Activation of Phospholipase C {beta}2 by the {alpha} and {beta}{gamma} Subunits of Trimeric GTP-Binding Protein

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Activation of phospholipase C β_2 by the α and $\beta\gamma$ subunits of trimeric GTP-binding protein

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ABSTRACT **Cotransfection assays were used to show that the members of the GTP-binding protein G_q class of α subunits could activate phospholipase C (PLC) β_2. Similar experiments also demonstrated that $G\beta_1\gamma_1$, $G\beta_1\gamma_5$, and $G\beta_2\gamma_5$ could activate the β_2 isoform of PLC but not the β_1 isoform, while $G\beta_2\gamma_1$ did not activate PLC β_2. To determine which portions of PLC β_2 are required for activation by $G\beta\gamma$ or $G\alpha$, a number of PLC β_2 deletion mutants and chimeras composed of various portions of PLC β_1 and PLC β_2 were prepared. We identified the N-terminal segment of PLC β_2 with amino acid sequence extending to the end of the Y box as the region required for activation by $G\beta\gamma$ and the C-terminal region as the segment containing amino acid sequences required for activation by $G\alpha$. Furthermore, we found that coexpression of $G\alpha_{16}$ and $G\beta_1\gamma_1$ but not $G\beta_1\gamma_5$ in COS-7 cells was able to synergistically activate recombinant PLC β_2. We suggest that $G\alpha 16$ may act together with free $G\beta_1\gamma_1$ to activate PLC β_2, while $G\alpha_{16}$ may form heterotrimeric complexes with $G\beta_1\gamma_5$ and be stabilized in an inactive form. We conclude that the regions of PLC β_2 required for activation by $G\beta\gamma$ and $G\alpha$ are physically separate and that the nature of the $G\beta$ subunit may play a role in determining the relative specificity of the $G\beta\gamma$ complex for effector activation while the nature of the $G\gamma$ subunit isoform may be important for determining the affinity of the $G\beta\gamma$ complex for specific $G\alpha$ proteins.**

Heterotrimeric GTP-binding proteins (G proteins) mediate intracellular changes generated by ligand binding to different members of highly diverse families of cell-surface receptors (1, 2). The G proteins are made up of three subunits—α, β, and γ—and these in turn are assembled from among the products of 16 different $G\alpha$ genes, four different $G\beta$ genes, and at least six different $G\gamma$ genes to generate a large variety of heterotrimers (3). The different combinatorial forms are thought to be important in forming intracellular networks that process information by coupling ligand binding at specific receptors to the activation of intracellular effectors. The effectors may be enzymes that generate "second messengers" or ion channels that are gated by activated G proteins (1–3). Thus, for example, when heterotrimeric G proteins that include members of the G_q α-subunit class are activated by an appropriate receptor, they bind GTP and presumably transiently dissociate from their cognate $\beta\gamma$ subunits. The GTP bound G_q α-subunit family members have been shown to activate an inositol phospholipid-specific phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce two second messengers—diacylglycerol and inositol 1,4,5-trisphosphate (4–6).

There are a variety of classes of PLC enzymes and each of these has been found to occur in a number of isoforms (7, 8). Three isoforms of the PLC β class have been identified and members of the G_q class of α subunits including $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$ were all found to activate the PLC β_1 isoform in a variety of assay systems (6, 9, 10). Members of the G_q α class have also been found to activate the PLC β_2 isoform (9, 10). Less is known about the functions of the free $\beta\gamma$ complex. $\beta\gamma$ subunits have been implicated in a number of activities including the activation of cardiac potassium channels, of phospholipase A2 (11, 12) and of the β-adrenergic receptor kinase (13–15). Specific $\beta\gamma$ subunits have also been shown to interact with different isoforms of adenylyl cyclase to modulate its activation by members of the $G\alpha_s$ class of proteins (16). Furthermore, cotransfection of cells resulting in the production of excess recombinant $G\alpha$ subunits titrated the effects of the free $\beta\gamma$ complex (17). Recently purified retinal $\beta\gamma$ complexes were shown to stimulate PLC β_2 (18) and recombinant $\beta_1\gamma_1$ and $\beta_1\gamma_2$ complexes were found to specifically activate the PLC β_2 isoform but not PLC β_1 in COS-7 cells transfected with expression vectors carrying the appropriate cDNAs (19). Heterotrimeric G proteins that include $G\alpha$ subunits of the G_q class are generally resistant to inactivation by pertussis toxin; on the other hand, G proteins that include members of the G_i or G_o class of α subunits are sensitive to pertussis toxin inactivation (3). In a model system, receptor-mediated release of $\beta\gamma$ subunits from the pertussis toxin-sensitive heterotrimeric complex was found to result in activation of PLC β_2, and pertussis toxin blocked this activation (19). Thus, both $\beta\gamma$ complexes and activated forms of G_q α subunits can activate specific isoforms of PLC β.

All of the inositol phospholipid-specific PLC enzymes whose amino acid sequences are known have been found to include homologous regions designated the X and Y boxes (see Fig. 2) that are thought to be associated with their catalytic activity (8). The PLC β subclass can be distinguished from other phospholipases by the presence of a long stretch of sequence at the C-terminal end of the molecule following the Y box of the enzyme (20). This region was found to be required for activation of PLC β_1 by $G\alpha_q$. The C-terminal region can be further subdivided into two sequences: the P box, which was shown to be required for the association of PLC β_1 with the particulate fraction, and the G box, which was required for interaction of PLC β_1 with $G\alpha_q$ (20). The sequences that make up the G box region in PLC β_1 and PLC β_2 are homologous, suggesting that this is the region of the enzyme that is required for activation by $G\alpha$ subunits in both of these isoforms. In this report, we use the cotransfection assay to demonstrate that all of the members of the G_q class of α subunits can activate PLC β_2 as well as PLC β_1. Furthermore, by making chimeras of PLC β_1 and PLC β_2 and deletion mutations of PLC β_2 we identified the region of PLC β_2 required for activation by $\beta\gamma$ subunits. Using the transfection assay we found that the nature of the β subunit is important for the activation of PLC β_2 and that certain $\beta\gamma$ complexes can act synergistically with specific $G\alpha_q$ subunits to activate PLC β_2.

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Abbreviations: G protein, GTP-binding protein; PLC, phospholipase C.

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MATERIALS AND METHODS

Transfection of COS-7 Cells with Expression Vectors and Analysis of Inositol Phosphates. COS-7 cells (1×10^5) were seeded in each well of a 12-well plate. A mixture of plasmid DNAs and 5 μl of Lipofectin (Bethesda Research Laboratories) was added to the cells. Five hours later, transfection was stopped by adding an equal volume of Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum and the cells were further cultured overnight. Then they were labeled with 10 μCi of [^{3}H]inositol (1 Ci = 37 GBq) (New England Nuclear) in inositol-free DMEM supplemented with 10% dialyzed fetal bovine serum for another 24 hr. In Fig. 5*B*, cells were labeled with [^{3}H]inositol 5 hr after transfection. The levels of inositol phosphates were determined after cells had been labeled for 24 hr as described (6).

Construction of PLC β_2 Mutants and Chimeras. A restriction map of the cDNAs corresponding to PLC β_1, PLC β_2, PLC β_2 mutants and chimeras are illustrated in Fig. 2. P2ΔC was obtained by cleaving the PLC β_2 plasmid with *Kpn* I and *Afl* II and religating with an adapter that has a stop codon in-frame. P2ΔC1 was made by cutting the PLC β_2 plasmid with *Kpn* I and *Afl* II and religating with a DNA segment synthesized by PCR corresponding to the cDNA sequence of PLC β_2 from nucleotide 2519 (corresponding to the *Kpn* I site) to nucleotide 3310. A stop codon and an *Afl* II site were designed into the 3′ primer. Pch1 was constructed by cutting PLC β_2 plasmid with *Kpn* I and *Afl* II and religating with a PCR segment corresponding to the sequence of PLC β_1 cDNA from nucleotides 2525–3900. Pch2 was generated by digestion of PLC β_1 plasmid with *Mlu* I and *Xho* I, followed by ligation with a PCR fragment corresponding to the sequence found from nucleotide 2011 to nucleotide 3793 in the PLC β_2 cDNA. Another chimera, Pch3, was made by cutting PLC β_1 plasmid with *Cla* I and *Afl* II, followed by ligation with a PCR fragment corresponding to the sequence found from nucleotide 16 to nucleotide 942 in the PLC β_2 cDNA. PCR was performed with a GeneAmpli kit (Perkin–Elmer).

SDS/PAGE. Cells were lysed in SDS sample buffer and loaded onto an SDS/polyacrylamide gel. After electrophoresis, the proteins were electroblotted to a nitrocellulose membrane and the recombinant proteins were detected by specific antibodies.

RESULTS

We have found (6, 21) that COS-7 cells that were cotransfected with cDNAs that express different α subunits of the G_q class together with cDNA constructs that express PLC β_1 released more inositol phosphates than cells transfected with either cDNA alone. These results suggest that all of the members of the G_q class of α proteins can activate the β_1 isoform of PLC; some fraction of the recombinant Gα proteins can presumably exist in the GTP-bound form or can be activated by endogenous receptors. In similar cotransfection assays with cDNA encoding PLC β_2 (Fig. 1*B*) G_q α-subunit recombinant proteins were also found to activate PLC β_2. The levels of expression of these Gα proteins were estimated by Western analysis using an antibody raised against a common sequence shared by all four Gα proteins and all of the recombinant proteins were clearly expressed at detectable levels (Fig. 1*A*). In cotransfection experiments with PLC β_2 and members of the G_i class of α-subunit proteins, there was no apparent activation of PLC β_2. We have previously demonstrated that the other portion of the heterotrimeric G protein, the $\beta\gamma$ complex, can specifically activate the PLC β_2 isoform but not the PLC β_1 isoform (19). Thus, in cotransfection experiments both specific Gα and G$\beta\gamma$ proteins are able to activate PLC β_2 isoforms.

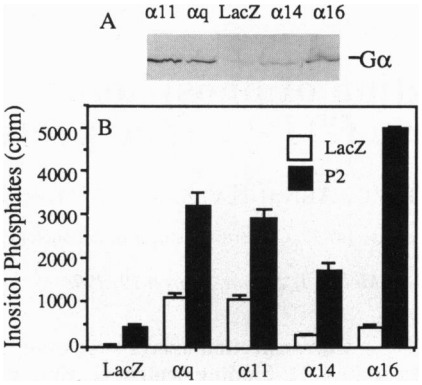

FIG. 1. (*A*) Expression of recombinant Gα subunits in COS-7 cells. Cells were transfected with cDNAs as indicated. The cells (1×10^5 cells for the LacZ, α14, or α16 transfectants; 2×10^4 cells for the α11 or αq transfectants) were solubilized in SDS sample buffer 48 hr posttransfection. The expression of Gα subunits was analyzed by Western blot with a polyclonal antibody recognizing a 15-amino acid peptide common to all the G_q class. (*B*) Activation of PLC β_2 by the members of the $G\alpha_q$ class. COS-7 cells were cotransfected with cDNA (0.5 μg per well) encoding $G\alpha_q$ (αq), $G\alpha_{11}$ (α11), $G\alpha_{14}$ (α14), $G\alpha_{16}$ (α16), or β-galactosidase (LacZ) and cDNA (0.5 μg per well) encoding PLC β_2 (P2) or LacZ. The levels of inositol phosphates were determined 48 hr after transfection. The level of inositol phosphates (800 cpm) in LacZ-transfected cells was subtracted.

The ability of the $G\alpha_q$ subunit protein to activate the PLC β_1 isoform depends on the presence of the C-terminal region of the PLC β protein (20). To delineate possible subdomains or regions of the PLC β_2 protein that might be responsible for activation by $\beta\gamma$ subunits or by the α subunits, we prepared a series of deleted and chimeric cDNA molecules designed to incorporate different regions of PLC β_1 and PLC β_2. A comparison of homologous regions of the two PLC cDNAs is shown in Fig. 2. The results of cotransfection of these modified PLC constructs with the α or $\beta\gamma$ subunits of the G proteins are shown in Figs. 3 and 4 and are tabulated in Fig. 2. The chimera Pch1, which consists of two-thirds of the PLC β_2 cDNA and the C-terminal one-third of PLC β_1 cDNA, showed high basal activity upon transfection. Nonetheless, Pch1, PΔC, and PΔC1 were markedly activated when cotransfected with cDNAs that expressed the $\beta_1\gamma_1$ subunits (Figs. 3*A* and 4*A*). These constructs all include the region that corresponds to the N-terminal two-thirds of PLC β_2, suggesting that this region may be required for activation by G$\beta\gamma$. In similar experiments, cotransfection with $G\alpha_q$ or $G\alpha_{16}$

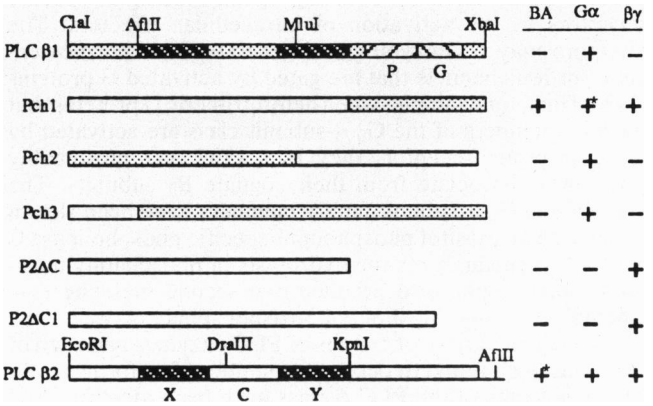

FIG. 2. Schematic representation of PLC molecules, their mutants, and chimeras. Boxes represent coding nucleotide sequence with the important restriction sites denoted. A summary of the basal activity (BA) and activation by G-protein α subunits (Gα) or G-protein $\beta\gamma$ subunits ($\beta\gamma$) is tabulated on the right. * indicates only slightly higher than controls. See Figs. 3–5 for details.

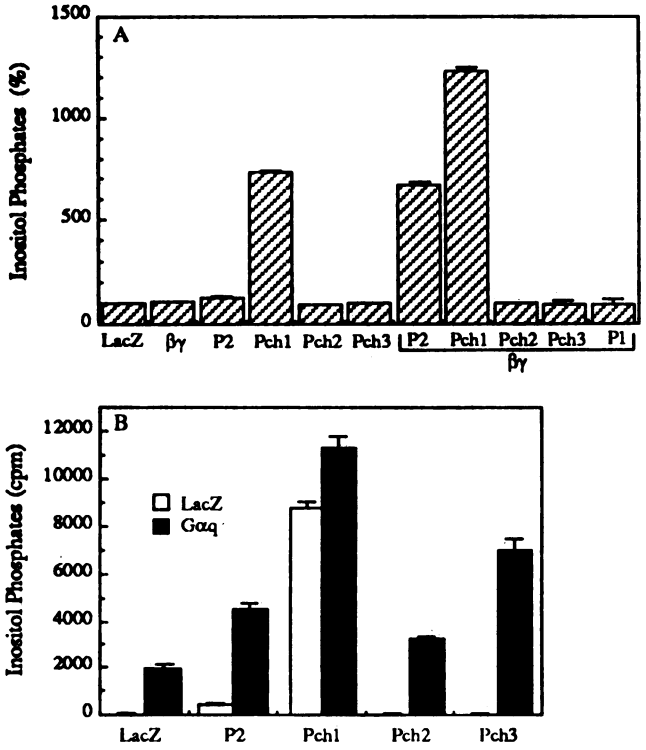

FIG. 3. Activation of PLC β_1 and PLC β_2 chimeras by $G\alpha_q$ or $G\beta_1\gamma_1$. (*A*) Cells were transfected or cotransfected with cDNAs (1.0 μg of DNA per well containing 0.33 μg of each component except LacZ, which was used to make up the total) encoding $G\beta_1\gamma_1$ ($\beta\gamma$), PLC β_1 (P1), PLC β_2 (P2), and β-galactosidase (LacZ). (*B*) Cells cotransfected with cDNA (0.5 μg per well) corresponding to PLC β_2 (P2) or chimeras and cDNA (0.5 μg per well) encoding $G\alpha_q$ (solid bars) or β-galactosidase (open bars). After 48 hr, the levels of inositol phosphates were determined. The level of inositol phosphates (1245 cpm) in cells transfected with LacZ was taken as 100% in *A* and was subtracted in *B*.

was shown to activate the chimeras Pch2, Pch3, and to some extent Pch1 but showed no activation of the truncated P2ΔC or P2ΔC1 forms (Figs. 3*B* and 4*B*). The recombinant protein products of cotransfection in each of the experiments were monitored by using specific antisera and the differences in activation could not be accounted for by differences in the

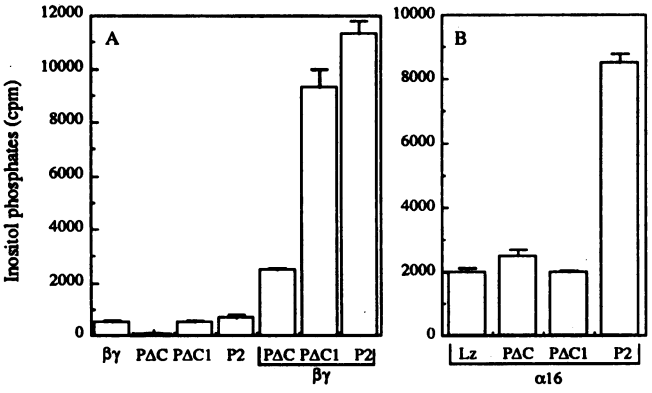

FIG. 4. Activation of PLC β_2 deletion mutant P2ΔC and P2ΔC1 by $G\beta_1\gamma_1$ or $G\alpha_{16}$. Cells were transfected or cotransfected with cDNAs (1.2 μg of DNA per well containing 0.4 μg of each component except Lz) corresponding to PLC β_2 (P2), P2ΔC (PΔC), P2ΔC1 (PΔC1), $G\beta_1\gamma_1$ ($\beta\gamma$), β-galactosidase (Lz), and $G\alpha_{16}$ (α16) as indicated. Levels of inositol phosphates were determined after 48 hr. The level of inositol phosphates (1355 cpm) in cells transfected with LacZ was subtracted in *A*.

levels of production of the recombinant proteins. Thus, we conclude that the C-terminal one-third region of the protein is required for activation of PLC β_2 by the G_q class of α subunits, while the amino acid sequence extending from the N terminus to the end of the Y box region (Fig. 2) contains the region required for activation of PLC β_2 by $\beta\gamma$ subunits. The chimera Pch3 could not be activated by $G\beta\gamma$, suggesting that the N-terminal 250 amino acids of PLC β_2 are not sufficient to endow the chimera with the ability to be activated by $G\beta\gamma$.

There are a large variety of $\beta\gamma$ heterotrimers that can form in different cells. To examine the question of the role of different combinations of $G\beta$ and $G\gamma$ in the activation of PLC β_2, a variety of experiments were done to express some of the various combinations of β and γ subunits together with PLC β as shown in Fig. 5*A*. In all cases, the $G\beta\gamma$ subunits activated only the PLC β_2 isoform and the $G\beta_1\gamma_1$ and $G\beta_1\gamma_5$ subunit combinations were found to be most effective. There is evidence that $G\beta_2$ and $G\gamma_1$ do not form an effective heterodimer (22). $G\beta_2\gamma_5$ does show activation of PLC β_2, albeit at a much lower level than the $G\beta_1\gamma_5$ homologue. Using specific antisera, we could measure the relative levels of expression of the recombinant $G\beta_1$ and $G\beta_2$ proteins (Fig. 5*A Inset*). We could not account for the differences in activation by the differences in the levels of protein expressed in the assay.

The transfection assay mirrors the interactions that are known to occur among the heterotrimeric G proteins. Thus, the presence of free $\beta\gamma$ subunits can activate PLC β_2; however, the introduction of high levels of recombinant $G\alpha$

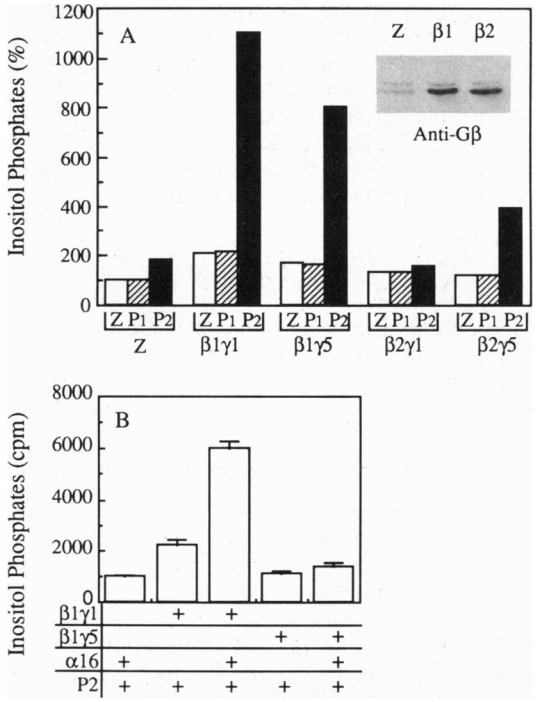

FIG. 5. Activation of PLC β_2 by $G\beta_1\gamma_{1,5}$ and $G\alpha_{16}$. (*A*) COS-7 cells were cotransfected with cDNAs (0.33 μg of DNA per well per component) encoding β-galactosidase (Z), PLC β_1 (P1), or PLC β_2 (P) and combinations of $G\beta_1$ and $G\gamma_{1,-5}$. (*B*) Cells were transfected with combinations of cDNA (0.25 μg per well per component) encoding $G\beta_1\gamma_1$, $G\beta_1\gamma_5$, α_{16}, and PLC β_2 (P2) as indicated. Total (1 μg per well) cDNA per transfection was kept constant with the LacZ cDNA. Levels of inositol phosphates were determined 48 hr (*A*) or 24 hr (*B*) after transfection. The level of inositol phosphates in cells transfected with LacZ was subtracted. (*Inset*) Cells transfected with β-galactosidase (Z), $G\beta_1\gamma_1$, or $G\beta_1\gamma_5$ were analyzed by Western blot and detected by an antibody raised against the C terminus of $G\beta_1$, which is identical to $G\beta_2$.

subunits can titrate the $\beta\gamma$ effect (17, 19). Since PLC β_2 can be activated by both α and $\beta\gamma$ subunits, we could test the relative ability of different combinations of $\beta\gamma$ subunits in the presence of a specific α subunit to activate PLC β_2. Fig. 5*B* shows that as a result of transfection $G\alpha_{16}$ activates PLC β_2 by itself as does $G\beta_1\gamma_1$; when they are cotransfected $G\beta_1\gamma_1$ and $G\alpha_{16}$ show synergistic activation of PLC β_2—i.e., the level of activation is higher than the sum of activity seen with either one alone. On the other hand, while $G\beta_1\gamma_5$ alone is able to activate PLC β_2 when $G\beta_1\gamma_5$ is coexpressed with $G\alpha_{16}$, the level of activation of PLC β_2 is lower than the sum of the activation seen with $G\alpha_{16}$ and $G\beta_1\gamma_5$ alone. We believe that the transfection assay reflects the relative affinities of the α subunits and of PLC β_2 for different $\beta\gamma$ combinations. Thus, we suggest that $G\beta_1\gamma_1$ and $G\alpha_{16}$ interact with different portions of PLC β_2 and when they are both present at sufficient levels they can provide maximal activation of the enzyme. The affinity of $G\beta_1\gamma_1$ for $G\alpha_{16}$ may be relatively low; thus, when they are both present each can act independently on the PLC β_2 substrate. On the other hand, if the affinity of $G\beta_1\gamma_5$ and of $G\alpha_{16}$ is greater than its ability to associate with PLC, then $G\beta_1\gamma_5$ could form stable complexes with $G\alpha_{16}$ and decrease the levels of activation of PLC by both of the components of the heterotrimeric G proteins. Since the only difference between these experiments is in the nature of the γ subunit—i.e., $G\beta_1\gamma_1$ vs. $G\beta_1\gamma_5$—we suggest that the Gγ subunits may play an important role in determining the relative affinity of G$\beta\gamma$ for Gα.

We have also found an intrinsic PLC activity in COS-7 cells that can only be clearly demonstrated in the simultaneous presence of high levels of $\beta_1\gamma_1$ and $G\alpha_{16}$ (data not shown). PLC β_2 could not be detected in COS-7 cells and cotransfection of exogenous PLC β_1 together with G$\beta\gamma$ and $G\alpha_{16}$ gave no further increase in activity. Thus, we suggest that the intrinsic activity may correspond to another isoform of the PLC β enzyme family, perhaps PLC β_3, which can be detected with a PLC β_3-specific antibody in COS-7 cells (data not shown). PLC β_3 can be activated by $\beta_1\gamma_1$ subunits and $G\alpha_{16}$ in much the same way as PLC β_2 (23, 24).

DISCUSSION

All of the data presented in this paper and in our previous report (20) support the suggestion that the Gα subunits of the G_q family function to activate isoforms of PLC β by interacting with the C-terminal region of the enzyme. The homology found in the amino acid sequences in the C-terminal region, particularly in the elements that we have defined as the G box, may account for the direct interaction and activation of the enzymes with the Gα proteins (20). Current data suggest that the $\beta\gamma$ subunits interact with the N-terminal region of PLC β_2. Some of the $\beta\gamma$ complexes that include β_1 rather than β_2 are much more efficient at activating PLC β_2. Interestingly, the presence of the $G\alpha_{16}$ and the $\beta_1\gamma_1$ subunits in COS-7 cells led to a marked synergistic effect on PLC β_2 activation, suggesting that $G\alpha_{16}$ and free $\beta\gamma$ subunits can modulate the activity of PLC β_2 simultaneously. This synergistic effect is not seen when the cotransfection is done with the $\beta_1\gamma_5$ subunit and $G\alpha_{16}$. We interpret these results to indicate that the $G\alpha_{16}$ subunit may have a lower affinity for $\beta_1\gamma_1$ than for $\beta_1\gamma_5$. Thus, interaction of the $\beta\gamma$ complex with the GDP-bound form of the α subunit would stabilize the inactive heterotrimeric complex. When the interaction between Gα and $\beta\gamma$ is weak, it is possible that the $\beta\gamma$ complex may exist free and that the α subunit may exist in equilibrium with bound GTP or may be activated by endogenous receptors in the cell to bind GTP. Our results provide a qualitative picture of the effects and the relative abilities of $G\alpha_{16}$ to interact with different $\beta\gamma$ subunit combinations and with effectors. Current work in a number of laboratories with purified proteins will allow direct measurements of these affinities and provide a rigorous test of the hypothesis that relative affinities between combinatorial forms of heterotrimeric G-protein components and between these components and isoforms of specific effectors generate intracellular signal processing circuits. Nonetheless, the cotransfection experiments suggest that the nature of the Gβ subunits may play a role in determining the preference of the $\beta\gamma$ complexes for effectors, while the nature of the Gγ subunits may be important for determining the relative affinity of the $\beta\gamma$ complexes for a specific Gα protein.

Schultz and co-workers (25, 26) in a series of elegant experiments using antisense to eliminate specific G-protein components have shown that different receptors may sequester and act through specific combinations of α, β, and γ subunits of G proteins that form heterotrimers required for receptor-specific gating of ion-channel activity. On the effector side of the circuit, a variety of different kinds of PLC activation responses can result depending on the nature of the PLC β isoform and the specific β, γ, or α subunits that are released by receptor interaction with ligand. These results taken together clearly have implications for the nature of the signaling circuits that are generated in differentiated cell types (3). Cells expressing specific isoforms of PLC β may generate different levels of inositol phospholipids and diacylglycerol in response to receptors that activate certain Gα proteins or that release specific $\beta\gamma$ subunits from other G-protein complexes. Furthermore, the activity of a variety of receptors can be integrated depending on the nature of the PLC β isoform; thus, either activated α subunits alone may activate the PLC enzyme or $\beta\gamma$ subunits alone or both types of subunits when released simultaneously can generate high levels of PLC function in the presence of the appropriate PLC isoform. Bourne and colleagues (17) have suggested that the simultaneous availability of free $\beta\gamma$ subunits and α subunits may be required for maximal activation of the target enzyme in certain specific cells. The requirement for specific kinetic characteristics in an information-processing circuit may account for the evolution of differentially expressed isoforms in a variety of cell types.

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