Regulation of Phosphoinositide-specific Phospholipase C Isozymes

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The hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP$_2$), by a specific phospholipase C (PLC) is one of the earliest key events in the regulation of various cell functions by more than 100 extracellular signaling molecules (1–4). This reaction produces two intracellular messengers: diacylglycerol and inositol 1,4,5-trisphosphate, which mediate the activation of protein kinase C (PKC) and intracellular Ca$^{2+}$ release, respectively. Furthermore, a decrease in the amount of PIP$_2$ itself in the cell membrane is likely an important signal because the activities of several proteins are modulated by this phospholipid (5). PIP$_2$ is a cofactor for phosphatidylinositol-specific phospholipase D (PLD) and a substrate for phosphoinositide 3-kinase (PI 3-kinase), both of which are also receptor-activated effector enzymes. In addition, PI 3-kinase modulates actin polymerization by interacting with various actin-binding proteins and serves as a membrane-attachment site for many signaling proteins that contain pleckstrin homology (PH) domains. Consequently, the activity of PLC is strongly regulated in cells through various distinct mechanisms that link multiple PLC isoforms to various receptors.

PLC Isoforms and Structural Organization

The 10 mammalian PLC isozymes (excluding alternatively spliced forms) identified to date are all single polypeptides and can be divided into three types, $\beta$, $\gamma$, and $\delta$, of which four PLC-$\beta$, two PLC-$\gamma$, and four PLC-$\delta$ proteins are known (1–5). The $\delta$-type isoforms are smaller ($M$, 85,000) than the PLC-$\beta$ and PLC-$\gamma$ ($M$, 140,000–155,000) isoforms. Lower eukaryotes such as yeast and slime molds contain only $\delta$-type isoforms, suggesting that $\beta$- and $\gamma$-type isoforms in higher eukaryotes evolved from the archetypal PLC-$\delta$. Two regions of high sequence homology (40–60% identity), designated X and Y, constitute the PLC catalytic domain (1–5) (Fig. 1). A PH domain is located in the NH$_2$-terminal region, preceding the X domain, in all three types of PLC. Whereas PLC-$\beta$ and PLC-$\delta$ isoforms contain a short sequence of 50–70 amino acids that separates the X and Y regions, PLC-$\gamma$ isoforms have a long sequence of $\sim$400 amino acids that contains Src homology (SH) (two SH2 and one SH3) domains. PLC-$\gamma$ isoforms contain an additional PH domain that is split by the SH domains. PH ($\sim$100 residues), SH2 ($\sim$80 residues), and SH3 ($\sim$50 residues) domains are shared by many signaling proteins; whereas PH domains mediate interaction with membrane surface by binding to PIP$_2$, SH domains mediate interactions with other proteins by binding to phosphorylated tyrosine residues (SH2) or proline-rich sequences (SH3).

The three-dimensional structure of a PLC-$\beta$1 molecule lacking the PH domain has recently been determined (6). As expected, the X and Y regions are tightly associated. The structure also revealed two accessory modules, an EF-hand domain and a C2 domain, the latter of which was previously suggested to mediate the Ca$^{2+}$-dependent binding to lipido-velices. On the basis of the structural information, a catalytic mechanism comprising two steps, tether and fix, was proposed. The PH domain of PLC-$\beta$1 would tether the enzyme to the membrane by specific binding to PIP$_2$, and the C2 domain would fix the catalytic domain in a productive orientation on the membrane. The EF-hand domain would serve as a flexible link between the PH domain and the rest of the enzyme. Calcium is required for the function of the C2 domain. Another Ca$^{2+}$ ion located at the active site, together with His$_{311}$ and His$_{356}$, directly participates in catalysis, consistent with the fact that all eukaryotic PLC isoforms require Ca$^{2+}$ for activity, that the two histidines equivalent to His$_{311}$ and His$_{356}$ are completely conserved among all PLC isoforms, and that mutation of either of the two histidine residues results in enzyme inactivation (7).

The multidomain structure observed with PLC-$\beta$1 is likely to be common to all mammalian PLC isoforms (Fig. 1). However, PLC-$\beta$ and PLC-$\gamma$ isoforms contain additional regulatory COOH-terminal and SH domains, respectively. These regulatory domains are responsible for the fact that different PLC isoforms are linked to receptors through distinct mechanisms. Furthermore, the COOH-terminal domain of PLC-$\beta$ isoforms might contribute to the tethering of the enzyme to the membrane surface, given that truncation of this domain completely blocked membrane association of PLC-$\beta$1 (8). The SH domains of PLC-$\gamma$ appear to play a critical role in mitogenic signaling independently of PLC activity; catalytically inactive mutants of PLC-$\gamma$ (containing mutations at the essential His residues) elicited a mitogenic response when microinjected into NIH 3T3 cells, and mitogenic activity was localized to the SH region (7, 9).

Activation of PLC-$\beta$ by G Proteins

The $\alpha$ subunits ($\alpha_{161}$, $\alpha_{111}$, and $\alpha_{16}$) of all four members of the $G_{\alpha}$ subfamily of heterotrimeric G proteins activate PLC-$\beta$ isoforms but not PLC-$\gamma$1 or PLC-$\delta$1 (1–4, 10) (Fig. 2). The receptors that activate this $G_{\alpha}$-PLC-$\beta$ pathway include those for thromboxane $A_{2}$, bradykinin, bombesin, angiotensin II, histamine, vasopressin, acetylcholine (muscarnic m1 and m3), $\alpha_{1}$-adrenergic agonists, thyroid-stimulating hormone, C-C and C-X-C chemokines, and endothelin-1 (4, 11).

The GTP$\gamma$S-activated $G_{\alpha}$ or $G_{\alpha}13$ subunits stimulate PLC-$\beta$ isoforms with the rank order of potency PLC-$\beta1 \geq PLC_{\beta3} > PLC_{\beta2}$ (4, 5). PLC-$\beta4$ is also activated by $G_{\alpha}$ subunits; however, because the basal activity of this enzyme is inhibited by ribonucleotides, including GTP$\gamma$S, accurate estimation of the extent of activation is difficult (12). All four $G_{\alpha}$ subunits are palmitoylated at residues Cys$^{9}$ and Cys$^{13}$ (13). Removal of the two palmitate groups affects neither the capacity of the proteins to activate PLC-$\beta$ nor their association with the cell membrane. $G_{\alpha}$, which is detected only in hematopoietic cells and is distantly related to the more widely expressed $G_{\alpha}$ (amino acid sequence identity of 55%), activates PLC-$\beta1$, $\beta2$, and $\beta3$ in a manner essentially indistinguishable from that of $G_{\alpha}$ (14). However, the $\alpha$ subunits can be discriminated by certain receptors (11).

The receptor-mediated activation of PLC-$\beta$ has been studied in detail by reconstituting the m1 muscarinic acetylcholine receptor, G protein, and PLC-$\beta$ in lipid vesicles (15, 16). The muscarinic agonist carbachol stimulated PLC activity 90-fold, and each member of the $G_{\alpha}$ family mediated this activation. The intrinsic GTPase activity of purified $G_{\alpha}$ was low but was stimulated >50-fold by the presence of PLC-$\beta1$, that is PLC-$\beta1$ is a GTPase-activating protein for $G_{\alpha}$ (16). In the reconstituted system,
PLC-β1 also increased the rate of GTP hydrolysis by Gαβγ up to 60-fold in the presence of carbachol, which alone stimulated activity 6–10-fold (16). These results indicate that the receptor and PLC-β1 coordinately regulate the amplitude of the PLC signal and the rate of signal termination.

The Gβγ dimer also activates PLC-β isozymes (1–4, 15). The sensitivity of PLC-β isozymes to Gβγ subunits differs from that to Gα subunits and decreases in the order PLC-β3 > PLC-β2 > PLC-β1, 4, 5. The ability of Gβγ subunits to activate PLC-β2 in response to ligation of the luteinizing hormone receptor, V2 vasopressin receptor, β1- and β2-adrenergic receptors, m2 muscarinic acetylcholine receptor, and the receptors for the chemotactants interleukin 8 (IL-8), formyl-Met-Leu-Phe, and complementation factor 5a was recently suggested that Gβγ subunits may independently modulate a single isozyme-dependent production of inositol 1,4,5-trisphosphate (4). A specific autophosphorylated site (for example, Tyr1021 of the β-type PDGF receptor) is recognized by one of the SH2 domains of PLC-γ. Mutation of the PLC-γ-binding Tyr residue to Phe in the receptors for PDGF, epidermal growth factor, and nerve growth factor prevents association of the receptor with PLC-γ and abolishes the growth factor-dependent production of inositol 1,4,5,6-tetrakisphosphate (4).

Phosphorylation of PLC-γ1 by all growth factor receptors occurs at identical sites: tyrosines 771, 783, and 1254. Phe substitution at Tyr783 completely blocks the activation of PLC by PDGF in NIH 3T3 cells (1–5). Tyrosine phosphorylation of PLC-γ1 appears to promote its association with unidentified components of the cytoskeleton; the SH3 domain of PLC-γ1 is responsible for targeting the enzyme to the actin microfilament network. Whether this cytoskeletal association serves to bring the enzyme into contact with its substrate or whether it promotes interaction with another protein component essential for its activation is unknown. Autophosphorylation of growth factor receptors and subsequent tyrosine phosphorylation of substrate proteins, including PLC-γ1, require the presence of H2O2, whose concentration increases transiently and which appears to function as an intracellular messenger in growth-factor-stimulated cells (23). This requirement probably reflects that the activation of a receptor PTK by the binding of a growth factor is insufficient to increase the steady-state level of protein tyrosine phosphorylation. Concurrent inhibition of protein tyrosine phosphatases by H2O2 is also necessary.

Nonreceptor PTKs also phosphorylate and activate PLC-γ isozymes in response to the ligation of certain cell surface receptors. Such receptors include the T cell antigen receptor, membrane immunoglobulin (Ig) M, the high affinity IgG receptor, the IgG receptors, the IgA receptor, CD20, CD38, the α2-macroglobulin receptor, integrins, and several receptors for cytokines such as ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin M, IL-1, IL-4, IL-6, and IL-7 (4, 24–26). These receptors, most of which comprise multiple polypeptide chains, do not themselves possess PTK activity, but they activate a wide variety of nonreceptor PTKs such as the members of Src, Syk, and Jak/Tyk families. The activated PTKs often phosphorylate one of the receptor com-

**Fig. 1.** Linear representation of the various domains identified in the three types of PLC isozymes. Catalytic domains X and Y as well as PH, EF-hands, C2, and SH (SH2 and SH3) domains are indicated.

**Fig. 2.** Receptor-induced activation of PLC-β isozymes by Gα subunits and Gβγ subunits.
ponents to which PLC-γ then binds via its SH2 domains and becomes phosphorylated by the PTK. PLC-γ1 associates directly with Src and Syk in cells, and in vitro it is phosphorylated by various soluble PTKs including Src, Fyn, Lck, Lyn, and Hck (4, 5). Tyrosine phosphorylation of PLC-γ1 has also been shown to be elevated in cells that express SV40 middle T antigen, that are strained mechanically, or that are exposed to electroconvulsive shock (27–29).

Tyrosine phosphorylation of PLC-γ has also been observed in response to the ligation of several heptahelical, G protein-coupled receptors, including m5 muscarinic acetylcholine receptor in Chinese hamster ovary cells, the angiotensin II and thrombin receptors in vascular smooth muscle cells, and platelet-activating factor (4, 30, 31). Src appears to be responsible for the phosphorylation of PLC-γ1 in vascular smooth muscle cells and platelets; electroporation of antibodies to Src inhibited the tyrosine phosphorylation of PLC-γ1 elicited by angiotensin II or platelet-activating factor. Although activation of Src family PTKs in response to stimulation of a variety of G protein-coupled receptors has been demonstrated (32), the mechanism by which the enzymes are coupled to the receptors is not clear. One possible mechanism is through a member of the recently identified proline-rich PTK (Pyk) family; stimulation of receptors coupled to the G proteins Gα or Gβγ in neuronal cells resulted in tyrosine phosphorylation of Pyk-2, binding of the SH2 domain of Src to the phosphorylated Pyk-2, and activation of Src (33).

**PTK-independent Activation of PLC-γ**

PLC-γ isoforms can be activated directly by several lipid-derived second messengers in the absence of tyrosine phosphorylation. Phosphatidic acid produced by the action of PLD activates purified PLC-γ1 by acting as an allosteric modifier (34). PLC-γ isoforms are also stimulated by arachidonic acid (AA) in the presence of the microtubule-associated protein tau (in neuronal cells) or tau-like proteins (in non-neuronal cells) (35). The effect of tau and AA was specific to PLC-γ isoforms and was markedly inhibited by phosphatidylethanolamine (PC). These observations suggest that the activation of PLC-γ1 by tau or tau-like proteins might be facilitated by a concomitant decrease in PC concentration and an increase in AA concentration, both of which occur in cells upon activation of an 85-kDa cytosolic phospholipase A2 (cPLA2). This enzyme is coupled to various receptors and preferentially hydrolyzes PC containing AA. Therefore, activation of PLC-γ isoforms may occur secondarily to receptor-mediated activation of cPLA2. Several studies are consistent with the notion that stimulation of PLC by endogenously released AA occurs in cells.

Ligation of a variety of receptors results in the activation of PI 3-kinase, which phosphorylates the D3 position of PIP2 to produce phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 activates purified PLC-γ isoforms specifically by interacting with their SH2 domains. In addition, incubation of NIH 3T3 cells with PIP3 resulted in a transient increase in the intracellular Ca2+ concentration, an effect that was blocked in the presence of a PLC inhibitor. Thus, receptors coupled to PLD, cPLA2, or PI 3-kinase may activate PLC-γ isoforms indirectly, in the absence of tyrosine phosphorylation, through the generation of lipid-derived second messengers (Fig. 4).

**Activation of PLC-δ**

Although four distinct PLC-δ isoforms are known, the mechanism by which these isoforms are coupled to membrane receptors remains unclear. A new class of GTP-binding protein, termed Gαδ and containing 75–80-kDa α and 50-kDa β subunits, has been shown to be associated with agonist-bound α2-adrenergic receptors (α2-AR). The Gαδ subunit, a multifunctional protein that also possesses tissue transglutaminase activity (37), activates purified PLC-δ1 and forms a complex with PLC-δ1 in cells stimulated via α1-AR (38). Furthermore, overexpression of Gαδ in COS cells enhanced the activation of PLC induced by ligation of the α1-AR (37). These results suggest that Gαδ directly couples α1-AR to PLC-δ1. It is not yet known whether other PLC-δ isoforms are also activated by Gαδ, what other receptors couple to Gαδ, and how the tissue transglutaminase activity of Gαδ is related to its PLC-δ1-activating function. The GTPase-activating protein for the small GTP-binding protein RhoA (RhoGAP) also activates purified PLC-δ1; PLC-δ1 activation was thus suggested to occur downstream of RhoA activation (39).

All PLC isoforms are activated by Ca2+ in vitro, but PLC-δ isoforms are more sensitive to Ca2+ compared with the other isoforms. Furthermore, PLC-δ can be tethered to PIP3-containing membranes via its PH domain in the absence of other signals. An increase in the intracellular concentration of Ca2+ to a level sufficient to fix the C2 domain of PLC-δ might therefore trigger its activation. Thus, activation of PLC-δ isoforms might occur secondarily to receptor-mediated activation of other PLC isoforms or Ca2+ channels.

**Nuclear PLC**

PLC signaling also appears to occur in the nucleus (40). PLC-β1 is the major PLC isoform that has been detected in the nucleus of various cells. The amount of nuclear PLC-β1 protein, which appears to be activated independently of its plasma membrane counterpart by an unknown mechanism, increases during cell growth and decreases during differentiation (41–44). The changes in the amount of nuclear PLC-β1 correlate with changes in the amount of PIP3 hydrolyzed in the nucleus. Studies of cells lacking PLC-β1 as a result of gene ablation revealed that it is essential for the onset of DNA synthesis in response to insulin-like growth factor I (45). The COOH-terminal region downstream of the Y domain was also shown to be necessary for translocation of PLC-β1 to the nucleus (8).
Inhibition of PLC via Protein Kinases A and C

The activation of PKC or CAMP-dependent protein kinase (PKA) attenuates the PLC signaling pathway in a variety of cells. The proposed targets for phosphorylation by these kinases include cell surface receptors, G proteins, and PLC itself. PLC-β1 is rapidly phosphorylated in cells treated with phorbol ester and is phosphorylated at Ser241 by PKC in vitro; however, phosphorylation had no effect on either the basal or Gqα-stimulated activities of PLC-β1 (1).

In human Jurkat T cells, activation of PKA or PKC results in an increase in phosphorylation of Ser887 by PKC and a concomitant decrease in the tyrosine phosphorylation of PLC-1, the latter of which might be responsible for the decreased PLC activity apparent in Jurkat cells treated with PKA- or PKC-stimulating agonists (1).

The interaction of PLC and PKA was studied in COS cells transfected with cDNAs encoding PLC-β2, G protein subunits, and PKA (46). Expression of the catalytic subunit of PKA specifically inhibited Gβγ stimulation of PLC-β2 activity, without affecting Gαi-induced activation. The effect of PKA was not mimicked by PKC isozymes. Furthermore, PKA directly phosphorylated serine residues of PLC-β2 both in vivo and in vitro.

Genetic Mapping and Disruption of PLC Genes and the Relation of PLC to Human Disease

The 8-type PLC, which is the only known PLC in yeast and slime mold, has been disrupted in these lower eukaryotes by gene targeting (47, 48). Both mutants were viable. Whereas the yeast mutant showed increased sensitivity to various stresses, the slime mold mutant appeared normal, including with regard to such phenotypic aspects as growth, development, and chemotaxis. Chromosome positions for 10 mouse PLC genes and 8 human homologs have been targeted in mouse. The homozygous mutants for PLC-2 activity, without affecting Gqα-induced activation. The effect of PKA was not mimicked by PKC isozymes. Furthermore, PKA directly phosphorylated serine residues of PLC-β2 both in vivo and in vitro.

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