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REGULATION OF PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C*

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■ **Abstract** Eleven distinct isoforms of phosphoinositide-specific phospholipase C (PLC), which are grouped into four subfamilies (β, γ, δ, and ε), have been identified in mammals. These isozymes catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to inositol 1,4,5-trisphosphate and diacylglycerol in response to the activation of more than 100 different cell surface receptors. All PLC isoforms contain X and Y domains, which form the catalytic core, as well as various combinations of regulatory domains that are common to many other signaling proteins. These regulatory domains serve to target PLC isozymes to the vicinity of their substrate or activators through protein-protein or protein-lipid interactions. These domains (with their binding partners in parentheses or brackets) include the pleckstrin homology (PH) domain [PtdIns(3)P, $\beta\gamma$ subunits of G proteins] and the COOH-terminal region including the C2 domain (GTP-bound α subunit of G_q) of PLC-β; the PH domain [PtdIns(3,4,5)P_3] and Src homology 2 domain [tyrosine-phosphorylated proteins, PtdIns(3,4,5)P_3] of PLC-γ; the PH domain [PtdIns(4,5)P_2] and C2 domain (Ca^{2+}) of PLC-δ; and the Ras binding domain (GTP-bound Ras) of PLC-ε. The presence of distinct regulatory domains in PLC isoforms renders them susceptible to different modes of activation. Given that the partners that interact with these regulatory domains of PLC isozymes are generated or eliminated in specific regions of the cell in response to changes in receptor status, the activation and deactivation of each PLC isoform are likely highly regulated processes.

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INTRODUCTION

The cellular responses elicited by the interaction of many extracellular signaling molecules with their cell surface receptors are triggered by the rapid hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. This reaction is catalyzed by phosphoinositide-specific phospholipase C (PLC) isozymes and results in the generation of two intracellular messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]. These messengers then promote the activation of protein kinase C and the release of Ca^{2+} from intracellular stores, respectively. Ins(1,4,5)P_3 is further converted by the actions of several distinct kinases and phosphatases to a variety of inositol phosphates, some of which are also implicated in intracellular signaling.

PtdIns(4,5)P_2 is a precursor not only of Ins(1,4,5)P_3 and DAG but also of PtdIns(3,4,5)P_3, which is produced by the action of PtdIns 3-kinase (1). PtdIns-(3,4,5)P_3 accumulates in the plasma membrane of cells that have been activated by the interaction of ligands with protein tyrosine kinase (PTK)– or G protein–coupled receptors, and it modulates the activities of various effector molecules such as the small GTP-binding protein Rac and the serine-threonine kinase known as protein kinase B or Akt (1). This phospholipid also appears to function in the Ca^{2+} signaling pathway triggered by activation of PLC-γ isozymes (see below). PtdIns(4,5)P_2 also directly modulates the activities of many enzymes, including protein kinase C, phospholipase D (PLD), and phospholipase A_2 (PLA_2) (2, 3), as well as regulates actin polymerization-depolymerization through interaction with actin regulatory proteins (4). Finally, PtdIns(4,5)P_2 binds to pleckstrin homology (PH) domains and thus serves as a membrane docking site for a host of PH domain–containing proteins (5).

Consistent with the important roles of PtdIns(4,5)P_2 and its derivatives in intracellular signaling, the conversion of this lipid to Ins(1,4,5)P_3 and DAG and to PtdIns(3,4,5)P_3 as well as its synthesis from and degradation to phosphatidylinositol monophosphate is subject to strict regulation (6). The PLC isozymes

responsible for the generation of Ins(1,4,5)P_3 and DAG have been classified on the basis of amino acid sequence into four major types. These groups also differ in the mechanisms by which the isozymes are activated in response to ligand interaction with various receptors. In this review, I focus on recent progress in characterization of the mechanisms that underlie receptor-dependent hydrolysis of PtdIns(4,5)P_2.

STRUCTURAL ORGANIZATION OF PLC ISOZYMES

The PLC family comprises a diverse group of enzymes that differ in structure and tissue distribution. Until recently, 10 mammalian PLC isozymes had been identified and divided into three types: four PLC-β, two PLC-γ, and four PLC-δ proteins (7, 8). An isoform originally termed PLC-α was subsequently shown to be a proteolytic fragment of PLC-δ1 (7). However, the coding sequence for a new type of PLC, termed PLC-ε, was recently identified (9, 10), bringing the total number of mammalian isozymes to 11 (excluding the products of alternative RNA splicing). PLC-ε is a homolog of PLC 210, which was initially identified from a *Caenorhabditis elegans* cDNA library and contains several domains not apparent in any other known PLC isoforms (11).

Whereas the molecular size of the PLC-δ isozymes is only ~85 kilodaltons (kDa), those of the β- and γ-type enzymes are in the range of 120–155 kDa, and that of PLC-ε is 230–260 kDa. Lower eukaryotes such as yeast and slime molds contain only δ-type isozymes, which suggests that the β-, γ-, and ε-type isoforms present in higher eukaryotes evolved from the archetypal PLC-δ. The amino acid sequences of PLC isozymes are relatively nonconserved with the exception of two regions, known as the X and Y domains, that form the catalytic core (7, 8) (Figure 1). The amino acid sequence similarity in the X and Y domains is ~60% among all 11 mammalian isozymes, and it is substantially greater among members of the same PLC type. The sequence between the X and Y domains comprises 40 to 110 residues in the β- and δ-type isozymes and 190 residues in PLC-ε. In γ-type isozymes, this region is much larger (~400 residues) and contains two Src homology 2 (SH2) domains and one SH3 domain, which bind phosphotyrosine-containing sequences and proline-rich sequences, respectively.

The β-, γ-, and δ-type isozymes all contain an NH_2-terminal PH domain, an ~100-residue module that is present in many signaling proteins and that binds to polyphosphoinositides [PtdIns(3)P, PtdIns(4,5)P_2, PtdIns(3,4)P_2, and PtdIns-(3,4,5)P_3] and Ins(1,4,5)P_3 (1, 5). Determination of the three-dimensional structure of a PLC-δ1 mutant lacking the PH domain (12) also revealed the presence of two additional modules: an EF-hand domain located between the PH and X domains, and a C2 domain, which is sometimes represented as part of an extended Y domain. Both of these domains are also present in β- and γ-type isozymes, but only the C2 domain is present in PLC-ε. PLC-γ isozymes contain an additional PH domain that is split by the SH domains. Furthermore, β-type isozymes

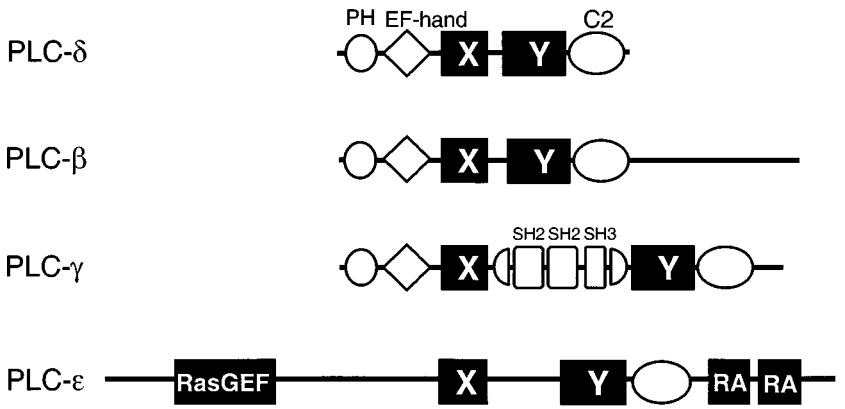

Figure 1 Domain organization of the four types of PLC isozymes. The X and Y catalytic domains as well as the PH, EF-hand, C2, SH2, SH3, RasGEF, and RA domains are indicated.

(with the exception of a splice variant of PLC-β4) contain a long COOH-terminal sequence (~450 residues) downstream of the Y domain, whereas this region is almost nonexistent or short in γ- and δ-type isozymes. PLC-ε differs from the other three types of isozymes in that it does not contain a PH domain but possesses an NH_2-terminal Ras guanine nucleotide exchange factor (RasGEF)–like domain and at least one and perhaps two COOH-terminal Ras binding (RA) domains (9, 10). The RasGEF-like and RA domains are also present in PLC-210 of *C. elegans* (11).

Several mammalian PLC isoforms exist in splice variants. The primary transcripts of the PLC-β1 and PLC-β4 genes are alternatively spliced in the region corresponding to the COOH-terminal portion of the proteins downstream of the C2 domain (13, 14). Three splice variants (ALT I, II, and III) of PLC-δ4 have been identified (15, 16). Whereas ALT I and ALT II each contain additional amino acids between their X and Y domains, the difference in ALT III occurs within the X domain and the protein is consequently catalytically inactive. The PH domain of ALT III binds PtdIns(4,5)P_2 with high affinity, and this protein has been shown to modulate PLC-δ activity by competing for PtdIns(4,5)P_2 with the PH domains of other PLC-δ isozymes (see below).

Another type of catalytically inactive PLC-like protein, p130, was identified as an Ins(1,4,5)P_3-binding protein (17). This protein, also known as PLC-L, contains all of the domains present in the δ-type isozymes but is catalytically inactive because it lacks several critical conserved amino acids in the X domain. Because the PH domain of p130 binds Ins(1,4,5)P_3 with high affinity but does not interact with PtdIns(4,5)P_2, this protein does not affect PtdIns(4,5)P_2 hydrolysis catalyzed by other PLC isozymes but rather inhibits agonist-induced mobilization of intracellular Ca^{2+} by competing for Ins(1,4,5)P_3 with the Ins(1,4,5)P_3 receptor (17).

STRUCTURE AND REACTION MECHANISM OF PLC

The three-dimensional structures both of a PLC-δ1 mutant lacking the PH domain (12) and of the PH domain of this isozyme (18) have been determined. Together, these structures revealed a four-module organization of the enzyme consisting of the PH domain, the EF-hand domain, the catalytic domain (comprising tightly associated X and Y domains), and the C2 domain (19). The PH domain of PLC-δ1 binds to Ins(1,4,5)P_3 and to PtdIns(4,5)P_2 (20, 21). The binding of PLC-δ1 to its lipid substrate and, consequently, its catalytic activity are dependent on the concentration of PtdIns(4,5)P_2, and the catalytic activity of this isozyme is inhibited by Ins(1,4,5)P_3.

The crystal structure of the PH domain of rat PLC-δ1 complexed with Ins(1,4,5)-P_3 revealed that the 4- and 5-phosphoryl groups of Ins(1,4,5)P_3 interact with the side chains of Lys32 and Lys57 and with those of Lys30, Arg40, and Lys57, respectively, thus explaining the mechanism by which these phosphoryl groups contribute to high-affinity binding (18). As would be expected on the basis of this structural determination, replacement of any of these basic amino acids in PLC-δ1 reduced both the extent of the interaction of the enzyme with PtdIns(4,5)P_2-containing lipid vesicles and its catalytic activity. The PH domain of PLC-δ1 was thus proposed to tether the enzyme to the cell membrane by specific binding to PtdIns(4,5)P_2, thereby allowing it to catalyze the hydrolysis of many substrate molecules without dissociating from the lipid surface, a process referred to as processive catalysis (12). Because the PH domain of PLC-δ1 also binds Ins(1,4,5)P_3, this latter molecule may function as a feedback regulator of catalysis.

Although PLC-β and PLC-γ isozymes also possess a PH domain at their NH_2-termini, the basic amino acids (Lys30, Lys32, Arg40, and Lys57) located inside the inositol phosphate binding pocket of PLC-δ1 are not well conserved in these proteins. Consistent with this sequence difference, the NH_2-terminal PH domains of PLC-γ isozymes bind to PtdIns(3,4,5)P_3 but not to PtdIns(4,5)P_2 (22), which suggests the possibility of recruitment of these isozymes by PtdIns(3,4,5)P_3 (see below). The PH domains of PLC-β isozymes associate with phospholipid bilayers; however, the binding affinity is not affected by the presence or absence of PtdIns(4,5)P_2, which suggests that this lipid does not recruit PLC-β isozymes to the cell membrane (23). The PH domain of PLC-β1 has recently been shown to specifically bind PtdIns(3)P, and this interaction appears to be responsible for the membrane recruitment of this isozyme in cells in which PtdIns 3-kinase is activated (22). It therefore appears that the PH domains of the different types of PLC isozymes interact with distinct types of inositol-containing lipid: PtdIns(3)P for PLC-β, PtdIns(4,5)P_2 for PLC-δ, and PtdIns(3,4,5)P_3 for PLC-γ. Furthermore, the PH domains of PLC-β1 and PLC-β2 appear to be the sites of interaction of these enzymes with the $\beta\gamma$ subunits of G proteins (24). It has been suggested that PLC-β is recruited to the membrane surface by the cooperative action of PtdIns(3)P and G$\beta\gamma$ subunits, which bind to separate sites within the PH domain (see below).

All four types of PLC require Ca^{2+} for catalytic function; the δ-type isozymes are most sensitive to this cation. Structural and mutational studies have identified residues within the catalytic domain that likely contribute to substrate recognition, Ca^{2+} binding, and catalysis (25) (Figure 2). Measurement of the activities of mutant rat PLC-δ1 enzymes with PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 showed that Lys438, Ser522, and Arg549 are important for preferential hydrolysis of polyphosphoinositides, whereas replacement of Lys440 selectively affected hydrolysis of only PtdIns(4,5)P_2; these observations implicate Lys438, Ser522, and Arg549 in interactions with the 4-phosphate and Lys440 in interactions with the 5-phosphate (25). These four amino acid residues are conserved in PLC-β and PLC-γ isozymes. The crystal structures of complexes of PLC-δ1 with Ca^{2+} and Ins(1,4,5)P_3 reveal complex interactions of Ca^{2+} with several negatively charged residues (Glu3441, Asp343, and Glu390) and Asn^{312} in the active site as well as with the 2-hydroxyl

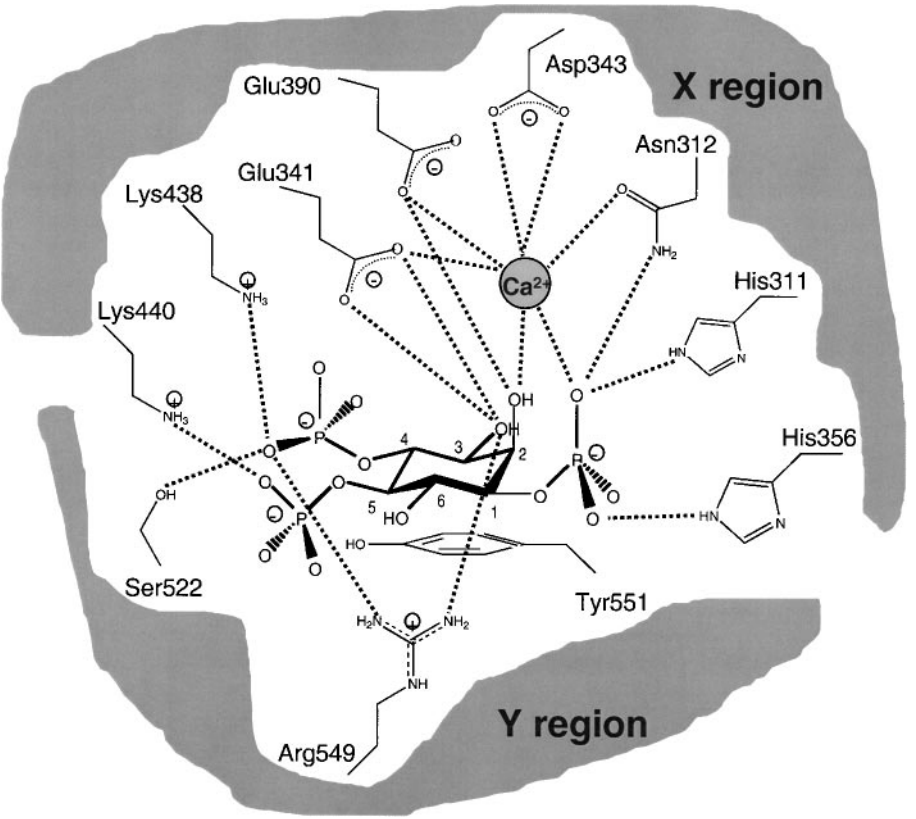

Figure 2 Schematic representation of the catalytic domain of rat PLC-δ1. Side chains of amino acid residues that contact either Ca^{2+} or hydroxyl or phosphate moieties of Ins(1,4,5)P_3 are indicated. (Adapted from Reference 12.)

group of Ins(1,4,5)P_3 (12, 26). The importance of all four of these amino acid residues in coordination of Ca^{2+} was demonstrated by analysis of the PLC activity of the respective point mutants at various concentrations of Ca^{2+} (25).

Hydrolysis of phosphoinositides by mammalian PLC enzymes produces cyclic inositol phosphates as minor products. These enzymes catalyze the conversion of PtdIns to inositol phosphate with overall retention of the stereochemical configuration at the phosphorus atom (27). Thus, a sequential reaction mechanism has been proposed that involves general acid-base catalysis with formation of cyclic inositol phosphate in a phosphotransfer step, followed by conversion of the cyclic molecule to an acyclic inositol phosphate in a phosphohydrolysis step (28). Structural and mutational studies indicate that His311 and His356 of rat PLC-δ1 act as general acid-base catalysts (29, 30). In addition, Glu341 and Glu390, both of which interact with Ca^{2+}, also form hydrogen bonds with the 2-hydroxyl group of inositol to facilitate the phosphotransfer step (25). Tyr551 has also been implicated in hydrophobic interaction with the inositol ring (12). Each of the three loops that form the rim of the entrance to the active site contains a hydrophobic residue (Leu320, Phe360, and Trp555) at the rim (12). Mutational analysis indicates that this hydrophobic ridge provides a membrane interaction site that is necessary for penetration of the catalytic domain into a phospholipid membrane (25).

The C2 domains of PLC isozymes comprise ~120 residues, and such domains are present in many proteins that interact with lipid membranes. Many, but not all, C2 domains bind Ca^{2+} and mediate Ca^{2+}-dependent interaction of proteins with phospholipids. The C2 domain of PLC-δ1 has been estimated to contain three to four Ca^{2+} binding sites (31). Deletion analysis of PLC-δ1 indicates that the C2 domain is important for activity, consistent with the previous suggestion that calcium ions bound to the C2 domain of PLC function as bridging elements in the interaction of the enzyme with acidic phospholipids (12). Calcium ions bound to the C2 domain of PLC-δ1 were recently shown to enhance enzyme activity by promoting the formation of an enzyme-phosphatidylserine-Ca^{2+} ternary complex, thereby increasing the affinity of the enzyme for substrate vesicles (33). In contrast, the C2 domains of PLC-β isozymes exhibit no apparent affinity for membrane bilayers in the presence of Ca^{2+}, but they do interact with activated α subunits of G_q proteins (34) (see below).

The role of the EF-hand domains of PLC isozymes is not clear. Such domains are also Ca^{2+} binding motifs that usually each bind one calcium ion, although naturally occurring variants that do not bind Ca^{2+} have been identified. Indeed, crystallographic data suggest that the EF-hand domain of PLC-δ1 does not bind Ca^{2+} but rather serves as a flexible link between the PH domain and the rest of the enzyme, thus allowing the C2 and catalytic domains to interact with the membrane after binding of the PH domain to PtdIns(4,5)P_2 (25, 26). However, more recent evidence indicates that the EF-hand domain of PLC-δ1 does bind Ca^{2+} and that the bound Ca^{2+} is necessary for the efficient interaction of the PH domain with PtdIns(4,5)P_2 (35).

ACTIVATION OF PLC-β ISOZYMES BY HETEROTRIMERIC G PROTEINS

Activation of PLC-β by $G_q\alpha$ Subunits

Heterotrimeric G proteins consist of α, β, and γ subunits that are stably associated in the inactive, GDP-bound state. Physical interaction between a G protein and an agonist-occupied receptor triggers the exchange of GDP for GTP on the α subunit and the subsequent dissociation of this subunit from the tightly associated $\beta\gamma$ dimer. Both the Gα-GTP and G$\beta\gamma$ entities participate in intracellular signaling (Figure 3). The intrinsic GTPase activity of the Gα subunit mediates

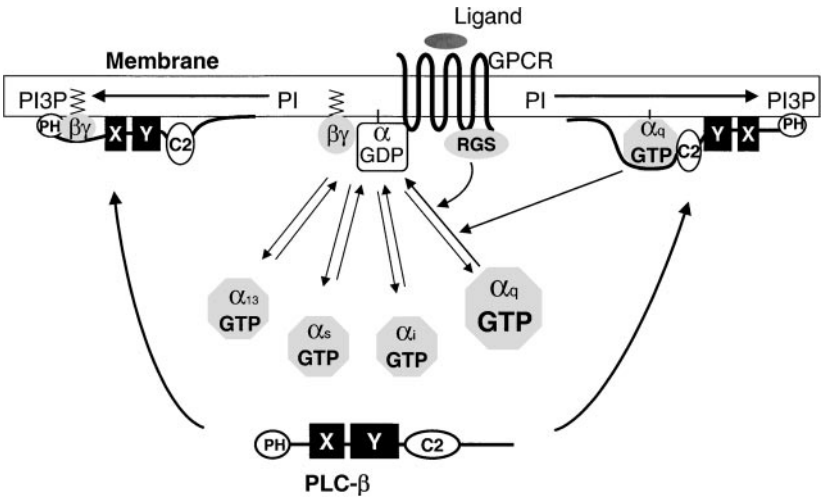

Figure 3 GPCR-mediated activation of PLC-β isozymes. An agonist-occupied GPCR induces the exchange of GDP for GTP on the G protein α subunit and the subsequent dissociation of this subunit from the membrane-associated G$\beta\gamma$ dimer. In contrast to the depiction in the middle portion of the figure, the dissociated Gα subunits remain localized at the membrane as a result of their covalent lipid modification. The GTP-bound α subunits of the G_q subfamily mediate the targeting of PLC-β to the membrane and activate it as a result of interaction with the C2 domain and downstream COOH-terminal region of the enzyme (*right*). The membrane localization of PLC-β isozymes might be further promoted by interaction of the PH domain with PtdIns(3)P (PI3P) generated in response to ligation of the GPCR. Hydrolysis of GTP to GDP at the active site of Gα subunits by their intrinsic GTPase activity results in the reassociation of α with the $\beta\gamma$ subunits and reformation of the inactive heterotrimer. RGS proteins and the COOH-terminal region of PLC-β stimulate the GTPase activity of $G_q\alpha$, leading to inhibition of the GPCR signal. The membrane-anchored G$\beta\gamma$ dimer also recruits PLC-β by interacting with the PH and Y domains (interaction with the Y domain is not shown) (*left*); this recruitment is also aided by the presence of PtdIns(3)P in the membrane. The EF-hand domain of PLC-β is not shown.

the rate-limiting hydrolysis of GTP to GDP and results in the reassociation of this subunit with the $G\beta\gamma$ complex and consequent inactivation of signaling. On the basis of their amino acid sequences and effector interactions, G protein α subunits have been divided into four subfamilies: $G_s\alpha$, $G_i\alpha$, $G_q\alpha$, and $G_{12}\alpha$. The α subunits (α_q, α_{11}, α_{14}, and α_{16}) of all four members of the G_q subfamily activate PLC-β isozymes but not PLC-γ, PLC-δ, or PLC-ε (9, 36, 37). The four mammalian PLC-β isozymes differ in their tissue distribution as well as in their ability to be activated by G proteins. Expression of PLC-β2 is restricted to hematopoietic cells and that of PLC-β4 is limited to the retina and certain neuronal cells, whereas PLC-β1 and PLC-β3 are expressed more widely. G protein–coupled receptors (GPCRs) that activate the $G_q\alpha$–PLC-β signaling pathway include those for thromboxane A_2, bradykinin, bombesin, angiotensin II, histamine, vasopressin, acetylcholine (muscarinic m1 and m3), α_1-adrenergic agonists, thyroid-stimulating hormone, C-C and C-X-C chemokines, and endothelin-1.

$G\alpha_q$ and $G\alpha_{11}$ subunits activated by the nonhydrolyzable GTP analog guanosine 5′-*O*-(3′-thiotriphosphate) (GTP-γ-S) stimulate PLC-β isoforms according to the rank order PLC-β1 $\geq$ PLC-β3 $>$ PLC-β2 (38, 39), which is consistent with the observation that the affinities of GTP-γ-S-activated $G\alpha_q$ for PLC-β1 and PLC-β3 are similar [dissociation constants (K_d) of 40–60 nM] to each other and substantially higher than that for PLC-β2 (K_d of 400 nM) (40). PLC-β4 is also activated by $G_q\alpha$ subunits; however, because the basal activity of this enzyme is inhibited by ribonucleotides, including GTP-γ-S, accurate determination of the extent of activation is difficult (41). All four members of the $G_q\alpha$ subfamily activate PLC-β1 (42). Furthermore, $G\alpha_{16}$, which is expressed only in hematopoietic cells and is distantly related to the more widely expressed $G\alpha_q$ (amino acid sequence identity of 55%), activates PLC-β1, -β2, and -β3 in a manner essentially indistinguishable from that of $G\alpha_q$ (43). However, certain receptors discriminate among the α subunits of the G_q subfamily. The receptor for macrophage chemotactic protein-1, for example, couples to $G\alpha_{14}$ and $G\alpha_{16}$ but not to $G\alpha_q$ or $G\alpha_{11}$ (44).

The activated $G\alpha_q$ subunit appears to interact with the COOH-terminal region of PLC-β1 downstream of the Y domain; this region contains the C2 domain (residues 663–802) followed by a sequence (residues 803–1216) that is unique to this subfamily of PLC isozymes. The results of deletion analysis suggested that the region downstream of residue 845 is required for binding of and stimulation by $G\alpha_q$ (45, 46). Secondary structure predictions indicate that this region (residues 846–1216) is composed predominantly of α helices, and it contains a high proportion of basic residues that appear in clusters (47). Basic residues in two such clusters (residues 911–928 and 1055–1075) are important for stimulation by $G\alpha_q$ (47). The activated $G\alpha_q$ subunit also interacts with the C2 domains of PLC-β isoforms (34). Unlike the C2 domain of PLC-δ1, which binds Ca^{2+} and serves as a membrane attachment module (33), the C2 domains of PLC-β1 and PLC-β2 do not bind to membranes. However, they each bind GTP-γ-S-activated $G\alpha_q$ with high affinity (K_d, 18 nM); they bind the inactive, GDP-bound $G\alpha_q$ with a much lower affinity (K_d values of 120 and 60 nM for PLC-β1 and -β2, respectively) and do not associate

with the GTP-γ-S-activated $G\alpha_i$ (34). In contrast, the C2 domain of PLC-δ1 does not exhibit any measurable affinity for GTP-γ-S-activated $G\alpha_q$.

Although G protein subunits are not intrinsically hydrophobic, they are bound to the cell membrane as a result, at least in part, of covalent lipid modification. Gγ subunits are prenylated at their COOH termini, a modification that promotes both the association of the $\beta\gamma$ dimer with membranes as well as the interaction of $\beta\gamma$ with Gα subunits and effectors. Activated, GTP-bound $G_q\alpha$ subunits remain attached to the cell membrane even after separation from the $\beta\gamma$ complex; this association is attributable to the fact that their NH_2-terminal regions are hydrophobic and contain two cysteine residues that are palmitoylated (48). The membrane-embedded $G_q\alpha$ subunits are likely recognized by PLC-β isozymes that are loosely associated with the cell membrane as a result of the interaction of their COOH-terminal basic residues with acidic phospholipids (47) (Figure 3).

The membrane localization of PLC-β isozymes is likely further promoted by the presence of PtdIns(3)P, which binds to the PH domain of PLC-β1 (Figure 3). The evidence for a specific interaction of the PH domain of PLC-β1 with PtdIns(3)P has been provided by direct lipid binding studies and by the observation that the lysophosphatidic acid–induced membrane localization of a fusion construct containing green fluorescent protein and the PH domain of PLC-β1 is prevented by a PtdIns 3-kinase inhibitor (22). The amount of PtdIns(3)P is low in mammalian cells and does not change markedly in response to receptor activation. It is therefore possible that PtdIns(3)P contributes to the membrane association of PLC-β1 only under specific circumstances. Nevertheless, treatment of cells with a PtdIns 3-kinase inhibitor results in a rapid reduction in the abundance of PtdIns(3)P, which is indicative both of rapid turnover of this lipid and of a role for PtdIns 3-kinase in its production. PtdIns 3-kinase isoforms containing a p110β or p110γ catalytic subunit are activated by G$\beta\gamma$ (49). Given that G$\beta\gamma$ subunits also bind to the PH domains of PLC-β isozymes, this interaction may contribute to the membrane localization of these isozymes (see below).

PLC-β isozymes also might be targeted to the membrane environment through interaction with adapter proteins known as Na^+/K^+ exchanger regulatory factors (NHERFs) (50). These proteins contain two PDZ domains, one of which interacts with receptors such as the β_2-adrenergic receptor and purinergic P_2Y1 receptor. Interaction with a PDZ domain is mediated through a COOH-terminal sequence, (Ser/Thr)-X-(Val/Leu)-COOH, of PDZ binding proteins. All four PLC-β isozymes contain this PDZ binding motif. PLC-β3 binds specifically to NHERF2, and expression of NHERF2 in cells of the COS line potentiated the activation of PLC-β by carbachol (50).

Activation of PLC-β by G$\beta\gamma$ Subunits

With the exception of PLC-β4, PLC-β isozymes are also activated by G$\beta\gamma$ dimers (39, 41, 51, 52) (Figure 3). The relative sensitivity of PLC-β isozymes to G$\beta\gamma$

subunits differs from that to $G_q\alpha$ subunits; PLC-β1 is the least sensitive to $G\beta\gamma$ (39, 52). Direct binding measurements indicate that, although the $G\beta\gamma$ dimer interacts with PLC-β1, -β2, and -β3, it exhibits a high affinity only for PLC-β2 (40). The activation of PLC-β2 by $G\beta\gamma$ in response to ligation of the luteinizing hormone receptor, V2 vasopressin receptor, β_1- and β_2-adrenergic receptors, m2 muscarinic acetylcholine receptor, and the receptors for the chemoattractants interleukin-8, formyl-Met-Leu-Phe, and complement factor 5a has been demonstrated in transfected COS cells (53). Some of these receptors also stimulate PLC-β through $G_q\alpha$ subunits. Although the concentrations of $G\beta\gamma$ required for maximal activation of PLC-β isoforms in vitro are much larger than the effective concentrations of $G_q\alpha$ subunits, the maximal extents of activation are similar. Thus, both $G_q\alpha$ and $G\beta\gamma$ subunits likely contribute to activation of PLC-β in cells. It was suggested that $G\beta\gamma$ is the predominant mediator of PLC-β activation and that the role of $G_q\alpha$ subunits is to specify the receptors that couple to the enzyme (54). However, a variety of physiological stimuli that activate PLC-β isozymes in normal platelets were not able to activate these isozymes in platelets derived from α_q knockout mice; α_q is the only member of the $G_q\alpha$ subfamily expressed in normal platelets (55). Thus, $G\alpha_q$ appears essential for PLC-β activation and cannot be replaced by $G\beta\gamma$ in this regard.

The region of PLC-β that interacts with $G_q\alpha$ subunits differs from that responsible for interaction with $G\beta\gamma$. Thus, COOH-terminal truncation of PLC-β2 generated enzymes that were activated by $G\beta\gamma$ but not by $G\alpha_q$ (56). The PH domain of PLC-β2 exhibits high affinity for $G\beta\gamma$ subunits bound to membranes (23). A chimeric PLC-δ1 molecule in which the PH domain of this isozyme was replaced with that of PLC-β2 was as sensitive to activation by $G\beta\gamma$ as was intact PLC-β2, whereas native PLC-δ1 is not responsive to $G\beta\gamma$ subunits (24). Furthermore, $G\beta\gamma$ induced the membrane translocation of a fusion protein containing green fluorescent protein and the PH domain of PLC-β1 by a mechanism that also required the production of PtdIns(3)P (22), which suggests that $G\beta\gamma$ and PtdIns(3)P bind to different regions of the PH domain. $G\beta\gamma$ subunits appear to interact with more than one region of PLC-β, however, given that the site of interaction of PLC-β2 with $G\beta\gamma$ was also mapped to the sequence that spans residues Glu574 and Lys583, located in the Y region (57). The region of $G\beta$ subunits that interacts with effector molecules such as PLC-β and adenylate cyclase overlaps with that responsible for binding to $G\alpha$ subunits, which explains why the $\beta\gamma$ complex is able to interact with $G\alpha$ or with effectors but not with both simultaneously (58). PLC-β2 actually contacts multiple sites within $G\beta$.

Mammalian cDNAs that encode 7 distinct $G\beta$ subunits and 12 $G\gamma$ subunits have been isolated. Although some of these subunits are expressed only in specific tissues and selectivity is apparent in the interaction of $G\beta$ and $G\gamma$ subunits, the formation of many different $G\beta\gamma$ dimers is possible. Among several such combinations of $G\beta$ and $G\gamma$ subunits tested, all except $\beta_1\gamma_1$ activated purified PLC-β3 with similar potencies (59). In cells, however, not all available $G_q\alpha$ subunits and $G\beta\gamma$ combinations appear to function in activation of PLC. The results of

experiments with antisense oligonucleotides directed to mRNAs encoding various G protein subunits suggested that the m1 muscarinic acetylcholine receptor interacts only with G protein complexes composed of the subunits α_q, α_{11}, β_1, β_4, and γ_4 in order to activate PLC in RBL-2H3 cells, despite the fact that the subunits α_{14}, β_2, β_3, γ_2, γ_3, γ_5, and γ_7 are also expressed in these cells (60).

The G protein β_5 subunit differs from the other β subunits in that it shares only 53% amino acid sequence identity with these other highly conserved β isoforms, and in that when complexed with Gγ, it appears to interact selectively with Gα_q-coupled receptors as a result of its specific association with Gα_q (61). Furthermore, the β_5 subunit discriminates among PLC-β isozymes independently of Gα_q. Thus, when recombinant G$\beta_5\gamma$ and G$\beta_1\gamma$ dimers were tested for their ability to activate PLC-β1, PLC-β2, and PLC-β3, G$\beta_1\gamma$ activated all three PLC-β isozymes whereas G$\beta_5\gamma$ activated PLC-β1 and PLC-β2 but not PLC-β3 (62).

Deactivation of PLC-β Signaling

G protein–initiated signaling is turned off by hydrolysis of the GTP bound to the Gα subunit, a reaction catalyzed by the intrinsic GTPase activity of the α subunit itself, and the subsequent reassociation of the GDP-bound α subunit with the $\beta\gamma$ subunits. This deactivation process was studied in detail by reconstituting the m1 muscarinic acetylcholine receptor, G protein, and PLC-β1 in lipid vesicles (63). The muscarinic agonist carbachol induced a 90-fold increase in PLC activity in the presence of the $G_q\alpha$ subfamily (63). Although the intrinsic GTPase activity of purified Gα_q was low ($\sim$0.8 min^{-1}), the presence of PLC-β1 induced a $>$50-fold increase in this activity; that is, PLC-β1 is a GTPase-activating protein (GAP) for Gα_q. In the reconstituted system, PLC-β1 also increased the rate of GTP hydrolysis by Gα_q by a factor of up to 60 in the presence of carbachol, which alone stimulated GTPase activity by a factor of 6 to 10. More recent kinetic analysis suggests that PLC-β acts directly on Gα_q to stimulate hydrolysis of bound GTP, and that the other components of the reconstituted system (the m1 muscarinic receptor, G$\beta\gamma$, and phospholipids) promote only GDP-GTP exchange during steady-state hydrolysis (64). A fragment of the COOH-terminal region of PLC-β1 (residues 903–1042) has also been shown to exhibit GAP activity (65). These results indicate that the receptor and PLC-β1 coordinately regulate the amplitude of the PLC signal and the rate of signal termination (Figure 3).

The GTPase activity of Gα_q is also stimulated by a family of regulatory proteins termed RGS (regulators of G protein signaling). Members of this protein family were first identified genetically as negative regulators of G protein signaling in lower eukaryotes, but more than 20 mammalian isoforms have been identified to date (66). Biochemical evidence indicates that RGS proteins block G protein function predominantly by acting as GAPs to limit the lifetime of the active, Gα_q-GTP complex, resulting in inhibition of PLC-β activity (67–69) (Figure 3). RGS proteins exhibit GAP activity toward G_i and G_q class α subunits but not toward $G_s\alpha$ or $G_{12}\alpha$ subunits (67, 68), and the extent of the RGS protein effect depends on

the interaction of these proteins with the receptor rather than on their interaction with a specific Gα subunit (69).

PLC-β Signaling in the Nucleus

PLC signaling occurs not only at the plasma membrane but also in the nucleus (70, 71). PLC-β1 is the major PLC isoform in the nucleus of various cells (72, 73), and the COOH-terminal region of the protein is required for nuclear localization (47). The amount of nuclear PLC-β1 protein, which appears to be activated independently of its counterpart at the plasma membrane by an unknown mechanism, increases during cell growth and decreases during differentiation (74). The changes in the abundance of nuclear PLC-β1 correlate with those in the amount of PtdIns(4,5)P_2 hydrolyzed in the nucleus (70). However, the roles of the DAG and Ins(1,4,5)P_3 so generated remain unclear. Translocation of protein kinase C to the nucleus has been demonstrated coincident with the generation of DAG in this organelle. Analysis of cells lacking PLC-β1 as a result of gene ablation revealed that this isozyme is essential for the onset of DNA synthesis in response to insulin-like growth factor I (74).

ACTIVATION OF PLC-γ ISOZYMES

Activation of PLC-γ by Receptor Protein Tyrosine Kinases

Polypeptide growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF), vascular endothelial growth factor, glial cell–derived growth factor, and hepatocyte growth factor, induce PtdIns(4,5)P_2 turnover by activating PLC-γ1 in a wide variety of cells (53). However, insulin and colony-stimulating factor-1, whose receptors are structurally similar to those of PLC-activating growth factors, generally do not activate PLC-γ1 (75, 76).

The binding of a growth factor to its receptor results in dimerization of receptor subunits, stimulation of the intrinsic tyrosine kinase activity of the receptor, and autophosphorylation of the receptor on specific tyrosine residues. These phosphorylated residues initiate cellular signaling by acting as high-affinity binding sites for the SH2 domains of various effector proteins. The selectivity of the receptor-effector interaction is determined not only by the residues surrounding the phosphorylated tyrosine of the receptor but also by the structure of the SH2 domain of the effector. In the PDGF receptor (β chain), for example, eight autophosphorylation sites have been identified that mediate the specific binding of Src family tyrosine kinases (Tyr579 and Tyr581), growth factor receptor–bound 2 (Grb2) (Tyr716), the 85-kDa subunit of PtdIns 3-kinase (Tyr740 and Tyr751), Ras GAP (Tyr771), SH2 domain–containing protein tyrosine phosphatase 2 (SH-PTP2) (Tyr1009), and PLC-γ1 (Tyr1021) (77). In addition, Tyr766 of the FGF receptor and Tyr785 of the NGF receptor (Trk) specifically bind PLC-γ1 (78, 79).

However, in the case of the EGF receptor, which contains five autophosphorylation sites located in the COOH-terminal region, such individual sites are not strictly required for the recognition of and association with different SH2 domain–containing proteins. Thus, PLC-γ1 is able to bind to any of these five phosphorylated tyrosine residues (80, 81).

The individual roles of the two SH2 domains of PLC-γ1 in interaction of the enzyme with the PDGF receptor were investigated by functional inactivation of each domain and expression of the mutant proteins in a PLC-γ1-deficient fibroblast cell line (82, 83). The mutant protein in which the COOH-terminal SH2 (C-SH2) domain was disabled was bound to and phosphorylated by the PDGF receptor, and it catalyzed the production of inositol phosphates to an extent only slightly less than that observed with the wild-type enzyme. In contrast, the mutant in which the NH_2-terminal SH2 (N-SH2) domain was impaired did not bind to the PDGF receptor and consequently was neither phosphorylated nor activated. These results suggest that the N-SH2 domain, but not the C-SH2 domain, of PLC-γ1 is required for PDGF-induced enzyme activation.

Phosphorylation of PLC-γ1 by PDGF, EGF, FGF, and NGF receptors occurs at identical sites: tyrosine residues 771, 783, and 1254 (84). The role of tyrosine phosphorylation of PLC-γ1 was investigated by substituting phenylalanine for tyrosine at each of these three sites and expressing the mutant enzymes in cells of the NIH 3T3 line (85). Replacement of Tyr783 with Phe prevented the activation of PLC by PDGF; however, like the wild-type enzyme, the mutant PLC-γ1 associated with the PDGF receptor. Although mutation of specific autophosphorylation sites of various receptors—including Tyr1021 in the PDGF receptor (β chain) (86), Tyr766 in the FGF receptor (87), and Tyr785 in the NGF receptor (88) as well as Tyr1169 in the vascular endothelial growth factor receptor (Flt) (89), Tyr1015 in the rat glial cell–derived growth factor receptor (Ret) (90), and Tyr1356 of the hepatocyte growth factor receptor (Met) (91)—prevented receptor association with PLC-γ1 and growth factor–induced production of Ins(1,4,5)P_3, the mutant receptors still mediated growth factor–dependent tyrosine phosphorylation of PLC-γ1. Thus, the growth factor–induced activation of PLC-γ1 requires not only PLC-γ1 tyrosine phosphorylation but also the association of the enzyme with the growth factor receptor. Such receptor association is likely required to target the tyrosine-phosphorylated (activated) enzyme to the membrane environment, where its substrate molecules reside.

PDGF-induced generation of PtdIns(3,4,5)P_3 also appears to contribute to the translocation of PLC-γ1 to the plasma membrane, resulting in more efficient hydrolysis of PtdIns(4,5)P_2 (92–94). Thus, prevention of PtdIns(3,4,5)P_3 generation in PDGF-stimulated NIH 3T3 cells by exposure to a specific inhibitor of PtdIns 3-kinase (wortmannin or LY294002) resulted in an $\sim$40% decrease in both intracellular generation of Ins(1,4,5)P_3 and the release of intracellular Ca^{2+} (93). Given that PtdIns(3,4,5)P_3 binds to both the NH_2-terminal PH domain (92) and the C-SH2 domain of PLC-γ1 (93, 94), both of these domains likely contribute to the PtdIns(3,4,5)P_3-dependent translocation of the enzyme to the membrane.

A model depicting these various steps in the activation of PLC-γ by growth factors is depicted in Figure 4. Growth factor stimulation thus triggers autophosphorylation of the receptor protein tyrosine kinase (PTK) on tyrosine residues, which provides a docking site for the N-SH2 domain of PLC-γ1 and thereby allows the receptor PTK to catalyze the phosphorylation of the bound enzyme. In addition, interaction with the receptor promotes the targeting of PLC-γ1 to its PtdIns(4,5)P_2 substrate molecules in the membrane. Membrane association is also facilitated by interaction of the PH and C-SH2 domains of PLC-γ1 with PtdIns(3,4,5)P_3 molecules in the membrane that are produced in response to growth factor stimulation. Although Figure 4 shows phosphorylated PLC-γ located in the vicinity of its substrate as a result of simultaneous association with the receptor PTK and PtdIns(3,4,5)P_3, it is also possible that the binding of the N-SH2 domain to the receptor and that of the C-SH2 or PH domain to PtdIns(3,4,5)P_3 constitute distinct means of targeting PLC-γ1 to the membrane. Little is known of the roles of the EF-hand, SH3, and C2 domains in receptor-mediated activation of PLC-γ. The SH3 domain of PLC-γ1 has been shown to bind to and stimulate the guanine nucleotide exchange activity of the protein SOS1 (95).

Autophosphorylation of growth factor receptors and subsequent tyrosine phosphorylation of substrate proteins, including PLC-γ1, both require H_2O_2, whose concentration increases transiently and which appears to function as an intracellular messenger in growth factor–stimulated cells (96). In addition to the activation of a receptor PTK by the binding of a growth factor, concurrent inhibition of protein

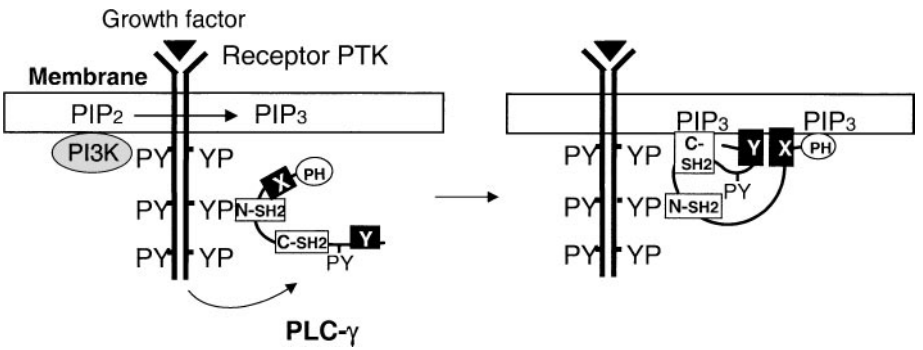

Figure 4 Growth factor receptor–mediated activation of PLC-γ. (*Left*) Growth factor stimulation triggers autophosphorylation of the receptor PTK on tyrosine residues, which then function as docking sites for many SH2 domain–containing proteins, including PtdIns 3-kinase (PI3K) and PLC-γ. The receptor PTK phosphorylates and activates PLC-γ and PtdIns 3-kinase, the latter of which then catalyzes the conversion of PtdIns(4,5)P_2 (PIP_2) to PtdIns(3,4,5)P_3 (PIP_3). (*Right*) Phosphorylated PLC-γ likely undergoes substantial conformational changes and is maintained in proximity to the membrane through association both of its N-SH2 domain with the receptor PTK and of its PH or C-SH2 domains with PtdIns(3,4,5)P_3. PY and YP, phosphotyrosine. The EF-hand, SH3, and C2 domains of PLC-γ are not shown.

tyrosine phosphatases by H_2O_2 may be necessary to increase the steady-state level of protein tyrosine phosphorylation (97).

Antigen Receptor–Induced Activation of PLC-γ by Nonreceptor Protein Tyrosine Kinases

Hematopoietic cells express a variety of antigen and immunoglobulin (Ig) receptors that are able to bind specifically to a wide spectrum of ligands. Such binding results in a rapid activation of signaling events such as the tyrosine phosphorylation and subsequent activation of PLC-γ1 or PLC-γ2. These receptors, which include the T cell antigen receptor (TCR), the B cell antigen receptor (BCR), the high-affinity IgE receptor (FcεRI), and the IgG receptors (FcγRs), comprise multiple polypeptide chains but do not possess intrinsic PTK activity; rather, they activate members of distinct families of nonreceptor PTKs such as the Src, Syk, and Tec families. The ligand-binding extracellular portion of these receptors consists of variable chains that confer specificity, and these chains are noncovalently associated with invariant subunits that serve to couple each receptor to intracellular signaling molecules such as nonreceptor PTKs, Ras, and PtdIns 3-kinase. This coupling is mediated by the presence in the invariant chains of the immune receptor tyrosine-based activation motif (ITAM), with the consensus sequence Asp-X_2-Tyr-X_2-Leu-X_7-Tyr-Asp-X-Leu (where X is any amino acid). Phosphorylation of the two tyrosine residues within this motif by members of the Src family of nonreceptor PTKs generates docking sites for SH2 domain–containing molecules that propagate signals from the activated receptors (98).

The extracellular domain of the TCR is formed by α and β (or γ and δ) subunits, which contain the variable domains responsible for recognition of small peptides bound to major histocompatibility complex class I or class II molecules. The noncovalently associated invariant chains of the TCR include the CD3 complex (CD3γ, CD3δ, and two copies of CD3ε) and the ζ chain homo dimer. Each of the CD3 subunits contains one copy of the ITAM sequence, whereas the ζ chain contains three copies of this motif (Figure 5). The binding of antigen by the TCR triggers activation of Lck and Fyn, two members of the Src family of PTKs, by an undefined mechanism. Either one or both of these enzymes phosphorylates the tyrosine residues located within the ITAM sequences of the CD3 and ζ chains. These pairs of phosphorylated tyrosine residues serve as binding sites for the tandem SH2 domains of ZAP-70, a member of the Syk family of PTKs. Lck or Fyn then phosphorylates the bound ZAP-70, resulting in its activation. Together with Lck and Fyn, activated ZAP-70 phosphorylates various downstream substrates, including PLC-γ1, LAT (linker for activation of T cells), and SLP-76 (SH2 domain–containing leukocyte protein of 76 kDa).

LAT is a palmitoylated, transmembrane domain–containing protein that is predominantly localized to membranes (99). In response to TCR stimulation, LAT is phosphorylated on at least four tyrosine residues: Tyr132, Tyr171, Tyr191, and Tyr226 (100). Phosphorylated LAT associates in vivo with PLC-γ1, the adapter

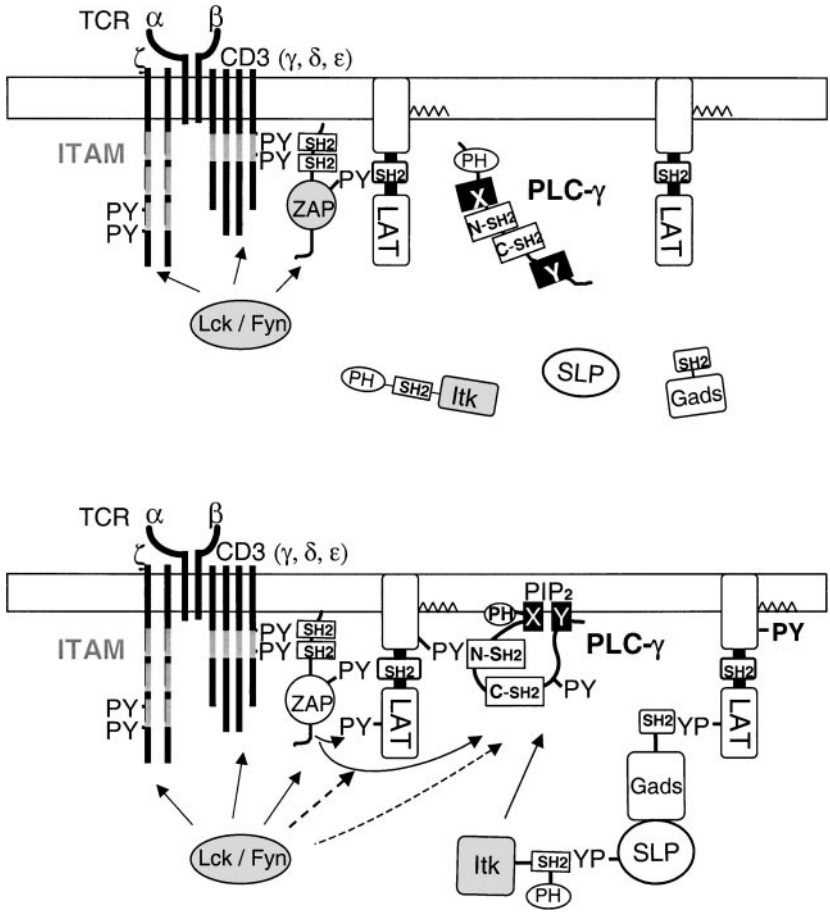

Figure 5 TCR-induced activation of PLC-γ1. (*Top*) Ligation of the TCR triggers the activation of Lck and Fyn by unknown mechanisms. Either or both of these Src family PTKs then phosphorylates tyrosine residues within ITAM sequences located in TCRζ and CD3 chains. Two phosphorylated tyrosine residues with this motif serve as binding sites for the tandem SH2 domains of ZAP-70. Lck or Fyn then phosphorylates the bound ZAP-70, resulting in its activation. (*Bottom*) Together with Lck and Fyn, activated ZAP-70 phosphorylates various downstream substrates, including membrane-bound LAT and SLP-76. The interaction of the N-SH2 domain of PLC-γ1 with a phosphorylated tyrosine residue of LAT serves to position the unphosphorylated enzyme close to activated ZAP-70 and Lck or Fyn, resulting in the phosphorylation and activation of PLC-γ1 and in its localization in the vicinity of its substrate. Phosphorylated LAT also associates with Gads, which might in turn associate with Itk-bound SLP-76; the close proximity of Itk and PLC-γ1 may result in the phosphorylation by Itk of PLC-γ1. Two LAT molecules are shown to avoid overcrowding; this does not imply that PLC-γ1 and Gads necessarily associate with separate LAT molecules. The EF-hand, SH3, and C2 domains of PLC-γ1 are not shown.

protein Gads, and other proteins as a result of interaction of its phosphorylated tyrosine residues with their SH2 domains. As in its interaction with the PDGF receptor, only the N-SH2 domain of PLC-γ1 is necessary for binding to phosphorylated LAT (101). The Tyr132 residue of LAT mediates binding of PLC-γ1, whereas Tyr171 and Tyr191 are responsible for binding Gads. Gads is abundant in T cells and, through its COOH-terminal SH3 domain, associates with the adapter protein SLP-76, thus linking SLP-76 to LAT (102). Studies with a LAT-deficient Jurkat T cell line indicate that LAT is essential for signaling through PLC and Ras (103). Engagement of the TCR on the mutant cells resulted in normal tyrosine phosphorylation of the ζ chain and ZAP-70; however, the extent of tyrosine phosphorylation of PLC-γ1 was markedly reduced and PLC-γ-mediated PtdIns(4,5)P_2 hydrolysis and mobilization of intracellular Ca^{2+} were not apparent. T cells also express two members, Itk and Rlk, of the Tec family of nonreceptor PTKs, which contain an NH_2-terminal PH domain followed by SH3, SH2, and kinase domains. T cells from mice lacking both Itk and Rlk also exhibit a reduced extent of PLC-γ1 activation in response to TCR stimulation (104).

On the basis of these various observations, it has been proposed that activated ZAP-70 phosphorylates LAT, and that phosphorylated LAT first recruits unphosphorylated PLC-γ1 and positions it close to activated ZAP-70 and subsequently localizes the phosphorylated PLC-γ1 in proximity to its substrate (Figure 5). The tyrosine phosphorylation of PLC-γ1 may also be mediated to a certain extent by Fyn or Lck or by ZAP-70 without the help of LAT. SLP-76 is also required for TCR-induced phosphorylation of PLC-γ1 and activation of the Ras signaling pathway, but not for the tyrosine phosphorylation of most proteins in response to TCR stimulation (105). Although the mechanism by which SLP-76 contributes to PLC-γ1 activation is not clear, the recruitment of Itk by phosphorylated SLP-76 might serve to position Itk in close proximity to LAT-associated PLC-γ1 (106).

The BCR is composed of an IgM molecule (consisting of two heavy chains and two light chains) noncovalently associated with two heterodimers of the invariant Igα and Igβ subunits, each of which contains one copy of the ITAM sequence. Engagement of the BCR results in the activation of Lyn, the most abundant Src family PTK in B cells, by an unknown mechanism that involves autophosphorylation on Tyr416 (located within the catalytic domain of the human protein) (107). Activated Lyn phosphorylates the pair of tyrosine residues in the ITAM sequences of Igα and Igβ, which results in the recruitment of Syk through its tandem SH2 domains. The ITAM-bound Syk is then phosphorylated on Tyr519 (located in the activation loop) by Lyn. This sequential activation of Lyn and Syk is similar to that of Lck-Fyn and ZAP-70 in T cells. However, in Lyn-deficient B cells, Syk is still partially activated and transmits signals to PLC-γ2 (107), which suggests that Syk activation, unlike that of ZAP-70, is not strictly dependent on members of the Src family.

PLC-γ1 is tyrosine phosphorylated after recruitment through its SH2 domains to phosphorylated Syk when reconstituted in COS cells (108). However, PLC-γ2,

not PLC-γ1, is expressed in B cells. Furthermore, PtdIns(3,4,5)P_3 and Btk, a Tec family PTK, have been implicated in BCR-induced PLC-γ signaling (109, 110). BCR engagement activates PtdIns 3-kinase, resulting in the conversion to PtdIns (3,4,5)P_3 of a small fraction of presumably the same pool of PtdIns(4,5)P_2 as that targeted by PLC-γ2. The resulting PtdIns(3,4,5)P_3 interacts with the PH domain of Btk, thereby promoting its membrane targeting and activation either by autophosphorylation or through its phosphorylation mediated by Lyn and Syk. The importance of Btk PH domain function is evidenced by the fact that spontaneously arising mutations in the region of the Btk gene encoding this domain cause X-linked agammaglobulinemia in humans and mice (111). Activated Btk is thought to contribute to PLC-γ2 activation, at least in part, by catalyzing its tyrosine phosphorylation. Thus, treatment of B cells with wortmannin inhibits the tyrosine phosphorylation of PLC-γ2. PtdIns(3,4,5)P_3 may also influence PLC-γ2 activity directly through its interaction with the PH and SH2 domains of the enzyme, similar to its role in the PDGF-induced activation of PLC-γ1 (Figure 4). Consistent with this scenario, engagement of both the BCR and the IgG receptor FcγIIb, the latter of which recruits the PtdIns(3,4,5)P_3 phosphatase SHIP and thereby prevents PtdIns(3,4,5)P_3 accumulation, blocks the activation of PLC-γ2 (110).

A protein known as B cell linker (BLNK) or SLP-65 is also an essential component of the PLC-γ2 activation pathway in B cells (112). Phosphorylation of BLNK by Syk induces its translocation to the cell membrane. The phosphorylation of BLNK does not appear to require Btk, given that it occurs normally in Btk-deficient cells. On the basis of these various observations, a model that integrates the roles of PtdIns(3,4,5)P_3, Btk, Syk, and BLNK in PLC-γ2 activation has been proposed (Figure 6). Phosphorylated, membrane-bound BLNK is thought to recruit PLC-γ2 to the membrane, where it is phosphorylated and activated by membrane-associated Btk. Given that the SH2 domain of Btk, in addition to its PH domain, is required for full activation of PLC-γ2 in B cells, this domain likely plays a role in the membrane localization of Btk by binding to tyrosine-phosphorylated BLNK.

The products of PtdIns 3-kinase also contribute to PLC-γ activation in response to ligation of Fc receptors, a family of receptors that bind to soluble Ig and immune complexes via the Fc region of Ig. For example, PtdIns 3-kinase is required for the translocation and tyrosine phosphorylation of PLC-γ1 (but it has no effect on PLC-γ2 activation) in response to antigen stimulation of FcεRI in RBL2H3 mast cells (113). In platelets, which express a single class of FcγR (FcγRIIA), inhibition of PtdIns 3-kinase prevents PLC-γ2 activation induced by cross-linking of this receptor (114). Whereas inhibition of PtdIns 3-kinase does not affect tyrosine phosphorylation of PLC-γ2, it blocks the stable association of this isozyme with the membrane-cytoskeleton interface that occurs at an early stage of platelet activation. Both SLP-76 and BLNK may also play important roles in FcγR-mediated PLC-γ activation (115).

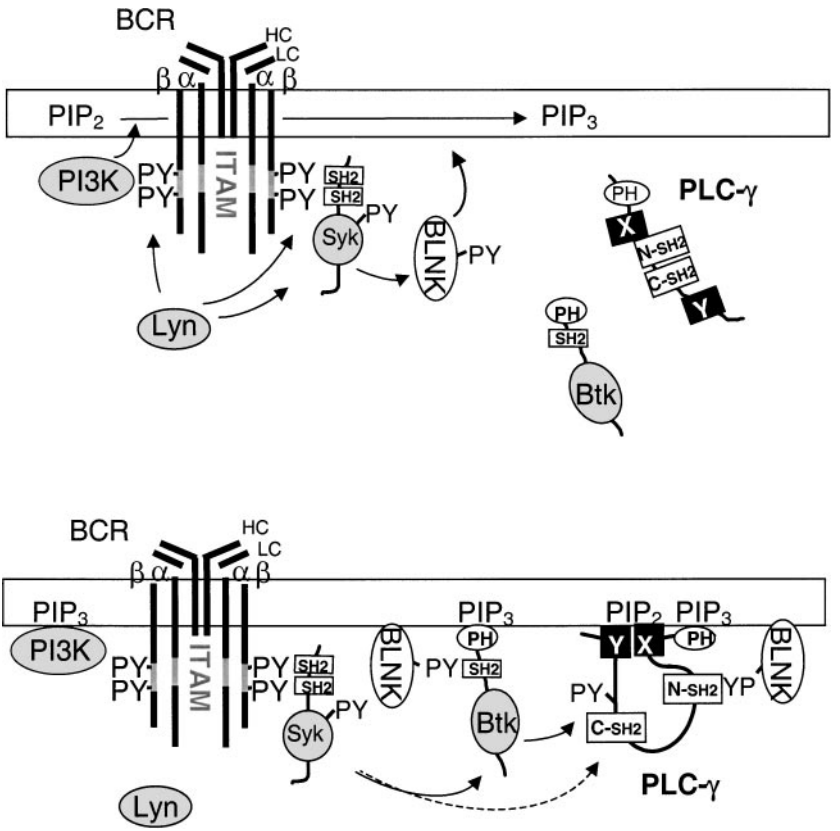

Figure 6 BCR-induced activation of PLC-γ2. (*Top*) BCR engagement triggers the activation of Lyn by an unknown mechanism. Activated Lyn phosphorylates tyrosine residues within ITAM sequences located in the Igα and Igβ chains. The two phosphorylated tyrosines within this motif serve as binding sites for the tandem SH2 domains of Syk, and the ITAM-bound Syk is phosphorylated (activated) by Lyn. Activated Syk then phosphorylates the cytosolic protein BLNK, thereby inducing its translocation to the cell membrane. BCR engagement also induces activation of PtdIns 3-kinase, which catalyzes the conversion of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. (*Bottom*) PtdIns(3,4,5)P_3 recruits Btk and PLC-γ2 to the membrane by binding to their PH domains. The membrane-bound, phosphorylated BLNK also contributes to recruitment of Btk and PLC-γ2 through the binding of its phosphorylated tyrosine residues to the SH2 domains of these enzymes. The recruited Btk is phosphorylated (activated) by Syk, and activated Btk phosphorylates PLC-γ2. Syk also might directly phosphorylate PLC-γ2. Two BLNK molecules are shown to avoid overcrowding; this does not imply that Btk and PLC-γ2 necessarily associate with separate BLNK molecules. The EF-hand, SH3, and C2 domains of PLC-γ2 are not shown. HC and LC, heavy and light chains, respectively, of IgM.

G Protein–Coupled Receptor Activation of PLC-γ via Nonreceptor Protein Tyrosine Kinases

Although G protein–coupled receptors (GPCRs) lack intrinsic PTK activity, tyrosine phosphorylation of PLC-γ occurs in response to ligation of several such receptors, including those for acetylcholine (muscarinic), angiotensin II, thrombin, platelet-activating factor, and ATP (116–119). c-Src appears to be responsible for the phosphorylation of PLC-γ1 in vascular smooth muscle cells and platelets; the introduction of antibodies to c-Src into these cells by electroporation inhibited the tyrosine phosphorylation of PLC-γ1 elicited by angiotensin II or platelet-activating factor, respectively.

Activation of many GPCRs also induces tyrosine phosphorylation of receptor PTKs such as those for PDGF, EGF, and insulin-like growth factor I (120). G$\beta\gamma$ subunits have been implicated in the mechanism of GPCR-induced transactivation of receptor PTKs. Overexpression of G$\beta\gamma$ subunits in COS cells was sufficient to increase EGF receptor phosphorylation (120). In cultured cells from vascular smooth muscle, angiotensin II induced association of c-Src with the EGF receptor in the presence of EGF receptor kinase inhibitors, which suggests that activation of c-Src precedes EGF receptor transactivation (121). The activated α subunits of G_i or G_s proteins were recently shown to bind directly to the catalytic domain of c-Src and to increase its kinase activity, whereas neither the α subunits of G_q or G_{12} nor G$\beta\gamma$ subunits exhibited this effect (122). Transactivation of a receptor PTK results in the formation of a structural scaffold for the assembly of a signaling complex (including PLC-γ) that resembles that formed in response to interaction of the receptor PTK with its ligand (Figure 7).

In some instances, the GPCR itself contributes to a signaling complex that includes c-Src or Janus kinase (JAK). In HEK-293 and COS cells, the agonist-occupied β_2-adrenergic receptor forms a complex with activated c-Src in a manner that is dependent on receptor desensitization (120). This indirect interaction is mediated by the binding of c-Src to the receptor-bound adapter protein β-arrestin, and it results in the targeting of both c-Src and the receptor to clathrin-coated pits. Thus, the binding of β-arrestin to β_2-adrenergic receptors, which terminates receptor coupling to G proteins, also initiates a second wave of signal transduction in which the desensitized receptor functions as a structural component of a PTK cascade. Whether a similar PTK cascade operates for other GPCRs that induce PLC-γ activation remains unknown.

Stimulation of the angiotensin II receptor (AT_1 receptor) in vascular smooth muscle cells activates JAK2 by inducing its tyrosine phosphorylation and association with the receptor. This association requires a Tyr-Ile-Pro-Pro (YIPP) motif that is located in the intracellular COOH-terminal domain of the receptor and which is identical to the binding site in the PDGF receptor for the SH2 domain of PLC-γ1 (117). JAK2 does not contain an SH2 domain; rather, its interaction with the AT_1 receptor appears to be mediated by the action of SH-PTP2 as a linker. Stimulation of the AT1 receptor induces phosphorylation of the tyrosine residue in

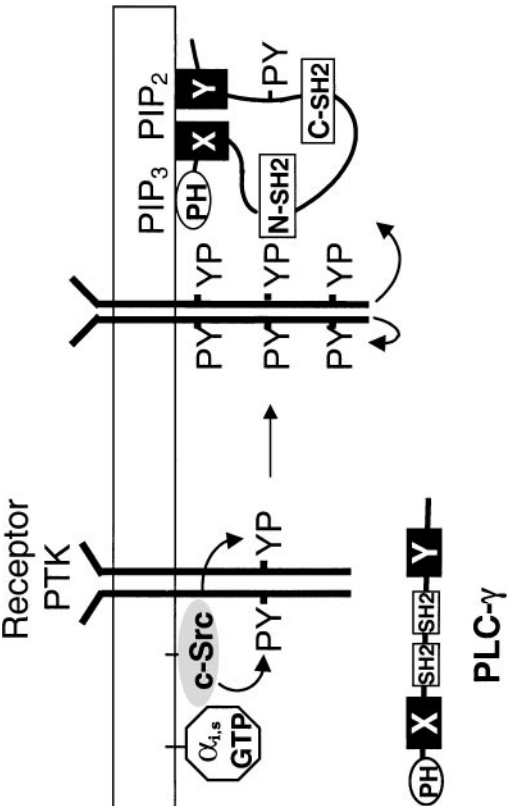

Figure 7 GPCR-induced activation of PLC-γ by PTKs. An agonist-occupied GPCR induces the exchange of GDP for GTP on the α subunit of G_s or G_i and the subsequent dissociation of the α subunit from the membrane-associated $G\beta\gamma$ dimer. The GTP-bound α_s or α_i subunit activates c-Src by inducing its autophosphorylation, and the phosphorylated enzyme then associates with and phosphorylates a receptor PTK. The phosphorylated receptor PTK provides a structural scaffold for the assembly of a signaling complex that includes PLC-γ (see Figure 4).

the YIPP motif, and the phosphorylated tyrosine residue serves as the binding site for PLC-γ1 (123). Both PLC-γ1 and the SH-PTP2–JAK2 complex thus appear to bind to the same site on the AT_1 receptor.

Activation of PLC-γ by Other Lipid-Derived Messengers

Alternative mechanisms for the activation of PLC-γ that do not rely on tyrosine phosphorylation appear to exist. Phosphatidic acid has been shown to activate both tyrosine-phosphorylated and unphosphorylated forms of PLC-γ1 to similar extents by increasing their affinity for substrate vesicles (124, 125). Given that phosphatidic acid is an immediate product of phosphatidylcholine hydrolysis by phospholipase D (PLD), activation of PLD in cells may also result in activation of PLC-γ (Figure 8).

Unsaturated fatty acids, such as arachidonic acid (AA), also stimulate PLC-γ activity independently of tyrosine phosphorylation in the presence of various splicing variants of the microtubule-associated protein tau (126). Although tau is expressed exclusively in neurons, nonneuronal cells also contain a protein that, together with AA, activates PLC-γ. This activating protein was recently identified as a 680-kDa molecule termed AHNAK (giant in Hebrew) (127). AHNAK

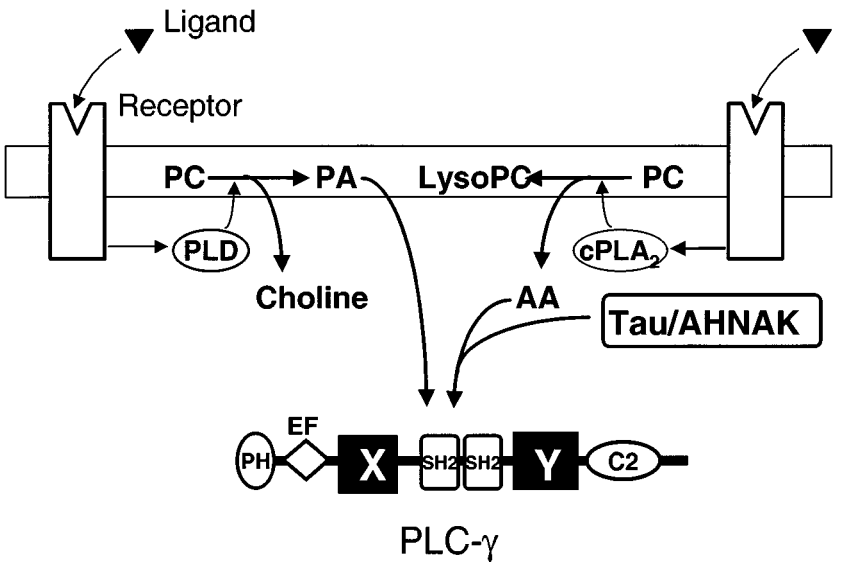

Figure 8 Receptor-mediated activation of PLC-γ by phosphatidic acid generated by PLD (*left*) and by the concerted action of tau (or AHNAK) and AA generated by cytosolic phospholipase A_2. Although PLC-γ is not shown in close proximity to the membrane, its activation by the products of lipid hydrolysis is achieved by increasing its affinity for the membrane surface. $cPLA_2$, cytosolic phospholipase A_2; PA, phosphatidic acid; PC, phosphatidylcholine.

contains ~30 repeats of a motif of 128 amino acids. A glutathione S-transferase fusion protein containing one of the repeated motifs activated PLC-γ1 at nanomolar concentrations in the presence of AA, which suggests that an intact AHNAK molecule contains multiple sites capable of activating PLC-γ.

Activation of PLC-γ by AHNAK appears highly similar to that by tau, even though the only similarity in the primary structures of these two proteins is a high percentage of proline residues. These two activators competed with each other in the activation of PLC-γ, further supporting the notion that they share a common mechanism of activation. Tau interacts with all three of the activation reaction components: PLC-γ, AA, and PtdIns(4,5)P_2. Both tau and AHNAK coimmunoprecipitate with PLC-γ, but not with PLC-β or PLC-γ isozymes.

Although the concentration of AA in resting cells is relatively low, large amounts of this fatty acid are released from phosphatidylcholine by the action of cytosolic PLA_2 in response to cell stimulation. It is therefore likely that certain stimuli that induce activation of cytosolic PLA_2 may also indirectly trigger the activation of PLC-γ in the presence of tau or AHNAK (Figure 8). Activation of cytosolic PLA_2 requires mobilization of intracellular Ca^{2+}, and thus may occur secondarily to PLC activation; the subsequent activation of PLC-γ by the AA produced as a result of the action of cytosolic PLA_2 may thus contribute to a positive feedback loop in the hydrolysis of PtdIns(4,5)P_2. On the other hand, PtdIns(4,5)P_2 is a potent activator of $cPLA_2$ (3), so that hydrolysis of PtdIns(4,5)P_2 by PLC attenuates the activity of cytosolic PLA_2, constituting a negative feedback loop in terms of AA generation.

ACTIVATION OF PLC-δ ISOZYMES

Although four distinct PLC-δ isoforms have been identified, the mechanisms by which these enzymes are coupled to membrane receptors remain unclear. The sensitivity of PLC-δ isozymes to Ca^{2+} is greater than that of the other isozymes. Calcium promotes both the association of the PH domain with PtdIns(4,5)P_2 as the result of its binding to the EF-hand domain (35) as well as the formation of a C2 domain–phosphatidylserine-Ca^{2+} ternary complex as the result of its binding to the C2 domain (33). An increase in the intracellular concentration of Ca^{2+} might therefore be sufficient to trigger activation of PLC-δ. This hypothesis is supported by observations with permeabilized (128) and intact (129) PC12 cells; in the latter experiments, PLC-δ1 overexpressed in PC12 cells was activated by capacitative Ca^{2+} entry in response to the activation of PLC-β by bradykinin.

Another potential regulator of PLC-δ isozymes is a recently discovered type of GTP-binding protein, termed high-molecular-weight G protein or G_h (75–80 kDa) (130). This protein, which also possesses tissue transglutaminase activity, forms a complex with PLC-δ1 in cells stimulated through α_1-adrenergic or oxytocin receptors, and it increases the activity of purified PLC-δ1 in the presence of GTP-γ-S (130, 131). Furthermore, G_h coimmunoprecipitates with the α, but not with the β,

subtype of thromboxane A_2 receptor and enhances thromboxane-dependent PLC activity when transiently expressed in cells (132). By analogy with heterotrimeric G proteins, the GTP-bound form of G_h was initially proposed to function as an activator of PLC-δ1 (130). However, free or GDP-bound G_h, but not GTP-bound G_h, was recently shown to be associated with PLC-δ1 (133), which suggests that the activity of PLC-δ1 might be inhibited by G_h in unstimulated cells and that such inhibition might be attenuated by the binding of G_h to GTP in response to receptor occupancy.

The G_h-interacting (α_1-adrenergic, oxytocin, thromboxane) receptors are also coupled to G_q proteins and activate PLC-β isozymes. A model for receptor-induced activation of PLC-δ that incorporates these various observations is shown in Figure 9. Ligation of the GPCR thus induces the dissociation of PLC-δ from G_h by stimulating GDP-GTP exchange. Concurrently, the agonist-bound receptor activates PLC-β through G_q and thereby induces the mobilization of intracellular Ca^{2+}. The increase in the cytosolic Ca^{2+} concentration then induces the translocation of free PLC-δ to the membrane, where it exerts its catalytic action.

ACTIVATION OF PLC-ε

The recently identified human PLC-ε has been detected in two alternatively spliced forms with molecular sizes of 260 kDa (2303 residues) and 230 kDa (1998 residues) (9, 10). The mRNAs encoding these enzymes are present in a wide variety of human tissues, most abundantly in the heart. Both the 260- and 230-kDa forms of PLC-ε contain not only the conserved catalytic (X and Y) and regulatory (C2) domains common to other PLC isozymes, but also RasGEF and RA domains (one RA domain for the 260-kDa form and two such domains for the 230-kDa form) that are specific to this type of PLC. PLC-ε enzymes appear not to contain PH and EF-hand domains.

The presence of RasGEF and RA domains suggests that PLC-ε might be a GDP-GTP exchange factor for, as well as an effector of, Ras. Indeed, expression of wild-type PLC-ε promotes generation of the GTP-bound form of Ras in cultured cells (9, 10). This exchange activity was shown to be attributable to the RasGEF domain of PLC-ε rather than to indirect effects of second messengers produced by this enzyme, given that expression of a PLC-inactive mutant also increased the amount of GTP-Ras. Furthermore, Ras is an activator of PLC-ε, as revealed by the observations that the RA domain of PLC-ε binds GTP-Ras with high affinity (K_d, 40 nM), but it does not bind GDP-Ras; the presence of prenylated Ras in substrate vesicles increases the activity of PLC-ε; and coexpression of activated Ras with PLC-ε (260-kDa form) induces translocation of the latter from the cytosol to the membrane (10). Stimulation of cells with EGF, which increases the abundance of GTP-Ras, also induces the membrane translocation of PLC-ε (10). Therefore, the notion that the increase in PLC activity in growth factor–stimulated cells is solely attributable to the activation of PLC-γ might need to be amended to incorporate

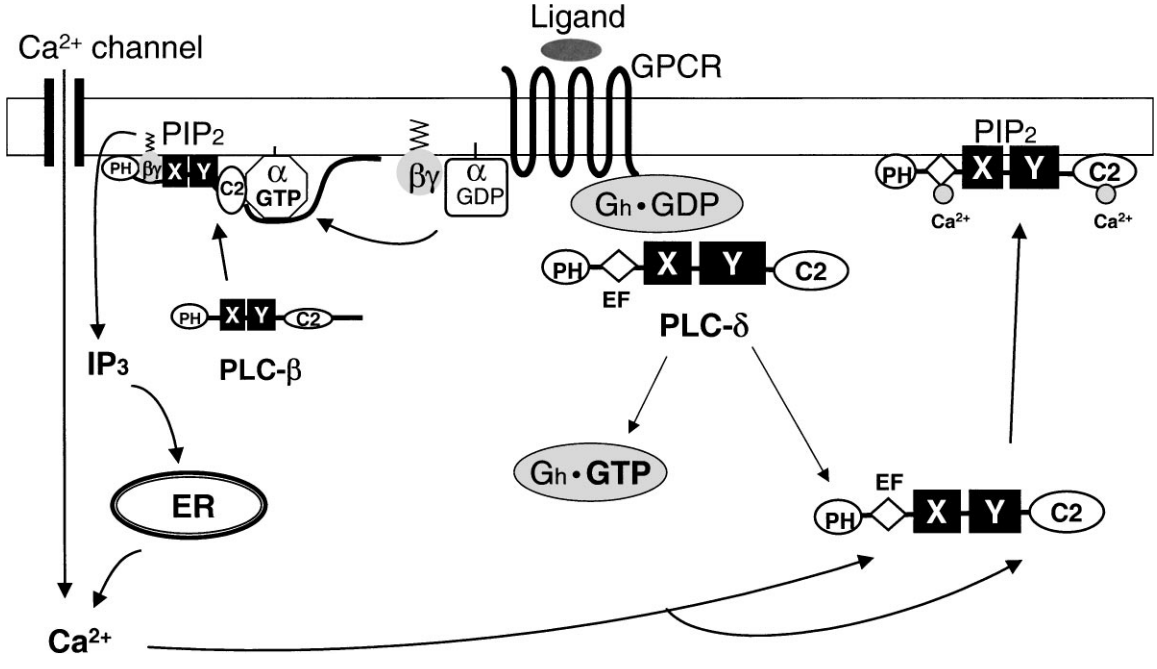

Figure 9 GPCR-induced activation of PLC-δ isozymes. An agonist-occupied GPCR induces the activation of PLC-β through $G_q\alpha$ or $G\beta\gamma$ subunits (see Figure 3), resulting in hydrolysis of PtdIns(4,5)P_2. The Ins(1,4,5)P_3 (IP_3) so produced induces the mobilization of Ca^{2+} from the endoplasmic reticulum (ER) and subsequent capacitative Ca^{2+} entry. Concurrent with PLC-β activation, the GPCR promotes GDP-GTP exchange on the G_h protein bound to the unidentified region of PLC-δ, resulting in dissociation of the GTP-bound protein from PLC-δ. The EF-hand and C2 domains of the released PLC-δ then associate with Ca^{2+} and the enzyme translocates to the membrane for catalytic action.

a contribution of PLC-ε. The fact that both activator and effector domains of Ras exist in the same molecule of PLC-ε suggests a possible mechanism for the concerted action of these domains (Figure 10). Thus, whereas the growth factor–induced activation of PLC-ε might be terminated by the hydrolysis of Ras-bound GTP to GDP, the GDP-Ras might be immediately converted to GTP-Ras by the action of the RasGEF domain of PLC-ε, thereby prolonging the receptor-mediated activation of PLC-ε.

PLC-ε also appears to be activated by $G\alpha_{12}$ (Figure 10). Coexpression of constitutively active (GTPase-defective) mutants of $G\alpha_{12}$ with PLC-ε (230-kDa form) specifically increased PLC activity, whereas none of the other Gα subunits tested

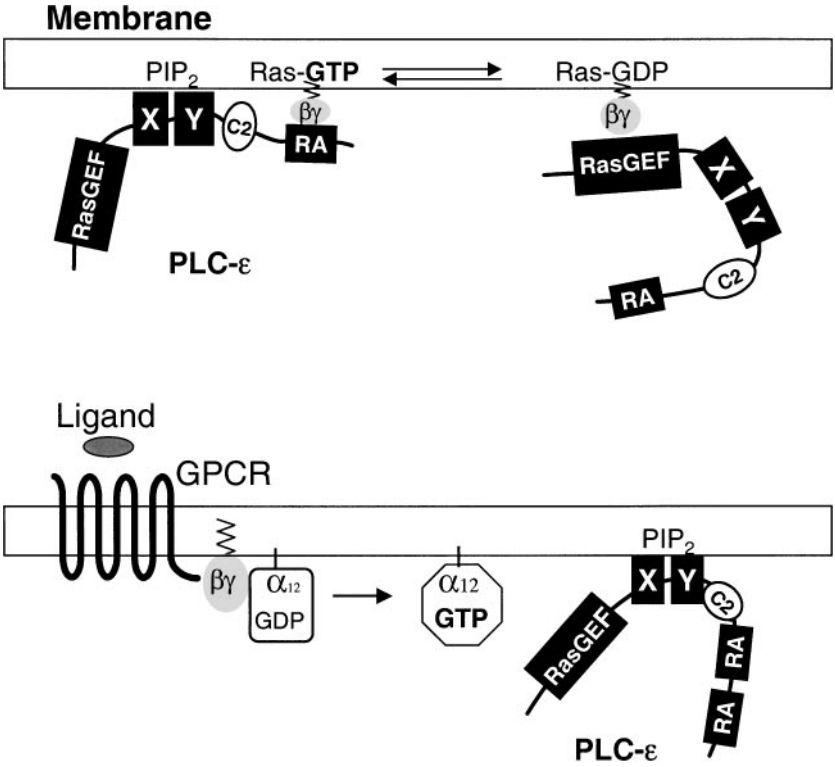

Figure 10 Receptor-mediated activation of PLC-ε. (*Top*) Ras-GTP, produced in response to receptor occupancy with ligand, promotes the translocation (activation) of PLC-ε to the membrane by binding to its RA domain. The activation signal is turned off by the hydrolysis of Ras-bound GTP catalyzed by the intrinsic GTPase activity of Ras. The resulting GDP-Ras might then be converted to GTP-Ras by the action of the RasGEF domain of PLC-ε. (*Bottom*) An agonist-occupied GPCR induces the exchange of GDP for GTP on an α subunit of the G_{12} subfamily of G proteins. The GTP-bound α subunit then positions PLC-ε closer to the membrane and activates it by interacting with an unidentified region of the enzyme.

had any such effect (9). Thus, the receptors for thrombin and lysophosphatidic acid, which are coupled to the G_{12} subfamily of G proteins, might be expected to activate PLC-ε.

CONCLUDING REMARKS

Substantial progress has been achieved over the last several years with regard to our knowledge of the mechanisms by which signals are conveyed from receptors at the plasma membrane to the various PLC isozymes. This progress is largely attributable to our understanding of the modular function of regulatory domains that recognize either membrane-associated proteins or lipid molecules. One overriding principle that has emerged is that receptor-induced recruitment of PLC enzymes to the vicinity of the cell membrane achieved through such regulatory domains appears key for regulation of PLC. Allosteric mechanisms also appear to contribute to receptor-mediated PLC activation, but these are less well understood. The mechanisms depicted in Figure 3 and Figures 4 to 7 for the activation of PLC-β by heterotrimeric G proteins and that of PLC-γ by PTKs, respectively, appear well founded, although additional factors that play minor roles might be uncovered in the future. In contrast, the mechanisms shown in Figures 8 and 9 for the activation of PLC-γ by lipid messengers and that of PLC-δ by Ca^{2+} and G_h, respectively, require further confirmation. Given that PLC-ε was identified only recently, its activation by $G\alpha_{12}$ or Ras (Figure 10) or by other mechanisms requires substantial further study.

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