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The Phospholipase C Isozymes and Their Regulation

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Abstract

The physiological effects of many extracellular neurotransmitters, hormones, growth factors, and other stimuli are mediated by receptor-promoted activation of phospholipase C (PLC) and consequential activation of inositol lipid signaling pathways. These signaling responses include the classically described conversion of phosphatidylinositol(4,5)P₂ to the Ca²⁺-mobilizing second messenger inositol(1,4,5)P₃ and the protein kinase C-activating second messenger diacylglycerol as well as alterations in membrane association or activity of many proteins that harbor phosphoinositide binding domains. The 13 mammalian PLCs elaborate a minimal catalytic core typified by PLC- δ to confer multiple modes of regulation of lipase activity. PLC- β isozymes are activated by G α_q - and G $\beta\gamma$ -subunits of heterotrimeric G proteins, and activation of PLC- γ isozymes occurs through phosphorylation promoted by receptor and non-receptor tyrosine kinases. PLC- ϵ and certain members of the PLC- β and PLC- γ subclasses of isozymes are activated by direct binding of small G proteins of the Ras, Rho, and Rac subfamilies of GTPases. Recent high resolution three dimensional structures together with biochemical studies have illustrated that the X/Y linker region of the catalytic core mediates autoinhibition of most if not all PLC isozymes. Activation occurs as a consequence of removal of this autoinhibition.

Keywords

Phospholipase C; Inositol lipid signaling; Heterotrimeric G protein; Ras GTPase; Tyrosine kinase; X/Y-linker-mediated autoinhibition

3.1 Introduction

The physiological effects of many hormones, neurotransmitters, growth factors, and other extracellular stimuli are initiated through receptor-promoted inositol lipid signaling. The ground-breaking work of Hokin and Hokin in the 1950s/1960s (Hokin and Hokin 1953) and of Berridge, Michell, Nishizuka, and many other investigators in the 1970s/1980s established the importance of membrane inositol lipids in hormone action (Berridge 1987;

Michell 1975; Nishizuka 1992). Receptors for extracellular stimuli promote activation of phospholipase C (PLC), which converts (Fig. 3.1) phosphatidylinositol (4,5)bisphosphate (PtdIns(4,5)P₂) into the Ca²⁺-mobilizing second messenger, inositol (1,4,5)trisphosphate (Ins(1,4,5)P₃), and the protein kinase-activating second messenger, diacylglycerol (DAG). Although PLC-catalyzed formation of second messengers from PtdIns(4,5)P₂ constitutes one of the major mammalian cell signaling responses, inositol lipids themselves also carry out important signaling functions. Indeed, PtdIns(4,5)P₂ selectively binds to PH, FYVE, and PX domains (Lemmon 2003), and the activities and/or subcellular localization of a broad range of proteins involved in cell signaling (e.g. PTEN and Ca²⁺, K⁺, and Na⁺ channels), actin assembly and remodeling, and vesicle trafficking are modified (Ling et al. 2006; Suh and Hille 2008; Yin and Janmey 2003).

Receptor-mediated regulation of inositol lipid signaling historically has been considered to occur through two major mechanisms (Exton 1996; Rhee 2001). First, PLC- γ isozymes are activated by a panoply of growth factors and immunological stimuli that signal through receptor and non-receptor tyrosine kinases. Second, the G protein-coupled receptors (GPCR) represent one of the largest classes of proteins in the mammalian genome, and a large proportion of these receptors produce their major cellular responses through activation of PLC- β isozymes. Although these two major components of inositol lipid signaling make enormous contributions to the cell “signalsome”, the existence of at least 13 different mammalian PLC isozymes suggests much more extensive modes of regulation (Harden and Sondek 2006). Indeed, multiple Ras superfamily GTPases directly activate PLC- ϵ , as well as certain of the G α_q - (e.g. PLC- β_2) and tyrosine kinase- (e.g. PLC- γ_2) activated PLCs (Harden et al. 2009), and the inositol lipid signaling field continues to expand in surprising directions. This chapter focuses on the mammalian PLC isozymes, their complex modes of regulation, and their physiological functions.

3.2 Phosphoinositide-specific PLC

PLC enzymes were purified initially several decades ago from a variety of tissues and were shown to exist in at least three major isozyme forms based on size and immunoreactivity (Hofmann and Majerus 1982; Ryu et al. 1986, 1987a, 1987b; Takenawa and Nagai 1981). These included enzymes that are activated by tyrosine phosphorylation (Meisenhelder et al. 1989; Wahl et al. 1989) or by G proteins (Morris et al. 1990). The deduced amino acid sequences obtained from initial cloning of the cDNAs of several of these enzymes revealed the existence of PLC- β , - δ , and - γ isozymes (Suh et al. 1988). Multiple subtypes eventually were shown to exist in each of these isozyme classes, but a protein originally designated as PLC- α proved to not be a PLC. Additional PLC isozymes (- ϵ , - ζ , - η) were subsequently discovered and cloned (Harden and Sondek 2006), and a total of 13 isozymes in six mammalian PLC family members are currently recognized (Fig. 3.2).

3.3 Catalytic Function and Structure of Conserved Core Domains of PLC

PLC enzymes are calcium-dependent phosphodiesterases that preferentially hydrolyze PtdIns(4,5)P₂ into DAG and Ins(1,4,5)P₃ (Fig. 3.1). The core structure of these isozymes (Fig. 3.2) consists of an N-terminal PH domain, an array of four EF-hands, a catalytic triose phosphate isomerase (TIM) barrel comprised of two halves (X and Y boxes), and a C-terminal C2 domain (Katan and Williams 1997). Additional regulatory domains evolved that engender unique regulatory mechanisms to individual isozymes (Harden and Sondek 2006). The structure of PLC- β_2 (Fig. 3.3) highlights the conserved core structure found in all PLC isozymes.

The conserved tertiary structure of PH domains is composed of a sandwich of seven β -strands capped on one end by a C-terminal α -helix and on the other end by three loops,

which diverge in both length and amino acid sequence (Rebecchi and Scarlata 1998). Although the structural folds of PH domains are generally conserved, they carry out diverse functions. Thus, the N-terminal PH domains of isozymes of the PLC- δ and PLC- γ families bind PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, respectively (Essen et al. 1996; Falasca et al. 1998; Singh and Murray 2003), whereas the PH domain of PLC- β 2 binds Rac GTPases with high affinity (Illenberger et al. 2003a; Snyder et al. 2003). The PH domain of PLC- δ 1 is tethered to the rest of the protein via a flexible linker and is highly mobile (Essen et al. 1996; Ferguson et al. 1995). In contrast, the PH domain of PLC- β 2 makes direct contacts with the other domains of the catalytic core and remains tightly associated during activation (Hicks et al. 2008; Jezyk et al. 2006).

Typical EF-hands are calcium-binding motifs composed of two helices (E and F) joined by a loop and divided into pairwise lobes (Kawasaki and Kretsinger 1994). The electron density of the loops connecting the secondary elements of the EF hands is incomplete in the structures of both PLC- δ 1 and PLC- β 2 indicating a high degree of flexibility in this region (Essen et al. 1996; Hicks et al. 2008; Jezyk et al. 2006). Little evidence exists for Ca²⁺-promoted regulation of PLC isozymes through the EF-hands, and Ca²⁺ is not bound in the EF-hands in the structures of PLC- δ 1, PLC- β 2, or PLC- β 3. In contrast, the structure of PLC- β 3 in an activated complex with G α_q highlights a novel function for EF hands in G protein-dependent signaling (Waldo et al. 2010). A cassette uniquely present between the third and fourth EF hands of the four mammalian PLC- β isozymes contains the structural determinants necessary for PLC- β -promoted enhancement of GTP hydrolysis by its activator G α_q .

The C2 domain folds into an eight β -stranded antiparallel sandwich, with three loops at one end of the sandwich forming calcium binding sites (Nalefski and Falke 1996). The C2 domains of PLC- δ 1 and PLC- β 2 exhibit very similar structures tightly packed against the TIM barrel, likely to maintain the structural integrity of the catalytic core (Essen et al. 1996; Jezyk et al. 2006). The C2 domain of PLC- δ 1 binds Ca²⁺ and promotes translocation of the isozyme to the plasma membrane (Essen et al. 1997). However, the residues coordinating Ca²⁺ in PLC- δ 1 are not generally conserved among other PLC isozymes, and it is unclear whether Ca²⁺ regulates any of the other mammalian isozymes in a similar fashion. Although not formally part of the C2 domain, conserved sequences found at its N- and C-terminal ends in PLC- β isozymes provide the major binding surface for activated G α_q (Waldo et al. 2010).

3.4 Mechanism of PtdIns(4,5)P₂ Hydrolysis

The catalytic TIM barrel is the most highly conserved region among PLC isozymes with 60–70% sequence identity. The X and Y boxes fold together in an alternative pattern of α -helices on the outside and β -strands in the inner part of the barrel to constitute the active site of the lipase (Essen et al. 1996; Wierenga 2001). The structure of PLC- δ 1 first illustrated the organization of the active site of a PLC and revealed the mechanism for PtdIns(4,5)P₂ hydrolysis (Essen et al. 1996).

The active site is formed as a solvent-accessible depression at the C-terminal ends of the β -strands (Figs. 3.3 and 3.4). Indeed, a ridge of hydrophobic residues, Leu320, Tyr358, Phe360, Leu529, and Trp555 surrounding the active site of PLC- δ 1 facilitates insertion of the catalytic domain into the lipid bilayer (Essen et al. 1996). A PLC- δ 1 mutant containing alanine substitution of these bulky nonpolar residues exhibited activity similar to wild-type PLC- δ 1 when PtdIns(4,5)P₂ hydrolysis was measured in detergent-mixed micelles but was a much less effective enzyme in assays of PtdIns(4,5)P₂ hydrolysis using phospholipid vesicles (Ellis et al. 1998). These residues are conserved across all PLC isozymes and

assume the same orientation in the structures of PLC- β 2 and PLC- β 3 (Hicks et al. 2008; Jezyk et al. 2006; Waldo et al. 2010).

Eukaryotic PLC enzymes preferentially hydrolyze PtdIns(4,5)P₂, but also hydrolyze PtdIns(4)P and to a much lesser extent PtdIns (Ryu et al. 1987b). Ins(1,4,5)P₃ buried at the bottom of the active site in PLC- δ 1 provided the initial structural snapshot of substrate recognition (Essen et al. 1996). An extensive network of H-bonds and salt-bridge interactions formed through the side chains of Lys438, Lys440, Ser522, and Arg549 with the 4' and 5' phosphorylated hydroxyl groups of the inositol ring favors interaction with lipids phosphorylated at both positions. The aromatic ring of Tyr551 also is parallel with the inositol ring and forms numerous van der Waals contacts with it (Fig. 3.4).

The essential Ca²⁺ cofactor is ligated by surrounding acidic residues, Asn312, Glu341, Asp343, and Glu390, and PLC activity is completely lost after, for example, mutation of Glu341 to Gly (Cheng et al. 1995). Ligation of Ca²⁺ with the 2'-hydroxyl group of the inositol ring also is essential for PLC activity (Essen et al. 1996). In contrast, prokaryotic PLC enzymes utilize basic amino acids to fulfill the functional requirements of the Ca²⁺ cofactor and thus are Ca²⁺-independent (Heinz et al. 1998).

PtdIns(4,5)P₂ hydrolysis follows a general acid/base catalytic scheme (Fig. 3.4). Ca²⁺ lowers the pK_a of the 2-hydroxyl group of the inositol ring to facilitate its deprotonation, and Glu341 is a putative general base carrying out nucleophilic attack on the 1-phosphate (Ellis et al. 1995, 1998). This initial step in hydrolysis leads to formation of a cyclic intermediate, which is stabilized by His311 and Ca²⁺ through ligation of the 1-phosphate. His356 then utilizes a proton from water to promote nucleophilic attack on the pentavalent cyclic intermediate and DAG and Ins(1,4,5)P₃ are formed (Cheng et al. 1995; Ellis et al. 1995; Essen et al. 1996; Heinz et al. 1998). All of the residues participating in substrate specificity, Ca²⁺ coordination, and the catalytic reaction are strictly conserved across the PLC family (Fig. 3.4) and are positioned in similar orientation in PLC- β 2 structures (Jezyk et al. 2006; Hicks et al. 2008), indicating that the mechanism of PtdIns(4,5)P₂ hydrolysis is conserved throughout eukaryotic PLC enzymes.

3.5 PLC Subfamilies and Their Regulation

3.5.1 PLC- δ Isozymes

PLC- δ is found in early eukaryotes, including yeast (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) (Andoh et al. 1995; Payne and Fitzgerald-Hayes 1993; Yokoo et al. 1993) and slime mold (*Dictyostelium discoideum*) (Drayer and van Haastert 1992). Three PLC- δ isozymes (PLC- δ 1, - δ 3, and - δ 4) exist in mammals (Harden and Sondek 2006) ("PLC- δ 2" proved to be a species homologue of PLC- δ 4 (Irino et al. 2004)). Most, if not all, cells express at least one of these isoforms, and all three are broadly, if not ubiquitously, expressed (Suh et al. 2008). PLC- δ 1 is mainly a cytoplasmic protein, whereas PLC- δ 3 is detected in membrane fractions. PLC- δ 4 is principally located in the nucleus where its expression is directly linked with the cell cycle.

3.5.1.1 Regulation—Activation of PLC- δ 1 occurs through association with membrane surfaces driven by PtdIns(4,5)P₂ binding by the PH domain and Ca²⁺ binding by the C2 domain. The structure of the isolated PH domain of PLC- δ 1 in complex with Ins(1,4,5)P₃ highlighted its capacity to bind phospholipids with high affinity and therefore serve as a plasma membrane anchor for PLC- δ isoforms (Ferguson et al. 1995). PH domains are typically highly polarized with a positively charged surface interacting with the negatively charged inner face of the plasma membrane. Consistent with this idea, the binding site for the soluble head group of PtdIns(4,5)P₂, Ins(1,4,5)P₃, is located opposite to the C-terminal

α -helix and at the center of the positively charged region of the PH domain of PLC- δ 1. The residues coordinating Ins(1,4,5) P_3 in the β 1/ β 2 and β 3/ β 4 loops are poorly conserved among PH domains. Moreover, these residues, which ligate PtdIns(4,5) P_2 with high affinity in PLC- δ 1 are not conserved in the PH domains of the isozymes of the other PLC subfamilies, strongly suggesting that the N-terminal PH domain is not functionally redundant (Harden and Sondek 2006).

Several studies have highlighted the functional role of the PH domain in modulation of lipase activity of PLC- δ isoforms. These isozymes carry out a scooting mode of substrate hydrolysis facilitated by the PH domain. Binding of PtdIns(4,5) P_2 to the PH domain anchors PLC- δ 1 at the plasma membrane and multiple PtdIns(4,5) P_2 molecules are processively hydrolyzed by the catalytic site (Lomasney et al. 1996). Thus, PLC- δ isoforms hydrolyze numerous PtdIns(4,5) P_2 molecules during a single binding event at the membrane interface. This two-substrate process also allows feedback regulation of enzymatic activity through decreases in local PtdIns(4,5) P_2 concentration. Experimental evidence for this model includes observation of a concentration-dependent increase in PLC- δ 1 activity with increases in the mole fraction of PtdIns(4,5) P_2 , but not PtdIns(4)P (Lomasney et al. 1996). Truncation of the PH domain, substitution at residues of the PH domain that ligate PtdIns(4,5) P_2 , or competitive addition of Ins(1,4,5) P_3 all impair PtdIns(4,5) P_2 -stimulated PLC- δ 1 activity, but do not affect function of the catalytic site (Yagisawa et al. 1998).

The PH domain of PLC- δ 1 is the prototypical PtdIns(4,5) P_2 -binding module, and the high affinity and selective interaction between PtdIns(4,5) P_2 and this PH domain has been exploited to generate fluorescent probes for monitoring local phospholipid signaling. Thus, the PH domain of PLC- δ 1 fused to green fluorescent protein (GFP) reveals the localization and dynamics of PtdIns(4,5) P_2 in living cells (Stauffer et al. 1998). More recent studies utilized an enhanced GFP-tag to delimit the cellular localization of PtdIns(4,5) P_2 to distinct regions of the plasma membrane, such as membrane ruffles. PtdIns(4,5) P_2 associates with the cytoskeleton and binds and modulates many actin-regulatory proteins, including gelsolin, cofilin, profilin, the Arp2/3 complex, and Wiskott-Aldrich syndrome protein (Nebt et al. 2000). Indeed, PtdIns(4,5) P_2 acts as a second messenger regulating adhesion between the plasma membrane and cytoskeletal structure (Raucher et al. 2000).

The C2 domain has been proposed to mediate Ca^{2+} -stimulated membrane association of PLC- δ isoforms, and the crystal structure of PLC- δ 1 reveals the existence of three Ca^{2+} binding regions (CBRs) in the loops of the C2 domain (Essen et al. 1997; Grobler et al. 1996). Membrane binding studies with isolated C2 domains from PLC- δ 1, - δ 3, and - δ 4 indicate that Ca^{2+} binding switches the electrostatic potential to favor non-specific electrostatic interactions with the plasma membrane (Ananthanarayanan et al. 2002). The C2 domains of PLC- δ 1 and - δ 3 also form a protein- Ca^{2+} complex with the anionic lipid phosphatidylserine (PS) and therein target these isozymes to specific regions of the plasma membrane (Lomasney et al. 1999). In contrast, the C2 domain of PLC- δ 4 lacks two of the four aspartic acid residues coordinating Ca^{2+} and does not exhibit Ca^{2+} -dependent translocation to PS-enriched membrane regions (Ananthanarayanan et al. 2002).

To date, Ca^{2+} is the only regulator that directly enhances the activity of PLC- δ isoforms. Reconstitution assays using permeabilized cells depleted of PLC isozymes illustrated that the lipase activity of PLC- δ 1, but not PLC- β 1 or PLC- γ 1, is stimulated in a concentration dependent manner by physiological concentrations (10nM to 10 μ M) of Ca^{2+} , indicating that Ca^{2+} alone is sufficient to promote increased lipase activity of PLC- δ isoforms (Allen et al. 1997; Kim et al. 1999). Whether Ca^{2+} -mediated activation is further potentiated through interaction of these isozymes with other regulators has not been clearly established.

Several lines of evidence suggest that PLC- δ isoforms are regulated by G-protein mediated signaling. For example, stimulation of the $\alpha 1$ -adrenergic receptor has been proposed to directly regulate PLC- $\delta 1$ through activation of the atypical G-protein transglutaminase II, also called $G\alpha_H$ (Feng et al. 1996). Additional studies indicate that GPCR indirectly activate PLC- δ through signaling pathways selective for other PLC isoforms. For example, inositol phosphate production downstream of the angiotensin II type 1 receptor requires activation of PLC- β isoforms via $G\alpha_{q/11}$ and is potentiated by interaction between RalA and PLC- $\delta 1$ (Godin et al. 2010; Sidhu et al. 2005). Thus, PLC- $\delta 1$ may serve as an amplifier of signaling initiated or mediated by other PLC isoforms (Guo et al. 2010).

3.5.1.2 Physiology—PLC- $\delta 1$ null mice exhibit a hairless phenotype that is reminiscent of nude mice in which loss of function of Foxn1, a member of the winged helix/forkhead family of transcription factors, leads to hair loss and an inborn dysgenesis of the thymus (Nehls et al. 1994). In fact, PLC- $\delta 1$ is a Foxn1-inducible gene that regulates the expression of hair keratins, although the molecular mechanism underlying this effect remains to be identified.

PLC- $\delta 1$ null mice also display symptoms of skin inflammation (Ichinohe et al. 2007). Exogenous expression of PLC- $\delta 1$ attenuates LPS-induced upregulation of IL-1 β , a pro-inflammatory cytokine that typically induces expression of IL-6, which in turn promotes keratinocyte proliferation. It is likely that the lack of PLC- $\delta 1$ in keratinocytes results in aberrant production of IL-1 β and subsequent upregulation of IL-6 expression, leading to skin inflammation and epidermal hyperplasia. Together, these results suggest that PLC- $\delta 1$ regulates homeostasis of the immune system in the skin.

Although the localization of PLC- $\delta 1$ is primarily cytoplasmic, PLC- $\delta 1$ contains both nuclear export and import sequences that allow it to shuttle between the nucleus and the cytoplasm (Yamaga et al. 1999). PLC- $\delta 1$ accumulates in the nucleus at the G₁/S boundary of the cell cycle, and its accumulation is positively correlated with the level of nuclear PtdIns(4,5)P₂ (Stallings et al. 2005). Depletion of PLC- $\delta 1$ in the nucleus delays the completion of S phase and transition into G₂/M phase leading to decreased cell proliferation and growth rate (Stallings et al. 2008). Levels of cyclin E, a key regulator of the G₂/M transition, are also elevated. Therefore, PLC- $\delta 1$ modulates nuclear phospholipid metabolism critical for cell cycle progression. Consistent with this idea, PtdIns(4,5)P₂ inhibits histone H1-mediated basal transcription initiated by RNA polymerase II through a direct interaction with its C-terminal tail (Yu et al. 1998).

Several studies also implicate PLC- $\delta 1$ in neurodegenerative disorders (Shimohama et al. 1993). Specifically, PLC- $\delta 1$ accumulates in the neurofibrillary tangles of Alzheimer patients (Shimohama et al. 1991). Overexpression of PLC- $\delta 1$ protein also is associated with high PLC activity in Alzheimer patients, suggesting that PLC- $\delta 1$ -mediated phospholipid turnover plays an important role in the development of this neurodegenerative disease. A recent study indicated a direct link between activation of the N-methyl-D-aspartic acid receptor under oxidative stress conditions and an increase in PLC- $\delta 1$ protein levels (Nagasawa et al. 2004). This result provides a putative molecular link between neurons responding to oxidative stress and the accumulation of neurofibrillary tangles and senile plaques in Alzheimer's disease.

PLC- $\delta 1$ was recently identified as a tumor suppressor located at chromosome 3p22, an important tumor suppressor locus. Down-regulation of PLC- $\delta 1$ in esophageal squamous cell carcinoma is associated with promoter hypermethylation and frequent allelic loss at the PLC- $\delta 1$ locus (Fu et al. 2007). Epigenetic regulation of PLC- $\delta 1$ also was linked to cancer progression in other tissues. For example, PLC- $\delta 1$ expression is greatly reduced through

hypermethylation of its gene in both gastric cancer cell lines and primary tumors. This gene silencing is associated with later stages of gastric cancer (Hu et al. 2009). Moreover, this study showed that PLC- $\delta 1$ decreases cell motility, which is consistent with the idea that PLC- $\delta 1$ regulates proteins, such as actin-regulated protein, Rho GTPases, or metalloprotease proteins that in turn modulate cytoskeletal rearrangement. Together, these studies indicate that PLC- $\delta 1$ is frequently silenced by epigenetic alteration in a tumor-specific manner. Another study reported that expression of mRNA for PLC- $\delta 1$ and - $\delta 3$ directly correlates with the metastatic state of human breast cell lines and that PLC- $\delta 1$ and - $\delta 3$ are more highly expressed in transformed cell lines (Rebecchi et al. 2009). This study also supported an important role for PLC- $\delta 1$ and - $\delta 3$ in cell growth and migration.

Disruption of the PLC- $\delta 3$ gene in mice has not resulted to date in a reported abnormality. However, embryonic lethality occurs after disruption of both PLC- $\delta 1$ and PLC- $\delta 3$ due to developmental failure of the placenta (Nakamura et al. 2005). The labyrinth trophoblast layer of the placenta was poorly vascularized in the PLC- $\delta 1$ /PLC- $\delta 3$ double knock-out mouse and exhibited reduced cell proliferation and abnormal cell death (Nakamura et al. 2005).

Disruption of the PLC- $\delta 4$ gene leads to male infertility, whereas female mice remain fertile. *In vitro* fertilization studies indicated that sperm from PLC- $\delta 4$ -deficient mice were unable to maintain a sustained influx of Ca^{2+} , which is a critical component of the interaction of the sperm with the zona pellucida in the acrosome reaction (Fukami et al. 2003). Therefore, PLC- $\delta 4$ is important in the early steps of fertilization.

3.5.2 PLC- β Isozymes

The four PLC- β isozymes differ in expression pattern and regulation. While PLC- $\beta 1$ is highly expressed in the cerebral cortex and hippocampus (Homma et al. 1989), PLC- $\beta 2$ expression is largely, but not entirely, limited to hematopoietic cells (Park et al. 1992). PLC- $\beta 3$ is broadly expressed (Jhon et al. 1993), while PLC- $\beta 4$ expression is enriched in the cerebellum and the retina (Adamski et al. 1999). Historically, PLC- β isozymes have been structurally characterized by the unique presence of a C-terminal (CT) coiled-coil domain thought to be important for dimerization, membrane association, and activation by $\text{G}\alpha$ -subunits (Ilkaeva et al. 2002; Singer et al. 2002).

3.5.2.1 Regulation—PLC- β isozymes are effectors of heterotrimeric G-proteins downstream of GPCR belonging to the rhodopsin superfamily of seven transmembrane receptors. These isozymes are activated by $\text{G}\alpha$ -subunits of the G_q subfamily (Smrcka et al. 1991; Taylor et al. 1991; Waldo et al. 1991) as well as by $\text{G}\beta\gamma$ (Boyer et al. 1992; Camps et al. 1992) and mediate the physiological actions of many extracellular stimuli.

The G_q family consists of four different $\text{G}\alpha$ -subunits ($\text{G}\alpha_q$, $\text{G}\alpha_{11}$, $\text{G}\alpha_{14}$, $\text{G}\alpha_{16}$) of closely related sequence (Hepler and Gilman 1992). All four of these G proteins markedly activate PLC- β isozymes in intact cells as well as in assays with purified components using phospholipid vesicles. All four PLC- β isozymes are activated by $\text{G}\alpha_q$, although there may be selectivity for PLC- $\beta 1$ and - $\beta 3$ over PLC- $\beta 2$ (Paterson et al. 1995; Smrcka and Sternweis 1993). The interface between $\text{G}\alpha_q$ and PLC- β isoforms was originally thought to be contained within the isozyme-specific CT domain (Ilkaeva et al. 2002; Paulssen et al. 1996; Singer et al. 2002), but the recent crystal structure of an activated complex of $\text{G}\alpha_q$ with PLC- $\beta 3$ (Waldo et al. 2010) revealed a novel interface outside the CT domain. Thus, $\text{G}\alpha_q$ interacts with a unique extension of the C2 domain which forms a helix-turn-helix. This motif is found as a highly conserved insert in all PLC- β isozymes including the two PLC- β s of *C. elegans*; conversely, it is not found in other PLC isozymes. A similar structure also is present in other $\text{G}\alpha_q$ effectors, including p63RhoGEF (Lutz et al. 2007) and G protein-

coupled receptor kinase 2 (Tesmer et al. 2005). In all three effectors, the helical motif interacts with switch 2 and $\alpha 3$ from $G\alpha_q$ and is necessary and sufficient to confer $G\alpha_q$ binding.

The CT domain of PLC- $\beta 3$ is not necessary for binding of $G\alpha_q$ (Waldo et al. 2010) as was suggested in earlier studies, and the role of this domain in signaling by the PLC- β subgroup of isozymes remains incompletely understood. Clearly, this domain is polybasic and is important for membrane association. A three-dimensional structure of the CT domain of avian PLC- $\beta 2$ highlights three α -helices packed together to form a coiled-coil likely important for dimerization (Singer et al. 2002). However, the functional significance of this dimerization remains unclear. As is the case with the PH domain in PLC- δ isoforms, the polybasic CT domain of PLC- β isoforms likely provides an anchor point for interaction with the plasma membrane and almost certainly works in coordination with G protein binding to orient the active site for efficient enzymatic activity.

PLC- $\beta 1$ was the first GTPase-activating protein (GAP) identified for heterotrimeric G proteins (Berstein et al. 1992) and all PLC- β isoforms robustly stimulate the hydrolysis of GTP by $G\alpha_q$ -subunits of the Gq family (Biddlecome et al. 1996; Ross 2008). Not only does this activity result in rapid turn-off of $G\alpha_q$ -promoted signaling once agonist-dependent stimulation of GPCR is terminated, it also markedly alters the dynamics of PLC- β -mediated signaling nodes. The rates of activation and deactivation are robustly increased and signaling acuity is sharpened. Indeed, the magnitude of signaling may paradoxically increase as a consequence of a phenomenon known as “kinetic scaffolding” (Ross 2008). $G\alpha_q$ remains in a signaling complex with the activated GPCR through multiple cycles of activation/deactivation and the steady state amount of $G\alpha_q$ in the GTP state is increased. The recent crystal structure of the PLC- $\beta 3 \cdot G\alpha_q$ complex indicates that the structural requirements for GAP activity exist within a unique insertion between the third and fourth EF hands (Waldo et al. 2010). This eight amino acid sequence is conserved in all PLC- β isoforms but not in other PLC isozymes. An asparagine (Asp260 in PLC- $\beta 3$) in this loop directly interacts with the catalytic Gln209 of $G\alpha_q$ and stabilizes the pentameric transition state necessary for GTP hydrolysis. The same mechanism independently evolved in the very large family of regulator of G-protein signaling (RGS) proteins (Tesmer et al. 1997).

Overexpression of PLC- $\beta 2$ with $G\beta\gamma$ -subunits resulted in large increases in inositol phosphate accumulation, and reconstitution assays with purified avian and mammalian PLC- β isozymes illustrated that $G\beta\gamma$ stimulates inositol lipid signaling by directly binding to PLC- β isozymes (Boyer et al. 1992; Camps et al. 1992; Smrcka and Sternweis 1993). This effect is more prominent with PLC- $\beta 2$ and PLC- $\beta 3$ than with PLC- $\beta 1$ and PLC- $\beta 4$. Indeed, physiological signaling through PLC- $\beta 1$ and PLC- $\beta 4$ appears to be mostly, if not entirely, mediated through the G α -subunits of the Gq family. In contrast, inositol lipid signaling downstream of Gi-linked GPCR (e.g. receptors for chemotactic peptides in neutrophils) occurs through $G\beta\gamma$ -mediated activation of PLC- $\beta 2$ and/or PLC- $\beta 3$. The binding interface between PLC- β isoforms and $G\beta\gamma$ has not been firmly established and may include both the N-terminal PH domain as well as part of the catalytic TIM barrel (Barr et al. 2000; Wang et al. 2000b). Interestingly, PLC- $\beta 2$ and PLC- $\beta 3$ can be simultaneously activated by $G\alpha_q$ and $G\beta\gamma$, and studies with purified proteins illustrate that the combined presence of activated $G\alpha_q$ and $G\beta\gamma$ results in supra-additive stimulation of PLC- $\beta 3$ (Philip et al. 2010). This cooperative activation apparently accounts for synergistic activation of PLC- β often observed in cells during simultaneous activation of Gq- and Gi-activating GPCR (Rebres et al. 2011).

Members of the Rac subfamily of small GTPases also activate PLC- $\beta 2$ (Illenberger et al. 1998) and potentially PLC- $\beta 3$, but no detectable binding is observed with PLC- $\beta 1$ or PLC-

$\beta 4$ (Illenberger et al. 2003a; Snyder et al. 2003). Biochemical approaches illustrated that the PH domain of PLC- $\beta 2$ is both necessary and sufficient for Rac binding (Illenberger et al. 2003a; Snyder et al. 2003). A structure of a complex of GTP-bound Rac2 with PLC- $\beta 2$ confirmed that the switch regions of Rac directly engage the PH domain of PLC- $\beta 2$ (Jezyk et al. 2006). Rac-dependent activation of PLC- $\beta 2$ occurs through recruitment to the plasma membrane since the active site of the lipase observed in a structure of PLC- $\beta 2$ alone (Hicks et al. 2008) is super-imposable with that of Rac2-bound isozyme (Jezyk et al. 2006). Recent studies quantified fluorescence recovery after photobleaching to illustrate that, whereas Rac recruits PLC- $\beta 2$ to specific regions of the plasma membrane, recruitment promoted by G $\beta\gamma$ is more diffuse throughout the plasma membrane (Gutman et al. 2010; Illenberger et al. 2003b).

Hydrolysis of PtdIns(4)P and PtdIns(4,5)P₂ by nuclear PLC- $\beta 1$ established the existence of nuclear inositol lipid signaling (Martelli et al. 1992). PLC- $\beta 1$ apparently is the most abundant PLC- β isozyme in the nucleus, although the presence (in decreasing order of abundance) of PLC- $\beta 3$, - $\beta 2$, and - $\beta 4$ has also been detected (Cocco et al. 1999). Although both the significance and regulation of nuclear signaling of these lipases needs further clarification, several studies suggest a role for PLC- $\beta 1$ in controlling cell cycle progression, specifically at the G2/M boundary (Faenza et al. 2000; Fiume et al. 2009).

3.5.2.2 Physiology—PLC- $\beta 1$ -null mice experience sudden death preceded by epileptic seizures. This phenotype resembles responses observed with GABA_A receptor antagonists, suggesting that PLC- $\beta 1$ is essential for the normal function of inhibitory neuronal pathways (Kim et al. 1997). A recent study suggested that PLC- $\beta 1$ regulates the plasticity of M1-muscarinic receptor expression in the adult neocortex, resulting in an imbalance between the muscarinic and dopaminergic systems, as often seen in schizophrenia (McOmish et al. 2008).

PLC- $\beta 2$ -deficient mice are viable but display a reduction in chemoattractant-stimulated inositol phosphate accumulation, intracellular Ca²⁺ levels, superoxide production, and cell surface MAC-1 expression, suggesting that PLC- $\beta 2$ is critical for chemoattractant-elicited signals in leukocytes (Jiang et al. 1997). Neutrophils lacking PLC- $\beta 2$ exhibit increased rates of chemotaxis, which is consistent with the idea the PLC- $\beta 2$ -dependent signaling negatively influences chemotaxis (Li et al. 2000).

PLC- $\beta 3$ null mice exhibit higher sensitivity to morphine, suggesting that PLC- $\beta 3$ suppresses μ -opioid receptor signaling possibly downstream of G $\beta\gamma$ (Xie et al. 1999). Conversely, PLC- $\beta 3$ (-/-) mice do not respond to sensory stimuli that induce itch responses (Han et al. 2006). PLC- $\beta 3$ deficiency leads to premature death in mice and is associated with lymphomas and carcinomas (Xiao et al. 2009). PLC- $\beta 3$ -deficient mice also develop myeloproliferative disease due to the loss of Stat5 regulation by a PLC- $\beta 3$ • SHP-1 complex (Xiao et al. 2009).

PLC- $\beta 4$ null mice develop ataxia (Jiang et al. 1996), motor defects, and impaired visual processing (Kim et al. 1997). A recent genome-wide profiling study of pancreatic cancer revealed a genetic substitution of Arg254 in PLC- $\beta 4$ (Jones et al. 2008). This substitution diminishes GAP activity of PLC- β isozymes leading to aberrant G α_q -mediated signaling (Waldo et al. 2010).

3.5.3 PLC- γ Isozymes

Two PLC- γ isozymes (PLC- $\gamma 1$ and PLC- $\gamma 2$) exist in mammals. Whereas PLC- $\gamma 1$ is found ubiquitously, PLC- $\gamma 2$ expression primarily is restricted to cells of the haematopoietic system (Homma et al. 1989). PLC- γ isozymes are structurally characterized by a large

insertion between the two halves of the catalytic TIM barrel consisting of a split PH domain, two SH2 domains, and a SH3 domain. They are primarily regulated through phosphorylation by receptor and non-receptor tyrosine kinases in a mechanism that involves the unique domain insert of the linker region.

3.5.3.1 Regulation—Almost all growth factor receptors with intrinsic tyrosine kinase activity (RTKs) have been linked to stimulation of PLC- γ isozymes (Kamat and Carpenter 1997). Agonist binding stimulates dimerization and tyrosine autophosphorylation of these RTKs on the cytoplasmic side of the receptor, which creates docking sites for SH2-containing proteins like PLC- γ (Hubbard and Till 2000). Specifically, autophosphorylation of Tyr766 in fibroblast growth factor receptor 1 (Mohammadi et al. 1991), Tyr992 in epidermal growth factor receptor (Rotin et al. 1992), and Tyr1021 in platelet-derived growth factor receptor (Larose et al. 1993) confer a high-specificity interaction with the N-terminal SH2 (nSH2) domain of PLC- γ 1 (Bae et al. 2009; Poulin et al. 2000). This interaction is essential for both membrane recruitment (Matsuda et al. 2001; Todderud et al. 1990) and tyrosine phosphorylation of PLC- γ 1 (Larose et al. 1993; Poulin et al. 2000).

Activated RTKs phosphorylate PLC- γ 1 at five residues, Tyr472, Tyr771, Tyr775, Tyr783, and Tyr1254 (Bae et al. 2009; Matsuda et al. 2001; Todderud et al. 1990) leading to increased catalytic activity (Gresset et al. 2010). Recent studies highlight differences between requirements for tyrosine phosphorylation *in vitro* versus *in vivo*. Specifically, reconstitution assays with purified protein indicated that only phosphorylation of Tyr783 increases PLC- γ 1 activity (Gresset et al. 2010), whereas phosphorylation at both Tyr775 and Tyr783 was necessary to increase lipase activity in intact cells (Matsuda et al. 2001). Although the nature of this discrepancy remains unclear, evolutionary analysis suggests that Tyr783 is the primary regulatory residue in PLC- γ 1 since its conservation extends to *C. elegans*, whereas Tyr775 first appears in arthropods (*Aedes aegypti*), then in chordates (*Xenopus laevis*). Phosphorylation of Tyr775 in intact cells likely acts in concert with Tyr783 to sustain PLC- γ 1 activity.

The molecular details linking tyrosine phosphorylation and increased lipase activity of PLC- γ isozymes were recently described (Gresset et al. 2010). Phosphorylation of Tyr783 results in high affinity interaction with the C-terminal SH2 (cSH2) domain, which in turn results in a conformational change responsible for removal of autoinhibition. PLC- γ 1 and PLC- γ 2 share high sequence conservation, and although not fully elucidated, the mechanism of phosphorylation-dependent activation of PLC- γ 2 is likely to be analogous to that of PLC- γ 1.

PLC- γ 1 and PLC- γ 2 are also activated downstream of cytosolic tyrosine kinases in large signaling complexes located at the plasma membrane (Marrero et al. 1996; Park et al. 1991; Roifman and Wang 1992; Venema et al. 1998). For example, in haematopoietic cells most receptors that activate PLC- γ isozymes do so through non-receptor tyrosine kinases coupled to proteins containing immunoreceptor tyrosine-based activation motifs (ITAMs) of the consensus sequence DX₂YXLX₆₋₁₂YDXL (X = any amino acid). PLC- γ 1 is the predominant isoform activated in T cells downstream of the T-cell antigen receptor (TCR, CD3) (Secrist et al. 1991; Weiss et al. 1991). The Src-family tyrosine kinases Lck, and to a lesser extent Fyn (Shiroo et al. 1992), phosphorylate the ITAM motifs within the TCR complex. This recruits T-cell-specific ZAP-70 into the signaling complex through its tandem SH2 domains (Chan et al. 1991). ZAP-70 phosphorylates tyrosines in two adaptor proteins, LAT (Zhang et al. 1998) and SLP-76 (Bubeck-Wardenburg et al. 1996), which act as scaffolding proteins. PLC- γ 1 is recruited to this membrane complex through its N-terminal SH2 domain binding LAT, while SLP-76 interacts with PLC- γ 1 through its SH3 domain (Braiman et al. 2006; Stoica et al. 1998; Yablonski et al. 2001). Although the kinases

responsible for directly phosphorylating PLC- γ 1 remain undefined, phosphopeptide mapping studies indicate the major sites of PLC- γ 1 tyrosine phosphorylation in human T cells are the same as those described for cells treated with growth factors (Park et al. 1991).

Cross-linking of the B-cell antigen receptor results in tyrosine phosphorylation and activation of PLC- γ 2 rather than PLC- γ 1 (Coggeshall et al. 1992). Cytosolic tyrosine kinases, e.g. Lyn, Syk, or Btk, are recruited to a signaling complex and phosphorylation of PLC- γ 2 ensues (Kim et al. 2004). Phosphorylation of both Tyr753 and Tyr759 in PLC- γ 2 (the equivalent of Tyr775 and Tyr783 in PLC- γ 1) appear to be essential for functional B-cell signaling.

PLC- γ isozymes are also activated by mechanisms that do not require tyrosine phosphorylation. Activation of PtdIns 3-kinase generates PtdIns(3,4,5)P₃, which functions as a specific ligand for the N-terminal PH domain of PLC- γ isozymes and mediates translocation to the plasma membrane (Falasca et al. 1998). Studies using truncation constructs of PLC- γ 1 indicate that PtdIns(3,4,5)P₃ also binds to the cSH2 domain of PLC- γ 1 (Bae et al. 1998) providing an additional anchor point to the plasma membrane. However, the mechanistic details of such activation remain unknown.

Rac GTPases directly activate PLC- γ 2, but not PLC- γ 1 (Piechulek et al. 2005). Rac-dependent activation requires the split PH domain of PLC- γ 2 (Walliser et al. 2008), and Rac2 is the most potent GTPase activator (Piechulek et al. 2005). Structural studies revealed that the switch regions of activated Rac2 directly engage the β 5-strand and α -helix of the isolated split PH domain of PLC- γ 2 through hydrophobic interactions (Bunney et al. 2009). Interestingly, this interface is distinct from the engagement of activated Rac1 with the N-terminal PH domain of PLC- β isozymes (Jezyk et al. 2006). The mechanism leading to increased PLC- γ 2 activity after Rac binding also apparently involves translocation to the plasma membrane and removal of auto-inhibition mediated by the X/Y-linker (Everett et al. 2011).

3.5.3.2 Physiology—Animals homozygous for the PLC- γ 1 null allele die by embryonic day 9 due to generalized growth failure (Ji et al. 1997). Closer examination of PLC- γ 1 embryos indicated that the embryonic lethality might be attributed to the loss of both erythroid progenitors and endothelial cells, necessary for vasculogenesis and erythropoiesis (Liao et al. 2002). Vascular endothelial growth factor (VEGF) activates PLC- γ 1 via the RTKs FLT-1 and FLK-1, and VEGF is produced and secreted by myocardiocytes during development to enhance cardiac vascularization (Rottbauer et al. 2005). A zebrafish model also suggests that VEGF signaling through PLC- γ 1 modulates cardiac contractility since zebrafish deficient in functional PLC- γ 1 lose ventricular contractility and are defective in vasculogenesis (Rottbauer et al. 2005).

Several studies implicate PLC- γ 1 as a critical component of cellular transformation downstream of EGFR/erbB2 activation. Increased expression of PLC- γ 1 occurs in a number of EGF-dependent breast cancer tissues (Arteaga et al. 1991). Overexpressed PLC- γ 1 also was highly phosphorylated in correlation with upregulation of both EGFR and erbB2, suggesting that PLC- γ 1 activity drives breast tumor formation.

PLC- γ 2 null mice remain viable after birth but exhibit strong deficiencies in signaling responses of B cells to immunoglobulins (Wang et al. 2000a). Collagen-induced platelet aggregation is also compromised in PLC- γ 2-deficient mice.

3.5.4 PLC- ϵ

PLC- ϵ initially was discovered in *C. elegans* (Shibatohge et al. 1998), and the mammalian homologue was later cloned independently by three research groups (Kelley et al. 2001; Lopez et al. 2001; Song et al. 2001). A single isoform of PLC- ϵ exists. It is expressed relatively ubiquitously with highest levels found in heart, liver, and lung (Kelley et al. 2001; Lopez et al. 2001; Song et al. 2001). PLC- ϵ is a complex signaling protein since in addition to the core lipase domains it contains an N-terminal cysteine-rich domain, an N-terminal CDC25 domain, and two C-terminal Ras-associating domains (Wing et al. 2003a). PLC- ϵ provides a unique signaling node that integrates phospholipid signaling with pathways involving heterotrimeric and Ras family G proteins.

3.5.4.1 Regulation—CDC25 domains typically exhibit guanine nucleotide exchange factor (GEF) activity, resulting in exchange of GTP for GDP on small GTPases of the Ras family (Boguski and McCormick 1993). The GTPase specificity of the CDC25 domain is not well-defined, but several studies suggest that PLC- ϵ acts as a GEF for Rap1 and/or Ras (Jin et al. 2001; Satoh et al. 2006). Therefore, pathways downstream of these GTPases are activated as a consequence of activation of PLC- ϵ .

PLC- ϵ possesses tandem Ras-associating (RA) domains at its C-terminus (Shibatohge et al. 1998). RA domains are known effector sites for members of the Ras subfamily of small GTPases, and both H-Ras and Rap1 were shown in early studies to bind to the RA2 domain of PLC- ϵ in a GTP-dependent manner (Kelley et al. 2001; Lopez et al. 2001; Shibatohe et al. 1998; Song et al. 2001). Binding of H-Ras to the RA1 domain also was observed albeit with an affinity much lower than measured for the RA2 domain (Kelley et al. 2001). A later study confirmed the capacity of the RA2 domain to bind both H-Ras and Rap1b, whereas the RA1 domain exhibited binding to neither protein (Wohlgemuth et al. 2005). The RA2 domain binds H-Ras with an affinity eightfold higher than for Rap1 (Bunney et al. 2006). The activity of the CDC25 domain of PLC- ϵ produces GTP-bound Ras GTPases. Therefore, upstream activators position PLC- ϵ for CDC25-dependent activation of GTPases that in turn bind the second RA domain of the C-terminus of the isozyme to produce long-lasting activation through a feed-forward mechanism.

Cellular studies indicate that EGF increases PLC- ϵ activity through H-Ras and Rap1-promoted translocation to proximal membranes, which is dependent on the C-terminal RA domain (Kelley et al. 2001; Song et al. 2001). Whereas H-Ras mediated translocation of PLC- ϵ to the plasma membrane, Rap1 promoted translocation to the perinuclear region (Song et al. 2001). The detailed mechanism for Ras-mediated activation of PLC- ϵ remains unknown, but it likely involves RA2 domain-dependent translocation of PLC- ϵ to the plasma membrane and orientation of the active site for substrate hydrolysis.

Early studies illustrated that co-transfection of PLC- ϵ with a GTPase-deficient mutant of $G\alpha_{12}$ (but not $G\alpha_{13}$) leads to increased lipase activity, suggesting that $G\alpha_{13}$ -coupled GPCR, such as lysophosphatidic acid and thrombin receptors, signal to this lipase (Lopez et al. 2001). Subsequent studies revealed that both $G\alpha_{12}$ and $G\alpha_{13}$ mediate activation of PLC- ϵ (Wing et al. 2001), but this effect is not direct. Rather, $G\alpha_{12/13}$ activates RhoGEFs, e.g., p115RhoGEF or LARG, which activate Rho, and then Rho directly binds to and activates PLC- ϵ (Seifert et al. 2004; Wing et al. 2003b). Thus, C3 botulinum toxin, which ADP-ribosylates and inactivates Rho, blocks PLC- ϵ -mediated inositol lipid signaling responses in intact cells promoted by activation of GPCR, $G\alpha_{12/13}$, or Rho (Hains et al. 2006). GTP-dependent activation of PLC- ϵ can be recapitulated with purified PLC- ϵ and Rho in a phospholipid vesicle reconstitution system, and is independent of the Ras/Rap binding RA-domains since it occurs with truncation mutants of PLC- ϵ lacking the entire C-terminal region (Seifert et al. 2008). Although activation requires binding of Rho in the catalytic core

of PLC- ϵ and is lost when a unique 62-residue insert within the Y box of PLC- ϵ is removed, the mechanism of this activation remains undefined.

One complexity of Rho dependent-activation of PLC- ϵ is that it robustly occurs through activation of two different classes of GPCRs that nonetheless converge on a common mechanism. Thus, $G\alpha$ subunits of the G_{12} family of G proteins activate Rho, via activation of p115RhoGEF or LARG, whereas $G\alpha$ subunits of the G_q family of G proteins do so by activating p63RhoGEF (Aittaleb et al. 2010). The physiological significance of GPCR simultaneously signaling through PLC- β - and PLC- ϵ -dependent pathways is unclear, but obviously provides opportunities for cross-talk and synergy between pathways. Kelley and his colleagues illustrated that inositol lipid signaling downstream of certain GPCR, e.g. thrombin and lysophosphatidic acid receptors, in Rat-1 cells involves both PLC- β - and PLC- ϵ -dependent responses; PLC- β 3 mediates acute phospholipid signaling, whereas PLC- ϵ mediates sustained signaling (Kelley et al. 2006). Although PLC- ϵ also is activated by cotransfection with $G\beta\gamma$, this effect apparently does not occur via a direct interaction and its mechanism and potential importance remain uncertain (Wing et al. 2001).

Members of the Ras and Rho subfamilies of GTPases activate PLC- ϵ by binding to two distinct regions of the isozyme, and assays in intact cells as well as in reconstitution assays with purified components indicate that at least additive effects on PLC- ϵ activity occur during simultaneous activation by H-Ras and RhoA (Seifert et al. 2008). The fact that PLC- ϵ is more sensitive to stimulation by H-Ras following RhoA binding also suggests the potential for signaling synergy between these two GTPases. Indeed, cooperative activation of Rho and Ras/Rap through PLC- ϵ clearly occurs. For example, thrombin induces astrocyte proliferation by stimulating $G\alpha_{12/13}$ -dependent activation of a RhoGEF, which activates Rho, which in turn activates PLC- ϵ (Citro et al. 2007). The CDC25 domain of PLC- ϵ promotes activation of Rap1, which in turn leads to activation of ERK and DNA synthesis.

Activation of the β_2 -adrenergic receptor or forskolin-promoted activation of adenylyl cyclase leads to PLC- ϵ -dependent increases in inositol lipid hydrolysis (Schmidt et al. 2001). This effect is mediated by cyclic AMP-dependent activation of a RapGEF, which in turn activates Rap1B (Evellin et al. 2002), and consequently, activates PLC- ϵ through binding to the RA2 domain.

3.5.4.2 Physiology—Targeted disruption of PLC- ϵ in mice leads to developmental defects of the aortic and pulmonary cardiac valves (Tadano et al. 2005). This phenotype is similar to that of mice deficient in heparin-binding epidermal growth factor, an agonist of the EGF receptor (Jackson et al. 2003), suggesting that growth factor-mediated activation of PLC- ϵ is important in heart development. PLC- ϵ ($-/-$) mice exhibit reduced cardiac contraction in response to activation of β -adrenergic receptors, which renders these animals susceptible to hypertrophy (Wang et al. 2005). The involvement of PLC- ϵ in the action of catecholamines occurs downstream of the activation of adenylyl cyclase. That is, β_1 -adrenergic receptor-promoted elevation of cyclic AMP levels activates the RasGEF, Epac, which activates Rap2B and subsequently, PLC- ϵ (Oestreich et al. 2007, 2009).

Targeted inactivation of PLC- ϵ in the skin resulted in dampened cell proliferation and markedly reduced incidence of squamous tumors in a chemical carcinogenesis model (Bai et al. 2004). The idea that PLC- ϵ might be a tumor suppressor gene also is supported by the observation that the PLC- ϵ gene was significantly down-regulated in patients with sporadic colorectal cancer (Wang et al. 2008).

Positional cloning also identified PLC- ϵ as a targeted gene in nephrotic syndrome (Hinkes et al. 2006). Patients with severe kidney disease have missense or truncating mutations in the

PLC- ϵ gene that lead to loss of PLC- ϵ function; normal glomerular development is arrested and early-onset nephrotic syndrome occurs. Knockdown of PLC- ϵ in zebrafish resulted in lack of development of a functional kidney barrier, suggesting that a role of PLC- ϵ in podocyte development is conserved evolutionarily (Hinkes et al. 2006).

3.5.5 PLC- ζ Isozymes

PLC- ζ was first isolated and cloned from human and mouse testis and exists as a gamete-specific PLC only expressed in spermatids (Saunders et al. 2002). Fluorescence microscopy studies indicate that PLC- ζ accumulates in the pronucleus (Yoda et al. 2004). It is the smallest PLC isozyme, and is the only one that lacks an N-terminal PH domain. The absence of this domain suggests that PLC- ζ is more closely related to plant PLCs (Mueller-Roeber and Pical 2002; Tasma et al. 2008) than the mammalian PLC- δ isozymes; it is 33% identical with PLC- $\delta 1$ (Saunders et al. 2002).

3.5.5.1 Regulation—Enzymatic characterization of PLC- ζ using purified proteins indicates that the EC_{50} of PLC- ζ for Ca^{2+} -dependent $PtdIns(4,5)P_2$ hydrolysis is ~ 100-fold lower than that of PLC- $\delta 1$, suggesting that PLC- ζ exhibits the highest sensitivity to Ca^{2+} of all PLC isoforms (Kouchi et al. 2004). PLC- ζ activity was stimulated with Ca^{2+} concentrations as low as 10nM and reached a maximum at 1 μ M. Deletion of the EF-hands or the C2 domain abrogated this Ca^{2+} -dependent PLC- ζ activation, indicating a potential role for these domains in regulating PLC- ζ activity (Kouchi et al. 2004, 2005; Nomikos et al. 2007). However, structural knowledge from PLC- $\delta 1$ (Essen et al. 1996) and PLC- $\beta 2$ (Hicks et al. 2008; Jezyk et al. 2006) illustrate a supportive role of the EF-hands and the C2 domain in overall structural integrity of the PLC isozymes, and it is likely that their absence in PLC- ζ would result in impaired substrate hydrolysis.

PLC- ζ contains two nuclear localization signals: the first (residues 299–308 (KFKILVKNRK)) is in the C-terminus of the X domain, and the second (residues 374–381 (KKRK RKMK)) is located in the X/Y-linker (Kuroda et al. 2006). Point mutations within the two regions abrogate the nuclear localization of PLC- ζ , but the mutant proteins retain capacity to initiate Ca^{2+} signaling. It is unclear how PLC- ζ targets to the plasma membrane where substrate $PtdIns(4,5)P_2$ resides.

3.5.5.2 Physiology—Injection of RNA encoding PLC- ζ in mouse eggs induces intracellular Ca^{2+} oscillations and egg activation (Saunders et al. 2002). Additional fluorescent studies indicate that PLC- ζ located in the perinucleus disperses to the cytoplasm upon nuclear envelope breakdown and translocates back into the nucleus after cleavage in a cell-cycle dependent manner (Sone et al. 2005). The shuttling of PLC- ζ in and out of the nucleus coincides with Ca^{2+} oscillations. PLC- ζ is associated with the perinucleus in interphase, and no Ca^{2+} is detected; PLC- ζ translocates to the cytoplasm in the mitotic phase, and Ca^{2+} oscillations are initiated.

3.5.6 PLC- η Isozymes

A novel class of PLC isozymes that includes two isoforms, PLC- $\eta 1$ and PLC- $\eta 2$, was discovered in 2005 (Hwang et al. 2005; Nakahara et al. 2005; Stewart et al. 2005; Zhou et al. 2005). Both PLC- η isoforms are enriched in neuron-enriched regions of the brain, suggesting a role for these proteins in neuronal development (Nakahara et al. 2005; Zhou et al. 2005). PLC- η isozymes are structurally similar to PLC- δ isozymes with the addition of an extended C-terminus after the C2 domain that includes a putative PDZ domain-interacting sequence at the end (Zhou et al. 2005).

3.5.6.1 Regulation—The extent and identity of upstream regulators of PLC- η isoforms has not been fully established. However, coexpression of PLC- η 2 with G $\beta\gamma$ in COS-7 cells resulted in increases in inositol lipid hydrolysis (Zhou et al. 2005). Moreover, purified PLC- η 2 is robustly activated by G $\beta\gamma$ in reconstitution assays with model phospholipid vesicles, and therefore, this isoform signals downstream of GPCR (Zhou et al. 2008). The N-terminal PH domain and the C-terminal extension are dispensable for G $\beta\gamma$ -mediated activation of PLC- η 2, and therefore, the interface for G $\beta\gamma$ lies within the catalytic core of PLC- η 2. Deletion of the N-terminal PH domain resulted in appearance of PLC- η 2 in the cytosol, suggesting that the PH domain of PLC- η 2 functions as a localization signal for the plasma membrane, analogous to the PH domain of PLC- δ isoforms (Nakahara et al. 2005). Further studies are necessary to confirm the role of the PH domain of PLC- η isoforms as a membrane anchor and to determine its specificity of interaction with phospholipids.

3.5.6.2 Physiology—PLC- η 2 knockout mice are viable and no detectable abnormalities have been identified to date (Kanemaru et al. 2010). Transcriptional reporter assays, *in-situ* hybridization, and immunohistochemistry illustrate that PLC- η 2 is highly expressed in the habenula and retina. Further analysis highlighted the previously unappreciated role of PLC- η 2 in the development and maturation of the retina (Kanemaru et al. 2010).

3.6 Auto-inhibition of PLC Isoforms by Their X/Y-linker

All PLC isoforms are soluble enzymes while their substrate, PtdIns(4,5)P₂, is membrane-bound. Therefore, recruitment to plasma (and other) membranes is a required step in the action of these signaling proteins. Many of the activators discussed above are membrane-associated proteins, but the molecular details that accompany membrane association and activation of PLC isoforms remain unclear. Nonetheless, biochemical and structural studies suggest a central role of the X/Y-linker in autoinhibition of most if not all PLC isoforms. The most parsimonious model for activation centers on mechanisms that remove this autoinhibition (Fig. 3.5).

Multiple biochemical experiments indicate that disruption of the X/Y-linker of PLC isoforms enhances their enzymatic activities. Specifically, limited proteolysis within PLC- δ 1 (Ellis et al. 1993), PLC- β 2 (Schnabel and Camps 1998), PLC- γ 1 (Fernald et al. 1994), and PLC- ζ (Kurokawa et al. 2007) targets the X/Y-linker and resulted in PLC isoforms that exhibit higher enzymatic activity. Similarly, independently expressed polypeptide chains encompassing the N-terminus to the X-box and the Y-box to the C-terminus of PLC- β 2 (Zhang and Neer 2001) or PLC- γ 1 (Horstman et al. 1996) reassemble in the absence of their respective X/Y-linker regions and reconstitute as functional isoforms that exhibit higher basal activity than the holoenzymes. Moreover, systematic deletion of the X/Y-linker dramatically enhances the lipase activity of PLC- β 2, - β 3, - δ 1, - ϵ , - γ 1, and - γ 2 both in intact cells and with purified proteins (Gresset et al. 2010; Hicks et al. 2008; Waldo et al. 2010). The X/Y-linker-deleted versions of several PLC isoforms retain capacity to be activated further by G proteins (Hicks et al. 2008), indicating that deletion of the X/Y-linker does not impair the structural integrity or the signaling capacity of the remainder of the protein. Together, these results indicate that the X/Y-linker mediates auto-inhibition of PLC isoforms.

A regulatory role of the X/Y-linker is supported by several high-resolution structures that highlight interactions between a portion of the X/Y-linker and the active site of PLC- β isoforms (Hicks et al. 2008; Jezyk et al. 2006; Waldo et al. 2010). The absence of conformational rearrangements of the catalytic core of PLC isoforms in the high-resolution structures of activated complexes of PLC- β isoforms with Rac1 or G α_q (Jezyk et al. 2006;

Waldo et al. 2010) also is consistent with activation occurring as a direct consequence of the removal of the auto-inhibitory X/Y-linker.

Although biochemical and structural studies highlight a common inhibitory role of the X/Y-linker in all PLC isozymes, how is this function effected by the very divergent primary sequences of the various X/Y-linkers?

PLC- δ and - β isozymes share a high density of negative charge in their X/Y-linker, but they diverge structurally. The entire X/Y-linker of PLC- δ 1 is disordered with no electron density visible in the structure of PLC- δ 1 (Essen et al. 1996). In contrast, a small portion of the X/Y-linker was visible in the different structures of PLC- β isozymes (Hicks et al. 2008; Jezyk et al. 2006; Waldo et al. 2010). For example, 22 of the 70 residues of the X/Y-linker are ordered in the inactive form of PLC- β 2 (Hicks et al. 2008). Fourteen of these residues form an α -helix that runs perpendicular to the TIM barrel and the last eight residues lay on the surface of the catalytic cleft and form a small 3_{10} helix that makes direct hydrogen-bond contacts with active site residues. These interactions are conserved in the structure of an activated complex between PLC- β 2 and Rac1 (Jezyk et al. 2006). The PLC- β 3 structure in complex with $G\alpha_q$ also displays residues making direct contacts with the active site (Waldo et al. 2010). The persistence of a small ordered portion directly occluding the active site in both the absence and presence of G protein-binding suggests that binding of activators is not sufficient to remove auto-inhibition. Consistent with this idea, superposition of the three structures of PLC- β isozymes highlights no conformational changes within the catalytic TIM barrel in the absence or presence of activators (Hicks et al. 2008; Jezyk et al. 2006; Waldo et al. 2010).

The most parsimonious model (Fig. 3.5) for activation of these lipases involves an interfacial mechanism driven by electrostatic repulsions between the negatively charged X/Y-linker and the proximal membranes. Thus, activators do not induce general conformational changes, but rather, recruit and optimally orient PLC isoforms at the plasma membrane to facilitate electrostatic repulsion and removal of the X/Y-linker from the active site. Consistent with this model, removal of monovalent acidic phospholipids from the monolayer decreases by threefold the initial rate of $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis by PLC- β 1 and - δ 1 (Boguslavsky et al. 1994), indicating that the presence of negative charges in the inner leaflet of the membrane is essential for maximal activities in PLC- β and - δ isozymes. Furthermore, Rac-mediated activation of PLC- β 2 was abolished in detergent-mixed micelles, but not in phospholipid vesicles, which is also consistent with the idea that recruitment to the membrane is critical in interfacial activation (Hicks et al. 2008).

Although three-dimensional structures are not yet available for PLC- ϵ , its mechanism of activation is apparently similar to that of PLC- β and - δ isozymes. Thus, removal of the X/Y-linker robustly activates PLC- ϵ but does not prevent activation by its upstream regulators, RhoA and H-Ras (Seifert et al. 2008).

PLC- ζ is the only PLC isozyme with a highly basic X/Y-linker, although two splice forms of this isozyme contain an insertion in the X/Y-linker that is highly acidic. Conflicting results suggest that the basic patches of the X/Y-linker of PLC- ζ serve as a membrane targeting signal to facilitate interaction with phospholipids (Nomikos et al. 2007) or as a nuclear localization signal (Kuroda et al. 2006). Thus, interfacial activation as described for PLC- β and - δ isozymes likely applies to PLC- ζ isozymes harboring the acidic insertion. Alternatively, PLC- ζ isozymes lacking the acidic insertion might be solely regulated by proteolytic processing of their X/Y-linker to enhance PLC- ζ catalytic activity (Kurokawa et al. 2007).

PLC- γ isoforms contain a unique X/Y-linker composed of modular domains—a split PH domain, tandem SH2 domains, and an SH3 domain. These isoforms are recruited to the plasma membrane by their activators, either receptor or cytosolic tyrosine kinases, through engagement of the nSH2 domain. Although structural data are not available, the cSH2 domain mediates auto-inhibition of PLC- γ isoforms since its deletion recapitulates the high degree of constitutive activation observed after removal of the entire X/Y-linker (Gresset et al. 2010).

PLC- γ isoforms also harbor a unique mechanism of activation since they link tyrosine phosphorylation with enhanced catalytic activity. Interfacial activation is unlikely to be important for PLC- γ isoforms. Instead, tyrosine kinases phosphorylate PLC- γ isoforms at a specific tyrosine within the X/Y-linker, Tyr783 in PLC- γ 1 (Tyr759 in PLC- γ 2), and a high affinity interaction with the phosphotyrosine and the cSH2 domain ensues (Gresset et al. 2010). Substitution of the invariant Arg β 5 within the cSH2 domain that coordinates the phosphate oxygens of the phosphorylated tyrosine and is critical for high affinity binding of the phosphorylated tyrosine to the SH2 domain (Booker et al. 1992; Waksman et al. 1992) eliminates phosphorylation-mediated increases in PLC- γ activity (Gresset et al. 2010). In addition, increased PLC- γ isoform activity is associated with a large conformational rearrangement of the X/Y-linker with respect to the rest of the protein (Gresset et al. 2010). Overall, PLC- γ isoforms have elaborated on the common mechanism of auto-inhibition observed in other PLC isoforms and now couple tyrosine phosphorylation and release of a highly complex X/Y-linker from its autoinhibited state.

3.7 Conclusion

Activation of receptors for hundreds of extracellular signaling molecules promotes activation of PLC by mechanisms involving heterotrimeric and Ras superfamily G proteins, tyrosine kinases, Ca²⁺, and/or other stimuli. Although the importance of the PLC isoforms was first established for Ca²⁺- and PKC-mediated cell signaling, PLC-mediated changes in membrane phosphoinositide levels alter the activities of many membrane, cytoskeletal, and cytosolic proteins. Thus, PLC isoforms function as major signaling nexuses from which a panoply of downstream signals radiate. How these signaling nodes are organized spatially and functionally is largely unknown but assuredly occurs in a cell-specific manner. The existence of 13 different isoforms that are differentially regulated and co-expressed across tissues adds complexity to understanding of these signaling networks. The core function of PLC isoforms as major cell signaling proteins is well-established, and beginning insight into the physiological roles played by individual PLC isoforms has accrued recently from genetic studies. However, much is yet to be learned about how these proteins function in the larger context of human physiology and pathophysiology. Identification of pharmacological agents that selectively inhibit the function of individual PLC isoforms would provide important new reagents for the study of these signaling proteins.

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Fig. 3.1.

The enzyme activity of phospholipase C. Phospholipase C (*PLC*) isozymes convert membrane phosphatidylinositol (4,5)bisphosphate (PtdIns(4,5)P₂) into the Ca²⁺-mobilizing second messenger inositol(1,4,5) trisphosphate (IP₃) and the protein kinase C-activating second messenger diacylglycerol (*DAG*). PtdIns(4,5)P₂ also acts as a second messenger that binds to a broad range of membrane, cytoskeletal, and cytosolic proteins to change their activities

Fig. 3.2.

The mammalian PLC isozymes and their modes of regulation. The human PLC isozymes were aligned based upon conservation of protein sequence, and a dendrogram that clusters similar sequences within shared branches is presented. The common core of these isozymes includes a pleckstrin homology (PH) domain (*purple*), a series of four EF-hands (*yellow*), a catalytic TIM barrel (*pink*), and a C2 domain (*green*). The four PLC- β isozymes contain a long C-terminal (CT) domain (*light blue*). The two PLC- γ isozymes contain conserved domains inserted within the TIM barrel that include a split PH domain, two Src-homology 2 (SH2) domains and a single Src-homology 3 (SH3) domain. PLC- ϵ contains a guanine nucleotide exchange domain (RasGEF) that activates Rap1 and possibly other GTPases and two C-terminal Ras-association (RA) domains that bind activated Ras GTPases. A cysteine-rich (C) domain of unestablished function occurs at the N-terminus. PLC- ζ is the only mammalian PLC that lacks a PH domain. PLC- η isozymes contain a serine/proline (S/P) rich region in the C-terminus. The PLC-like (PLC-L) proteins exhibit the common core of other PLC isozymes but are catalytically inactive due to mutations of critical residues in the active site. Established modes of regulation are indicated for each of the PLC isozyme classes

Fig. 3.3.

Three-dimensional structure of PLC-β2. *Left panel*, A ribbon diagram is illustrated of the three dimensional structure (PDB 2ZKM) of PLC-β2 solved at 1.6 Å resolution by Hicks and coworkers (Hicks et al. 2008). The PH domain (*purple*), EF hands (*yellow*), TIM barrel (*red*) and C2 domain (*green*) are colored as in Fig. 3.2. The Ca²⁺ co-factor (*orange sphere*) within the active site and the X/Y linker region (*cyan*) that occludes the active site also are shown. The approximate membrane-binding surface is indicated. *Right panel*, The structure is rotated 90° with respect to the *left panel*. This view emphasizes occlusion of the active site within the TIM barrel by the X/Y linker

Fig. 3.4.

Mechanism of PLC-catalyzed PtdIns(4,5)P₂ hydrolysis. *Top panel*, The catalytic site of PLC-δ1 (Essen et al. 1996) is shown. The residues that ligate the soluble head group (Ins(1,4,5)P₃) of the substrate are colored in *light blue*. The residues that ligate the essential Ca²⁺ cofactor (*yellow sphere*) are colored in *magenta*. The residues that are essential for the acid-base mechanism of catalysis are colored in *salmon*. The oxygen atoms of the side chains are colored in *red*, and the nitrogen atoms of the side chains are colored in *blue*. *Bottom panel*, The mechanism of PtdIns(4,5)P₂ hydrolysis as proposed by Essen et al. (1996) is presented

Fig. 3.5.

General model of auto-inhibition and activation of PLC isozymes. The model presents the general mechanism whereby a G protein, e.g., Gαq or Rac1, activates a PLC-β isozyme. *Left side*, The G protein (*green toroid*) is shown in an inactive GDP-bound state, and PLC-β is presented as a gold toroid except for its C-terminal (CT) domain (*light pink*) and X/Y linker (*red cylinder and dotted lines*). The CT domain of PLC-β basally associates with membranes, and the X/Y linker blocks the active site. *Right side*, GTP binding activates the G protein, the active G protein forms a complex with the main portion of PLC-β, the lipase active site is anchored and oriented at the membrane surface, and the X/Y linker is repulsed by the membrane surface therein freeing the active site to hydrolyze PtdIns(4,5)P₂ into diacylglycerol (DAG) and Ins(1,4,5)P₃ (IP₃)