mice. These results reveal oligomeric Aβ induces early synaptic deficits in AD through the pre-synaptic mGluR5-PLCβ pathway [416]. Using RNAi, it was demonstrated that both PLCβ1 and PLCβ4 were activated by Aβ identifying the PLCβ-subtype involved.

In sporadic AD, gene dosage of apolipoprotein E type 4 (ApoE4) is a major risk factor and is also associated with decreased levels in $PI(4,5)P_2$ [414]. From studies with ApoE4 knockin and ApoE-null mice, degradation of Synj1 mRNA is dysfunctional and results in increased Synj1 protein leading to decreased $PI(4,5)P_2$ levels. Not surprisingly, in animal studies, increasing $PI(4,5)P_2$ concentration by genetic reduction of *Synj1* has been shown to be neuroprotective in AD animal models [413, 415].

Thus several lines of evidence indicate that Aβ42 causes PLC activation either through the metabotropic glutamate receptor 5 at the pre-synapse or through increases in cytosol Ca^{2+} through NMDA receptors. The resulting decrease in $PI(4,5)P_2$ levels at the pre-synapse or the post-synapse has multiple consequences including deficits in the TRPM7 channel activity, increased Aβ40:Aβ42 ratio and suppression of excitatory neurotransmitter release. Multiple PLCs appear to be involved; PLCβ1 and β4 isoforms at the pre-synapse and PLCδ and PLCη enzymes, at the post-synapse.

It is well documented that neuroinflammation is also a pathological hallmark of AD; both beneficial and destructive activity of immune cells have been observed (reviewed in [410, 418, 419]). In contrast to neurons, in microglia, one PLCγ2 variant has been identified that is

protective against late stage AD as described in Section 5.2.2 [224, 225, 420]. Microglia are the immune cells (phagocytes) of the brain and this PLCγ2 variant (P522R) has a slight hypermorphic effect on enzyme activity [225]. It is unclear how this variant exerts its protective effects, but this subtle upregulation of PLCγ2 activity is thought to improve the immune function of microglial cell. Recent studies have indicated that the P522R variant might exert its beneficial effects by enhanced phagocytic or other endocytic activity and possibly also by increased acute inflammatory responses [303, 304, 421]. It is presumed that the microglia are in a more active state in the mouse when this variant is present, compared to the wild type protein [303]. This suggests that a weak lifelong activation, and not inhibition, of PLCγ2 in microglia could have a beneficial therapeutic effect. Consequently, inhibiting PLC activity in neurons prevents synaptic dysfunction, whereas a subtle upregulation of PLCγ2 activity in microglial cells has a protective effect.

7. PLCs as targets for small molecule modulators and other treatment options

Selective small molecule modulators or other selective compounds are critical in elucidating physiological and aberrant functions of specific proteins in cells and whole organisms. Furthermore, such compounds serve as candidates for drug development. Specific pharmacological inhibitors, promising drug compounds and FDA approved drugs have now been developed for many signaling components within cellular networks and in particular for protein kinases [422, 423]. PLC enzymes, however, notably lack not only potential drug molecules but, it appears, there is not even a fully validated, direct small molecule inhibitor suitable for research applications. This is even more surprising for a class of signaling proteins that are not intrinsically intractable and possess well-characterized enzymatic activity. Until relatively recently, the main limitations in inhibitor development were related to a lack of suitable high-throughput screening, difficulties of generating chemical probes based on $PI(4,5)P_2$ substrate and, importantly, a lack of motivation based on insufficient evidence linking changes in PLC function with a disease development. As discussed in previous Sections, significant progress has been made in generating supporting data for the involvement of PLC enzymes (in particular PLCγ; **Section 5.2**) in disease development, providing new impetus for drug discovery initiatives. Below, we outline the current issues and describe recent advances that address some of them.

7.1 Properties of compounds frequently used to study the PLC-pathway

Several compounds, structurally unrelated to $PI(4,5)P_2$, have been identified as potential PLC inhibitors and are widely used in research. These include the most frequently used aminosteroid U73122 [1-(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione] and edelfosine or ET-18-OCH3 (1-0-octadecyl-2-0-methyl-glycerol-3-phosphocholine) [424, 425]. However, mechanisms of action remain unclear and a number of reports, in particular for the most widely used U73122, suggest other targets; these include calcium pumps, ion channels and unrelated enzymes regulating lipid metabolism [426-436]. Initial assessments *in vitro* have suggested that the inhibitory effects of U73122 could be demonstrated only when using specific lipid composition and further studies have shown that treatment of purified PLC isoforms with this compound had surprisingly diverse effects on activity, including increased activity of PLCγ [437, 438]. Although there is no definitive answer that explains many different actions of U73122 in cells, it is possible that the effects

on the PLC-pathway are caused by the ability of this highly lipid-soluble cation to sequester anionic PI(4,5)P_2 substrate while the inhibition of numerous protein targets could be due to its chemical reactivity to alkylate cysteine -SH groups. Surprisingly, despite these well-documented shortcomings, U73122 is still used as an inhibitor of PLC enzymes.

Similar issues remain unresolved for the compound *m*-3M3FBS that could potentially act as a pan-PLC activator [439]. Specifically, in addition to unfavorable properties of the compound, there is no clear evidence for direct binding, the interaction sites on PLC proteins have not been defined and unrelated targets have been identified in cells [434, 440, 441].

Collectively, these observations highlight the need to reevaluate the early pharmacological modulators and generate new, specific compounds for PLC enzymes. Considering that many 3D-structures of PLCs have been solved **(Table 1)**, structural and mechanistic insights into modulatory action of the existing and new compounds will be important for further progress. Furthermore, as already shown for many target proteins involved in cellular signal transduction, these efforts will also facilitate rational drug design.

7.2. New assays suitable for high-throughput screening

For over 30 years the main methodologies to directly measure PLC activity have been based on radioactively-labelled inositol headgroup present in lipid substrates for the use *in vitro* or incorporated in cellular phosphoinositides by metabolic labelling of specific cell types; following hydrolysis by PLC enzymes, the radioactivity, now present in inositol phosphates, is redistributed to the water-soluble phase [442]. Some of the initial high-throughput screens also used radioactively labelled PI(4,5)P_2 for applications *in vitro* [443]. As discussed below, a second generation of assays, for measurements of PLC activity both *in vitro* and in cells, is now emerging; importantly, these assays are also suitable for high-throughput screening.

One of the technologies that has been used to develop new assays for sensitive measurements of PLC products generated in cells, specifically the inositol-1-P accumulated following the conversion from I(1,4,5)P_3 in the presence of LiCl, is based on homogeneous time-resolved fluorescence (HTRF) assay [444]. In this competitive immunoassay the inositol-1-P produced by cells competes with an inositol-1-P analogue coupled to a d2 fluorophore (acceptor) for binding to an anti-inositol-1-P monoclonal antibody labelled with Eu Cryptate (donor); this commercially available IP-One assay (Cisbio) can be conducted in a multi-well format. Direct comparison with the traditional methods and applications to different cellular systems revealed its suitability for measurements of PLC activity of different isoforms and stimulation by a range of cellular agonists [84, 217, 225, 445-447].

For *in vitro* assays, the main focus has been on the development of inositol lipid based chemical probes that could be used as a substrate in screens. Despite the initial difficulties, some promising, fluorogenic substrate analogues were reported [448-452]. One of the more recent examples is a soluble PI(4,5)P_2 analog with a cleavable tag (compound WH-15) that is hydrolyzed by PLCs to produce a blue fluorescent molecule [453-455]; a related compound (XY-69), that can be incorporated into lipid vesicles, has also been synthesised and used *in vitro* [83, 456]**.** The fluorogenic analogs, of which several are commercially available, will

continue to greatly facilitate high-throughput screening to identify highly specific and more potent PLC inhibitors.

7.3. PLC modulators and treatment options for specific disease contexts

As summarized in Table 3, a number of studies identified germline and somatic mutations that are linked to various inherited disorders and specific cancer types, respectively. Notably, for many of these diseases the current treatments remain ineffective and targeting PLC enzymes could address this unmet need. For example, in T-cell lymphomas (ATL) where *PLCG1* is the most frequently mutated gene **(**see **Section 5.2.1)**, conventional chemotherapy and other available standard therapies are usually ineffective or improve prognosis for only a small proportion of cases [457]. Additionally, PLC enzymes are involved in the control of many different functions some of which become affected in a range of diseases where mutations in respective PLCs have not been found so far. A number of examples illustrate aberrant signaling in a disease context that presumably involves PLCs in their wild-type form. In particular, alterations in the $I(1,4,5)P_3/Ca^{2+}$ signaling could contribute to the onset of disorders affecting neural, muscular, cardiac, immune, exocrine and endocrine functions [41]. One illustrative example of a PLC isoform where the mutated or rare variants, as well as the wild-type, are link to the disease is PLCγ2. As described in **Section 5.2.2**, germline mutations in PLCγ2 cause complex immune disorders, somatic mutations are implicated in cancer (CLL) resistance and one rare variant has a protective role in neurodegeneration. Additionally, the wild-type PLCγ2 is a component of critical, upregulated pathways in a number of disorders characterized by autoimmunity and inflammation with broad therapeutic need and of the pathway required for proliferation and survival of CLL cells where it could provide a first line target.

Diverse, and sometime opposing roles of different PLC enzymes in disease, together with their numerous and essential physiological roles, point out that for generation of pharmacological modulators that can be used as drugs, selective compounds are highly desirable. In general, a common, targetable site in signal transduction proteins doesn't preclude generation of selective inhibitors. For example, many kinase inhibitors target the common ATP-binding site while achieve selectivity for closely related isoforms or mutated variants by interacting with unique residues within or in the vicinity of the ATP-binding site [422]. It remains to be seen whether such selectivity can be achieved when targeting the active site in different PLC enzymes and their variants. Nevertheless, recent structural insights for PLCβ and PLCγ enzymes revealed that the activation state of these PLCs is stringently regulated *via* diverse autoinhibitory interactions that are released by changes in unique allosteric networks **(Section 3.3)**. These observations suggest that the selectivity can be achieved by targeting autoinhibitory and allosteric sites; these are also frequently affected by disease-linked mutations.

Another possible route to achieve selectivity is by targeting known sites involved in regulatory protein-protein interactions. Despite the fact that the protein–protein interfaces can be very difficult to "drug", this option has been successfully explored for specific targeting of PLCβ and PLCγ enzymes and there are already examples where the generated modality is evaluated in specific cellular contexts. Notably, cyclic peptides YM-254890 and FR900359

disrupt the interaction and activation of PLCβ isoforms by Gαq while M119 and gallein, structurally related to fluorescein, similarly affect regulation of these isoforms by Gβγ [445, 458-460]. In addition to their use as pharmacological tools, their potential use to treat various diseases, including melanoma and opioid analgesia, is being assessed in preclinical studies [446, 461-464]. Although all these inhibitors bind to the G-protein subunits and not to PLCβ enzymes, they provide proof of principle for targeting regulatory protein-protein interactions that control PLC activity. Recently, this approach has been applied in the context of trafficking of IL-17-producing effector T cells, termed Th17 cells, that trigger inflammation in diseases such as systemic lupus erythematosus [465]. An important step in this process is activation of PLCγ1 by the death receptor CD95 on Th-17 cells [466]. Unlike in TCR signaling mediated by adapter proteins (described in **Section 5.2.1**), the CD95 receptor and PLCγ1 interact directly; interaction between the two proteins involves the calcium inducing domain (CID) of CD95 and the SH3 domain of PLCγ1. A known drug molecule (ritonavir) and a synthetic peptidomimetic of the key region in CID (compound DB550) disrupt the CD95–PLCγ1 interaction, selectively inhibit the CD95-mediated non-apoptotic pathway and appear to alleviate clinical symptoms in lupus-prone mice [465]. The approach to target the SH3 domain of PLCγ1 has been further extended to identify additional inhibitory compounds [467].

With further development of other potential treatments, such as gene editing, the options for targeting PLC enzymes in disease will not be limited to pharmacological modulators. In the future, this could be particularly relevant for dominantly inherited, monogenic diseases caused by mutations in PLC enzymes and/or rare variants with a protective role (see Table 3).

8. Perspectives

We have come a long way since PLC was discovered as an enzyme responsible for generating second messengers, $I(1,4,5)P_3$ and DAG. The presence of multiple PLCs regulated by a wide range of stimulatory inputs is now well established. Despite this wealth of understanding, many questions remain unanswered. To gain a deeper knowledge, more full-length structures of PLCs, in both their inactive and active forms, and their structures when bound to interacting proteins, including known activators, adapters and scaffolds, would provide a better understanding of their regulation. Why is this important? PLCs are emerging targets for several diseases and therefore, designing drugs to target specific intra-molecular or regulatory interactions would get around the issue of specificity. A pan-inhibitor of PLC would be useful as a research tool but not as a therapeutic agent.

Another exciting aspect is specific localization of PLC activity in different cell types. Much work needs to be done to visualise PLC functioning as a component of a signaling complex in living cells; here advances in new technologies are required that allow dynamic protein-protein and protein-lipid interactions at the membrane to be mapped. In particular, understanding how the substrate, $PI(4,5)P_2$, is presented to the PLC in the membrane is essential as well as understanding of the role of membrane composition and organisation in regulating PLC signaling.

Although our current understanding provides a basis for comprehensive charts of PLC signaling connectivity, there are some long-standing and new issues that need to be resolved. These include an understanding of how Ca^{2+} regulates the activity of PLCδ and PLCη enzymes and the identification of the substrate(s) for PLC-XD enzymes. The discovery of these minimalist enzymes with the strong possibility that their substrate is likely to be PI rather than $PI(4,5)P_2$ is an exciting prospect. To date, PI is regarded merely as a precursor to make the phosphorylated derivatives of PI. The possibility that PI itself has a signaling function would open a new era for PI signaling.

The reinforced prospect that PLC enzymes could be therapeutic targets will not only require more vigorous efforts to generate modalities suitable for treatment but also appropriate cellular and animal models for preclinical studies. Understanding the role of individual PLCs at the level of an organism will remain an important direction in the context of both, physiology and pathology.

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FIGURE LEGENDS

Figure 1. Products of PLC activation and their molecular functions in cell signaling

In response to many external stimuli, PLC enzymes hydrolyzes $PI(4,5)P_2$, to generate two new signaling molecules, $I(1,4,5)P_3$ and DAG. $I(1,4,5)P_3$ releases Ca^{2+} from ER stores and is also utilized to make IP_6 and other polyphosphorylated inositol derivatives (IP_7 and IP_8) collectively referred to as IPn. DAG binds to proteins with C1 domains and can bind and/or activate these proteins. DAG can be metabolized to PA by phosphorylation or to 2-AG by DAG lipase to generate further signaling molecules. $PI(4,5)P_2$ is both a signaling molecule in its own right and the precursor of $PI(3,4,5)P_3$, generated by phosphorylation by phosphoinositide 3-kinases, another signaling molecule. In the Figure, activation of cell-surface receptors is depicted in purple, the PLC reaction is boxed in blue, the signaling metabolites in yellow and the regulatory protein targets of the lipids are boxed in green. Abbreviations: PLC, phospholipase C; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; DAG, 1,2-diacylglycerol; $I(1,4,5)P_3$, inositol 1,4,5-trisphosphate; IPn, inositol polyphosphates; PA, phosphatidic acid; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; 2-AG, 2-arachidonoyl glycerol; PKC, protein kinase C; PKD, protein kinase D; RasGrp, Ras guanyl nucleotide-releasing protein; Munc13, mammalian uncoordinated-13; TRPC, Transient receptor potential cation channels;

Figure 2. Classification and domain organization of eukaryotic PLCs

(A) The well-established PLC superfamily of classical PLCs includes six families of multi-domain enzymes. The basic structure of these enzymes consists of an N-terminal pleckstrin homology (PH) domain (missing in PLCζ), four tandem EF hand repeats, a βα TIM barrel catalytic domain and a C2 domain (green). The catalytic barrel structure is formed from the X region and the Y region (light green) and the linker between the two is referred to as the X–Y linker. Although these domains are common to the superfamily, except for the catalytic domain, their functional properties vary. For example, the N-terminal PH domain in PLCδ binds $PI(4,5)P_2$ while in PLCβ it contributes to interactions with regulatory proteins (Rac and Cdc42 GTPases and possibly also Gβγ). Uniquely, the EF-hands in PLCβ have a GAP function for Gαq. The PLC enzymes, except PLCδ and PLCζ, have additional regulatory regions (purple). PLCβ and PLCη have extended C-terminal regions. In PLCβ this region, the C-terminal domain is highly structured (proximal and distal CTD) and involved in interaction with Gαq and cellular membrane; PLCβ isoforms also have a PDZ-binding motif (PBM) at the C-terminus that mediates interactions with scaffold proteins. PLCγ contains a split-PH domain, nSH2, cSH2 and an SH3 domain within its X–Y linker; these domains provide interactions sites with RTKs (nSH2), intramolecular interactions with the key pY (cSH2) and Rac (split-PH). PLCε has an N-terminal Cdc25 domain (acting as GEF for Rac) and two C-terminal RA domains (binding Rac and Ras GTPases). In families with more than one isoform, binding properties with interaction partners are not always conserved across all isoforms and the representation here covers all well-supported interactions regardless of conservation. Red arrows represent the main sites of interaction for the specified ligands while blue arrows indicate GAP and GEF substrates. Other regulatory elements, for example

regions involved in autoinhibition, are embedded within various linkers and domains and are discussed separately.

(B) Atypical PI-PLCs are single domain enzymes and consist of a βα barrel involved in catalysis. Based on similarity with the X region in the catalytic domain from classical enzymes (also referred to as X-domain or XD) (green), these PI-PLCs have been designated as PLC-XD.

Figure 3. Substrate hydrolysis catalysed by PI-PLCs

(A) Structure of the catalytic domain from PLCδ1 (left) and a model of PLC-XD3 based on *L. monocytogenes* PI-PLC (right). Two histidine residues important for catalysis and conserved in all classical enzymes (corresponding to H311 and H356 in PLCδ1) are similalry positioned in a number of bacterial enzymes as well as in the PLC-XD3 model (H37 and H114); these hitidine residues are shown in red. Some other residues implicated in catalysis (N312 and E341 in PLCδ1 and D38 in the model) are shown in yellow. Residues involved in substrate binding and calcium binding (not shown) have been defined but the similarity is restricted to a few residues interacting with the inositol moiety.

(B) The two-step reaction, phosphotransferase reaction (1) followed by hydrolysis of a cyclic IP intermediate (2), is common to classical and most minimalist PI-PLC studied so far. These enzymes utilize a general acid general base mechanism. While in bacterial enzymes, such as *B. cereus* and *L. monocytogenes* PI-PLC, the two key histidines have a role as general acid/base, in the classical enzymes it is likely that only one histidine residue (H356 in PLCδ1) has this function; the second histidine (H311 in PLCδ1) has been implicated in stabilization of the transition state while another residue (E341 in PLCδ1) is likley to act as a general acid/base. The substrate shown here is PI(4,5)P_2, the preferred substrate for classical PLCs; the substrate preference for PLC-XD enzymes is not clear and may be limited to PI, similarly to bacterial enzymes.

Figure 4. Autoinhibition and activation in classical PLCs

(A) Regulation of PLCβ. In the absence of extracellular stimulation, PLCβ is in an inactive form where mainly three elements (proximal and distal CTD and X-Y linker) contribute to autoinhibition. Several types of regulatory proteins interact with PLCβ enzymes leading to activation. In the case of activation by Gαq, the binding of PLCβ to this protein and positioning at membrane proximity overcome the autoinhibition. The PLC-core domains are shown in green, with the catalytic domain in light green, and the domains unique to PLCβ in purple.

(B) Regulation of PLCγ. This family is mainly activated by phosphorylation of a specific Tyrosine (Y) residue within the regulatory region; an array of regulatory domains is placed within the X-Y linker. In the inactive form, two domains within the regulatory region (cSH2 and sPH) directly contribute to autoinhibition. Following phosphorylation, the critical pY residue binds to the cSH2 domain resulting in reposition of the regulatory region and release

of autoinhibition. The PLC-core domains are shown in green, with the catalytic domain in light green, and the domains unique to PLCγ in purple.

Figure 5. Mutations in the genes encoding PLCγ, PLCδ and PLCζ enzymes, linked to disease development

(A) Analyses of gain-of-function PLCγ variants across different pathologies and the mapping of specific alterations on the PLCγ1 structure have revealed that the majority resides within the autoinhibitory interfaces (about 75% of total observations). All domains forming two distinct interfaces, cSH2/C2 and sPH/TIM-barrel, harbour mutations (solid line, purple and green arrows). Their mechanism of activation is *via* release of the autoinhibition. It is likely that in several specific cases other functions of PLCγ can be affected, including changes in intramolecular allosteric networks, domain stability and interactions with regulatory proteins or cell membrane (dashed line, purple and green arrows). Examples illustrating these different mechanisms are listed (with the residue numbers in ascending order) in the corresponding boxes. The PLC-core domains are shown in green, with the catalytic domain in light green, and the domains unique to PLCγ in purple.

(B) Mutations in PLCδ1 and PLCζ1 enzymes occur in hereditary leukonychia and male infertility, respectively, resulting in loss-of-function. They include frameshift deletions/premature stop codons (grey bars) resulting in protein truncation and amino acid substitutions (red bars) that can compromise protein stability and/or enzyme activity. Specific mutations in PLCδ1 and PLCζ1 are listed (with the residue numbers in ascending order) in the corresponding boxes. The PLC domains are shown in green, with the catalytic domain in light green.

Table 1. List of available structures for phosphoinositide-specific PLCs

Description	PDB ID
Families of classical eukaryotic PLCs	
PLCδ	
PLCδ1- large part of the protein – lacking the PH domain; includes structures with Ca^{2+} and Ins(1,4,5,)P_3 ligands	2ISD 1DJG, 1DJH, 1DJI, 1DJW, 1DJX, 1DJY, 1DJZ, 1QAS, 1QAT
PLCδ1- PH domain with the Ins(1,4,5,)P_3 ligand	1MAI
PLCβ	
S. officinalis PLC21- C-terminal truncation/PLC-core with ligands	3QR0
L. pealei PLC21- C-terminal truncation/PLC-core	3QR1
M. gallopavo PLCβ- C-terminal region	1,AD
D. melanogaster NORPA- C-terminal region	6IRC, 6IRB
PLCβ2- C-terminal truncation/PLC-core	2ZKM
PLCβ2- C-terminal truncation/PLC-core in complex with Rac1	2FJU
PLCβ3- C-terminal truncation/PLC-core in complex with Gαq	3OHM
PLCβ3- Full-length in complex with Gαq	4GNK
PLCβ3- C-terminal truncation/PLC-core with further deletions in complex with Gαq and with Ins(1,4,5,)P_3 ligand	4QJ3, 4QJ4, 4QJ5
D. melanogaster NORPA- C-terminal region in complex with INAD PDZ45	6IRE
PLCγ	
PLCγ1- domains from the regulatory (γ-SA) region: single nSH2, cSH2, SH3 and sPH domain and the nSH2-cSH2 tandem; include structures with peptide ligands	5TNW, 2PLD, 2PLE, 5TO4, 5TQ1, 5TQS, 1YWP, 1HSQ, 2HSP, 1Y0M, 1YWO, 2FJL, 2FCI, 4K44, 4K45, 4EY0, 4FBN
PLCγ2- domains from the regulatory (γ-SA) region: single nSH2, cSH2, SH3 and sPH domain	2DX0, 2EOB, 2EQI, 2K2J, 2W2W
PLCγ1- nSH2-cSH2 tandem in complex with intracellular part of FGFR1	3GQI;
PLCγ1- cSH2 domain in complex with intracellular part of FGFR2	5EG3;;
PLCγ2- sPH domain in complex with Rac2	2W2X;;
PLCγ1- Near full-length, lacking segments from the N-terminus, C-terminus and the cSH2-SH3 linker; includes Ca^{2+} ligand	6PBC
PLCγ1- Full-length in complex with intracellular part of FGFR1	EMD-10288
PLCε	
PLCε1– single RA1 and RA2 domain	2BYE, 2BYF
PLCε1– RA2 domain in complex with Ras	2C5L;

Bacterial PI-PLCs	
Gram-Positive Bacteria	
B. cereus PI-PLC- includes structures with *myo*-Ins ligand and different protein variants	1PTD, 1PTG, 1GYM, 2PTD, 3PTD, 4PTD, 5PTD, 6PTD, 7PTD
B. thuringiensis PI-PLC- includes structures with metal ion ligands and different protein variants	1T6M, 3EA1, 3EA2, 3EA3, 2OR2
S. aureus PI-PLC- includes structures with *myo*-Ins ligand and different protein variants	3V16, 3V18, 4F2B, 4F2T, 3V1H, 4I9T, 4I8Y, 4I90, 4I9J, 4I9M, 4RV3, 4S3G
L. monocytogenes PI-PLC- includes structure with *myo*-Ins	1AOD, 2PLC
Gram-Negative Bacteria	
Str. antibioticus PI-PLC	3H4W, 3H4X
Pseudomonas sp. 62186 PI-PLC- includes structures with *myo*-Ins and Ca^{2+} ligands	5FYO, 5FYP, 5FYR

Table 2. Phenotypes observed in mice where PLCs are deleted in the whole animal or by targeted disruption of specific tissues.

Protein	mouse gene	Phenotype of knockout mice	Regulation	Comments
PLCβ1	*Plcb1*	Epileptic seizures from the 3rd week after birth [172];	Gαq family [468];	Widely expressed with high levels in the cerebral cortex and hippocampus; Coupled to M1 muscarinic cholinergic receptors [172, 173];
PLCβ2	*Plcb2*	Disruption of FMLP-stimulated superoxide in neutrophils [184]; Transduction of taste in taste neurons [469];	βγ subunits [470, 471]; Rac2 [472];	Expression limited to haematopoietic cells and Taste cells; Transduction of taste; Ca^{2+} stimulates the opening of TRPM5 channel [473];
PLCβ3	*Plcb3*	Premature death, enlarged spleens, myeloproliferative disease [193]; knockout mice more sensitive to opioids [192]; protection from atherosclerosis [195];	Gαq; βγ subunits;	PLC activity not required for myeloproliferative disease, the C-terminal extension binds to Stat5 and SHP-1 restraining Stat5 proliferative activity [193];
PLCβ4	*Plcb4*	Ataxia	Gαq;	Highly expressed in cerebellum and couples to the metabotropic glutamate receptor [172];
PLCδ1	*Plcd1*	Progressive hair loss [474] Inflammation of skin [475]	Ca^{2+} (μM range) [309]	Ca^{2+} mobilization and activation of NFAT impaired in PLCδ1-deficient primary keratinocytes; defects in skin stem cell lineages;
PLCδ3	*Plcd3*	No obvious abnormality;	Ca^{2+} (μM range)	Global knockout of both PLCδ1 and δ3 is embryonic lethal E11.5-E13.5 due to placental defects [338]; Can be rescued by expression of PLCδ1 in placental tissue but now reveals cardiomyopathy [322];
PLCδ4	*Plcd4*	Male infertility [340]	Ca^{2+} (nM range)	Required for sperm acrosome reaction induced by zona pellucida [340];
PLCγ1	*Plcg1*	Embryonic lethality at day E9 [229]; Conditional PLCγ1-deficient mice, T-cell aberrations [231].	Tyrosine phosphorylation;	Forebrain-specific-ablation causes hyperactivity due to impaired BDNF/TrkB receptor signaling in hippocampus, reversed by lithium chloride [265]; Specific ablation in neuronal precursors causes

				impaired netrin /DCC axon guidance of dopaminergic neurons [262];
PLCγ2	*Plcg2*	B cell development and downregulation of responses in platelets, mast cells and MK-cells. [194, 273]	Tyr phosphorylation; Rac2;	Rac2 intersects with the split PH domain [277, 278];
PLCε	*Plce1*	Cardiac dysfunction at 2 months of age and enhanced hypertrophy in response to chronic β-adrenergic stimulation [476]; In chemical carcinogen-induced skin tumours, papillomas formed in *Plce1 KO* mice fail to undergo malignant progression into carcinomas [371]; *Plce1 KO* mice show increased susceptibility to tumour formation suggesting a tumour suppressor role for PLCε [370].	Ras [154]; βγ;	
PLCη1	*Plch1*	Not available	Ca^{2+} (μM range) [477]	Provides a Ca^{2+}-dependent amplification;
PLCη2	*Plch2*	No obvious phenotype [384]	βγ subunits [161]; Ca^{2+} (μM range) [162];	Brain-specific; Highly expressed in the retina and habenula [384];
PLCζ	*PLCZ*	Male sterility;	Ca^{2+} (μM range)	Expressed only in testes and sperm; Mice knockouts are subfertile [374, 375];
PLCXD1	*Plcxd1*	Not available	Not known	
PLCXD2	*Plcxd2*	Not available	Not known	
PLCXD3	*Plcxd3*	Not available	Not known	Potential regulator of insulin secretion [393, 394]; Bipolar disorder [392]; Enriched in brain;

Table 3. Phenotypes observed in humans due to specific mutations in PLCs

Gene	Phenotypes in humans	Mutation	Comments
PLCB1	Early infantile epileptic encephalopathy; Infantile spasms and profound intellectual disability;	Homozygous deletion [175-177];	Only seven cases identified ;
PLCB3	Milder phenotype in cystic fibrosis patients;	Loss of function mutant (S845L) [200, 201];	Highly expressed in bronchial epithelial cells where secretion of IL-8 is reduced due to the mutation;
PLCB3	Spondylmetaphyseal dysplasia with corneal dystrophy;	Loss of function mutant (A878S) [202];	Postnatal growth deficiency; limb shortening; intellectual disability; patient fibroblasts show increased $PI(4,5)P_2$ levels and disorganization of the actin cytoskeleton;
PLCB4	Uveal melanoma that arise from melanocytes (tumour of the eye)	Gain of function mutation (D630Y); Driver mutation located to the Y-domain of the highly conserved catalytic core of PLCβ4 [210];	Cysteinyl leukotriene receptor 2 couples to Gαq and activates PLCβ4;
PLCB4	Auriculocondylar Syndrome;	Loss of function mutations or mutations in the catalytic X and Y domains of PLCβ4 [211-213];	Rare craniofacial disorder with specific malformation of the external ear; PLCβ4 is activated by endothelin receptor type A;
PLCD1	Hereditary leukonychia,	Protein truncation or single point mutation (C209R and A574T) [325-329];	a nail disorder characterized by nail plates whitening on all finger and toe nails referred to as porcelain nails;
PLCE	Nephrotic syndrome	Missense mutation in the X domain [367, 368];	Tyrosine-phosphorylated advillin recruits PLCε; Advillin knockdown inhibits EGF-stimulated PLCε;
PLCG1	Angiosarcoma	Somatic, gain-of-function point-mutations [216, 240-242];	
PLCG1	Range of T-cell lymphomas including: angioimmunoblastic T-cell lymphomas (AITL); adult T-cell leukemia/lymphoma	Somatic, gain-of-function point-mutations/deletions [222, 228, 243-260];	

	(ATLL or ATL); hepatosplenic T-cell lymphoma (HSTL); Mycosis Fungoides; Sezary Syndrome; peripheral T-cell lymphomas-not otherwise specified (PTCL-nos); follicular helper T-cell- derived lymphomas (TFH-derived PTCL); T-cell-prolymphocytic leukemia (TPLL)		
PLCG2	PLAID	Gain-of-function large deletions [215];	
PLCG2	APLAID	Gain-of-function point-mutations/deletions [217, 219, 280-282];	Related, ENU-generated mouse strains Ali5 and Ali14 [284, 285];
PLCG2	Inflammatory bowel disease (IBD);	Missense mutations [218];	
PLCG2	Steroid-sensitive nephrotic syndrome (SSNS);	Missense mutations [223]	
PLCG2	Ibrutinib resistant CLL;	Somatic, gain-of-function point-mutations/deletions [221, 289-297];	
PLCG2	Myelodysplastic syndrome (MDS)	Somatic, missense mutation [298];	
PLCG2	Alzheimer's disease and other dementia	Rare, protective variant P522R [220, 224, 299-302];	
PLCZ	Fertilisation failure after Intracytoplasmic sperm injection (ICSI);	Several mutations identified including the X-domain (R197H; H233L); Y-domain (H398P); C2 domain (I489F) [376-380, 478];	Reduced expression causes male infertility in humans;

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