Mammalian Phospholipase C

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Abstract
Phospholipase C (PLC) converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG and IP_3 each control diverse cellular processes and are also substrates for synthesis of other important signaling molecules. PLC is thus central to many important interlocking regulatory networks. Mammals express six families of PLCs, each with both unique and overlapping controls over expression and subcellular distribution. Each PLC also responds acutely to its own spectrum of activators that includes heterotrimeric G protein subunits, protein tyrosine kinases, small G proteins, Ca^{2+}, and phospholipids. Mammalian PLCs are autoinhibited by a region in the catalytic TIM barrel domain that is the target of much of their acute regulation. In combination, the PLCs act as a signaling nexus that integrates numerous signaling inputs, critically governs PIP_2 levels, and regulates production of important second messengers to determine cell behavior over the millisecond to hour timescale.
INTRODUCTION

In animals, phospholipase C (PLC) selectively catalyzes the hydrolysis of a relatively rare phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2), on the glycerol side of the phosphodiester bond. This reaction regulates literally dozens of cellular processes (Figure 1) and hundreds more indirectly. Although the essential pathway outlined in 1987 by Berridge (1) remains fundamentally accurate, things have become far more complex.

First, PIP2 regulates many cellular processes, including the activity of PLCs, and the PLC reaction is a major pathway for PIP2 depletion. Diverse proteins depend on PIP2 as a membrane anchor or an allosteric regulator (2, 3). PIP2 is also the substrate for synthesis of a far rarer lipid, phosphatidylinositol 3,4,5-trisphosphate (PIP3), which controls an additional array of crucial signaling reactions (4). PIP2 depletion by the PLC reaction is therefore of major importance to the cell and is tightly coordinated with PIP2 synthesis to regulate the local PIP2 concentration in both the plasma membrane and the nuclear membrane (2, 3).

PLC is more famous for creating two important second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which themselves have far-reaching regulatory and metabolic roles. DAG stimulates the activity of a variety of enzymes and structural proteins by binding to a conserved C1 domain; it is also the substrate for synthesis of phosphatidic acid, which is also a regulatory molecule. IP3 gates a Ca2+ channel in the endoplasmic reticulum that is a major regulator of the cytoplasmic concentration of Ca2+, which itself is the center of a major regulatory network. In addition, IP3 is the rate-limiting substrate for synthesis of inositol polyphosphates, which stimulate multiple protein kinases, transcription, and mRNA processing. Regulation of PLC activity is thus of crucial importance to the cell, and its regulation and coordination with other enzymes of these pathways are central to the control of cellular physiology. Several excellent reviews discuss these contexts of PLC biology (5–8).

The sheer number of functions exerted by the PLC reaction demands its strict regulation and localization and its ability to respond to multiple extracellular and intracellular inputs with

Figure 1

Signaling pathways involving PIP2. PLC hydrolyzes PIP2, which is both a signaling molecule in its own right and the precursor of another signaling molecule, PIP1. The PLC reaction creates two new signaling molecules, DAG and IP3, which are substrates for the formation of yet other signaling molecules, phosphatidic acid and inositol polyphosphates. In the figure, the PLC reaction is shown in red, other signaling metabolites are blue, and regulatory targets are green. Abbreviations: DAG, 1,2-diacylglycerol; IP1, inositol 1,4,5-trisphosphate; IPn, inositol polyphosphates; PA, phosphatidic acid; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C.
appropriate kinetics and absolute activities. This need has driven the evolution of six families of PLCs in animals, each with a distinct mode of regulation (Figure 2). Most PLC pre-mRNAs are also subject to differential splicing such that up to 30 PLC enzymes may exist in mammals (7).

In this review, we attempt to relate how individual PLCs are biochemically regulated to their individual regulatory functions in cells. This is not easy. The precise mechanism of PLC regulation is incompletely understood for all of the isoforms, although recent structural insights have at least clearly defined the question. Functional specialization of PLCs is also understood only at a fragmentary level, except that we now know many of the distinct inputs to individual PLC enzymes. We focus on mammalian PLCs, but PLCs in other eukaryotes catalyze the same reaction and execute some of the regulatory functions defined in metazoa. Prokaryotic PLCs share a conserved catalytic domain with the animal enzymes but act on PI rather than PIP2 and are thought to be degradative rather than regulatory.

**PHOSPHOLIPASE C CORE ENZYME**

The inositide-selective PLCs share a conserved core structure in addition to a variety of other domains specific for each family (Figures 2 and 3). The core enzyme is composed of a pleckstrin homology (PH) domain, four tandem EF hand domains, a split TIM barrel, and a C2 domain.
active site, all catalytic residues, and a Ca$^{2+}$ binding site are contributed by the TIM barrel. This domain is interrupted by an autoinhibitory insert, which differs among the families in sequence and size and is central to regulation of activity. The presence of this insert led to the naming of the N- and C-terminal halves of the TIM barrel as X and Y domains on the basis of initially recognized sequence similarity, and the insert is referred to as the X-Y linker.

The TIM barrel is the sole domain in many prokaryotic PLCs, whose preferred substrate is PI rather than PIP$_2$. This domain is therefore sufficient for catalytic activity, at least conceptually. The C-terminal portion of most bacterial PLCs diverges markedly in sequence from the eukaryotic PLCs and appears to lack two helices of the canonical TIM barrel (9), but a PLC from *Streptomyces antibioticus* is similar in sequence and structure to the animal PLCs even in this region (PDB 3H4W).

Although the noncatalytic domains within the core are conserved among the PLC families, their functions vary. The PH domain can bind activating and anchoring ligands such as PIP$_2$,
Ca²⁺, Gβγ, or the small GTPase Rac; it is absent in PLC-ζ. In PLC-β, the EF hands, which in most proteins are Ca²⁺-binding domains, accelerate GTP hydrolysis by Gαq. The C2 domain binds Ca²⁺ and the membrane surface (10, 11). This domain also contributes to the Gαq-binding interface in PLC-β, and a C-terminal extension of C2 is required for activation by Gαq (12). Clearly, the function of a domain in one PLC family cannot be inferred from the function of the same domain in another. In addition to the globular core structure, the individual PLC families contain diverse inserts and extensions that reflect their particular regulatory roles in cells, as described below.

**CATALYTIC ACTIVITY AND MECHANISM**

Because PLC operates on an insoluble substrate at a lipid-water interface, understanding of its mechanism has come primarily from a combination of structural analysis and mutagenesis, initiated largely by Katan, Williams, and coworkers studying PLC-δ (10, 13, 14). More recent information on the active site and on similarities between PLC-δ and PLC-β are reviewed by Gresset et al. (8).

Active-site residues and surrounding structures are conserved in all PLC families. Both prokaryotic and eukaryotic PLCs initially catalyze hydrolysis of DAG from inositol phosphate to form a weakly enzyme-bound inositol 1,2-cyclic phosphodiester. This intermediate, for PIP₂ hydrolysis, is then hydrolyzed to form IP₃ (14, 15). Two conserved His residues are required for this mixed acid/base-catalyzed reaction, and a Ca²⁺ ion is also required for PIP₂ hydrolysis by the eukaryotic PLCs (13–15). The active-site Ca²⁺ is coordinated by a clutch of four acidic residues, and mutation of any of them increases the concentration of Ca²⁺ required for catalysis (13). Overall, the active site is highly conserved. The most notable difference between the eukaryotic and prokaryotic enzymes is the positioning of basic residues in eukaryotic PLCs to interact with the two additional phosphate groups on the PIP₂ substrate.

Like other phosphodiesterases, mammalian PLCs are excellent catalysts, and measurement of their maximum catalytic capacity has been limited mostly by constraints of the assay. Our group has measured the specific activity of PLC-β3 at >1,000 s⁻¹ at 30°C. We estimate that the actual kcat is >5,000 s⁻¹ because our assays use a low substrate concentration, 1–4 mol% PIP₂ in vesicles composed of PE and PS, to approximate the PIP₂ concentration in the inner leaflet of the plasma membrane. This concentration is in the first-order domain, well below Km (16). Assays from other laboratories with other PLC isoforms give similar values (see, e.g., 13, 17).

Measuring PLC activity in vitro depends crucially on the medium in which the PIP₂ substrate is presented. Consequently, both absolute activities and relative (“-fold”) activations vary dramatically among laboratories. In general, highest activities are obtained when the substrate is presented in unilamellar vesicles without added detergents. We feel that maintaining the PIP₂ concentration at a low mole fraction of total phospholipid is the most reasonable surrogate for predicting cellular activity, but doing so often leads to rapid depletion of PIP₂ in the outer leaflet of the vesicles. Thus, without care, hydrolysis decreases with time as substrate is depleted and yields an underestimate of activity. This problem is worse than it initially appears because PLCs appear to follow “scooting” kinetics: A PLC molecule is more likely to diffuse over the surface of a single vesicle and hydrolyze available PIP₂ than to sample multiple vesicles (18, 19). Too short an assay time can also lead to underestimation of activity if activation, by a G protein, for example, takes many seconds. Some investigators have simply used very high PIP₂ concentrations, as much as half of total lipids, but this essentially places the substrate on an ion-exchange surface. There is no obvious solution except to rigorously compare the measured activity of pure protein with its behavior in a cell.
Cellular PLC activity is usually measured according to the release of \[^{3}H\]IP\(_3\) or its hydrolysis products by using cells that have been prelabeled with \[^{3}H\]inositol to assumed steady state. Because of the complex and highly regulated metabolic network that controls cellular concentrations of PIP\(_2\), monitoring and standardizing the specific activity of the substrate for such experiments are difficult (see Figure 1). Direct mass spectrometric analysis with internal standards is better, but upstream metabolism and downstream metabolism make even absolute mass changes hard to interpret in terms of PLC activity alone. When subcellular compartmentalization is considered, all bets are off (2). It is not just absolute activity that is in question. Upstream and downstream reactions can alter the apparent kinetics of PLC activation and deactivation within a cell, distorting the shape and duration of an activation transient.

A putative PLC inhibitor, U73122, has been used frequently to blank such assays or, more generally, to point to a role of PLC in a cellular process. Many groups have noted nonspecific effects of U73122, however, and Klein et al. (20) recently showed that U73122 acts as a nonspecific thiol reagent that can even stimulate some PLCs (see also 21). Edelfosine may be more selective (21, 22).

To circumvent these difficulties, several groups have designed fluorescence sensors for IP\(_3\), DAG, or PIP\(_2\) that can be monitored in a physiologically reasonable time frame and, in some cases, with subcellular spatial resolution (23–28). Signal amplitude and background remain problematic, and there is no means of absolute calibration, but such cellular determinations are crucial for understanding pathway dynamics. The DAG-sensitive TRPC6 cation channel can also be used as an electrical sensor for PLC activation. Signals are excellent, and the probe can be used in cells or in detached membrane patches, but again there is no absolute quantification of PLC activity, in part because of further metabolism of the DAG (29, 30). A different sensor strategy uses the PH domain of PLC-\(\delta\)-1 fused to a fluorescent protein to monitor relative amounts of substrate and product in subcellular locations (31–35). This PH domain binds both PIP\(_2\) and IP\(_3\). In a resting cell, most of the sensor is located at the plasma membrane, indicating binding to PIP\(_2\). Upon PLC stimulation, fluorescence moves to the cytoplasm, reflecting the ability of newly synthesized IP\(_3\) to compete with depleted PIP\(_2\) for sensor binding. Although this technique does not quantitate enzyme activity or flux, it does give kinetic descriptions of changes in flux with excellent signal and subcellular resolution.

**REGULATION OF MAMMALIAN PHOSPHOLIPASE C**

The mammalian PLC families have evolved to respond to diverse regulatory inputs, but they share the same conserved domains, active site, and catalytic mechanism. It is therefore tempting to suggest that there is a common mechanism that unites their activation processes in addition to their recruitment to the membrane and their requirement for Ca\(^{2+}\).

A general mechanism for PLC activation, first stated clearly by Hicks et al. (36), involves movement of the X-Y linker in the TIM barrel domain away from the active site, which it apparently occludes. The X-Y linker is evidently inhibitory because both proteolysis (37–39) and genetic deletion (36, 40, 41) of sections of this linker activate PLC-\(\beta\), -\(\gamma\), -\(\delta\), and -\(\epsilon\). Furthermore, when the N- and C-terminal portions of PLC-\(\beta\) and PLC-\(\gamma\) are expressed as separate proteins, they can bind to form an active enzyme that has elevated basal activity (42, 43). PLC-\(\zeta\), which has a positively charged X-Y linker rather than the more common negative charge cluster, is constitutively active, and removal of this linker causes inhibition (44).

Hicks et al. (36) also based their proposal on the observation that PLC-\(\delta\) and a fragment of PLC-\(\beta\) that lacks the C-terminal coiled-coil domain show very little if any change in overall tertiary structure upon activation, as indicated by X-ray crystallography (10, 12, 36, 45). This
suggested the need for a mechanism other than a conventional conformational isomerization of the core enzyme. This idea was supported by the fact that the X-Y linker regions of PLC-β and PLC-δ appear to be either highly mobile or disordered (or both) because neither is observed by X-ray diffraction.

The active site of the PLCs is shallow and must act on a substrate that cannot protrude far from the bilayer surface. Covering the active site with a movable lid may be the most effective way of turning it off. It is tempting to imagine that the X-Y linker plays this role, maintaining the enzyme in an autoinhibited state. Movement of an autoinhibitory structure allows regulation over a wide dynamic range and is often used for regulation of enzymes whose unstimulated activities must be highly suppressed but whose stimulated activities are high.

Note that, although deletion of the X-Y linker elevates basal activities, these constitutively activated enzymes can still respond further to their individual activators. This suggests the existence of still unknown regulatory mechanisms.

**PHOSPHOLIPASE C-β**

The four mammalian PLC-β genes encode enzymes that are stimulated by Gα subunits of the Gq family, Gβγ subunits, and Ca²⁺. Individual PLC-β isoforms are also regulated by the small GTP-binding protein Rac, phosphatidic acid, and phosphorylation catalyzed by several protein kinases. The isoforms differ somewhat in their tissue and cell distribution, with PLC-β4 expressed preferentially in neural tissue, PLC-β1 somewhat less so, PLC-β2 expressed preferentially in immune cells, and PLC-β3 expressed more widely. It is common for a single cell type to express two or three PLC-βs. Two splice products of each gene are known at the mRNA level (7). The two PLC-β1 proteins, whose extreme C termini are encoded by different exons, assume different subcellular distributions. Structurally, the PLC-βs are unique in having a C-terminal three-stranded coiled coil, which is required for stimulation by Gαq (see below).

**Regulation by Heterotrimeric G Proteins**

The PLC-βs are stimulated most strongly by Gαq subunits, up to several hundredfold (46, 47), with EC₅₀ values as low as 1–2 nM (16). PLC-β2 is the least sensitive to Gαq but is still activated more than tenfold. Gαq and Gα₁₁ activate the PLC-βs equally well both in cells and after purification, and there is no evidence that the other Gαq family members, Gα₁₄ and Gα₁₆, differ appreciably. (We refer to Gq below without distinguishing among the four Gq isoforms.)

PLC-β2, -β3, and -β1 are also activated by the Gβγ subunits, in declining order of effect, and thereby respond to Gq-coupled receptors (see sidebar, Why Gq Is the Cellular Source of Gβγ Subunits). PLC-β2 is the most Gβγ-sensitive isoform, and a strong Ca²⁺ response to stimulation of a Gq-coupled receptor suggests its mediation by PLC-β2. PLC-β4 does not respond to Gβγ detectably (48). The absolute extent of activation and the selectivity of an isoform for Gβγ, Gαq, or both will vary according to details of the assay (46, 47) and cell type. Typically, cellular responses mediated through Gβγ and PLC-β2 are smaller than those mediated by Gαq and are frequently somewhat longer lasting, consistent with in vitro assay data.

**Stimulation by Gαq**. The mechanism whereby Gαq activates PLC-β remains controversial despite recent important advances in understanding the structure of the PLC-β-Gαq complex. Waldo et al. (12) solved the structure of a fragment of PLC-β3 that lacks the C-terminal coiled-coil domain bound to Gαq, which was activated by the GTP analog GDP/Al₃⁻/P⁻₄ (Figure 4). This complex showed three principal sites of contact between PLC-β3 and Gαq. The switch
WHY G\textsubscript{i} IS THE CELLULAR SOURCE OF G\textsubscript{\beta\gamma} SUBUNITS

Why is G\textsubscript{\beta\gamma}-mediated signaling almost always initiated by G\textsubscript{i}- or G\textsubscript{o}-coupled receptors? It is not because they bind different G\textsubscript{\beta\gamma} dimers. The answer is that only G\textsubscript{i} is abundant enough to make it work. G\textsubscript{\beta\gamma} acts on its effectors—PLCs, ion channels, adenylyl cyclases, PI-3-kinase, etc.—at concentrations at least tenfold higher than do most G\textsubscript{x} subunits. In isolation, EC\textsubscript{50} values for G\textsubscript{\beta\gamma} are usually in the 20–200 nM range or even higher, depending on the effector protein and the assay. Common EC\textsubscript{50} values for G\textsubscript{i}, G\textsubscript{q}, or G\textsubscript{12/13} are often 1–5 nM, and they are expressed at appropriately low levels. Thus, even complete activation of one of these G proteins would provide inadequate G\textsubscript{\beta\gamma}. G\textsubscript{s} are expressed at higher levels than are other G protein heterotrimers such that only they have the stoichiometric capacity to release significant amounts of G\textsubscript{\beta\gamma}. In addition, GTP-activated G\textsubscript{x} subunits generally bind G\textsubscript{\beta\gamma} less tightly than do G\textsubscript{s}, G\textsubscript{q}, or G\textsubscript{12/13}. The stoichiometric difference is therefore amplified. When overexpressed, however, the other G\textsubscript{x} subunits and their receptors are capable of initiating G\textsubscript{\beta\gamma}-mediated signals.

regions 1 and 2 of G\textsubscript{x} contact both the EF hand domain and the linker region between the TIM barrel and C2 domains, and a helix-turn-helix motif (termed H\textsubscript{1}/H\textsubscript{2} just C-terminal to the C2 domain of PLC-\textbeta\textsubscript{3} binds to a shallow surface on G\textsubscript{x} that also binds other effectors. Mutagenesis demonstrated the importance of these contact points, the C2 domain interactions were predicted by previous studies (49, 50), and a chimeric PLC-\textdelta with the helix-turn-helix extension added to its C2 domain became somewhat sensitive to stimulation by G\textsubscript{x} (12).

Surprisingly, the core PLC domains did not show a conformational rearrangement or local rearrangement of the active site upon G protein binding when compared with the presumably nonactivated structure of isolated PLC-\textbeta\textsubscript{2} (36). Furthermore, the structure of isolated PLC-\textbeta\textsubscript{2} was essentially identical to that of presumably activated PLC-\textbeta\textsubscript{2} bound to Rac1 (45). Following Hicks et al. (36), the authors proposed that G\textsubscript{x} does not promote conformational isomerization of PLC-\textbeta but only anchors and orients it at the membrane surface such that the autoinhibitory X-Y linker is moved away from the active site. They further suggested that the X-Y linker is pushed away because its negative charge is repelled from the negatively charged membrane surface. A caveat to this mechanism, discussed below, is that the PLC-\textbeta\textsubscript{3} fragment used in this and the following study lacks the coiled-coil domain, which is required for efficient activation of PLC-\textbeta\textsubscript{s} by G\textsubscript{x} (2). A second, probably more minor, caveat is that the G\textsubscript{x} construct lacks correct N-terminal modification and activates PLC-\textbeta with low potency, <1% of wild type in our hands.

An alternative mechanism of activation was proposed by Lyon et al. (51), who solved structures of a slightly longer fragment of two invertebrate PLC-\textbeta\textsubscript{s} in the absence of G\textsubscript{x}. In this nonactivated structure, the H\textalpha\textsubscript{1}/H\textalpha\textsubscript{2} region is rearranged, and a more C-terminal helix, H\textalpha\textsubscript{2}, folds back onto the PLC core enzyme in close proximity to the X-Y linker (nearby 50 Å from its location in the complex with G\textsubscript{x}) (Figure 4). The H\textalpha\textsubscript{1} region is disordered. Comparison of this structure with that of the PLC-\textbeta-G\textsubscript{x} complex suggests that G\textsubscript{x} binds the H\textalpha\textsubscript{1}/H\textalpha\textsubscript{2}/H\textalpha\textsubscript{2} region to move it away from its TIM barrel contact. Mutagenesis aimed at disrupting the binding of H\textalpha\textsubscript{2} to the core enzyme decreased the thermostability of full-length mammalian PLC-\textbeta\textsubscript{3}, suggesting that this helix is strongly associated with the catalytic core in the absence of G\textsubscript{x}-mediated activation. Furthermore, deletion of H\textalpha\textsubscript{2} from a PLC-\textbeta\textsubscript{3} fragment that lacked the coiled-coil domain increased basal activity threefold, suggesting that this region is somehow involved in autoinhibition of the enzyme. More convincingly, mutation of residues on either side of the proposed interface between H\textalpha\textsubscript{2} and the TIM barrel markedly increased activity of intact PLC-\textbeta\textsubscript{3}. On the basis of these data, the authors proposed that H\textalpha\textsubscript{2} stabilizes the X-Y linker.
Conformational changes in PLC-\(\beta\) upon G\(\alpha_q\) binding. The structures of Sepia officinalis PLC21 (PDB 3QR0) and the complex of human PLC-\(\beta_3\) with G\(\alpha_q\) (PDB 3OHM) are overlaid from the N terminus through the C2 domain, with color coding as in Figures 2 and 3. G\(\alpha_q\) is depicted as a green surface. Note the close similarity in structure between the core domains of the two PLCs. In PLC21, H\(\alpha_1\) and the N-terminal end of H\(\alpha_2\) are disordered (dotted line), and H\(\alpha^2'/\alpha_3\) (red helices) packs against the core enzyme. In the complex of PLC-\(\beta_3\) and G\(\alpha_q\), H\(\alpha_1'/\alpha_2\) (pink helices) makes extensive contact with G\(\alpha_q\), which rotates this region \(\sim 50\) Å away from the core enzyme. The other G\(\alpha_q\)-interacting regions in the EF hand and C2 domains of PLC-\(\beta_3\) are shown in dark green. H\(\alpha_3\) is absent in the PLC-\(\beta_3\)-G\(\alpha_q\) complex. Structural alignment of the PLCs was done in Pymol. Neither protein contains the coiled-coil domain.

In an autoinhibitory conformation and that G\(\alpha_q\) binding to H\(\alpha_1/\alpha_2\) moves H\(\alpha^2'\) to cause, or contribute to, PLC-\(\beta\) activation.

In addition to the domains present in the crystal structures described above, the C-terminal coiled-coil domain is required both for stimulation of PLC-\(\beta\) by G\(\alpha_q\) and for G\(\alpha_q\) GTPase-activating protein (GAP) activity (52–54). Its parallel effects on both activities suggest that the coiled-coil domain contributes to G\(\alpha_q\)-PLC-\(\beta\) binding. This idea is consistent with observations that the isolated coiled-coil domain inhibits both GAP activity and PLC stimulation in vitro (55), it acts as a dominant inhibitor of G\(\alpha_q\) signaling in cells (56, 57), and it displays low but clearly measurable G\(\alpha_q\) GAP activity (55). The isolated coiled-coil domain crystallizes as an antiparallel dimer of a three-stranded coiled coil (58). The validity of the structure is supported by the observation that inhibitory mutations in the coiled-coil domain clustered in two areas of the tertiary structure, the dimer interface and a slight concavity on the dimer surface, even though they do not cluster in the primary sequence (54). The precise function of the coiled-coil domain remains...
unclear. A truncated PLC-β3 that terminates shortly after the C2 domain can still bind Goq, and in the absence of phospholipids, its affinity for Goq is not notably less than that of the full-length protein (although its phospholipase activity is insensitive to Goq) (12). These data led Sondek and coworkers (8, 12) to suggest that the only function of the coiled-coil domain is to provide membrane anchorage, perhaps as a means for local concentration or to orient the PLC core with respect to the bilayer. This mechanism seems inconsistent with the fact that removal of the coiled-coil domain has no effect on PLC-β stimulation by Gβγ, Rac, or Ca2+ when the PIP2 substrate is presented in the context of a phospholipid bilayer (52, 53, 59). We think it more likely that the coiled-coil domain interacts directly with Goq, forming yet another contact point, although this speculation will not be tested until the structure of a complex of the two intact proteins is known.

Stimulation by Gβγ. Activation of PLC-β by Gβγ apparently involves binding through two distinct interfaces on the core enzyme and does not require the coiled-coil domain or the C2-coiled-coil linker. There is no X-ray structure of a PLC-β-Gβγ complex, however, and both proposed binding sites remain somewhat problematic. Fluorescence resonance energy transfer assays suggest that Gβγ binds directly to the PH domain (60). Removal of the PH domain blocks response to Gβγ, although the response of the truncated PLC to Goq is unaltered (53). More convincingly, a chimeric PLC in which the PH domain of PLC-δ1 replaces that of PLC-δ2 responds to Gβγ (61). The PLC-β2 PH domain is therefore essential for response to Gβγ, even if it is not an actual binding site. If there is direct binding, the Gβγ binding site on the PH domain is unlikely to resemble that on the PH domain of GRK2 (62) because it would cause steric conflict between Gβγ and the TIM barrel. Scarlata et al. (63) proposed that Gβγ binding moves the PH domain with respect to the TIM barrel, which would solve this problem. Their suggestion drew on the apparent flexibility of the PH domain of PLC-δ1 (10) and on changes in intramolecular fluorescence resonance energy transfer that they observed when PLC-β2 was activated by Gβγ. This proposal also allows Gβγ to bind to a PH domain surface away from the binding site for Rac, consistent with the idea that Gβγ and Rac1 can bind simultaneously to PLC-β2 (64) (see below). Arguing against this idea is the observation that the domain interface between the TIM barrel and the PH domain is apparently immobile in several crystal structures, including the complex with Rac1, but none of these contain Gβγ (12, 36, 45, 51).

The second putative Gβγ binding site lies on the C-terminal half of the TIM barrel domain (65). This interaction was inferred from the ability of peptide fragments of this region in PLC-β2 to block PLC stimulation by Gβγ and from the cross-linking of these peptides to isolated Gβγ (66). Furthermore, mutation of PLC-β2 in this region markedly reduced stimulation by Gβγ (67). This site is essentially on the opposite side of the TIM barrel from the PH domain, and it is unlikely that Gβγ binds both here and to the PH domain unless the PH domain reorients dramatically. This TIM barrel interaction is provocative in that it lies near the autoinhibitory X-Y linker and near the Hα2′ extension of the C2 domain that may stabilize the linker (51). If Gβγ binds here, it could easily interact with the X-Y linker, although no structural data directly support this idea. It would also suggest that Gβγ and Goq activate by structurally similar mechanisms, consistent with the idea that they stabilize the same active state of PLC-β (46) (see below).

Other Regulatory Inputs
PLC-βs respond to multiple inputs in addition to G proteins. All four PLC-βs are stimulated by Ca2+ over a physiologically important concentration range, although cellular effects of Ca2+ have not been studied in detail with respect to regulation by the other ligands.
PLC-β2 is stimulated directly by the small GTP-binding proteins Rac1, Rac2, and Cdc42 (68, 69), and PLC-β3 responds weakly (64, 70). Rac binds to the PH domain of PLC-β2 (70), and the structure of the Rac1-PLC-β2 complex has been solved (45). The structure of the PLC-β2 core in this complex (PH, EF hands, TIM barrel, C2) is essentially identical to that of isolated PLC-β2 (36), suggesting that Rac does not induce a conformational change in PLC. Illenberger et al. (64) found that Rac1 can activate a C-terminally truncated PLC-β2 in the presence of Gβγ, suggesting that the binding sites for Rac and Gβγ do not overlap. Stimulation by the two ligands was about additive, but it was not clear that Gβγ was saturating in this experiment.

Last, PLC-β1, -β2, and -β3 are phosphorylated by protein kinases A, C, and G and calmodulin-stimulated kinase II (71–77). All reported phosphorylation sites, one each for PLC-β1a and PLC-β2a and three for PLC-β3, are serine residues that are not conserved among different isoforms. Phosphorylation has been reported either to inhibit activity, to attenuate stimulation by G protein, or both (73, 75–79). Activation by phosphorylation has not been reported, and we have not detected effects of PLC-β phosphorylation on Gq GAP activity.

Scaffolding PLC-β
One way in which multiple and overlapping signals to and from PLC-βs are organized is through the action of scaffolding proteins. Several scaffolding proteins bind PLC-βs and other membrane proteins with which they functionally, if not physically, interact. The effects of scaffolding proteins on the regulation of PLC-βs are important in several cases. Their quantitative effects on activity, stimulation by various ligands, and selectivity among ligands have been more difficult to estimate, but removal of the scaffolds frequently has pronounced effects on cellular signaling. The classic PLC-β scaffold is InaD, a penta-PDZ-domain protein that organizes the fundamental signaling complex in the Drosophila visual photoreceptor cell (80). InaD binds the Drosophila PLC-β, NorpA, and protein kinase C on a membrane surface that is dominated by rhodopsin, and loss of InaD by mutation causes functional blindness. All four mammalian PLC-β isoforms also have a consensus PDZ-binding peptide at their C termini (on at least one mRNA splice product), and about half of the Gq-coupled receptors also have a C-terminal PDZ-binding motif (80, 81). Therefore, it is not surprising that at least three PDZ domain–containing proteins scaffold a PLC-β in some fashion, most frequently keeping PLC-β in contact with its upstream receptor. PAR3, with three PDZ domains and a PKC-binding domain, binds PLC-β3 (82). NHERF2 (Na+/H+ exchanger regulatory factor 2), with two PDZ domains, binds either PLC-β1 or PLC-β2 and an LPA receptor (83, 84). PAR3 may act similarly to NHERF2 (85). NHERF3 binds PLC-β3 and a somatostatin receptor (86). Shank2, which forms a homodimer with one PDZ domain per monomer, also binds PLC-β3 (87). In addition, Shank2 is bound by yet another scaffolding protein, Homer, which also regulates the G, GAP activity of PLC-β3 (81, 88, 89) (see below). WDR36, a protein based on WD40 repeats, binds PLC-β and at least two G protein–coupled receptors (GPCRs) (90). These scaffolding interactions certainly influence the regulation of PLC-β. In addition, it is hard to imagine that the scaffolds that bind the PLC-βs do not also impact their subcellular localization and, perhaps, the consequent channeling of the IP3 and DAG products to their sites of action.

PLC-β in the Nucleus
Most PLC-β protein is associated with the plasma membrane, but a substantial amount can also be found in cytosolic fractions of some cells. The shorter PLC-β1 splicing product localizes preferentially to the nucleus, and the longer is found in both cytoplasmic and nuclear fractions (91, 92). Although nuclear PLC-βs may play roles in cell growth and differentiation, their mechanism
of regulation and how they influence nuclear processes are less well understood. Some effects result from the second messenger activities of DAG and IP₃ (see 93 for a review); others may reflect the role of IP₃ as a substrate for generation of polyphosphoinositides, which control mRNA processing, chromatin structure, and other nuclear events (93–95). Specific nuclear functions of PLC-β have been reviewed elsewhere (92, 93, 96, 97).

Additive and Synergistic Regulation of PLC-βs

The diversity of inputs to the PLC-βs raises the question of how their outputs are determined: additive, less than additive (adaptive), or greater than additive (synergistic). Substantial synergism can result in coincidence detection, a process in which only both inputs received simultaneously can elicit a significant physiological response. In many mammalian cells, simultaneous stimulation of a Gq-coupled receptor and a Gi-coupled receptor produces synergistic signals measured as PIP₂ hydrolysis or IP₃-driven Ca²⁺ signals (see 46 for references). The response to both ligands can be more than fourfold higher than the sum of the responses to either receptor alone. Seaman’s group (98, 99) showed that such synergism requires expression of PLC-β3 but not that of PLC-β1 or PLC-β4, and Philip et al. (46) found that purified PLC-β3 produces up to a tenfold synergistic output to Gaq and Gβγ. Synergism displayed relatively broad concentration optima for Gaq and Gβγ that can easily account for the synergistic effects displayed in cells. PLC-β3 thus behaves as a coincidence detector for Gi and Gq signaling. PLC-β2, which also responds well to both Gβγ and Gaq, was reported not to display synergism (46), and results of cellular studies were ambiguous (99). More recent experiments have detected weak (≤twofold) synergism between Gβγ and Gaq (A.C. Dugas & E.M. Ross, unpublished data), only about 20% of that displayed by PLC-β3. Synergism itself may be regulated by yet a third input.

The synergistic response of PLC-β3 to Gβγ and Gaq can be formally explained by a two-state allosteric mechanism, which also explains why the other PLC-β isoforms do not display synergism (46). The two-state model formally holds that the active state of the PLC is the same whether its formation is driven by Gβγ, Gaq, or both. Henis and coworkers (100, 101) have shown that Rac, Gaq, and Gβγ cause distinct kinetic patterns both of lateral diffusion and of membrane association/dissociation for PLC-β2 (100, 101). Although these observations do not prove that each ligand has a distinct mode of activation or drives creation of a distinct active state, they do caution against assuming a single mechanism. Learning the mechanism whereby Gaq and Gβγ (and Rac) activate PLC-βs will therefore also be crucial for a detailed understanding of their synergism on PLC-β3.

Ca²⁺ at the active site is a general amplifier of PLC-β activity, and relative (“-fold”) stimulation by Ca²⁺ is similar regardless of what other stimuli are present (46). Over the presumed physiological range (50 nM–2 μM free Ca²⁺), stimulation of activity is five- to tenfold. In this sense, Ca²⁺ can also be said to act synergistically with respect to other stimuli.

PLC-βs Are GTPase-Activating Proteins for Gaq

Although Gaq stimulates the activity of PLC-β, PLC-β also regulates Gaq by accelerating its deactivation. PLC-β accelerates hydrolysis of Gaq-GTP, and consequent Gaq deactivation, about 1,000-fold (102). PLC-β is thus a GAP for Gaq, and PLC-β1 was the first GAP for a heterotrimeric G protein to be discovered (103). All four PLC-β isoforms display Gaq GAP activity and act on both Gaq and Gβγ. GAP activity on Gaq and Gβγ has not been tested to our knowledge, but cellular signaling behaviors are consistent with their similar sensitivity. It is difficult to quantitate the Gaq GAP activity of PLC-β in cells or membranes, but comparison of the GTPase activity and
guanine nucleotide binding in membranes from PLC-β-deficient mutants indicates that PLC-β exerts GAP activity in cells (12, 104–106).

The structural basis of GAP activity was recently elucidated by Waldo et al. (12). In the Gαq-PLC-β structure, an asparagine residue on a loop from the EF hand domain interacts directly with the conserved switch II region of Gαq, which is adjacent to the bound GTP. This interaction stabilizes switch II and orients residues necessary for hydrolysis, ultimately stabilizing a transition-state structure in GTP that leads to hydrolysis (reviewed in 107). This mechanism is functionally and structurally similar to that used by RGS proteins, the other major class of G protein GAPs. Although there is no primary sequence similarity between the PLC-βs and RGS proteins, their similarities include a loop that extends from PLC to the switch II region of Gαq, the specific contacts between the molecules, and the use of an asparagine residue to make the principal contact (12, 107, 108). This mechanism does not require participation of the C-terminal coiled-coil domain, but Gδ GAP activity is not observed when the coiled-coil domain is absent or mutated, perhaps because it is required for high-affinity binding to Gδ on a membrane surface (54). Alternatively, the GAP activity of the coiled-coil domain (55) may indicate a second interaction necessary for maximal acceleration of GTP hydrolysis.

The principal function of PLC-β’s GAP activity seems to be to terminate Gq-PLC-β signaling immediately after dissociation of agonist from the upstream receptor (109). Isolated Gq hydrolyzes bound GTP slowly, with a half-time of about 30 s at physiological temperature, which would cause significant delay in signal termination when the receptor is deactivated. The Gq GAP activity of PLC-β allows it to deactivate with less than 0.1 s decay time. In design terms, PLC-β’s GAP activity decreases the sampling interval at which the PLC interrogates the incoming signal from the receptor. To extend this metaphor, the value of having GAP activity intrinsic to the effector instead of residing in a different protein is that GTP-activated Gαq can exist in a more stable state until PLC-β is engaged. Then, perhaps with the help of structural or kinetic scaffolding (see below), PLC will remain active until the receptor is deactivated, but no longer.

The ability of PLC-β to accelerate Gq deactivation more than 1,000-fold raises the question of how Gq manages to signal through PLC-β at all, given the relatively slow rates of receptor-promoted GDP/GTP exchange measured previously. In the case of PLC-β, several plausible explanations exist (see 109 for review). We initially suggested that because GTP hydrolysis by the receptor-Gαq-PLC-β complex is fast, the receptor may remain bound during the lifetime of the GTP-activated state and thus be available to catalyze a new round of GDP/GTP exchange without the potentially rate-limiting step of diffusion-limited rebinding. This mechanism, known as kinetic scaffolding for its ability to maintain association of receptor, Gαq, and PLC, is supported by the observation that PLC-β also increased the rate of GTP binding in a reconstituted three-protein system (16, 110, 111). It is consistent with the multipoint Gαq-PLC-β binding described by Waldo et al. (12) because the contact between Gαq and the helical extension of the C2 domain may tether the proteins together even after Gq deactivation weakens the interface between the switch regions of Gq and the EF hand and C2 domains of PLC. In addition to kinetic scaffolding, steady-state kinetic analysis of GTPase cycle turnover suggests that PLC-β actually potentiates the intrinsic activity of the receptor as a nucleotide exchange catalyst (112). Other possibilities involve the ability of Gβγ to inhibit GAP activity (53, 113).

PHOSPHOLIPASE C-γ

The two PLC-γ isoforms are characterized by a large multidomain insert in the X-Y linker that is central to their regulation (Figure 2). A so-called split PH (sPH) domain is composed of residues at either end of the linker that fold into the tertiary structure of a standard PH domain (114). Two
SH2 domains, referred to as nSH2 and cSH2, and one SH3 domain emerge from the sPH domain such that the total insert is similar in size to the core enzyme. The sPH, nSH2, cSH2, and SH3 bind numerous cellular scaffolding and regulatory proteins within diverse multiprotein signaling complexes. At least in part, phosphorylation activates PLC-γ by removing autoinhibition by the X-Y linker structure. There is no information available on the tertiary structure of the complete insert or on its relationship to the core enzyme. Most inferences about regulation of PLC-γ activity thus stem from correlation of specific contacts with changes in function.

PLC-γ1 and PLC-γ2 are similar in structure, and their regulation appears to be similar in most cases. PLC-γ1 is widely expressed, whereas PLC-γ2 is expressed primarily in the immune cell lineage, but many cells express both isoforms (reviewed in 115). To generalize, PLC-γ1 appears to be important primarily for control of cell growth and differentiation in response to both receptor tyrosine kinases and soluble tyrosine kinases that are recruited to the plasma membrane by assorted inputs. In immune cells, PLC-γ2 acts downstream of soluble tyrosine kinases recruited by T and B cell receptors and modulates more acute responses (116). In cells that express both enzymes, each can exert nonoverlapping functions, and one enzyme generally cannot compensate for depletion of the other (117, 118).

**PLC-γ Regulation by Tyrosine Protein Kinases**

The major and best-studied mechanism of activation of PLC-γ is through phosphorylation of a single conserved tyrosine residue (Tyr783 in PLC-γ1, Tyr759 in PLC-γ2) (119). Depending on the cell, the stimulus, and the PLC-γ isoform, phosphorylation can be catalyzed by receptors for epidermal growth factor (EGF) (the prototype; 120), platelet-derived growth factor (PDGF), nerve growth factor (NGF), or fibroblast growth factor (FGF) and by multiple soluble tyrosine protein kinases. These soluble kinases may be recruited by cell surface receptors, such as the T or B cell receptor or FcεR1, or they may be activated by extended signaling pathways initiated by GPCRs, integrins, or other tyrosine kinase receptors.

Activation of PLC-γ by receptor tyrosine kinases begins when, in response to agonist binding, the receptor autophosphorylates to provide phosphotyrosine docking sites that recruit PLC-γ. A receptor may have one or more docking sites that allow efficient PLC-γ phosphorylation, and mutation of these phosphorylated tyrosine residues prevents PLC-γ activation even though the receptor may be capable of phosphorylating other target proteins. The PLC-γ nSH2 mediates association with the receptor, and mutating the nSH2 domain, but not cSH2, to prevent phosphotyrosine recognition abolishes PLC-γ phosphorylation.

PLC-γs are typically phosphorylated on multiple Tyr residues with varying effects, but it was shown as early as 1991 that phosphorylation of Tyr783 is both necessary and sufficient for stimulation of lipase activity (119). This result has now been confirmed with numerous kinases (reviewed in 8). Tyr783 lies between the cSH2 and SH3 domains. Poulin et al. (121) showed that the binding of cSH2 to pTyr783 is required for activation, and Gresset et al. (40) directly confirmed binding. These authors also showed that binding causes a significant conformational change in the intact PLC-γ1 molecule, readily detectable by changes in Stokes radius. They then used a series of deletion mutations to argue that the conformational change reflects a reorientation of the X-Y linker structure with respect to the core enzyme. This reorientation presumably allows the substrate access to the otherwise occluded active site.

Receptors phosphorylate several other Tyr residues in PLC-γ in addition to Tyr783. Additional phosphorylation depends on cell type and stimulus. Its effects can include efficiency of interaction and the recruitment of other signaling proteins, but in many cases its functions have not been assigned (5).
PLC-γ is also activated by phosphorylation catalyzed by soluble tyrosine protein kinases such as Btk and Tec, usually in the context of membrane-bound, multiprotein complexes. These complexes may be constitutive or dependent on incoming signals for their formation. Upstream tyrosine kinases that probably do not phosphorylate PLC-γ directly include Syk and Zap70, and many others have been implicated. A good feeling for the complexity and level of current mechanistic understanding of these processes can be gained from reviews on PLC-γ signaling in specific cell types or pathways (see, e.g., 116, 118, 122, 123). The tyrosine protein phosphatases that deactivate PLC-γ have not been identified, nor has regulation of dephosphorylation, although deactivation can occur in cells within minutes after removal of a stimulus.

The SH3 domain of PLC-γ is involved in formation of multiprotein complexes that contain both upstream regulators and downstream effectors. Targets of the SH3 domain, which binds proline-rich sequences, may include several adaptor and scaffolding proteins (SWIP, Cbl, Lcp2), cytoskeleton components (microtubule-associated proteins, cortical actin complexes, dynamin), and diverse signaling proteins (Akt, TrpC3 channels, SHIP1, PIKE). Caveats regarding specificities, physiological importance, and the roles of other associated proteins apply to most of these observations.

**PLC-γ Regulation by Rac and PIP3**

Prompted by suggestions of Rac-mediated PLC-γ2 activation in B lymphocytes, Piechulek et al. (124) showed that PLC-γ2, but not PLC-γ1, is directly stimulated by the small GTPases Rac1 and Rac2. Stimulation is independent of tyrosine phosphorylation. Because only PLC-γ2 is sensitive to Rac, this path to PLC activation exists only in immune cells. Rac2 regulates PLC activity by binding to the sPH domain in the X-Y linker (124, 125). However, the Rac binding surface is different from that on the N-terminal PH domain of PLC-β2 (126). It is tempting to suggest that binding of Rac to the split PH domain moves the X-Y linker away from its autoinhibitory conformation, but this idea remains to be tested. Stimulation by Rac also involves recruitment of PLC-γ2 to sites of Rac activation on the plasma membrane (124, 127).

Last, both PLC-γ isoforms are stimulated by PIP3, an important lipid messenger that regulates growth, differentiation, and motility (128–130). PIP3 is synthesized by PI 3-kinases, a family of enzymes that are regulated by tyrosine phosphorylation, Gβγ subunits, and scaffolding proteins. Stimulation of PLC-γ by PIP3 means that PLC-γ can integrate inputs from multiple tyrosine kinases and Gβγ-coupled receptors that mediate diverse cell functions. Relatively high concentrations of PIP3 are required to activate PLC-γ1 maximally in vitro (∼100 μM), and little activation was noted below 40 μM. Such low potency suggests that there is a threshold concentration below which PIP3 has no effect, but it is also consistent with recruitment or activation of PLC-γ by PIP3 in specific domains of the plasma membrane. Recruitment appears to be a significant component of stimulation by PIP3 (129). The response of PLC-γ to PIP3 apparently depends both on the N-terminal PH domain and on the SH2 domains in the X-Y linker. It is not clear how stimulation by PIP3 and tyrosine phosphorylation are integrated in cells, but effects of the two stimuli are probably at least additive. Despite the obvious importance of PIP3 regulation of PLC-γ, it has not yet been well studied. This probably reflects the difficulty in differentiating direct stimulation of PLC-γ by PIP3 from (a) stimulation by tyrosine protein kinases that act downstream of PIP3, (b) stimulation by a tyrosine kinase that also stimulates a PI 3-kinase, or (c) a feedforward mechanism in which the tyrosine kinase both stimulates PLC-γ directly and stimulates a PI 3-kinase leading to PLC-γ stimulation by PIP3. Such experiments will require working in cells that are engineered both to lack endogenous PLC-γ and to express a PLC-γ mutant sensitive to only one input.
PHOSPHOLIPASE C-δ

The three PLC-δ genes (numbered 1, 3, and 4) appear to be the progenitor animal PLCs, in that they most closely resemble those in fungi and other single-cell eukaryotes (6). The PLC-δ proteins consist of only the core domains (Figure 2), and their only well-defined regulatory ligands are Ca\(^{2+}\) and PIP\(_2\). They are expressed in almost all cells in mammals, although usually at low protein concentrations (7). A catalytically inactive paralog of PLC-δ, PRIP, is thought to act as a scaffolding protein (131, 132).

Depending on cell type, the PLC-δs are generally found distributed between the cytoplasm and various membrane fractions, and subcellular sorting and membrane recruitment are thought to be the principal mechanisms for their regulation. The PH domains of the PLC-δ family are unique in that they bind PIP\(_2\) tightly (34, 133–136). PIP\(_2\) binding drives their membrane association and promotes PIP\(_2\) hydrolysis. Depletion of plasma membrane PIP\(_2\) in cells drives PLC-δ\(_1\) from the plasma membrane to the cytosol, and mutation of PIP\(_2\)-interacting residues in the PH domain blocks membrane association and activity (19, 135, 137). PIP\(_2\)-mediated membrane binding is not sufficient to activate PLC-δ, however, and Ca\(^{2+}\), in addition to Ca\(^{2+}\) at the active site, is required for maximal activity.

Ca\(^{2+}\) in the 10–100 nM range is the second significant activator of PLC-δ (138–141). Three or four Ca\(^{2+}\) ions bind to the C2 domain of PLC-δ, in addition to the Ca\(^{2+}\) at the active site in the TIM barrel domain (11, 141, 142). Ca\(^{2+}\) binding promotes movement of PLC-δ, particularly PLC-δ\(_1\), from cytosol to the plasma membrane and also increases PLC-δ’s catalytic activity in vitro. A general model for PLC activation proposed by Sondek and coworkers (12, 36) suggests that these two functions are linked, i.e., that stronger membrane binding displaces the autoinhibitory X-Y linker to allow faster catalysis.

The PLC-δs are differentially distributed between nucleus and cytoplasm, and that distribution is coupled to the cell cycle. PLC-δ\(_4\) is constitutively nuclear, but it accumulates primarily during the G\(_1\)-S interface and is apparently degraded subsequently (143). PLC-δ\(_1\) also accumulates in the nucleus to some extent during G\(_1\)-S, but it then moves back to the plasma membrane under the direction of a constitutive nuclear export signal (144, 145). Stallings et al. (144) suggested that transient nuclear accumulation of PLC-δ\(_1\) reflects its binding to nuclear PIP\(_2\), which increases at G\(_1\)-S, because a mutation that blocks high-affinity PIP\(_2\) binding also inhibited nuclear accumulation.

How cells use PLC-δ as a signaling molecule is unclear on the basis of these biochemical behaviors. Observation of animals or cells with deleted or inhibited expression of PLC-δ isozymes has confirmed their importance but has not shed much light on how they act in particular signaling circuits (reviewed in 7, 8). A common speculation, reviewed by Rebecchi & Pentyala (6), is that PLC-δs intensify and prolong Ca\(^{2+}\) signals by responding to elevated cytosolic Ca\(^{2+}\) that has been evoked by the action of other PLCs. The extent to which this occurs is hard to quantitate, and it is unknown how this potentiation might be regulated. Interactions of PLC-δ with other proteins have also been observed, but their effects are small relative to the extent of activation caused by Ca\(^{2+}\) (146–149).

PHOSPHOLIPASE C-ε

PLC-ε was first discovered as a binding partner of Ras in Caenorhabditis elegans (150) and was quickly shown to have a single ortholog in mammals that is expressed in most if not all cell types (151–153). Since then, PLC-ε has been found to act downstream of almost every conceivable signaling pathway: GPCRs coupled to G\(_q\), G\(_s\), G\(_{12}\), and G\(_{13}\); several tyrosine kinase receptors; and other inputs to Ras, Rap, and Rho signaling modules. This rich literature was recently reviewed...
carefully by Smrcka at al. (154). PLC-ε is the direct target of Ras, Rap, and Rho. It can therefore respond to all the varied inputs that regulate these GTP-binding proteins. In most cases, details of the intermediary reactions are not clear, but the phenomena are observed in multiple tissues (see 154, 155 for reviews). In addition to its ability to hydrolyze PIP₂, PLC-ε also activates Rap/Ras. PLC-ε can thus integrate concurrent primary signals while modulating their input over time.

The GTP-bound forms of Rap and Ras stimulate PLC-ε by binding to the second of its two C-terminal Ras association (RA) domains (151, 152, 156). (The role of the first is unclear.) Depending upon cellular context, any of the signaling pathways that activate Ras or Rap can cause PLC-ε activation. These include tyrosine protein kinases [presumably acting on Ras GDP/GTP exchange factors (GEFs)], G_s-coupled receptors (presumably acting via Epac, a cyclic AMP–sensitive Rap GEF) (157), and G_i-coupled receptors (presumably acting via Gβγ and poorly defined intermediary pathways) (158, 159). Even G_s-coupled receptors can stimulate PLC-ε in cells via a pathway that involves Ca^{2+}, activation of adenyl cyclase, and consequent Epac activation (160).

The structural mechanism of PLC-ε stimulation by Ras or Rap is not known. By analogy to the coiled-coil domain of PLC-β, the C-terminal RA domains may act as a membrane-linked lever to relocate the catalytic domain with respect to the membrane surface and thus alter the position of the autoinhibitory X-Y linker (12), or they may bend the region just past the C2 domain to alter its interaction with the linker (51). They might also position their protein ligands near the catalytic domain and directly influence the active site or X-Y linker. Mutating the region past the C2 domain of PLC-ε on the basis of results with PLC-β could clarify these questions until structures are known.

In addition to stimulating the catalytic activity of PLC-ε, Ras and Rap also participate in recruiting PLC-ε to specific organelles. Song et al. (152) showed that either expression of an activated mutant of Ras or stimulation of the EGF receptor, which activates Ras via the Ras GEF son of sevenless (SOS), promotes the movement of PLC-ε to the cell periphery (152). Recruitment requires the RA2 domain, as does activation (156). In contrast, activated Rap recruits PLC-ε to a region near the nucleus, presumably the Golgi, where Rap is concentrated (161). How this process is regulated is unknown, but it may occur when Rap is activated downstream of cell surface receptors. An intriguing aspect of this movement is that a mutant PLC-ε that lacks the N-terminal CDC25-homology region is still recruited by Rap in response to EGF, but its dwell time in the perinuclear region is brief, and it quickly returns to a more diffuse cytoplasmic distribution. Activation of this mutant’s phospholipase activity is also brief (161).

The ability of the CDC25-homology domain of PLC-ε to act as a Ras GEF and thus activate Ras was demonstrated shortly after its discovery (153, 161). Its importance in specific signaling pathways and how it relates to control of phospholipase activity remain to be established. The ability of PLC-ε to prolong the response to stimulation of growth factor pathways suggests that its GEF activity toward Rap or Ras is involved in a positive feedforward loop (153, 161). These ideas and their supporting and detracting evidence are reviewed by Smrcka et al. (154).

A fascinating correlate to this idea of feedforward signaling comes from studies of the activation of PLC-β and PLC-ε by a panel of GPCRs in Rat1 fibroblasts. Using siRNAs to deplete either PLC-ε or PLC-β3, Kelley et al. (162) showed that the phospholipase activity of PLC-β3 peaked rapidly within the first minute of exposure to agonist and then decayed to near baseline within 2 min. In contrast, stimulation of PLC-ε by these same receptors lasted over 1 h. It was not clear from this work that the GEF activity of the CDC25-homology domain is involved in the extended time course of PLC-ε signaling, nor is it clear whether these receptors activate PLC-ε through Ras/Rap or through Rho. Regardless, both this work and earlier work indicate that one major function of PLC-ε is to signal over many tens of minutes.
The Rho GTPases also bind and activate PLC-ε directly. The binding site for Rho has not been established, but activation by Rho requires a region near the C-terminal end of the X-Y linker that is unique to PLC-ε (163). Rho binding does not require the RA domains (163) or the PH domain, which in PLC-β2 contains the binding site for Rac (164). Neither Rac nor CDC24, other members of the Rho family, activates PLC-ε. Rho binding increases the catalytic activity of PLC-ε up to sixfold in purified preparations in addition to any subcellular recruitment effect. It is tempting to speculate that this region is part of, or adjacent to, the autoinhibitory structure and that Rho binding somehow moves it out of the way. In cells, PLC-ε responds to multiple G12/13-coupled receptors (153, 165), presumably as a result of the activation of Rho by Gα12/13. Gα12/13 stimulates a family of Rho GEFs, at least one member of which is present in most cells (166). It is plausible that p63RhoGEFs, which are stimulated by Gαq, can also cause PLC-ε activation.

PHOSPHOLIPASE C-ζ

PLC-ζ is expressed uniquely in vertebrate sperm and is both necessary and sufficient to initiate the train of cytoplasmic Ca2+ spikes that begins after sperm-egg fusion (167). These Ca2+ spikes are necessary for the maturation and development of the fertilized egg (see 168, 169 for reviews). Microinjection of PLC-ζ mRNA into unfertilized eggs can initiate the spike train in the absence of sperm, and injection of inactive protein or of RNA that encodes a catalytically inactive PLC-ζ has no effect (167, 170). Loss of PLC-ζ or mutation that destroys catalysis causes male infertility (171, 172). The cellular physiological role of PLC-ζ is thus well established.

The regulation of PLC-ζ is less well understood than is its cellular function in reproduction, and PLC-ζ may be constitutively active upon its injection into the egg. Whether its activity correlates with different phases of the Ca2+ spike train is unknown, as is whether PIP2 availability and/or an alternative Ca2+ trigger is involved.

PLC-ζ is unique among the animal PLCs in that it lacks a PH domain, the site of many known regulatory interactions. In this way it resembles PLCs in green plants (173), but it is more closely related in sequence to mammalian PLCs, particularly PLC-δ. Its only known regulatory ligand is Ca2+. It is sensitive to low concentrations of Ca2+, with an EC50 of about 30 nM (174, 175), tenfold lower than typical for mammalian PLCs. Kouchi et al. (174) speculated that this enhanced sensitivity to Ca2+ reflects binding to one of the EF hand domains, probably the first, but this idea has not been tested directly. The EF hand mutations analyzed by Kouchi et al. also decreased maximal activity, and the importance of the EF hands to acute regulation is therefore uncertain.

In addition to lacking a PH domain, PLC-ζ differs from the other mammalian PLCs in that the X-Y linker peptide in the TIM barrel is positively charged. Its charge may contribute to its binding to the plasma membrane and its affinity for substrate (176). As discussed above, the X-Y linker in the other PLCs is either negatively charged or, in the case of PLC-γ, a large multidomain structure; removal of these linkers generally causes activation. In PLC-ζ, however, deletion of the linker significantly decreases enzymatic activity (44), and proteolytic cleavage of the linker does not activate the enzyme (177). This behavior is consistent with its constitutive activity.

The cationic linker in PLC-ζ also contains a class I nuclear localization signal (NLS) that provides a mechanism for turning off the Ca2+ spikes once fertilization has started to proceed. Termination of the spike train correlates with and requires NLS-dependent movement of PLC-ζ from cytoplasm to pronucleus, followed at longer times by its degradation (178–181). As the pronucleus develops, PLC-ζ leaves the cytoplasm for the nucleus, and Ca2+ spiking gradually ceases. Mutation of the NLS allows PLC-ζ to remain in the cytoplasm, and spiking continues after pronucleus formation.
PHOSPHOLIPASE C-η

The PLC-η family, first identified in 2005 (182–185), includes proteins derived from two genes, PLC-η1 and -η2, with PLC-η2 represented by multiple RNA splicing products. PLC-η is most closely related to PLC-δ, although it contains a significant C-terminal extension whose function is unknown. The PLC-ηs are expressed almost exclusively in brain, spinal cord, and retina, but a unique function for PLC-η in neurons is not known. PLC-η2 knockout mice are not reported to display an obvious phenotype, but this may reflect compensation by PLC-η1, which was expressed at elevated levels in the PLC-η2 knockout mice (186).

PLC-η is stimulated by Ca\(^{2+}\), which allows it to promote a further PLC signal once another input has caused either release of Ca\(^{2+}\) from the endoplasmic reticulum or Ca\(^{2+}\) influx from outside the cell (187, 188). Zhou et al. (189) also found that PLC-η2 is stimulated by Gβγ subunits, potentially allowing its regulation through Gi-coupled receptors. The extent of stimulation by Gβγ was somewhat smaller than that displayed by PLC-β2 but was still more than twofold above basal levels under those assay conditions. Cotransfection with cDNAs for PLC-η2 and Gβγ led to elevated turnover of [\(^{3}H\)]inositol, supporting the validity of this interaction. Observed stimulation of PLC-η1 by Gβγ was minimal.

SUMMARY POINTS

1. PLC simultaneously regulates the concentrations of three important cellular signaling molecules: the PIP\(_2\) substrate and the IP\(_3\) and DAG products. All three molecules have multiple targets and are further metabolized to other signaling species.

2. Mammals express six families of PLCs, each with its own regulatory domains. Each PLC responds to its own spectrum of regulatory inputs: monomeric and heterotrimeric G proteins, lipids, Ca\(^{2+}\), and both tyrosine and serine phosphorylation. One cell typically expresses several different PLCs that act in combination.

3. PLCs can be activated more than 100-fold by a combination of membrane recruitment and displacement of an autoinhibitory region that blocks the active site.

FUTURE ISSUES

1. How are PIP\(_2\) levels sensed and regulated in cells? When and how is PIP\(_2\) synthesis coordinated with PLC-catalyzed PIP\(_2\) hydrolysis?

2. Do individual PLC pathways act independently of each other? Is there cross talk, and how is it mediated? Do PLC-δ and PLC-ζ act only to prolong and/or intensify Ca\(^{2+}\) transients initiated by other PLCs?

3. There is almost no known inhibitory regulation of PLC activity. Why? Have we missed it, or is substrate depletion the only major inhibition?

4. Is there a general mechanism of activation of the six PLC families, or is each mechanism idiosyncratic?

DISCLOSURE STATEMENT

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