Phosphorylation of the G Protein $\gamma_{12}$ Subunit Regulates Effector Specificity*

Hiroshi Yasuda†, Margaret A. Lindorfer, Chang-Seon Myung, and James C. Garrison§

From the Department of Pharmacology, Health Sciences Center, University of Virginia, Charlottesville, Virginia 22908

Although the G protein $\beta\gamma$ dimer is an important mediator in cell signaling, the mechanisms regulating its activity have not been widely investigated. The $\gamma_{12}$ subunit is a known substrate for protein kinase C, suggesting phosphorylation as a potential regulatory mechanism. Therefore, recombinant $\beta_1\gamma_{12}$ dimers were overexpressed using the baculovirus/Sf9 insect cell system, purified, and phosphorylated stoichiometrically with protein kinase C $\alpha$. Their ability to support coupling of the $G_{i_1}$ $\alpha$ subunit to the A1 adenosine receptor and to activate type II adenyl cyclase or phospholipase C-$\beta$ was examined. Phosphorylation of the $\beta_1\gamma_{12}$ dimer increased its potency in the receptor coupling assay from 6.4 to 1 nM, changed the $K_{ac}$ for stimulation of type II adenyl cyclase from 14 to 37 nM, and decreased its maximal efficacy by 50%. In contrast, phosphorylation of the dimer had no effect on its ability to activate phospholipase C-$\beta$. The native $\beta_1\gamma_{10}$ dimer, which has 4 similar amino acids in the phosphorylation site at the N terminus, was not phosphorylated by protein kinase C $\alpha$. Creation of a phosphorylation site in the N terminus of the protein (Gly$^4$-Lys$^5$) resulted in a $\beta_1\gamma_{10 Goodman}$ dimer which could be phosphorylated. The activities of this $\beta_1\gamma_{10 Goodman}$ dimer were similar to those of the phosphorylated $\beta_1\gamma_{12}$ dimer. Thus, phosphorylation of the $\beta_1\gamma_{12}$ dimer on the $\gamma$ subunit with protein kinase C $\alpha$ regulates its activity in an effector-specific fashion. Because the $\gamma_{12}$ subunit is widely expressed, phosphorylation may be an important mechanism for integration of the multiple signals generated by receptor activation.

Most cells possess multiple signaling pathways to receive signals from the hormones, autacoids, neurotransmitters, and growth factors in their environment. One of the best charac-terized signal transduction systems is used by receptors coupled to the heterotrimeric G proteins (1–5). Receptors activate this system by stimulating the release of bound GDP from the G protein $\alpha$ subunit leading to exchange of GDP for GTP in the protein’s nucleotide binding site. Binding of GTP induces a conformational change in the $\alpha$ subunit, simultaneously activating the protein and markedly decreasing its affinity for the $\beta\gamma$ dimer (1). Both the GTP-bound form of the $\alpha$ subunit and the released $\beta\gamma$ subunit are capable of activating multiple effectors to generate intracellular messages (3, 4, 6, 7). The mechanisms that regulate the lifetime of the active, GTP-bound form of the $\alpha$ subunit have been studied extensively. All $\alpha$ subunits have an intrinsic GTPase activity, which hydrolyzes bound GTP to GDP (1, 3, 4), returning the molecule to its basal state and increasing its affinity for GDP and the $\beta\gamma$ subunit (1, 3, 4, 8). Both changes induce formation of the stable, hetero-trimeric form of the G protein. Interestingly, the GTPase activity of many $\alpha$ subunits can be increased by a class of proteins termed RGS molecules (9, 10) and by certain effectors such as PLC-$\beta$ (11).

Although the activity of the $\alpha$ subunit is regulated by multiple mechanisms, regulation of the activity of the $\beta\gamma$ dimer is not well characterized. Recently, the $\gamma_{12}$ subunit has been shown to be a substrate for protein kinase C (12, 13), suggesting that dimers containing this $\gamma$ subunit may be subject to regulation by phosphorylation. The $\gamma_{12}$ subunit is widely expressed (12–15) and, given the extensive role of the $\beta\gamma$ subunit in cell signaling (6, 16), its phosphorylation may have important consequences. To examine the effects of $\gamma_{12}$ subunit phosphorylation on its activity, we purified recombinant $\beta_1\gamma_{12}$ dimers from baculovirus-infected Sf9 insect cells, phosphorylated them with PKC $\alpha$ and $\beta_1$, and tested their activity in three assays of $\beta\gamma$ function. We examined the ability of phosphorylated dimers to support coupling of the $G_{i_1}$ $\alpha$ subunit to the A1 adenosine receptor and to activate two effectors, type II adenyl cyclase or phospholipase C-$\beta$. Phosphorylation of the $\beta_1\gamma_{12}$ subunit had no effect on its ability to activate PLC-$\beta$, but increased its potency in the receptor coupling assay and markedly inhibited its ability to activate adenyl cyclase. These results suggest that phosphorylation reduces the ability of the $\beta\gamma$ signal to increase cyclic AMP levels and favors activation of other effectors.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Baculoviruses for the $\beta$ and $\gamma$ Subunits—Full-length clones encoding the human $\gamma_{12}$ and $\gamma_{10}$ proteins were identified in the EST data base and obtained from Research Genetics, Inc ($\gamma_{12}$, GenBank™ N42722; $\gamma_{10}$, GenBank™ U31383). To minimize the length of the construct 5′ from the ATG start codon, the end of the $\gamma_{12}$ CDNA was modified using the polymerase chain reaction (PCR). For the $\gamma_{12}$ cDNA, the primers used were: (sense primer: 5′-CCCCGGAT-GTCGACGACAAACGACCA-3′; antisense primer: 5′-ATAGAGACTGAG-GAGCTCAT-3′). The PCR products were subcloned into the pCNTR shuttle vector, the $\gamma_{12}$ coding sequence excised from pCNTR with SmaI and XhoI, and ligated into these sites in the baculovirus transfer vector, pVL1393. The native $\gamma_{10}$ CDNA was excised from the pT7T3D plasmid with EcoRI, further digested with BanII and Asp700 and subcloned into the pCNTR shuttle vector. The $\gamma_{10}$ coding sequence was excised from this article were defrayed in part by the payment of page charges. This advertisement must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* This work was supported by National Institutes of Health Grants PO1-CA 40042 and RO1-DK-19552. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a fellowship from the Virginia Affiliate of the American Heart Association. Present address: Dept. of Medicine, University of Tokyo, Tokyo 112-8688, Japan.

‡ Supported by a fellowship from the Virginia Affiliate of the American Heart Association. Present address: Dept. of Medicine, University of Tokyo, Tokyo 112-8688, Japan.

§ To whom correspondence should be addressed: Box 448, Health Sciences Center, University of Virginia, Charlottesville, VA 22908. Tel.: 804-924-5618; Fax: 804-982-3878; E-mail: jcg8w@virginia.edu.

The abbreviations used are: G proteins, guanine nucleotide-binding regulatory proteins; Sf9 cells, Spodoptera frugiperda cells (ATCC number CRL 1711); CHAPS, 3-(3-cholamidopropyl)dimethylammoniom-1-propanesulfonate; GENAPOL® C-100, polyoxyethylene(10)dodecyl ether; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; PLC, phospholipase C; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1-bis(hydroxy-methyl)ethyl]glycine; NTA, nitrilotriacetic acid; GTP$\gamma$S, guanosine 5′-O-(thiotriphosphate).

This paper is available on line at http://www.jbc.org

21958
pCNTR with BamHI and XbaI and ligated into these sites in the pVL1393 transfer vector. The N terminus of the γ12 protein was modified to have a protein kinase C phosphorylation site by mutagenesis of the γ12 cDNA using PCR. The primers used were: (sense primer: 5' GCGAAGGCTACG-3'; antisense primer: 5'-CTCCTG- CAGTTGCTGAAGGAAATTC-3'). The PCR reaction was subcloned into pCNTR, digested with BamHI and EcoRI, and ligated into these sites in the pVL1393 transfer vector. To add the hexahistidine-FLAG affinity tag to the 5' end of the βi subunit, the polymerase chain reaction was used to add XbaI and BamHI restriction sites to the 3' and 5' ends of the βi coding region. The primers used were: (sense primer: 5'-TCTAGAATGGAACGGGCTAG-3'; antisense primer: 5'-GGAGCTTGTGATCGGATTCA-3'). The reaction products were digested with XbaI and BamHI and ligated into the pDouble Trouble (pDT) vector, which adds the nucleotide sequences for the hexahistidine and FLAG affinity tags to the 5' end of the βi coding region (17). The βiHR coding region was excised from pDT with HindIII and BamHI and subcloned into the pCNTR shuttle vector. The βiHR coding sequence was excised from pCNTR with BamHI and ligated into the BamHI site of pVL1393. The pVL1393 transfer vectors containing these four constructs were sequenced to verify the fidelity of the βi and γ sequences. Recombinant baculoviruses were constructed by co-transfecting each transfer vector with Sindbis virus vector DNA into Sf9 insect cells using the Qiagen BaculGold® kit as described (18). The recombinant baculoviruses were purified by one round of plaque purification using standard techniques (19). The construction of the recombinant baculoviruses for the Gs and γ subunits and the A1 adenosine receptor have been described (20, 21).

Expression and Purification of Recombinant G Protein α and ϒ Subunits—G protein α and γ subunits were overexpressed by infecting suspension cultures of SF9 insect cells with recombinant baculoviruses (22, 23). The Gα subunit was purified to homogeneity as described (22). The recombinant βγ dimers were extracted from SF9 cells and purified using DEAE chromatography and an α subunit affinity column (23). Phosphorylation of Purified γ Subunits by PKC—The purified γmγc subunit was incubated for 30 min at 30 °C in 50 mM Tris, pH 7.5, 1 mM β-mercaptoethanol, 10 mM MgCl2, 0.4 mM CaCl2, 40–100 μM ATP, recombinant PKC α or β, 40 μg/ml phosphatidylinositol, and 0.8 μg/ml diolein. Usually, 25 μg of βγ dimer was incubated with 0.74 unit of PKC α to achieve stoichiometric phosphorylation of the γ subunit. Control reactions contained deionized water in the place of PKC. The stoichiometry of the phosphorylation reaction was measured by incubating 40 μM [γ-32P]ATP (500–2000 cpm/mmol) in the reaction mix and subjecting the phosphorylated βγ subunits to Tricine/SDS-PAGE (24). The resolved γ subunit was cut from the dried gel and the amount of radioactive phosphate incorporated estimated by scintillation counting. After the phosphorylation reaction and before use in the assays, the βγ subunit was repurified from the reaction mixture by loading it onto a Nickel-NTA-agarose from Qiagen; and washing with 15 ml of 20 mM Hepes, pH 8.0, 150 mM NaCl, 1 mM MgCl2, 0.5% (w/v) Triton X-100, 0.17 mM EDTA, 3 mM EGTA, 17 mM NaCl, 67 mM KCl, 0.83 mM MgCl2, 1 mM diethylthioctetate, and 1 mg/ml bovine serum albumin. The stoichiometry only reached 0.5 mol/mol after 60 min of incubation. The reaction buffer (25 mM Hepes, pH 8.0, 10 mM phosphocreatine, 10 units/ml creatine phosphokinase, 0.4 mM 3-isobutyl-1-methylxanthine, 10 mM MgSO4, 0.5 mM ATP, and 0.1 mg/ml bovine serum albumin) was preincubated at 30 °C for 20 min. Production of cyclic AMP was initiated by addition of the reconstituted membranes to the reaction buffer and the incubation continued for 10 min at 30 °C. Reactions were stopped by the addition of 0.1 N HCl and cyclic AMP measured using an automated radioimmunoassay (33).

Electrophoresis—Tricine/SDS-polyacrylamide gels were run according to the procedure of Schagger and von Jagow (24). The separating gel contained 16.5% total acrylamide, 0.4% bisacrylamide, and 10% (v/v) glycerol. The stacking gel contained 4% total acrylamide and 0.1% bisacrylamide. Gels were run at constant voltage (~100 volts) at 10 °C for 4–5 h. The proteins were stained with silver by the method of Morrissey (34), with the modification that the dithiothreitol incubation was reduced to 15 min.

Calculation and Expression of Results—Experiments presented under “Results” are representative of three or more similar experiments. Data expressed as dose-response curves were fit to rectangular hyperbolas using the fitting routines in the GraphPad Prizm® software. Statistical differences between the curves were determined using the individual data points from multiple experiments to calculate the F statistic as described (35).

Materials—All reagents used in the culture of SF9 cells and for the expression and purification of G protein α and βγ subunits have been described in detail (20, 23). The baculovirus transfer vector, pVL1393, was a gift of Dr. David Baltimore of the California Institute of Technology. 10% GENAPOL® C-100, CHAPS, microcystin, and the α and β isoforms of PKC from Calbiochem; Ni2+-NTA-agarose from Qiagen; H1 and H2 phosphatidylinositol bisphosphatase from NEN Life Science Products; PMA from Sigma; the pCNTR shuttle vector from Prime to 3 Prime, Inc. (Boulder, CO). All other reagents were of the highest purity available.

RESULTS

Stoichiometry of Phosphorylation of the γ12 and the γ12γ4γ2 Subunits—Recent experiments have demonstrated that the bovine γ12 subunit is a substrate for protein kinase C (12), but the functional significance of this phosphorylation event has not been extensively studied. As this newly discovered γ12 subunit is widely expressed (12–15), its phosphorylation may have important consequences. To examine the effects of γ12 subunit phosphorylation on its activity, we purified recombinant β12γ12 dimers from SF9 insect cells, phosphorylated them with PKC α and γ, and measured their activity in assays of βγ function. The human γ12 subunit was rapidly phosphorylated by PKC α to a stoichiometry of about 1 mol/mol as shown in Fig. 1. A and B. Protein kinase C β was less effective than PKC α; the stoichiometry only reached 0.5 mol/mol after 60 min of incubation. Addition of a hexahistidine-FLAG affinity tag to the β1 subunit.
Phosphorylation of \( \gamma_{12} \) Subunit

FIG. 1. Phosphorylation of \( \beta_{11HF} \gamma_{12} \) by protein kinase C. A, purified \( \beta_{11HF} \gamma_{12} \) dimer was incubated with protein kinase C \( \alpha \) for the indicated times. The autoradiograph shows the time course of the phosphorylation of the \( \gamma_{12} \) subunit by PKC \( \alpha \) (upper panel) and PKC \( \beta_1 \) (lower panel). Aliquots were withdrawn from the kinase reactions at the indicated times for analysis by tricine/SDS-PAGE as described under “Experimental Procedures.” B, stoichiometry of the phosphate incorporated into the \( \gamma_{12} \) subunit measured as described under “Experimental Procedures.” C, reaction mixtures were subjected to gel electrophoresis to visualize the purity of proteins (silver stain) and the subunits phosphorylated (autoradiograph). The left panel shows the reaction mixture before and after purification of the phosphorylated \( \beta_{11HF} \gamma_{12} \) on the NTA-agarose column. The right panel shows that only the \( \gamma_{12} \) subunit is phosphorylated. Experiments are representative of 10 similar experiments.

Effect of Phosphorylation of the \( \gamma \) Subunit on Receptor Coupling—Having established the ability of protein kinase C to phosphorylate the two recombinant \( \gamma \) subunits, we examined the effects of phosphorylation on the function of the dimer. The \( \beta \gamma \) subunits play several important roles in the signaling mechanisms used by receptors to activate effectors. In combination with the \( \alpha \) subunit, they participate in forming the high affinity agonist binding conformation of the receptor (21, 38, 39); they stabilize the basal state of the system by increasing the affinity of the \( \alpha \) subunit for GDP (7, 8); and when released from the \( \alpha \) subunit, they activate effectors such as type II adenyl cyclase and phospholipase C-\( \beta \) (6, 7). We first examined the effect of \( \gamma \) subunit phosphorylation on the ability of the \( \beta \gamma \) dimer to support establishment of the high affinity, agonist binding conformation of a G protein-coupled receptor. In membranes from S9 cells overexpressing recombinant A1 adenosine receptors, about 90% of the receptors are in a low affinity conformation. Reconstitution of pure \( \alpha \) and \( \beta \gamma \) subunits into these membranes establishes high affinity agonist binding and provides a sensitive assay for receptor-\( \alpha \beta \gamma \) interactions (21). Fig. 3 shows that the dimers used in this study, \( \beta_1 \gamma_{12}, \beta_2 \gamma_{10}, \beta_1 \gamma_{10G4K} \), and \( \beta_{11HF} \gamma_{12} \), were able to re-establish the high affinity, agonist binding conformation of the receptor with a potency and efficiency equal to the well studied and highly effective \( \beta_2 \gamma_2 \) dimer (26). All dimers tested support coupling with a \( K_{\text{on}} \) of 0.5–1.0 nm (see Fig. 3 legend). Thus, the newly discovered \( \gamma_{10} \) and \( \gamma_{12} \) subunits are able to couple very effectively to the \( \gamma_4 \) subunit and the A1 adenosine receptor when dimerized with the \( \beta_4 \) subunit. Importantly, neither the hexahistidine-FLAG tag added to the N terminus of the \( \beta_4 \) subunit nor the G4K mutation made in the N terminus of the \( \gamma_{10} \) subunit affect the ability of these recombinant \( \beta \gamma \) dimers to induce the high affinity
conformation of the A1 adenosine receptor.

The data in Fig. 4 illustrate the ability of phosphorylated and unphosphorylated forms of the $\beta_1$HF$\gamma_{12}$ dimer to support reconstitution of the high affinity binding state of the A1 adenosine receptor. Phosphorylation of the $\beta_1$HF$\gamma_{12}$ subunit (open circles) increased the potency of the dimer in this assay from about 6.4 to 1 nM (Fig. 4A). This difference was significant ($p < 0.001$). Interestingly, phosphorylation of the $\gamma_{12}$ subunit has been reported to increase the affinity of the $\beta\gamma$ dimer for the $\alpha$ subunit (12), a result consistent with the increased potency seen in Fig. 4A. A similar result was obtained when the phosphorylated and unphosphorylated forms of the $\beta_1$HF$\gamma_{10G4K}$ dimer were tested (Fig. 4B). This small difference in potency was also significant ($p < 0.05$). The observation that the phosphorylated $\beta_1$HF$\gamma_{10G4K}$ dimer does not shift the curve as greatly as the phosphorylated $\beta_1$HF$\gamma_{12}$ dimer may be due to the fact that the stoichiometry of phosphorylation is only about 0.5 mol/mol (see Fig. 2 and text). It is important to note that these differences were observed only when microcystin was included in the binding assay buffer, suggesting that the SF9 cell membranes contain a protein phosphatase able to dephosphorylate $\gamma_{12}$. To examine this possibility, we incubated $^{32}$PO$_4$-labeled $\beta_{1HF}\gamma_{12}$ with the SF9 cell membranes at 30 °C in the presence or absence of 100 nM microcystin, removed aliquots from the incubation medium over a 60-min time period, and resolved the proteins on a Tricine/SDS gel. In the absence of microcystin, the amount of radioactivity in the $\gamma_{12}$ subunit decreased more than 80% over the 60-min incubation. Little dephosphorylation occurred when microcystin was included in the 30 °C incubation or if the mixture was held at 0 °C without microcystin (data not shown). This result confirms the existence of an effective phosphatase for the $\gamma_{12}$ subunit. The insect cell phosphatase must be analogous to mammalian protein phosphatases 1 and 2, which are very sensitive to microcystin (27). Finally, controls were performed to determine whether the steps used to phosphorylate and re-purify the $\beta\gamma$ dimers decreased their ability to couple to the adenosine receptor (see "Experimental Procedures"). The activity of a pure $\beta_1$HF$\gamma_{12}$ dimer used directly in the binding assay was about 10% higher than dimers subjected to a mock-phosphorylation incubation and re-purified on the Ni$^{2+}$-NTA column prior to assay (compare maximal binding in Figs. 3 and 4).

Effect of $\gamma$ Subunit Phosphorylation on Effector Activity—The data in Fig. 5A show the ability of the native and modified $\beta\gamma$ dimers used in this study to activate PLC-\(\beta\) in an in vitro assay using $[\text{H}]$IP$_3$ incorporated into phospholipid vesicles as substrate. Note that $\beta_1\gamma_{12}$, $\beta_1\gamma_{10}$, $\beta_1$HF$\gamma_{10G4K}$ and $\beta_{1HF}\gamma_{12}$ are equally as effective as the $\beta_1\gamma_{12}$ dimer. All four forms of the protein stimulated the release of $[\text{H}]$inositol 1,4,5-trisphosphate with a $K_{act}$ of 6–8 nM and were equally effective (~8-fold increase in activity). The data in Fig. 5B demonstrate that there is no difference in the ability of either the phosphorylated or unphosphorylated $\beta_1$HF$\gamma_{12}$ dimers to activate PLC-\(\beta\). The phosphorylated or unphosphorylated forms of the $\beta_1$HF$\gamma_{10G4K}$ dimers were also tested and no differences were observed (data not shown). As can be seen by comparison of the maximal activities shown in Fig. 5, A and B, the phosphorylation protocol caused about a 40% decrement in $\beta\gamma$ activity in the PLC assay.

The $\beta\gamma$ dimer causes a synergistic activation of type II adenyl cyclase in the presence of GTP$\gamma$S-activated G\(_{\alpha}\) subunit (40). Therefore, we examined the effect of phosphorylation on

![Fig. 3. Ability of native and modified $\beta_1\gamma_{12}$ and $\beta_1\gamma_{10}$ dimers to support the high affinity, agonist binding state of the adenosine A1 receptor. SF9 cell membranes expressing recombinant bovine A1 adenosine receptors were reconstituted with 10 nM G\(_{\alpha}\) subunit, the indicated concentrations of the defined $\beta\gamma$ dimers, and high affinity $^{125}$I-aminobenzyladenosine binding measured as described under "Experimental Procedures." The ratio of receptor::$\beta\gamma$ was approximately 1:20–3:33. The $K_{act}$ as determined by fitting each data set to a rectangular hyperbola ranged from 0.5 to 1.0 nM. The $K_{act}$ values are not significantly different. The results are representative of three similar experiments performed in triplicate.](image1)

![Fig. 4. Comparison of the ability of phosphorylated and unphosphorylated $\beta_1$HF$\gamma_{12}$ and $\beta_1$HF$\gamma_{12}$ dimers to support high affinity agonist binding to the adenosine A1 receptor. A, SF9 cell membranes expressing recombinant bovine adenosine A1 receptors were reconstituted with 6 nM G\(_{\alpha}\) subunit and the indicated concentrations of unphosphorylated (closed circles) or phosphorylated (open circles) $\beta_1$HF$\gamma_{12}$ dimers. Formation of the high affinity, agonist binding state of the receptor was measured as described under "Experimental Procedures." Phosphorylation of the $\beta_1$HF$\gamma_{12}$ dimer caused a significant increase in potency from 6.4 to 1 nM ($p < 0.001$). B, an analogous experiment performed with unphosphorylated (closed circles) or phosphorylated (open circles) $\beta_1$HF$\gamma_{10G4K}$ dimers. Phosphorylation of the $\beta_1$HF$\gamma_{10G4K}$ dimer caused a significant increase in potency from 3.4 to 1.8 nM ($p < 0.05$). The binding reactions were performed in the presence of 100 nM microcystin. Data points are the mean ± S.D. of three independent experiments, each performed in triplicate. Rectangular hyperbolas fit to the data and statistical differences between the curves determined as described under "Experimental Procedures."](image2)
the ability of the $\beta_{1HF}\gamma_{12}$ and $\beta_{1HF}\gamma_{10G4K}$ dimers to stimulate recombinant, type II adenylyl cyclase in Sf9 cell membranes. Fig. 6A shows that the unphosphorylated forms of all the $\beta\gamma$ dimers used in this study effectively stimulate type II adenylyl cyclase. As before, the $\beta_{1}\gamma_{12}$, $\beta_{1HF}\gamma_{12}$, and the $\beta_{1HF}\gamma_{10G4K}$ dimers are all as effective as the $\beta_{1}\gamma_{2}$ dimer. The $\beta_{1}\gamma_{10}$ dimer had activity equal to the $\beta_{1HF}\gamma_{10G4K}$ dimer in this assay (data not shown). The $K_{cat}$ for all the dimers ranges from 3 to 14 nm. Each of these dimers was purified with the $\alpha$ subunit affinity column and assayed directly. Thus, the newly discovered $\gamma_{12}$ and $\gamma_{10}$ subunits are able to effectively stimulate type II adenylyl cyclase when combined with the $\beta_{1}$ subunit. Moreover, neither the hexahistidine-FLAG tag on the $\beta_{1}$ subunit nor the G4K mutation in the $\gamma_{10}$ subunit greatly affected the ability of these subunits to stimulate adenylyl cyclase.

Interestingly, the phosphorylated forms of the $\beta_{1HF}\gamma_{12}$ and $\beta_{1HF}\gamma_{10}$ dimers were significantly less potent and effective in their ability to activate adenylyl cyclase (Fig. 6B). Note that the phosphorylated forms of the $\beta\gamma$ dimers are less active than the unphosphorylated forms over the concentration range of 1–40 nm. Fitting the data to rectangular hyperbolas estimates that phosphorylation of the dimers decreases their activity about 50% and shifts their $K_{cat}$ from 14 to 37 nm (see legend). As before, controls were performed to determine whether the steps needed to phosphorylate and re-purify the $\beta\gamma$ dimers decreased their activity. The protocol causes about a 15% decrement in activity (compare the maximal activities in Fig. 6, A and B). To verify the effect of $\gamma_{12}$ subunit phosphorylation on cyclase activity, we prepared dimers with different stoichiometries of phosphorylation and assayed their activity. As shown in Fig. 7, varying the stoichiometry of $\gamma_{12}$ subunit phosphorylation resulted in a gradual reduction in the dimer’s ability to stimulate type II adenylyl cyclase from about 17 nmol/min/mg of protein to about 10 nmol/min/mg of protein. Each of the four curves in Fig. 7 is significantly different from the other (see legend). This result clearly demonstrates that phosphorylation of the serine residue at the N terminus of the $\gamma$ subunit can reduce its ability to stimulate type II adenylyl cyclase.

Previous work has demonstrated that activation of PKC with phorbol 12-myristate 13-acetate (PMA) in Sf9 cells expressing type II adenylyl cyclase results in phosphorylation of the enzyme and an increase in both basal and forskolin-stimulated cyclase activity (31). Moreover, direct phosphorylation of the type II adenylyl cyclase expressed in Sf9 cell membranes with PKC alters its responsiveness to both $\alpha$ and $\beta\gamma$ subunits (41). Thus, our finding that phosphorylation of $\gamma_{12}$ by the same kinase reduces the ability of the $\beta\gamma$ dimer to stimulate type II
was incubated without PKC at 30 °C for 30 min; 0.25 mol of PO₄/mol pretreatment of cyclase infected Sf9 cells with PMA did result in agents were 0.2, 1.0, 1.2, and 6.25 nmol of cAMP/min/mg of the activities of the control membranes treated with these open circles were prepared as follows: 0 mol of PO₄/mol (open diamonds), the dimer was incubated with PKC α for 2 min at 30 °C; 1.0 mol of PO₄/mol (open triangles), the dimer was incubated with PKC α for 30 min at 30 °C. The βγ dimers were purified from the PKC reaction mixture and their ability to stimulate recombinant type II adenylyl cyclase measured as described under "Experimental Procedures." Rectangular hyperbolas were fit to the data and statistical differences between the curves determined as described under "Experimental Procedures." Each curve was significantly different from the others (p < 0.001). The results are representative of four similar experiments performed in duplicate. adenyl cyclase frames an interesting problem. To determine the effect of a phosphorylated β₁γ₁₂ dimer on the activity of type II adenyl cyclase in PMA-treated cells, we infected Sf9 cells with a recombinant baculovirus encoding type II adenyl cyclase for 48 h, added 1 μM PMA to the medium for 30 min before harvest, and prepared membranes as described under "Experimental Procedures." The type II adenyl cyclase activity in the control and PMA-treated membranes was compared following treatment with vehicle, 100 nM forskolin, activated Gαs, and Gαs plus 20 nM βγ. The activities of the control membranes treated with these agents were 0.2, 1.0, 1.2, and 6.25 nmol of cAMP/min/mg of protein, respectively. In keeping with previous results (31), pretreatment of cyclase infected Sf9 cells with PMA did result in a 10–20% increase in the rates of basal, 100 nM forskolin, Gαs, and Gαs plus 20 nM βγ-stimulated cyclic AMP synthesis relative to control membranes. Next, dose-response curves were performed with both membrane preparations using phosphorylated and unphosphorylated β₁H₁₂γ₁₂ dimers in the presence of activated Gαs. Interestingly, in the membranes from PMA-treated cells, the phosphorylated dimers were still markedly less effective than unphosphorylated dimers at stimulating type II adenyl cyclase. In membranes from control cells, phosphorylation of the dimer reduced the stimulation of cyclase by the following percentages: at 10 nM βγ, by 31%; at 20 nM βγ, by 40%; and at 40 nM βγ, by 45% (n = 5). In membranes from PMA treated Sf9 cells, the reductions were very similar: at 10 nM βγ, by 33%; at 20 nM βγ, by 40%; and at 40 nM by βγ, 55% (n = 5). Thus, in a cell expressing the γ₁₂ subunit, its phosphorylation by PKC would still inhibit the ability of a βγ dimer to stimulate type II adenyl cyclase activity, even though basal or Gαs-stimulated cyclase activity itself might be slightly elevated by kinase activation.

**DISCUSSION**

Identification of the diversity in the family of G protein γ subunits has prompted studies to determine whether the differences in these proteins translate to specificity in transmembrane signaling (42). In this regard, one major finding of this study is that, when combined with the βγ subunit, the newly discovered γ₁₂ and γ₁₀ subunits are equal in potency and efficacy to the well studied β₁γ₂ dimer. Both the β₁γ₁₂ and β₁γ₁₀ dimers were fully effective in the receptor coupling assay using the Gαs subunit and the A1 adenosine receptor and able to maximally activate type II adenyl cyclase and PLC-β. As these γ subunits are widely expressed in brain and peripheral tissues (12, 14, 15, 37), they are likely to play important roles in signaling by a large number of G protein coupled receptors. A second important finding is that phosphorylation of the β₁γ₁₂ dimer with protein kinase C has distinct effects on the activity of the molecule, increasing its potency in the receptor coupling assay and inhibiting its ability to stimulate type II adenyl cyclase. Previous studies have determined that the phosphorylation site in γ₁₂ is Ser² at the N terminus of the molecule (12, 13). This finding is consistent with our observations that the phosphorylation site created in the γ₁₂GTPGαd subunit makes it a substrate for protein kinase C and that phosphorylation regulates its activity. Although not fully explored, the finding that the dephosphorylation of γ₁₂ is blocked by microcystin suggests that the protein phosphatases that dephosphorylate the protein in the intact cell are most likely protein phosphatase 1 and/or 2A. Overall, the protein kinases and phosphatases regulating the phosphorylation state of the γ₁₂ subunit are those known to participate in responses to receptors generating diacylglycerol and Ca²⁺ (27, 43–45), suggesting an important role for this event in cell signaling.

The finding that phosphorylation of the γ subunit can reduce the ability of the βγ dimer to stimulate one effector without changing its activity on other effectors adds complexity to the regulation of this signal. Previously, the known mechanisms for regulating βγ activity only involved sequestration by either G protein α subunits or phosducin (3, 6, 7). Because the GDP bound form of the α subunit has a higher affinity for the βγ subunit, an important mechanism for regulating the activity of the βγ subunit is the return of the active, GTP-bound α subunit to its basal state (1, 3, 4, 7). Indeed, overexpression of α subunits is an effective means of decreasing the activity of βγ subunits in cultured cells (46, 47). Phosducin also has a nanomolar affinity for the βγ subunit (48) and, whereas its role in cell function is still emerging, it appears to inhibit the βγ dimer by sequestration of the protein following dissociation of the αβγ heterotrimer (49). Because the βγ dimer is needed for coupling the α subunit to receptors (21, 38, 39), the continued activation of α subunits is decreased. Accordingly, overexpression of phosducin in cultured cells can inhibit the ability of released βγ to activate PLC-β or type II adenyl cyclase (50).

Interestingly, phosphorylation of Ser⁷₃ in phosducin via the cyclic AMP-dependent protein kinase decreases its affinity for the βγ dimer (49, 51), and treatment of cells overexpressing phosducin with dibutyryl cyclic AMP can relieve its inhibitory effects (50). In this context, the finding that phosphorylation of the γ₁₂ subunit with protein kinase C can inhibit the activity of the βγ dimer toward certain effectors offers new paradigms for understanding the regulation of this important signal.

The observation that phosphorylation of the γ₁₂ subunit on Ser⁷ inhibits the ability of the dimer to stimulate type II adenyl cyclase suggests that the N-terminal region of the γ subunit is important for interaction with this effector. In support of this concept, pilot experiments demonstrated that a peptide mimicking the N-terminal 21 amino acids of the γ₂...
protein can inhibit the ability of βγ12 to stimulate cyclase. Thus, the negatively charged phosphate group at the N terminus of the protein may inhibit the interaction of the dimer with the cyclase molecule. Two other functional domains of the γ subunit have been intensively studied using both biochemical assays and site directed mutagenesis. The C-terminal 15 amino acids and the prenyl group are important for interaction with the plasma membrane, the α subunit, and the receptor (4, 5, 26, 52, 53), and the central region of the molecule is important for specific interaction with the β subunits (54, 55). The importance of these interaction sites is clearly supported by the x-ray structures of the αβγ heterotrimer, which indicate a stretch of 15 amino acids beginning at Arg26 of γ1 that interact with the β subunit and likely contacts between the C-terminal domain of the γ subunit and the membrane-α subunit interface (56, 57). Overall, these findings are interesting because they indicate that three different domains of this small protein are used for interactions with other proteins. Comparison of the N-terminal sequences of the 11 γ subunits through the first helical region (Val526 in γ1 (57)) shows that amino acid identity varies from 15 to 85%. Thus, the diversity of this domain may be important for specificity in effector signaling.

The domains in the PLC-β molecule that interact with the βγ dimer have been examined using a number methods. Multiple lines of evidence indicate that the βγ dimer binds near the Y domain in the catalytic core of PLC-β (58). Refinement of this location using overexpression of glutathione S-transferase fusion proteins containing small regions of the molecule suggests that the amino acids between Leu580 and Val641 are involved in binding the βγ dimer (59). Experiments using small peptides indicate a potential βγ binding domain in the 10 amino acids between Glu572 and Lys583 (60). The domains in the β subunit that interact with effectors appear to be similar to those responsible for binding the α subunit (7). However, the domains in the γ subunits responsible for interaction with PLC-β have not been clearly identified. The finding that the ability of the βγ subunit to stimulate PLC-β is not affected by phosphorylation suggests that the central or C-terminal regions in the protein are more likely to interact with PLC-β than the N-terminal domain. Alternatively, the negative charge introduced by phosphorylation of the protein may not inhibit binding of the dimer to PLC-β.

It is especially interesting that phosphorylation of the γ12 subunit increases the affinity of the receptor-αβγ interaction, since the structure of the heterotrimer shows no interaction between the N terminus of the γ subunit and the α subunit (56, 57). In addition, the N-terminal region of the γ subunit is predicted to be some distance from the receptor and the membrane (57). A similar situation occurs with phosducin, where phosphorylation changes its affinity for the βγ dimer (49, 51), yet the phosphorylation site is not directly in contact with either the β or γ subunit (61). One possible explanation of this result is that phosphorylation may cause an indirect effect on receptor-heterotrimer interactions by an induced conformational change in the βγ dimer. However, a definitive answer should emerge from direct structural analysis of the receptor-αβγ interaction.

The finding that phosphorylation of β1γ12 markedly decreases its ability to stimulate type II adenyl cyclase and focuses the βγ signal toward other effectors is likely to be a broadly important regulatory mechanism. The γ12 subunit is widely expressed and has been demonstrated to be phosphorylated by PKC in intact cells following receptor activation (12, 13). The type II adenyl cyclase is expressed at high levels in the brain and the type IV adenyl cyclase, with nearly identical regulatory properties, is widely expressed in peripheral tissues such as lung, heart, kidney, and liver (62). Thus, the phosphorylation of the γ12 subunit has the potential to affect the interplay of the Ca2+ and cyclic AMP signaling networks in most cells. As one example, in vascular smooth muscle cells where phosphorylation of the γ12 subunit occurs following application of the contractile agonists vasopressin or angiotensin II (12), this mechanism may augment the ability of Ca2+ to cause contraction by blunting a rise in cyclic AMP, which relaxes smooth muscle (63). A similar mechanism could be used in neural networks to amplify the effects of a Ca2+ signal and blunt those of cyclic AMP.

The fact that only the γ12 subunit has a protein kinase C phosphorylation site in its N terminus is intriguing. Whether other γ subunits can be phosphorylated is an important issue. However, the γ12 subunit is highly expressed in all regions of the brain (14) and in most peripheral tissues (12). Thus, γ12 may be the γ subunit in the βγ dimers used in many important signaling systems. Our findings that the β1γ12 dimer is equal in activity to the better studied β2γ12 dimer support this conclusion. However, the γ12 subunit may also be used by cells for undiscovered or specialized signaling roles where phosphorylation is critical to its activity. In this regard, in Swiss 3T3 and C6 cells, dimers containing γ12 appear to be localized to actin stress fibers, whereas those containing the γ5 subunit are found in focal adhesions. Moreover, the β1γ12 dimers appear to bind much more tightly to purified actin filaments than do dimers containing the γ5 subunit (15). These findings suggest that the cytoskeleton may be an important site for γ12 function and may lead to discovery of new roles for the βγ dimer in cell signaling.

The observation that phosphorylation can change the effect of the βγ dimer on certain effectors may have consequences for many signaling systems not studied in this report. The βγ dimer is emerging as an important regulatory signal for a large number of effectors including: K− and Ca2+ channels (64), the β adrenergic receptor kinase (65), phosphatidylinositol 3-kinase (66–68), mitogen-activated protein kinase (16), and novel kinases such as p21-activated protein kinase (69). It will be important to determine whether phosphorylation alters the activity of the β1γ12 dimer toward any of these signaling molecules.

Acknowledgments—We thank Dr. Joel M. Linden for the 125I-L-aminobenzyladenosine, the pDT vector, and help with statistical analysis; Dr. Ravi Iyengar for the baculovirus encoding type II adenylyl cyclase; and Dr. T. K. Harden for the turkey phospholipase C-β. We also acknowledge Rimma Khazan for technical assistance, the University of Virginia Biomolecular Research Facility for DNA sequencing, and the Diabetes Core Facility for [32P]ATP and the cAMP assays.

REFERENCES

1. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
2. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. F. (1994) Annu. Rev. Biochem. 63, 101–132
3. Neer, E. J. (1995) Cell 80, 249–257
4. Sprang, S. R. (1997) Annu. Rev. Biochem. 66, 639–678
5. Bourne, H. R. (1997) Curr. Opin. Cell Biol. 9, 134–142
6. Clapham, D. E., and Neer, E. J. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 167–203
7. Hamm, R. E. (1998) J. Biol. Chem. 273, 669–672
8. Higashi, J., Ferguson, K. M., Sternweis, P. C., Smigel, M. D., and Gilman, A. G. (1987) J. Biol. Chem. 262, 762–766
9. Dohman, H. G., and Thorner, J. (1997) J. Biol. Chem. 272, 3871–3874
10. Berman, D. M., and Gilman, A. G. (1998) J. Biol. Chem. 273, 1269–1272
11. Berstein, G., Blank, J. L., Hoon, D., Exton, J. H., Rhee, S. G., and Ross, E. M. (1992) Cell 70, 411–418
12. Morishita, R., Nakayama, H., Itoe, T., Matsuda, T., Hashimoto, Y., Okano, T., Fukada, Y., Mizuno, K., Ohno, S., Kizawa, O., Kato, K., and Asano, T. (1995) J. Biol. Chem. 270, 29469–29475
13. Asano, T., Morishita, R., Ueda, H., Asano, M., and Kato, K. (1998) Eur. J. Biochem. 251, 314–319
14. Morishita, R., Saga, S., Kawamura, N., Hashizume, Y., Inagaki, T., Kato, K., and Asano, T. (1997) J. Neurochem. 68, 820–826
