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# Structure, Function, and Control of Phosphoinositide-Specific Phospholipase C

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**Rebecchi, Mario J., and Srinivas N. Pentyala.** Structure, Function, and Control of Phosphoinositide-Specific Phospholipase C. *Physiol Rev* 80: 1291–1335, 2000.—Phosphoinositide-specific phospholipase C (PLC) subtypes $\beta$, $\gamma$, and $\delta$ comprise a related group of multidomain phosphodiesterases that cleave the polar head groups from inositol lipids. Activated by all classes of cell surface receptor, these enzymes generate the ubiquitous second messengers inositol 1,4,5-trisphosphate and diacylglycerol. The last 5 years have seen remarkable advances in our understanding of the molecular and biological facets of PLCs. New insights into their multidomain arrangement and catalytic mechanism have been gained from crystallographic studies of PLC-$\delta_1$, while new modes of controlling PLC activity have been uncovered in cellular studies. Most notable is the realization that PLC-$\beta$, -$\gamma$, and -$\delta$ isoforms act in concert, each contributing to a specific aspect of the cellular response. Clues to their true biological roles were also obtained. Long assumed to function broadly in calcium-regulated processes, genetic studies in yeast, slime molds, plants, flies, and mammals point to specific and conditional roles for each PLC isoform in cell signaling and development. In this

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review we consider each subtype of PLC in organisms ranging from yeast to mammals and discuss their molecular regulation and biological function.

## I. INTRODUCTION

Phosphoinositide-specific phospholipase C (PLC) isozymes found in eukaryotes comprise a related group of proteins that cleave the polar head group from inositol phospholipids. Under the control of cell surface receptors, these enzymes hydrolyze the highly phosphorylated lipid phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$], generating two intracellular products: inositol 1,4,5-trisphosphate ($InsP_3$), a universal calcium-mobilizing second messenger, and diacylglycerol (DAG), an activator of protein kinase C.

Historically, the PLC isozymes have been studied since the 1950s. Early observations by the Hokins (142), and later by Michell (243) and others, led to the recognition of PLC as a key enzyme in agonist-stimulated phosphoinositide metabolism and calcium signaling. The direct link between PLC and the release of intracellular calcium stores was finally forged with the publication of a seminal paper in 1983 by Streb et al. (351) describing the second messenger properties of $InsP_3$ (reviewed in Ref. 25).

In the late 1980s and early 1990s, three mammalian PLC subtypes, $\beta$, $\gamma$, and $\delta$, were isolated and their corresponding cDNA sequences determined (300). Paralleling this work, a number of PLC regulators were identified, especially the GTP-binding (G) $\alpha_q$ related subunits (349) (reviewed in Ref. 91) and protein tyrosine kinases (reviewed in Ref. 45). Isolation and identification of these components allowed investigators to test whether already recognized regulatory mechanisms controlled the PLC subtypes. Their results gave rise to the current G protein and tyrosine kinase models of PLC regulation.

PLCs are soluble multidomain proteins ranging in molecular masses from 85 to 150 kDa. Four $\beta$-, two $\gamma$-, four $\delta$-isoforms, and numerous spliced variants have been described in mammals. Those found in yeasts, slime molds, filamentous fungi, and plants closely resemble mammalian $\delta$. Comparisons of their DNA sequences suggest an evolutionary relationship in which PLC-$\delta$ appeared first in primitive single-celled eukaryotes. The PLC-$\beta$ and -$\gamma$ subtypes arose later, after the split between fungi or plants and animals, but before the parazoan-eumetazoan split, about 940 million years ago (198); their delayed appearance coincides with the diversification of other signaling components, such as G$\alpha$ subunits and protein kinase C. Later duplications of each PLC subtype led to the appearance of the numerous isoforms in animals.

At present, many of the players in phosphoinositide/calcium signaling are identified, some with three-dimensional pictures. On a cellular level, questions of which PLC isozymes go with which regulators are mostly answered. Despite this progress, our understanding of how and where PLC isozymes work in living cells is limited. New information suggests a higher level of organization than is implied by the current regulatory schemes, giving rise to a number of questions: Are these freely diffusing effector proteins or part of a highly organized network? Do these enzymes only act at the plasma membrane? Might they act in concert? Where is their substrate localized and how is it supplied? Finally, what are the physiological functions of the many isoforms and how is their expression controlled? In our review we attempt to address these questions (for other recent reviews see Refs. 91, 182, 299, 335).

## II. STRUCTURE AND CATALYTIC FUNCTION

The sequences of the eukaryotic PLC contain a string of modular domains organized around a catalytic $\alpha/\beta$-barrel formed from the characteristic X- and Y-box regions (392). They include a pleckstrin homology (PH) domain, EF-hand motifs, and a single C2 domain that immediately follows the Y-box region (see Fig. 1). Additional regulatory motifs are present in the $\beta$- and $\gamma$-subtypes, but absent in PLC-$\delta$. To simplify the discussion of common domains, we draw comparisons to $\delta_1$, the only eukaryotic PLC for which the three-dimensional structure is known (Fig. 1).

### A. Catalytic $\alpha/\beta$-Barrel

In the crystallographic structure of PLC-$\delta_1$, the catalytic domain is formed from the X and Y regions, 147 and 118 residues, respectively (88, 90). The domain is comprised of alternating $\alpha$-helices and $\beta$-strands and resembles an incomplete triose phosphate isomerase (TIM), $\alpha/\beta$-barrel. Like similar structures, the catalytic residues of PLC-$\delta_1$ are located at one end of the barrel. In this case, the site which is partly rimmed by hydrophobic residues is formed by a shallow cavity at the carboxy-terminal end. The unfinished lip of the barrel forms a spoutlike structure that may allow entry and egress of substrate or product at the membrane surface. The intervening sequence joining the X and Y halves of the barrel (43 residues) is highly disordered and not an integral part of the structure, although it may have an important regulatory function (see discussion below).

Eukaryotic and prokaryotic forms of PLC catalyze hydrolysis of the O-P bond connecting phosphoinositol to

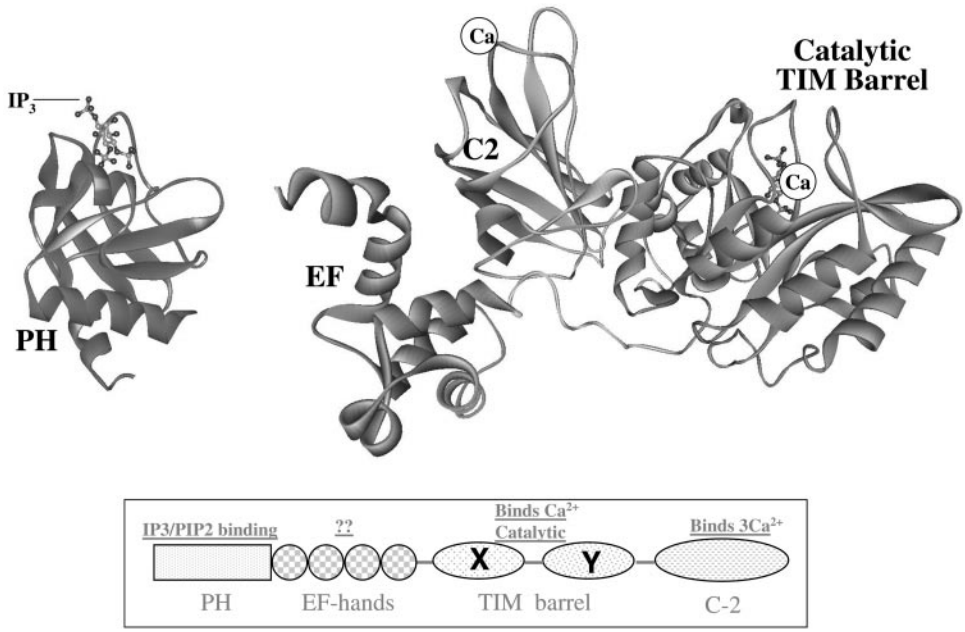



FIG. 1. Crystal structure of phospholipase C (PLC)-$\delta_1$. Structures of the enzyme (88, 98) lacking the PH domain and the $\delta_1$ PH domain were solved separately. Four $Ca^{2+}$ bind PLC-$\delta_1$, three to the C2 domain, and one to the catalytic TIM barrel. *Box*: linear representation of different domains of $\delta_1$ and their binding partners. $IP_3$, inositol 1,4,5-trisphosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate.

DAG. The requirement for inositol is absolute, because the substrate, through the 2-position hydroxyl, participates in nucleophilic attack on the phosphorous, resulting in a cyclic intermediate. Catalysis proceeds by an in-line sequential mechanism involving the cyclic 1,2-phosphodiester intermediate, which can be further hydrolyzed to *myo*-inositol 1-phosphomonoester (41, 146, 147, 221, 378). Eukaryotic forms readily hydrolyze this intermediate, although the relative amounts of the cyclic and noncyclic product depend on the particular isozyme, substrate, pH, and calcium concentration (188).

Various features of the polar head group affect substrate preference. Although the prokaryotic forms of PLC prefer phosphatidylinositol (PI) and PI-glycans, the eukaryotic enzymes have an order of preference that is generally $PI(4,5)P_2$ > phosphatidylinositol 4-phosphate [PI(4)P] > PI. Unlike their secreted prokaryotic counterparts, they are incapable of cleaving the polar head group of PI-glycan anchors. Neither forms are capable of hydrolyzing the 3-phosphorylated phosphoinositides.

Within the PLC-$\delta_1$ catalytic site, a network of hydrogen bonds and salt-bridges ligate the inositol ring substituents and generally account for the observed substrate preference. Lys-438 and Lys-440 of the first half of the $\alpha/\beta$-barrel, and Ser-522 and Arg-549 of the second half, ligate the phosphomonoesters at positions 4 and 5 of the $PI(4,5)P_2$ polar head group. These are conserved in the $\beta$- and $\gamma$-isozymes. Interestingly, single amino acid substitutions of Arg-549 do not abolish catalytic activity but switch substrate preference from $PI(4,5)P_2$ to PI (52, 383).

The catalytic residues, conserved in all eukaryotic PLCs, include His-311, His-356, Glu-341, Asp-343, and Glu-390 (89). A single calcium ion is bound to the active site coordinated by the side chains of Asn-312, Glu-341, Asp-343, and Glu-390 of PLC-$\delta_1$. The 2-position hydroxyl of the inositol ring and the exocyclic phosphodiester oxygen also appear to contact this metal which plays an essential role in catalysis, lowering the $pK_a$ of the attacking hydroxyl and the negative charge of the transition state. In the current model of the reaction (89), an active site base, possibly Glu-390 in a charge relay system with His-392, strips a proton from the 2-position hydroxyl of the inositol ring, promoting intramolecular attack on the phosphorous and cyclization. His-311 is too far removed to abstract a proton but instead stabilizes the developing charge on the initial pentacovalent transition state. His-356 participates in general acid/base catalysis, protonating the DAG leaving group during the formation of the cyclic 1,2-phosphoinositol intermediate. Acting as a general base, this residue then abstracts a proton from water which attacks the cyclic phosphodiester intermediate. Consistent with this model, amino acid substitutions of His-311 and His-356, as well as the calcium binding residue Glu-341, have been shown to reduce or abolish catalytic activity (52, 85).

Although other domains in PLC have the potential to bind calcium, the single catalytic calcium ion seems to be the only essential metal. This is supported by studies of a PLC-$\delta_1$ mutant missing other calcium binding sites located in the C2 domain (121). This mutated enzyme which has the same activation constant ($K_{act}$) for calcium (~1.4 $\mu$M) as the wild-type PLC. The in vitro results also agree with calcium activation constants obtained in permeabilized cells (2). Interestingly, the dissociation constant ($K_d$) for calcium binding to the catalytic site of PLC-$\delta_1$, measured by isothermal titration calorimetry, is ~ 30–50 $\mu$M,

in the absence of phospholipid (121). Although this is substantially greater than its $K_{act}$, the crystallographic structure shows that the PI(4,5)$P_2$ polar head group helps coordinate the metal ion, accounting, at least in part, for the weak affinity measured in the absence of substrate. The affinities of the β- and γ-catalytic sites for calcium have yet to be determined, but their $K_{act}$ is generally less than those reported for δ (see Refs. 165 and 379 for examples). Thus the β- and γ-, but not the δ-isoforms, are expected to be active at resting cytoplasmic calcium concentrations.

### B. Hydrophobic Rim

Surrounding the active site is a ridge of hydrophobic residues, Leu-320, Tyr-358, Phe-360, Leu-529, and Trp-555 (88); a similar ridge or rim is found in the prokaryotic forms (130). Such a ridge could insert into the membrane surface in a process required for full enzymatic activity. This proposal is based on studies of PLC-$\beta_1$, -$\beta_2$, -$\gamma_1$, and -$\delta_1$ in which raising the surface pressure of phospholipid monolayers to levels equivalent to, or slightly beyond, the packing densities found in membrane bilayers profoundly inhibits catalytic activity (33, 160, 161, 294). One notable exception is the PLC-β isoform found in turkey erythrocytes, which exhibits a pressure optimum that is nearly equivalent to bilayer packing density.

Inhibition by lateral pressures implies that the enzyme must do work to penetrate the membrane surface, bringing the substrate into register with catalytic residues. The presence of hydrophobic residues surrounding the active site further suggests that this ridge inserts into the acyl-chain region. Mutagenesis of the rim, involving replacement of bulky nonpolar residues with alanine, reduces the effects of increased surface pressure in monolayers, without affecting PI(4,5)$P_2$ hydrolysis in detergent-mixed micelles (84). These results point to an important interaction between the hydrophobic rim and the membrane bilayer and are consistent with a hydrophobic insertion model (a smaller area of penetration reduces the negative slope of the pressure/activity relation). Nevertheless, the identity of the protein sequence and the depth to which it penetrates are unknown. Moreover, these enzymes need only dip between the polar head groups to effectively engage substrate.[1] At this depth they would still experience the lateral pressures exerted in the monolayer experiments.

[1] It is worth noting that the hydrophobic residues found in this region of PLC-δ have been replaced by polar amino acids in the equivalent sequences of the β- and γ-subtypes.

### C. X/Y-Spanning Sequence (Z Region)

Among the PLC subtypes, sequences linking the X- and Y-box regions are poorly conserved and are not required for catalysis (39, 83, 318), suggesting a role in subtype-specific regulation. These regions are also susceptible to proteolysis (59, 83, 318), consistent with the idea that they are highly flexible.

Unlike the β- and δ-subtypes, the X/Y-spanning polypeptide in PLC-γ (also known as the Z region) is extensive, consisting of multiple adaptor domains (Table 3). The γ-sequences contain two Src homology (SH)2, an SH3, and a single PH domain that engage both protein and lipid binding partners. Although these domains are critical to extrinsic regulation of the γ-isoforms, they also exert an intrinsic control on catalytic activity (see sect. IV). In contrast, the comparable sequences of PLC-β and -δ subtypes lack any identifiable regulatory motifs. Nonetheless, the relatively short sequences in the β- and δ-subtypes also appear to exert an intrinsic control over the catalytic core (318), raising the possibility that Z-region sequences are key to a general mechanism for controlling PLC catalytic activity.

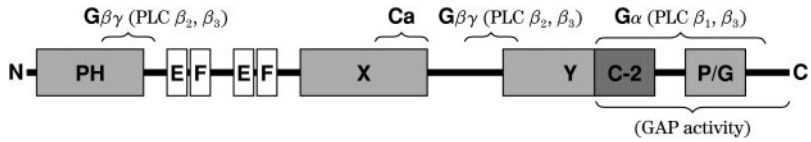
### D. PH Domain

The PH domain was originally described as a novel protein motif of ~100-amino acid residues, repeated twice in the protein, pleckstrin (platelet and leukocyte C kinase substrate) (128, 239). These motifs have now been identified in >100 other proteins (reviewed in Refs. 219, 296). Most can be grouped by function into a few classes: Ser/Thr protein kinases, Tyr protein kinases, small G protein regulators, endocytic GTPases, phosphoinositide-metabolizing enzymes, and cytoskeletal-associated proteins. Many feature a catalytic site (e.g., protein kinase) and additional adaptor domains. Because PH domains lack any obvious catalytic properties and are found in proteins associated in some way with the membrane, it was suggested that these domains function as adaptors or tethers, linking their host proteins to the membrane surface (99). Principal binding partners are phosphoinositides and the βγ-subunits of heterotrimeric G proteins.

Most eukaryotic PLCs contain a single PH domain of ~130 residues located in the amino-terminal region. An additional PH motif, found in the Z region of PLC-$\gamma_1$ and -$\gamma_2$, is split by two SH2 and a single SH3 domain. PH sequences are not well conserved among the PLCs, suggesting their association with subtype-specific regulation. Interestingly, some PLCs lack any PH domain (Tables 1 and 2; see PLC in higher plants and PLC-$\beta_4$ spliced variants).

The δ PH domain binds the polar head group of PI(4,5)$P_2$ (112, 220) and is archetypical. The domain is

TABLE 1. *Phospholipase C-β isozymes*

| Organism | Residues | Sequence Features* |
|---|---|---|
| | |   |
| Mammals | | |
| PLC-$\beta_1$ | | |
| 1a(H) | 1,216 | Sequence diagram based on this isoform |
| 1b(B) | 1,173 | 75 Carboxy-terminal residues replaced with 32 amino acid sequences |
| PLC-$\beta_2$(H) | 1,183 | Most similar to $\beta_1$ except within carboxy-terminal extension |
| PLC-$\beta_3$(H) | 1,217 | Most similar to $\beta_1$ |
| PLC-$\beta_4$ | | |
| a(R) | 1,176 | |
| b(R) | 1,022 | 162 Amino acids deleted from carboxy terminus and replaced by unique 10-amino acid sequence |
| Retinal variants(R) | | |
| I (a, b) | 907, 919 | All retinal variants lack PH domain and the first EF-hand motif |
| II (a, b) | 1,011, 1,023 | (Ib and IIB retinal variants have a 12-amino acid sequence inserted in the region connecting X & Y boxes) |
| III | 1,022 | Unique 14 amino-terminal amino acids |
| Turkey | | |
| PLC-βtk | 1,211 | Most similar to $\beta_2$ |
| *Xenopus* | | |
| PLC-β(x) | 1,210 | Most similar to $\beta_3$ |
| *Drosophila* | | |
| norpA | 1,095 | |
| Type I | | Most similar to $\beta_4$; eye specific |
| Type II | | Differences in residues 130–155 (present in brain, leg thorax, and male abdomen) |
| PLC-21 | | |
| Type I | 1,305 | Most similar to $\beta_1$; head specific |
| Type II | 1,312 | 7 amino acids missing from the carboxy terminus of type 1; body specific |

Phospholipase C (PLC)-βS (1,355) from *Sponge* and PLC-βH1 (1,012) and PLC-βH2 (1,364) from *Hydra* were also identified, but little else is known. (H), human; (B), bovine; (R), rat. * A general linear cartoon of the PLC subtype that indicates the order of appearance of the various domains (392). It neither accurately reflects the precise length of each nor provides information about sequence similarity. Among PLC subtypes, the X and Y regions are highly conserved, and the C2 domain less so. On the other hand, the PH and EF-hand regions show poor sequence conservation.

required for PLC-$\delta_1$ to processively hydrolyze its substrate (59, 228, 402), suggesting that it tethers the enzyme to the membrane surface during catalysis.[2] This is further supported by equilibrium binding measurements (52, 58, 280, 295). Photolabeling studies of the whole protein also point to a single high-affinity PI(4,5)$P_2$ binding site (180, 361).

The crystallographic structure of the PLC-$\delta_1$ PH domain, bound to Ins(1,4,5)$P_3$ (Fig. 1), provides a molecular view of this high-affinity site (98). The whole structure is highly dipolar, with the positively charged surface surrounding the binding cavity where nine residues ligate the 4 and 5 position phosphomonoesters through hydrogen bonds and salt bridges.

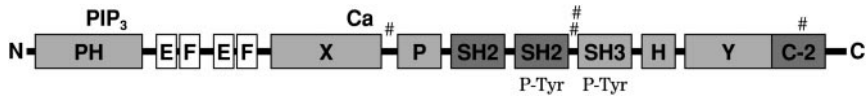
This remarkable specificity for PI(4,5)$P_2$ is also found in the related, but noncatalytic, Ins$P_3$/PI(4,5)$P_2$ binding protein (IP3BP130) (179, 413) and the PH domain of PLC-$\delta_4$ (254). Comparable sequences of the primitive δ-isoforms (in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Dictyostelium discoidium*) bear little resemblance to their mammalian counterparts.

Instead of PI(4,5)$P_2$, PLC-γ isoforms bind the higher order polyphosphoinositide, phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)$P_3$], in vitro (10, 94) and in living cells (94), forging a direct link between PI 3-kinase activation and recruitment of PLC-γ. The $K_d$ for binding the amino-terminal PH domain of PLC-$\gamma_1$ to this lipid is ~1 μM (94). Mutating a sequence localized to the putative loop between β-strands 3 and 4 of the $\gamma_1$ PH domain, a region also important in the binding of PI(4,5)$P_2$ by PLC-$\delta_1$, blocks binding.

Although the PH domains of the β-isoforms may also serve as membrane tethers, they are not polyphosphoinositide specific. PLC-$\beta_1$ and -$\beta_2$ bind strongly to membranes regardless of the presence of these lipids (165, 309). Moreover, PLC-$\beta_1$, -$\beta_2$, and -$\beta_3$ fail to bind Ins$P_3$ or other

[2] In the processive mode of catalysis, the enzyme hydrolyzes numerous substrate molecules on the membrane surface before returning to the bulk solution (see Ref. 113 for a recent review). By definition, in this mode the protein determinants for binding the membrane surface must be distinct from the active site residues. The faster the $k_{cat}$ and the slower the off rate from the membrane surface, the more efficiently the enzyme works.

TABLE 2. *Phospholipase C-γ isozymes*

| Organism | Residues | Sequence Features* |
|---|---|---|
| | |  |
| Mammals | | |
| PLC-$\gamma_{1(H)}$ | 1,290 | P-Tyr sites: 771, 783, 1254; sequence diagram based on this isoform |
| PLC-$\gamma_{2(R)}$ | 1,265 | 25 Residues are deleted from carboxy terminus (Tyr-phosphorylation 1,254 site missing) |
| *Drosophila* | | |
| PLC-γD | 1,220 | 70 Residues deleted from the carboxy terminus (Tyr-phosphorylation sites unknown) |
| Sponge | | |
| PLC-γS | 1,283 | Most similar to PLC-$\gamma_1$ (Tyr-phosphorylation sites unknown) |



Two forms, PLC-$\gamma_{1a}$ (1,010) and $\gamma_{1b}$ (1,067), from *Xenopus* were also identified, but little else is known of these forms. (H), human; (R), rat. * See legend to Table 1 for definition of asterisk. # Known sites of tyrosine phsophorylation.

inositol polyphosphate or polyphosphoinositide analogs, with measurable affinity (361) (see *point 1* in NOTE ADDED IN PROOF). The isolated PH domains, like the full-length enzymes, bind with moderate affinity to artificial membrane bilayers, but with relatively little specificity for the phospholipid head group (387). These nonspecific lipid interactions can account, at least in part, for the membrane binding of the intact enzyme (see also sect. II*G*).

PH domains of PLC and other host proteins also bind to so-called WD-40 proteins, which fold into β-propeller structures whose surfaces harbor binding sites for other proteins (326). One of these, the G-β subunit, has special relevance for the PLC-$\beta_2$ and -$\beta_3$ isoforms that are activated by Gβγ heterodimers. Although the affinities of most PH domains for these G subunits are low (296), their colocalization with PLC at the membrane surface could translate relatively weak binding into specific lateral interactions. Using resonance energy transfer to measure these interactions, we obtained evidence of specificity in the binding of Gβγ subunits to the PH domains of several PLC isotypes (387). The order of affinity is PLC-$\beta_2 > \beta_1$ and $\delta_1$. Moreover, the $\beta_2$ PH domain is sufficient for Gβγ binding and enzyme activation, since exchanging the PH domain of PLC-$\delta_1$, a Gβγ-insensitive subtype for the corresponding domain of $\beta_2$, results in a chimera that is highly activated by Gβγ subunits (385). These data are consistent with an earlier study that mapped the sequence for Gβγ activation to the amino-terminal two-thirds of the PLC-$\beta_2$ isoform (395). Although the PH domain seems sufficient, other Gβγ interaction sites have also been considered. One promising candidate encompasses the sequence 580–641 of PLC-$\beta_2$, located within the well-conserved Y half of the catalytic α/β-barrel (202, 313, 406). These observations support the view that PLC-β isoforms engage Gβγ subunits through multiple sites, but the critical determinants reside in the PH domain.

### E. EF-Hands

PLC isoforms have up to four EF-hand motifs, each consisting of a helix-loop-helix structure. In PLC-$\delta_1$, as in other EF-hand proteins such as calmodulin and tropinin C, the motifs are divided into pairwise lobes (88). This striking similarity to calmodulin extends to their main chain conformations that are nearly superimposable. As originally noted (88), the conformation of the second lobe and the EF-hand/C2 interface correspond closely to the calcium-saturated form of calmodulin bound to its target polypeptide. The interesting juxtaposition to the catalytic α/β-barrel suggests the second lobe is not part of a calcium switch, but is instead an integral part of the enzyme's core structure. Indeed, deletions in this region completely inactivate mammalian PLC-$\delta_1$ (257).

The first two EF-hands present in mammalian, yeast (22, 107, 283, 337, 411), and *D. discoideum* isoforms (79) possess residues that would appear capable of binding calcium or magnesium ions, whereas EF-hands 3 and 4 do not. There is no evidence, however, that the first two motifs actually bind metal ions, since none is found in crystals of the enzyme soaked in calcium or its analogs (90); calcium binding to these motifs is also not discernible in solution, whereas a single calcium binding site, corresponding to the catalytic α/β-barrel, is readily detected (121). Furthermore, EF-hands 1 and 2 do not influence calcium sensitivity, since substitution of the putative binding residues in PLC-δ from *D. discoideum* is without effect (78). This rule may extend to other PLC subtypes as well, since β- and γ-isoforms retain the four helix-loop-helix motifs but lack residues critical to metal binding.

Although the EF-hand region may have an important regulatory function, it has yet to be identified. In fact, the first two EF-hands, as well as the amino-terminal PH domain, have been dispensed with entirely in higher plants. Of four *Arabidopsis* PLC sequences, two are also missing a portion of the third EF-hand

(125, 139). Similarly, two spliced variants of PLC-$\beta_4$ from mammalian retina lack the PH domain and first EF-hand motif (102).

### F. C2 Domain

C2 motifs, ~120 residues in length, have been identified in numerous proteins, many of which function in lipid-signaling pathways, including PLC (reviewed in Refs. 258, 303). The C2 domain from PLC-$\delta_1$ (88, 90, 120), like that of synaptotagmin-I (SytIA) (325, 357), consists of eight antiparallel $\beta$-strands arranged as a sandwich; their main chains can be superimposed. In the $\delta_1$-domain, three loops at one end of the $\beta$-sandwich form the binding sites for up to three calcium ions (90). The calcium binding regions of the $\delta_1$ C2 domain, designated (CBR) 1(643–653), 2 (675–680), and 3 (706–714), are well conserved in the various $\delta$-isoforms found in organisms ranging from yeast to humans, suggesting that some important function has been retained.

Because each coordination complex is completed by water, the binding affinity is assumed to be weak in the absence of membranes, in agreement with the low calcium affinity measured by isothermal calorimetric titration (121). Moreover, disruption of these calcium sites in PLC-$\delta_1$ fails to affect the calcium-dependent hydrolysis of $PI(4,5)P_2$ in detergent micelles and phosphatidylcholine bilayers. Despite this evidence, additional ligands (lipid or protein) could contribute to the coordination of calcium in living cells, forming a stable complex that modulates PLC catalytic activity at lower calcium concentrations.[3]

More recent results indicate that PLC-$\delta_1$ forms a functional ternary complex with phosphatidylserine (PS) and calcium ($EC_{50}$ ~ 8 $\mu$M) through its C2 domain, in vitro (227). The inability of phosphatidic acid (PA) to substitute suggests the involvement of specific phosphoserine determinants. Importantly, the complex is highly activating, but only when the membrane concentration of $PI(4,5)P_2$ is limiting (~1 mol%). These results support the tether and fix model (88), wherein the PH domain bound to $PI(4,5)P_2$ tethers the enzyme, whereas the low-affinity binding of the C2 domain orients and fixes the catalytic core to the membrane surface. Thus PS and calcium bound to the rigid C2 domain could enhance surface sampling by the tethered catalytic core, facilitating processive substrate hydrolysis when the density of substrate is on the order of 1 mol% or less. While attractive, this idea remains to be tested.

[3] The relatively low affinities of the C2 domain sites for calcium suggest that this domain does not respond to calcium levels normally attained in the bulk cytoplasmic space (121). It is worth pointing out, however, that such high concentrations may be achieved, albeit transiently, near the open mouths of calcium channels (60). In this regard, a relevant parallel might be synaptotagmins, which sense the transiently high calcium levels produced by similar channel openings.

Both $\beta$- and $\gamma$-subtypes also contain C2 domain motifs, yet the key residues involved in calcium ligation are not conserved, a situation reminiscent of some PKC and synaptotagmin subtypes whose C2 domains are also unable to bind calcium. Although these domains may have been retained as an integral part of the PLC catalytic core, they could also function in recognition of other regulatory lipids and proteins. The later possibility is consistent with our recent finding that the PLC-$\beta_1$ C2 domain binds specifically to GTP-charged $\alpha_q$, its physiological activator (386). As discussed below, the C2 domain of PLC-$\beta$ appears to operate in concert with the carboxy-terminal extension to effectively engage this protein. Whether comparable determinants are present in the C2 domains of the $\gamma$-isoforms is not known.

### G. Carboxy-Terminal Extension

A single C2 domain and short peptide cap the carboxy-terminal ends of $\gamma$- and $\delta$-isozymes, whereas $\beta$-subtypes have extensions of ~400-amino acid residues that contain sequences important to membrane binding, nuclear localization, and their activation by G protein subunits (165, 185, 274, 393). Deletion of this entire carboxy-terminal region from PLC-$\beta_1$ does not destroy catalytic activity but abolishes activation by $G\alpha_q$ and related proteins in vitro (274) and in living cells (393) (see sect. III). Deletions of the carboxy-terminal extensions of PLC-$\beta_1$ or -$\beta_2$ or portions thereof, also block binding to acidic phospholipids in vitro (165), association of PLC-$\beta_1$ with the cell's particulate fraction (185, 393), and transfer to the nucleus (185). The carboxy-terminal region is also required to stimulate the intrinsic GTPase activity of $G\alpha_{q/11}$ and to link the $\beta$-isoforms to membrane-associated scaffolding proteins (see sect. III*E*).

## III. THE PHOSPHOLIPASE C-$\beta$ ISOZYMES

Four $\beta$-isotypes and additional spliced variants have been identified in mammals. $\beta$-Homologs also have been found in turkey (PLC-$\beta_{tk}$)(382), *Drosophila* (NorpA and PLC-21)(32, 191, 330, 421), *Xenopus* (105), sponge, and hydra (198) (Table 1); each isoform has distinctive sequences outside the canonical X and Y regions.

PLC-$\beta$ isoforms are regulated by heterotrimeric GTP-binding proteins in a manner that generally fits the adenylyl cyclase control paradigm (see Refs. 91, 92, 335 for previous reviews), yet the coupling of G proteins to PLC involves some features of note. Of special interest is the high GTPase stimulating (GAP) activity of PLC itself, which appears to require an opposing activity of agonist-bound receptor to generate an $InsP_3$/calcium signal (see sect. III*B5*).

Although PLC regulation has been intensively stud-

ied, a number of questions remain concerning the determinants of specificity in the coupling of different receptor subtypes to individual β-isoforms and the lateral organization of these signaling components on the plasma membrane surface. Their presence in the nucleus and participation in the nuclear PI cycle further reflects our limited understanding of what they do. While transgenic animal experiments have provided some insights, they have also raised many questions concerning the biological role of each isoform. Especially relevant are recent studies of NorpA in *Drosophila* phototransduction and the phenotypes of transgenic mice lacking particular PLC isoforms or their regulatory G protein subunits. The various signaling and developmental phenotypes associated with their disruption suggest a few well-circumscribed functions for each isoform.

### A. Tissue Distribution and Expression

Mammalian PLC β-isoforms are differentially distributed, with each pattern of expression reflecting, to some degree, the functions identified in transgenic work. PLC-$\beta_1$ is most widely expressed, with the highest concentrations found in specific regions of the brain (114, 145, 246, 302, 353). This PLC is prominent in the pyramidal cells of the hippocampus and, to a lesser extent, in the granule cells of the dentate gyrus, the reticular, mediodorsal, and anteromedial thalamic nuclei (307). PLC-$\beta_1$ mRNA levels are highest in the cerebellar Purkinje and granule cells, frontal and pyriform cortex, hippocampus, and dentate gyrus; hindbrain structures have relatively low levels of this isoform.

PLC-$\beta_1$ exists as alternatively spliced variants $\beta_{1a}$ and $\beta_{1b}$ (14). The $\beta_{1b}$-variant replaces 75 carboxy-terminal residues of the original PLC-$\beta_1$ cDNA with a unique 32-amino acid sequence. Both are abundant in brain, although the $\beta_{1a}$-variant more so. Neither variant is detected in kidney or stomach. Interestingly, most cell lines fail to express any detectable PLC-$\beta_1$; exceptions include C6Bu-1, PC-12, and NIH-3T3 cells. One of these, the rat C6Bu-1 glioma cell line, expresses both spliced variants (15). Whether the variants are coexpressed in specific neuronal tracts is not yet known.

PLC-$\beta_2$, first isolated from an HL-60 cDNA library (200, 272), is expressed at highest levels in cells of hematopoeitic origin (211). This pattern of expression is consistent with the part PLC-$\beta_2$ plays in leukocyte signaling and host defenses (see sect. III*G*).

PLC-$\beta_3$ protein, originally isolated from rat brain, is widely expressed, with the highest concentrations found in brain, liver, and parotid gland (166). In brain, its mRNA is discretely distributed, with the highest levels found in cerebellar Purkinje and granule cells, and the pituitary gland (362).

PLC-$\beta_4$ was first isolated from cerebellum (244, 245) and retina (173, 210). Its mRNA is highly concentrated in cerebellar Purkinje and granule cells (308, 362), the median geniculate body (308), whose axons terminate in the auditory cortex, and the lateral geniculate nucleus, where most retinal axons terminate in a visuotopic representation of each half of the visual field. This pattern of expression may be highly relevant to the phenotypes of PLC-$\beta_4$ null animals (see sect. III*G*).

Several alternatively spliced variants of PLC-$\beta_4$ have been identified. One PLC-$\beta_4$ protein, designated the "b" form, is missing the carboxy-terminal 162 amino acids (190). The sequence is replaced by a unique 10-residue peptide. This isoform, which is also found in brain, is recovered exclusively in the cytoplasmic fraction, unlike the "a" variant.

Additional variants have been described in the retina (101, 102). They are divided into two groups: PLC-$\beta_4$ class "I" and "II," each containing a and b variants that lack the PH domain and first EF-hand motif. These sequences are replaced by a unique amino-terminal region in class II forms. The Ib and IIb variants have an additional 12-amino acid insert within the sequence connecting the X and Y halves of the α/β-barrel, the result of alternative splicing. More recently, another retinal variant has been described that contains 14 unique amino-terminal residues (3); we term this form variant III (see Table 1).

The *Drosophilia* PLC-$\beta_4$ homolog, NorpA, was originally described as an eye-specific gene product (32), but hybridization with cRNA probes in Northern blots showed that the gene encodes at least four transcripts ranging in size from 5 to 7.5 kb (421). These transcripts are expressed in adult body and early stages of development. The various size transcripts of the *NorpA* gene are accounted for by alternative splicing of two forms of exon 4 which encode slightly different sequences between residues 130 and 155 at the carboxy-terminal boundary of the PH domain motif (191). Termed subtypes 1 and 2, NorpA subtype 1 is eye specific, whereas type 2 is diffusely present in brain and leg and is at high levels in thorax and abdomen. Subtype 1 is required for normal photoreceptor cell development, as well as the normal phototransduction process itself, whereas specific functions have yet to be assigned to the second subtype.

*Drosophila* PLC-21 differs from NorpA (330) and is expressed as two alternatively spliced variants. More recently, β-homologs have been identified in sponge (PLC-βS) and hydra (PLC-$\beta H_1$ and $\beta H_2$) (198). Although their domain organization seems identical to the mammalian and *Drosophila* isoforms, there is currently no information on their distribution or function.

### B. Control by G protein-Coupled Receptors

PLC-β isoforms function as effector enzymes for receptors belonging to the rhodopsin superfamily of trans-

membrane proteins that contain seven transmembrane spanning (heptahelical) segments (169). They are activated by a wide range of stimuli, from photons and tiny odorant molecules, to full-sized proteins and require specific combinations of G$\alpha$ and G$\beta\gamma$ subunits to couple to their effectors. In the standard G protein model of PLC activation, binding of agonist triggers receptor-catalyzed exchange of GTP for bound GDP on the $\alpha$-component of the heterotrimer. The GTP-charged subunit then dissociates in the plane of the membrane, and either the $\alpha$-subunit monomer, the $\beta\gamma$-heterodimer, or both bind to PLC-$\beta$, increasing its catalytic activity and thereby amplifying the initial receptor stimulus (Fig. 2). Because the evidence supporting this model has been extensively reviewed before, we focus on some of the unsettled issues and some of the newer developments that have led to a more complete view of how these enzymes participate in signaling events.

### 1. Activation by G$\alpha$ subunits

Before the identification of specific PLC isotypes or the G proteins involved, both pertussis toxin (PTX)-sensitive and -insensitive G proteins were implicated in phosphoinositide/calcium signaling (36, 93). Differential sensitivity to PTX suggested that G protein-activated forms of PLC might be heterogeneous, which was substantiated by the identification of the four different mammalian $\beta$-isotypes. Concurrent with their identification, the $G_q$ subfamily of PTX-insensitive $\alpha$-subunits ($\alpha_q$, $\alpha_{11}$, $\alpha_{14}$, $\alpha_{15}$, and $\alpha_{16}$) were isolated and characterized. $\alpha_q$ and $\alpha_{11}$ are found in nearly all tissues (350), whereas $\alpha_{14}$,$\alpha_{15}$, and $\alpha_{16}$ are generally restricted to cells of hematopoeitic origin (390).

When reconstituted into artificial vesicles, these G$\alpha$ subunits activate PLC-$\beta$ isoforms but fail to stimulate PLC-$\gamma_1$ or $\delta_1$ (91, 334); comparable results are obtained in cotransfection experiments. Nonetheless, the $\beta$-isoforms are not uniformly responsive to these subunits, with PLC-$\beta_2$ being considerably less sensitive in vitro (134, 166, 310, 343). Coexpression of PLC-$\beta$ isotypes 1, 2, or 3 and various $\alpha_q$ subfamily members produces a similar pattern (173, 199, 208, 395). Interestingly, a recently identified $\beta_4$-variant, which is missing a portion of the carboxy-terminal region (G box), is insensitive to $\alpha_q$ stimulation (190). Hence, PLC-$\beta_1$, -$\beta_3$, -$\beta_{tk}$, and most PLC-$\beta_4$ variants seem to be controlled by $\alpha_q$-related proteins, whereas the less sensitive $\beta_2$-isoform is not. Nonetheless, this simple state of affairs is unlikely to pertain when these proteins are expressed in their natural setting, as discussed below.

Although the PLC-$\beta$ isoforms are differentially sensitive to G$\alpha_q$-related subunits, the subunits themselves are nearly interchangeable in their activation of individual isoforms, whether examined by reconstitution in artificial membranes (134, 208, 255, 394) or in cotransfection experiments (173, 199, 208, 397). This promiscuity is also observed, with few exceptions in the coupling of various receptor types to PLC through $\alpha_q$-related subunits (399).

### 2. Activation by $\beta\gamma$-subunits

$\beta\gamma$-Heterodimers are now recognized as regulators of many effectors, including selective potassium and calcium ion channels, several isotypes of adenylyl cyclase, and PLC (reviewed in Ref. 61). The realization that these heterodimers activate PLC helps explain the disruption of

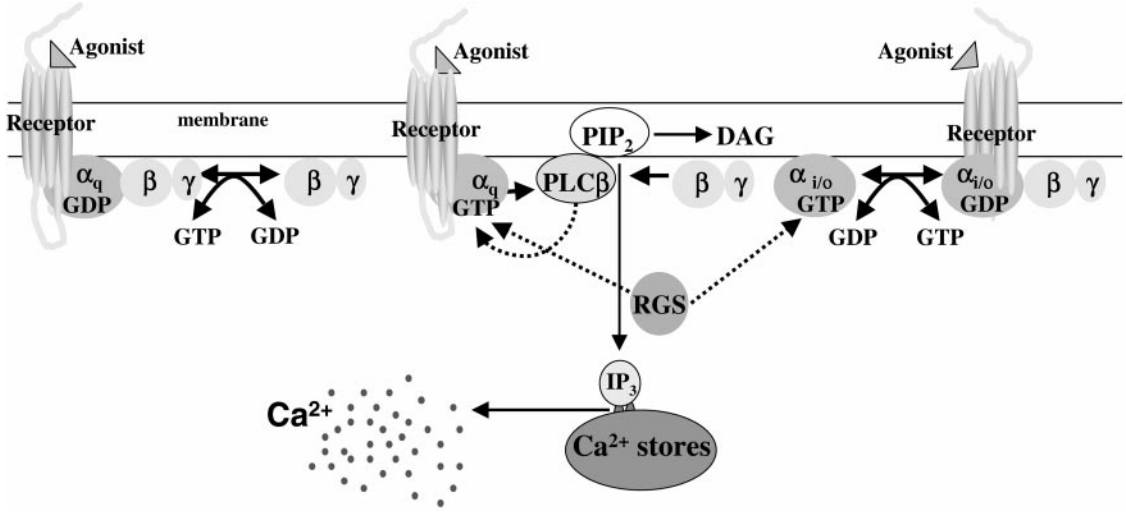



FIG. 2. Regulation of PLC-$\beta$ by G protein-coupled receptors (GPCR). Both $G_q$ and $G_{i/o}$ proteins regulate the function of PLC-$\beta$. The $\alpha_q$ subunit of $G_q$ activates PLC directly, whereas $\beta\gamma$-subunits typically released from $G_{i/o}$ also activate PLC. Hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) generates diacylglycerol (DAG) and $IP_3$, which in turn releases calcium from internal stores. RGS proteins act as receptor-specific down-modulators of the G$\alpha$ subunits. Recent evidence also shows that PLC-$\beta$ isozymes act as GTPase-activating proteins. Hence, the continued receptor-catalyzed charging of $\alpha_q$ with GTP is required to maintain the activated state of PLC-$\beta$.

phosphoinositide/calcium signaling by PTX and integrates toxin-sensitive G proteins of the $G_i$ subfamily into the overall scheme of PLC regulation.

When reconstituted into artificial and biological membranes, G$\beta\gamma$ subunits strongly activate mammalian PLC-$\beta_2$, -$\beta_3$ (42, 43, 273), and -$\beta_{tk}$ (37, 382). PLC-$\beta_1$ is weakly stimulated (42), whereas PLC-$\beta_4$ is completely insensitive (209). Both $\beta_3$ and $\beta_{tk}$ can be stimulated by $\alpha_q$ in the presence of saturating amounts of free G$\beta\gamma$, suggesting the formation of a ternary complex (343, 382). Although the dual activation of these PLC isoforms resembles the regulation of adenylyl cyclase, the effector types differ significantly since the G$\beta\gamma$-sensitive cyclase isoforms, AC II and IV, require concurrent activation by both G$\beta\gamma$ and GTP-charged $\alpha_s$ (363).

Although PLC-$\beta_3$ is strongly activated by G$\beta\gamma$ in vitro, this isoform is only weakly stimulated in coexpression experiments in COS cells (170). The reason for this discrepancy is unclear, but other work demonstrates that some receptors are coupled through PTX-sensitive pathways to this isoform (250–252). In smooth muscle, adenosine A1, $M_2$ muscarinic, somatostatin, and $\mu$-, $\delta$-, and k-opioid receptors can couple through $G_i$ and $G_o$ to PLC, although the extent varies, with some receptors coupling through G$\alpha_q$ related proteins as well. These cells contain the PLC-$\beta$ isoforms 1, 2, and 3. Interestingly, the $G_i/G_o$-mediated component is blocked by antibodies against PLC-$\beta_3$, but not $\beta_2$ or $\beta_1$; the extent of inhibition correlates with the extent of PTX sensitivity. Presumably, high concentrations of free $\beta\gamma$-subunits arise from the normally abundant $G_i$ heterotrimers, thereby stimulating PLC-$\beta_3$. If true, then PLC-$\beta_2$, which is also present, should have been stimulated, but was not. These results imply that PLC-$\beta_2$ may be activated by G$\alpha_q$ or its associated G$\beta\gamma$ subunits, but not the G$\beta\gamma$ arising from $G_{i/o}$. This seems to contradict the trend determined in artificial and biological reconstitution assays. How these differences arise, when the PLCs are expressed in their natural settings, is unknown.

There are at least 16 distinct G$\alpha$, 6 G$\beta$, and 12 G$\gamma$ subunits, yielding more than 1,300 different heterotrimer combinations. Although not all combinations are possible, the number is huge, giving support to the notion of "right" $\beta\gamma$-subunit combinations. The possibility that specific $\beta\gamma$-combinations are required to activate particular PLC-$\beta$ isoforms has been investigated. Of the limited number of $\beta$- and $\gamma$-subunits tested, however, most are completely interchangeable when reconstituted with PLC in artificial vesicles (35, 368) or coexpressed in living cells (389). The few exceptions are G$\beta_5$, which is most effective in stimulating PLC-$\beta_2$ when cotransfected with the G$\gamma_2$ subunit (389), and retinal G$\beta\gamma$, which is less effective than other subunit combinations in stimulating PLC (368). The relevance of the G$\beta_5$ activity is questionable, since tissue expression of this subunit and PLC-$\beta_2$ do not coincide. Moreover, weak stimulation by the retinal heterodimer appears to be caused by the attachment of a farnesyl rather than a geranylgeranyl group to the retinal $\gamma$-subunit. Thus when normal patterns of expression and lipid modification are considered, there is little evidence of effector selectivity for $\beta\gamma$-subunit combinations; rather, this specificity appears at the level of receptor/G protein coupling. This raises the question of specificity in the pathway from receptor to PLC, and the origin of the $\beta\gamma$-subunits. What are the actual concentrations of $\beta\gamma$-subunits liberated by activated receptors in living cells? Are other factors necessary to enhance the potency of $\beta\gamma$-subunits? Are the right $\beta\gamma$-subunit combinations really necessary? Are these subunits sequestered with, or directed to, a particular isoform? These questions remain unresolved.

### *3. Evidence of combinatorial specificity*

Like the PLC-$\beta$ isoforms, G protein-coupled receptors also discriminate poorly among G$\alpha_q$ subfamily members and various $\beta\gamma$-subunit combinations when reconstituted into artificial membranes or overexpressed in cultured cells (399). Nonetheless, evidence of combinatorial specificity in receptor/effector coupling can be obtained in living cells expressing their normal receptor/G protein complement. The evidence is based mainly on antisense RNA experiments involving the suppression of individual G subunits or their combinations. In the case of the $M_1$ muscarinic receptor coupling to PLC, several preferred combinations have been identified (G-$\alpha_q$ or $\alpha_{11}$, $\beta_1$ or $\beta_4$, and $\gamma_4$) (76). Comparable results have been obtained for other signaling pathways. For example, efficient coupling of $M_4$ muscarinic and somatostatin receptors to the inhibition of voltage-gated calcium channels requires two completely different heterotrimers, $\alpha_{o1}\beta_3\gamma_4$ and $\alpha_{o2}\beta_1\gamma_3$, respectively (195).

What confers this remarkable specificity? Clearly, determinants intrinsic to receptor and effector are insufficient, since these bestow selectivity only among $\alpha$-subunit classes. Determinants found in other factors, such as the RGS proteins (see sect. III*B5*), also fail most tests of specificity, although combining different determinants (receptor/G protein/RGS/effector) may help in the selection process. Alternatively, a physical sorting mechanism may sequester specific G-subunit combinations with their cognate receptors. The findings that receptors, G$\alpha$ and $\beta\gamma$ subunits, PLC isoforms and their substrates, are laterally organized lend support to this idea (see sect. III*E*). Another plausible mechanism would involve unique phosphorylation states of the receptor itself, modulating its ability to couple through distinct heterotrimers and PLC-$\beta$ isoforms. Evidence for this mechanism was obtained in an examination of the PKA-mediated phosphorylation of

the $\beta_2$-adrenergic receptor and its coupling to $G_s$ and $G_i$ (reviewed in Ref. 218).

*4. How do G protein subunits activate PLC?*

Although regions of PLC-$\beta$ that are important to their interactions with G proteins have been identified (see sect. II), the molecular basis of their activation is still unknown. G$\alpha$ subunits do not serve as membrane tethering devices for the $\beta$-isozymes (165, 309), nor do they affect the penetration of the membrane by PLC (249), nor do they increase its sensitivity to calcium (28, 30). The same appears to be true for G$\beta\gamma$ subunits. This would suggest that the mechanisms involve acceleration of some step in the catalytic cycle itself, yet attempts to measure the effects of G-subunit activation on substrate or product affinities or the catalytic rate constants have been inconclusive. Part of the problem lies in deriving mechanistic inferences from the kinetics of lipid-hydrolyzing enzymes. These catalysts operate at a membrane/solution interface, forcing the investigator to deal with artificial membrane binding and exchange steps that may be independent of the affinities of the enzyme for substrate and product. Fortunately, water-soluble glycero-1-phosphoinositol 4,5-bisphosphate and similar compounds are now known to be hydrolyzed by PLC (398). This should remove some of the complications, permitting a mechanistic understanding of G protein stimulation.

*5. GTPase activating proteins and phosphoinositide signaling*

Members of the G$\alpha_q$ subfamily have a slow intrinsic GTPase activity in vitro ($\sim$0.8 $min^{-1}$) (103). This leisurely rate is increased dramatically by the so-called regulators of G protein signaling (RGS) proteins (reviewed in Ref. 24), and the PLC-$\beta$ isoforms themselves. First discovered in yeast (Sst2), at least 19 different RGS proteins have been identified in mammals. Of those tested, RGS2 (137), RGS3 (259), and RGS4 (133, 149) interact most effectively with G$\alpha_q$ and block activation of PLC-$\beta_1$. RGS4 activates $\alpha_q$ GTPase by $\sim$25-fold, whereas G$\alpha$-interacting protein (GAIP), which interacts strongly with $\alpha_i$-related subunits, stimulates only about 2-fold at the equivalent RGS4 concentrations (133). In artificial vesicles, RGS2, which is widely expressed (49), is more selective than RGS4 for $\alpha_q$, and is 10-fold more potent at inhibiting PLC-$\beta_1$ activation in vitro (137). Like RGS4, RGS2 is also an effective GTPase activating protein (GAP) for $\alpha_i$ and suppresses $G_i$-dependent signaling in living cells (157). Thus RGS proteins that exclusively recognize the $\alpha_q$-class have not emerged.

Nonetheless, some RGS proteins are able to suppress calcium signaling in an agonist-selective manner, suggesting they do discriminate among receptor/G$\alpha_q$ complexes. In permeabilized pancreatic acinar cells, RGS4 inhibits PLC activation and calcium release from internal stores, but the amounts required to suppress carbachol, bombesin, and CCK differ by more than 10-fold (400); similar results are obtained in intact cells where RGS4 decreases the frequency and amplitude of calcium oscillations and raises the threshold for stimulation. While RGS1 and RGS16 are also potent inhibitors of muscarinic receptor signaling, they weakly suppress CCK-induced calcium release. In this case, the degree of selectivity for muscarinic compared with CCK receptor is 100- to 1,000 fold. In contrast, RGS2 is equivalent in its suppression. This differential sensitivity is not due to the expression of different levels of receptor or $G_q$-related proteins.

In addition to RGS proteins, $\alpha_q$ GTPase is stimulated by PLC-$\beta_1$, a property that extends to all PLC-$\beta$ isoforms (E. Ross, personal communication). This activity was first demonstrated by reconstitution of $M_1$ muscarinic receptors, $G_q$, and PLC-$\beta_1$ (26). In these artificial membranes, muscarinic agonist stimulates exchange of GDP for GTP on G$\alpha_q$ , whereas PLC and agonist increase the steady-state GTPase activity up to 20-fold. Thus the GAP activity of PLC-$\beta_1$ is balanced by receptor-catalyzed GDP/GTP exchange activity, yielding a population of G$\alpha_q$-GTP that increases the steady-state PLC activity by 90-fold (28) which explains the old observation that hydrolysis-resistant analogs, but not GTP itself, supports PLC/receptor coupling in biological membranes. Interestingly, G$\beta\gamma$ subunits also play an important role, suppressing the GAP activities of both PLC and RGS (54). This would further expand the number of points where G$\beta\gamma$ subunits could mediate or regulate PLC/receptor coupling.

The remarkable activities of PLC and RGS proteins, and the opposing exchange activity of the receptor, have important implications for the kinetics of signal generation and termination, the stimulus threshold, and the specificity of receptor/G protein coupling to PLC. Their GAP activities suggest PLC and RGS proteins rapidly dissipate the amplification cascade, and control the "noise" of agonist-independent activation, raising the threshold for stimulus-response coupling. Beyond their capability as noise suppressors, RGS proteins have been found to act on specific receptor/$\alpha_q$ complexes rather than the isolated $\alpha$ subunits, helping to account for the agonist-specific nature of the PI calcium signal. Moreover, differential RGS expression could enforce a tissue-specific response pattern to a given set of agonists. Depending on the complement of receptors and RGS proteins, multiple different receptors could generate temporally distinct calcium signals in the same cell, while drawing on a common pool of G protein subunits and effector enzymes. The spatial distribution and sensitivity of elementary calcium release and refilling events could further magnify these differences.

### C. Serine/Threonine Phosphorylation

Phosphorylation of PLC-$\beta$ by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) link the $\beta$-isoforms to heterologous and homologous receptor pathways that modulate phosphoinositide/calcium signals. Reciprocal and synergistic links between cAMP and $InsP_3$/calcium paths have long been recognized. Most notable are the findings that calcium stimulates some forms of adenylyl cyclase (356), permitting a wide range of cellular responses to agonists that engage these pathways.

The results of most studies show that increasing cAMP suppresses PLC activation, although a few have reported potentiation (reviewed in Ref. 106). The ability of cAMP to inhibit PLC is thought to occur at the level of PLC-$\beta$ phosphorylation. For example, PKA directly phosphorylates PLC-$\beta_2$, in vitro and in cotransfection experiments, thereby inhibiting its activation by G$\beta\gamma$ subunits (224). One of the putative phosphorylation sites, Ser-954, is located in the carboxy-terminal P-box region. Similar results have been reported for PLC-$\beta_3$ (1). Interestingly, phosphorylation by PKA uncouples receptors that activate PLC-$\beta_3$ through $G_{i/o}$, while preserving the activation by receptors that utilize $G_q$. The results are entirely consistent with the independent and simultaneous activation of this PLC isoform by G$\beta\gamma$ and $\alpha_q$, previously observed in vitro (343). On the other hand, PKA-mediated phosphorylation has been reported to partially block the activation of PLC-$\beta_3$ by G$\alpha_q$ (416). Here the PLC-$\beta_3$ phosphorylation has been mapped to a single site, Ser-1105, located in the G-box region. Mutation of this Ser to Ala confers resistance to PKA inhibition. Whether this might be related to activation by G$\beta\gamma$ subunits derived from $G_q$, rather than the G$\alpha_q$ itself, is unclear.

Various heptahelical receptors linked to PLC can be downmodulated by PKC through the generation of DAG and the rise in cytoplasmic calcium (106). This rapid desensitization may occur at the levels of receptor, G protein, or effector, or some combination thereof. Importantly, the PLC-$\beta$ isoforms themselves are substrates for PKC. Studies of PC12, C6Bu1, and NIH-3T3 cells, which contain PLC-$\beta_1$, -$\gamma_1$, and -$\delta_1$, show that treatment with tetradecanoylphorbol 13-acetate (TPA) stimulates phosphorylation of PLC-$\beta_1$, but not the other subtypes (312). Phosphorylation of PLC-$\beta_1$ by PKC in vitro results in a stoichiometric incorporation of phosphate at Ser-887, but without any measurable effect on PLC activity. This result was surprising since PKC-mediated desensitization seems to correlate with the level of PLC-$\beta$ phosphorylation. More recently, significant inhibition of the partially purified enzyme was reported, but G$\alpha_q$ stimulation was unaffected (223). Suppression of this calcium-stimulated G$\alpha_q$ independent activity requires an as yet unidentified cofactor. Comparable results have been reported for PLC-$\beta_{tk}$ reconstituted in erythrocyte membranes (104). Here the stimulation by $P_{2Y}$ agonist and guanosine 5′-*O*-(3-thiotriphosphate) (GTP$\gamma$S) is partly suppressed by phosphorylation. On artificial surfaces, however, activation of the purified enzyme by G$\alpha_q$ is hardly affected and stimulation by G$\beta\gamma$ subunits is completely insensitive. These results would suggest that other factors, such as the receptor and RGS proteins, are necessary to observe the PKC-mediated inhibition.

PLC-$\beta_2$, which is closely related to $\beta_{tk}$, is not phosphorylated when coexpressed with PKC isoforms, which themselves fail to inhibit PLC activation by G$\beta\gamma$ (224). On the other hand, PKC-dependent phosphorylation of PLC-$\beta_3$ has been observed. Phosphorylation correlates with uncoupling from platelet-activating factor receptors, which are linked to $G_q$, but not formyl-Met-Leu-Phe (FMLP) receptors, which are linked to $G_{i/o}$. These results suggest the same covalent modifications of PLC produce functionally distinct consequences that depend on the particular receptor and its cognate G protein. This might appear to contradict the generally held idea of heterologous desensitization, in which engagement of second messenger-activated protein kinases by one receptor should desensitize other receptors, assuming a common set of consensus phosphorylation sites. Yet heptahelical receptor desensitization is mediated by many different protein players and can occur at many steps on the path to PLC activation, some of which are receptor specific. Differential phosphorylation of the receptors or associated proteins (RGS proteins, $\beta$-arrestins), could affect how receptor-specific signaling components are uncoupled from their effector, laterally segregated, and internalized (218).

### D. Polyphosphoinositide Synthesis: the Need to Resupply

During the sustained phases of receptor activation of PLC-$\beta$ isoforms, the mass of $InsP_3$ produced often exceeds the fall in cellular $PI(4,5)P_2$ levels by severalfold. In some cases, levels of this lipid fail to decrease or even rise. The entire agonist-sensitive pool (estimated at ~80% of total) is metabolized several times per minute (391). Thus newly formed $PI(4,5)P_2$ must be continuously supplied to PLC.

This resupply of substrate requires numerous enzymes and at least two compartments coupled by PI transfer protein (PI-TP), which passively exchanges PI and phosphatidylcholine (PC) between membrane surfaces (see Ref. 65 for a recent review). PI is needed to supply PI 4- and PI(4) 5-kinase pathways, which must also

be active to continuously deliver polyphosphoinositides.[4] Although de novo PI synthesis, which occurs in the endoplasmic reticulum (ER), is also a universal feature of the cellular response, it is significantly delayed (up to 5 min) relative to the acute demand (see Ref. 20). Hence, an internal reservoir of preexisting PI, probably located in the ER and Golgi, is rapidly tapped by PI-TP during the initial phase of agonist stimulation.

Although PI-TP seems to operate passively, the inositol lipid kinases that process PI are highly activated (347). PI(4)P 5-kinase (type I) binds to and is stimulated by low-molecular-weight GTPases of the rho family that are engaged by many different classes of receptor. This activation may be relevant to resupplying substrate, since enhanced production of $PI(4,5)P_2$ is suppressed by rho-family inhibitors (317). Other factors influence PI(4)P 5-kinase as well. For example, these kinases are dramatically activated in both artificial and biological membranes by PA (248), a product of DAG kinase and PLC, or phospholipase D (see *point 2* in NOTE ADDED IN PROOF).

Both metabolic labeling (347) and pharmacological studies (391) indicate that PI 4-kinase is also highly stimulated during agonist activation of PLC. The mechanism of activation could involve PKC since the appropriate inhibitors block, while phorbol esters increase PI 4-kinase activity (347). Whether this enhanced flux from PI to $PI(4,5)P_2$ takes place throughout the plasma membrane compartment, or is highly localized within microdomains, is unknown. Interestingly, agonist-occupied receptors have been shown to direct PI-TP to the membrane, presumably to sites of PLC activation (183), thereby enhancing the potential for localized metabolism. To test this concept, the dynamic distributions of $PI(4,5)P_2$ and the enzymes that metabolize it must be examined in living cells and compared with agonist-occupied receptor. Recent advances in polyphosphoinositide detection will be key to this effort (346). Lateral segregation of these PLC-$\beta$ isoforms by scaffolding proteins (293) and the actin cytoskeleton (375), and the concentration of polyphosphoinositides in caveolae (287), suggest this is likely (see sect. III*E*).

### E. Scaffolding and Lateral Organization

In addition to the numerous membrane anchoring and adaptor domains that have been described, recent evidence points to new members of this functional class that assemble and organize various signal-transducing components at membrane surfaces (282). These include A-kinase anchoring protein (AKAP), 14–3-3, caveolin, and postsynaptic density disc-large ZO-1 (PDZ) proteins, which recognize complementary sequence motifs in the modular units comprising many different signaling components, including Tyr and Ser/Thr protein kinases, protein phosphatases, ion channels, and PLC. In the case of PDZ, specific carboxy-terminal sequences are recognized by different PDZ domains, permitting the organization of a wide variety of proteins.

Scaffolding proteins serve at least four purposes: *1*) they help to circumvent the impractically slow diffusion of multiple signal-transducing components that would otherwise limit both the speed and order of reaction; *2*) they provide a spatial domain to signaling; *3*) they enhance specificity of otherwise weak lateral protein/protein and protein/lipid interactions; and *4*) they integrate signals from disparate pathways. These characteristics are exemplified in the photoreceptor response of *Drosophila*, in which PLC plays a key role.

Proof that PLC isozymes are organized with other proteins into functional arrays has been obtained in *Drosophila* mutants lacking the inactivation, no after-potential (*InaD*) gene (367), a retinal membrane-associated protein containing five (PDZ) domains. Each domain recognizes a phototransduction component including $G_q\alpha$, NorpA (PLC-$\beta$), PKC (InaC), TRP, and TRPL. The lateral organization of these proteins may be important to the speed and efficiency of opening and closing of these channels which require PLC-generated DAG, PKC, and *InaD* (321). These proteins colocalize with *InaD* on the membrane surfaces of wild-type fly rhabdomeres but are either randomly distributed on the membrane (TRP) (53, 367) or located in the cytoplasm (PLC and PKC) (367) in flies lacking *InaD*. These mutants also exhibit a prolonged recovery time following a single flash of light. Similar results have been reported for *Calliphora* (blowfly) (152).

In *Drosophila* experiments, the NorpA mutation C1094S erases a critical residue in the carboxy-terminal sequence (Phe-Cys-Ala) required for binding its cognate PDZ domain of *InaD* (329). The mutation produces a broadening of the electroretinogram (ERG), indicating that both activation and deactivation are slowed. The slower rate of activation observed in the NorpA mutant is not observed in the *InaC* (PKC) null mutants. The results suggest that only the NorpA/*InaD* binding is critical for opening Trp channels, whereas a combination of calcium, the PLC product, DAG, and activated PKC are required to close the channels.

Other PLC-$\beta$ isoforms may be similarly organized. Mammalian PDZ domain proteins, typified by PSD-95 (Dlg homolog in *Drosophila*), target ion channels to synaptic terminals and dendrites where they are organized into signaling domains (327). Although a PLC-binding PDZ homolog of *InaD* remains to be identified in mammals, a

[4] In theory, a coordinated decrement in phosphatase activity could also contribute to net production of these lipids, but this requires a futile cycle of kinase and phosphatase activities. Although the relative phosphatase rates are difficult to determine, the best estimate suggests their contribution is minor.

set of such proteins is likely to exist, since all the PLC-$\beta$ isoforms contain a carboxy-terminal sequence that should recognize this structural motif (293).

In fact, most PLC-$\beta$ isoforms are strongly associated with the membrane/particulate fraction and can only be extracted with high salt concentrations or detergent. The relatively weak binding to phospholipids, measured in vitro, cannot explain this observation, but their strong association with a scaffolding protein could. The results suggest that proteins related to InaD could bind PLC-$\beta$ to the membrane, organizing receptors, G proteins, and other components on a nanometer scale while the cytoskeleton could further organize these complexes over larger distances (see Ref. 375).

In addition to scaffolding proteins, plasma membrane lipid may contribute to the lateral organization of signal molecules through the formation of micro-domains. These regions, typically enriched in cholesterol and glycosphingolipids, appear to exist in what has been termed the liquid ordered state ($l_o$) (40). Although most of the plasma membrane is in a conventional liquid crystalline state, these more ordered regions attract extrinsic proteins modified with saturated acyl chains, myristoyl or palmitoyl groups, or PI-glycans. In most studies, they have been physically defined by their detergent insolubility and relatively low buoyant density. Although these properties provide a broad functional definition, it is now clear that this portion of the membrane represents a heterogeneous collection of different domains, each having distinct lipid and protein constituents.

The best characterized of these are caveolae, which typically present as small invaginations of the plasma membrane, ~50–100 nm in diameter (4). Previous morphological studies have implicated these structures in transcytosis of macromolecules, potocytosis, and the lateral segregation of signaling molecules. This later function has been mainly inferred from copurification and enrichment of many different signaling proteins with a low-density, detergent-resistant membrane fraction, containing a 22-kDa integral membrane scaffolding protein, caveolin, which appears to bind and organize many of the components (268). In a few cases, morphological evidence is also available to confirm their association with caveolae.

Most of the components related to PLC activation: receptors, G proteins, PKCs, and polyphosphoinositides are associated with detergent-resistant membrane fractions (DRGs) including caveolae (4). This has led to the idea that phosphoinositide/calcium signaling is initiated at these sites. Strong evidence supporting this hypothesis has been obtained in A431 cells (287). Here, at least half of the recovered PI(4,5)$P_2$ cofractionates with caveolin-enriched low-density membranes. Treatment with epidermal growth factor (EGF) or bradykinin specifically reduces this polyphosphoinositide pool by half at 5 min, the earliest time point measured. Additional evidence is also obtained with the cholesterol-depleting drug methyl-$\beta$-cyclodextrin. This drug disrupts hormone activation of PLC and delocalizes PI(4,5)$P_2$, caveolin, and $G_q$ distributions in subcellular fractions (288). Thus the concentrations of membrane cholesterol and, by implication, the conservation of $l_o$ domains are necessary for transmembrane signaling.

While these results suggest that polyphosphoinositides and other signaling components might be preassociated, neither PLC-$\beta$ nor -$\gamma$ isoforms are recovered in these fractions (148).[5] Moreover, it is unclear whether the polyphosphoinositide pools that were measured reflect the initial or late response to agonist. Evidence obtained with bradykinin ($B_2$) (73) and muscarinic ($M_2$) (100) receptors shows these proteins are only sequestered in caveolae well after agonist stimulation. Importantly, the time course of $B_2$ receptor sequestration is much slower [half time ($t_{1/2}$) ~2–5 min] than the peak of $InsP_3$ production $t_{1/2}$ ~15 s. Thus the activation of PLC-$\beta$ isoforms may take place elsewhere, while the recruitment of receptor/G protein complexes into $l_o$ domains and polyphosphoinositide degradation therein, could be part of the late response to agonist, perhaps leading to receptor internalization, as part of a heterologous/ homologous desensitization mechanism. Clearly more work is needed before the operation and function of these membrane domains can be fully understood in the context of PLC-generated signals.

### F. Nuclear Targeting

The $\beta$-isoforms operate not only at the plasma membrane, but in the nucleus as well, where a small but significant fraction of cellular polyphosphoinositides are located, along with all the enzymes required for their synthesis and transport (81a). This nuclear phosphoinositide cycle, which operates independently of the plasma membrane, is agonist sensitive, showing remarkable changes in the levels of PI(4,5)$P_2$ and PI(4)P. Concomitant generation of nuclear DAG triggers the translocation of PKC isoforms to this compartment (260).

How do the relatively large PLC molecules gain access to the nucleus? All PLC-$\beta$ isoforms appear to have a nuclear localization signal sequence that is located in the carboxy-terminal extension and is necessary for their nuclear importation (185). These enzymes can be imported when artificially overexpressed. In contrast, nuclear levels of endogenous PLC change in an isoform- and variant-specific manner, suggesting that other factors are critical to their normal transport to, or retention in, the nucleus. For example, in promyelocytic leukemia (HL-60) cells, a

[5] This contrasts with adenylyl cyclase, which is found in low-density membrane fractions, although not necessarily in caveolae (150).

significant fraction of PLC-$\beta_3$ is detected in nuclei of undifferentiated cells (27). During terminal myeloid differentiation, the $\beta_2$-isoform appears in the nucleus, where its levels increase in parallel to the $\beta_3$-isozyme. The levels of both decline after 72 h. In Friend erythroleukemia cells, nuclear PLC-$\beta_1$ is downregulated during differentiation (238). Importantly, no changes in total cellular PLC levels are observed, indicating that the changes are due primarily to translocation. Interestingly, nuclear overexpression of PLC-$\beta_1$, which is normally downregulated in these cells, blocks differentiation (238). Differences in nuclear localization are also observed among PLC-$\beta$ variants. In the case of alternatively spliced variants, PLC-$\beta_1$ a and b, the a variant is preferentially cytoplasmic while the b variant is predominantly nuclear (15).

Further supporting an important role for PLC in the nucleus, a clear correlation is established between nuclear PLC-$\beta_1$ and insulin-like growth factor I (IGF-I) stimulated changes in nuclear phosphoinositides in Swiss 3T3 cells. IGF-I activates the nuclear isoform independently of PLC present in the plasma membrane fraction. Moreover, when PLC-$\beta_1$ expression is blocked with antisense RNA, the onset of DNA synthesis induced by this growth factor is inhibited (232). In the converse experiment, nuclear overexpression of PLC-$\beta_1$ enhances cell division, suggesting that its activation is both a necessary (for IGF-I) and sufficient signal.

These observations all point to distinct PLC-mediated signaling in the nucleus, but there are many gaps in our understanding. What controls nuclear import and retention of PLC? What is their mechanism of activation? How are their substrates disposed within this compartment? Do these signals directly regulate gene expression or other nuclear functions? Clearly, these are areas for intensive investigation.

### G. Studies in Transgenic Animals

Transgenic mice and flies lacking individual $\beta$-isoforms have been produced, with the absence of each enzyme resulting in a well-defined phenotype. As with most knockout experiments, however, it is often difficult to sort out the enzyme's role in a specific process from more subtle effects on development. Nonetheless, the experimental results provide important insight into how these enzymes are integrated into animal physiology.

In mice, the absence of PLC-$\beta_1$ leads to sudden death due to epileptic-like seizures (186). Their spontaneous seizures and hypersensitivity to convulsing drugs suggest that PLC-$\beta_1$ is necessary for the development and/or maintenance of brain inhibitory pathways. Indeed, somatostatin-containing hilar interneurons are selectively lost from the hippocampus of animals which experience spontaneous seizures. In the temporal lobe, cerebellum, and hippocampus, PLC activation by muscarinic agonists is substantially decreased, whereas the hippocampal response to metabotropic glutaminergic agonist is markedly enhanced; no change is observed in the response to serotonin-type 2 receptor agonist. These results are consistent with the codistribution of PLC-$\beta_1$ and $M_1$ or $M_3$ muscarinic receptors. This general decrease in muscarinic cholinergic tone may decrease inhibitory interneuron firing. This coupled with unopposed glutaminergic stimulation may account for the seizures. Because PLC-$\beta_1$ is prominently expressed in hippocampal pyramidal cells, which have numerous muscarinic cholinergic connections, memory and learning deficits are also predicted, but these possibilities have not been investigated.

A very different phenotype is observed in mice lacking PLC-$\beta_2$, which is highly expressed in cells of the immune system (171). Chemokine receptors for C5a, fMLP (170), CKR-1, monocyte chemoattractant peptide-1 (201), and interleukin-8 (396) are known to couple to PLC-$\beta_2$ through a $G_{i/o}$. These animals show no outward differences from their wild-type littermates and hematopoiesis appears normal, yet chemokine signaling is partly disrupted. Neutrophils from these animals fail to react to the chemoattractant fMLP with the usual spectrum of PTX-sensitive responses: PLC activation, calcium mobilization, superoxide production, and MAC-1 upregulation, yet the response to lipopolysaccharides remains intact.

Although PLC activation and other responses are abolished in leukocytes, chemotaxis is enhanced rather than inhibited. In particular, the chemotactic responses of eosinophils are dramatically increased. Moreover, enhanced responses to bacterial and viral challenges are observed in the intact animal. These results suggest that alternative pathways couple chemokine receptors to cell locomotion, whereas the pathways dependent on PLC-$\beta_2$ antagonize the process. The alternative pathways or the mechanisms underlying this antagonism are unknown.

Mice lacking PLC-$\beta_4$ have a number of specific deficits, especially in motor coordination (186). These animals are hypokinetic and exhibit a waddling gait that is not due to muscle weakness or bone deformity, pointing to a defect in the cerebellum (186). Although this structure develops more slowly in the mutant mice, it is reportedly normal at maturity. The authors suggest that altered signaling patterns may account for this phenotype, consistent with the reduction in PLC stimulation by metabotropic glutaminergic and muscarinic agonists that is observed in cerebellar slices from these animals. Whether the effects of the knockout are due mainly to a developmental deficit is unclear, but a similar phenotype, observed in $\alpha_q$ null mice, was traced to a defect in cerebellar development.

Mice lacking $\alpha_q$ but not $\alpha_{11}$ have a defect in motor coordination that is apparently caused by supernumerary climbing fiber innervation of each cerebellar Purkinje cell

(267). Interestingly, $\alpha_q$ is normally concentrated in dendritic spines projecting from the surfaces of Purkinje cells (231). The postnatal failure to cull extra climbing fibers, which may be essential for motor learning (48), is also observed in animals lacking metabotropic receptor type 1 (181) or PKC-$\gamma$ (47). The phenotypes suggest that the mGluR1 receptor is coupled via $\alpha_q$ to PLC-$\beta_4$ which produces the DAG stimulation needed to activate PKC-$\gamma$ at a critical stage in cerebellar development.

In addition to ataxia, PLC-$\beta_4$ null mice have defective visual responses (172). Although individual rod photoreceptors of these animals respond normally to a light stimulus, the integrated retinal response is impaired, as judged from changes in the ERG, especially the absence of the scotopic b wave that originates from rod bipolars. This ERG change is not accounted for by any gross developmental defect, implying that the many retinal variants of PLC-$\beta_4$ function in signal processing within the retina.

The defects appear to extend to higher levels, as well, since shuttle box behavioral experiments demonstrate that these mice are impaired in their visual-mediated learning but respond normally to sound. These characteristics are completely consistent with the anatomical distribution of PLC-$\beta_4$ in the lateral geniculate nucleus, the principal subcortical site for processing signals from the retina. The results suggest that this isozyme, or variants thereof, play a critical role, not in photon detection per se, but in processing the information content of the light stimulus, both in the retina and in higher centers. This contrasts with the role of the PLC-$\beta_4$ homolog NorpA in *Drosophila*.

Unlike vertebrate photoreceptors, invertebrates utilize PLC as the main effector in photon detection. Flies lacking NorpA are blind (32), as are flies lacking the $\alpha_q$ homolog dgq (A variant), which activates NorpA (217). Stimulation of this PLC somehow opens transient receptor potential (Trp and TrpL) calcium and cation channels in the fly photoreceptor. The subsequent increase in calcium leads to rapid deactivation. Although the mechanisms that couple NorpA activation to channel opening are ill-defined, the calcium-dependent deactivation process is better understood and involves PLC-generated DAG, PKC, calmodulin, and InaD, as well as rhodopsin kinase, arrestins, and a photoconversion reaction that returns metarhodopsin to its resting state (321); defects in any of these proteins lead to retinal degeneration.

In contrast to the other $\beta$-subtype null mutations, homozygous disruption of the mouse PLC-$\beta_3$ gene is lethal by *day 2.5* of development (384); much earlier than the PLC-$\gamma_1$ null mutants, which perish by embryonic *day 9* (see below). Homozygous PLC-$\beta_3$ knockout embryos are characterized by disorganized development, low cell numbers, and failure to form a blastocoel. Normally, PLC-$\beta_3$ expression is high in unfertilized oocytes and very early embryos but disappears by the primitive streak stage, suggesting that the maternal pool of the enzyme has an important role in early development. Consistent with this idea, an early report showed that PLC activity was required for continued division of the blastomere (124).

Unlike PLC-$\beta_3$, the other $\beta$-isoforms do not appear to be essential. Their individual absence gives rise to a phenotype reflecting the specialized role of each isoform. Further functional differentiation of these isoforms may be uncovered when alternatively spliced exons are similarly targeted. On the other hand, many of the $\beta$-isoforms are widely coexpressed, and the possibility for adaptation and redundancy in the signaling systems they support cannot be discounted. A next important step will be crossing of the null animals to determine to what degree these isozymes are truly redundant.

## IV. THE PHOSPHOLIPASE C-$\gamma$ ISOZYMES

Receptors for growth factors, immunoglobulins, and cytokines recruit and activate numerous effector proteins including the phosphoinositide metabolizing enzymes, PI 3-kinase and PLC-$\gamma$. Their activation mobilizes internal calcium stores and engages multiple protein kinase pathways that control or modulate cell division, transformation, differentiation, shape, motility, and apoptosis. Recruitment of PLC-$\gamma$ isoforms results in their tyrosine phosphorylation by protein kinase activities intrinsic to, or secured by, the receptor. This phosphorylation is necessary but not sufficient for activation, leaving a major gap in our understanding of PLC regulation. By linking the PI 3-kinase product PI(3,4,5)$P_3$ to PLC activation, recent studies have filled this gap and provided a mechanism for tethering PLC-$\gamma$ isoforms to the membrane after their dissociation from the receptor complex. Moreover, the intersection of the PI 3-kinase and PLC pathways lays down an additional path to activate the $\gamma$-isoforms, in the absence of direct tyrosine phosphorylation. PI 3-kinase products also figure prominently in the newly recognized distal actions of PLC-$\gamma$, in which $\beta$- and $\gamma$-isoforms are integrated into a single cellular response, suggesting that parsing of these subtypes among broad classes of receptors is incorrect.

Although critical aspects of PLC-$\gamma$ regulation are understood, their biological purpose is not. Long assumed to function broadly in many basic cellular processes, new findings, especially from transgenic experiments, indicate that these enzymes play decisive but narrowly defined roles, most easily discerned during development.

### A. Expression in Adults and During Development

Two types of mammalian PLC-$\gamma$ have been identified (Table 2): $\gamma_1$, which is ubiquitously expressed (145, 301), and $\gamma_2$, whose pattern of expression, although widespread, is

highest in cells of hematopoeitic origin (86, 145). In adult rat brain, PLC-$\gamma_1$ protein and mRNA are diffusely distributed (114, 307). Protein expression is highest in neurons, followed by oligodendrocytes and astrocytes (246). Although relatively high levels of mRNA are found in the hippocampus, olfactory bulb, and cerebellum, significantly higher levels are observed in embryonic cortical structures (307). Unlike $\gamma_1$, PLC-$\gamma_2$ mRNA is selectively expressed in the anterior pituitary and in cerebellar Purkinje and granule cells, especially those located in regions, connected to the vestibular nucleus, that are associated with cerebellar control of eye movement, posture, and gait (362).

During rat brain development (by embryonic *day 17*), PLC-$\gamma_1$ protein is localized to the processes of radial glia (404). These glia may serve as guides for neuroblast migration during histogenesis of the cortex. By postnatal *day 7*, expression of PLC-$\gamma_1$ becomes more widespread and prevalent in cortical neurons. By *day 14*, the PLC-$\gamma_1$ content of radial fibers has declined to undetectable levels. The remaining patterns eventually dissipate in the newborn, replaced by astrocyte staining in the cerebral white matter and hippocampus, consistent with the overall decline of PLC-$\gamma_1$ expression in neurons of the adult brain. These observations imply that a set of fine-tuned mechanisms is in place to regulate PLC-$\gamma$ expression during embryonic and postnatal development; recent work points to control at the level of transcription.

Distinct *cis* regulatory elements have been found in the genes encoding PLC-$\gamma_1$ and -$\gamma_2$ (213). These upstream sequences serve as binding sites for positive regulators of transcription, whose activity coincides with increased expression during the differentiation of myoblasts to myotubes (215) and in colon carcinomas (214, 264). On the other hand, evidence for posttranscriptional regulation of this isoform has been obtained as well (216).

A *Drosophila* PLC-$\gamma$ homolog has also been isolated (87). Its mRNA, which is widely distributed in the embyro, is increased during formation of the blastoderm and other stages of development. Interestingly, mutations within the open reading frame of this *Drosophila* homolog are associated with a developmental derangement known as small wing (*sl*) (364) (see sect. IV*I*).

In addition to altered gene expression, persistent changes in the subcellular distribution of the $\gamma$-isoforms, especially translocation to the nuclear compartment, may be relevant to their long-term effects. For example, nuclei from regenerating liver (261) and highly transformed cells (74) have high levels of PLC-$\gamma_1$. During myeloid differentiation of HL-60 cells, the nuclear levels of PLC-$\gamma_1$ and -$\gamma_2$ are upregulated and remain high in terminally differentiated cells (27). These observations suggest that nuclear transfer of PLC-$\gamma$, like other PLC isoforms, is generally restricted but may be enhanced during growth stimulation, transformation, or differentiation. Unlike the $\beta$-isozymes, however, the regions required for nuclear transfer of the PLC-$\gamma$ isoforms have yet to be identified.

### B. Activation by Receptor Tyrosine Kinase

Growth factor receptors possessing intrinsic protein tyrosine kinase activity, such as platelet-derived growth factor (PDGF), EGF, and nerve growth factor (NGF) receptors bind and phosphorylate the $\gamma$-isoforms (reviewed in Ref. 178). These receptors generally dimerize after engagement of their protein ligands, triggering autocatalyzed transphosphorylation. The receptor pTyr sites provide a set of specific docking platforms to recruit various effector proteins, including PI 3-kinase and PLC-$\gamma$ isoforms, which contain SH2 or PTB domains. Binding to these autophosphorylaton sites can be independent, mutually exclusive, or reinforcing (135, 289). Secured through these adaptor domains, the recruited proteins are themselves phosphorylated at specific tyrosine residues. Subsequent release leads to association with downstream binding partners simultaneously activating divergent pathways, including production of PI(3,4,5)$P_3$, generation of Ins$P_3$, elevation of cytoplasmic calcium, and activation ras/raf/MEK/MAPK and other protein kinase cascades that alter gene expression (Fig. 4). All these early events, especially PLC-$\gamma$ activation, are readily reversible (for examples, see Refs. 366 and 203).

Critical tyrosine phosphoacceptor sites are located within the large insert spanning the X and Y halves of the catalytic $\alpha/\beta$-barrel (Z region). PLC-$\gamma_1$ is phosphorylated by receptor tyrosine kinases (RTKs) at Y771 and Y783 that are conserved in the sequence of PLC-$\gamma_2$ (189, 380). One additional site, Y1254, is located at the carboxy terminus but has no clear role and is absent from the $\gamma_2$-isoform. While Y771 and Y783 are most rapidly phosphorylated in response to EGF or PDGF receptor kinases, only Y783 is required for activation of PLC-$\gamma$ in living cells (187). Y771 appears to serve an inhibitory function, since its removal enhances PLC activation. In fact, only 1 mol pTyr is found per mole of enzyme isolated from growth factor-stimulated cells. Interestingly, the single amino acid substitutions of Y771 and Y783 produce different growth and cytoskeletal phenotypes in fibroblasts overexpressing these mutations (285), suggesting that distinct biological functions are associated with each site.

Many RTK, like those for NGF (226) and PDGF (370), possess a single autophosphorylation site that binds to the SH2 domains of PLC-$\gamma_1$ with moderate to high affinity. In contrast, the EGF receptor contains multiple potential binding sites with equivocal affinities for PLC (345). Deletion of these PLC recognition sites blocks RTK-catalyzed tyrosine phosphorylation of PLC-$\gamma_1$ and its activation. Conversely, receptors that naturally lack PLC-specific autophosphorylation sites, like the insulin

receptor, fail to bind or activate PLC in vitro or in living cells (262). In agreement with the idea that binding to the receptor is a critical step, the affinities of the PLC-$\gamma_1$ SH2 domains for the autophosphorylation sites of different receptors correlate with the degree of PLC activation and the initial rise in cytoplasmic calcium concentration (266).

Binding of SH2 domains to pTyr sites on the RTK appears to be driven by enhanced rates of association rather than slower dissociation (96, 270). Thus increased affinity of the receptors for effector proteins does not result in formation of a longer lived complex, but rather an increased turnover of protein substrate. This is a particularly important feature of RTKs, since the effector proteins must dissociate from the receptor to interact with downstream signaling components.

It is also important to note that protein tyrosine phosphatases (PTPases) influence tyrosine protein kinase pathways, in both a positive and negative sense, and thereby PLC-$\gamma$. Numerous PTPases have been discovered, some with transmembrane receptor features and others with the properties of soluble cytoplasmic proteins. Whether engaged by their own ligand or recruited to activated receptors, these proteins are implicated in nearly all signaling pathways. It seems likely that some PTPases are recruited to activated receptor complexes where they dephosphorylate effector molecules, such as PLC-$\gamma$. This is consistent with the observations that basal PTPase activity is constitutively high, that most of the PLC bound to RTK is in the dephospho-tyrosine state, and that PTPase activity must be suppressed before full tyrosine kinase activity is realized. Among the mediators of PTPase suppression, reactive oxygen species (ROS) have emerged as both important regulators of PTPases (71) and modulators of PLC-$\gamma$ activation (11).

### C. Serine/Threonine Phosphorylation

In addition to its tyrosine residues, PLC-$\gamma_1$ is also phosphorylated on select serine and threonines in response to growth stimulants (192, 381). The major site, serine-1248, can be phosphorylated by either PKA or PKC. In vitro, the enzyme is phosphorylated on serine-1248 by PKA at nearly 1 mol phosphate/mol protein, but this has no effect on catalytic activity. Stimulation of C6Bu1 cells with cAMP-elevating agents increases the serine phosphorylation of PLC-$\gamma_1$, but not $\beta$- or $\delta$-isozymes (193). In Jurkat T cells, ligation of the T-cell antigen receptor (TCR) complex results in phosphorylation of PLC-$\gamma_1$ at serine-1248, as does incubation with stimulators of PKC or PKA (276). Although PLC-$\beta_1$ and -$\delta_1$ also contain significant levels of phosphoserine and phosphothreonine, stimulation does not change the content in these cells. Interestingly, prior incubation with phorbol 12-myristate 13-acetate (PMA) or forskolin, which stimulate PKC and PKA, respectively, suppresses TCR-stimulated tyrosine phosphorylation of PLC-$\gamma_1$ and its subsequent activation, suggesting an important negative-feedback role.

Although phosphorylation by PKC is suspected to be part of a negative-feedback loop, Ser/Thr phosphorylation of PLC-$\gamma$ is not sufficient; rather, it is likely that Ser/Thr phosphorylation of the receptor and associated proteins represents the critical step in desensitizing various effector pathways, including PLC-$\gamma$. In support of this idea, it was shown that suppression of EGF receptor coupling to PLC-$\gamma_1$ by phorbol esters is a function of receptor downmodulation rather than direct blockade of PLC activity (69, 153).

PKC activation does not always result in downmodulation. For example, the coupling of PDGF receptor to PLC-$\gamma$ activation is enhanced by PKC activation, whereas activation of PLC by GPCR is suppressed (127). These various observations point to a complex regulation of PLC by PKC and other serine/threonine protein kinases. Sorting out which phosphorylation events are critical will be a demanding task.

### D. Tyrosine Phosphorylation and the Control of Catalytic Activity

Despite a decade of research, it is unclear how tyrosine phosphorylation leads to PLC-$\gamma$ activation. When isolated, the tyrosine and nontyrosine phosphorylated forms of PLC-$\gamma_1$ and -$\gamma_2$ have nearly the same catalytic activity measured in vitro. This observation led to the early proposal that $\gamma$-isoforms are under the control of negative modulators that preferentially reduce the activity of the nonphosphorylated form (298). Alternatively, activation of the phosphorylated form may require binding to specific proteins or lipids, missing from in vitro assays. A number of positive and negative modulators have been considered.

#### *1. Negative modulators: actin-binding proteins*

Among the best studied negative modulators are actin-binding proteins, many of which also bind to polyphosphoinositides. In vitro, actin-binding proteins, such as profilin, suppress activity of the nonphosphorylated form of PLC-$\gamma$ more so than the phosphorylated form (117, 344), suggesting that these proteins represent the missing modulator. Indeed, it is generally observed that proteins which link the actin cytoskeleton to membrane surfaces, including vinculin (116) and $\alpha$-actinin, (111) or that sever actin filaments, like gelsolin (164, 222), bind PI(4,5)$P_2$ and negatively modulate PLC (162). These and other observations have helped fuel speculation concerning the role of PLC-$\gamma$ and its substrate in the polymerization of actin (see sect. IV*I3*).

Although in vitro results seem compelling, a specific role for these proteins in PLC regulation is far from clear. Curiously, overexpression of CapG, another actin/$PI(4,5)P_2$ binding protein, enhances the sensitivity of PLC to stimulation by PDGF without affecting its basal activity (354). In contrast, this same actin-binding protein inhibits bradykinin stimulation of PLC-$\beta$ (355). While these results implicate actin-binding proteins as modulators, it has yet to be established how these proteins influence PLC activity in living cells.

### *2. Positive modulators: $PI(4,5)P_2$ and $PI(3,4,5)P_3$*

Both substrate and nonsubstrate phosphoinositides activate PLC-$\gamma$ isoforms in a manner that suggests multiple binding sites for these lipids. To study these interactions, many investigators have used mixed micelles of detergent and lipid to systematically vary the surface and bulk concentrations of substrate without many of the problems associated with bilayers. With a sufficiently slow enzyme (low $k_{cat}$), and a sufficiently rapid rate of substrate and product exchange, a "surface continuum" can be created. With the use of this methodology, tyrosine-phosphorylation has been shown to increase the catalytic activity of PLC-$\gamma_1$ under conditions where the enzyme processively hydrolyzes its substrate in mixed micelles (379). Phosphorylation enhances the apparent affinity of the enzyme for the $PI(4,5)P_2$/detergent surface, although the degree of activation is rather modest. The results are consistent with a two-substrate model in which $PI(4,5)P_2$ binds a noncatalytic site, tethering the catalytic domain to the membrane surface and increasing the number of substrate molecules it hydrolyzes before returning to the bulk solution, which is very similar to the model proposed for PLC-$\delta_1$ (see sect. II). Enhanced affinity for $PI(4,5)P_2$ is also consistent with the observation that actin-binding proteins, which can sequester limiting amounts of this lipid, favor the activity of the tyrosine-phosphorylated enzyme.

More recent work establishes the nonsubstrate lipid, $PI(4,5)P_3$, as a positive and highly specific modulator of PLC-$\gamma$. This lipid binds directly to the amino-terminal PH (94) and the carboxy-terminal SH2 (292) domains of PLC-$\gamma_1$, tethering the enzyme to the membrane surface, and dramatically increasing $PI(4,5)P_2$ hydrolysis, even in the absence of tyrosine phosphorylation (10). Consistent with this idea, low concentrations of PI 3-kinase inhibitors suppress PDGF-stimulated generation of $InsP_3$ (10, 94) and cytoplasmic calcium transients (292), yet tyrosine phosphorylation is unaffected (94). Likewise, overexpression of a PI 3-kinase regulatory subunit, containing a dominant interfering mutation, also blocks PLC activation (94), while overexpressing the catalytic subunit of PI 3-kinase increases the basal levels of PLC product (10). In an important series of experiments, mutations of the PI 3-kinase specific pTyr docking site on the PDGF receptor suppress the generation of $InsP_3$ and completely blocks mobilization of calcium (292). In contrast, PDGF receptors that exclude PLC-$\gamma$, but bind PI 3-kinase, are unable to activate PLC-$\gamma$ indirectly through $PI(3,4,5)P_3$, showing that engagement of both pathways is required to generate an $InsP_3$/calcium signal.[6] Thus recruitment to the autophosphorylated receptor is only a first step in the process leading to PLC activation.

In vitro, PLC-$\gamma_2$, like -$\gamma_1$, is activated by $PI(3,4,5)P_3$ (10); results in living cells suggest a similar mode of regulation. In myeloid FDC-P1 cells, macrophage colony-stimulating factor induces transient tyrosine phosphorylation of PLC-$\gamma_2$, but activation requires concurrent stimulation of PI 3-kinase (34). Comparable results have been obtained for T- and B-cell receptor stimulation of PLC-$\gamma_1$ and -$\gamma_2$ as well (see sect. IV*E*).

Despite these significant advances, the molecular basis for PLC-$\gamma$ activation is still uncertain. High-affinity binding of the amino-terminal PH and/or the SH2 domain to $PI(3,4,5)P_3$ should increase PLC activity simply by tethering the enzyme to the membrane surface, allowing it to processively cleave $PI(4,5)P_2$, an intrinsically more efficient process. Nonetheless, allosteric modulation is also likely.

Whatever the nature of the allosteric modulation, the Z region is likely to play a critical role. Indeed, this region seems to be an intrinsic negative modulator of catalysis (see sect. II*C*). Based on the pH dependence of catalysis, Roberts and co-workers (420) have proposed that the protein exists in two alternative conformations, one of which has a closed "lid" that blocks the active site (420). The lid presumably corresponds to the Z region. $PI(3,4,5)P_3$, whether bound to the PH or SH2 domain, could lift the lid favoring the "open" active state. Clearly more data on the conformations of PLC-$\gamma$ and their relationships to phosphorylation state and $PI(3,4,5)P_3$ binding are needed.

## E. Regulation by Immunoglobulin/Cytokine Receptors

Various components of the immune system express receptors that regulate proliferation, differentiation, and apoptosis. These include B- and T-cell receptor com-

[6] Although this may be true of PDGF receptor in Hep G2 cells, the degree of dependence on PI 3-kinase may vary considerably (see Ref. 292). Other pathways, some of which are independent of tyrosine protein kinases, could also generate $PIP_3$ and thereby activate PLC-$\gamma$. Especially relevant is PI 3-kinase p110$\gamma$ which is activated by G$\beta\gamma$ subunits. Generation of $PIP_3$ by $\beta\gamma$-stimulated PI 3-kinase isoforms could forge a link between these G subunits and PLC-$\gamma$. This may also explain the PTX sensitivity of EGF-stimulated PLC-$\gamma_1$ activity in hepatocytes (408) and pancreatic acinar cells (286).

plexes, receptors for the Fc regions IgE, IgG, IgA, and IgM, and for cytokines such as interleukin (IL)-1, -4, -5, and -7. Beyond the immune system, most cells express receptors for cytokines, like IL-1. Unlike RTKs, these receptors consist of multiple transmembrane polypeptide chains that lack intrinsic tyrosine kinase activity but oligomerize to form a functional receptor unit. Receptor engagement triggers the recruitment of nonreceptor tyrosine kinases (NRTKs), including Src, Jak/Tyk, and Syk/Zap70 family members. Like RTKs, the recruited NRTKs are themselves phosphorylated, recruiting other effector proteins that contain SH2 domains, including PLC-$\gamma_1$ and -$\gamma_2$, which are tyrosine phosphorylated at sites also targeted by RTKs (275, 277) (Fig. 3).

### *1. PLC activation in B and T cells*

As part of the humoral immune response, antigens bind B-cell receptors (BCRs), leading to the recruitment of Src-related kinases, Lyn, Fyn, and Blk, which tyrosine phosphorylate specific activation motifs within the complex. Syk/Zap70 and Bruton's tyrosine kinase (Btk)/Tec family tyrosine kinases are subsequently recruited and phosphorylated as well, leading to the activation of PLC-$\gamma$ and PI 3-kinase (reviewed in Ref. 205). Src and Syk-related kinases appear to mediate the tyrosine phosphorylation of partially overlapping sets of proteins in response to BCR engagement (360); one of the Syk-specific substrates is PLC-$\gamma_2$.

Both Syk and Btk are required for B-cell development and for optimum stimulation of PLC-$\gamma_2$ (184). Cells deficient in either kinase show a reduced tyrosine phosphorylation of PLC-$\gamma_2$ in response to BCR stimulation, along with decreased PLC activation and calcium mobilization (359, 360). Antigen or anti-IgM stimulates tyrosine phosphorylation and activation of PLC-$\gamma_2$ in DT-40B cells, leading to calcium mobilization and apoptosis, a cultured cell phenomenon reflecting the normal elimination of self-reactive B cells that occurs in vivo. Cells deficient in either Syk or PLC-$\gamma_2$ fail to generate InsP, to mobilize calcium, or to die (358).

While both Syk and Btk are believed to directly phosphorylate PLC-$\gamma_2$ and stabilize its binding to the plasma membrane, a simple sequential recruitment model is unlikely to explain the requirements for multiple different regulators of this isoform. More likely, recruitment is accompanied by essential lateral interactions with receptor components, activated protein tyrosine kinases, PI(3,4,5)$P_3$, and adaptor proteins that help organize the signaling complex.

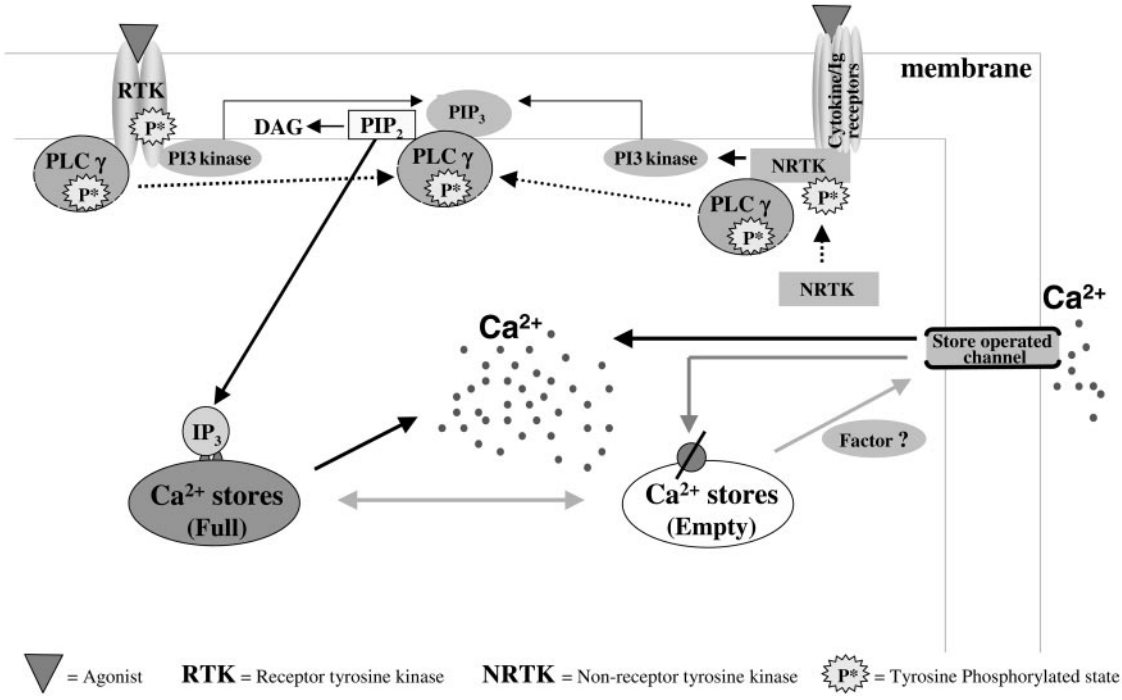



FIG. 3. Regulation of PLC-$\gamma$ by tyrosine protein kinases and phosphatidylinositol trisphosphate ($PIP_3$). PLC-$\gamma$ is under the control of both RTKs and NRTKs. Growth factors, antigen, and cytokine receptors recruit and activate PLC-$\gamma$ and phosphatidylinositol 3-kinase (PI 3-kinase) among other signal-transducing proteins. Phosphorylation of critical tyrosine residues in PLC-$\gamma_1$ and -$\gamma_2$, catalyzed by kinases intrinsic to or recruited by the receptor, leads to PLC activation. The PI 3-kinase product $PIP_3$, which is also crucial, serves to anchor and activate the enzyme after its dissociation from the tyrosine protein kinase. GPCRs also activate NRTKs and PI 3-kinases, thereby controlling PLC-$\gamma$ function. The emptying of the $Ca^{2+}$ stores by $IP_3$ triggers the influx of external $Ca^{2+}$ through store-operated channels, raising $Ca^{2+}$ levels and replenishing these compartments.

One such adaptor, BLNK, is essential for PLC-$\gamma_2$ activation (158). This protein, which associates with the BCR, is phosphorylated on multiple tyrosine residues by Syk, thereby providing docking sites for both PLC-$\gamma_2$ and Btk.

PLC-$\gamma_1$ is activated by a comparable mechanism, although the relative contribution of this isoform to the overall cellular response is unclear. CD-38 ligation results in tyrosine phosphorylation and activation of PLC-$\gamma_1$ in immature B cells, a process that requires Syk and PI 3-kinase (333). Direct phosphorylation by Syk is suggested by the finding that PLC-$\gamma_1$ forms a complex with this kinase following BCR or $H_2O_2$/pervanadate stimulation (206, 331).

In T cells, PLC-$\gamma$ isoforms are regulated by analogous mechanisms. Stimulation of the T-cell receptor (TCR) initiates a tyrosine kinase cascade involving src family members Lck, Blk, or Fyn, as well as coreceptors like LAT, a major Zap70 kinase substrate (419). After TCR engagement, numerous effectors are recruited to tyrosine-phosphorylated LAT, including PI 3-kinase and PLC-$\gamma_1$. Optimal tyrosine phosphorylation of these substrates requires SLP-76, an adaptor protein which has a proline-rich sequence for binding Grb2-SH3 domain and participates in the multimolecular complex organized by LAT (401). SLP-76, which is tyrosine phosphorylated, associates with Vav, a rho family activator (GEF), and probably recruits the tyrosine kinases that phosphorylate PLC.

New insights have recently emerged concerning the integration of the $\gamma$-isoforms into the immune cell response. Especially pertinent is the recognition that PLC-$\gamma$ contributes importantly to the late as well as the early rise in cytoplasmic calcium. It has been shown that both initial and the late phases of Ins(1,4,5)$P_3$ generation in B cells reflect the persistent tyrosine phosphorylation and activation of PLC-$\gamma$, which requires the Tec family kinase Btk (109). Persistent PLC activation and influx of extracellular calcium require continued production of PI(3,4,5)$P_3$, a result that supports the direct activation of PLC-$\gamma$ isoforms by PI(3,4,5)$P_3$ demonstrated in vitro (see above). Based mainly on the suppression of calcium influx by PI 3-kinase inhibitors, Scharenberg and Kinet (315) have proposed that Tec kinases, like BTK, and PI(3,4,5)$P_3$ persistently activate PLC-$\gamma_2$ to sustain the levels of Ins(1,4,5)$P_3$ and thereby maintain intracellular stores in a near-empty state. Signals from the empty internal stores should sustain the opening of store-operated calcium channels in the plasma membrane, accounting for the late phase of calcium influx. Although this idea remains to be fully tested, it successfully explains the complex pattern of phosphoinositide/calcium signaling that is generally observed.

### *2. Signaling in platelets and basophils*

In a manner reminiscent of BCR and TCR signaling, collagen engages a complex of platelet glycoproteins, including integrin $\alpha_2\beta_1$, CD36, glycoprotein VI, and Fc receptor $\gamma$-chain (31). Although the precise composition of the complex is unclear, intracellular tyrosine kinases are subsequently recruited, particularly Syk (290). Kinase recruitment leads to tyrosine phosphorylation of numerous proteins, including PLC-$\gamma_2$ (29, 155, 407), whereas tyrosine kinase inhibitors block the activation of PLC, suppressing collagen-induced calcium mobilization (242) and platelet aggregation (155). Interestingly PLC-$\gamma_1$, though present, is not phosphorylated. Collagen stimulates multiple different tyrosine kinases, of which Syk appears to be responsible for PLC-$\gamma_2$ phosphorylation, activation, and calcium release (29, 155, 242). Clustering of platelet Fc$\gamma$IIa, which triggers platelet aggregation and secretion, engages PI 3-kinase and PLC-$\gamma_2$, leading to the production of PI(3,4,5)$P_3$ and Ins$P_3$ (119). Activation and membrane translocation of PLC-$\gamma_2$ are abolished by PI 3-kinase inhibitors, which is overcome by addition of exogenous PI(3,4,5)$P_3$, suggesting that PLC-$\gamma_2$ is coactivated by both tyrosine phosphorylation and this PI 3-kinase product. In addition to activating PLC-$\gamma$, PI(3,4,5)$P_3$ also stimulates influx of extracellular calcium, but this influx does not depend on emptying internal platelet stores (229).

Similar results have been reported in the rat basophil cell line RBL-2H3 (18). When polyvalent antigens engage IgE-armed Fc$\epsilon$RI complexes on the surfaces of basophil or mast cells, internal calcium stores are released, triggering an explosive degranulation that is part of the allergic response. Fc$\epsilon$RI stimulates both PLC-$\gamma$ isoforms, although they translocate to different regions of the cell (17). Like in B and T cells, activation of PLC-$\gamma$ and the sustained rise in cytoplasmic calcium are suppressed by PI 3-kinase inhibitors. On the other hand, PI 3-kinase-dependent calcium influx is not store related, suggesting that its lipid products regulate calcium permeability through multiple different mechanisms. Although engagement of immune regulatory receptors typically triggers a protein tyrosine kinase cascade, there may be alternative routes that lead to activation of Tec kinases and PLC-$\gamma$. Both $G_q$-related subunits (21) and G$\alpha_{12}$ (174) bind to and activate the Tec kinase Btk, suggesting a potential pathway between G protein-coupled receptors and PLC-$\gamma$ in B and T cells. Indeed, the G protein-coupled receptor for thromboxane $A_2$ induces the association of G$\alpha_{12}$ with BTK, stimulating this tyrosine protein kinase in MEG-01 human leukemia cells. Whether thromboxane $A_2$ also activates PLC-$\gamma$ is unknown.

## F. Regulation by Heptahelical Receptors

PLC-$\gamma$ isoforms are also under the control of G protein-coupled, heptahelical receptors. Although there are a number of examples, we focus on ANG II receptors,

which are best studied for their links to PLC-γ. These receptors engage many of the same pathways stimulated by growth factors, antigens, and cytokines, including the ras/MAPK/ERK pathways, thereby explaining the mitogenic and other long-term effects of this and other GPCR agonists (reviewed in Refs. 122 and 230). One of these routes to ras engagement leads from GPCRs to RTKs, through src-related protein tyrosine kinases. Once phosphorylated, activated RTKs function as if bound with growth factor, recruiting the usual set of signaling molecules, including PLC-γ. The phenomenon seems quite general, since heptahelical receptors coupled to either $G_{i/o}$ or $G_q$ *trans*-activate RTKs (68). In select cases, *trans*-activation is also associated with a rise in cytoplasmic calcium and/or activation of PKC. Presumably, PLC-β subtypes contribute to the initial calcium/DAG signal, whereas PLC-γ isoforms are downstream of receptor *trans*-activation.

A prime example of this highly integrated response is found in vascular smooth muscle (VSM) where ANG II stimulates acute vasoconstriction and long-term vascular remodeling. Operating through the $AT_1$ receptor, this agonist stimulates an array of effectors including $PLA_2$, PLD, adenylyl cyclase, and the PLC-β and -γ isoforms (23). Activation of PLC-β is linked to $G_q$, whereas stimulation of PLC-γ seems to depend on protein tyrosine kinases (234). Consistent with engagement of the PLC-γ isoforms, ANG II stimulation of PLC is suppressed by genistein, whereas introduction of neutralizing antibodies against src produces a similar inhibition (236), suggesting that src, or a related protein tyrosine kinase, is required to couple the ANG receptor to PLC. Nonetheless, this idea has been challenged. In one of the notable exceptions, ANG II and other calcium-mobilizing agonists fail to stimulate tyrosine phosphorylation of PLC-$\gamma_1$, although calcium transients are observed (77). This rise in cytoplasmic calcium is dependent, in part, on protein tyrosine kinase activity, however.

The exact mechanism by which the $AT_1$ receptor activates PLC-γ has yet to be firmly established, but multiple pathways are possible. Clearly the $AT_1$ receptor engages the ras pathways, in part, by *trans*-activating RTKs (82). In the case of ANG II, the rise in cytoplasmic calcium somehow triggers c-*src* to associate with and, presumably, *trans*-activate the EGF receptor. Whether this results in PLC-γ phosphorylation is not clear. Recently, it has been demonstrated that some of the same Janus tyrosine kinases (JAKs) that bind to antigen and cytokine receptors are also recruited directly to a comparable sequence present in the receptors for ANG II (235). Specifically, this site is capable of binding a complex of SHP-2 phosphotyrosine phosphatase/JAK2 tyrosine kinase. Recently, Venema et al. (376) have shown that this same sequence is tyrosine phosphorylated by a src-like kinase in ANG II-stimulated VSM cells, leading to association of the $A_1$ receptor and PLC-γ (376).

Other pathways could also link GPCRs to PLC-γ, most notably through focal adhesion kinases, such as $p125^{FAK}$ and Pyk2. These kinases are activated by many GPCRs, creating a scaffold for the recruitment of other signaling molecules, including, perhaps, PLC-γ. Whether or how this might contribute to PLC-γ activation is unknown.

Although the $A_1$ receptor clearly couples to PLC-β, resulting in a rise in $InsP_3$ and calcium, the contribution of PLC-γ to the calcium response has been a matter of confusion. In human aortic smooth muscle, tyrosine kinase inhibitors fail to suppress PLC activity or the associated calcium transients induced by ANG II (316). Here $AT_1$ receptor signaling is directed mainly through PLC-$\beta_1$, since antibodies against this isoform, but not $\gamma_1$, block PLC activation and calcium release. Based on these observations, it would appear that the γ-subtype does not initiate the $InsP_3$/calcium signals triggered by ANG II. Supporting this view, new results show that PLC-$\gamma_1$ is mainly responsible for the delayed phase of the $InsP_3$/calcium response to angiotensin in aortic smooth muscle (369). While the acute increase in $InsP_3$ and calcium is suppressed by antibodies against PLC-$\beta_1$ and $G_{q/11}$, neutralizing PLC-$\gamma_1$ antibodies block the late (>30 s), sustained elevation of $InsP_3$, an effect that correlates with the pTyr state of PLC-$\gamma_1$. By maintaining the $InsP_3$-sensitive stores in a near-empty state, activated PLC-γ could persistently open store-operated calcium channels (Fig. 3), accounting for the late phase of calcium, as suggested for B and T cells (315). Although this is an attractive notion, the sustained rise in calcium clearly has other components, some of which may release calcium independently of $InsP_3$, presumably via ryanodine receptor-mediated mechanisms. Also notable are the voltage-dependent calcium channels whose activation by ANG II also requires tyrosine and PI 3-kinase activities (324). Enhanced influx through these channels could also promote calcium-induced calcium release.

Generalizing from the $A_1$ receptor and a limited number of other examples, it seems that GPCR engage both tyrosine and PI 3-kinases to amplify and extend the initial $InsP_3$/calcium signal through PLC-γ. In some cases, such as the ANG II receptor, this involves a hierarchal arrangement of PLC-β and -γ, the former initiating and the latter sustaining the calcium signal (Fig. 4). Hence, the $InsP_3$/calcium responses to GPCR agonists closely resemble those of antigen, cytokine, and growth factor.

### G. PI-Transfer Protein

Like GPCR activation of the β-isozymes, the local level of substrate is likely to be limiting during growth

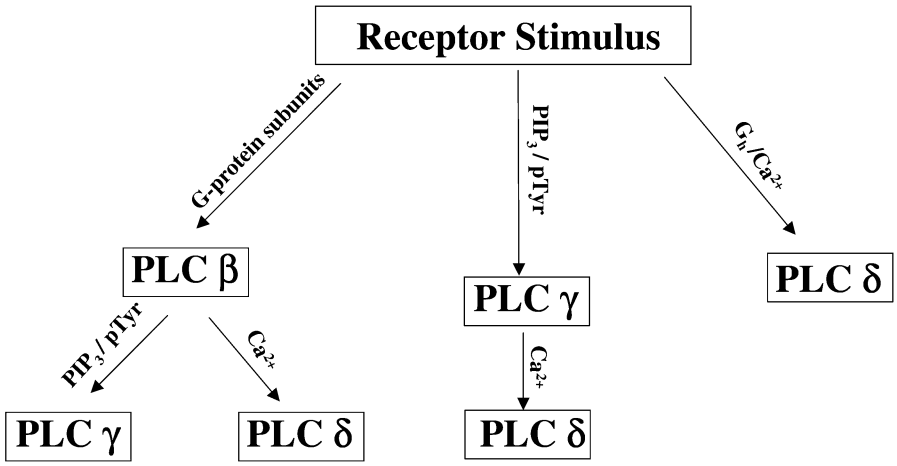



FIG. 4. Hierarchy of PLC subtypes in cell signaling. Various receptor stimuli seem to engage the activities of multiple PLC subtypes. The initial activation by GPCRs may rapidly desensitize PLC-$\beta$ isoforms. The simultaneous engagement of PLC-$\gamma$ through the action of NRTKs and PI 3-kinase production, however, could sustain the phosphatidylinositol/$Ca^{2+}$ signal. Similarly, PLC-$\delta$ isoforms, responding to the rise in $Ca^{2+}$ and costimulation by TGII/$G_h$, could amplify and extend the $Ca^{2+}$ signals initiated by either $\beta$- or $\gamma$-isoforms. The unique responsiveness of PLC-$\delta$ to the levels of $PIP_2$ (+) and $IP_3$ (−) could contribute to the frequency and amplitude of cytoplasmic $Ca^{2+}$ oscillations.

factor stimulation of PLC-$\gamma$. Evidence supporting this idea has been obtained in A431 cells permeabilized with streptolysin-O, where PI-transfer protein (PITP) is required for EGF stimulation of PLC-$\gamma_1$ and PI 4-kinase (183). This result implies that continued synthesis of polyphosphoinositides from PI is necessary, implicating activated inositol lipid kinases, which are known to be activated by EGF receptor (64). Like the GPCRs, RTK-stimulated Rho pathways also contribute to PI(4)P 5-kinase activation (417), whereas DAG, acting through PKC, may stimulate PI 4-kinase. Further enhancing the spatial restriction of the resynthesis, PITP and PI 4-kinase appear to colocalize with the RTK and PLC-$\gamma$ (183).

### H. Subcellular Distribution and Translocation

PLC-$\gamma$ isozymes translocate from the cytosol to the membrane fraction after receptor engagement, although most of the enzyme is not directly associated with the receptor. For growth factors, like EGF, this redistribution is rapid (<1 min), and reversible, accounting for nearly 70% of the enzyme in A431 cells (366). Of the PLC recovered in the particulate fraction (EGF-stimulated hepatocytes), one-half is resistant to extraction by nonionic detergents, which also accounts for most of the tyrosine-phosphorylated enzyme (409). Resistance to detergent extraction suggests an association with the cytoskeleton, which agrees with early observations in rat embryo fibroblasts, showing that PLC-$\gamma_1$ is bound to actin stress fibers and concentrated in regions of focal contacts where these fibers engage the plasma membrane (240). More recent studies also show that PLC-$\gamma_1$ is rapidly concentrated in actin-supported membrane ruffles of fibroblasts stimulated by EGF (75) or PDGF (414).

PLC-$\gamma_2$ appears to have a subcellular distribution distinct from $\gamma_1$. In mast cells, both PLC-$\gamma_2$ and -$\gamma_1$ are tyrosine phosphorylated following Fc$\epsilon$R1 receptor engagement (see sect. IV*B*), yet the later translocates to the plasma membrane where it is concentrated in ruffles, whereas PLC-$\gamma_2$ remains in the subplasma membrane and perinuclear regions of the cell (17). The two isotypes are further distinguished by wortmannin, an inhibitor of PI 3-kinase and other inositol lipid kinases, which blocks the redistribution of PLC-$\gamma_1$ leaving $\gamma_2$ unaffected. Clearly, determinants unique to each isoform must be involved, but their nature is unknown.

Several domains that direct PLC-$\gamma$ to appropriate intracellular sites have been identified. Although the SH2 domains recruit to pTyr proteins, as described above, the SH3 and PH domains direct PLC-$\gamma$ to other binding partners. The $\gamma_1$ SH3 domain, which binds strongly to proline-rich sequences, may direct PLC-$\gamma_1$ to actin filaments and could contribute to its codistribution with other actin-based structures (19). This domain may also mediate association of PLC-$\gamma_1$ with endocytic vesicles, since it binds strongly to the proline-rich region of dynamin (314, 322), a microtubule-associated GTPase involved in endocytosis. The amino-terminal PH domain, which binds $PI(3,4,5)P_3$, is also critically important in directing PLC-$\gamma_1$ to actin-supported modifications of the plasma membrane (94). This translocation to ruffles is blocked by overexpression of a dominant negative PI 3-kinase mutant, or by treatment with wortmannin. Thus a number of binding partners, both protein and lipid, are likely to govern the distribution of the $\gamma$-isoforms.

### I. Functional Studies of the PLC-$\gamma$ Isozymes

#### *1. Cell cycle control*

Because PLC-$\gamma$ isoforms are recruited, phosphorylated, and activated by receptors for antigens, immunoglobulins, cytokines, and growth factors, functional studies have focused on their role in cell growth. Although some studies have implicated PLC-$\gamma$, many have concluded these isozymes are not essential. Many of the early

studies involved deletion or mutation of the RTK autophosphorylation sites that engage PLC-$\gamma$. For example, mutation of these sites in the FGF (247) and PDGF receptors (370) prevented activation of PLC but failed to suppress FGF- or PDGF-stimulated mitosis. Conversely, overexpression of PLC-$\gamma_1$ did not enhance DNA synthesis (233).

These observations seemed to exclude an essential role in growth factor-stimulated mitosis, yet other studies suggested PLC-$\gamma_1$ strongly influences the process. Many of these experiments involved introduction of neutralizing antibodies or portions of PLC-$\gamma$ or the entire enzyme. In fibroblasts, microinjection of native PLC-$\gamma_1$ or a catalytically inactive mutant stimulated entry into the cell cycle (151, 341, 342). The SH3 domain, but not SH2 domain of the Z region, was necessary, although not sufficient for the response (151). Similarly, microinjection or expression of the entire Z region stimulated proliferation of fibroblasts (340) and PC-12 cells (9), implicating PLC-$\gamma$ in cell cycle control.

Contrary results however have been reported. In these experiments, the Z region and its component domains function as dominant negative regulators, disrupting growth factor signaling and mitosis. Microinjection of either of the SH2 domains blocks PDGF-stimulated c-*fos* expression and DNA synthesis in fibroblasts (305). Similarly, these SH2 domains inhibit PLC-$\gamma$ activation during fertilization and delay the rise in cytoplasmic calcium (46). Likewise, introduction of myristoylated peptides based on Z-region sequences block PLC activation by PDGF, EGF, bombesin, or serum and suppress cell growth (143). Introduction of the entire Z region into NIH-3T3 fibroblasts and Madin-Darby canine kidney (MDCK) cells blocks PDGF- and EGF-stimulated entry into the S phase of the cell cycle (388). This blockade correlated with DAG/PKC-dependent gene expression.

Other observations also support a positive role in mitosis. Microinjection of neutralizing antibodies specific for the $\gamma_1$-isozyme also blocks proliferation induced by oncogenic ras, although antibodies against ras fail to prevent proliferation induced by microinjected PLC-$\gamma_1$ (339), suggesting that this enzyme operates downstream of ras to induce mitosis. Interestingly, PLC-$\gamma_1$ also associates with the upstream regulators of ras, Grb2 and SOS, during growth factor activation (284), establishing a plausible link between this enzyme and the ras/raf/MEK/MAP kinase pathway.

Although these results favor a role for PLC-$\gamma_1$ in receptor-stimulated mitosis, there are many conflicting reports. In particular, disparities between those studies reporting growth inhibitory effects of the Z-region domains, and those showing growth-promoting effects, remain unresolved. Significant weaknesses are also inherent in the designs of these experiments. The most critical issue concerns specificity. Because SH2, SH3, and other domains operate cooperatively to reenforce specific binding to other pathway components, their independent overexpression is liable to engage proteins unrelated to the physiological binding partners of PLC-$\gamma_1$. Likewise, excessive amounts of the whole enzyme may promote promiscuous interactions.

On the other hand, many of the studies that separated PLC activation from the mitogenic potential RTKs have comparable weaknesses. In these experiments, erasing the PLC-$\gamma$ recognition site on the receptor creates an artificial situation in which recruitment and upregulation of redundant pathways cannot be discounted. Because receptors normally engage a signaling network rather than a set of linear pathways, the quality and dynamics of all the signals emanating from such disabled receptors are likely to be quite different.

In an alternative approach, the biological function of PLC-$\gamma$ has been examined using transgenic mice in which one or both copies of the $\gamma_1$-gene are disrupted (168). Animals heterozygous for the null allele develop normally, whereas homozygous mice die by embryonic *day 9*. These embryos are small but are otherwise normal in appearance. Although further work is needed to examine the cause of their mortality, it is clear that PLC-$\gamma_1$ is indispensable at a time of active fetal growth but indispensable for what, is not known. By this stage of development considerable growth has occurred and organs are already well formed. Thus the results are compelling evidence against an essential and generalized role in mitosis; rather, the effect on growth seems highly conditional.

To further address the role of this isoform in growth control, fibroblasts cultured from the $\gamma_1$-null embryos have been studied (167). These cells fail to mobilize calcium in response to PDGF or EGF, yet they divide normally in response to serum; in fact, the incorporation of radioactive thymidine into newly synthesized DNA is more robust.[7] Many biochemical responses to these growth factors also remain intact, including MAP kinase activation and induction of c-*fos*. Interestingly, cells lacking PLC-$\gamma_1$ grow to higher densities before becoming contact inhibited, but this appears to be a function of their rounded morphology and not the loss of contact inhibition, suggesting a role in cytoskeletal regulation rather than cell cycle control. These observations echo the re-

[7] An alternative explanation of the transgenic phenotype invokes redundant/adapted pathways in the $\gamma_1$-null embryo. Functional redundancy and adaptation are observed in many basic cell processes. For example, multiple members of the src family are capable of replacing each other in propelling cells through the $G_2$ phase of the cell cycle (304). In the case of the PLC-$\gamma_1$ null mice, a clear candidate is PLC-$\gamma_2$, which is widely expressed. The presence of this alternative isotype could account for the PDGF-induced calcium mobilization observed in fibroblasts cultured from the $\gamma_1$-null embryos (136). Thus it is still possible for PLC-$\gamma_1$ to play an important role earlier in development, but one that is masked by other PLC isozymes or other signaling pathways.

sults obtained by mutating the PLC-$\gamma$ recognition sites of RTKs. Nonetheless, as in previous work, these immortalized cell lines harbor other, growth-related genetic defects that permit their adaptation to continuous culturing; such defects may mitigate against the loss of PLC.

As discussed previously, comparable results have been obtained in B cells lacking the $\gamma_2$-isoform. These cells lose their apoptotic response to surface IgM but otherwise grow normally in culture (358), suggesting that PLC-$\gamma_2$ plays a more important part in the cell death program than the cell cycle.

Taken as a whole, it would appear that, under most circumstances, PLC-$\gamma$ isoforms do not play a central role in mitosis. Whether these effector enzymes mainly modulate, or are redundant to other pathways, is unclear. Either possibility would be consistent with the role PLC-$\gamma$ isoforms play in differentiation and development, as discussed below.

### *2. Cell transformation*

Although the relationship to mitosis is unsettled, there is a good correlation between the transformed phenotype, the levels of PLC-$\gamma_1$, and tumor progression. For example, expression of PLC-$\gamma_1$ is dramatically increased during the progression of breast cancer (263) and in polyps from patients with familial adenomatous polyposis (278), a condition that often leads to colorectal cancer (264).

Consistent with the idea that PLC-$\gamma_1$ permits the progression of some tumors, overexpression of PLC-$\gamma_1$ in cultured fibroblasts promotes anchorage-independent growth, reduces their serum requirements, increases their overall growth rate, and disrupts the normal cell cycle (338). These same fibroblasts induce tumors in nude mice, suggesting that this enzyme, which is expressed at high levels in many human tumors, can promote cell transformation. Of course, the same concerns apply to this experiment as discussed above.

An important role for PLC-$\gamma_1$ in transformation is also suggested by the finding that $\beta$-PDGF receptor-dependent transformation of fibroblasts requires only the receptor autophosphorylation site that binds PLC (70). The only other site capable of sustaining the transformed phenotype is the docking site for PI 3-kinase p85 subunit. On the other hand, PLC-$\gamma$ stimulation is not essential for $\alpha$-PDGF receptors to promote anchorage-independent cell growth, but it is needed for $\alpha$-PDGF-dependent focus formation (415).

Interestingly, the transforming potential of EGF and NGF receptors is inversely related to their relative affinities for PLC-$\gamma_1$ (266). PLC-$\gamma_1$ has a much higher affinity for docking sites in the NGF receptor's cytoplasmic domain than the corresponding sites in the EGF receptor. In cells expressing EGF receptors bearing PLC binding sites from the NGF receptor, more robust and sustained increases in PLC products, MAP kinase activities, and cytoplasmic calcium levels are observed. These effects correlate positively with enhanced thymidine incorporation but inversely with transformation, as assessed morphologically, and in focus-forming assays performed in culture and in soft agar. This difference in transforming potential may be due to enhanced receptor dephosphorylation observed when the EGF receptor contains a high-affinity PLC binding site; the activation/deactivation kinetics of various downstream targets, including MAP kinase, are also affected. Wild-type EGF receptor normally has a prolonged phospho-state lasting hours, whereas the chimeric receptor possessing the high-affinity site loses the phospho-state in ~5 min, presumably due to greater stimulation of PLC-$\gamma_1$ and generation of $InsP_3$, $Ca^{2+}$, and DAG. Unable to maintain its active state, the EGF-stimulated chimeric receptor fails to promote the transformation phenotype, an example where the duration of the signal is as important as intensity in shaping the biological response.

### *3. Control of the actin cytoskeleton*

The influence of PLC-$\gamma$ on cell transformation could be related to its effect on the actin cytoskeleton, especially since polyphosphoinositides and their metabolism have profound effects on cell shape and motility (163). Their interactions with proteins that directly regulate actin polymer assembly have led to the idea that polyphosphoinositides are key to the remodeling of actin-based structures, such as focal adhesions and contacts, stress fibers, filopodia, and lamellipodia. When triggered by soluble and matrix-based stimuli, these dynamic structures alter cell shape, adherence, and purposeful movement. PI-metabolizing enzymes, including PLC-$\gamma$, have been implicated.

Assembly/disassembly of actin-based structures is coupled to the activation of receptor tyrosine kinases, integrins, and GPCRs by rho/rac/cdc42 family of low-molecular-weight GTPases (reviewed in Ref. 123). Rho regulates the maturation of focal adhesions, while rac and cdc42 control the formation and activity of lamellipodia and filopodia. Importantly, the three GTPases function in a coordinated and hierarchal arrangement with GPCRs, like bradykinin, activating cdc42, which activates rac, a GTPase under the control of RTKs and ras. Rho is conditionally activated or inhibited by rac and stimulated by lysophosphatidic acid. These interactions result in a coordinated remodeling of different actin-supported structures. A host of signaling molecules operate both upstream and downstream of these LMW GTPases (297). These include protein tyrosine kinases, such as FAKs and src, Ser/Thr protein kinases, typified by rho kinase, as well as various inositol lipid kinases (57, 62, 126, 306).

Reports that both PLC-γ subtypes are activated by $PI(3,4,5)P_3$ suggest the possibility that inositol lipid kinase pathways, controlled by rho-related GTPases, coordinate the actions of PLC-γ. Indeed, the consistently observed translocation of PLC-$\gamma_1$ to membrane ruffles and its association with the actin cytoskeleton point to a role in actin remodeling.

A) FOCAL ADHESIONS. Focal adhesions connect the internal actin cytoskeleton with the extracellular matrix (ECM) through integrins whose affinity for the matrix is highly regulated (320). Mature focal adhesions are composed of clustered integrins and actin-associated proteins, such as vinculin, α-actinin, and talin, that link stress fibers to the plasma membrane. When isolated, these specialized membrane microdomains are found to contain an array of active regulatory enzymes including FAKs, ras, src, PI(4)P 5-kinase, PI 3-kinase, and PLC-$\gamma_1$ (175). The PLC substrate $PI(4,5)P_2$ is also found in these focal adhesions (111) and is essential for their normal assembly (116). What role the γ-isoforms may play in formation and stability of focal adhesions, however, remains unclear.

B) FOCAL COMPLEXES AND MOTILITY. Focal complexes, unlike focal adhesions, are highly dynamic structures involved in cell locomotion (123). Although focal complexes are composed of the same constituent proteins, they are much smaller than adhesions and are not involved in stress fiber formation. These complexes also form connections with the ECM through integrins. In motile cells, these connections are transient, alternatively broken through cyclical changes in integrin affinity for the ECM as the cells, following a chemical or matrix gradient, extend lamellipodia and other structures through rac and cdc42-dependent actin polymerization. Alternatively, stable adhesion complexes can be torn from the surface of the cell's posterior edge, leaving behind a trail of integrins (269). In the former mechanism, dissociated integrin molecules may be recycled through an endocytic pathway from the back to the leading edge of the cell (207). These cyclical changes in integrin affinity imply control through inside-out signaling pathways that could involve both PLC-γ and inositol lipid kinases, suggesting an important role for this PLC subtype in cell motility.

Such a relationship has been established between activation of PLC-γ and growth factor-induced motility in fibroblasts. Here overexpression of the PLC-$\gamma_1$ Z region, acting as a dominant negative suppressor, or introduction of antisense-oligonucleotides specific for PLC-$\gamma_1$ suppress both EGF-stimulated PLC activity and chemotaxis (50). Similarly, EGF receptors missing the autophosphorylation sites required to dock PLC-$\gamma_1$ are unable to transduce a cell migration signal. In a comparable study of PDGF stimulation, erasure of the receptor autophosphorylation sites for docking PLC-$\gamma_1$ and PI 3-kinase suppresses chemotaxis, as does overexpression of a dominant negative form of PLC-$\gamma_1$ lacking key phosphorylation sites (Y783F and Y771F) (204). Because PLC-γ isoforms may also be recruited and activated by $PI(3,4,5)P_3$, an order of events can be suggested that is relevant to cytoskeletal remodeling and motility. Upon binding of growth factors or other stimulants that engage inside-out signaling pathways, PI 3-kinase is activated, generating $PI(3,4,5)P_3$. The 3-phosphorylated lipid directs tyrosine phosphorylated PLC-γ isoforms to sites of cytoskeletal remodeling. This is consistent with the observation that PLC-$\gamma_1$, or its amino-terminal PH domain, translocates in a PI 3-kinase-dependent manner to the membrane ruffles of motile cells stimulated by IgE (17) or PDGF (94). How this might affect integrin binding to the ECM and the actin cytoskeleton is unknown. It is worth noting, however, that the PLC substrate, $PI(4,5)P_2$, and the PI 3-kinase product $PI(3,4,5)P_3$ mediate the binding of integrins to proteins that modulate their binding to the actin cytoskeleton and extracellular matrix.

Although the preceding discussion ties PLC-$\gamma_1$ activation to the control of actin-based structures, there is considerable evidence that its role is not generally essential. For example, cells expressing basic fibroblast growth factor (bFGF) receptors missing the PLC-$\gamma_1$ docking platform do not generate an $InsP_3$/calcium signal in response to bFGF, yet they migrate in response to a chemotactic gradient (63). Similarly, execution of the early developmental program in the mouse, which involves extensive cell migration and cytoskeletal remodeling, seems relatively unperturbed by disruption of both PLC-$\gamma_1$ alleles (168).[8] Obviously other signaling components are engaged. Hence, like mitosis, PLC-$\gamma_1$ plays a conditional role. Whether this involves the modulation of actin remodeling initiated by other pathways or a redundancy in control is unknown.

### *4. Development*

It is evident from transgenic studies that PLC-$\gamma_1$ is dispensable in very early embryonic development, where compartmentation, differentiation, and their associated cellular processes have already functioned efficiently (see above). The previous finding that mesodermal induction occurs without PLC-γ coupling to the FGF receptor (311) is consistent.

In the mammalian PC-12 cell line, PLC-γ may function in neuronal differentiation, but its role is only apparent when connections to other pathways are simultaneously broken. In PC-12 cells, binding of NGF stimulates

[8] Fibroblasts derived from PLC-$\gamma_1$ null embryos exhibit membrane ruffling in response to PDGF (136) and fill the wounded sites in monolayer cultures (167). Interestingly, these fibroblasts have a more rounded morphology than their normal counterparts, implying that their control of cytoskeletal structures and adhesion is abnormal.

auto- and *trans*-phosphorylation of its receptor which then recruits an array of signaling proteins including SHC, PI 3-kinase, phosphotyrosine phosphatase (PTP1D), and PLC-$\gamma_1$ resulting in neurite outgrowth, a measure of differentiation (265). Removal of the receptor's docking site for PLC-$\gamma_1$ or PI 3-kinase has only marginal effects on the differentiation program, however (226, 265, 348). Only the SHC site, which engages the ras pathway, is essential, since its loss severely depresses neurite outgrowth (265). Conversely, loss of both the PI 3-kinase and PLC-$\gamma_1$ binding sites, but retention of the SHC site, permits stimulated outgrowth. Removal of the SHC and PI 3-kinase sites, but retention of the PLC-$\gamma_1$ site, is permissive, while SHC and $\gamma_1$-sites together enhance differentiation; similar results were reported elsewhere (348). Although the SHC and PLC-$\gamma$ pathways are largely redundant in PC-12 cells, this may not be the case in other neurons or other differentiation programs. Indeed, $\gamma_1$-expression in developing mammalian brain is highly regulated (as described previously); it would be surprising were it not to play a dominant role in the differentiation programs of at least a few specialized neurons.

A clearer picture has emerged from studies of fruit flies. Here the *Drosophila* small wing (*sl*) gene encodes a homolog of PLC-$\gamma$ (87). While the homozygous null mutation is not lethal, inactivating mutations in *sl* result in wing defects and the appearance of additional R7 photoreceptors (364). Development of these photoreceptors in *Drosophila* is under the control of several RTK, each of which signals through the ras/raf/MEK/MAPK pathway, as well as other overlapping or redundant ras-independent paths (reviewed in Ref. 422). Results obtained with *sl*/ras pathway double mutants suggest that PLC-$\gamma$ suppresses RTK signaling by downmodulating connections to the ras-dependent pathways. Loss of PLC-$\gamma$ leads to overstimulation of the ras/raf/MEK/MAPK module and disruption of the normal developmental program. This is consistent with the finding that reduced *DER* (EGF receptor homolog) expression in the *sl* mutant background rescues their eye defect. Similary, the ras pathway mutant sevenless, which would otherwise lack R7, mitigates the supernumerary R7 *sl* phenotype. A partial loss of functional mutation in the MAPK homolog, rolled (*rl*), also reduces the number of ommatidia containing extra R7 cells, again implicating the ras/raf/MEK/MAPK module.

The *DER/sl* mutant phenotypes are consistent with the findings in mammalian cells that PLC-$\gamma$, operating through PKC and/or other kinases, modulates growth or differentiation signals by engaging negative-feedback pathways (266, 323).[9] Thus a similar situation could pertain, where the ability of PLC-$\gamma$ to influence cell fate seems limited to wing development and a special collection of photoreceptor cells in which PLC-$\gamma$-dependent pathways strongly modulate RTK output.

## V. THE PHOSPHOLIPASE C-$\delta$ ISOZYMES

A single $\delta$-related gene is found in simple organisms, such as yeast and cellular slime molds, whereas numerous $\delta$-isoforms and alternatively spliced variants have been described in higher plants and mammals (Table 3). Although much is known of their structure and chemistry, particularly the mammalian $\delta_1$-isoform, their biology has remained obscure. Until very recently, most clues to function have been discovered in nonmammalian organisms. In this section, we review the studies of PLC-$\delta$ in yeast, cellular slime molds, plants, and mammals and discuss the ideas pertaining to its regulation and biological role.[10]

### A. Yeast PLC

*S. cerevisiae* (107, 283, 411), *S. pombe* (6), and the pathogenic yeast *Candida albicans* (22) contain a single gene, *PLC1*, encoding a protein of ~100 kDa, which is most closely related to the mammalian PLC-$\delta$ isoforms which are ~85 kDa. The larger size is accounted for by additional sequences located amino-terminal to the PH domain (Table 3). Similar to its mammalian counterparts, Plc1p is a calcium-dependent enzyme, with a marked preference for PI(4,5)$P_2$ (107). An understanding of PLC1 function has come from studying mutant yeast strains carrying either a disruption in the gene or a mutation that confers temperature sensitivity (*ts*).

In *S. cerevisiae*, deletion of *PLC1* slows growth, which ceases all together at temperatures above 34°C (107, 411). At these temperatures, null *PLC1* mutants fail to complete cytokinesis and become multi-budded (107). Interestingly, chromosomes are also missorted when *ts* mutants of Plc1p are grown just below the nonpermissive temperature (283). Although this could imply direct participation in cytokinesis or chromosome sorting, evidence against a direct role has been obtained. When ts-plc1p strains are rapidly shifted to the nonpermissive temperature, the cells are blocked at all stages of growth (410). The random nature of the blockade suggests that active plc1p is conditionally required throughout the cell cycle; failure to complete cytokinesis or correctly sort chromosomes could be an effect secondary to the inhibition of other processes.

[9] The effect, however, is receptor specific. Unlike the EGF receptor that is downmodulated, signaling by the PDGF receptor is enhanced when PLC and PKC are persistently activated (127).

[10] PLC-$\delta$-related isoform was recently identified in hydra (198), and multiple $\delta$-isoforms were discovered in *Neurospora crassa* and other filamentous fungi (176), but the information concerning their regulation and function is insufficient to warrant discussion.

TABLE 3. *Phospholipase C-δ isozymes*

| Organism | Residues | Sequence Features* |
|---|---|---|
| | |   |
| Mammals | | |
| PLC-$\delta_1$(H) | 756 | Sequence diagram based on this isoform |
| PLC-$\delta_2$(B) | 764 | Most similar to $\delta_1$ |
| PLC-$\delta_3$(H) | 736 | String of 13 acidic residues (Glut) in the loop region of TIM barrel |
| PLC-$\delta_4$(R) | 772 | Similar to $\delta_2$; alternatively spliced forms exist |
| Alt1(R) | 786 | 32 Residues added after residue 487 in loop between X and Y |
| Alt2(R) | 782 | 14 Residues added after residue 487 in loop between X and Y |
| Alt3(R) | 817 | 63 Residues substituted for the 32 found in Alt1; catalytically inactive |
| Alt4(R) | 771 | 16 Residues substituted for 17 starting at residue 479 of $\delta_4$ |
| $IP_3$-BP(R) | 1,096 | Catalytically inactive (closely related to $\delta_1$) |
| Plants | | |
| Several forms | 600–650 | All subtypes lack the PH domain and first two EF-hand motifs |
| Slime mold | | |
| *DdPLC* | 801 | Most similar to PLC-$\delta_1$ |
| Yeast | | |
| *PLC1p* | 869 | Additional sequence amino terminal to the PH domain |
| Hydra | | |
| PLC-δH | 738 | Most similar to PLC-$\delta_1$ |

(H), human; (B), bovine; (R), rat. * See legend to Table 1 for definition of asterisk.

In addition to temperature, this growth defect is dependent on the genetic background of the strain and nutrients present in the medium. PLC1 null mutants grow poorly in media where they must utilize galactose, raffinose, or glycerol, or where nitrogen is limiting, but they grow normally on glucose-containing media (107), suggesting a complex relationship between PLC activity, carbon sources, and nitrogen sensing. This is consistent with previous work showing that $InsP_3$ and DAG levels are increased when starved yeast are placed in nitrogen-containing medium (319). While PLC activation was originally associated with glucose sensing (177), later work unequivocally demonstrated that this carbohydrate does not stimulate $InsP_3$ and DAG formation, although it does induce cell cycle entry (129, 319).

More recent results suggest a set of pathways that link PLC and nutrient sensing to cell cycle control. In these experiments, the temperature-sensitive growth defect exhibited by the *PLC1* null mutant (Δplc1) is suppressed by PHO81, an inhibitor of cyclin-dependent protein kinase (Pho80p/Pho85p Cdk), as well as a related gene, *SPL2* (108). The relationship of PLC1 to growth is not simple, however, since mutations in the two suppressors alone fail to mimic loss of this gene. Moreover, double mutants Δplc1/Δspl2 or Δpho81 exhibit a more severe growth defect than Δplc1 alone. The results imply that *PLC1*, PHO81, and SPL2 have some overlap in function and may participate in convergent pathways regulating growth at elevated temperatures or under restrictive nutrient conditions.

PLC1 seems to function similarly in *S. pombe*. Here, the growth-inhibited phenotype of plc1p mutants, selected in high phosphate minimal medium, is suppressed by lowering the concentrations of phosphate and *myo*-inositol (95). The finding that reduced inositol suppresses the plc1p growth defect is of note, since inositol is key to controlling the transcription of numerous genes required for phospholipid biosynthesis. When inositol levels are low, syntheses of PC, PS, and phosphatidylethanolamine are enhanced (44). This transcriptional regulation is also closely linked to production of chaperones and the response to unfolded ER proteins in *S. cerevisiae* (332). It is possible that *PLC1*-generated signals are required to match membrane biosynthesis to the production of ER-resident chaperones when yeast are grown at elevated temperatures. Restricting inositol may supplant these signals.

Recent work in *S. cerevisiae* also implicates *PLC1* in the TOR2 (targets of rapamycin) signaling network, which coordinates mitogenic and protein synthetic pathways, with organization of the actin cytoskeleton (365). TOR1 and TOR2, putative PI and protein kinases, are required for translational initiation; TOR2 is also necessary for cell cycle-dependent organization of the actin cytoskeleton. When overexpressed, PLC1 or MSS4, a PI(4)P 5-kinase, suppresses mutations in TOR2 that impact on actin organization and protein synthesis (131). In contrast, PKC1 suppresses only the actin defect. Interestingly, overexpression of Pkc1p and Pkc1-regulated MAP kinase (Mpk1p) also rescues a *ts* tor2 mutant defective in rho-dependent actin organization (132). Taken as a whole, these results suggest that Tor2p may regulate the supply of substrate to PLC via PI(4)P 5-kinase, which is somehow important for protein synthesis. On the other hand, DAG, derived from the PLC catalyzed reaction, may acti-

vate a PKC/MAPK cascade, thereby regulating actin organization.

In *S. cerevisiae*, Plc1p modulates pseudohyphal differentiation, which is also linked to nutrient detection (7). Gpr1p, a putative heptahelical receptor involved in nitrogen sensing, interacts with Plc1p, and with the G protein Gpa2p, which operates in a ras-independent, cAMP-dependent pathway to control filamentation and growth. Cells lacking Gpr1p, Plc1p, or Gpa2p fail to form pseudohyphae when nitrogen sources are removed. The filamentation defects of Gpr1 and Plc1 null strains are rescued by activating STE11–4 (mitogen-activated protein kinase pathway) or overexpressing Tpk2p, a catalytic subunit of cAMP-dependent protein kinase (cAMP pathway). The physical association of Plc1p with Gpr1p, measured by two-hybrid and coimmunoprecipitation, is independent of Gpa2, whereas Gpr1p/Gpa2p association depends on the presence of the phospholipase. These genetic and physical interactions between PLC1, GPR1, and GPA2 suggest Gpr1p and Gpa2p act in concert with Plc1p, but in parallel to ras. Thus Plc1p is an important component in a nitrogen-sensing signaling pathway that controls the switch to pseudohyphal development. Based on analysis of transcriptional regulation of filamentation, it appears that the MAPK pathway is strongly dependent on Plc1p, but not Gpr1p. In contrast, Gpr1p acts mainly through the cAMP pathway involving Gpa2p. Because Ras2p suppresses gpr1pΔ and gpa2Δ phenotypes, yet fails to rescue plc1p null mutants, Ras2p may also be downstream of Plc1p.

Taken together with the binding data, these results suggest that Plc1p acts upstream of the G protein, Gpa2p, mediating or regulating its interactions with its cognate heptahelical receptor, Gpr1p. It is proposed (7) that activation of Plc1p may hydrolyze $PI(4,5)P_2$ locally, exposing a binding site for Gpa2p in the carboxy-terminal region of Gpr1p. How these interactions are actually regulated by PLC catalytic activity is unknown.

In addition to its roles in nutrient sensing, growth control, and differentiation, PLC1 is also important in the response of yeast to stress. Indeed, the upstream regulatory region of the PLC1 gene appears to contain a heat shock promoter (107). As with any stress response gene, the production of plc1p is tightly controlled resulting in normally low levels of expression and activity. Further supporting this classification, 14–3-3 proteins, Rad24p and Rad25p, which are involved in radiation damage responses, have been identified as binding partners for plc1p (5). Consistent with its involvement in damage resistance, PLC1 null mutants, like rad24 null yeast, are hypersensitive to ultraviolet irradiation. It is also worth noting that PLC1 is required for sporulation (107), a process triggered when nutrient and environmental conditions no longer support growth.

Further supporting a stress response role, PLC1 modulates the nuclear export of mRNA, including those that are stress related (412). York, Wente, and co-workers (412) identified three sets of mutations that are lethal in combination with a *ts* mutation in gle1p, an essential component of the nuclear pore complex. *PLC1* was found to complement one of these groups. The other two involved mutations in IPK1, a nuclear inositol 1,3,4,5,6-pentakisphosphate ($InsP_5$) 2-kinase and GSL3, an $InsP_4$/$InsP_3$ kinase, or regulator thereof. The genetic interactions of *PLC1* and these inositol polyphosphate kinases are consistent with a functional connection whereby the InsPs, generated by Plc1p, are converted to inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$), a regulator of nuclear export. Because this inositol polyphosphate accumulates under stress conditions and is undetectable in PLC1 null mutants, York and co-workers (412) propose that $InsP_6$ is an important stress signal generated by a PLC/InsP-kinase pathway. Under stress conditions, the $InsP_6$ product may bind the pore complex and thereby modulate mRNA transport.

### B. Slime Mold PLC

The cellular slime mold *Dictyostelium discoideum* has been studied extensively as a model for cellular differentiation and chemotaxis (72, 271). *D. discoideum* normally exists as free living haploid ameba. When their food supply is exhausted, the ameba stops dividing and excretes a chemoattractant that prompts their aggregation, slug formation, and differentiation, with eventual development of a fruiting body consisting of spores atop a cellular stalk. Among the most important signals that initiate the change from single to multicellular organism is cAMP, a substance normally considered an intracellular second messenger. Acting as a chemoattractant, cAMP, generated in pulses by the ameboid cells, is essential to normal aggregation and development of the multicellular slug, stalk, and fruiting body. cAMP binds to heptahelical receptors, like cAR1, whose occupancy leads to activation of adenylyl and guanylyl cyclases, as well as influx of extracellular calcium (373). $G\alpha_2$, one of many $G\alpha$ subunits essential for normal development, couples cAR1 to stimulation of these effector pathways.

A single PI-PLC gene, *DdPLC* in *D. discoideum*, encodes a 91-kDa protein with strong homology to mammalian PLC-$\delta$ (80). Although its mRNA and activity increase in ameba during starvation and later during development, neither overexpression of *DdPLC* (80) nor disruption of its gene (79) noticeably affects growth or development. Surprisingly, cAMP still increases $InsP_3$ in *DdPLC* null cells, but this is due to activation of an inositol polyphosphate phosphatase rather than PLC activation (371, 372), leaving open the question of *DdPLC* function.

New findings point to an important, albeit condi-

tional, role for *DdPLC* in *D. discoideum*. Conditioned media factor (CMF), a protein secreted by starved ameba and implicated in cell density sensing (118), binds its own GPCR, which leads to PLC activation, decreasing the threshold for sensing cAMP (38). Thus the activated enzyme and its products enhance the sensitivity of dispersed ameba to cAMP gradients, like those found in the slime mold's natural habitat. CMF receptor signals through another G protein, $G\alpha_1$, which is required to couple these receptors to *DdPLC* (38). CMF activation of *DdPLC* also suppresses the intrinsic GTPase activity of $G\alpha_2$, the G protein that couples cAR1 to its effectors, thereby preserving the activated state of $G\alpha_2$ and enhancing sensitivity to cAMP. This explains why ameba lacking PLC fail to aggregate at low cell densities, even in the presence of CMF. How *DdPLC* activation effects GTPase suppression is unknown.

Although $G\alpha_1$ is required to couple CMF receptor to PLC, $G\beta\gamma$ could be the main positive regulator of PLC in *D. discoideum*, since cells lacking the single $\beta$-gene fail to increase PLC activity in response to CMF, whereas cells lacking $G\alpha_1$ have high basal PLC activity and behave as though stimulated by this factor (38). Reconstitution studies have yet to be performed, so this positive connection between $G\beta\gamma$ and *DdPLC* could be indirect.

## C. Plant PLC

Multiple different PLC-δ-related proteins have been identified in higher plants where phosphoinositide/calcium signaling systems, activated by auxins, oligosaccharide elicitor, and light are already well-established phenomena (81). δ-Related PLC are found in *Arabidopsis thaliana* (125, 139), *Glycine max* (soybean) (328), *Solanum tuberosum* (196), and *Pisum sativum* (pea). They lack the amino-terminal PH domain and the first two EF-hand motifs. Of the *Arabidopsis* PLC sequences, two are also missing a portion of the third EF-hand as well. So far, no $\beta$- or $\gamma$-related subtypes have been identified in plants.

Although plant PLC lack some of the motifs found in their mammalian counterparts, particularly the amino-terminal region, the overall properties of the enzymes appear unchanged. These proteins are recovered in the particulate fractions of plant tissues, readily hydrolyze $PI(4,5)P_2$, and respond to calcium in the range of 0.1–10 $\mu$M. (196, 328).

The connection between PLC and stress responses is most dramatically demonstrated in higher plants where environmental factors markedly alter PLC expression. In *Arabidopsis*, mRNA encoding AtPLC1S, one of a number of δ-related isotypes, is concentrated in shoot and leaf. Its mRNA levels increase markedly when the plants are exposed to drought, cold, osmotic and salt stresses, as well as absicissic acid, which is known to induce the expression of stress-related genes in higher plants (139). These observations are consistent with the enhanced phosphoinositide metabolism and calcium mobilization observed under similar conditions. In contrast, another form of PLC in *A. thaliana*, AtPLC2, is constitutively expressed in vegetative and floral tissues (138) and is not affected by these environmental stresses, implying that each isotype serves a different purpose. Similar results are found in potato, where three PLC-δ isotypes are differentially expressed in leaves, flowers, tubers, and roots (196). As in *A. thaliana*, PLC gene expression is differentially altered by stress with mRNA levels encoding two δ-isoforms changing dramatically, but inversely in wounded or wilted leaves. When subjected to long-term stresses, the levels of all three isoforms change in different directions, suggesting a complex relation between PLC activity and adaptation of plants to their environment.

## D. Mammalian PLC-δ

Four different subtypes have been described in mammals; at least one of these, PLC-$\delta_4$, is differentially expressed as spliced variants (Table 3). This section focuses mainly on the best understood isoform, PLC-$\delta_1$.

### *1. PLC -$\delta_1$*

Among the main δ-isoforms found in mammalian tissues, PLC-$\delta_1$ is the most abundant and widely expressed, although its levels are relatively low compared with $\beta$- and $\gamma$-subtypes (352). $\delta_1$-mRNA levels are highest in skeletal muscle, spleen, testis, and lung (145). Many different cultured cell lines also express PLC-$\delta_1$ protein; $GH_4$, PC12, and C6 glioma cells have notably high levels. In adult rat brain, PLC-$\delta_1$ is concentrated in astroglial cells, whereas much lower levels are present in neurons (56, 246). During embryonic rat brain development, PLC-$\delta_1$ is diffusely distributed (404). By postnatal *day 14*, moderate levels of $\delta_1$ are detected in astrocytes, which rise rapidly thereafter. The function of PLC-$\delta_1$ in astrocytes, however, is unknown.

Apart from the CNS, surprisingly little information is available on the tissue levels and distributions of PLC-$\delta_1$ or the other δ-isoforms during mammalian development, even though levels differ significantly among adult tissues. With the sequencing of the human gene encoding the entire PLC-$\delta_1$ protein (15 exons spanning 22 kb) (159), more information should be forthcoming on the molecular mechanisms underlying its controlled expression in various tissues and during development.

### *2. Subcellular distribution*

PLC-$\delta_1$ is recovered mostly in the cytoplasmic fraction following the disruption of tissues or cultured cells.

This is due, in large part, to its relatively weak affinity for most membrane components other than polyphosphoinositides which are rapidly degraded under most conditions. Once the cell is disrupted and its contents diluted, dissociation from the remaining $PI(4,5)P_2$ should be complete within seconds. Thus polyphosphoinositide degradation, as well as simple dilution of the membrane components, can account for the appearance of this protein in the cytoplasmic fraction.

The fact that $PI(4,5)P_2$ is its only known membrane tether implies a codistribution between PLC-$\delta_1$ and its substrate, linking this isozyme to the many processes controlled by polyphosphoinositides. The strong association between receptor signaling at the cell surface and PLC activation has fostered the assumption that these enzymes and their substrates are all concentrated in the plasma membrane, which is clearly not true (see sects. III and IV). Because PLC-$\delta_1$ may function considerably downstream from receptor engagement (see discussion below), there seems no need to restrict PLC-$\delta_1$ to this compartment, since $PI(4,5)P_2$ and related lipids are produced at many sites throughout the cell. Thus PLC-$\delta_1$ should be tethered to various intracellular membranes and structures, following the distribution of this polyphosphoinositide. Recent subcellular localization studies have begun to address this issue.

Because endogenous PLC-$\delta_1$ and related subtypes are expressed at very low levels, investigators have resorted to microinjecting the whole protein or introducing expression plasmids to detect these proteins by specific monoclonal antibodies in single cells. Using the former approach, and indirect immunofluorescence, Katan and co-workers (279) showed in MDCK cells that PLC-$\delta_1$ associates with the cell periphery and areas of cell-cell contact (279). The prominent edge pattern, indicative of plasma membrane localization, is eliminated if the injected protein is missing the PH domain or part of its $PI(4,5)P_2$ binding site. The isolated $\delta_1$ PH domain microinjected into MDCK cells behaves like the intact enzyme (403). Again, much of the injected protein localizes to the periphery. However, many cells show a more complex distribution that includes internal structures that have yet to be clearly defined (see *point 3* in NOTE ADDED IN PROOF).

Studies of living cells have confirmed and extended the immunofluorescence work. Expressing a chimera of green fluorescent protein (GFP) linked to the PLC-$\delta_1$ PH domain in cultured cell lines, several groups have shown that most of the fusion protein localizes to the plasma membrane from which it transiently dissociates during stimulation by platelet activating factor (346) and ANG II (374). Point mutations that abolish $PI(4,5)P_2$ binding prevent its association with the cell periphery. What underlies the PH-GFP translocation is not entirely clear. The distribution does correlate with polyphosphoinositide levels, suggesting that translocation from plasma membrane to cytosol is caused by their hydrolysis, but competition from inositol polyphosphates, especially $InsP_3$ or $InsP_4$, cannot be discounted. Indeed, it can be demonstrated that much of the observed redistribution of PH-GFP chimera is caused by the rise in $InsP_3$ and its high affinity for the PH domain (140). One other problem that cannot be ignored is the potential for these domains to interfere with normal signaling pathways. Although it is unlikely that these domains sequester a significant fraction of the cellular polyphosphoinositides, when expressed at high levels they do block cell signaling (see *point 4* in NOTE ADDED IN PROOF).

Intact PLC-$\delta_1$ linked to GFP behaves like its PH domain (110). PLC-$\delta_1$-GFP expressed in MDCK cells associates with the plasma membrane, and like PH-GFP, it dissociates following a stimulus that degrades $PI(4,5)P_2$ (osmotic stress). As predicted by prior studies, the PH domain is critical for membrane targeting and the observed translocation. Our own observations, obtained in NIH-3T3 cells, are similar, although a uniformly fluorescent plasma membrane is not observed. Rather, PLC-$\delta_1$ and its PH domain are discretely localized to actin-supported modifications of the membrane, suggesting some segregation of the polyphosphoinositides within these compartments (361a).

It is important to note that PLC-$\delta_1$ is actively excluded from the nucleus (405), unlike $\delta_4$ (see below) and PLC-$\beta$. Treatment of MDCK cells expressing PLC-$\delta_1$/GFP with leptomycin B, an inhibitor of sequence-dependent nuclear export, results in nuclear accumulation of this isoform. Sequence encompassing residues 164–177 of the EF-hand region have been identified as the potential export signal; a similar sequence is also found in PLC-$\delta_3$. Given the size of the PLC-$\delta_1$-GFP chimera (~112 kDa), active nuclear import must also take place, yet a specific import sequence has not been identified. In contrast, PLC-$\beta$ isoforms contain import sequences within their carboxy-terminal extensions (see sect. III). How transport of any PLC relates to cell/nuclear physiology is, as yet, unknown.

### *3. PLC-$\delta_1$ is activated by an atypical G protein*

Unlike the other PLC isoforms, neither protein phosphorylation cascades nor heterotrimeric G protein subunits, including $G\alpha_q$ and related G proteins, significantly affect PLC-$\delta_1$ activity. Among other modes of regulation that have been considered, compelling evidence points to an atypical GTP-binding protein, $G_h$, that appears to couple PLC-$\delta_1$, but not PLC-$\beta$, to a select set of heptahelical receptors (291).

$G_h$ is a widely expressed, multidomain enzyme that hydrolyzes GTP and ATP and functions as a transglutaminase (TGase, TGII) (156), which catalyzes the transamidation of glutamine residues with polyamines, or the

cross-linking of proteins through N$\epsilon$ ($\gamma$-glutamyl) lysine bridges. It is also capable of hydrolyzing these linkages. Binding of GTP inhibits transamidating activity; the sites for GTP and ATP appear to be separate.

A relatively large, soluble protein (74–87 kDa), TGII/$G_h$ is found in membrane and cytosolic compartments. Interestingly, TGII/$G_h$ is also present in nuclear membranes where it may associate with the pore complex (336). Its expression is highly regulated. TGII/$G_h$ levels are increased by such factors as IL-6, interferon-$\beta$, retinoic acid (156), and ionizing radiation (197). TGII/$G_h$ levels also rise during differentiation (377).

TGII/$G_h$ has been implicated in PLC activation pathways controlled by $\alpha_1$-adrenergic receptor subtypes $\alpha_{1b}$- and $\alpha_{1d}$-receptors in heart and liver (51, 67, 256), as well as oxytocin receptors in myometrium (13). In one of the earliest studies, epinephrine was shown to stimulate binding of TGII/$G_h$ to a 69-kDa form of PLC (67). Stimulation seemed to involve an increased sensitivity of PLC to calcium, leading to its activation by 0.1–5 $\mu$M calcium in the presence of GTP. This "novel" form of PLC is now recognized as an active fragment of PLC-$\delta_1$, which is also $G_h$ sensitive (97). Presumably, the 69-kDa form is missing the protease-sensitive PH domain and a portion of the EF-hand region, implying that they are unnecessary for association with or activation by $G_h$.

Further evidence for physiological interactions between $G_h$ and PLC-$\delta_1$ is supported by the findings that $G_h$, or a small peptide corresponding to the $G_h$ sequence 654–673, binds to and activates intact PLC-$\delta_1$ in cell lysates (97). This same sequence, which is located near the carboxy terminus of $G_h$, is also required for coupling $\alpha_{1b}$-adrenergic receptors to PLC-$\delta_1$ (154).

TGII/$G_h$ has other binding partners as well, including a 50-kDa protein that suppresses its GTPase activity (12). Incubation of TGII/$G_h$ with GTP induces dissociation of this protein, suggesting a function analogous to G$\beta$, although it does not seem to regulate receptor/TGII interactions; no "G-$\gamma$" equivalent has been identified. A model that loosely parallels the regulated GTPase cycle of heterotrimeric G proteins has been proposed, wherein activated $\alpha_1$-adrenergic receptor enhances GTP binding to TGII/$G_h$, leading to dissociation from its 50-kDa binding partner and activation of PLC-$\delta_1$ (156). This simple model may require some modifications, since the recent work of Murthy et al. (253) has brought into question the role of GTP. While confirming that TGII/$G_h$ binds strongly to PLC-$\delta_1$, they report its association with PLC is disrupted (not promoted) by GTP or a nonhydrolyzable GTP analog. Their results suggest binding of guanine nucleotide to TGII/$G_h$ causes a conformational change that releases, and thereby activates, PLC.

Although TGII/$G_h$ is implicated in coupling adrenergic receptor subtypes to PLC, it is not the major link. Indeed, cotransfection of $\alpha_{q/11}$ is much more effective in coupling $\alpha_{1b}$-adrenergic receptors to PLC-$\beta$ than is cotransfection of TGII/$G_h$ in coupling to PLC-$\delta$ (51). Using neutralizing antibodies against TGII/$G_h$ and $G_{q/11}$, Zhang et al. (418) showed that the latter is the dominant link between $\alpha_{1b}$-adrenergic receptor and PLC ($\beta$), while the contribution of TGII/$G_h$ coupling to PLC-$\delta_1$ is a relatively minor component of agonist-stimulated phosphoinositide hydrolysis (418). Moreover, the effect of TGII/$G_h$ expression on adrenergic-stimulated PI hydrolysis (attributed to $\delta_1$) is bimodal, with activation at low and inhibition at high levels of TGII/$G_h$. In contrast, muscarinic-stimulated hydrolysis (presumably mediated by PLC-$\beta$) was unaffected by the level of TGII/$G_h$. Murthy et al. (253) also find that coexpression of TGII/$G_h$ with PLC-$\delta_1$ lowers the basal activity of PLC attributed to the $\delta_1$-isoform (253). These reports contrast with those obtained in vitro (97), where only activation is observed. Taken as a whole, these results suggest a complex modulatory role for TGII/$G_h$ in the coupling of some heptahelical receptors to PLC.

The relationships between $\delta_1$-modulating and transamidating activities of $G_h$ have been studied as well. Although activation of the TGase is not required for coupling of $\alpha_1$-adrenergic receptors to PLC (51), there appears to be an inverse relation between activation of PLC by GTP-charged $G_h$ and its intrinsic transamidation activity, which is suppressed by GTP and $\alpha_1$-adrenergic stimulation (256). Thus the regulation of PLC-$\delta_1$ and $G_h$/TGII could be reciprocal, but more work is needed to clarify the relationships among the G states of TGII, their transamidating potential, and the activated state of PLC-$\delta$.

### *4. Is PLC-$\delta_1$ a calcium signal amplifier?*

The observation that PLC-$\delta$ isoforms are activated by calcium concentrations in the range of $10^{-7}$ to $10^{-5}$ M has led to the suggestion that these enzymes amplify rather than initiate calcium mobilizing signals. In contrast, PLC-$\beta$ and -$\gamma$ isoforms have been placed more proximal to the receptor. Consistent with this arrangement, the specific catalytic activity of PLC-$\delta$ is 50- to 100-fold greater than the calcium-stimulated activities of the $\beta$- and $\gamma$-isoforms, measured in the absence of activating G protein subunits, tyrosine protein kinases or PI(3,4,5)$P_3$.

Results of reconstitution experiments also point to PLC-$\delta$ as the calcium-responsive subtype. In permeabilized PC-12 cells, raising calcium from 0.1 to 1 $\mu$M stimulates PLC-$\delta_1$ (2). In contrast, PLC-$\beta_1$ and -$\gamma_1$ are unaffected. These cells normally contain high levels of the $\delta_1$-isoform and respond to a micromolar rise in cytoplasmic calcium with a marked stimulation of inositol lipid hydrolysis. This calcium-stimulated activity can be attributed to PLC-$\delta_1$, since loss of activity correlates with loss of the enzyme, which is regained after addition of the pure recombinant protein to the permeabilized cells. Similar results are obtained when exogenous PLC-$\delta_1$ is intro-

duced into permeable HL-60 cells. As predicted, introduction of 0.1–1 $\mu$M $InsP_3$, which competes with $PI(4,5)P_2$ for binding to the $\delta_1$ PH domain, suppresses calcium-stimulated $PI(4,5)P_2$ hydrolysis, providing a mechanism to dampen amplification.

Measurements of the $InsP_3$ and $Ca^{2+}$ levels in intact cells also support the idea that PLC-$\delta_1$ amplifies the PLC-$\beta$ generated signal. In Chinese hamster ovary (CHO) cells, increased expression of PLC-$\delta_1$ raises the level of InsP generated in response to thrombin (16). In PC-12 cells, expression of PLC-$\delta_1$ increases $InsP_3$ production, the rise in cytoplasmic calcium, and secretion of norepinephrine stimulated by bradykinin (194). These increases require enhanced influx of extracellular calcium, mainly through store-operated channels. The situation seems analogous to the PLC-$\gamma$ isoforms, which extend the PLC-$\beta$-generated calcium signal through a similar mechanism (see sect. IV and Fig. 4).

Is the rise in calcium alone sufficient for optimal activation of PLC-$\delta_1$? Thus far, three studies have compared receptor-specific activation to simply raising cytoplasmic calcium. In frog oocytes expressing thrombin and PDGF receptors, microinjection of PLC-$\delta_1$ antibody specifically inhibits thrombin, but not PDGF-induced calcium mobilization, as measured by release of radio-calcium (55). This implies that a PDGF-stimulated calcium rise is insufficient to activate PLC-$\delta_1$. In CHO cells, overexpression of PLC-$\delta_1$ enhances the amounts of InsP generated by ionomycin, but this increment is much smaller than the increase observed during thrombin stimulation (16). Similar results are obtained in bradykinin-stimulated PC-12 cells expressing high levels of PLC-$\delta_1$ (194). Here, raising calcium with high extracellular potassium, thapsigargin, or ionomycin induces a measurable increase in $InsP_3$, yet this increment is substantially less than that observed with a maximum dose of bradykinin. While these observation are consistent with an important role for PLC-$\delta_1$, they also suggest that other receptor-coupled pathways contribute indirectly to PLC-dependent amplification, such as parallel stimulation of $PI(4,5)P_2$ synthesis. Indeed, PITP markedly enhances calcium stimulation of PLC-$\delta_1$ (2), implying that PI is transferred to sites of active $PI(4,5)P_2$ synthesis and PLC-catalyzed hydrolysis.

### 5. A connection to rho-regulated pathways?

A novel form of RhoGAP associates strongly with PLC-$\delta_1$ in cell lysates (144). This soluble protein stimulates PLC-$\delta_1$ catalytic activity up to 10-fold at low levels of calcium ($\sim$0.1 $\mu$M) but has no effect on PLC-$\beta_1$ or -$\gamma_1$ activities. The amounts of rhoGAP needed for stimulation are low and stoichiometric, suggesting the formation of a high-affinity complex. The results are consistent with a role for PLC-$\delta_1$ in regulating the actin-cytoskeleton, especially at focal adhesions and membrane ruffles (see also sect. IV). Interestingly, *Clostridium botulinum* toxin, which preferentially targets rho, stimulates partially purified preparations of PLC-$\delta$ (141), although the degree of stimulation is modest. The results suggest that rho tonically inhibits PLC-$\delta$, whereas rhoGAP, which downmodulates rho, activates. Whether this is related to rho-regulated processes is unknown.

### 6. Other mammalian PLC-$\delta$ subtypes

Although our understanding of PLC-$\delta_1$ function is limited, even less is known about other $\delta$-subtypes. PLC-$\delta_2$ was isolated and cloned from bovine cerebral cortex (241). The enzyme consists of 764 residues, has a predicted molecular mass of 87 kDa, and is active when expressed in COS-1 cells. Otherwise, there is little information concerning its function. A more recent immunohistochemical analysis of cryosections of the mouse cerebellum shows that Purkinje cells express relatively high levels of this enzyme (237), but the physiological meaning is unclear.

A cDNA encoding PLC-$\delta_3$ has been isolated from human fibroblasts and its protein sequence has been published (115). The enzyme, which is 736 residues, is expressed at very low concentrations in most cells and tissues. Higher concentrations are found in kidney, cardiac muscle, and aorta (281). On a subcellular level, PLC-$\delta_3$ is found primarily in the particulate fraction, whereas $\delta_1$ is principally cytosolic, implying different modes of membrane binding. Interestingly, the loop (X/Y spanning) region of $\delta_3$ is unique among the $\delta$-isoforms in its preponderance of acidic residues. A string of 13 acidic residues (mostly Glu) is found, reminiscent of the PEST sequences observed in the $\beta$-isoforms. Like PLC-$\delta_1$, $\delta_3$ is activated by physiological levels of calcium, although $\delta_3$ is less sensitive when the substrate is presented as a bilayer (281). Another noteworthy difference is the marked sensitivity of PLC-$\delta_1$ catalytic activity to spermine and sphingosine, compared with the insensitivity of PLC-$\delta_3$ (281).

PLC-$\delta_4$ and several alternatively spliced variants have been identified (Table 3) (212). RT-PCRs of mRNA from various tissues shows high levels of $\delta_4$-message in brain, skeletal muscle, testis, and kidney. Antisera specific for Alt 1 and 2 variants recognize a 93-kDa form in testis. Another alternatively spliced form, Alt3, has been isolated (254). Expression of this protein is generally low relative to the other $\delta_4$-variants, although a strong signal is detected in Western blots of heart muscle. Interestingly, this variant has a truncated X-box region and appears to be catalytically inactive, both in vitro and in living cells. Like the other PLC-$\delta_4$ variants, much of the $PI(4,5)P_2$ binding PH domain sequence is conserved. As in PLC-$\delta_1$, this domain is responsible for the moderately high-affinity polyphosphoinositide binding of PLC-$\delta_4$-Alt3, but unlike

$\delta_1$, $InsP_3$ binding is not detected, implying intriguing differences between the two isoforms.

PLC-$\delta_4$-Alt3 may be a natural negative regulator of other PLC-$\delta_4$ variants (254). Coexpression of PLC-$\delta_4$ and PLC-$\delta_4$-Alt3, or its PH domain, suppresses PLC activity, but Alt3, lacking a PH domain, is ineffective. Comparable results are obtained in vitro. Mutation R36G, which corresponds to an arginine essential for $PI(4,5)P_2$ binding by the $\delta_1$ PH domain, lowers the affinity of the whole enzyme for $PI(4,5)P_2$, and prevents the suppression of PLC activity, suggesting that Alt3 and its PH domain suppress activity by competing for a limited pool of this lipid.

In vitro, however, suppression of PLC-$\delta_4$ activity appears to involve a complex with PLC-$\delta_4$-Alt3. Significant inhibition is observed at a molar ratio of 1:1, whereas higher ratios are required to inhibit other PLC-$\delta$ isoforms. No inhibition of PLC-$\beta_1$ or -$\gamma_1$ is observed. The stoichiometric nature of the inhibition suggests a direct association of the variant with PLC-$\delta_4$, possibly through its PH domain, suggesting PLC-$\delta_4$-Alt3 is a negative regulator. This association may require binding of the Alt3 PH domain to $PI(4,5)P_2$ as well. A recently described $InsP_3$-binding protein, closely related to PLC-$\delta_1$ and lacking catalytic activity (179), might function like PLC-$\delta_4$-Alt3, targeting PLC-$\delta_1$ or other PLC isoforms.

Still another PLC-$\delta_4$ variant, which we refer to as Alt-4, was cloned from a regenerating liver cDNA library at the same time as PLC-$\delta_4$ (225). Alt4 mRNA is highly expressed in intestine and in regenerating liver tissue, but poorly in other tissues.[11] Expression is higher in transformed cell lines; its level, which is cell cycle dependent, can be induced by serum stimulation (225). Alt4 also appears to be one of several PLCs previously isolated from the nuclei of regenerating liver (8). In fibroblasts, nuclear levels of Alt4 increase dramatically at the transition from $G_1$ to S phase (225). These levels are maintained through metaphase. In contrast, nuclear PLC-$\beta_1$ levels are constant throughout the cell cycle, whereas PLC-$\gamma_1$ and -$\delta_1$ remain in the extranuclear compartment. One interesting feature of the $\delta_4$-variants is the location of the alternatively spliced sequences that correspond to the loop regions. These differences may well confer further functional and regulatory specificity.

## VI. SUMMARY AND CONCLUSIONS

Through the work of many investigators, we have learned how PLC isoforms act as catalysts, discovered what proteins and lipids regulate their activities, and gleaned some hints of their diverse biological roles. Crystallographic studies of PLC-$\delta_1$ catalytic core and its constituent domains have offered us a molecular view of the reaction and provided a template for interpreting the structure and function of similar modules in the other PLC subtypes. The current challenge is to understand the nature and dynamics of the membrane/enzyme microinterface and their relation to the cycle of substrate binding and product release; in the case of $\beta$- and $\gamma$-isoforms, how engagement by G protein subunits or PI 3-kinase products stimulate activity.

Although subtype-specific activation of PLC-$\beta$, -$\gamma$, and -$\delta$ isoforms by G protein, tyrosine protein kinase, and calcium are distinguishable, recent studies have broadened our understanding of how each is regulated. In the case of PLC-$\beta$, stimulus threshold and receptor-specific coupling seem to be modulated by regulators of G protein signaling (RGS) and the $\beta$-isoforms themselves, which enhance the GTP hydrolyzing activities of $G\alpha_q$ and related subunits. To elicit a signal from PLC, heptahelical receptors must continuously charge $G\alpha_q$ with GTP, suggesting the formation of a ternary complex of receptor, G protein, and enzyme, all in the face of continued suppression by RGS. To sustain the agonist-dependent signal, new substrate must be continuously supplied as well, a process that requires the concerted actions of PITP and inositol lipid kinases. These findings suggest the need to spatially restrict diffusion of the various signaling components, even the enzymes that synthesize and deliver substrate. Indeed, PLC-$\beta$ isozymes are laterally organized by scaffolding proteins that could facilitate the speed and specificity of their engagement with receptors and G proteins. Moreover, a significant fraction of the agonist-sensitive polyphosphoinositide pool is found in caveolae, cholesterol-enriched membrane rafts believed to harbor other signaling proteins.

New work has also clarified how PLC-$\gamma$ isoforms are regulated and where they should be placed within pathways initiated by antigens, immunoglobulins, cytokines, growth factors, and GPCR agonists. The discovery that $PI(3,4,5)P_3$ recruits and stimulates the $\gamma$-isoforms ties together the two major polyphosphoinositide pathways and provides an activation mechanism that is both distinguishable from, and synergistic with, phosphorylation by tyrosine protein kinases. Importantly, it is now recognized that PLC-$\gamma$ isoforms are integrated into response pathways involving other PLCs, especially the $\beta$-isoforms, where they operate to prolong the calcium response. With the knowledge of many of the protein and lipid binding partners of the $\gamma$-isoforms, it is now conceivable to investigate how and where these components are organized in living cells and to test whether these enzymes modulate basic or specialized cellular responses.

PLC-$\delta$, the evolutionary precursor of the other sub-

[11] Antibodies against the other variants of $\delta_4$ did not detect this form in regenerating liver or intestine (212). This negative result could have been due to loss of Alt4 during the steps taken to enrich for PLC-$\delta_4$ and other variants. Alternatively, antibodies prepared against PLC-$\delta_4$ or other variants may have failed to cross-react.

types, arose in the earliest eukaryotes. In yeast and higher plants, δ-isoforms are implicated in the response to nutritional and environmental stresses, especially cyclin-dependent growth control and nuclear mRNA export, but the details of what regulates this PLC are lacking. In the cellular slime mold, PLC-δ sensitizes free-living ameba to chemoattractants required for the stress-induced switch from unicellular to multicellular life-style. Thus the primitive δ-isoforms function as stress response proteins, helping these organisms adapt to a changing environment.

Among the isoforms in mammals, $\delta_1$ is the most widely expressed. Although best studied for its structure, its mode of regulation is not clear. It can tether, by its own PH domain, to membrane surfaces enriched in $PI(4,5)P_2$, where the enzyme can respond to calcium transients and a specialized GTP-binding protein ($G_h$) that is also a transglutaminase. Whether other mammalian δ-isoforms are similarly regulated is unknown, but the multiplicity of isotypes and their spliced variants imply a differential set of functions. It has yet to be determined if some of these are stress related.

Transgenic experiments indicate that each isoform is critical to a select set of functions in developing and adult animals. Although the β-isoforms operate broadly in cell signaling, their individual loss is only appreciated in a few well-defined processes, such as phototransduction and visual signal processing. Similarly, PLC-γ isoforms have specialized roles whose essential nature is only detected at later stages of development. Considering their relatively late appearance in animal evolution, this degree of specialization is not unexpected, yet these experiments only hint at the multiple roles each isozyme plays.

While enormous progress has been made in uncovering the how, what, and where of the PLCs, many questions remain unanswered. Most of these concern their true function. For instance, what are the many PLC-δ isoforms and their variants doing in higher plants and animals? Are they signal amplifiers? Do they participate in calcium oscillations? How does tyrosine phosphorylation of PLC-γ lead to its activation? What are PLC-β isoforms doing in the nucleus? Further work should yield some fascinating surprises and new insights.

## NOTE ADDED IN PROOF

*1*) PLC-β1 appears to bind PI-3P (293b). *2*) *PI*(4)P 5-kinase-α is recruited to membrane ruffles where it is activated by the low-molecular-weight GTPase ARF6 (145a). *3*) ARF-stimulated PI(4)P and $PI(4,5)P_2$ synthesis is essential for stability of the Golgi apparatus where it functions in organizing a spectrin-based membrane scaffold (116a). *4*) High levels of PHδ1-GFP suppress exocytosis (142a) and actin binding to the plasma membrane (293a). In the latter case, actin cables are disrupted and fibroblast morphology is altered. When expression levels are low, however, most cells retain their network of actin cables and have a normal appearance (361a). Thus it is extremely important, as with any indicator, to prevent this fusion protein from significantly buffering the free $PI(4,5)P_2$ concentration.

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