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Purification from Sf9 Cells and Characterization of Recombinant $G_{q\alpha}$ and $G_{11\alpha}$

ACTIVATION OF PURIFIED PHOSPHOLIPASE C ISOZYMES BY G_α SUBUNITS*

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Members of the $G_{q\alpha}$ subfamily of heterotrimeric guanine nucleotide-binding proteins (G proteins) activate phospholipase C (PLC). The complementary DNAs (cDNAs) for the G protein α subunits $G_{q\alpha}$ and $G_{11\alpha}$ were expressed in insect (Sf9) cells using recombinant baculovirus. Active, nonaggregated, and membrane-associated protein was generated only when the α subunit cDNA was expressed together with cDNAs encoding G protein β and γ subunits. Recombinant α subunits ($rG_{q\alpha}$ and $rG_{11\alpha}$) were purified by three-step procedures, as was a PLC-activating α subunit(s) endogenous to Sf9 cells. Guanosine 5′-3-(thio)triphosphate (GTPγS) activated $rG_{q\alpha}$ and $rG_{11\alpha}$ with an apparent $K_{0.5}$ of 30 μM; similarly high concentrations of the nucleotide were required to observe [^{35}S]GTPγS binding to $rG_{q\alpha}$. Activated $rG_{q\alpha}$ and $rG_{11\alpha}$ each stimulated all three isoforms of purified PLC-β with the rank order of potency PLC-β1 = PLC-β3 ≥ PLC-β2; both α subunits also stimulated PLC-β1 and PLC-β3 to a much greater extent (10-fold) than they did PLC-β2. In contrast, activated $rG_{q\alpha}$ and $rG_{11\alpha}$ failed to stimulate either PLC-δ1 or PLC-γ1. Recombinant $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, $G_{o\alpha(A)}$, $G_{s\alpha}$, and $G_{z\alpha}$ all failed to stimulate any of the isoforms of PLC. The apparent affinities of $rG_{q\alpha}$ and $rG_{11\alpha}$ for PLC-β1 and their capacities to activate the enzyme were similar to values observed for purified brain $G_{q\alpha/11\alpha}$. Purified brain βγ subunits also stimulated the three isoforms of PLC-β. The capacities of $rG_{q\alpha}$ and $rG_{11\alpha}$ to activate PLC-β1 and PLC-β3 greatly exceeded those of βγ, whereas $G_{q\alpha}$, $G_{11\alpha}$ and βγ were roughly equiefficacious with PLC-β2; the α subunits were more potent than βγ in all cases. The effects of α and βγ together were nonadditive for both PLC-β1 and PLC-β2. These results demonstrate that $G_{q\alpha}$ and $G_{11\alpha}$ specifically and selectively stimulate β isoforms of PLC and confirm the idea that these members of the $G_{q\alpha}$ subfamily of G proteins are physiological regulators of this signaling pathway.

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins)[1] serve an essential role in cell physiology by transducing signals from a broad class of cell surface receptors to specific effector proteins at the plasma membrane (1–5). G protein subunits are designated α (39–46 kDa), β (37 kDa), and γ (8 kDa), and to date, at least 21 unique α, 4 β, and 6 γ subunits have been identified (2, 5). Some of these G proteins are known to regulate specific effectors in response to activation by defined receptors. For example, G_s and G_{olf} activate isozymes of adenylylcyclase (1, 6); G_{t1} and G_{t2} activate cGMP-specific phosphodiesterases in retinal rods and cones (7); and G_s, G_{i1}, G_{i2}, G_{i3}, and G_o can modulate the activity of certain ion channels (8).

A wide variety of neurotransmitters, hormones, and growth factors activates PLC to hydrolyze the membrane lipid phosphatidylinositol 4,5-bisphosphate and thereby generate two second messengers, Ins(1,4,5)P_3 and diacylglycerol (9, 10). Compelling evidence indicates a role for one or more G protein(s) in this signaling pathway (11, 12). In a limited number of cells, receptor-mediated activation of PLC is inhibited by prior treatment with pertussis toxin, suggesting a role for a protein of the G_i or G_o type. However, in most cells regulation of PLC by G proteins is insensitive to bacterial toxins. Eight G protein α subunits that are not substrates for ADP-ribosylation by bacterial toxins have been identified recently, either by molecular cloning (13–17) or biochemically (18–21). These include members of the G_q subfamily (G_q, G_{11}, G_{14}, G_{15}, G_{16}), the G_{12} subfamily (G_{12} and G_{13}), and G_z (2, 5).

Recent reports also indicate that at least some members of the G_q subfamily can stimulate PLC activity (21–25). A mixture of $G_{q\alpha}$ and $G_{11\alpha}$ has been purified from bovine brain (19) and rat liver (20); a closely related protein has also been isolated from turkey erythrocytes (21). Reconstitution of these activated proteins with purified mammalian PLC-β1 or turkey erythrocyte PLC markedly stimulates enzymatic activity (21–23). Nevertheless, the close structural similarities between $G_{q\alpha}$ and $G_{11\alpha}$ (88% amino acid identity) make their biochemical resolution difficult (19, 20, 26). Strathmann and Simon (13) isolated the full-length cDNAs for both $G_{q\alpha}$ and $G_{11\alpha}$; these have been expressed in mammalian cells and shown to encode proteins that activate PLC-β1 (27). To obtain pure, resolved $G_{q\alpha}$ and $G_{11\alpha}$ for functional studies and biochemical characterization, we have synthesized these pro-

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[1] The abbreviations used are: G protein(s), heterotrimeric guanine nucleotide-binding regulatory protein(s); G_α, the α subunit of a G protein; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; PLC, phospholipase C; PLC-β, -γ, and -δ, the β, γ, and δ isoforms of PLC; $InsP_3$, inositol trisphosphate; PIP_2, phosphatidylinositol bisphosphate.

teins in Sf9 insect cells using a baculovirus expression system. We now describe the purification and characterization of these proteins and their capacity to activate different purified isoforms of PLC.

MATERIALS AND METHODS

Construction of $G_{q\alpha}$ and $G_{11\alpha}$ Transfer Vectors—All methods used to construct the plasmids for expression of G protein α subunits have been described by Sambrook *et al.* (28). An Sf9 insect cell/baculovirus expression system (29) was used; the pVL1393 or pVL1392 transfer vector containing a polyhedron promoter and an ampicillin resistance gene was chosen for expression purposes. Full-length cDNAs encoding $rG_{q\alpha}$ and $rG_{11\alpha}$ were generated as described (13). To construct the expression vector for $rG_{q\alpha}$, pVL1393 was cleaved with *Bam*HI and *Sma*I; the $G_{q\alpha}$ cDNA was cleaved with *Bam*HI and *Ssp*I to yield a 1.2-kilobase fragment containing the entire coding sequence. The $G_{q\alpha}$ fragment was purified by gel electrophoresis, ligated with digested pVL1393, and transformed into competent *Escherichia coli* as described (28). Plasmid DNA from positive colonies was checked for the presence of the $G_{q\alpha}$ coding sequence by restriction mapping, and pVL1393/$G_{q\alpha}$ plasmid DNA from positive clones was amplified and purified by centrifugation through a CsCl gradient (28). To construct the expression vector for $G_{11\alpha}$, pVL1392 (identical to pVL1393, except the polycloning site is in an inverted orientation) was cleaved with *Eco*RI and *Sma*I. The $G_{11\alpha}$ cDNA was then cleaved with *Xho*I and filled in with the Klenow fragment of DNA polymerase to yield a linearized fragment with a blunt end. This DNA was cleaved with *Eco*RI to yield a 1.2-kilobase fragment that was purified by gel electrophoresis. The recovered $G_{11\alpha}$ and pVL1392 fragments were ligated and transformed as described above.

Preparation of Virus and Expression of Recombinant Proteins in Sf9 Cells—Virus encoding $rG_{q\alpha}$ or $rG_{11\alpha}$ was generated as described previously (30). Purified pVL1393/$G_{q\alpha}$ or pVL1392/$G_{11\alpha}$ was mixed with linearized AcRP23-LacZ virus and transfected (using lipofectin; Bethesda Research Laboratories) into a monolayer culture of Sf9 cells grown in IPL-41 medium. Following initial infection (overnight), the medium was replaced, and recombinant virus was amplified for 2–3 days. Recombinant virus was then plaque-purified as described (29). Purified virus was amplified and medium was saved as high titer stock. The remaining monolayer of cells was collected and screened by Western blotting with appropriate antisera (see below) for the presence of expressed recombinant G_α subunits.

Sf9 Cell Culture—Stock cultures of Sf9 cells (50 ml) were grown in suspension in IPL-41 medium (GIBCO) containing 1% Pluronic F68, 10% fetal calf serum (heat-inactivated), fungizone, and gentamicin. Large scale cultures (8–12 × 1 liter) were grown in IPL-41 medium containing 1% fetal calf serum, 1% lipid mix (GIBCO), gentamicin, and fungizone. Cells were maintained in room air at 27 °C with constant shaking (125 rpm). Generally, cells were seeded at a density of 0.5×10^6 cells/ml and allowed to multiply for 3 days to $4–6 \times 10^6$ cells/ml before subsequent passage.

Measurement of Phospholipase C Activity—In general, measurement of phospholipase C activity was as described previously (22); the final assay volume was 60 μl. Substrate was provided as mixed phospholipid vesicles containing PIP_2 (Sigma) and phosphatidylethanolamine (Sigma) in a ratio of 1:10 with 5,000–10,000 cpm of [^{3}H]PIP_2 (Du Pont-New England Nuclear) per assay. The final concentration of PIP_2 was usually 50 μM (3,000 pmol) or 75 μM (4,500 pmol) unless otherwise stated. Assays were performed in a final buffer containing 50 mM sodium Hepes (pH 7.2), 3 mM EGTA, 0.2 mM EDTA, 0.83 mM $MgCl_2$, 20 mM NaCl, 30 mM KCl, 1 mM dithiothreitol, 0.1 mg/ml ultrapure albumin (bovine), 0.16% sodium cholate, and 1.5 mM $CaCl_2$ (to yield approximately 150 nM free Ca^{2+}).

To perform the assay, four solutions containing the reaction components were prepared separately: 1) G_α subunit mix (10 μl/assay); 2) phosphatidylethanolamine:PIP_2 lipid mix (20 μl/assay); 3) PLC mix (20 μl/assay); and 4) Ca^{2+} mix (10 μl/assay). G_α subunits were first activated in incubation buffer 1 (50 mM sodium Hepes (pH 7.2), 1 mM EDTA, 3 mM EGTA, 5 mM $MgCl_2$, 2 mM dithiothreitol, 100 mM NaCl, and 1% sodium cholate) with 1 mM GTPγS for 1 h at 30 °C (unless otherwise stated) and then stored on ice. This solution contained G_α subunits at a concentration six times higher than that desired in the final assay. Appropriate amounts of lipids (stored in chloroform at −20 °C) were dried under nitrogen at room temperature prior to sonication in incubation buffer 2 (50 mM sodium Hepes (pH 7.2), 3 mM EGTA, 1 mM dithiothreitol, 80 mM KCl). PLC was also prepared in incubation buffer 2 containing bovine serum albumin (1 mg/ml) and, when appropriate, GTPγS (0–3 mM). Ca^{2+} mix was prepared as a 9 mM solution of $CaCl_2$ in incubation buffer 2. Before assay, 10 μl of G_α mix was added to each tube on ice, followed by 10 μl of Ca^{2+} mix. The lipid and PLC solutions were then added together (20 μl of each), and tubes were transferred to a 30 °C water bath for the indicated times. Assays were terminated by the addition of 200 μl of 10% trichloroacetic acid. Tubes were then immediately transferred to an ice bath, followed by the addition of 100 μl of bovine serum albumin (10 mg/ml). Centrifugation at $2,000 \times g$ for 10 min separated unhydrolyzed [^{3}H]PIP_2 (pellet) from [^{3}H]$InsP_3$ (supernatant). Radioactivity in the supernatant was measured by liquid scintillation counting. With 50 μM PIP_2 as substrate, the assay was linear for 10 min at 30 °C; assays were usually run for 3–5 min. In most cases PLC activity is expressed as pmol of $InsP_3$/min/ng PLC. To quantify purification of G_α subunits (see Tables I and II), PLC activity is expressed as pmol of $InsP_3$/min/mg of protein sample containing $rG_{q\alpha}$ or $rG_{11\alpha}$; 2.9 ng of partially purified bovine brain PLC-β1 was used in these assays. Purified recombinant PLC-β1 (see below) was used for other experiments.

Purification of $rG_{q\alpha}$, $rG_{11\alpha}$, and Endogenous $G_{q\alpha}$-like Activity from Sf9 Cells—Starting material for purification was derived from 12-liter cultures of Sf9 cells infected with 2–5 plaque-forming units/cell of either $rG_{q\alpha}$ or $rG_{11\alpha}$ viruses; cells were also infected with approximately equal numbers of virions encoding G protein β2 and γ2 subunits (31). Endogenous $G_{q\alpha}$-like activity (Sf9 $G_{q\alpha}$) was purified from cells infected with only β2 and γ2 viruses. Cells were harvested by centrifugation ($750 \times g$), and pellets were suspended in 400 ml of ice-cold lysis buffer consisting of buffer A containing 25 mM NaCl, 10 mM NaF, 30 μM $AlCl_3$, and a mixture of protease inhibitors (0.02 mg/ml phenylmethylsulfonyl fluoride, 0.03 mg/ml leupeptin, 0.02 mg/ml 1-chloro-3-tosylamido-7-amino-2-heptanone, 0.02 mg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone, and 0.03 mg/ml lima bean trypsin inhibitor). (Buffer A is 50 mM sodium Hepes, pH 7.2, 1 mM EDTA, 3 mM EGTA, 5 mM $MgCl_2$, 3 mM dithiothreitol, and 0.1 mM GDP.) The cell suspension was subjected to nitrogen cavitation (Parr bomb) at 500 p.s.i. for 45 min at 4 °C. Cell lysates were then centrifuged at $500 \times g$ for 10 min to remove intact cells and nuclei. Supernatants were centrifuged at $100,000 \times g$ for 30 min, and the membrane pellets derived therefrom were suspended in 300 ml of lysis buffer with a Dounce homogenizer (20 strokes). The membranes were finally frozen in liquid nitrogen and stored at −80 °C. For purification of recombinant G proteins, membranes were thawed and diluted to 5 mg of protein/ml with buffer A containing fresh protease inhibitors and then extracted by the addition of sodium cholate to a final concentration of 1% with constant stirring for 60–90 min at 4 °C. The extracted membranes were centrifuged at $100,000 \times g$ for 30 min, and 300 ml of supernatant (cholate extract) was collected.

Phenyl-Sepharose Hydrophobic Chromatography—A column of phenyl-Sepharose CL-4B (200 ml; Pharmacia LKB Biotechnology Inc.) was washed with equilibration buffer (buffer A containing 10 mM NaF, 30 μM $AlCl_3$, protease inhibitors, 400 mM NaCl, and 0.25% sodium cholate). The cholate extract (300 ml) was diluted with 900 ml of buffer A containing 575 mM NaCl, 10 mM NaF, 30 μM $AlCl_3$, and protease inhibitors and loaded onto the column. The resin was washed with 300 ml of equilibration buffer (without AlF_4^-), and protein was eluted from the column in 25-ml fractions using a 1,500-ml linear gradient of equilibration buffer (without AlF_4^-) containing descending concentrations of NaCl (400–0 mM) and ascending concentrations of sodium cholate (0.25–1.5%). Column fractions were assayed for their capacity to activate partially purified PLC-β1 from bovine brain (32, 33) and for their specific immunoreactivity (antiserum W082 for $rG_{q\alpha}$ (34), B825 for $rG_{11\alpha}$, and Z811 for Sf9 $G_{q\alpha}$; see below). Immunoreactive fractions (generally fractions 22–28) were pooled, concentrated to 10 ml by ultrafiltration, and diluted with 90 ml of Mono Q equilibration buffer (buffer A containing 0.1 mM GDP, protease inhibitors, and 1% octyl glucoside). This solution was concentrated to 20 ml and was loaded directly onto a Mono Q column.

Mono Q Anion Exchange Chromatography—A 10-ml Mono Q anion exchange column for FPLC (Pharmacia) was equilibrated with 5 volumes of Mono Q equilibration buffer (buffer A containing 1% octyl glucoside). The sample was loaded under pressure at a flow rate of 0.5 ml/min. The column was washed with an additional 10 ml of Mono Q equilibration buffer, and bound protein was eluted in 50 3-ml fractions using a linear gradient of NaCl (25–300 mM for $rG_{11\alpha}$; 25–400 mM for $rG_{q\alpha}$). The concentration of NaCl was then increased to 1,000 mM over an additional 10 fractions (fraction 60) and held constant to fraction 65. Fractions were assayed for specific immuno-

reactivity, as well as their capacity to activate PLC-β1, and peak fractions were pooled (typically 25–30 ml) and loaded onto a $\beta\gamma$-agarose column.

$\beta\gamma$-Agarose Affinity Chromatography—$\beta\gamma$-Agarose affinity resin (5 ml; Ref. 19) was equilibrated with 20 ml of buffer B (buffer A containing 100 mM NaCl, 10 mM NaF, 30 μM $AlCl_3$, and 0.2% Lubrol). To facilitate dissociation of residual $\beta\gamma$ from G_α subunits, 10 mM NaF and 30 μM $AlCl_3$ (final concentrations) were added to the sample, which was incubated at 22 °C for 30 min. The $\beta\gamma$-agarose was added to the sample and mixed for an additional 30 min at 22 °C. EDTA (20 mM final) was then added directly to the sample to chelate Mg^{2+} and promote binding of α to the affinity resin. After mixing overnight at 4 °C, the slurry was transferred to a 10-ml disposable column (Bio-Rad); the flow-through was collected and passed again over the packed resin. The column was washed at 4 °C with 15 column volumes (collected as 5-ml fractions) of buffer A containing 0.2% Lubrol, followed by 15 column volumes of buffer C (buffer A containing 0.1% Lubrol and 400 mM NaCl) and 3 column volumes of buffer D (buffer A containing 0.2% sodium cholate and 100 mM NaCl). All nonspecifically bound protein appeared to wash off the resin under these conditions. The column was then warmed to 22 °C, and specifically bound protein was eluted (collected at 4 °C) by the addition of 5 column volumes of buffer E (buffer A containing 100 mM NaCl and 1% sodium cholate). To ensure that all α subunits were eluted from the column, the resin was washed further with 5 column volumes of buffer F (buffer A containing 100 mM NaCl, 1% sodium cholate, 10 mM NaF, 30 μM $AlCl_3$) followed by 5 column volumes of buffer G (buffer A containing 100 mM NaCl, 1% sodium cholate, 10 mM NaF, 30 μM $AlCl_3$, and 50 mM $MgCl_2$). Fractions were assayed for both specific immunoreactivity and their capacity to activate PLC-β1; they were also stained with silver nitrate after SDS-polyacrylamide gel electrophoresis. Fractions containing Lubrol could not be assayed directly because of the detergent's marked capacity to inhibit PLC. Peak fractions were frozen in liquid nitrogen and stored at −80 °C.

Measurement of [^{35}S]GTPγS Binding to $rG_{q\alpha}$—Purified $rG_{q\alpha}$ was stored in buffer E containing 100 μM GDP. To remove free nucleotide, samples of $rG_{q\alpha}$ were thawed and gel filtered by centrifugation. Disposable columns (10 ml; Bio-Rad) containing 2 ml of G-50 resin were equilibrated with buffer E without GDP and spun at 500 × *g* for 4 min at 4 °C. Samples containing $rG_{q\alpha}$ were applied to the columns and spun again exactly as described. Recovered $rG_{q\alpha}$ was then incubated in buffer C containing 200 μM GTPγS (12,000 pmol/60 μl) and tracer amounts of [^{35}S]GTPγS (1000–1500 cpm/pmol). Binding proceeded for various times at 30 °C. At the times indicated, 60-μl samples were gel filtered by centrifugation; the flow-through containing GTPγS-bound $rG_{q\alpha}$ was collected, and the amount of bound [^{35}S]GTPγS was quantified by liquid scintillation counting. Recoveries of $rG_{q\alpha}$ following gel filtration were monitored by the capacity of $rG_{q\alpha}$ (activated with GTPγS) to stimulate PLC-β1 before and after gel filtration. Recoveries of $rG_{q\alpha}$ after two consecutive gel filtrations approximated 12%. Accounting for the loss of $rG_{q\alpha}$ during gel filtration, the fractional occupancy (*i.e.* the molar ratio of bound GTPγS to $rG_{q\alpha}$) was estimated to be 0.6. The fractional binding of GTPγS to $rG_{q\alpha}$ was also evaluated by a second method. $rG_{q\alpha}$ was reconstituted with purified recombinant type 1 muscarinic cholinergic receptor and brain $\beta\gamma$ in lipid vesicles as described previously (35); carbachol was added to stimulate binding of [^{35}S]GTPγS to a determined stoichiometry. The capacity of this activated $rG_{q\alpha}$ to stimulate PLC-β1 was compared with that of known amounts of $rG_{q\alpha}$ (determined by protein assays), which had been activated with unlabeled GTPγS. Based on this comparison, the fractional binding of GTPγS to purified $rG_{q\alpha}$ appeared to approach 1. Efforts to quantitate GTPγS binding to $rG_{q\alpha}$ with filter binding assays were unsuccessful because of unidentified technical constraints.

Antisera—Rabbit anti-$G_{q\alpha}$ serum W082 was raised to a synthetic 19-amino acid peptide representing an internal sequence (amino acids 115–133) unique to $G_{q\alpha}$ (34). Rabbit anti-$G_{11\alpha}$ serum B825 was raised to a synthetic 20-amino acid peptide representing an internal sequence (amino acids 114–133) unique to $G_{11\alpha}$. Rabbit anti-$G_{q\alpha/11\alpha}$ serum Z811 was raised to a synthetic 15-amino acid peptide representing the carboxyl terminus shared by $G_{q\alpha}$ and $G_{11\alpha}$. Methods for generation of these antisera have been described (34).

Miscellaneous Procedures—Reconstitution of cyc$^-$ S49 cell membranes with G_α subunits was performed as described previously (36). SDS-polyacrylamide gel electrophoresis of proteins was performed as described by Laemmli (37); protein concentrations were determined by staining with Amido Black (38). Staining of protein with silver nitrate following SDS-polyacrylamide gel electrophoresis was performed as described by Wray *et al.* (39); immunodetection of proteins after Western blotting was performed as described by Mumby *et al.* (40) using the ECL chemiluminescence detection system (Amersham Corp.). Other proteins were purified as described: bovine brain $G_{q\alpha/11\alpha}$ (19, 22); recombinant PLC-β1 and recombinant PLC-β2 from HeLa cells (25); and PLC-β3 (32, 41), PLC-γ1 (33), and PLC-δ1 from brain (42). $rG_{i\alpha1}$, $rG_{i\alpha2}$, $rG_{i\alpha3}$, $rG_{o\alpha(A)}$, $rG_{s\alpha(s)}$, and $rG_{z\alpha}$ (purified from *E. coli*) were kindly provided by Drs. M. E. Linder (University of Texas Southwestern Medical Center) and Patrick Casey (Duke University), and purified bovine brain $\beta\gamma$ was kindly provided by Ethan Lee (University of Texas Southwestern Medical Center).

RESULTS

Expression of $G_{q\alpha}$ and $G_{11\alpha}$—Initial efforts to synthesize $G_{q\alpha}$ in bacterial expression systems failed. Although immunoreactive recombinant protein was detectable in cell lysates, the concentration was modest, and all soluble recombinant protein was aggregated and inactive. We subsequently expressed $rG_{q\alpha}$ and $rG_{11\alpha}$ in Sf9 cells using recombinant baculovirus. Although the concentration of expressed protein was again low (estimated to be 0.05% and 0.01% for $G_{q\alpha}$ and $rG_{11\alpha}$, respectively, of detergent-extractable membrane protein), some portion of the expressed material had the capacity to stimulate brain PLC-β1. Based on electrophoresis and immunostaining, $rG_{11\alpha}$ was expressed as a single protein with an apparent molecular mass of 42 kDa. In contrast, $rG_{q\alpha}$ was visualized as a pair of proteins with apparent molecular masses of 42 and 43 kDa. The latter observation is presumably the result of unexpectedly efficient reading of the altered polyhedron initiator codon contained upstream of the inserted $G_{q\alpha}$ sequence in the original pVL1393 expression vector. If a particular sequence is in-frame with this altered start site, current expression of a slightly longer polyhedron fusion protein along with the normal protein is reported to be a common problem (43).

Initial studies were designed to determine what fraction of expressed recombinant protein was active and soluble (Fig. 1). When $G_{q\alpha}$ was expressed alone, the majority of the immunoreactive material was cytosolic; however, gel filtration revealed that most of the protein was inactive and aggregated (Fig. 1). Although much less immunoreactivity was associated with the membrane fraction, activity (capacity to activate PLC-β1) was roughly equal in the cytosol and membrane extracts. Attempts to purify the soluble $rG_{q\alpha}$ were unsuccessful because of further aggregation and loss of activity. To circumvent this problem, $rG_{q\alpha}$ was coexpressed with other G protein subunits: β2 (rβ2) and γ2 (rγ2) (31). Under these conditions, the majority of the active $rG_{q\alpha}$ was associated with membranes and was not aggregated after extraction with sodium cholate (Fig. 1); most of the $G_{q\alpha}$ that remained in the cytosol was aggregated and inactive (Fig. 1). To generate material for purification, $rG_{q\alpha}$ and $rG_{11\alpha}$ were coexpressed with rβ2 and rγ2 to produce a heterotrimer. Maximal levels of expressed protein were observed 48–60 h after infection with the three viruses.

Purification of $rG_{q\alpha}$, $rG_{11\alpha}$, and Endogenous Sf9 $G_{q\alpha}$—Cholate extracts of membranes from triply infected Sf9 cells (12-liter cultures) were used as the starting material for purification. However, uninfected, lacZ-infected, and lacZ plus rβ2/rγ2-infected Sf9 cells possess significant amounts of PLC-activating material because of the presence of endogenous $G_{q\alpha}$-like proteins (Sf9 $G_{q\alpha}$). This activity accounts for perhaps 10–20% of the initial total value in preparations of $G_{q\alpha}$ and up to 40% of the initial total with $G_{11\alpha}$. Sf9 $G_{q\alpha}$ was readily recognized by antiserum Z811. $rG_{q\alpha}$, $rG_{11\alpha}$, and Sf9 $G_{q\alpha}$ were each purified by monitoring specific immunoreactivity and the capacity of GTPγS-activated protein to stimulate purified brain PLC-β1 in reconstitution assays. A representative pu-

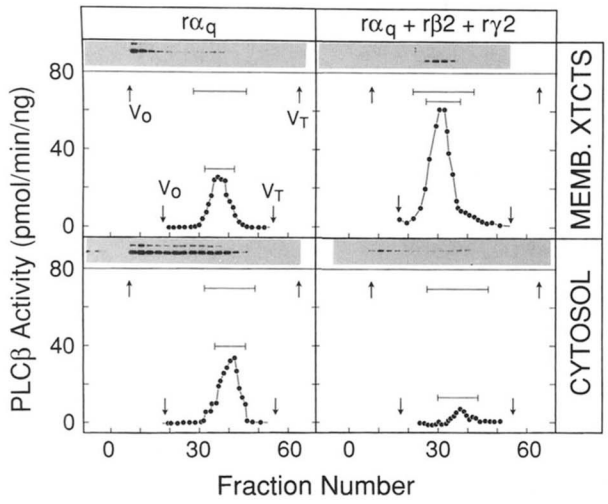

FIG. 1. **Effect of expression of G protein $\beta\gamma$ subunits on the activity and distribution of $rG_{q\alpha}$ in Sf9 cells.** Sf9 cells were infected either with virus encoding $rG_{q\alpha}$ alone (*left*, *top* and *bottom panels*) or with different viruses encoding $G_{q\alpha}$, β_2, and γ_2 subunits (*right*, *top* and *bottom panels*). Cytosol and cholate extracts of membranes were isolated as described under "Materials and Methods." Membrane extracts (*top*, *left* and *right panels*) and cytosol (*bottom*, *left* and *right panels*) were chromatographed on an AcA-34 gel filtration column in the presence of 10 mM NaF, 10 mM $MgCl_2$, and 30 μM $AlCl_3$, and individual fractions were assayed for their capacity to stimulate PLC-β1 (2.9 ng). Column fractions were also tested for immunoreactivity with anti-$G_{q\alpha}$ antiserum (W082) (see associated autoradiograms). V_0 marks the void volume of the column, and V_T marks the total volume. The *lines* marking the peak fractions of PLC activity correspond to the *underlined* immunoreactive fractions. PLC-β activity in this and all subsequent figures is expressed as pmol of $InsP_3$/min/ng of PLC-β.

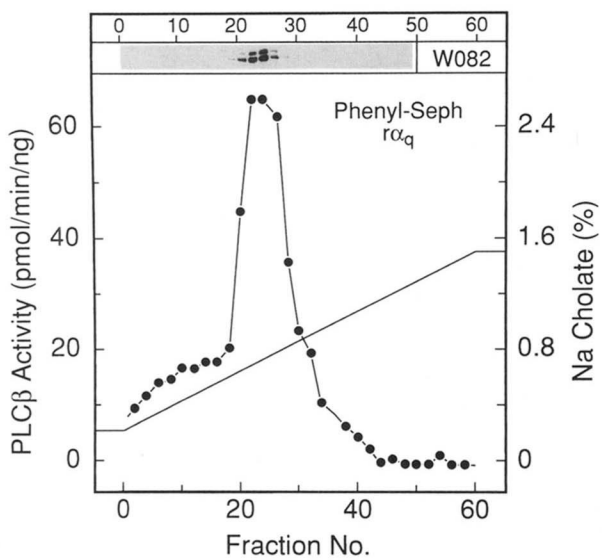

FIG. 2. **Phenyl-Sepharose hydrophobic chromatography of $rG_{q\alpha}$.** Cholate extracts from a 12-liter preparation of Sf9 cells infected simultaneously with viruses encoding $G_{q\alpha}$, β_2, and γ_2 subunits were chromatographed on a phenyl-Sepharose column. Individual column fractions were assayed for their capacity to stimulate PLC-β1 (2.9 ng) and for their immunoreactivity with anti-$G_{q\alpha}$ antiserum (W082).

rification of $rG_{q\alpha}$ is shown in Figs. 2, 3 (*top*), and 4. $rG_{q\alpha}$, $rG_{11\alpha}$, and Sf9 $G_{q\alpha}$ were each purified similarly with a three-step procedure involving hydrophobic chromatography (phenyl-Sepharose; Fig. 2), anion exchange chromatography (Mono Q; Fig. 3), and affinity chromatography ($\beta\gamma$-agarose; Fig. 4). The elution of $G_{11\alpha}$ from a Mono Q anion exchange column is shown in Fig. 3 (*bottom*), which also shows the resolution of $rG_{11\alpha}$ from Sf9 $G_{q\alpha}$. In contrast to the situation with $rG_{11\alpha}$, $rG_{q\alpha}$ was expressed at sufficiently high levels to obscure the activity associated with Sf9 $G_{q\alpha}$ on the Mono Q column (Fig. 3, *top*). Because of this, only the active fractions of $rG_{q\alpha}$ with the earliest elution times were pooled to ensure complete resolution of $rG_{q\alpha}$ from Sf9 $G_{q\alpha}$. Although most of the endogenous and recombinant $\beta\gamma$ eluted before the α subunits on the Mono Q column, residual $\beta\gamma$ was resolved from free $rG_{q\alpha}$ during subsequent affinity chromatography using $\beta\gamma$-agarose (Fig. 4). All final pools of $rG_{q\alpha}$, $rG_{11\alpha}$, and Sf9 $G_{q\alpha}$ could be activated by either GTPγS or AlF_4^- plus Mg^{2+} (Fig. 4 and data not shown). The purification schemes for $rG_{q\alpha}$ and $rG_{11\alpha}$ are summarized in Tables I and II, respectively. Because of interference from endogenous Sf9 $G_{q\alpha}$ during the early steps of purification and the negative influence of high concentrations of $\beta\gamma$ on the capacity of $rG_{q\alpha}$ and $rG_{11\alpha}$ to activate PLC-β1, accurate assessment of fold purification is not possible. A 12-liter culture of infected cells yielded approximately 15 μg of pure $rG_{11\alpha}$ in the peak fraction, whereas a similar preparation of $rG_{q\alpha}$ yielded 125 μg of protein.

SDS-polyacrylamide gel electrophoresis of purified brain $G_{q\alpha/11\alpha}$ (19, 22), $rG_{q\alpha}$, $rG_{11\alpha}$, and Sf9 $G_{q\alpha}$ is shown in Fig. 5. Following treatment with *N*-ethylmaleimide, all proteins migrate with an apparent molecular mass of approximately 40–42 kDa. As discussed above, $rG_{q\alpha}$ was expressed as two proteins with molecular masses of approximately 42 kDa (75%

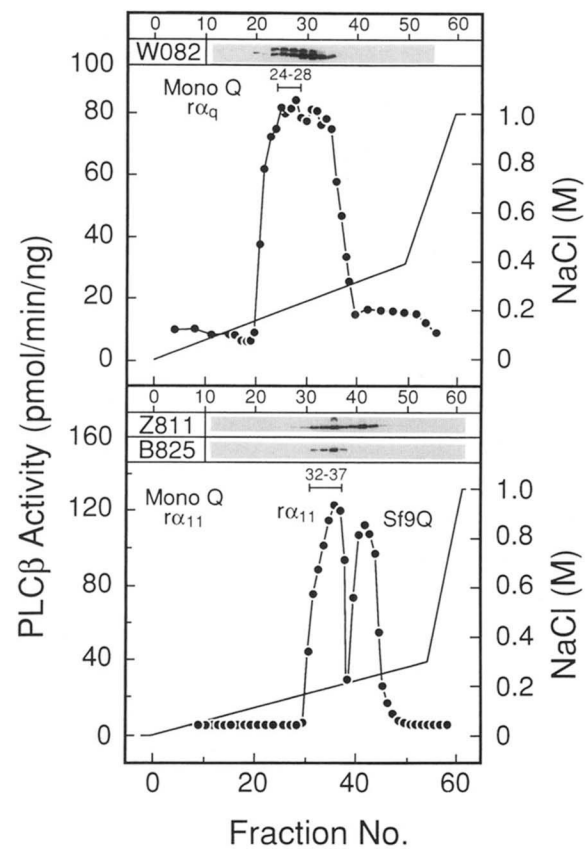

FIG. 3. **Mono Q anion exchange chromatography of $rG_{q\alpha}$ and $rG_{11\alpha}$.** Fractions containing rG_α protein from the phenyl-Sepharose column were chromatographed on a Mono Q FPLC column as described under "Materials and Methods." *Top panel*, $rG_{q\alpha}$; *bottom panel*, $rG_{11\alpha}$. Individual fractions were assayed for their capacity to stimulate PLC-β1 and for their immunoreactivity with selective antisera. W082 is an anti-$G_{q\alpha}$ antiserum, B825 is an anti-$G_{11\alpha}$ antiserum, and Z811 is an anti-$G_{q\alpha}$ and $G_{11\alpha}$ antiserum that recognizes the carboxyl terminus of both α chains.

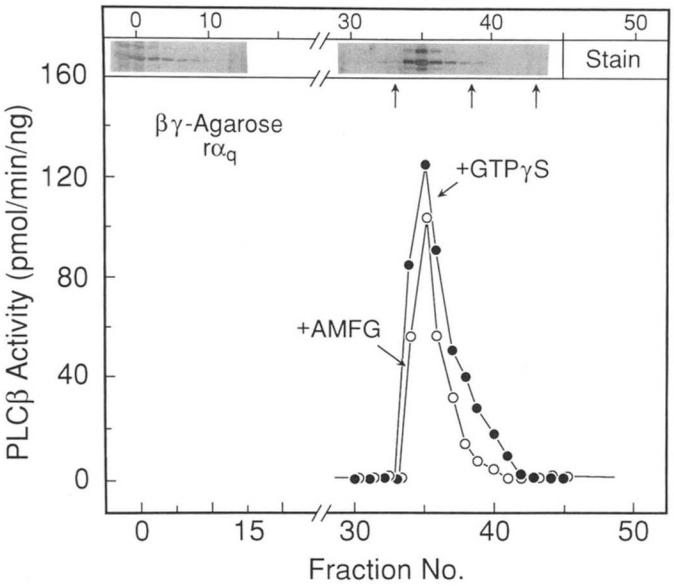

FIG. 4. **βγ-Agarose affinity chromatography of $rG_{q\alpha}$.** Fractions containing $rG_{q\alpha}$ from the Mono Q column were pooled and chromatographed on βγ-agarose as described under "Materials and Methods." Individual column fractions were assayed for their capacity to stimulate PLC-β1 (2.9 ng), and proteins were visualized by staining with silver nitrate (see associated gel). Samples were activated with either 1 mM GTPγS or $AlCl_3$ (30 μM), NaF (10 mM), $MgCl_2$ (5 mM), and GDP (0.1 mM).

TABLE I
Purification of $rG_{q\alpha}$

Step	Volume	Protein	Stimulated PLC activity
	ml	*mg*	*nmol $InsP_3$/min/mg protein*
Cholate extract	1,200	1,630	9.8[a]
Phenyl-Sepharose	175	119	24[a]
Mono Q	18	11.2	104
βγ-Agarose	5	0.125	14,600

[a] PLC activity includes that stimulated by endogenous (Sf9 cell) PLC-activating G proteins.

TABLE II
Purification of $rG_{11\alpha}$

Step	Volume	Protein	Stimulated PLC activity
	ml	*mg*	*nmol $InsP_3$/min/mg protein*
Cholate extract	1,200	1,520	13[a]
Phenyl-Sepharose	150	228	73[a]
Mono Q	18	5.1	1,070
βγ-Agarose	3	0.015	49,300

[a] PLC activity includes that stimulated by endogenous (Sf9 cell) PLC-activating G proteins.

of the total) and 43 kDa (25%). Endogenous Sf9 $G_{q\alpha}$ also appears to contain two proteins. All proteins were judged to be more than 90% pure based on silver staining. $rG_{q\alpha}$ contained a minor 40-kDa contaminant that is likely an endogenous $G_{i/o\alpha}$-like protein, based on its immunoreactivity with a nonspecific G_α antiserum (data not shown). $rG_{11\alpha}$ contained a minor, unidentified 42.5-kDa contaminant. Antiserum W082, which was generated with a synthetic peptide corresponding to an internal sequence specific for $G_{q\alpha}$ (34), recognizes brain $G_{q\alpha/11\alpha}$, $rG_{q\alpha}$, and Sf9 $G_{q\alpha}$ but fails to recognize $rG_{11\alpha}$. Antiserum B825 was raised against a synthetic peptide representing an internal sequence specific for $G_{11\alpha}$ (overlapping the

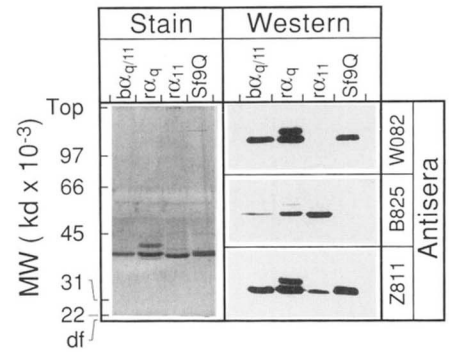

FIG. 5. **SDS-polyacrylamide gel electrophoresis and immunoblot analysis of purified bovine brain $G_{q\alpha/11\alpha}$ ($bG_{q\alpha/11}$), $rG_{q\alpha}$, $rG_{11\alpha}$, and endogenous Sf9 cell PLC-activating proteins (*Sf9Q*).** Approximately 75 ng of each preparation was treated with *N*-ethylmaleimide and then resolved on 9.5% polyacrylamide gels. The *left panel* is a silver stain of the proteins, and the *right panels* are immunoblots using the indicated antisera. *Sf9Q* refers to the endogenous PLC-activating $G_{q\alpha}$-like protein(s); $b\alpha_{q/11}$ refers to brain-derived native $G_{q/11\alpha}$.

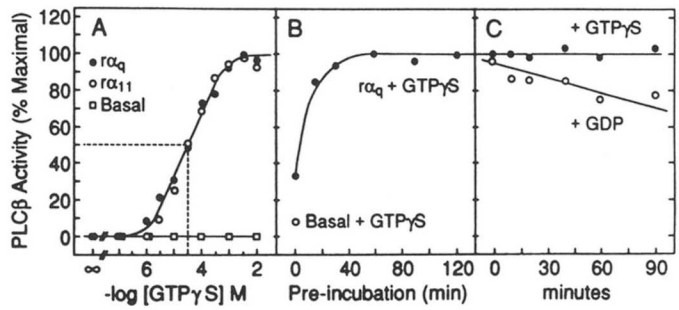

FIG. 6. **Activation of $rG_{q\alpha}$ and $rG_{11\alpha}$ by GTPγS.** *Panel A*, concentration dependence for activation of $rG_{q\alpha}$ and $rG_{11\alpha}$ by GTPγS. Assay buffer (□) or buffer containing either 10 nM $rG_{q\alpha}$ (●) or 10 nM $rG_{11\alpha}$ (○) was incubated with the indicated concentrations of GTPγS for 1 h at 30 °C; samples were then tested for their capacity to stimulate rPLC-β1 (1 ng). *Panel B*, time course for activation of $rG_{q\alpha}$ by GTPγS. Assay buffer (○) or buffer containing 10 nM $rG_{q\alpha}$ (●) was incubated with 1 mM GTPγS for the indicated times at 30 °C; samples were then tested for their capacity to stimulate rPLC-β1 (1 μg). *Panel C*, deactivation of $rG_{q\alpha}$. $rG_{q\alpha}$ (10 nM) was activated with 0.05 mM GTPγS for 1 h at 30 °C. At time = 0, samples were incubated at 30 °C with either 0.05 mM GTPγS (●) or 0.05 mM GTPγS plus 5 mM GDP (○). Aliquots were tested for their capacity to stimulate rPLC-β1 (1 ng) at the indicated times.

homologous region of $G_{q\alpha}$); this antiserum recognizes both $rG_{q\alpha}$ and $rG_{11\alpha}$ but fails to recognize Sf9 $G_{q\alpha}$. Antiserum Z811, raised against a synthetic peptide corresponding to the 15 carboxyl-terminal amino acid residues that are shared by $G_{q\alpha}$ and $G_{11\alpha}$, recognizes all α subunits in the four preparations.

Characterization of Purified $rG_{q\alpha}$ and $rG_{11\alpha}$—Both $rG_{q\alpha}$ and $rG_{11\alpha}$ are activated by GTPγS, although high concentrations of the nucleotide are required ($K_{0.5}$ = 30 μM) (Fig. 6*A*); maximal activation is achieved with 1 mM GTPγS. Despite the high concentration of nucleotide, complete activation of $rG_{q\alpha}$ by GTPγS occurred only after 60 min of incubation; activated protein is stable for at least an additional 60 min at 30 °C (Fig. 6*B*). Once achieved, activation of $G_{q\alpha}$ by GTPγS is reversed only slowly (by the addition of excess GDP) (Fig. 6*C*).

In keeping with the high concentration of GTPγS required to activate $rG_{q\alpha}$ and $rG_{11\alpha}$, it was technically difficult to measure the binding of [^{35}S]GTPγS to $rG_{q\alpha}$. For reasons that are unclear, assays based on the adsorption of the protein-nucleotide complex to filters were not successful. However,

the complex of [^{35}S]GTPγS with $rG_{q\alpha}$ could be isolated by gel filtration. Binding was detected in the presence of concentrations of GTPγS which are capable of activating $G_{q\alpha}$; data obtained using 200 μM [^{35}S]GTPγS are shown in Fig. 7. The rate of binding of this concentration of GTPγS to $rG_{q\alpha}$ appeared to be slightly slower than that for activation of the protein by 1 mM GTPγS, but they were clearly comparable. Because of limiting amounts of protein and the very large quantities of isotope required to perform these experiments, detailed characterization of the binding reaction was not undertaken. Nevertheless, using two independent measures (see "Materials and Methods"), the molar ratio of bound nucleotide to $rG_{q\alpha}$ (*i.e.* fractional occupancy) was estimated to range from 0.6 to 1.0. Measurement of GTPγS binding to $rG_{11\alpha}$ was not attempted because of the small amount of protein available.

$rG_{q\alpha}$ and $rG_{11\alpha}$ were purified, in part, based on their capacity to stimulate partially purified PLC-β1 from bovine brain. Studies were carried out to determine the relative capacity of $rG_{11\alpha}$, $rG_{q\alpha}$, bovine brain $G_{q\alpha/11\alpha}$, and other rG_{α} subunits to activate purified forms of PLC-β, including recombinant PLC-β1, recombinant PLC-β2, and native PLC-β3. Pertussis toxin blocks hormonal activation of phospholipase C in certain tissues, suggesting the involvement of a G_i- or G_o-like protein in those pathways (11, 12). As such, purified pertussis toxin-sensitive, GTPγS-activated α subunits ($rG_{i\alpha1}$, $rG_{i\alpha2}$, $rG_{i\alpha3}$, and $rG_{o\alpha A}$; purified from *E. coli*) were tested for their capacity to stimulate rPLC-β1, rPLC-β2, and native PLC-β3; other *E. coli*-derived G_{α} subunits that are unaffected by pertussis toxin and the brain βγ subunit complex were also tested (Table III). $rG_{q\alpha}$, $rG_{11\alpha}$, and brain $G_{q\alpha/11\alpha}$ each clearly activates all three isoforms of PLC-β; in contrast, the other α subunits fail to elicit any response (Table III). Purified bovine brain βγ also stimulates the activity of all three types of PLC-β (Table III; see also Refs. 41, 44–49). The capacity of $rG_{q\alpha}$ and $rG_{11\alpha}$ to stimulate PLC is apparently specific for the PLC-β isoforms; that is, no activated α subunit stimulates purified bovine brain PLC-γ1 or PLC-δ1 (Table IV). To address issues of specificity more completely, the relative capacity of $rG_{s\alpha(s)}$, $rG_{q\alpha}$, and $rG_{11\alpha}$ to activate adenylylcyclase in cyc$^-$ S49 cell membranes was also compared. $rG_{q\alpha}$ and $rG_{11\alpha}$ both fail to activate adenylylcyclase under conditions in which $rG_{s\alpha(s)}$ is clearly effective (data not shown). $rG_{q\alpha}$ and $rG_{11\alpha}$ also fail to inhibit $rG_{s\alpha(s)}$-mediated stimulation of adenylylcyclase. Similar results were obtained with recombinant types I and II adenylylcyclases.

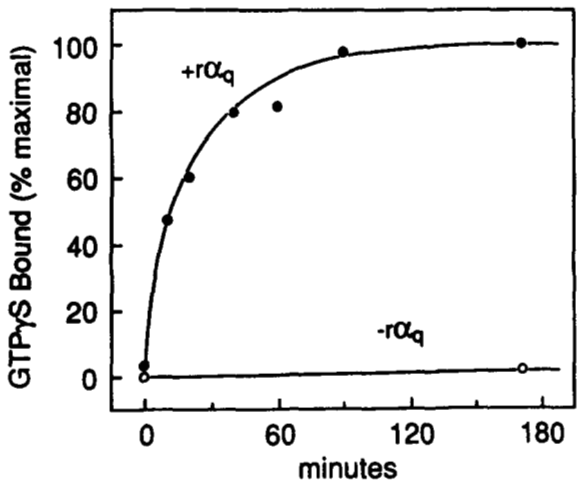

FIG. 7. **Binding of [^{35}S]GTPγS to $rG_{q\alpha}$.** $rG_{q\alpha}$ (4.5 pmol/60 μl) was incubated at 30 °C for the indicated times with 200 μM [^{35}S]GTPγS (1,500 cpm/pmol). Samples containing $rG_{q\alpha}$ (●) or buffer (○) were rapidly filtered by centrifugation through a 1.4-ml G-50 column. Fractional occupancy (the molar ratio of GTPγS bound to $rG_{q\alpha}$) was estimated to range from 0.6 to 1.0 (see "Materials and Methods").

TABLE III
Effect of G protein subunits on phospholipase C-β isoforms

Addition	Concentration	rPLC-β1 activity	rPLC-β2 activity	Brain PLC-β3 activity
		pmol $InsP_3$/min/ng PLC		
None		17	3.5	2.6
$G_{q\alpha/11\alpha}$	10 nM	246	22	95
$rG_{q\alpha}$	10 nM	305	40	246
$rG_{11\alpha}$	10 nM	332	39	259
$rG_{i\alpha1}$	100 nM	20	3.4	6.9
$rG_{i\alpha2}$	100 nM	18	2.9	3.6
$rG_{i\alpha3}$	100 nM	21	2.9	4.0
$rG_{o\alpha(A)}$	100 nM	21	3.6	5.7
$rG_{s\alpha\text{-}s}$	100 nM	21	4.0	5.7
$rG_{z\alpha}$	100 nM	25	6.2	4.5
βγ	3 μM	65	45	35

TABLE IV
Lack of stimulation of PLC-γ1 and PLC-δ1 by G protein subunits

Addition	Concentration	Brain PLC-δ1 activity	Brain PLC-γ1 activity
		pmol $InsP_3$/min/ng PLC	
None		14.1	6.5
$rG_{q\alpha/11\alpha}$	30 nM	11.0	8.0
$rG_{q\alpha}$	30 nM	8.8	8.1
$rG_{11\alpha}$	30 nM	9.1	7.6
$rG_{i\alpha1}$	100 nM	6.9	8.2
$rG_{i\alpha2}$	100 nM	8.3	7.0
$rG_{i\alpha3}$	100 nM	7.1	7.5
$rG_{o\alpha(A)}$	100 nM	8.8	7.2
$rG_{s\alpha(s)}$	100 nM	7.0	7.3
$rG_{z\alpha}$	100 nM	10.0	9.1
βγ	3 μM	16.6	9.4
Ca^{2+} (free)	0	2.0	0.5
	50 nM	3.7	3.5
	150 nM	14.1	6.5
	10 μM	369	12.7
	100 μM	372	14.0
	1 mM	365	11.4

The relative capacities of $rG_{11\alpha}$, $rG_{q\alpha}$, and Sf9 $G_{q\alpha}$ to activate purified isozymes of PLC-β are shown in Fig. 8. Both $rG_{11\alpha}$ and $rG_{q\alpha}$ activate each of the three forms of PLC-β; the two α subunits display essentially identical properties. In contrast, Sf9 $G_{q\alpha}$ stimulates only weakly. $rG_{q\alpha}$ and $rG_{11\alpha}$ stimulate PLC-β1 and PLC-β3 to a much greater extent than they do PLC-β2 (Fig. 8, *A* and *B*; see also Ref. 50). Under the assay conditions employed, $rG_{q\alpha}$ and $rG_{11\alpha}$ display similar or slightly lower half-maximal concentrations for activation of rPLC-β1 and PLC-β3 (~2 nM) than they do for rPLC-β2 (~5 nM) (Fig. 8).

Both $rG_{q\alpha}$ and $rG_{11\alpha}$ are at least as potent as purified bovine brain $G_{q\alpha/11\alpha}$ as activators of rPLC-β1 (Fig. 9). It should be noted, however, that the potencies of these proteins are sensitive to the choice of detergent used in the assay; that is, in the presence of 0.16% (final) octyl glucoside, the $K_{0.5}$ for activation of PLC-β1 by G_{α} and βγ subunits is 3–10-fold lower than that observed in the presence of 0.16% cholate (data not shown; see Ref. 41). Nevertheless, the rank order for G_{α} subunit-mediated activation of rPLC-β isozymes, as well as the extent of activation, is preserved in the presence of octyl glucoside (data not shown).

Studies were also carried out to test whether βγ subunits

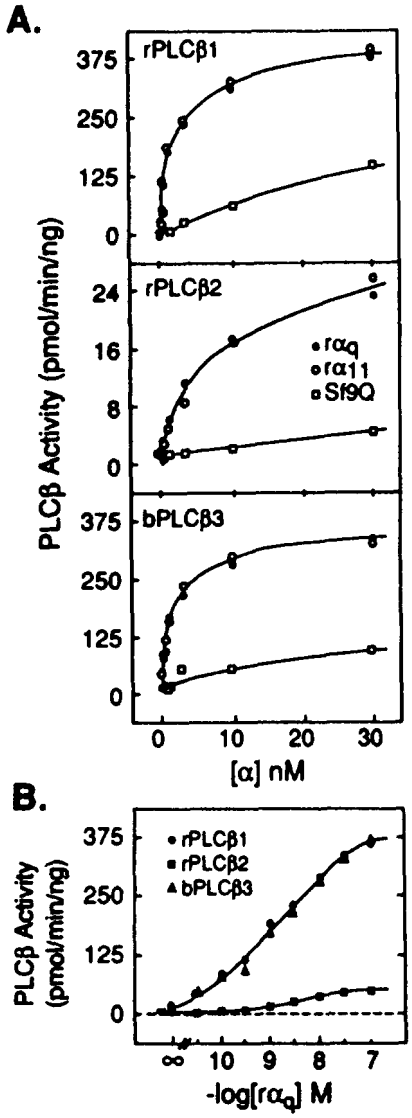

FIG. 8. **Activation of PLC-β isoforms by $rG_{q\alpha}$, $rG_{11\alpha}$, and Sf9 $G_{q\alpha}$.** *Panel A*, $rG_{q\alpha}$ (●), $rG_{11\alpha}$ (○), and Sf9 $G_{q\alpha}$ (*Sf9Q*; □) were each activated for 1 h with 1 mM GTPγS at 30 °C prior to reconstitution with rPLC-β1 (1 ng) (*top panel*), rPLC-β2 (8 ng) (*middle panel*), or rat brain PLC-β3 (0.32 ng) (*bottom panel*). *Panel B*, comparison of the activation of PLC-β1 (●), PLC-β2 (■), and PLC-β3 (△) by $rG_{q\alpha}$. G protein was activated with 1 mM GTPγS for 1 h at 30 °C prior to reconstitution with rPLC-β1 (1 ng). Data shown (*panels A* and *B*) are duplicate determinations from a single experiment and are representative of at least two such experiments. The data in *panel B* and those from additional experiments were subjected to a nonlinear least squares fit to the four-parameter logistic equation $Y = [A/1 + (C/X)^B)] + D$, and the derived mean $EC_{50} \pm$ S.E. values for activation of PLCβ isoforms by $rG_{q\alpha}$ were as follows: 2.0 ± 0.4 nM (n = 6) for rPLC-β1; 4.7 ± 0.7 nM (n = 3) for rPLC-β2; and 2.5 ± 0.9 nM (n = 3) for brain PLC-β3.

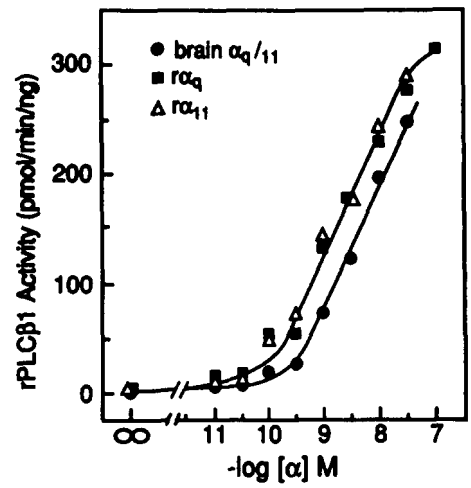

FIG. 9. **Activation of rPLC-β1 by $rG_{q\alpha}$, $rG_{11\alpha}$, and bovine brain $G_{q\alpha/11\alpha}$.** $rG_{q\alpha}$ (■), $rG_{11\alpha}$ (△), and brain $G_{q\alpha/11\alpha}$ (●) were activated with 1 mM GTPγS for 1 h at 30 °C prior to reconstitution with rPLC-β1 (1 ng).

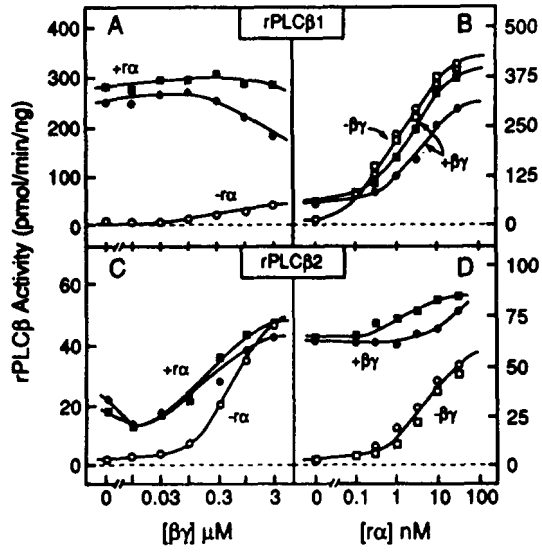

FIG. 10. **Effects of $rG_{q\alpha}$, $rG_{11\alpha}$, and G protein $\beta\gamma$ subunits on rPLC-β1 and rPLC-β2 activities.** *Panel A*, the capacity of the indicated concentrations of bovine brain $\beta\gamma$ to stimulate rPLC-β1 was determined in the absence of α subunit (○) or in the presence of either 10 nM $rG_{q\alpha}$ (■) or 10 nM $rG_{11\alpha}$ (●). *Panel B*, the capacity of the indicated concentrations of $rG_{q\alpha}$ (*squares*) or $rG_{11\alpha}$ (*circles*) to stimulate rPLC-β1 activity was determined in the absence of $\beta\gamma$ (*open symbols*) or in the presence of 2 μM bovine brain $\beta\gamma$ (*closed symbols*). *Panels C* and *D*, the same experiments were performed with rPLC-β2, rather than rPLC-β1. $rG_{q\alpha}$ and $rG_{11\alpha}$ were activated with 1 mM GTPγS for 1 h at 30 °C prior to the assays, and GTPγS was present in all assays at a final concentration of 1 mM.

influence activation of rPLC-β1 or rPLC-β2 by $rG_{q\alpha}$ or $rG_{11\alpha}$ (Fig. 10). Purified brain $\beta\gamma$ subunits stimulate all three isoforms of PLC-β (Table III). In the case of rPLC-β1, activated $rG_{q\alpha}$ and $rG_{11\alpha}$ each stimulates the enzyme to a much greater extent and at lower concentrations than does brain $\beta\gamma$ (Fig. 10, *A* and *B*); levels of stimulation in the presence of both G_α and $\beta\gamma$ are not additive (Fig. 10, *A* and *B*). In the case of rPLC-β2, $\beta\gamma$ subunits stimulate the enzyme to levels similar to those observed with either $rG_{q\alpha}$ or $rG_{11\alpha}$, but higher concentrations of $\beta\gamma$ than α are required (Fig. 10, *C* and *D*). The combined effects of G_α and $\beta\gamma$ are again not additive.

DISCUSSION

Following expression of $rG_{q\alpha}$ and $rG_{11\alpha}$ in Sf9 cells, modest quantities of active protein can be purified using a three-step procedure; however, unusual measures are required. When expressed under conditions that have previously favored synthesis of active recombinant G_α subunits in bacteria or insect cells (18, 51–53), $rG_{q\alpha}$ and $rG_{11\alpha}$ aggregate with loss of activity. Only when these subunits are expressed together with G protein β and γ subunits are reasonable quantities of active, nonaggregated protein generated. Such restrictions complicate the purification of free α subunits and dictate the use of

uncommon reagents (*i.e.* $\beta\gamma$-agarose and viruses encoding β and γ). Nevertheless, if these reagents are available, modest quantities of pure, active α subunit can be obtained.

The observation that purified $rG_{q\alpha}$ and $rG_{11\alpha}$ stimulate purified recombinant isozymes of phospholipase C both confirms and extends previous reports using purified components from native sources (21–23, 26). Since these studies involved the use of a mixture of native $G_{11\alpha}$ and $G_{q\alpha}$ and/or native PLC, the availability of resolved, pure forms of $rG_{q\alpha}$, $rG_{11\alpha}$, and PLC isozymes has allowed us to define specific interactions between these proteins unambiguously. The major findings from these studies can be summarized as follows. 1) $rG_{q\alpha}$ and $rG_{11\alpha}$ each regulates the activity of the three β isoforms of PLC but not PLC-γ1 or PLC-δ1. 2) $rG_{q\alpha}$ and $rG_{11\alpha}$ share indistinguishable properties for activation of these enzymes. 3) Of the several G proteins tested, only members of the $G_{q\alpha}$ subfamily of α subunits are capable of activating PLC; $\beta\gamma$ can also stimulate PLC-β activity. 4) The responses of PLC-β2 to $rG_{q\alpha}$, $rG_{11\alpha}$, and $\beta\gamma$ differ from those of PLC-β1 and PLC-β3. 5) In the absence of receptor, $rG_{q\alpha}$ and $rG_{11\alpha}$ have an unusually poor apparent affinity for GTPγS. 6) Recombinant $G_{q\alpha}$ and $G_{11\alpha}$ from Sf9 cells and native $G_{q\alpha/11\alpha}$ from bovine brain have similar affinities for PLC-β isoforms and capacities to activate the enzymes.

$rG_{q\alpha}$ and $rG_{11\alpha}$ Regulate the Activity of the Three β Isoforms of PLC but Not PLC-γ1 or PLC-δ1—Despite the relatively low sequence homology among the noncatalytic domains of PLC-β1, PLC-β2, and PLC-β3 (12, 54), $rG_{q\alpha}$ and $rG_{11\alpha}$ stimulate the activity of each of the enzymes. Previous reports (24, 25) indicated that $G_{q\alpha}$ activated PLC-β1 but not PLC-β2. The present results indicate that $rG_{q\alpha}$ and $rG_{11\alpha}$ can indeed stimulate rPLC-β2, albeit to a significantly lesser extent than either PLC-β1 or PLC-β3 (see also Ref. 50). In addition, activation of rPLC-β2 requires higher concentrations of $rG_{q\alpha}$ and $rG_{11\alpha}$ than is the case for rPLC-β1 and PLC-β3. It is clear that the concentrations of $G_{q\alpha}$ used previously (25) were insufficient to activate PLC-β2. $rG_{q\alpha}$, $rG_{11\alpha}$, and $\beta\gamma$ do not stimulate PLC-γ1 and PLC-δ1, confirming the specificity for regulation of the PLC isoforms by G protein subunits and defining differential pathways for regulation of the PLC subfamilies. The γ subfamily of PLC is regulated by certain tyrosine kinase receptors (12, 55), although a role for G proteins in this pathway cannot be completely ruled out (56). To date, there is no evidence to support the idea of receptor-directed regulation of the δ subfamily of PLC. However, PLC-δ1 seems particularly responsive to Ca^{2+} (at least under the present assay conditions; see Table IV), suggesting that this enzyme may be regulated primarily by changes in cytosolic concentrations of Ca^{2+}.

$rG_{q\alpha}$ and $rG_{11\alpha}$ Share Indistinguishable Properties for Activation of PLC-β—$rG_{q\alpha}$ and $rG_{11\alpha}$ activate the three isoforms of PLC-β in an indistinguishable manner, suggesting that the two G proteins can serve interchangeably as physiological regulators of these enzymes. These observations are reminiscent of the apparent lack of specificity for activation of cardiac K^+ channels by $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ (8, 57). The physiological significance of this is not clear. Specificity could exist at the level of receptor-G protein coupling. However, type 1 muscarinic receptors stimulate guanine nucleotide exchange with both $G_{q\alpha}$ and $G_{11\alpha}$ in reconstitution studies (35). It will be important to determine whether various PLC-linked receptors demonstrate equal or dissimilar affinities for $G_{q\alpha}$ and $G_{11\alpha}$, as well as other members of the $G_{q\alpha}$ family. Alternatively, specificity could be dictated by differential tissue distribution of G_α subunits and/or the PLC isoforms. Both $rG_{q\alpha}$ and $rG_{11\alpha}$ are expressed widely in a variety of tissues (13, 34), but little information is available as to whether these two α subunits are expressed simultaneously in the same cells. In the case of the enzymes, PLC-β3 and PLC-β1 seem to be expressed broadly, whereas PLC-β2 has a much more limited distribution (41, 50).

Of the G Proteins Tested, Only Members of the $G_{q\alpha}$ Subfamily of G Protein α Subunits Are Capable of Activating PLC—$\beta\gamma$ subunits, but apparently not other α subunits, have the capacity to stimulate PLC-β activity. Other G_α subunits, including $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, $G_{o\alpha(A)}$, $G_{z\alpha}$, and $G_{s\alpha(s)}$ from *E. coli*, fail to activate any of the PLC isozymes.[2] These findings are significant, since pertussis toxin blocks receptor-mediated activation of PLC in a limited number of tissues (11, 12), and none of the currently identified pertussis toxin substrates can activate the available isoforms of PLC. An unidentified isoform of PLC might be regulated by a pertussis toxin-sensitive α subunit, or an undefined toxin-sensitive α subunit could be involved. Alternatively and perhaps most reasonably, this pathway could be regulated primarily by G protein $\beta\gamma$ subunits liberated in the plasma membrane by activation of appropriate receptors (see 11, 41, 44–49). Consistent with this idea is the finding that $\beta\gamma$ subunits stimulate PLC-β2 to a similar or greater extent than either $rG_{q\alpha}$ or $rG_{11\alpha}$, albeit with lower potency (Fig. 10 and Refs. 41, 46). Unlike the situation with adenylylcyclase (58), activation of PLC-β isoforms by $\beta\gamma$ does not require α subunits.

The Responses of PLC-β2 to G Protein Subunits Differ from Those of PLC-β1 and PLC-β3—PLC-β1 and PLC-β3 are stimulated to a much greater extent than is PLC-β2 by $rG_{q\alpha}$ and $rG_{11\alpha}$, and slightly lower (2–3-fold) concentrations of $rG_{q\alpha}$ and $rG_{11\alpha}$ are required to stimulate PLC-β1 and PLC-β3 than PLC-β2. Thus, it is possible that these α subunits preferentially activate PLC-β1 and PLC-β3 but do not readily stimulate PLC-β2. As discussed above, this scenario is consistent with the notion that PLC-β2 is regulated significantly by G protein $\beta\gamma$ subunits (41, 44–49).

In the Absence of an Appropriate Receptor, $rG_{q\alpha}$ and $rG_{11\alpha}$ Have an Unusually Poor Affinity for GTPγS—In contrast to most other G_α subunits, $G_{q\alpha}$ and $G_{11\alpha}$ have an unusually poor apparent affinity for GTPγS in the absence of an appropriate agonist-receptor complex (19, 26). Here we demonstrate that $rG_{q\alpha}$ can bind GTPγS fairly rapidly (compared with other G protein α subunits) but only in the presence of high concentrations of nucleotide. Roughly 30 μM concentrations of GTPγS are required to activate $G_{q\alpha}$ or $G_{11\alpha}$ half-maximally. Similar values for most other G protein α subunits are in the 3–10 nM range. The rate of reversal of GTPγS-mediated activation of $G_{q\alpha}$ by GDP is slow (although faster than that observed with several other α subunits), suggesting that GTPγS does not dissociate rapidly once bound. The simplest explanation for these observations is that the kinetics of association of GTPγS with $rG_{q\alpha}$ is limited by the rate of dissociation of GDP from the protein (as with other G protein α subunits) and that the poor apparent affinity of the nucleotide for the protein is dictated by an extremely slow rate of association. Thus, we envision an occluded nucleotide binding

[2] A technical point should be raised concerning the use of *E. coli*-derived α subunits. Even though 10-fold higher concentrations of these proteins were tested, we cannot rule out the possibility of reduced potency of these proteins because they lack certain post-translational modifications (*e.g.* the addition of fatty acids) that may be functionally significant (31, 60). Nevertheless, these recombinant proteins do activate appropriate effectors (adenylylcyclase, ion channels) when used in the 1–100 nM concentration range. We have also determined that myristoylated forms of recombinant $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{o\alpha(A)}$ derived from *E. coli* (1 μM) fail either to stimulate directly or to inhibit $G_{q\alpha}$-directed activation of the three β isoforms of PLC.

site, even when the protein is nucleotide-free. Others have demonstrated that receptors can influence the association of nucleotides with G protein α subunits (35, 59), in addition to well documented effects of receptor on the rate of nucleotide dissociation. We suggest that such effects are particularly marked with $G_{q\alpha}$ and $G_{11\alpha}$, resulting in a more than 1,000-fold increase in the affinity of the proteins for GTPγS in the presence of an agonist-receptor complex. Although the physiological significance of these observations is as obscure as the mechanism, the phenomenon appears to make activation of these G proteins exquisitely dependent on receptor, ensuring minimal activation of PLC by $G_{q\alpha}$ in the absence of a proper stimulatory ligand.

Recombinant Forms of $G_{q\alpha}$ and $G_{11\alpha}$ Derived from Sf9 Cells and Native $G_{q\alpha/11\alpha}$ Activate PLC-β Isoforms Similarly—r$G_{q\alpha}$ and r$G_{11\alpha}$ obtained from the Sf9 cell expression system have similar if not higher apparent affinities for PLC-β1 than does bovine brain $G_{q\alpha/11\alpha}$, and the three α subunit preparations activate the three isoforms of PLC-β to similar extents. In contrast, studies with $G_{s\alpha}$ synthesized in *E. coli* revealed that the recombinant protein had a 30-fold lower apparent affinity for adenylylcyclase when compared with the native protein (51). G protein α subunits synthesized in bacteria lack one or more post-translational modifications (51); α subunits derived from eukaryotic expression systems are presumably modified properly (53, 60). Thus, G protein α subunits expressed in Sf9 cells are covalently modified with fatty acids; palmitate is incorporated into $G_{q\alpha}$, $G_{s\alpha}$, and members of the $G_{i\alpha}$ subfamily, whereas myristate is also incorporated into the $G_{i\alpha/o\alpha}$ proteins (61). It will be important to determine if these modifications influence the functional interactions of $G_{q\alpha}$ and $G_{11\alpha}$ with the plasma membrane or with other proteins in the signal transduction pathway.

The $G_{q\alpha}$ subfamily consists of at least five members, including $G_{q\alpha}$, $G_{11\alpha}$, $G_{14\alpha}$, $G_{15\alpha}$, and $G_{16\alpha}$. Whereas $G_{q\alpha}$, $G_{11\alpha}$, and $G_{14\alpha}$ are very similar proteins (79% amino acid identity), $G_{15\alpha}$ and $G_{16\alpha}$ are more distantly related (57 and 58% amino acid identity, respectively). $G_{15\alpha}$ and $G_{16\alpha}$ are also distinct in that they are expressed only in hematopoietic cells, whereas $G_{q\alpha}$, $G_{11\alpha}$, and $G_{14\alpha}$ are widely distributed. Despite this, $G_{14\alpha}$, $G_{15\alpha}$, and $G_{16\alpha}$ (synthesized in COS-7 cells) all activate PLC-β1 and PLC-β2 when the phospholipases are either expressed concurrently or added as purified proteins to membranes containing the expressed G_α subunit (24, 62). It will be important to define the extent of functional interactions among these α subunits, PLC isoforms, cell surface receptors, and $\beta\gamma$ subunits by reconstitution of purified components. Further studies will also be required to determine if members of the $G_{q\alpha}$ subfamily activate effectors other than PLC.

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