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REGULATION OF PHOSPHOLIPASE C ISOZYMES BY RAS SUPERFAMILY GTPASES

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■ **Abstract** The physiological effects of many extracellular 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 PtdIns(4,5)P_2 to the Ca^{2+}-mobilizing second messenger Ins(1,4,5)P_3 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. Here we discuss how the family of PLCs elaborates a minimal catalytic core typified by PLC-δ to confer multiple modes of regulation on their phospholipase activities. Although PLC-dependent signaling is prominently regulated by direct interactions with heterotrimeric G proteins or tyrosine kinases, the existence of at least 13 divergent PLC isozymes promises a diverse repertoire of regulatory mechanisms for this class of important signaling proteins. We focus here on the recently realized and extensive regulation of inositol lipid signaling by Ras superfamily GTPases directly acting on PLC isozymes and conclude by considering the biological and pharmacological ramifications of this regulation.

INTRODUCTION

Inositol lipid-specific phospholipase C (PLC)* isozymes are key signaling proteins in the cellular action of many hormones, neurotransmitters, growth factors, and other extracellular stimuli. The ground-breaking work of Hokin & Hokin in the 1950s/1960s and of Berridge, Michell, Nishizuka, and many other investigators in the 1970s/1980s established the importance of membrane inositol lipids in hormone action (1–3). The standard paradigm is that receptors for extracellular stimuli activate PLC, which converts PtdIns(4,5)P_2 into the Ca^{2+}-mobilizing second messenger, inositol (1,4,5)P_3, and the protein kinase-C-activating second messenger, diacylglycerol.

*The following abbreviations are used: DAG, diacylglycerol; EGF, epidermal growth factor; FYVE, Fab1p/YOTB/Vps27p/EEA1; GAP, GTPase activating protein; GDI, guanine

Although PLC-catalyzed formation of second messengers from PtdIns(4,5)P_2 constitutes one of the major mammalian cell signaling responses, inositol lipids themselves also subserve important signaling functions. Work in the late 1980s illustrated that conversion of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 by PtdIns 3-kinase occurred downstream of the activation of certain cell surface receptors (4–6). PtdIns(3,4,5)P_3 selectively binds to conserved domains, e.g., C2, PH, PTB, and PX domains, and coordinates the activity of many signaling proteins by promoting membrane association and/or directly regulating functional activity (Figure 1).

High affinity interaction of phosphoinositides with conserved binding domains in proteins is not restricted to PtdIns(3,4,5)P_3. Indeed, PtdIns(4,5)P_2 selectively binds to some (but not all) PH, FYVE, and PX domains (7, 8), and a polybasic motif in N-Wasp and other proteins has been proposed to provide multivalent cooperative binding surfaces for PtdIns(4,5)P_2 (9). Thus, the cell signaling roles subserved by PtdIns(4,5)P_2 expand well beyond the historical view of this phosphoinositide as a substrate for formation of diacylglycerol and Ins(1,4,5)P_3. The range of proteins selectively liganded by PtdIns(4,5)P_2 is large and includes proteins involved in cell signaling (e.g., PTEN and Ca^{2+}, K^+, and Na^+ channels), proteins involved in actin assembly and remodeling, and proteins involved in vesicle trafficking. Therefore, PLC-promoted depletion of membrane PtdIns(4,5)P_2 produces cell signaling responses that are independent of, or synergize with, the bifurcating signaling pathways emanating from PtdIns(4,5)P_2-derived second messengers. Indeed, receptor-mediated regulation of the activity of inwardly rectifying K^+ channels in cardiomyocytes (10), KCNQ K^+ channels in neurons (11, 12), N-type Ca^{2+} channels in sympathetic neurons (13), and Na^+ channels in epithelial cells (14) occurs through activation of Gq-regulated PLC isozymes and consequential depletion of PtdIns(4,5)P_2. Similarly, activation of Fcγ receptors by IgE immune complexes triggers phagocytosis by a mechanism that employs PLC-mediated depletion of PtdIns(4,5)P_2, is independent of Ca^{2+} mobilization or activation of protein kinase C, and results in actin disassembly (15).

Phosphoinositides involved in cell signaling exist in discrete cellular pools (16). Although it has been suggested that the hormone-sensitive pool of PtdIns(4,5)P_2

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nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; GEF, guanine nucleotide exchange factor; GPCR, G protein–coupled receptor; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; NHERF, Na^+/H^+ exchanger regulatory factor; PDGF, platelet-derived growth factor; PDZ, PSD-95/Discs-large/ZO-1; PH, pleckstrin homology; PtdIns 3-kinase, phosphoinositide 3-kinase; PLC, phospholipase C; PTB, phosphotyrosine binding; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns, phosphatidylinositol; PTEN, phosphatase tensin homologue deleted on chromosome 10; PTH, parathyroid hormone; PX, phox; RA, Ras-associating; RBD, Ras-binding domain; RTK, receptor tyrosine kinase; SH, Src homology; TIM, triose phosphate isomerase; Wasp, Wiskott-Aldrich syndrome protein.

largely exists at the plasma membrane, this almost certainly is an oversimplification, and certain PLC isozymes, e.g., PLC-ε activated by Ras family GTPases, have been shown to signal at multiple subcellular compartments. Cell growth and differentiation, for example, are regulated by inositol lipid-signaling pathways in the nucleus in addition to signaling events at the plasma membrane (17). Microdomains, perhaps established with unique lipid and protein compositions (18, 19), provide close proximity of receptors with the proteins involved in both activation of PLC and the ensuing downstream signaling responses. Thus, extracellular stimuli may change activity of a PtdIns(4,5)P_2-regulated ion channel without global changes in cellular PtdIns(4,5)P_2 [or Ins(1,4,5)P_3 and diacylglycerol] levels. Observation of remarkably different capacities of different Gq-coupled receptors to regulate ion channel activities in cardiomyocytes and neurons supports this idea (13, 20). Earlier studies indicated that turnover of cellular hormone-sensitive pools of PtdIns(4,5)P_2 occurs within seconds (21). Moreover, whereas hormones may induce a sharp decrease in PtdIns(4,5)P_2 levels acutely, resynthesis of PtdIns(4,5)P_2 is regulated such that supply meets demand, and thus hormone signaling, at least as quantified by cellular Ins(1,4,5)P_3 production, is maintained (22). The mechanisms underlying this rapid flux of phosphoinositides during hormone action are not known. Moreover, quantification of overall cellular responses may not be completely reflective of those of individual receptor-related microdomains, and how the complexities of PtdIns(4,5)P_2 synthesis and hydrolysis are orchestrated with signaling responses at the cell surface is a substantial and important question to be answered.

The substrate selectivity of PLC isozymes in intact cells is not known, although several isozymes have been shown in vitro to hydrolyze both PtdIns(4,5)P_2 and PtdIns-4P, and in some cases PtdIns (23). PLC isozymes apparently do not hydrolyze 3-phosphorylated phosphoinositides, and given the approximately 100-fold excess of cellular PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3, it seems unlikely that PLC-catalyzed depletion of PtdIns(4,5)P_2 substrate is generally rate limiting in the formation of PtdIns(3,4,5)P_3 by PI-3 kinase (24). However, this is not formally known, and the segregation/scaffolding of signaling proteins in microdomains suggests that receptor-regulated activation of PLC may in restricted situations also modify PtdIns 3-kinase-related signaling. For example, the work of Howes and colleagues suggests that PtdIns(3,4,5)P_3 generation and downstream activation of Akt is influenced by the availability of PtdIns(4,5)P_2 (25).

The existence of a mammalian family of six major isoforms (PLC-β, -γ, -δ, -ε, -ζ, and -η; Figure 2) consisting of at least 13 different PLC isozymes suggests multiple activities and mechanisms of regulation in this group of signaling proteins. Hormone-mediated regulation of inositol lipid signaling historically has been considered to occur primarily through regulation of PLC-γ isozymes by tyrosine kinases and activation of PLC-β isozymes by Gαq- and G$\beta\gamma$-subunits of heterotrimeric G proteins. However, this view now is known to be simplistic. For example, the realization that multiple Ras family GTPases directly activate certain PLC isozymes has markedly expanded the number of signaling pathways

that impinge on PLCs and has significantly increased the potential biological actions subserved by these signaling proteins. In this review, we briefly consider the domain structure and overall regulation of the family of PLC isozymes with emphasis placed on regulation of PLC-β isozymes by heterotrimeric G proteins. A more detailed focus is then placed on recent studies that have revealed prominent regulation of PLC isozymes by Rac, Ras, and Rho GTPases.

THE FAMILY OF PLCs

Led by the work of Rhee and colleagues in the late 1980s, multiple PLC isozymes were purified and subsequently cloned (23, 26). These initially included members of the PLC-β, PLC-γ, and PLC-δ (an earlier described PLC-α proved not to be a functional phospholipase) classes of isozymes, and the mammalian family has expanded in the last half decade to include PLC-ε (27–29), PLC-ζ (30), and PLC-η (31–33). These proteins exhibit relatively low overall homology, but each PLC isozyme contains conserved catalytic core regions historically designated as the X and Y domains (see below). The intervening sequence between the X and Y domains is approximately 50–100 amino acids in most isozymes, but contains two Src homology 2 (SH2) domains and a single Src homology 3 (SH3) domain in PLC-γ isozymes. With the exception of PLC-ζ, all PLC isozymes contain a pleckstrin homology (PH) domain, which potentially binds membrane phosphoinositides or regulatory proteins.

The details of regulation of PLC-γ by receptor tyrosine kinases and other tyrosine kinases have been broadly studied and reviewed (23, 34). The tandem SH2 domain inserts of the catalytic core of this isozyme are key components in the mechanism of activation. Thus, PLC-γ binds to tyrosine kinases through the most amino-terminal SH2 domain, leading to tyrosine phosphorylation, e.g., of Y783 in human PLC-γ1, intermolecular interaction of the phosphorylated residue with the most carboxyl-terminal SH2 domain, and activation of the phospholipase (35).

A purified avian PLC-β isozyme was first shown to be subject to regulation by G proteins (36), and this enzyme (37) and mammalian PLC-β1 (38, 39) were subsequently utilized to illustrate that Gα-subunits of the Gq family are direct activators of PLC. This concept was extended to illustrate that PLC-β2, PLC-β3, and the avian PLC-β (which is a species ortholog of PLC-β2) are subject to direct regulation by G$\beta\gamma$ subunits of heterotrimeric G proteins (40–42). Gαq activates PLC-β isozymes through interaction with their long carboxyl-terminal domains (43, 44). In contrast, G$\beta\gamma$ has been proposed to regulate certain PLC-β isozymes through interaction with the PH domain (45, 46), with sequence in the catalytic core (47–49), or perhaps through simultaneous interactions within both regions.

For more details on the individual PLC isozymes and their widely studied regulation by tyrosine phosphorylation and by subunits of heterotrimeric G proteins, the reader is referred to a number of outstanding earlier reviews (23, 26, 34, 50–52). The remainder of the current review focuses on structural aspects of PLC

isozymes, on the relatively new concept of direct regulation of PLC isozymes by Ras family GTPases, and on the potential biological implications of certain of these new modes of PLC regulation.

STRUCTURE OF PLCs

Canonical Structure and Enzymatic Mechanism of PLC-δ

Crystal structures of two fragments of PLC-δ1 provide a framework for understanding the conserved core found in all PLC isozymes. A composite structure of the catalytic core of PLC-δ based on these structures is presented in Figure 3.

The first structure of a domain of PLC-δ was of the N-terminal PH domain of PLC-δ1 bound to Ins(1,4,5)P_3 and highlighted a β-sheet architecture capped at one end by a terminating α-helix typical of numerous other PH domains (53). Ins(1,4,5)P_3 is bound through loops within the β-sandwich on the side essentially opposite the capping α-helix. The residues within the PH domain of PLC-δ1 that coordinate Ins(1,4,5)P_3 are not conserved within either the PLC-β or PLC-ε isozymes, suggesting potentially different functions for these PH domains. Indeed, a variety of biophysical studies failed to show specific binding of phosphoinositides to the PH domains of PLC-β isozymes (47, 54, 55), and homology models of these PH domains illustrate absence of the extremely polarized electrostatic surface potential of the PLC-δ1 PH domain required for Ins(1,4,5)P_3 binding (56). Recent structural determination of a large fragment of PLC-β2 also indicates that its PH domain is incapable of binding phosphoinositides (M. Jezyk, T.K. Harden & J. Sondek, unpublished data). However, conflicting in vivo data suggest that PLC-β1 binds PtdIns(3)P through its PH domain in an interaction necessary for function (57). The PH domain of PLC-ε also lacks the polarized surface potential of the PH domain of PLC-δ1 (56). In contrast, the amino-terminal PH domain of PLC-γ specifically binds PtdIns(3,4,5)P_3 (58, 59).

The second high-resolution structure of PLC-δ1 encompassed the central catalytic core of the enzyme and highlighted a catalytic TIM barrel sequestered between a series of EF-hands from the N-terminal region and a C2 domain at the C terminus (60). The PH domain is highly mobile relative to this fragment and was not included to facilitate crystallization. The catalytic TIM barrel and the C2 domain are the most highly conserved regions among PLC isozymes. A large region of nonconserved sequence divides the TIM barrel of all PLC isozymes. The sequence historically considered to comprise the X box corresponds to the first half of the TIM barrel, whereas the sequence historically considered as the Y box subsumes the second half of the catalytic domain and the C2 domain. With the advent of the PLC-δ structure, the X and Y boxes now usually refer to the split TIM domain, and the C2 domain is typically designated separately.

The active site within the TIM barrel of PLC-δ1 is ringed with hydrophobic residues that penetrate into lipid bilayers to facilitate membrane interactions and

access to phosphoinositide substrates (61). Ca^{2+}, necessary for enzymatic hydrolysis of phosphoinositide substrate, also is ligated within the active site. All enzymatically active isoforms of PLC are dependent on Ca^{2+} for catalysis, and based on extensive biochemical analyses and structural conservation, all PLC isozymes hydrolyze phosphoinositides by a similar mechanism. PLC-δ1 also ligates Ca^{2+} through the loops of its C2 domain (62). By analogy to other well-characterized C2 domains, these bound cations most likely mediate interactions with negatively charged membranes to provide an additional point of attachment of the PLC-δ isozymes to the lipid bilayer. Other isoforms of PLC lack the residues required for Ca^{2+} ligation through the C2 domain and presumably supplant this mode of membrane attachment by other mechanisms, as discussed below. EF-hand domains in many other proteins also bind Ca^{2+}, but the residues necessary for this interaction are not well conserved within PLC isozymes, and the EF-hands within the structure of PLC-δ1 do not contain bound Ca^{2+}.

The general picture developed from 20 years of research on PLC-δ is of an enzyme exquisitely designed to interact directly with membranes through multiple contact points, the PH domain, the TIM barrel, and the C2 domain, and processively hydrolyze PtdIns(4,5)P_2 under the control of intracellular Ca^{2+} and phosphoinositide levels. This concept is generally described as membrane scooting, as originally studied in detail for soluble phospholipase A2 and other lipolytic enzymes where enzyme action is described kinetically by two successive equilibria (63). The first involves reversible association and penetration of the water-soluble enzyme into the interface, and the second occurs with a molecule of the interface-associated enzyme binding to a single substrate molecule in a reaction that is equivalent in two dimensions to classical Michaelis-Menten equilibrium.

A Diverging Class of Phospholipases

PLC-δ is the prototypical isoform of PLC and its mechanism of interaction with the lipid bilayer and phosphoinositide substrate is largely understood. Other PLC isozymes presumably arose from the core structure typified by PLC-δ and have modified its basic mechanism to fulfill specific intracellular signaling requirements. It is unclear whether PLC-δ plays an important role as a signaling protein that is "regulated" by extracellular stimuli. On the other hand, the core structure of PLC-δ has evolved in ways, e.g., the PH domain of PLC-β2 to bind GTP-Rac and G$\beta\gamma$ or a carboxyl-terminal extension in all PLC-β isozymes to bind Gαq, that provide precise mechanisms for regulation of inositol lipid hydrolysis by hormones and growth factors. Although the mechanism(s) of activation of PLC isozymes remain to be defined in molecular terms, anticipated solution of the crystal structures of activated forms of PLC-β, PLC-γ, and PLC-ε will provide this important understanding.

For instance, the crystal structure of a fragment of PLC-β2 that spans the PH domain to the C2 domain indicates that the catalytic site within the TIM barrel is occluded by a portion of the loop between the X and Y boxes (M. Jezyk,

T.K. Harden & J. Sondek, unpublished data). G$\beta\gamma$ has been implicated in binding this region (47–49) as well as the PH domain (45, 46), suggesting that G$\beta\gamma$ may relieve the steric inhibition of the active site by a two-point binding mechanism. For example, G$\beta\gamma$ might bind the PH domain of PLC-β isozymes to facilitate favorable orientation with membrane-resident phosphoinositide substrates, whereas G$\beta\gamma$ interaction with the inhibitory portion of the X/Y linker would move the linker to free the active site for substrate entry. This idea is consistent with observations of increased specific activity of PLC-β2 on proteolysis of the X/Y linker (64). A version of PLC-β2 lacking the C-terminal dimerization domain retained its monomeric form after the X/Y linker region was proteolytically cleaved by either trypsin or *Staphylococcus aureus* protease V8. The basal specific activity of the clipped version was increased by 30-fold relative to the unclipped form. Both forms were stimulated to similar absolute activities by G$\beta\gamma$, which therefore resulted in a 60-fold increase in activity of the unclipped form but only a 2-fold increase in the activity of proteolyzed PLC-β2. These results are consistent with the crystal structure of PLC-β2, and strongly suggest that the X/Y linker is autoinhibitory and that its removal by proteolysis relieves this inhibition. Moreover, we suggest that G$\beta\gamma$ stimulates activity of PLC-β2 by altering the inhibitory properties of the X/Y linker, presumably through direct interaction of G$\beta\gamma$ with the linker. This idea is supported by work by Gierschik and colleagues (65, 66) illustrating that a peptide corresponding to sequence in the X/Y linker of PLC-β2 enhances enzymatic activity of this isozyme. Presumably, this peptide interferes with intramolecular interactions between the X/Y linker and the active site, thereby favoring access of phosphoinositides by release of the linker.

Inhibition of phospholipase activity by direct occlusion of the active site may provide a general mechanism for regulating PLC isozymes. For instance, PLC-δ1 behaves similarly to PLC-β2 because proteolysis of PLC-δ1 results in cleavage between the X and Y boxes and a concomitant increase in the basal specific phospholipase activity of the cleaved isozymes (67). The X/Y linker region is disordered within the crystal structures of PLC-δ1; however, the active site of PLC-δ1 might be occluded by many differing conformations of the linker that would be difficult to observe by X-ray crystallography. Alternatively, the active site may be only partially occupied by the highly mobile linker. In either case, it would still be possible to regulate access to the active site and thereby regulate phospholipase activity. For example, it has been reported that calmodulin binds to the X/Y linker of PLC-δ1 and inhibits phospholipase activity (68). Perhaps, calmodulin stabilizes conformations of the X/Y linker that occlude the active site, leading to the observed inhibition of phospholipase activity.

In the same vein, fragments of PLC-γ1 (69) or PLC-β2 (70) that eliminate portions of the X/Y linker region exhibit increased basal specific activities. In fact, phosphorylation of Tyr 783 within the X/Y linker of PLC-γ1 allows this region to interact specifically with the second SH2 domain of PLC-γ1 and thereby stimulate the phospholipase activity of PLC-γ1 (35). In this case, phosphorylation and the

resulting intramolecular sequestration of the X/Y linker of PLC-γ1 may relieve the steric exclusion of the active site by the X/Y linker.

The linker between the X and Y boxes that constitute the catalytic TIM barrel of PLCs generally has been ignored, presumably because this region is not well conserved between the different isozyme groups, and no function has been ascribed to this part. However, this region exemplifies the concept of a diversely regulated group of PLCs diverging from a common ancestor that has altered primordial means of regulation to suit the specific requirements of various signaling cascades within metazoans. Other insights into the regulation of PLC isozymes can be anticipated as more knowledge about their molecular regulation becomes available.

Novel Structural Features of G Protein–Regulated Isozymes

The PLC-β and PLC-ε isozymes build on the minimal PLC-δ1-like core with several additional domains that confer additional modes of regulation by heterotrimeric and Ras family GTPases (Figures 2 and 4).

PLC-β isozymes are distinguished by a large C-terminal extension that is highly basic. This region promotes membrane association and is required for activation by Gα subunits of the Gq family (43, 44). Although the regulation of PLC-β isozymes by Gq has been widely studied, the mechanism whereby activation occurs remains unknown. The structure of the unique carboxyl-terminal domain of avian PLC-β was solved by Singer and coworkers (71) and highlights an unusually elongated α-helical bundle of three helices that homodimerizes along its long axis to bury $\sim$3200 Å^2 of solvent-exposed surface area. Extensive buried surface area is indicative of biologically relevant function. Although the biological ramifications are not yet understood, both the isolated C-terminal domain and full-length PLC-β isozymes migrate as dimers during gel exclusion chromatography. Similarly, mutations that map to the dimeric interface prevent stimulation of the phospholipase activity of PLC-β1 by Gαq and lend further credence to the suggestion that dimeric PLC-βs are functionally relevant.

Inability to purify Gαq in useful amounts has inhibited structural studies with this Gα-subunit, and consequentially, unambiguous understanding of the mechanism of activation of PLC-β isozymes by Gαq remains unrealized; however, some insight is possible. The first description of a GTPase activating protein (GAP) for Gα-subunits of heterotrimeric G proteins was made in the elegant studies of Ross and colleagues illustrating that PLC-β isozymes markedly stimulate the rate of hydrolysis of GTP by Gαq (72–75). As with activation of the enzyme, the capacity of PLC-β to act as a GAP for Gαq requires the carboxyl-terminal domain. Indeed, certain carboxyl-terminal domain constructs of PLC-β1 studied by Ross and colleagues displayed robust GAP activity against Gαq independent of any phospholipase activity (76), and the isolated carboxyl-terminal domain used in the structural studies of Singer and coworkers blocked capacity of PLC-β to act as a GAP of Gαq (71). Accordingly, insight into the potential Gαq/PLC-β interface was obtained by utilizing the solved structure of the dimeric carboxyl-terminal domain

of PLC-β as a template to dock homology-modeled Gαq (71). Regions of PLC-β implicated from mutagenesis studies to be critical for activation of the PLC, as well as for mediating GAP activity of PLC-β, modeled to the predicted Gαq/PLC-β interface or the surface necessary for PLC-β dimerization. These putative regions of interaction also represent regions of high sequence conservation within carboxyl-terminal domains of PLC-β isozymes. Conversely, mutations within Gαq that inhibit activation of PLC-β also map to the predicted Gαq/PLC-β interface.

Detailed enzymatic analyses indicate that Gαq undergoes multiple rounds of GTP loading and hydrolysis while simultaneously engaged to the M1-muscarinic cholinergic receptor and PLC-β (73, 74). Such complexes serve to increase the dynamic range and decrease the response times for signals initiated by G protein–coupled receptors (GPCRs), transduced by Gαq, and output through PLCs. Finally, given the increased awareness that many GPCRs homo- and heterodimerize (77), it is intriguing to speculate that Gαq-coupled GPCRs might engage supramolecular complexes consisting of homodimeric PLCs through pairs of Gαq subunits. The structural docking studies described above are consistent with the possible simultaneous engagement of two Gα subunits per PLC homodimer. Related to this issue, all PLC-β isozymes terminate in consensus sequences indicative of interaction with PDZ domains. These domains are typically associated with scaffolding proteins that function to assemble larger macromolecular complexes for efficient and tightly regulated signal transduction (78).

A dramatic example of insulated and highly efficient signal transduction mediated by scaffolded complexes involves the parathyroid hormone 1 receptor (PTH1R), a GPCR that forms a complex with PLC-β1 or PLC-β2 mediated by the Na^+/H^+ exchanger regulatory factor (NHERF) (Figure 5) (79, 80). The NHERFs contain two PDZ domains, the first of which binds the C-terminal portion of either PLC-β1 or -β2, whereas the second PDZ domain engages the carboxyl-terminal region of PTH1R (81, 82). Coexpression of the PTH1R with NHERF2 in PS210 cells derived from Chinese fibroblasts resulted in a 10-fold increase in total inositol phosphates in response to parathyroid hormone relative to similar conditions without NHERF2. The PTH1R stimulates adenylyl cyclase through Gs to produce cyclic AMP, but this activity was prevented by coexpression of NHERF2. Therefore, a scaffolding complex consisting of PTH1R, PLC-β, and NHERF not only serves to enhance the efficiency of signal transduction from the PTH1R to PLC-β isozymes but this multiprotein complex also serves to insulate signal transmission to favor signaling through PLC as opposed to signaling through adenylyl cyclase. This scaffolding has biological consequences. The PTH1R is expressed in brush border and basolateral membranes of renal proximal tubules, whereas NHERF1 is expressed only in brush border membranes. Consequently, PTH increases cyclic AMP accumulation only at basolateral membranes, whereas it activates protein kinase C at brush border membranes, consistent with increased phosphoinositide hydrolysis through a PTH1R/NHERF/PLC-β complex.

At least three distinct domains elaborate the core catalytic framework of PLC-ε (Figures 2 and 4). A carboxyl-terminal domain extends past the C2 domain to

contain two Ras-associating (RA) domains (27–29, 83). RA domains and the highly related Ras-binding domains (RBDs) are characteristically found in effectors of Ras family GTPases. As discussed in some detail below, Ras directly binds the RA domains to promote translocation of PLC-ε to membranes and increase inositol lipid hydrolysis by PLC-ε. Similarly, the RA domains of PLC-ε mediate interaction with activated forms of several Rap GTPases, which likely promote a different perinuclear localization of the isozyme (84, 85). PLC-ε also possesses a functional guanine nucleotide exchange domain with homology to yeast Cdc25 and mammalian Sos. This RasGEF domain directly binds Ras and Rap GTPases to catalyze the exchange of GTP for GDP, which leads to their activation. The combination of the RA domains and a RasGEF domain in PLC-ε adds considerable regulatory complexity to signaling cascades controlled by PLC-ε, which is both a downstream effector and upstream regulator of various Ras GTPases.

Other unique features of PLC-ε include a cysteine-rich region immediately upstream of the RasGEF domain and several large insertions interspersed between its four EF-hands. No functional significance has been ascribed to either feature, which have been commonly ignored or misaligned in sequence comparisons.

DIRECT REGULATION OF PLC ISOZYMES BY Ras SUPERFAMILY GTPases

Observations made nearly 20 years ago by Wakelam, Hall, and colleagues and by Cantley and coworkers suggested that Ras GTPases regulate inositol lipid signaling (86, 87). However, attention was largely and justifiably focused over the ensuing decade on the details of receptor-regulated inositol lipid signaling that involved the SH2 and SH3 domain-containing PLC-γ isozymes, which are substrates for tyrosine kinase receptors, and the PLC-β isozymes, which are directly activated by Gαq and G$\beta\gamma$ subunits of heterotrimeric G proteins. The idea that Ras family GTPases are important regulators of inositol lipid signaling resurfaced from the groundbreaking work of Illenberger, Gierschik, and colleagues in the late 1990s demonstrating direct activation of PLC-β2 by small GTPases of the Rac subfamily, and from the discovery of PLC-ε, which is regulated by Ras and Rho GTPases through independent mechanisms.

GTP-Dependent Regulation of PLC-β Isozymes by Rac

Illenberger et al. (88) reported in 1997 that cytosolic preparations of bovine neutrophils contain a soluble, heterodimeric complex that stimulates PLC-β2 in a guanine nucleotide-dependent manner. The carboxyl-terminal domain of PLC-β2, which is necessary for Gαq-dependent activation of the isozyme, was not necessary for GTPγS-dependent activation by the soluble fraction, suggesting that a novel mode of regulation of inositol lipid signaling exists in neutrophils.

The PLC-β2-stimulating activity was purified from the cytosol of bovine neutrophils and was shown to consist of protein species of approximately 23 kDa and 26 kDa (89). The 23 kDa proteins(s) was immunoreactive with antisera against RhoA and Cdc42, and two peptide fragments from the 23 kDa protein(s) were sequenced and corresponded to stretches of amino acids in RhoA and RhoC. Protein sequence obtained from the 26 kDa protein revealed its identity with the Rho regulatory protein LyGDI. Thus, the novel PLC-β2-stimulating activity of neutrophils was concluded to purify as a heterodimer of a Rho family GTPase(s) and the Rho GDI, LyGDI. Depletion of Rho GTPase(s) from the purified preparation using immobilized GST-LyGDI resulted in loss of GTP-dependent activation of PLC-β2. Whereas marked GTPγS-dependent activation of PLC-β2 was observed with purified prenylated recombinant Cdc42, purified recombinant LyGDI was without effect. Therefore, the PLC-β2-stimulating activity of neutrophil cytosol apparently was contributed entirely by the GTPase.

The order of potency of purified Rac family GTPases for stimulation of PLC-β2 was Rac2 $>$ Rac1 $\gg$ Cdc42, and the maximal effect observed with Cdc42 was less than that observed with Rac1 and Rac2 (90). Purified RhoA was inactive. Similar selectivities for activation were observed by Snyder et al. (91) in Cos-7 cells cotransfected with PLC-β2 and constitutively active Rac family GTPases. Carboxyl-terminal processing is necessary for phospholipase-stimulating activity because expression of Rac GTPases from a baculovirus in Sf9 insect cells produced PLC-β2-stimulating protein, whereas GTPases produced in bacteria were inactive in the vesicle reconstitution assay (90). Illenberger et al. (90) concluded that integrity of the effector binding region of Cdc42 is essential because point mutations of two different amino acids (F37 or Y40) known to be important in activation of other effector proteins by Rac resulted in loss of capacity to activate PLC-β2. Rac2 exhibited selectivity for activation of PLC-β isozymes, with PLC-β2 exhibiting the most robust Rac-regulated activity; PLC-β3 was less sensitive to activation, and PLC-β1 essentially was unaffected by Rac2.

Snyder and coworkers utilized surface plasmon resonance to study directly the binding of Rac GTPases to PLC-β isozymes immobilized on a sensor chip surface (91). Rac1, Rac2, and Rac3, but not Cdc42, RhoA, or a majority of known Rho-family GTPases bound to PLC-β2, and binding was completely dependent on loading of the GTPases with GTPγS. The Rac GTPases bound to PLC-β2 with greater affinity than PLC-β3, and no interaction with PLC-β1 was observed.

The Rac/PLC-β2 binding interface recently has been defined. Illenberger and coworkers took advantage of the lack of Rac-regulated activity of PLC-β1 to construct chimeras of PLC-β1 and PLC-β2 with the goal of gaining insight into the structural requirements for Rac-dependent regulation of PLC-β2 (90). Replacement of the PH domain-containing amino-terminal portion of PLC-β1 with the analogous region of PLC-β2 conferred Rac2-stimulated activity. Conversely, replacement of the PH domain of PLC-β2 with the PH domain from PLC-β1 resulted in loss of Rac2-dependent activity of the resulting chimera.

The suggestion that the PH domain of PLC-β2 is an effector site for Rac GTPases was confirmed and extended in surface plasmon resonance studies by Snyder and coworkers (91). Thus, Rac1, Rac2, and Rac3 bound in GTP-dependent fashion and with similar affinities to wild type PLC-β2, to PLC-β2 lacking the carboxyl terminus, or to the amino-terminal PH domain of PLC-β2. No binding was observed to a carboxyl terminal fragment of PLC-β2. The specificity of this interaction was verified by illustrating that whereas the PH domain of PLC-β2 bound Rac, no GTP-dependent binding of Rac was observed with the PH domain of PLC-δ1 or of G protein receptor kinase2. Conversely, G$\beta\gamma$ bound to the PH domain of G protein receptor kinase2, but failed to associate with the PH domain of PLC-β2 or PLC-δ1.

The physiological significance of Rac-mediated regulation of PLC-β isozymes is not known but several possibilities are considered in the final section of this review below. The PLC-β2 isozyme is the least responsive of four PLC-β isozymes to activation by Gαq, but is the most sensitive to activation by Rac and is as sensitive as PLC-β3 to activation by G$\beta\gamma$ (90). A yet to be experimentally confirmed signaling pathway may include GPCR-promoted activation of Gi, release of G$\beta\gamma$, G$\beta\gamma$-mediated activation of PtdIns 3-kinase leading to formation of PtdIns(3,4,5)P_3, activation of Rac GEFs, activation of Rac, and consequential activation of PLC-β2. Is PLC-β2 simultaneously regulated by G$\beta\gamma$ and Rac, and what are the mechanistic/physiological implications of such regulation? No obvious hints follow from the biochemical data extant. Thus, Rac and G$\beta\gamma$ independently bind and activate PLC-β2, and at least in phospholipid vesicle assays with the unusual G$\beta\gamma$-dimer, Gβ5γ2 simple additivity of stimulatory activities of G$\beta\gamma$ and Rac2 was observed (90).

The action of Rac GTPases in inositol lipid signaling almost certainly involves recruitment of PLC-β isozymes to membrane surfaces, and studies in live cells with green fluorescent protein (GFP)-tagged PLC-β2 illustrated that enzymatic activity and membrane localization of the phospholipase were markedly increased by coexpression with a GTPase-deficient mutant of Rac2 (92). Localization in various subcellular compartments may occur in a GTPase specific manner, and clearly, it will be important to assess the relative roles of Rac GTPases versus heterotrimeric G proteins in regulation of phospholipase C in living cells. Their relative actions likely will have both spatially and temporally distinct consequences.

PLC-ε as a Ras-Binding Protein

Ras is encoded by a single gene (*let-60*) in the nematode *Caenorhabditis elegans*, and cell signaling pathways involving LET-60 exhibit strong similarities to their counterparts in mammalian cells. With the reasoning that yeast two-hybrid screens for LET-60 binding proteins in *C. elegans* potentially reveal novel Ras effectors, Kataoka and coworkers (83) identified a novel PLC (designated PLC210) as a Ras-binding protein. Unique Ras-related signaling was suggested by the domain structure of this PLC, which contains the conserved catalytic core of a PLC

that is flanked by a carboxyl-terminal region harboring two RA domains and an amino-terminal region homologous to that of guanine nucleotide exchange proteins represented by yeast CDC25.

Fragments of PLC210 corresponding to the two individual RA domains (RA1 and RA2) were produced, and the direct binding of H-Ras was studied (83). The fragment representing RA1 bound H-Ras in a GTP-dependent manner and with an affinity similar to that observed with the binding of H-Ras to a fragment that encompassed both RA1 and RA2. In contrast, no binding was observed to the RA2 fragment, which suggested that RA1 was responsible for GTP-dependent binding of H-Ras to PLC210. Certain mutations in the effector domain of H-Ras resulted in loss of interaction with PLC210 in a two-hybrid assay, and activation of yeast adenylyl cyclase by GTP-bound Ras was inhibited by the carboxyl-terminal fragment of PLC210 containing RA1 and RA2. Both sets of results were consistent with the idea that *C. elegans* PLC210 is an effector of Ras GTPases.

Cloning of human and rat homologues (designated PLC-ε) of PLC210 revealed the domain structure of the first mammalian PLC containing a Ras GTPase binding domain. Song et al. (27) reported that a carboxyl-terminal fragment of human PLC-ε encompassing the RA1 and RA2 domains bound H-Ras and Rap1A in a GTP dependent manner. This fragment also competitively inhibited (Ki $=$ 40 nM) the capacity of GTP-bound H-Ras to activate yeast adenylyl cyclase in vitro. Yeast two-hybrid assays revealed that mutations in the effector domain of H-Ras that inhibited interaction with PLC210 also resulted in loss of interaction with the RA domain–containing carboxyl-terminal fragment of PLC-ε. Whereas Rap1A interacted with PLC-ε in the two-hybrid assay, no interaction with R-Ras, RalA, RhoA, Rac1, or Cdc42 was observed.

Kelley and coworkers (29) illustrated that coexpression of rat PLC-ε with a GTPase-deficient mutant of H-Ras in COS-7 cells resulted in a marked increase in inositol phosphate accumulation. PLC-ε constructs lacking the RA1, RA2, or both RA1 and RA2 domains exhibited lower basal activities and were essentially unresponsive to coexpressed Q61L H-Ras. Immunocytochemical experiments with COS-7 cells also revealed that GTPase deficient H-Ras promoted plasma membrane association of FLAG-tagged PLC-ε. Conflicting results exist regarding direct activation of PLC-ε in vitro. Song and coworkers (27) reported that lipidated H-Ras activated Sf9 insect cell-expressed human PLC-ε in a GTP-dependent fashion in reconstituted liposomes, whereas Kelley and coworkers (29) failed to observe activation of purified rat PLC-ε by H-Ras in a similar assay system.

GTP-dependent binding of H-Ras was observed with the RA2 domain of rat PLC-ε and with a carboxyl-terminal fragment containing both the RA1 and RA2 domains (29). In contrast to the results reported with the *C. elegans* PLC210 homologue of PLC-ε (83), the RA1 domain of mouse PLC-ε bound H-Ras with much lower affinity and exhibited no GTP-dependence in this interaction (29). Mutations of a lysine (K2150) in the RA2 domain of mouse PLC-ε that is conserved in the RA domains of other Ras-binding proteins, e.g., in RalGDS and AF6, as well as in PLC-ε of other species, resulted in loss of GTP-dependent binding of

H-Ras (29). Mutation of K2150 to alanine (K2150A) reduced H-Ras binding by greater than 50%, and mutation of this position to a negative amino acid (K2150E) completely abolished Ras binding. Mutation to alanine of an adjoining lysine (K2152) alone also resulted in reduced GTP-dependent binding of H-Ras, and combined mutation of K2150 and K2152 completely prevented H-Ras binding. Results from assays of inositol lipid hydrolysis in COS-7 cells cotransfected with H-Ras, and RA2 domain mutants of PLC-ε were consistent with the in vitro binding experiments. For example, the K2150A and K2152A mutations resulted in 50% and 90% decreases, respectively, in H-Ras-stimulated inositol phosphate accumulation.

While the RA domains of PLC-ε isozymes clearly mediate functionally relevant interaction with H-Ras and Rap GTPases, the mechanistic implications of the tandem array of RA domains complicates current understanding. The RA2 domain of rat PLC-ε bound to the E37G and D38N effector domain mutants of H-Ras, but bound minimally to the T35S and E37G effector domain mutants and not at all to the D38N or Y40C mutants (29). Activation of wild-type PLC-ε by these effector mutants of H-Ras in cotransfection studies in COS-7 cells paralleled the data obtained in in vitro binding experiments. An exception was observed with the N26G mutant, which retained binding affinity for the RA2 domain in vitro, but nonetheless only weakly stimulated PLC-ε in cotransfection experiments. A similar dissociation of inhibition of effector-stimulating activity from binding activity has been observed for the interaction of this mutant with Raf-1 (93).

Interaction of members of the Ras subfamily of GTPases with the carboxyl-terminal RA domain(s) of PLC-ε provides a mechanism whereby Ras-activating cell surface receptors can promote inositol lipid signaling. Thus, activation of tyrosine kinase receptors, such as those for EGF or PDGF, have been shown to promote PLC-ε-dependent signaling through a mechanism that involves activation of Ras and/or Rap and the RA2 domain of this PLC isozyme (84, 94). The work of Schmidt and colleagues (95–97) on cyclic AMP-regulated GEFs for Rap also has led to the description of a novel PLC-ε-dependent signaling pathway downstream of GPCR, e.g., β2-adrenergic or prostaglandin E_1 receptors, that activate the heterotrimeric G protein Gs, which in turn activates adenylyl cyclase. Thus, elevation of cyclic AMP levels activates the cyclic AMP-regulated GEF, Epac, which in turn activates Rap2B and PLC-ε. Whereas overexpression of Epac markedly enhanced this signaling response to extracellular stimuli that elevate cyclic AMP levels, inhibitors of protein kinase A have no effect. Conversely, GPCRs, e.g., M2 muscarinic, sphingosine 1-phosphate, or opiate receptors, that activate the heterotrimeric G protein Gi, and therefore inhibit adenylyl cyclase and cyclic AMP accumulation, decreased formation of GTP-bound Rap2B and consequentially inhibited inositol lipid signaling through endogenous PLC-ε in HEK-293 cells (98).

Rho-Mediated Activation of PLC-ε

Lopez et al. (28) cotransfected TSA201 cells with PLC-ε and GTPase mutants of various Gα-subunits and observed marked increases in inositol phosphate

accumulation when PLC-ε was coexpressed with Gα12, but not with Gαq or other Gα-subunits. Although no apparent activation of PLC-ε by Gα13 was observed by Lopez et al. (28), subsequent transfection studies by Wing et al. (99) using COS-7 cells revealed similarly robust PLC-ε-dependent stimulation of inositol phosphate accumulation by both Gα12 and Gα13. Furthermore, Gα12/13-stimulated lipase activity was retained in PLC-ε mutated in the RA2 domain such that Ras-regulated activity was lost.

Gα12 and Gα13 may prove to be direct activators of PLC-ε but this has not yet been reported. The fact that guanine nucleotide exchange proteins for Rho (RhoGEFs) comprise some of the very few confirmed effectors of Gα12/13 (100–103) suggests the possibility that Gα12/13 regulate PLC-ε through signaling pathways involving Rho GTPases. Indeed, Wing et al. (104) illustrated that RhoA, RhoB, and RhoC, but not Rac GTPases, markedly stimulate inositol phosphate accumulation in COS-7 cells cotransfected with PLC-ε. Rho-mediated activation of PLC-ε was retained with PLC-ε constructs that lacked the carboxyl-terminal RA domains and were therefore insensitive to activation by Ras. Moreover, truncated mutants of PLC-ε lacking either the CDC25 domain-containing amino terminus or RA domain-containing carboxyl terminus retained regulation by both Rho GTPases and Gα12/13, suggesting that these two different types of G proteins might regulate PLC-ε through the same regulatory pathway. The existence of an approximately 70–amino acid sequence in the catalytic core of species orthologs of PLC-ε that is not found in the otherwise highly conserved catalytic domain of all other PLC isozymes suggested that Rho and/or Gα12/13 might regulate PLC-ε through binding to this sequence. This insert, which mapped to a small surface-exposed loop in the PLC-δ structure, was deleted from a PLC-ε fragment that included the EF hand through the carboxyl terminus. Ras-dependent activation was retained in this mutant enzyme, but capacity to be regulated by Rho and Gα12/13 was lost.

Pull-down assays were utilized to test the hypothesis that Rho directly binds to PLC-ε (104). Both GST-RhoA and GST-H-Ras bound in a GTP-dependent manner to PLC-ε but not to PLC-β2, in lysates from transfected COS-7 cells. In contrast, GST-Rac, which bound to expressed PLC-β2 in a GTP-dependent manner, did not interact with PLC-ε. Interestingly, GTP-dependent binding of Rho was also observed with PLC-ε lacking the 70–amino acid insert, which suggests that whereas this unique region of PLC-ε is important in the mechanism of activation of PLC-ε by Rho, it does not fully account for Rho binding.

The idea that Rho directly regulates PLC-ε was verified using a purified fragment of PLC-ε comprised of the EF-hands through RA2 domain (105). Marked GTP-dependent activation of this purified PLC-ε was observed in lipid vesicles reconstituted with prenylated RhoA purified after expression from a baculovirus in Sf9 insect cells. Purified, lipidated Rac1 activated PLC-β2 under these conditions, but failed to activate PLC-ε. Thus, RhoA activates PLC-ε under conditions similar to those first used by Illenberger and coworkers (89) to demonstrate GTP-dependent activation of PLC-β2 by Rac. A purified PLC-ε construct lacking the unique 70–amino acid insert in the catalytic core was not activated by GTP-RhoA,

which is consistent with the results described above for a similar deletion mutant studied in COS-7 cells (105).

Unambiguous demonstration of direct activation of PLC-ε by Rho (105) and knowledge that activation of Gα12/13 results in direct activation of RhoGEFs (100–103), and consequently activates downstream Rho GTPases, suggested that activation of PLC-ε by Gα12/13 is not direct. Indeed, coexpression of the RGS domain of p115RhoGEF with PLC-ε inhibits the capacity of Gα12/13 or of GPCRs that activate Gα12/13, e.g., lysophosphatidic acid or thrombin receptors, to activate PLC-ε (94, 106, 107). Moreover, expression of C3 botulinum toxin, which ADP-ribosylates and inactivates Rho GTPases, blocked the capacities of Gα12, Gα13, lysophosphatidic acid receptors, and thrombin receptors to activate PLC-ε in cotransfection studies in COS-7 cells (107). In contrast, Gq-promoted activation of PLC-β isozymes by M1-muscarinic cholinergic or P2Y2 receptors was not affected by either the RGS domain of p115RhoGEF or expression of C3 toxin. Coexpression of G$\beta\gamma$ subunits of heterotrimeric G proteins with PLC-ε also results in stimulation of inositol lipid hydrolysis (99). It is not yet clear whether G$\beta\gamma$ directly activates PLC-ε, whether Ras family GTPases are involved in G$\beta\gamma$ action, and whether GPCRs activate PLC-ε through release of G$\beta\gamma$ from heterotrimeric G proteins.

PLC-ε as a Potential Upstream Regulator of Ras GTPases

The presence of a region in the amino terminus of *C. elegans* PLC210 with homology to Ras GEFs, e.g., *Saccharomyces cerevisiae* CDC25 and mammalian Sos, suggested that this isozyme exhibits novel bifunctional action by acting as a PLC as well as an upstream regulator of the activation state of Ras GTPases (83). This idea was tested directly in mammalian PLC-ε. An amino-terminal fragment that encompassed the CDC25 domain of human PLC-ε promoted GDP release from purified Rap1 but not from Rap2 or other Ras family GTPases, including H-Ras, M-Ras, RalA, Rit, Rin, or Rheb (84). Coexpression of full-length PLC-ε or the amino-terminal fragment with Rap1 resulted in increases in cellular Rap1-GTP levels and activation of the downstream B-Raf/MEK/ERK signaling pathway. Song and coworkers (85) illustrated that EGF promotes translocation of PLC-ε to the plasma membrane by a mechanism that is inhibited by dominant negative H-Ras. In contrast, overexpression of GTPase-deficient Rap1A resulted in a perinuclear localization of PLC-ε, and EGF also promoted perinuclear localization of PLC-ε after coexpression with wild-type Rap1A. Such data led to the conclusion that receptor-promoted translocation to different subcellular compartments occurs through functional interplay of an initial Ras-dependent activation of PLC-ε through the RA domains and a consequential CDC25 domain promoted formation of GTP-Rap1A, which also interacts with the RA domains and promotes translocation to a perinuclear localization.

The potential functional interaction of the RA and CDC25 domains in PLC-ε was further examined in BaF3 cells stably expressing a PDGF receptor mutated to

inhibit its capacity to activate PLC-γ (85). In wild-type PLC-ε-expressing cells, PDGF caused a rapid but transient increase in GTP-bound H-Ras, and induced a slower occurring but sustained increase in GTP-bound Rap1A. In contrast, PDGF-promoted activation of Rap1A also was transient in cells expressing PLC-ε lacking the amino-terminal CDC25 domain.

The results of Kataoka and coworkers (84, 85) are consistent with the idea that the sustained PDGF-promoted activation of Rap1A occurs downstream of activation of the CDC25 domain of PLC-ε. The observation that PDGF-stimulated inositol lipid hydrolysis was long-lived in cells expressing wild-type PLC-ε but transient in cells expressing PLC-ε lacking the CDC25 domain also is consistent with this idea. However, different conclusions about the Ras GTPase selectivity of the CDC25 domain of human PLC-ε were made by Lopez et al. (28). Expression of wild-type PLC-ε, of PLC-ε harboring mutations that inhibit PLC activity, or of a amino-terminal construct of the CDC25 of PLC-ε all resulted in marked activation of the Ras/MAP kinase signaling pathway. All three CDC25 domain-containing constructs markedly stimulated formation of GTP-bound H-Ras, although the effect on other members of the Ras subfamily of GTPases was not examined. Thus, although it is clear that Ras GTPases interact with the RA domain and therefore stimulate the activity of PLC-ε, the selectivity of the CDC25 domain for downstream Ras GTPases and the role(s) this amino-terminal domain of PLC-ε plays both in regulation of downstream signaling pathways, as well as regulation of the cellular distribution and activity of PLC-ε remain to be more clearly defined.

PERSPECTIVES AND PHARMACOLOGICAL IMPLICATIONS

A surprising integration has occurred between signaling pathways controlled by heterotrimeric G proteins and pathways controlled by small GTPases. For instance, it is now well established that activated Gα12/13 as well as Gαq directly bind to guanine nucleotide exchange factors to simulate the loading of GTP onto RhoA. The coordination of these two sets of GTPases is required for various cellular responses such as chemotaxis and neuronal remodeling. Similarly, scientific thinking has shifted from viewing PtdIns(4,5)P_2 as a substrate for the production of the important second messengers, Ins(1,4,5)P_3 and diacylglycerol, to the idea that PtdIns(4,5)P_2 per se is a critical membrane lipid that modulates enzymatic activities, alters channel conductance, and varies signaling networks by modifying the intracellular localization/activity of proteins containing PtdIns(4,5)P_2-binding modules. Signaling networks have become increasingly complex as exemplified by the tasks cells face in integrating information pertaining to phosphoinositide levels with the activation and regulation of heterotrimeric G proteins and small GTPases.

The PLC isozymes are emerging as central nodes in these complicated networks. In their most simplistic role, PLC isozymes preferentially hydrolyze PtdIns(4,5)P_2,

but as we have discussed in this review, Ras and Rho GTPases now join heterotrimeric G proteins in their capacities to directly bind PLC isozymes to enhance phospholipase activity. This interplay is by no means spurious, and model studies of the actin cytoskeleton and chemotaxis probably best illustrate this point. For example, GPCRs for thrombin and LPA both couple to Gα12/13 leading to activation of RhoA (102, 108). However, only activation by thrombin leads to RhoA-mediated retraction and rounding of 1321N1 human astrocytoma cells caused by actin stress fiber assembly downstream of active RhoA. These receptor-dependent morphological differences were attributed to differential modulation of intracellular PtdIns(4,5)P_2 pools by the two receptors. The thrombin receptor, but not the LPA receptor, activates Gαq to enhance PLC-catalyzed hydrolysis of PtdIns(4,5)P_2. Cell retraction and rounding could be recapitulated by microinjection of the PH domain of PLC-δ1 to sequester PtdIns(4,5)P_2, suggesting that a decrease in polyphosphosphoinositide mass is necessary and sufficient for stimulation of retraction and rounding. The most parsimonious explanation of these results is that PtdIns(4,5)P_2 modulates actin-capping and -severing proteins needed for rearrangement of the actin cytoskeleton. In this example, the PLC isozymes downstream of Gαq are not known, but one could imagine that if PLC-ε were involved, then RhoA might also further modulate phosphoinositide hydrolysis through direct activation of this PLC isozyme.

Cells must sense a chemical gradient during chemotaxis and move preferentially through it. This process requires chemotaxing cells to differentiate chemical gradients as shallow as 2% that vary in space and time. Therefore, cells by necessity require highly sensitive, quickly responsive, and tightly regulated signal transduction cascades to sense and respond to shallow and variable chemical gradients. This task is accomplished by connecting GPCR-initiated events with downstream signaling through Rho GTPases that control actin polymerization, cell morphology, and ultimately cell movement (109, 110). Appreciation is growing for the roles PLC isozymes play in chemotaxis. For example, activation of PLC-γ is required to produce free actin barbed ends and directional protrusions during activation of MTLn3 rat mammary adenocarcinoma cells by EGF (111). The actin-severing protein cofilin normally is inhibited by binding PtdIns(4,5)P_2, and PLC-γ reduces available PtdIns(4,5)P_2 levels, thereby activating cofilin to promote barbed ends that establish the initial cell protrusions leading to cell movement. In this respect, the process of chemotaxis is most aptly modeled as a stochastic process of actin polymerization and depolymerization whereby local signaling events manipulate phosphoinositide pools (112), most likely in microdomains (113), to allow the rapid, sensitive, and controlled polymerization of actin.

As we learn more about the dynamic signaling events required for chemotaxis, PLC isozymes will undoubtedly take a more central role in this process, as well as other processes, such as phagocytosis (15), that require dynamic reorganization of the actin cytoskeleton. The greater task will be to relate this cellular information to the understanding of disease processes dependent on the interplay of phosphoinositides and signaling cascades. For instance, mice lacking PLC-β2 and PLC-β3

possess hyperchemotactic leukocytes and develop spontaneous facial ulcers hyperinfiltrated with leukocytes (114, 115). How are signaling cascades altered in these mice to promote hyperchemotaxis and ulcers? Our working hypothesis is that PLC-β2 activation downstream of Rac2 serves to limit local pools of phosphoinositides required by RhoGEFs to activate Rac. In this way, PLC-β2 may serve as an integral part of a negative feedback loop to limit Rac activation. Loss of PLC-β2 should then be associated with increased levels of GTP-bound Rac, an idea we are now testing. In another example, PLC-$\varepsilon-/-$ mice were remarkably resistant to chemical-induced Ras-dependent squamous cell carcinomas (116). These results point to a critical role for the interplay of PLC-ε and Ras in controlling cellular proliferation and suggest that PLC-ε may be a therapeutic target for treatment of cancer.

In summary, PLC isozymes are important in many ways that transcend their historical placement in a linear pathway of hormone-regulated Ca^{2+} signaling. These enzymes process multifactorial inputs and coordinate multiple downstream biological responses unrelated to those classically associated with PLC action. The realization that Ras superfamily GTPases prominently regulate these enzymes in parallel with their activation by the more traditionally considered tyrosine phosphorylation or heterotrimeric G proteins adds remarkable and subtle complexities to the otherwise simple hydrolysis of PtdIns(4,5)P_2. An enormous number of biological responses are associated with Ras family GTPases, and it will be important to understand what roles PLCs play in our redefined world of hormone-regulated signaling.

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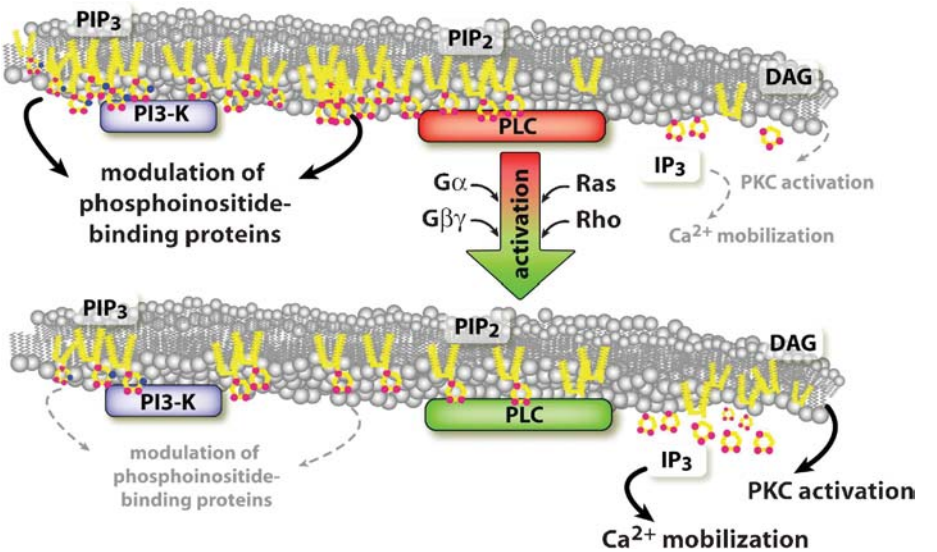

Figure 1 PLCs coordinate complex signaling networks through the regulated hydrolysis of PtdIns(4,5)P_2. Quiescent PLCs (*top*) favor high local concentrations of PtdIns(4,5)P_2 that modulate a variety of signaling proteins, such as actin-remodeling proteins and ion channels that directly bind PtdIns(4,5)P_2. In the same vein, phosphoinositide 3-kinase converts PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3, which modulates a second set of proteins with high affinity for this phosphoinositide. Various heterotrimeric and small GTPases directly activate PLCs (*bottom*) leading to the hydrolysis of PtdIns(4,5)P_2, the depletion of local phosphoinositides, and the production of the classical second messengers IP_3 and DAG, which promote high cellular concentrations of calcium and the activation of PKC. Therefore, by balancing local concentrations of PtdIns(4,5)P_2 with its various products, PLCs coordinate highly complex signaling networks.

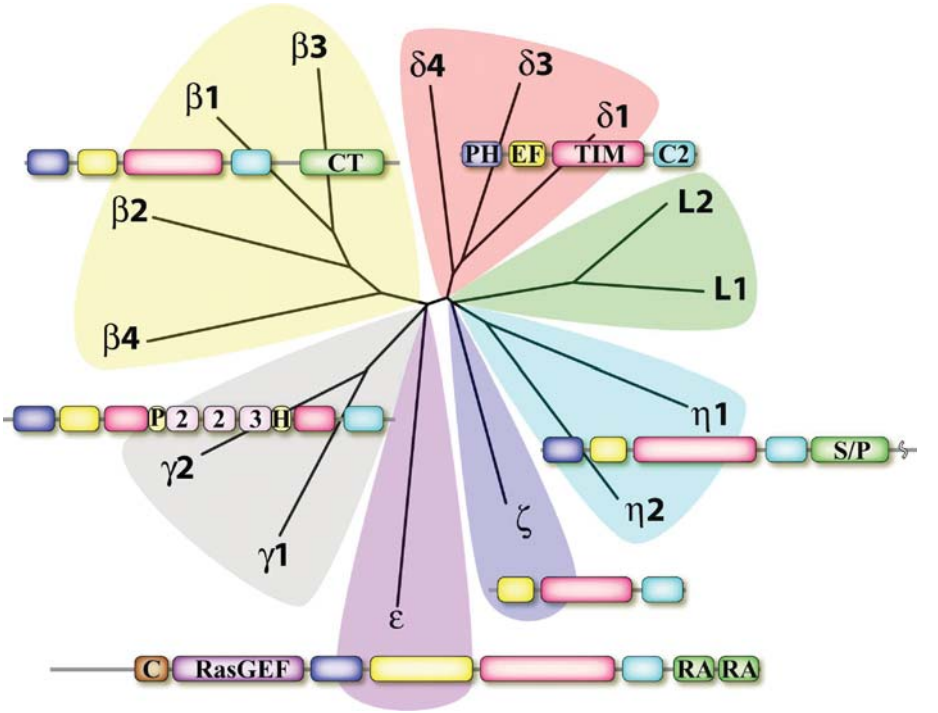

Figure 2 Dendrogram of PLC isozymes. The protein sequences of all human PLCs were aligned based on conservation, and the resulting pair-wise similarities were used as input to draw the dendrogram that clusters similar sequences within shared branches. Subfamilies of PLCs that share common functions are grouped by color. Overlaid on each colored cluster is a representative isozyme highlighting the domain architecture of the PLC subfamily. All PLCs except PLC-ζ share a common core typified by PLC-δ isozymes that contain a pleckstrin homology (PH) domain, a series of four EF-hand domains, a catalytic TIM barrel, and a C2 domain. Aside from these shared domains, the four PLC-βs contain a C-terminal (CT) domain necessary for dimerization and modulation by Gαq; the pair of PLC-γs contain an array of Src-homology (SH) 2 and 3 domains and a split PH domain inserted within the TIM barrel; PLC-ε contains an guanine nucleotide exchange domain (RasGEF) that activates Ras GTPases and two C-terminal Ras-association (RA) domains that bind activated Ras GTPases. An N-terminal cysteine-rich (C) domain in PLC-ε is uncharacterized but is suggestive of lipid binding based on weak homology to cysteine-rich, zinc-binding domains. PLC-η isozymes have been most recently identified and no role for the Ser/Pro (S/P)-rich region is known. PLC-ζ is sperm-specific and is the only PLC member to lack a PH domain. The PLC-like (PLC-L) proteins share the common architecture of the PLC-δs; however, the PLC-Ls are reported to be catalytically dead owing to mutations of critical residues in the active site. Several PLCs are expressed as splice variants (not shown) and domain architecture is drawn to scale.

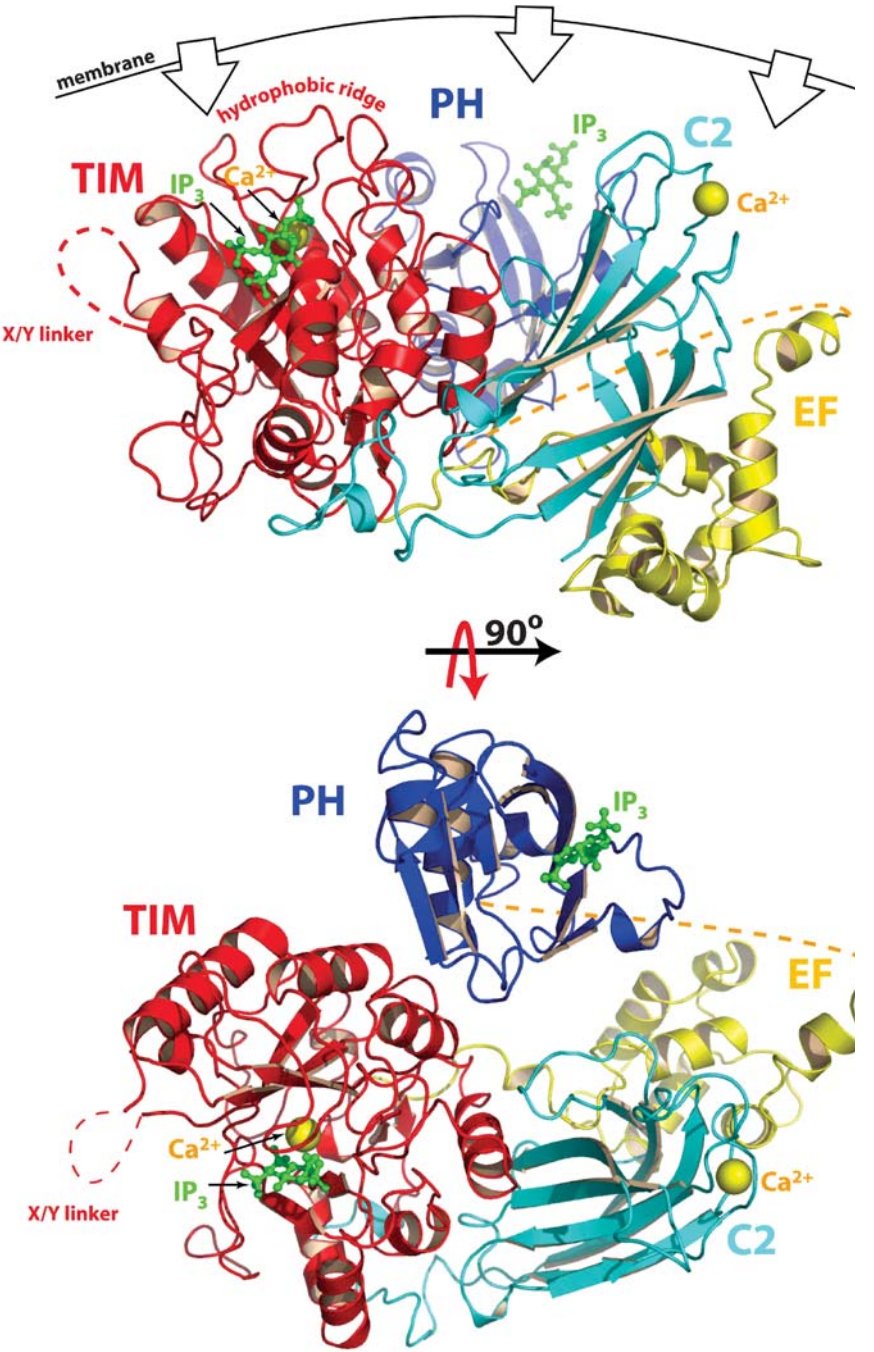

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Figure 3 Structure of PLC-δ1. Individual domains are color-coded and consist of an N-terminal pleckstrin homology (PH) domain, a series of four EF-hands, a catalytic TIM barrel, and a C2 domain. Inositol (1,4,5)-trisphosphate (IP3, *green*) is bound within the active site of the TIM barrel, as well as by the PH domain, indicating two points of attachment with lipid membranes. A third lipid-binding site is found within the C2 domain and is mediated by a bound calcium ion (*yellow*). A second Ca^{2+} is found within the active site and required for hydrolysis of phosphoinositide substrates. Dotted lines indicate disordered regions that include the X/Y linker within the TIM barrel and several of the N-terminal EF-hands. No structure of a full-length PLC-δ isozyme is available, and the illustration represents a composite of two crystal structures: one consisting of the PH domain of PLC-δ1 and the second consisting of the remaining portion of the protein. The two portions were superimposed on a structure of PLC-β2 (M. Jezyk, T.K. Harden & J. Sondek, unpublished data), which contains all shown domains. The bottom panel represents a 90° rotation of the molecule about the x-axis and the observer is looking onto the membrane-binding surface.

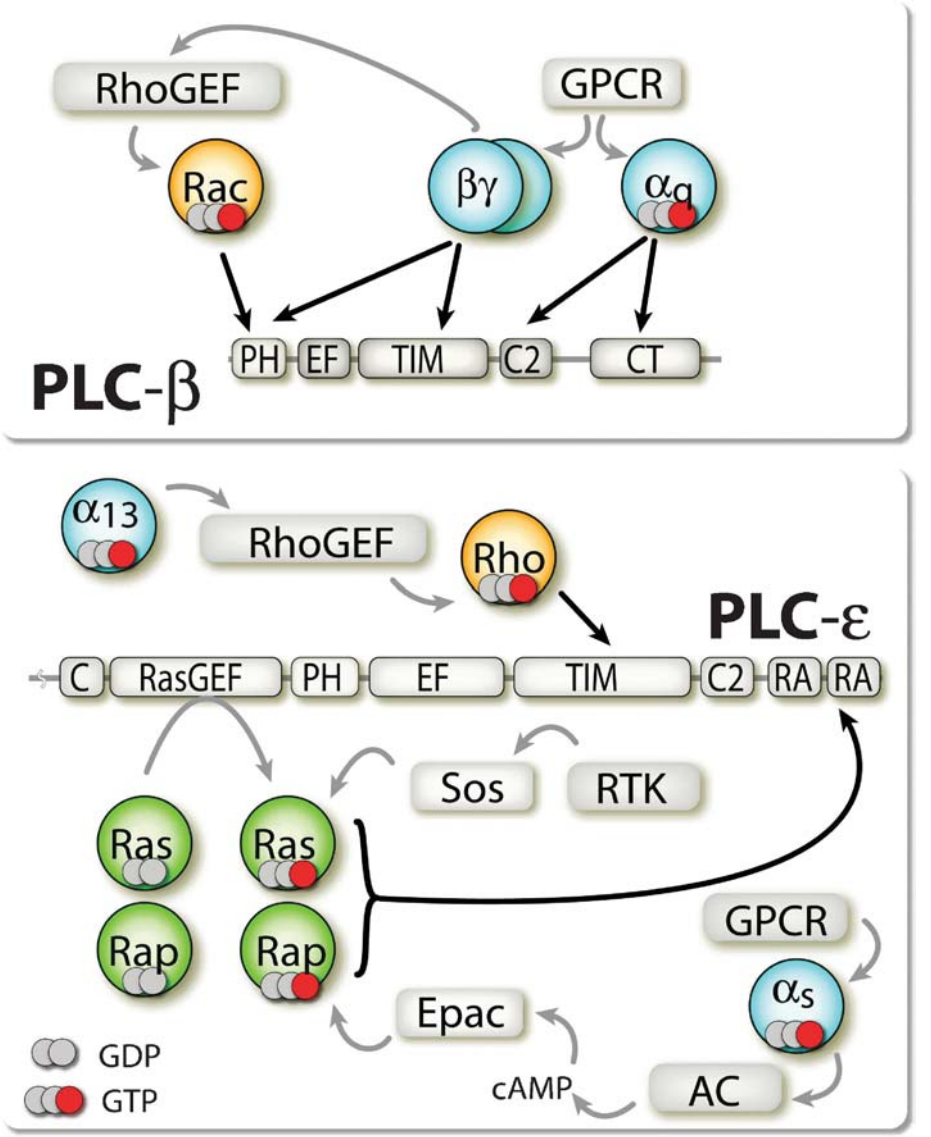

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Figure 4 PLC isozymes integrate multiple inputs from heterotrimeric G proteins and small GTPases. PLC-β isozymes (*top panel*) are differentially regulated by direct interaction with both Gβγ and GTP-bound Gα subunits of the Gq-subfamily of heterotrimeric G proteins. PLC-β2 and -β3 are also activated by GTP-bound Rac isozymes. Upstream, heterotrimeric G proteins are activated by GPCRs; the equivalent guanine nucleotide exchange factors for Rho-family GTPases such as Rac1 are RhoGEFs. Certain RhoGEFs, such as the P-Rex isozymes are also responsive to Gβγ, allowing for direct crosstalk between the two classes of GTPases prior to their activation of PLC-β isozymes. This crosstalk most likely has biological consequences for controlling events downstream of PLC-β activation. Similar crosstalk is focused through PLC-ε. More specifically, PLC-ε possesses a RasGEF domain that activates Ras and Rap isozymes; these activated GTPases directly stimulate the phospholipase activity of PLC-ε through direct binding to the RA domains in a classical feed-forward loop. Ras is also activated downstream of receptor tyrosine kinases, whereas Rap isozymes are activated downstream of GPCRs. The phospholipase activity of PLC-ε is also enhanced through the direct interaction with GTP-RhoA. Certain RhoGEFs that activate RhoA, such as p115-RhoGEF, are responsive to Gα12 and 13.

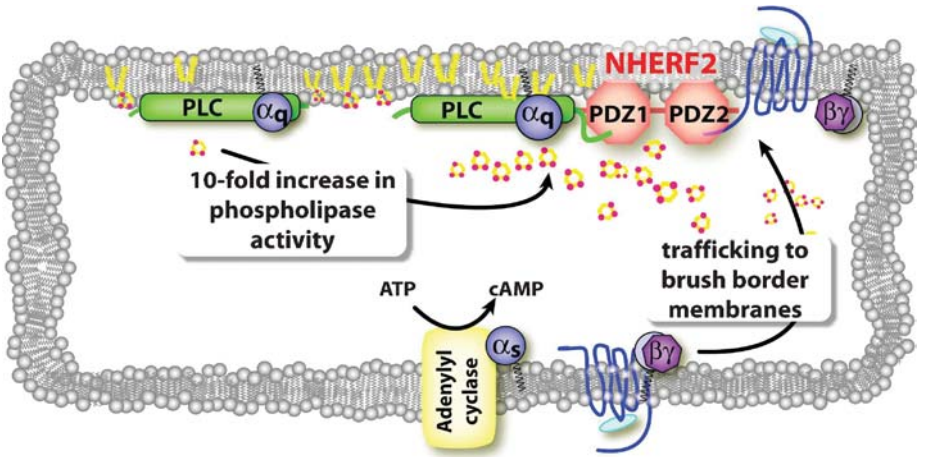

Figure 5 Supramolecular complexes mediated by scaffolding proteins localize PLCs and increase their efficiency within signaling cascades. In this example, the PDZ-1 and -2 domains of the scaffolding protein NHERF1 bind the immediate C-terminal regions of PLC-β1 and PTH1R, respectively, to form a supramolecular complex that directs PTH1R to brush border membranes in renal proximal tubules. The resulting complex is required to link agonist-mediated activation of PTH1R with the stimulation of PLC-β1 through Gαq.

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