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Dusaban, Stephanie S
Brown, Joan Heller

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PLC ϵ mediated sustained signaling pathways

Stephanie S. Dusaban ^{a, b}, Joan Heller Brown ^{a, *}

^a Department of Pharmacology, School of Medicine, University of California, San Diego, La Jolla, CA 92093, USA

^b Biomedical Sciences Graduate Program, University of California, San Diego, La Jolla, CA 92093, USA

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Ras
Golgi

Phospholipase C- ϵ (PLC ϵ) integrates signaling from G-protein coupled receptors (GPCRs) to downstream kinases to regulate a broad range of biological and pathophysiological responses. Relative to other PLCs, PLC ϵ is unique in that it not only serves a catalytic function in phosphoinositide hydrolysis but also functions as an exchange factor small the low molecular weight G-protein Rap1. PLC ϵ is selectively stimulated by agonists for GPCRs that couple to RhoA, which bind directly to the enzyme to regulate its activity. Rap1 also regulates PLC ϵ activity by binding to its RA2 domain and this generates a feedback mechanism allowing sustained signaling. As a result of its regulation by inflammatory ligands for GPCRs and its ability to promote chronic signals, PLC ϵ has been implicated in diseases ranging from cancer to ischemia/reperfusion injury. This review will discuss the regulation of PLC ϵ , molecular mechanisms that contribute to sustained signaling, and the role of the enzyme in various disease contexts.

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Introduction

Phospholipase C- ϵ (PLC ϵ) is the most recently discovered and arguably the most unique member of the PLC family of enzymes. PLCs are traditionally thought to catalyze hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate two important second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) (Bunney and Katan, 2006). IP₃ regulates release of intracellular

* Corresponding author.

E-mail address: jhbrown@ucsd.edu (J.H. Brown).

calcium stores while DAG activates protein kinase C (PKC) and protein kinase D (PKD), kinases that regulate a myriad of biological functions (Newton, 2009; Rozengurt, 2011). This review focuses on observations demonstrating that PLC ϵ is directly regulated by low molecular weight or small G-proteins and that its compartmentalization, coupled with its secondary function as a GTPase for Rap1, positions it as a signaling node that effects a variety of biological and pathophysiological responses through sustained DAG generation.

Discovery

PLC ϵ was discovered in 1998 and is the 13th isozyme of the PLC family of enzymes using a yeast two-hybrid screen in *Caenorhabditis elegans*. Kataoka's group first identified PLC210 or PLC ϵ as a Let-60 Ras-binding protein (Shibatohge et al., 1998). When the protein sequence was determined, it became apparent that PLC210 contained the conserved X and Y catalytic motif shared by the PLC family members (Shibatohge et al., 1998). However, PLC210 contains an extended N-terminal region, which makes it considerably larger (210 kDa) than other PLCs. Importantly, the N-terminus contains a CDC25-like domain homologous to the mouse and drosophila Son of Sevenless (SOS), suggesting that this domain functions as a guanine nucleotide exchange factor for Ras-like family members (Shibatohge et al., 1998). In addition, the C-terminus of PLC ϵ was determined to have two Ras associating (RA) domains. Thus, the identification of PLC ϵ and its novel domains suggested unique regulation and function of PLC ϵ compared to the other family members.

Regulation of PLC ϵ

Following its discovery, several groups demonstrated how the novel structure of PLC ϵ is able to integrate signals from large and small G-proteins to downstream pathways. In 2000, Lomasney's group cloned the human form of PLC ϵ and demonstrated its activation using heterologous expression of PLC ϵ and constitutively active heterotrimeric G proteins, including the G α_{12} and G α_{13} proteins, which leads to activation of RhoA (Lopez et al., 2001; Suzuki et al., 2009). Smrcka's group demonstrated that the small G-protein Ras also regulates PLC ϵ activity. When activated Ras was co-expressed with PLC ϵ in COS-7 cells, inositol phosphate production was increased 5.5-fold over basal (Kelley et al., 2001). Smrcka's group further determined that point mutation of a critical lysine residue in the RA2 domain of the enzyme abolished Ras binding to PLC ϵ in a GTP-dependent manner (Kelley et al., 2001).

In addition to Ras, other small G-proteins of the Ras family have been shown to directly interact with PLC ϵ to regulate its activity. These include Rap1, Rap2, and TC21 all of which when co-transfected with PLC ϵ in COS-7 cells increased inositol phosphate production (Kelley et al., 2004). Rap1, Rap2, and TC21 induced inositol phosphate production was shown to require the RA2 domain since mutation of the RA2 domain abolished responses to these proteins (Kelley et al., 2004). In HEK-293 cells and N1E-115 neuroblastoma cell, β 2-adrenergic stimulation of PLC ϵ mediated inositol phosphate production was observed and shown to occur through the ability of cAMP to activate Epac (exchange protein directly activated by cAMP), and hence Rap (Schmidt et al., 2001).

Harden's group unraveled another level of regulation of PLC ϵ . They identified Pleckstrin Homology (PH) and EF-hand domains within PLC ϵ , and hypothesized that since PH domains function as recognition motifs for G $\beta\gamma$, G $\beta\gamma$ might also regulate PLC ϵ (Wing et al., 2001). Indeed, using COS-7 cells, they showed that co-transfection of PLC ϵ with G $\beta\gamma$ resulted in inositol phosphate production to levels similar as that observed with G α_{12} and G α_{13} (Wing et al., 2001). The regulation of PLC ϵ by G $\beta\gamma$ was also demonstrated to be a distinct event from the activation by Ras since a PLC ϵ mutant that is unable to bind Ras still mediated G $\beta\gamma$ activation of inositol phosphate production by PLC ϵ (Wing et al., 2001).

Harden's group also observed PLC ϵ activation in COS7 cells heterologously expressing G $\alpha_{12/13}$ (Wing et al., 2001) in concordance with what was observed by the Lomasney group (Lopez et al., 2001). The G $\alpha_{12/13}$ proteins bind guanine nucleotide exchange factors (GEFs) for RhoA and hence signal through activation of RhoA (Siehler, 2009; Sternweis et al., 2007; Suzuki et al., 2009). The interaction of RhoA with PLC ϵ was determined to be responsible for the stimulatory effects seen with expression of the G $\alpha_{12/13}$ proteins. Interestingly the response to RhoA did not involve binding to the RA domains of the enzyme (Wing et al., 2003). Instead RhoA was shown to bind to a 65 amino acid residue insert within

the Y domain of PLC ϵ to directly regulate its activity (Wing et al., 2003). This insert is unique to PLC ϵ and not found in the other PLCs. Further studies examining the effects of receptor stimulation on inositol phosphate production showed that responses to lysophosphatidic acid (LPA) and PAR1 thrombin receptors were mediated through PLC ϵ whereas neither M1 muscarinic nor P2Y2 receptors co-expressed with PLC ϵ enhanced inositol phosphate production (Hains et al., 2006). Dependence of the LPA and thrombin responses on G $\alpha_{12/13}$ was demonstrated by inhibition with the GTPase-activating protein p115-RGS (Hains et al., 2006) and dependence on Rho using C3 (Hains et al., 2006). Work by our group further demonstrated using astrocytes from PLC ϵ WT and KO mice, that PLC ϵ is the primary PLC to mediate inositol phosphate production in response to PAR1, S1P, and LPA receptor activation but not in response to M3 muscarinic receptor activation (Citro et al., 2007).

Thus, work from several groups demonstrated unique regulation of PLC ϵ by binding of Ras family members to its RA domain or by binding of Rho subsequent to activation by G $\alpha_{12/13}$ -coupled receptors. Thus, there is a well-documented pathway for the Ras and Rho family of small G-proteins and the GPCRs that signal through them to generate important downstream signals through PLC ϵ mediated phosphoinositide hydrolysis.

PLC ϵ mediated sustained signaling

Not only is PLC ϵ a novel PLC family member because of its regulation by small G-proteins, but unlike the other PLCs, it mediates sustained signaling. Kelley et al. observed a temporal difference between the involvement of PLC β and PLC ϵ in PI hydrolysis (Kelley et al., 2006). Endothelin-1 (ET-1), LPA, and thrombin stimulated PI hydrolysis was compared in Rat-1 fibroblasts after PLC β 3 or PLC ϵ knockdown (Kelley et al., 2006). PLC β 3 knockdown only affected the acute (1–3 min) agonist induced response whereas PLC ϵ knockdown inhibited the ET-1, LPA, and thrombin-induced inositol phosphate accumulation observed at longer time (10–60 min) (Kelley et al., 2006). Work by our group further demonstrated that in primary astrocytes, PLC ϵ mediates sustained signaling through generation of DAG (as assessed by PKD activation) in response to ligands that activate receptors coupled to Rho/G $\alpha_{12/13}$ whereas activation of G α_q -coupled receptors mediate more transient responses (Dusaban et al., 2013).

The temporal difference in PLC β and PLC ϵ activation likely reflect the unique structure of PLC ϵ , specifically involvement of the RA and the CDC25 domain. As indicated above, the RA2 domain of PLC ϵ was found to bind to Ras family members. Kataoka's group demonstrated that binding to Ras and Rap1 resulted in differential localization of PLC ϵ . Co-expression of an activated HA-Ras mutant (HA-Ras^{G12V}) with GFP-tagged PLC ϵ resulted in PLC ϵ localization to the plasma membrane (Song et al., 2001) whereas PLC ϵ was localized to a perinuclear region when co-expressed with an activated form of Rap1 (Song et al., 2001).

The ability of Ras and Rap1 to localize PLC ϵ to different cellular compartments could lead to distinct temporal effects of these small G-proteins on PLC ϵ activation. Kataoka's group tested this hypothesis in BaF3 cells expressing a platelet-derived growth factor (PDGF) receptor mutated so as to only signal through PLC ϵ (Song et al., 2002). Stimulation of this mutant PDGF receptor leads to rapid and transient increase in Ha-Ras (1 and 5 min), whereas Rap1 was activated in a slower and more sustained manner (up to 20 min). Rap1 activation required the CDC25 domain of PLC ϵ , which has been shown to function specifically as a GEF for Rap1 but not other Ras or Rap1 family members (Jin et al., 2001; Song et al., 2002). Furthermore, PLC ϵ localization to the Golgi in response to Rap1 was sustained and presumably involved continued activation of Rap1 through the CDC25 domain because deletion of this domain resulted in transient PLC ϵ localization to the perinuclear compartment (Jin et al., 2001). In our studies using primary astrocytes, we observed that thrombin, which activates PAR1, stimulates transient Ras activation but leads to sustained activation of Rap1 (Citro et al., 2007). Furthermore, using astrocytes derived from PLC ϵ KO mice, we demonstrated Rap1 but not Ras activation to be PLC ϵ dependent (Citro et al., 2007). Our new unpublished studies provide additional evidence for feedback between the CDC25 and RA2 domains in leading to sustained signaling as shown in Fig. 1.

PLC ϵ localization to specific cellular compartments has biological implications. Smrcka's group demonstrated that in cardiac myocytes PLC ϵ is localized to a perinuclear compartment through scaffolding by A-kinase anchoring protein (AKAP) (Zhang et al., 2011, 2013). As a result of PLC ϵ localization, there is activation of protein kinase D (PKD) in the nucleus and subsequent induction of hypertrophic

Fig. 1. Schema of PLC ϵ mediated sustained signaling. In response to activation of GPCRs that couple to G $\alpha_{12/13}$, activated Rho binds to a unique insert in the Y domain of PLC ϵ to result in activation of downstream signaling kinases. The CDC25 domain of the enzyme, that functions as a Rap1 GEF, generates activated Rap1 that can then bind to the RA2 domain of PLC ϵ and result in sustained activation of the enzyme. It has also been shown that Rap1 is important for PLC ϵ 's localization to the Golgi and that this could mediate localized production of DAG and sustained activation of PKC and PKD.

genes (Zhang et al., 2013). We are currently testing the hypothesis that PLC ϵ localization to the Golgi is also important for sustained PKD activation and inflammatory responses such as COX-2 and interleukin expression in astrocytes.

PLC ϵ in physiology/pathophysiology

PLC ϵ in the heart

Smrcka's group found that PLC ϵ is upregulated in patients with heart failure (Wang et al., 2005). To study the role of PLC ϵ in cardiac function, they utilized global PLC ϵ knockout (KO) mice, which they generated through targeted deletion of Exon 6 of the PLC ϵ gene, resulting in loss of detectable PLC ϵ protein (Wang, 2006). PLC ϵ KO mice develop normally and exhibit no compensation in expression of the other PLCs including PLC β 1, PLC β 3, and PLC δ 1. It was noted, however, that the mice exhibited cardiac dysfunction beginning at 2 months of age and showed enhanced hypertrophy in response to chronic β -adrenergic stimulation (Wang, 2006).

To study the mechanism for decreased cardiac contractile function, adult mouse ventricular myocytes (AMVMs) were isolated from PLC ϵ wild-type (WT) and KO mice. The KO myocytes exhibited a decrease in Ca^{2+} -induced Ca^{2+} release (CICR) in response to β -adrenergic stimulation compared to WT myocytes (Oestreich et al., 2007). The β -adrenergic effect on CICR was mediated via Rap1 and the cAMP-responsive Rap guanine nucleotide exchange factor Epac (Oestreich et al., 2007). Further studies suggested that β -adrenergic stimulation activates PLC ϵ via Rap1 and Epac, resulting in downstream activation of PKC ϵ and CaMKII, which in turn regulate CICR (Oestreich et al., 2007).

PLC ϵ has also been shown by our laboratory to mediate cardioprotection against ischemia/reperfusion injury (Xiang et al., 2013). More specifically, RhoA activation, which is elicited by ischemia/reperfusion or ligands such as S1P, stimulates PLC ϵ in the heart leading to PKD activation (Means et al., 2007; Vessey et al., 2009, 2008; Xiang et al., 2013). The cofilin phosphatase Slingshot1L (SSH1L) is phosphorylated and inhibited, resulting in increased cofilin 2 phosphorylation. As a result, cofilin 2 and its putative pro-apoptotic partner Bax are unable to translocate to the mitochondria to induce cell death in response to oxidative stress. This pathway was fully elucidated in isolated myocytes and recapitulated in the isolated perfused subject to ischemia/reperfusion where treatment with S1P, acting through PLC ϵ signaling, prevents translocation of cofilin 2 and Bax to the mitochondria to protect the heart (Xiang et al., 2013).

PLC ϵ in central nervous system inflammatory processes

Insults to the central nervous system (CNS) trigger a response in astroglial cells, which is characterized by increased proliferation, migration, and inflammatory gene expression. As indicated earlier, thrombin and the lysophospholipids, LPA and S1P, which are generated during injury, are ligands for GPCRs that couple to activation of the low molecular weight G-protein RhoA and stimulate the novel PLC ϵ in astrocytes. We have previously demonstrated that PLC ϵ is the primary PLC that mediates inositol phosphate production in response to stimulation of mouse astrocytes with thrombin, LPA, and S1P (Citro et al., 2007). Thrombin activation of PLC ϵ also in turn mediates sustained Rap1 and ERK activation and subsequent DNA synthesis (Citro et al., 2007).

A recent publication by our group extended these observations and demonstrated, using WT and PLC ϵ KO astrocytes, that thrombin, LPA, and S1P require PLC ϵ to mediate expression of inflammatory genes including IL-1 β , IL-6, and COX-2 (Dusaban et al., 2013). Induction of inflammatory genes in response to GPCR ligands and RhoA mediated activation of PLC ϵ was found to involve PKD and NF- κ B translocation to the nucleus (Dusaban et al., 2013). This signaling cascade was also shown to be involved in *in vitro* scratch wounding and *in vivo* stab wound injury (Dusaban et al., 2013). Preliminary studies using the mouse EAE model of multiple sclerosis suggest that PLC ϵ may also be involved in development of clinical signs and in inflammatory gene expression associated with this disease (Dusaban et al., manuscript in preparation).

PLC ϵ in skin inflammation and cancer

As an effector of the proto-oncogene Ras, PLC ϵ has been linked to carcinogenesis. Kataoka's group generated a gene deletion mouse model in which the catalytic X domain of PLC ϵ was deleted yielding a shorter PLC ϵ protein that was catalytically dead with regard to hydrolysis of PIP $_2$. Using these mice, two stage skin chemical carcinogenesis was induced with 7,12-dimethylbenz(a)anthracene (DMBA) followed by 12-O-tetradecanoylphorbol-13-acetate (TPA). Compared to WT mice, PLC ϵ KO mice demonstrated delayed tumor development and reduction in the number of tumors (Bai et al., 2004). Kataoka's group also demonstrated that PLC ϵ KO mice are highly resistant to spontaneous intestinal tumorigenesis compared to WT mice (Li et al., 2009). Katan's group used a different PLC ϵ null mouse that was generated by disrupting two exons that prevented expression of any functional domains of PLC ϵ . They also tested the two stage skin chemical carcinogenesis model, and in contrast to the findings of the Kataoka group, determined that PLC ϵ KO mice exhibit an increase, rather than a decrease, in the number of tumors (Martins et al., 2014). They also generated a transgenic mouse using an RA2 mutant of PLC ϵ that is unable to bind Ras; these mice exhibited a greater number of tumors than the WT mice (Martins et al., 2014). They suggested that PLC ϵ has a tumor suppressor effect and that this is due to PLC ϵ inhibiting cell growth (Martins et al., 2014), consistent with their previous reports (Chan and Katan, 2013). Increased tumor development was also seen in PLC ϵ deficient mice tested in a chronic ultraviolet (UV) B-induced skin tumor model by the Kataoka group (Oka et al., 2010).

Further studies by Kataoka's group demonstrated that PLC ϵ plays a role in regulating inflammation in the tumor environment. Using an animal model for colorectal tumorigenesis, they discovered that adenomas from PLC ϵ KO mice exhibited significantly lower expression of several inflammatory genes including COX-2, Cxcl-1, and VEGF-A (Li et al., 2009). In the chronic ultraviolet (UV) B-induced skin

tumor development model used by the same group, there were also decreases in inflammatory markers like IL-1 β (Oka et al., 2010). Thus, while there are generally consistent observations on the role of PLC ϵ in inflammatory responses in several tumor models, the effect of PLC ϵ deletion in cancer progression is inconsistent among genetic models and laboratories; thus, PLC ϵ 's effects in carcinogenesis remains to be resolved.

Conclusion

This review focuses on the novel PLC ϵ and its ability to integrate heterotrimeric and small G-proteins to mediate sustained signaling. Sustained signaling to ERK is required for cell proliferation, while sustained activation of PKD appears to be important for cardiac hypertrophy and inflammatory gene expression. Central to this sustained signaling is the ability of PLC ϵ to activate Rap1, allowing feedback regulation through the enzymes RA2 domain. Significantly, PLC ϵ 's ability to activate downstream signaling kinases in a sustained way is also due to its localization to internal membrane structures. Its substrate at the Golgi is likely PI4P, not PIP $_2$ and accordingly its primary role would not be in generation of IP $_3$ and Ca $^{2+}$ mobilization but rather in DAG generation and activation of downstream kinases. Therapeutic targets of PLC ϵ localization or its sustained signaling could have benefits in treatment of hypertrophy, CNS diseases, and cancer.

Conflict of interest statement

The authors declare no conflict of interest.

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