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COMMENTARY

Regulation of Effectors by G-Protein α- and βγ-Subunits

RECENT INSIGHTS FROM STUDIES OF THE PHOSPHOLIPASE C-β ISOENZYMES

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ABSTEACT. Both the α- and βγ-subunits of heterotrimeric guanine nucleotide-dependent regulatory proteins (G-proteins) couple members of the heptahelical class of cell-surface receptors to a diverse range of signal-generating effectors including retinal cyclic GMP phosphodiesterase, ion channels, adenylylcyclases, phosphoinositide 3-kinase, and members of the β-class of inositol lipid-specific phospholipases C. Although the molecular details of the G-protein-regulated phospholipase C system were elucidated comparatively recently, these enzymes have become an important model for investigations of the process of G-protein effector coupling. A combination of molecular biological, biochemical, and structural studies using the phospholipase C-β enzymes has provided some important insights into the interplay between G-proteins and their effectors and promises to reveal the mechanisms by which G-protein α- and βγ-subunits selectively associate with and activate effectors. BIOCHEM PHARMACOL **54**;4:429–435, 1997. © 1997 Elsevier Science Inc.

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Members of the most abundant class of cell-surface receptors for agents including hormones, neurotransmitters, and growth factors belong to a structurally related family of membrane proteins with a single polypeptide chain folded into seven transmembrane-spanning α-helices linked by intra- and extracellular loops [1]. These receptors employ members of a versatile family of signal transducers, the G-proteins,§ to control the activity of effectors. These effectors, in turn, generate intracellular mediators or regulate ion flux in the cell [2–4]. Agonist-liganded receptors associate with G-protein heterotrimers composed of α-, β-, and γ-subunits. The receptors promote G-protein activation by stimulating release of GDP bound to the guanine nucleotide-binding site of the α-subunit, which is replaced by GTP causing dissociation of the heterotrimer into α-subunit monomers and βγ-subunit heterodimers. These dissociated subunits can then stimulate the activity of effector molecules in a manner that can be either mutually independent, synergistic, or antagonistic. The intrinsic GTPase activity of the α-subunit determines the lifetime of the active, dissociated state of the G-protein heterotrimer. Hydrolysis of bound GTP to GDP allows the α-subunit to re-associate with the βγ-dimer ready for another round of receptor-regulated activation. The structures of a G-protein αβγ heterotrimer and G-protein α-subunits in the GDP- and GTP-liganded states have been determined [5, 6]. G-proteins are classified by reference to their α-subunits, but all three polypeptide components belong to multi-gene families. To date, twenty α-, three β-, and seven γ-subunits have been identified in mammalian systems [7]. Selective assembly of G-protein heterotrimers from these separate components can therefore produce a large number of unique complexes that may differ in their interactions with receptors, effectors, or each other.

Despite this progress in understanding the coupling between heterotrimeric G-proteins and their effectors, many questions remain unanswered. Although G-protein α- and βγ-subunits are highly homologous in both primary sequence and (at least from the subset of data available) tertiary structure, the effectors they regulate are extremely diverse. What determinants allow these effectors to selectively interact with their G-protein activators, and how does G-protein binding lead to increased signal-transducing activity? Can interaction with effectors regulate G-protein activation through alterations in the α-/βγ-subunit interaction or through modulation of Gα GTP-binding or hydrolysis?

Most of the identified G-protein effectors are integral membrane proteins, and some of them are composed of multiple subunits. The PLC-β enzymes are an exception. These proteins can be expressed at high levels and purified

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§ *Abbreviations:* G-protein, heterotrimeric guanine nucleotide-dependent regulatory protein; PLC, inositol lipid-specific phospholipase C; PH domain, pleckstrin homology domain; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; MARKS protein, myristoylated alanine-rich C-kinase substrate protein; and GAPs, GTPase activating proteins.

as stable soluble proteins. The PLC-β enzymes belong to a larger family of inositol-lipid specific PLCs with well-defined functions. These enzymes share a modular domain organization [8–10]. A combination of mutagenesis studies and, more recently, structural determination has led to functional assignments for some of the modular domains shared by these proteins. Therefore, the PLC-β enzymes are an excellent model system that allows investigators to combine molecular biological, biochemical, and biophysical techniques for investigating G-protein–effector interactions.

REGULATION OF PLC-β ISOENZYMES BY G-PROTEIN α- AND βγ-SUBUNITS

The four known PLC-β isoenzymes are targets for activation by both the α family of G-protein α-subunits and by βγ-subunits. Members of the α_q family activate the PLC-β enzymes in a pertussis toxin-insensitive manner [11, 12]. These proteins are closely related in primary sequence but vary in their tissue distribution; α_q and α_{11} are widely expressed, α_{14} has a more restricted distribution, and α_{15} and α_{16} only appear to be expressed in cells of hematopoietic lineage [13, 14]. It is not clear whether the individual α_q-subunits differ in their ability to activate PLC-β isoenzymes. This possibility has been studied using both reconstitution assays with purified proteins and exogenously provided substrates, and by transient expression of PLC enzymes and G-protein subunits in COS-7 cells. Some experiments of the latter type suggest that there are differences in susceptibility of the individual PLC-β enzymes to activation by the various G_q family members [15]. However, experiments with purified PLC-β_1, -β_2, and -β_3 showed little difference in activation by purified α_q, α_{11}, and α_{16} [16–18]. It is clear that the individual G_q family members differ substantially in their coupling to cell-surface receptors [19, 20].

G-protein βγ-subunits activate the PLC-β enzymes both *in vitro* and in transient transfection assays [15, 21–25]. This finding most likely explains the sensitivity of receptor-regulated inositol lipid hydrolysis to inhibition by pertussis toxin. βγ-Subunits can activate all of the PLC-β isoenzymes, but PLCs-β_2 and -β_3 appear more sensitive than PLCs-β_1 and -β_4 [15, 18, 25]. α_q-Subunit activators of the PLC-β isoenzymes are considerably more potent (50- to 100-fold) than βγ-subunits. One reason for this difference could be that the βγ-subunits used in these studies were purified from bovine brain and, therefore, contain a mixture of βγ-dimers. A large number of βγ-dimers can potentially be assembled from individual members of the β- and γ-subunit multi-gene families, and it is possible that individual βγ-dimers may be more effective PLC-β activators. A limited number of studies have been done to test this idea. With the exception of the $\beta_1\gamma_1$-dimer, the incomplete variety of βγ-dimers tested appears to be equi-potent and effective PLC-β activators [24, 25]. In fact, the reduced potency of βγ-subunits (compared to α_q-subunits) as activators of the PLC-β enzymes may provide selectivity in receptor regulation of the PLC-β enzymes since presumably only activation of abundant G-protein heterotrimers (for example members of the pertussis-toxin-sensitive G_o and G_i families) would produce sufficient βγ-subunits for PLC-β activation.

PLC-β ENZYMES ARE GAPS FOR THEIR G-PROTEIN α-SUBUNIT ACTIVATORS

A unique characteristic of the G_q family of heterotrimeric G-proteins is that, when purified and reconstituted with appropriate receptors that promote the guanine nucleotide exchange step in the G-protein activation cycle, their intrinsic steady-state GTPase activities are much lower than those of members of the other G-protein families [3]. This finding was paradoxical since, in many systems, direct measurement of receptor-promoted PLC-mediated increases in intracellular Ca^{2+} revealed that, upon addition of a receptor antagonist, deactivation of the G_q/PLC-β system was extremely rapid. Regulation of PLC-β_1 has been studied in a reconstituted system containing purified M1 muscarinic cholinergic receptors and a purified mixture of α_q and α_{11} [26]. In this system, receptor-promoted binding of GTPγS to the G-protein and PLC-catalyzed PI(4,5)P_2 hydrolysis are closely coupled. When the non-hydrolyzable GTPγS is replaced by hydrolyzable GTP, PLC-β activation is greatly reduced. The reason for this apparent uncoupling between G-protein and effector is that PLC-β_1 is a GAP for its $\alpha_{q/11}$ activator. Promotion of GTP hydrolysis accelerates the deactivation rate of the $\alpha_{q/11}$-subunits and, in turn, accelerates the deactivation of PLC-β_1. The GAP activity of PLC-β_1 thus allows the inositol lipid signaling system to rapidly respond to receptor deactivation. In this way PLC-β activity is regulated not only by the rate of receptor-promoted GDP/GTP exchange in Gα, but also by the rate of GTP hydrolysis accelerated by the effector. More detailed studies of the kinetics of PI(4,5)P_2 hydrolysis by PLC-β_1 in this reconstitution system suggest that, in the presence of saturating agonist, receptor-G_q complexes can remain stable over multiple GTPase cycles [27].

The observation that PLC-β enzymes function as GAPs for their Gα-protein regulators offers another explanation for the much greater potency of α_q-subunits to activate PLC-β compared with βγ-subunits. Since PLC-β activation measurements usually employ non-hydrolyzable guanine nucleotide analogs to induce activation of α_q-subunits, the GAP effect is negated. Thus, the potencies of α_q-subunits in these assays are overestimated. When the GAP-dependent decrease in the level of α_q(GTP) is considered, the potencies of the two G-protein subunits are much closer [28].

STRUCTURAL DETERMINANTS OF PLC-β G-PROTEIN INTERACTIONS

Three classes of PLCs have been identified in mammalian systems: PLC-β, PLC-γ, and PLC-δ. The proteins contain

two highly conserved catalytic domains, X and Y, as well as other structural modules, but only the PLC-β family appears to be regulated by G-proteins [8]. The PLC-β family is distinguished from the -γ and -δ families by an extended COOH-terminus, and the role of this region in the regulation by G-protein α_q- and βγ-subunits has been studied in some detail. Truncation of PLC-β_1 and -β_2 immediately after the Y-domain produces catalytically active proteins that can be stimulated by G-protein βγ- but not α-subunits [29–31]. Over-expression of the COOH-terminus of PLC-β_1 in COS-7 cells blocks activation of co-transfected PLC-β_1 by co-expressed muscarinic cholinergic receptors and α_q-subunits. Two short peptides corresponding to a portion of this region of PLC-β_1 block activation of PLC-β_1 by α_q *in vitro*, whereas a peptide of identical amino acid composition but different sequence did not [29]. In broad agreement with a role for the PLC-β COOH-terminus in α_q-subunit regulation, several recombinantly expressed fragments of the COOH-terminus of PLC-β_1 function as GAPs for purified α_q in a reconstitution system with purified muscarinic cholinergic receptors. The PLC-β "tail" with the most effective GAP activity does not correspond to the region identified as a site of α_q interaction with PLC-β_1 using synthetic peptides. It is therefore possible that the G-protein PLC-β interactions that underlie stimulation of the $\alpha_{q/11}$ GTPase activity and activation of PLC catalytic activity involve different parts of the PLC-β COOH-terminus [27].

The site of interaction between the PLC-β enzymes and G-protein βγ-subunits is less well defined. Because, as discussed above, removal of the COOH-terminus of PLC-β_1 and -β_2 does not diminish the capacity of βγ-subunits to activate the enzymes, it is reasonable to presume that the remaining portion of the enzymes contains the βγ-subunit interaction site. Since the X and Y domains are common to all PLC enzymes, the most likely sites for βγ-subunit interaction are, therefore, the NH_2-terminus and the inter X-Y region. There is experimental evidence suggesting that both of these regions of the proteins are important for activation by βγ-subunits. Some studies have implicated the highly charged inter X-Y region in the regulation of PLC-β_2 by βγ-subunits. Peptide fragments from this region of PLC-β_2 expressed as fusion proteins bound to purified βγ-subunits *in vitro* and over-expression of this region of PLC-β_2 in COS-7 cells blocked activation of co-transfected PLC-β_2 [32].

There is indirect evidence that the NH_2-terminal region may interact with βγ-subunits. Analysis of the PLC-β structure identifies a PH domain located at the NH_2-terminus. PH domains are structural units found in a wide variety of proteins. Several lines of genetic, biochemical, and structural evidence suggest a role for PH domains in protein–membrane interactions [33]. The PH domains of some proteins act as specific binding sites for inositol-containing phospholipids (see below), whereas others may mediate protein interactions with βγ-subunits. The prototypic example of this latter function is the β-adrenergic receptor kinase (BARK), which has an NH_2-terminal PH domain that appears to mediate βγ-subunit-dependent recruitment of the enzyme to the plasma membrane [34]. As discussed above, the finding that removal of the COOH-terminus of the PLC-β proteins does not impair activation by βγ-subunits would be consistent with a role for the NH_2-terminus in this process. To date, there has not been a direct investigation of the role played by the NH_2-terminal PH domain of the PLC-β enzymes in activation by βγ-subunits.

HOW DO G-PROTEIN α- AND βγ-SUBUNITS ACTIVATE THE PLC-β ENZYMES?

The work described above suggests a preliminary model of PLC-β activation by G-protein subunits. This model is depicted in Fig. 1 and discussed in more detail below.

1. A *recruitment mechanism is not involved.* One possible mechanism by which G-protein subunits could regulate PLC-β activity is by regulating their association with membranes. Indeed, PLC-γ activation is thought to occur by its recruitment to the membrane surface through association with activated tyrosine kinase receptors [35]. This does not appear to be the case since G-protein α- and βγ-subunits do not affect binding of PLC-β_1 and PLC-β_2 to lipid bilayers of varying compositions [36].

   The PLC-β isoenzymes could also be recruited to the membrane by the presence of polyphosphoinositides similar to the membrane binding behaviors of PLC-δ [37] and turkey erythrocyte PLC-β [38], but direct binding studies show that PLC-β_1 and PLC-β_2 associate with micromolar affinities to membranes regardless of the presence of $PI(4,5)P_2$ [39]. This lack of binding specificity was, at first inspection, surprising since PLC-β isoenzymes contain PH domains, and in many proteins these domains bind strongly and specifically to $PI(4,5)P_2$. One example is PLC-δ_1, where it has been shown that its isolated PH domain binds to $PI(4,5)P_2$ and $Ins(1,4,5)P_3$ with affinities comparable to the intact protein [37]. The underlying differences in binding behavior of the PLC-δ and PLC-β proteins may lie in sequence differences in their respective PH domains. The recently described structure of a complex between the PLC-δ_1 PH domain and $Ins(1,4,5)P_3$ identifies amino acid residues that participate in ionic and hydrogen bonds with the inositide headgroup [40]. The PH domains of the PLC-β isoforms have substitutions of key residues that interact with $Ins(1,4,5)P_3$ in the PH domains of the δ-isoform, which presumably accounts for their inability to bind to $PI(4,5)P_2$ [36]. Interestingly, the sequence of the PH domain of turkey erythrocyte PLC is highly homologous to PLC-β_2 but yet it shows $PI(4,5)P_2$-dependent binding, suggesting that other regions of the protein play a role in $PI(4,5)P_2$ specificity [41].

   PLC-β isozymes require calcium for activity, and

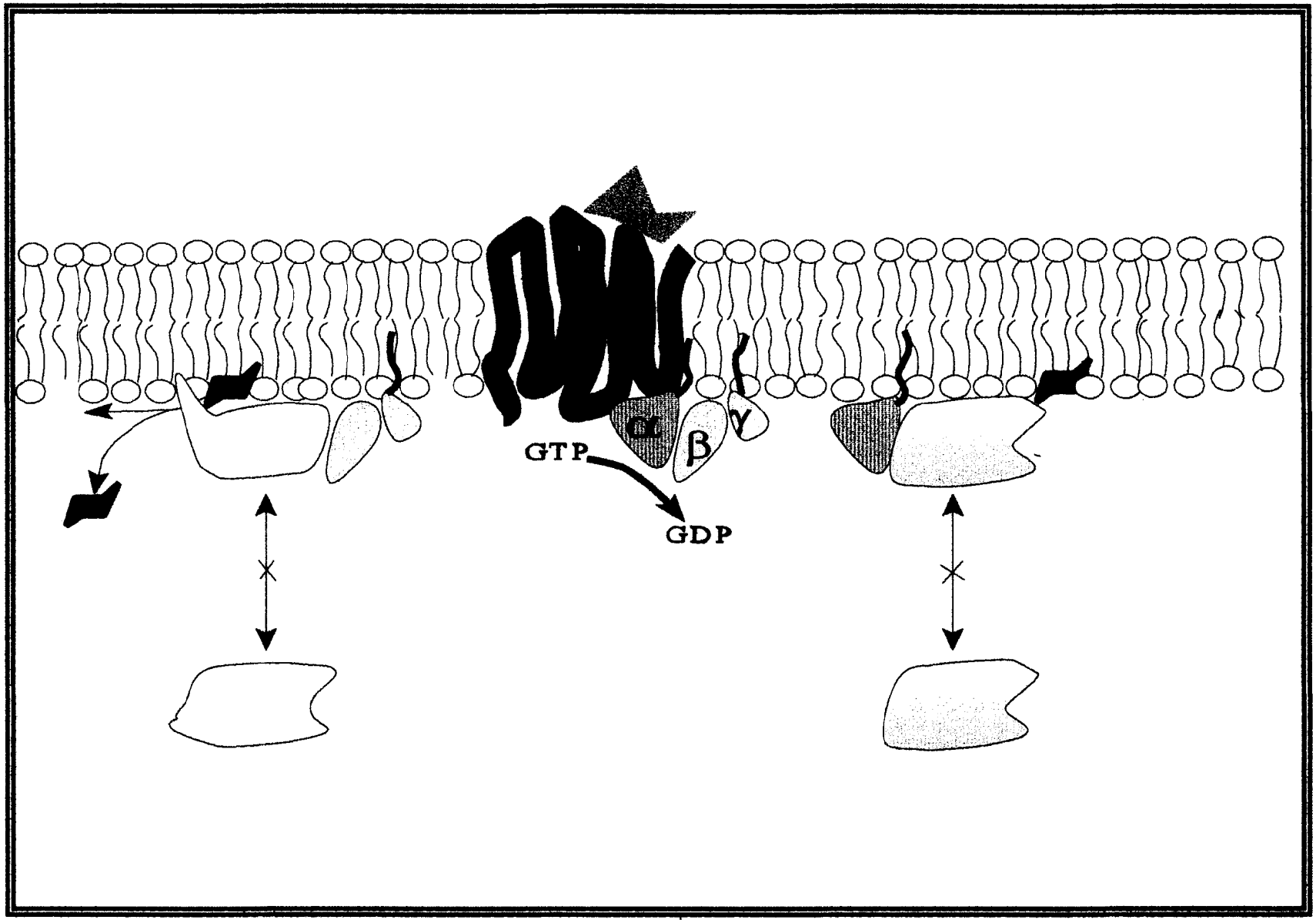

FIG. 1. Mechanism of activation of phospholipase C-β by G-protein subunits. The figure depicts an extracellular agonist binding to a 7-helical spanning transmembrane receptor, which then catalyzes the exchange of GDP for GTP on the α-subunits of heterotrimeric G-proteins. This exchange weakens the association between the α- and βγ-subunits. The dissociated α- and βγ-subunits can then interact with membrane-bound phospholipase C in different regions of the protein causing an increase in their catalytic rate. Although catalysis involves penetration of a portion of PLC-β into the head group region of the bilayer, this process is not promoted by interaction with G-protein subunits. G-protein α- and βγ-subunits do not promote membrane binding by the PLC-β enzymes.

regulation of membrane binding may therefore occur through their C2 or CALB domain located immediately after the catalytic Y-domain. This region of ~130 residues has been identified in over 40 proteins that interact with membrane surfaces, are activated by calcium, and play a role in transmembrane signaling and/or vesicular trafficking. Studies using synaptotagmin or a closely related C2 domain from cytosolic phospholipase A_2 indicate that this region functions as a Ca^{2+}-dependent lipid binding domain [42]. However, the C2 domains of PLCs-β_1 and -β_2 appear to be non-functional or their function is masked by stronger associations involving other regions of the protein because purified PLC-β_1 and -β_2 bind to phospholipid membranes of varying compositions in a Ca^{2+}-independent manner [36]. Interestingly, the crystal structure of PLC-δ_1 suggests a role of the C2 domain in orienting the enzyme to its substrate [10].

2. *Catalysis requires membrane surface penetration.* The idea that PLC must penetrate the membrane surface during catalysis comes from studies using substrate-containing phospholipid monolayers where it was observed that the activity of PLC-β_1 (and members of the -γ and -δ PLC classes) strongly decreases when the monolayer surface is compressed [43], suggesting that a small portion of the proteins must penetrate the lipid surface during catalysis. The crystal structure of the catalytic portion of PLC-δ identifies a region of the protein that could be involved in membrane penetration [10]. The ability of a portion of PLC to insert into membranes suggests that activation of the PLC enzymes could occur by agents that produced a local decrease in surface pressure. Since both G-protein α- and βγ-subunits bind to membrane surfaces with high affinity, this hypothesis offered a particularly attractive explanation for their ability to activate the PLC-β enzymes. However, monolayer studies and fluorescence energy transfer studies show that this is not the case (Boguslavsky V, Jenco J, Runnels L, Scarlata S, Morris AJ, and McLaughlin S, unpublished observations).
3. *α- and βγ-Subunits associate with different regions of membrane-bound PLC-βs.* Activation of membrane-bound PLC-β enzymes by α-subunits appears to involve the COOH-terminus of PLC-β [29–31], and although the precise structural determinants involved may be different, the α-subunit PLC-β interaction produces an enhancement of catalytic activity of both partners, resulting in increased phosphoinositide hydrolysis by the PLC enzymes and increased GTPase activity by the α-subunit. Activation by βγ-subunits apparently involves portions of the inter X-Y domain region and perhaps also the NH_2-terminus of the proteins [31, 32].
4. *Other regulatory factors?* There is increasing evidence that PLC activity and activation by G-proteins may be influenced by other factors. Recent studies indicate a potential role for modulation of substrate accessibility in the regulation of PLC-β activity. Binding of the MARKS protein to model membranes results in sequestration of acidic lipids [including the PLC-β substrate $PI(4,5)P_2$] in lateral domains. This results in inhibition of PLC-β activity. Phosphorylation of the MARKS protein by protein kinase C releases the protein from the membrane surface and concurrently attenuates this inhibition [44].

Covalent modification of the PLC-β enzymes by phosphorylation may also constitute an important regulatory mechanism. It has been demonstrated recently that PLC-β_2 is phosphorylated by protein kinase A, causing a decrease in βγ-subunit-stimulated activity [45]. PLC-β activity may also be regulated by self-association which, in turn, may be of importance to the mechanisms by which G-proteins activate the enzymes. For example, the COOH-terminal tails of PLC-β_1 inhibit the basal catalytic activity of wild-type PLC-β_1 *in vitro* in a manner that can be overcome by the addition of α_q [27]. Evidence for PLC-β_2 oligomerization comes from studies of Sternweis and co-workers* using a catalytically inactive mutant of PLC-β_2. Addition of this mutant to the native enzyme resulted in a decrease in basal $P(4,5)P_2$ hydrolysis and an increase in activation by βγ-subunits, leading to the speculation that association between the mutant and native enzymes can occur. The finding that, *in vitro*, G-protein βγ-subunits become more potent and effective activators of PLC-β_1 and PLC-β_2 as the concentration of these enzymes is increased would also be consistent with the idea that self-association of the PLC-β enzymes plays a role in the process by which they become activated by G-proteins [46].

FUTURE QUESTIONS

The identification and functional assignment of the modular domains shared by the G-protein α- and βγ-subunit-regulated PLC-β enzymes, coupled with the discovery of their GAP activity towards their α-subunit activators, suggest that a complex interplay between these proteins modulates PLC-β catalyzed formation of inositol-lipid-derived messenger molecules in agonist-stimulated cells. While biochemical studies have presented the cast of characters, biophysical studies, coupled with molecular biological techniques, may now be appropriate to address the importance of these interactions. For instance, it is not understood whether differences in potency or efficacy as PLC-β activators displayed by some G-protein subunits is a direct result of reduced affinity for the enzymes or due to a reduced ability of the G-protein to induce the activated state of the enzymes. Our understanding of the processes of GTP-binding and hydrolysis by G-protein α-subunits, association and dissociation of the G-protein heterotrimers, and PLC activation would benefit greatly from real time kinetic analysis. The idea that homo- and hetero-oligomerization of PLC-β may be important for G-protein regulation

* Paul C. Sternweis, personal communication. Cited with permission.

has not been fully explored. Although the PLC-β enzymes are sensitive to activation by both α- and βγ-subunits, it is unclear if these regulators can interact with the enzymes simultaneously and thereby act in an additive, synergistic (or perhaps antagonistic) manner to control PLC-β activity.

The functional roles of the many modular domains of PLC are now prime for study. The manufacture of PLC chimeras, and PLC deletion, substitution, and insertion mutants, coupled to structural and functional comparisons with homologous modules in other proteins, is an obvious way to address this question. A final note concerns the possible role of the membrane itself in modulation of PLC-β activity and the interaction of these enzymes with G-protein subunits. As noted above, studies using phospholipid monolayers indicate that PLC-β activity involves penetration of a portion of the protein into the bilayer. Other studies indicate that PLC-β activity varies widely with membrane composition. At present, it is not clear whether this variation is due to the presentation of the substrate on the surface, the binding and conformational changes of the protein on the surface, or changes in the physical properties of the bilayer such as hydration, compressibility, and surface dielectric. Biophysical studies using purified proteins and model membranes may best address these questions.

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