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Selective Activation of Phospholipase C by Recombinant G-protein α- and βγ-Subunits*

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Receptor activation of phospholipase C (PLC) via G-proteins occurs by pertussis toxin-sensitive and toxin-insensitive signaling pathways. The α-subunits of the G_q family are presumed to mediate the toxin-insensitive pathway, but the nature of the G-proteins mediating the toxin-sensitive pathway is not established. Recently, PLC-β has been shown to be activated by G-protein βγ-subunits of mixed or undefined composition. The relative activities of G-protein subunits that might activate PLC-β were examined using defined recombinant α- and βγ-subunits obtained from the baculovirus expression system by reconstituting the purified subunits with purified bovine brain PLC-β1 or turkey erythrocyte PLC-β in unilamellar phospholipid vesicles. Turkey erythrocyte $G\alpha_{11}$ and recombinant $G\alpha_{11}$ and $G\alpha_q$ obtained after expression in Sf9 cells activated both bovine brain PLC-β1 and turkey erythrocyte PLC-β. In contrast, under the same assay conditions, recombinant $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$ were without effect on either type of PLC. All types of βγ-subunits tested ($r\beta_1\gamma_2$, $r\beta_1\gamma_3$, $r\beta_2\gamma_2$, $r\beta_2\gamma_3$, bovine brain βγ or turkey erythrocyte βγ) inhibited $G\alpha_{11}$-mediated activation of PLC, presumably by promotion of formation of inactive heterotrimeric G-protein. All types of βγ-subunits also markedly stimulated the activity of turkey erythrocyte PLC-β but did not activate bovine brain PLC-β1. Of the four different βγ complexes of defined composition, three stimulated PLC with similar activities whereas $\beta_2\gamma_3$ was less effective. The data suggest that pertussis toxin-sensitive activation of PLC is mediated by the βγ-subunits of G-proteins acting on specific phospholipase C isoenzymes.

Membrane receptors for a large number of neurotransmitters, hormones, and growth factors activate phospholipase C leading to the breakdown of PtdIns(4,5)P_2[1] and generation of the intracellular messengers inositol 1,4,5-trisphosphate and diacylglycerol (1). Much diversity exists among the proteins comprising the transmembrane signaling mechanisms that generate these two messages. The phospholipase C enzymes include three major families comprised of the β, γ, and δ isoforms (2). Most growth factor receptors with intrinsic tyrosine kinase activity phosphorylate phospholipase C-γ on tyrosine residues leading to recruitment of this protein to a membrane signaling complex (3–5). In contrast, the β-isoforms of phospholipase C are regulated by certain members of the large family of heterotrimeric G-proteins through two different mechanisms (5, 6). The most ubiquitous of these is pertussis toxin-insensitive, while a less prominent pathway is inactivated by treatment of cells with pertussis toxin (7).

It is now clear that the toxin-insensitive mechanism is mediated by the α-subunits of the recently discovered G_q family of G-proteins (6). Experiments performed with purified proteins have demonstrated that the α-subunits of G_q and G_{11} can stimulate the phospholipase C-β isoenzymes (8–12). Similar conclusions have been reached in experiments using cultured cells transfected with cDNAs coding for the α-subunits of members of the G_q family (13, 14). Thus, the mechanism by which G_q activates phospholipase C-β appears to follow the well established model for G-protein-linked systems in which hormone-activated receptors promote release of an activated α-subunit, which then directly stimulates the effector protein (15).

The pertussis toxin-sensitive mechanism for activation of phospholipase C is less well understood. The major evidence that a member of the G_i family of G-proteins may be involved is that receptor-promoted activation of phospholipase C in some cells and by certain receptors is inhibited by pretreatment of cells with pertussis toxin (7, 16–19). The fact that only the three G_i and the G_o α-subunits have sites for ADP-ribosylation by pertussis toxin implicates these proteins in coupling receptors to activation of phospholipase C. However, there is no direct evidence that phospholipase C can be activated by α-subunits of G_i or G_o. While it could be argued that an unknown α-subunit or phospholipase C isoenzyme might be responsible for the observed toxin sensitivity, the recent discovery that G-protein βγ-subunits can activate phospholipase C suggests an alternative mechanism (20–22). Thus, heterotrimeric G_i or G_o could be activated by receptor occupation and release its βγ-subunits, and the βγ-subunits could stimulate the effector, phospholipase C. The existence of four different forms of G-protein β-subunits and up to seven forms of γ-subunits makes this a potentially complex system (6), since βγ-subunit dimers formed from different combinations of β and γ might differentially stimulate the phospholipase C isoenzymes.

The present work was carried out to answer two questions regarding the activation of phospholipase C by G-protein α- and βγ-subunits. First, considering the pertussis toxin-sensitive pathway, can purified G_i and G_o α-subunits activate various forms of phospholipase C reconstituted into phospholipid vesicles under conditions where these enzymes are markedly activated by members of the G_q family? Second, can G-protein

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[1] The abbreviations are: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; GTPγS, guanosine 5′-3-*O*-(thiotriphosphate); DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PLC, phospholipase C.

βγ-subunits of defined composition activate purified isoforms of phospholipase C and are there differences between their capacity to activate the phospholipase C isoforms? These questions were approached using purified bovine brain PLC-β1 and turkey erythrocyte PLC-β activated by different purified recombinant G-protein α- and βγ-subunits. The results suggest that the G_i and G_o α-subunits are incapable of activating either form of phospholipase C, that phospholipase C-β1 and the turkey erythrocyte PLC-β differ in their capacity to be activated by βγ-subunits, and that certain combinations of the βγ-subunits activate the turkey erythrocyte phospholipase C-β more effectively than other βγ-complexes.

EXPERIMENTAL PROCEDURES

Purification of Native βγ-Subunits—G-protein βγ-subunits were resolved from purified bovine brain G_o/G_i in the presence of $AlCl_3$, NaF, and $MgCl_2$ as described (23). G-protein βγ-subunits also were purified from turkey erythrocyte plasma membranes under non-activating conditions as we have reported (23). The properties of both preparations of βγ-subunits have been described in detail (21, 23).

Purification of Phospholipase C—Phospholipase C from turkey erythrocytes[2] was purified to homogeneity from the cytosolic fraction of turkey erythrocytes as previously described (24). The properties of this 150-kDa polyphosphoinositide-specific phospholipase C have been described in detail (21, 24, 25). Phospholipase C-β1 was purified from bovine brain by the method of Lee *et al.* (26) as previously described (21). The possibility of the presence of contaminant G-protein α-subunits in the phospholipase C and βγ-subunit preparations was ruled out by: (*a*) silver staining of gels after SDS-polyacrylamide gel electrophoresis; (*b*) lack of [^{35}S]GTPγS binding activity, and (*c*) lack of immunoreactivity against α-subunit common antibodies and $G\alpha_q$ and $G\alpha_{11}$ antipeptide antibodies (data not shown).

Purification of $G\alpha_{11}$—The 42-kDa protein conferring AlF_4^- activation to phospholipase C was purified from cholate extracts prepared from turkey erythrocyte plasma membranes as previously described in detail (12). This protein has been identified as $G\alpha_{11}$, based on complete homology of partial internal amino acid sequence of the purified protein with the sequence predicted from avian $G\alpha_{11}$ cDNA (27). The avian protein sequence is 98% homologous to mammalian $G\alpha_{11}$ sequence.

Expression and Extraction of Recombinant $G\alpha_{11}$ and $G\alpha_q$—Sf9 cells were infected at a multiplicity of infection of 1:1 for $G\alpha_{11}$ and 3:1 for $G\alpha_q$ with the corresponding viral stock as previously described (27, 28). Cells were harvested after 72 h and washed twice with ice-cold phosphate-buffered isotonic NaCl, pH 7.5. Cells were homogenized in 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 10 mM Tris, pH 7.2. After centrifugation at 500 × *g*, the supernatant was collected and centrifuged at 100,000 × *g* for 30 min at 4 °C. The membrane pellet was resuspended with 20 mM Tris, pH 7.4, 1 mM DTT, 10 mM $MgCl_2$, 10 mM NaF, 20 μM $AlCl_3$, 0.1 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride. Ten percent cholate was added to the suspension to obtain 1.2% final concentration. The suspension was stirred for 30 min at 4 °C and centrifuged at 100,000 × *g* for 30 min. Membrane extracts were collected and used without further purification in reconstitution experiments.

Expression and Purification of recombinant $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$—The α-subunits of the proteins G_{i1}, G_{i2}, G_{i3}, and G_o were expressed using the baculovirus expression system as described (28). The recombinant G-protein α-subunits were purified to homogeneity using DEAE, hydroxyapatite, and Mono P chromatography as described (28).

Expression and Purification of Recombinant βγ-Subunits—Sf9 cells were infected with the appropriate recombinant baculoviruses, cultured, and harvested exactly as previously described (29). Frozen, harvested cells were thawed in 15 times their wet weight of ice cold homogenization buffer (consisting of 10 mM Tris, pH 8.0 at 4 °C, 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml benzamidine, and 2 μg/ml each of aprotinin, leupeptin, and pepstatin A) and burst by N_2 cavitation after equilibration at 600 p.s.i. for 20 min. Lysed cells were mixed with an equal volume of homogenization buffer containing twice the desired final concentration of detergent and gently stirred for 1 h at 4 °C. Cholate (1%, w/v, final concentration), octylthioglucoside (Pierce, 1.1 or 0.4%, w/v) and Genapol C-100 (Calbiochem, 0.1%, w/v) proved equally effective for solubilization of the recombinant βγ dimers and behaved similarly in subsequent steps of purification. Genapol C-100 was chosen for the standard protocol. The detergent extract was centrifuged at 100,000 × *g* for 1 h, filtered through a 0.45-μm membrane, and applied at a flow rate of 5 ml/min to a 2 × 10-cm DEAE HR40 (Waters) column equilibrated with TED/CHAPS (50 mM Tris-Cl, 1 mM EDTA, 1 mM DTT, 0.6% CHAPS (w/v), pH 8.0 at 4 °C.). The flow rate was reduced to 3 ml/min, the column was washed with 36 ml (~2 column volumes) of TED/CHAPS, and eluted with a 96-ml linear gradient from 0 to 300 mM NaCl in TED/CHAPS. The bulk of the recombinant βγ dimers consistently eluted between 90 and 180 mM NaCl. These fractions were pooled and used for affinity purification of the recombinant βγ dimers using G-protein α-subunits immobilized on agarose beads.

Synthesis of the $G\alpha_{i2}$ affinity matrix was essentially as developed by Pang and Sternweis (30) and described by Mumby *et al.* (31). Briefly, ω-aminobutylagarose (Sigma, A-6142) was washed with 50 mM sodium phosphate, pH 7.0, and mixed with an equal volume of the same buffer containing 0.25 mM *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (Pierce Chemical Co.). After purging with N_2, the mixture was incubated for 30 min at room temperature and washed twice with 10 ml of phosphate buffer/ml of resin. Purified recombinant $G\alpha_{i2}$ (2.85 mg), prepared as described (28), was reduced in the presence of 1 mM DTT and 5 μM GDP and exchanged into thoroughly degassed 200 mM KPO_4, 200 mM NaCl, 5 μM GDP, 0.05% Genapol C-100, pH 8.0, using an HR10/10 Fast Desalting column (Pharmacia). The protein solution (~2 ml) was mixed with 1.5 ml of the activated resin, and the tube was purged with argon, sealed, and tumbled slowly for 1 h at 4 °C. After the coupling reaction, the resin was washed with 5 volumes of 50 mM $NaPO_4$ (pH 7), 1 mM EDTA, 300 mM NaCl, 2 μM GDP, 0.1% Genapol C-100, followed by 5 volumes of the same buffer containing 50 mM β-mercaptoethanol. The resin was stored in 50 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.1% Genapol C-100, 30% glycerol, 1 mM DTT, and 5 μM GDP at 4 °C (storage buffer).

The affinity resin was washed prior to use with 10 volumes of 20 mM HEPES (pH 8.0), 1 mM EDTA, 200 mM NaCl, 0.6% CHAPS, 3 mM DTT, 5 μM GDP (wash buffer). GDP was added to the pooled DEAE fractions to a final concentration of 5 μM, the DEAE pool was mixed with the affinity resin, and the mixture was gently tumbled for 1 h at 4 °C. Following a gentle centrifugation (50 × *g*, 5 min), the resin was resuspended in 10 ml of wash buffer and poured into a Poly-Prep column (Bio-Rad). The bed was washed twice with 2 ml of wash buffer, and twice with 2 ml of wash buffer with 400 mM NaCl (final concentration). For elution of the recombinant βγ dimers, 1 ml of elution buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 200 mM NaCl, 50 mM $MgCl_2$, 10 mM NaF, 30 μM $AlCl_3$, 3 mM DTT, 5 μM GDP, 0.6% CHAPS) was passed over the resin, flow was stopped, and an additional 2 ml of elution buffer was placed on the column and allowed to stand for 15 min. After collecting the first 2-ml fraction, an additional 6 ml of elution buffer was passed over the resin, the fractions pooled, concentrated to 0.1–0.5 mg/ml with a Centricon 30 (Amicon), aliquoted, and stored at −70 °C until use. The resin was then washed with 20 ml of wash buffer and placed in storage buffer (above).

Reconstitution and PLC Assay—G-protein α- and βγ-subunits were reconstituted into lipid vesicles by removal of detergent as described (21). Briefly, [^{3}H]PtdIns(4,5)P_2, phosphatidylethanolamine, and phosphatidylserine were combined in a 1:4:1 molar ratio, and phospholipids were dried under a stream of nitrogen and resuspended to 100 μM [^{3}H]PtdIns(4,5)P_2 (specific activity 10,000 cpm/nmol) by sonication in a medium containing 20 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 100 mM NaCl, 2 mM DTT, 0.1 mM benzamidine, and 0.8% sodium cholate. G-protein α- and/or βγ-subunits were combined with detergent-resuspended phos-

[2] The identity of the turkey erythrocyte phospholipase C is not clear because cDNA encoding for this protein has not been cloned. However, substantial internal amino acid sequence has been obtained from the purified protein. Ten peptides ranging in length from 6 to 20 amino acids have an overall identity to rat phospholipase C-β1, human phospholipase C-β2, and human phospholipase C-β3 of 51, 70, and 52%, respectively (G. L. Waldo and T. K. Harden, unpublished data). We have cloned cDNA encoding for turkey phospholipase C-β1, and the predicted sequence is >90% homologous to that of rat phospholipase C-β1 (A. J. Morris and T. K. Harden, unpublished data). This suggests to us that, although the avian protein may be more like phospholipase C-β2 than phospholipase C-β1 or phospholipase C-β3, it is probably an additional phospholipase C-β type, since based on the high homology between avian and rat phospholipase C-β1 we would expect a similar level of homology between avian and human homologues of phospholipase C-β2. In support of this idea, the purified avian phospholipase C does not react with an antisera generated against a carboxyl-terminal sequence from phospholipase C-β2. This antisera strongly reacts with recombinant phospholipase C-β2. In addition, the turkey erythrocyte phospholipase C comigrates with phospholipase C-β1 but not phospholipase C-β2 during SDS-polyacrylamide gel electrophoresis.

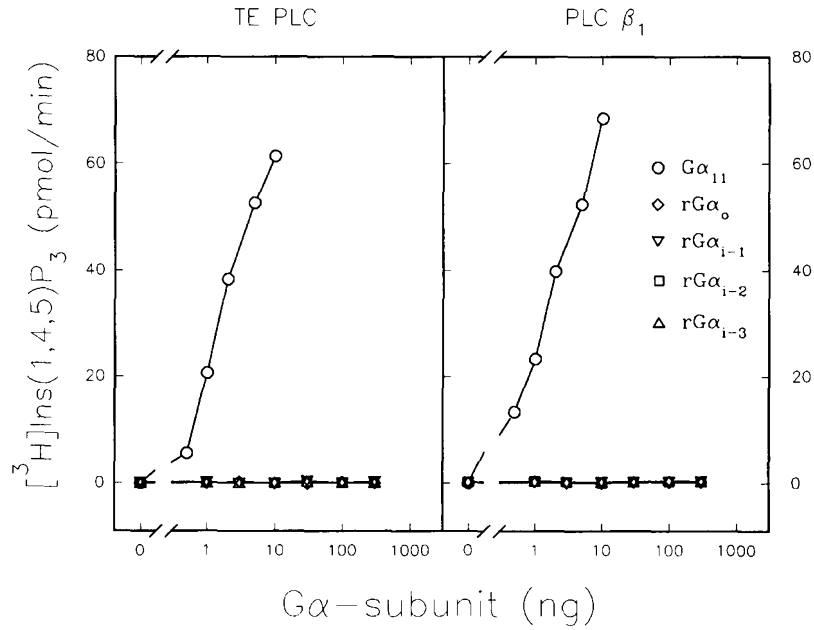

FIG. 1. **Effect of recombinant G-protein α-subunits on phospholipase C activity.** Phospholipid vesicles containing the indicated concentrations of purified $G\alpha_{11}$ (*circles*), $rG\alpha_o$ (*diamonds*), $rG\alpha_{i1}$ (*inverted triangles*), $rG\alpha_{i2}$ (*squares*), and $rG\alpha_{i3}$ (*triangles*) were prepared as described under "Experimental Procedures" and incubated for 15 min at 30 °C in the presence of two different isoforms of purified phospholipase C. *Left panel*, G-protein α-subunits with 10 ng of turkey erythrocyte phospholipase C-β; *right panel*, G-protein α-subunits with 10 ng of bovine brain PLC-β1. Data shown are from one experiment repeated three times with similar results.

pholipids and dialyzed for 16–20 h against the same buffer without cholate. This preparation consists of unilamellar vesicles of 60–120 nm diameter. Phospholipid vesicles were diluted 2.5-fold with dialysis buffer and phospholipase C assays were carried out as previously described in detail (21), with the following modifications: fatty acid-free BSA was used instead of BSA Fraction V, and the final concentration of free Ca^{2+} was increased to 3 μM. Reactions were started by the addition of 5–10 ng of phospholipase C and were terminated by extraction of phospholipids as previously described (21). $[^3H]Ins(1,4,5)P_3$ released to the aqueous phase was quantitated by scintillation spectroscopy. Under these conditions phospholipase C activity was linear for up to 15 min and less than 20–25% of substrate was hydrolyzed.

Data Presentation—Most of the figures are of data from representative experiments. The data presented are the mean or the mean ± S.D. from determinations in triplicate from experiments repeated at least three times using different vesicle preparations. S.D. was less than 10% of the mean for all data points.

Materials—$PtdIns(4,5)P_2$ was purified from a lipid extract obtained from bovine brain (type I Folch fraction obtained from Sigma). $[^3H]PtdIns(4,5)P_2$ was prepared from [^{3}H]inositol-labeled turkey erythrocytes as described (24). Phosphatidylethanolamine from bovine heart and phosphatidylserine from bovine brain were obtained from Avanti Polar Lipids, Inc. [^{3}H]Inositol (20 Ci/mmol) was from American Radiolabeled Chemicals Inc. All other reagents were from sources cited previously (21, 23, 28, 29).

RESULTS

To examine selectivity of activation of phospholipase C-β class isoenzymes, unilamellar $[^3H]PtdIns(4,5)P_2$-containing phospholipid vesicles were reconstituted with various amounts of G-protein α-subunits and with purified turkey erythrocyte phospholipase C-β[2] or phospholipase C-β1. Reconstitution of up to 300 ng of recombinant $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_o$ did not alter basal activity or confer AlF_4^--stimulated activity to either of the phospholipase C isoenzymes (Fig. 1). Although these recombinant proteins did not stimulate phospholipase C, they are as active as native proteins in other assays of activity (28). As we have reported previously (12, 21), reconstitution of purified turkey erythrocyte $G\alpha_{11}$ under the same conditions resulted in marked AlF_4^--promoted stimulation of both turkey erythrocyte phospholipase C-β and phospholipase C-β1 (Fig. 1). The apparent affinity for $G\alpha_{11}$ and the maximal stimulation observed were very similar for each phospholipase C-β isoform. Reconstitution of baculovirus-expressed $G\alpha_{11}$ and $G\alpha_q$ also conferred marked AlF_4^--stimulated activity to phospholipase C-β1 and the turkey erythrocyte enzyme (Fig. 2). Thus, whereas $G\alpha_{11}$ and $G\alpha_q$ markedly activate phospholipase C-β family isoenzymes, α-subunits of G_i or G_o apparently are not regulators of these effectors under the same assay conditions.

The lack of a direct effect of $G\alpha_i$ or $G\alpha_o$ on phospholipase C activity, together with the recent reports of marked stimulation of inositol lipid hydrolysis by G-protein βγ-subunits (20–22), suggests that the release of βγ-subunits from heterotrimeric G-proteins accounts for pertussis toxin-sensitive inositol lipid signaling. Using expressed and purified βγ-subunits of defined composition, we compared the activities of these proteins with purified βγ-subunits from bovine brain or turkey erythrocytes. As illustrated in the silver-stained gel shown in Fig. 3, the four purified recombinant βγ-subunit combinations all were essentially free of contaminating proteins.[3]

It was important to test the functionality of the recombinant βγ-dimers relative to the activity of native bovine brain or turkey erythrocyte βγ-subunits. $G\alpha_{11}$-mediated stimulation of phospholipase C is inhibited by low concentrations of βγ-subunits, presumably due to formation of inactive heterotrimeric G_{11} rather than by a direct effect of βγ-subunits on phospholipase C (12, 21). Therefore, the effect of the recombinant βγ-dimers on $G\alpha_{11}$-mediated activation of phospholipase C was examined. As illustrated in Fig. 4, all four recombinant βγ-dimers inhibited $G\alpha_{11}$-activated phospholipase C-β1 with similar potency and efficacy. Similar effects were observed when the turkey erythrocyte phospholipase C-β was used instead of phospholipase C-β1 (data not shown). All of the recombinant βγ-dimers used in this study were equally active in supporting pertussis toxin-induced ADP ribosylation of $G\alpha_i$-subunits[4] and in conferring high affinity binding characteristics to expressed adenosine receptors (32). It has been previously reported that the γ_2-subunit expressed in Sf9 cells incorporates [^{3}H]mevalonate and is carboxymethylated and therefore is likely to have undergone appropriate post-translational modification of the carboxyl terminus (29, 33). The fact that the recombinant βγ-dimers were purified by affinity chromatography on a G-protein α-subunit column is also consistent with this idea, since

[3] The turkey erythrocyte βγ preparation consists predominantly of β_1-, β_2-, and γ_7-subunits. β_3, γ_2, γ_3, and γ_5 were not detectable in the turkey erythrocyte preparation shown in Fig. 3. The bovine brain preparation consists of β1 and β2 and a mixture of four γ-subunits (21).

[4] S. G. Graber and J. C. Garrison, unpublished observations.

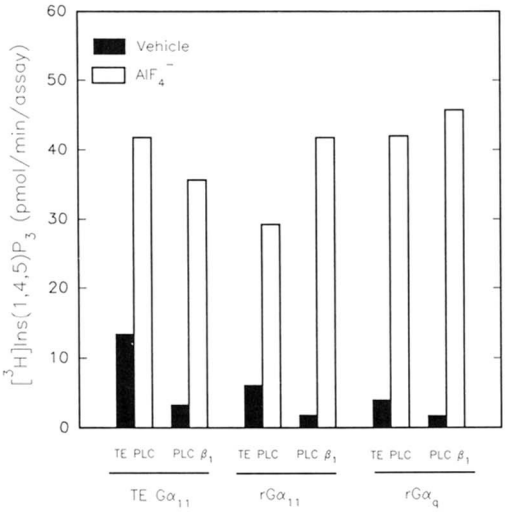

FIG. 2. **Activation of phospholipase C isoenzymes by recombinant $G\alpha_{11}$ and $G\alpha_q$.** Phospholipid vesicles containing purified turkey erythrocyte $G\alpha_{11}$ (*TE* $G\alpha_{11}$), recombinant $G\alpha_{11}$ (*r*$G\alpha_{11}$), or recombinant $G\alpha_q$ (*r*$G\alpha_q$) were prepared as described under "Experimental Procedures" and incubated for 15 min at 30 °C with 10 ng of either turkey erythrocyte phospholipase C-β (*TE PLC*) or purified bovine brain phospholipase C-β1 (*PLC* β_1). Incubations were in the absence (vehicle, *filled bars*) or in the presence (*empty bars*) of AlF_4^-. Results shown are the mean of duplicate assays from a representative experiment repeated at least three times using different phospholipid vesicle preparations.

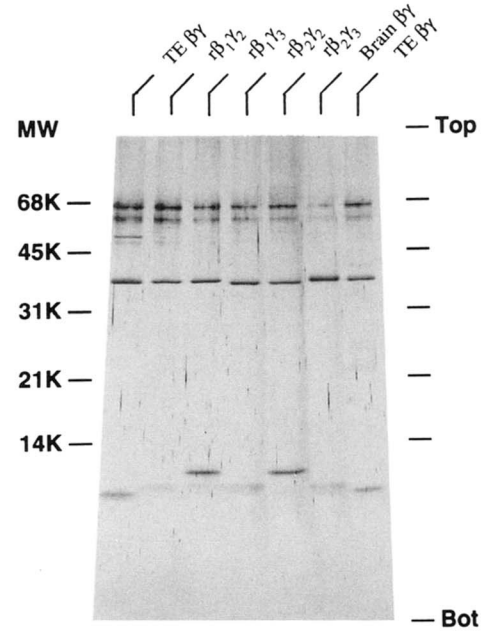

FIG. 3. **SDS-polyacrylamide electrophoresis of purified G-protein βγ-subunits.** One hundred ng of purified turkey erythrocyte βγ (*lane 1*), recombinant $\beta_1\gamma_2$ (*lane 2*), recombinant $\beta_1\gamma_3$ (*lane 3*), recombinant $\beta_2\gamma_2$ (*lane 4*), recombinant $\beta_2\gamma_3$ (*lane 5*), purified bovine brain βγ (*lane 6*), and turkey erythrocyte βγ (*lane 7*) were subjected to electrophoresis on a 10–20% polyacrylamide gradient gel containing 6 M urea and stained with silver. The mobility of the molecular mass markers is indicated on the *left*. The double bands with molecular sizes of about 68 kDa are artifacts of the silver-staining technique.

non-prenylated βγ-dimers do not associate tightly with α-subunits (34). It also has been reported that recombinant βγ-dimers purified after expression in Sf9 cells modulate adenylyl cyclase activity with properties similar to those previously observed with preparations of native βγ-subunits (34). Thus, based on a broad range of criteria, the purified recombinant βγ-subunits of defined composition possess the full complement of activities of their native dimer counterparts.

The relative capacities of the purified βγ-subunits of defined composition to activate phospholipase C were examined. Although the concentrations required were 30-fold higher than for the inhibition of $G\alpha_{11}$-stimulated phospholipase C, all four recombinant βγ-dimers directly activated turkey erythrocyte phospholipase C-β with a potency similar to that observed with bovine brain βγ (Fig. 5*A*) or turkey erythrocyte βγ-subunit (not shown). It is important to stress that all of the stimulating effects of βγ-subunits on phospholipase C occurred in the absence of any α-subunit. As indicated in Fig. 5*A*, the $\beta_2\gamma_3$ dimer consistently produced a sub-maximal activation of turkey erythrocyte phospholipase C-β when compared with the efficacy of the other βγ-dimers or with the bovine brain βγ-subunit preparation. Similar results were observed with two different preparations of purified $\beta_2\gamma_3$-subunit. Based on immunoblotting of the phospholipid vesicle preparations, the amount of $\beta_2\gamma_3$-subunit added in these experiments was the same as the amount of the other βγ-dimers (not shown). Furthermore, the similar activity of all four βγ-dimer combinations for reversal of $G\alpha_{11}$-stimulated enzyme activity and in other tests of functionality suggests that the low efficacy of $\beta_2\gamma_3$ for activation of phospholipase C is not caused by expression of an "inactive" dimer combination. Thus, some selectivity may exist among various βγ-dimers for activation of phospholipase C. In contrast to the effect of the βγ-subunits on turkey erythrocyte phospholipase C-β, none of these preparations activated phospholipase C-β1 at concentrations of βγ-subunit up to 300 ng/assay. Under the same assay conditions, AlF_4^--activated $G\alpha_{11}$ produced similar increases in the activity of both the turkey erythrocyte and bovine brain phospholipase C preparations (Fig. 5*B*).

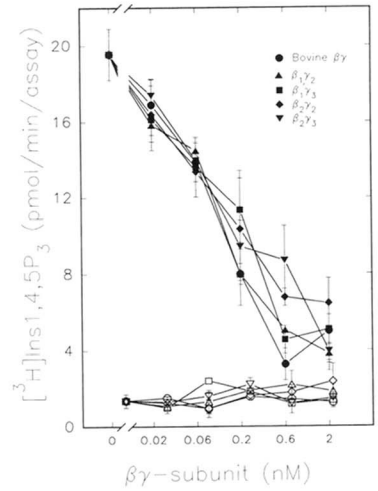

FIG. 4. **Effect of recombinant βγ-subunits on $G\alpha_{11}$ activation of phospholipase C.** Phospholipid vesicles containing 15 ng of $G\alpha_{11}$ and the indicated concentrations of bovine brain or recombinant βγ-subunits were prepared as indicated under "Experimental Procedures." Lipid vesicles were incubated for 15 min at 30 °C with 10 ng of PLC-β1 in the absence (*open symbols*) or in the presence (*filled symbols*) of AlF_4^-. Results shown are the mean ± S.D. of data obtained in one experiment carried out in triplicate, and repeated three times with similar results.

DISCUSSION

The marked progress in understanding receptor-regulated inositol lipid signaling has been primarily restricted to the pathways involving receptor-promoted activation of the G_q family of G-proteins and tyrosine phosphorylation of phospholipase C-γ. Although sensitivity to inactivation by pertussis toxin led to the idea that G_o or a form of G_i is involved in regulation of phospholipase C by a subset of receptors, *e.g.*, fMetLeuPhe receptors in neutrophils (7), neither the protein

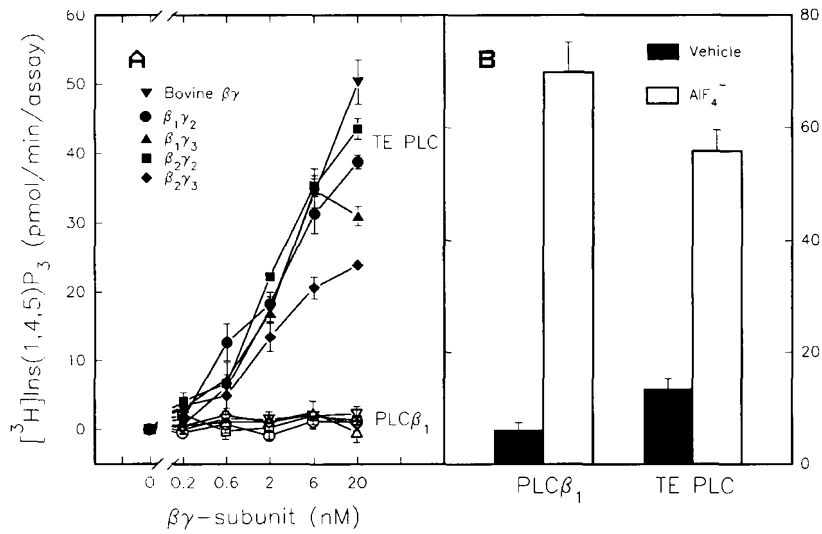

FIG. 5. **Differential activation of turkey erythrocyte and bovine brain phospholipases C by purified and recombinant G-protein βγ-subunits.** Phospholipid vesicles containing the indicated concentrations of G-protein subunits were prepared as indicated under "Experimental Procedures." *Left panel*, lipid vesicles containing the indicated concentrations of βγ-subunits were incubated with 10 ng of turkey erythrocyte phospholipase C-β (*filled symbols*) or with 10 ng of bovine brain PLC-β1 (*open symbols*). *Right panel*, lipid vesicles containing $G\alpha_{11}$ were incubated in the absence (*filled bars*) or in the presence of AlF_4^- (*open bars*). Incubations were for 15 min at 30 °C. Data shown are the mean ± S.D. from one experiment performed in triplicate and repeated at least six times using different phospholipid preparations.

components of this pathway nor the mechanism of their interaction have been unambiguously identified. It has been tacitly assumed that activation of phospholipase C occurs by receptor-promoted release of a GTP-liganded form of $G\alpha_i$ or $G\alpha_o$. However, direct support for this idea is limited to evidence supporting a role for $G\alpha_o$ in receptor-regulated inositol lipid signaling in frog oocytes (35).

The data reported here illustrate that purified recombinant $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$ have no effect on the activity of two different G-protein-regulated phospholipase C isoenzymes. These G-protein α-subunits are as active as native protein in tests of their capacity to interact with G-protein βγ-subunits[4] or to promote high affinity binding of agonists to adenosine receptors (32) and angiotensin receptors (28). Thus, two phospholipase C-β family isoenzymes that are markedly activated by $G\alpha_{11}$ and $G\alpha_q$ are not activated by the pertussis toxin-sensitive α-subunits extant. Our results do not rule out the possibility that other phospholipase C isoenzymes exist that are activated by these α-subunits; they also do not address the potential existence of yet to be identified pertussis toxin-sensitive α-subunits. However, these data, together with results reported by others indicating that various forms of G_i or G_o do not stimulate phospholipase C under conditions where members of the G_q family are very active (8–11), strongly support the idea that α-subunits of pertussis toxin-sensitive G-proteins are not important direct activators of phospholipase C.

The data reported here with recombinant βγ-subunits of various and defined composition are consistent with reports by this and other groups indicating that G-protein βγ-subunits are effective activators of some members of the phospholipase C family. The most parsimonious interpretation of these results is that pertussis toxin-sensitive inositol lipid signaling occurs as a consequence of receptor-promoted dissociation of G_o or a form of G_i into free α- and βγ-subunits. The βγ-subunit then activates phospholipase C. Very few studies have been reported examining the effect of βγ-subunits on phospholipase C in intact cells or membranes. Katz *et al.* (36) have recently reported that cotransfection of Cos-1 cells with βγ-subunit and phospholipase C-β2 resulted in a marked increase in inositol phosphate accumulation, and that cotransfection of M_2-muscarinic receptors and $G\alpha_{i2}$ in these cells resulted in carbachol-stimulated inositol phosphate production that was inhibited by treatment of cells with pertussis toxin. It will be important to obtain more information on direct involvement of βγ-subunits in this pathway in cells not over-expressing large amounts of signaling components.

As we previously had reported for βγ-subunits purified from bovine brain and turkey erythrocytes (21), four different recombinant βγ-subunits markedly activated the avian phospholipase C but had essentially no effect on bovine brain phospholipase C-β1 under the same conditions. The maximal activity observed with the avian phospholipase C in these studies was at least as large as that observed with maximally effective concentrations of $G\alpha_{11}$. Thus, whereas direct regulation of the avian enzyme by βγ-subunits is likely to be important, this seems much less likely with the phospholipase C-β1 isoenzyme. Park *et al.* (37) and Smrcka and Sternweis (38) also reported that βγ-subunit activated phospholipase C isoenzymes with the relative order of β3 > β2 > β1. Similarly, Carozzi *et al.* (39) recently have reported that under conditions where phospholipase C-β1 is marginally activated by transducin βγ-subunit, purified phospholipase C-β3 is markedly activated. Results from two recent reports studying the relative activities of phospholipase C-β1 and phospholipase C-β2 overexpressed in Cos cells are consistent with our results using purified proteins. Thus, Katz *et al.* (36) observed selectivity of activation by co-expressed $\beta_1\gamma_1$-subunit of phospholipase C-β2 over phospholipase C-β1. Similarly, Camps and co-workers (40) reported that reconstitution of transducin βγ-subunit into membranes prepared from Cos cells overexpressing phospholipase C-β2 resulted in a higher enzyme activity than reconstitution into membranes containing overexpressed phospholipase C-β1.

The full significance of βγ-subunit regulation of phospholipase C is not yet established. Although it is possible that βγ-subunit released from members of the G_q family could under certain situations activate phospholipase C in a physiologically important way, this does not appear to be an effect of major importance. The apparent affinity of $G\alpha_{11}$ for activation of the avian phospholipase C used in these studies is approximately 30-fold greater than the corresponding affinity of βγ-subunit for activation. Furthermore, βγ-subunits in stoichiometric concentrations with $G\alpha_{11}$ decrease rather than promote phospholipase C activity, apparently due to formation of inactive heterotrimeric G_{11}. However, it is also true that some phospholipase C-β isoenzymes, *e.g.*,phospholipase C-β2, are less sensitive to activation by $G\alpha_q$ and $G\alpha_{11}$ (41), and in cells expressing this but not other isozymes, regulation of inositol lipid signaling could potentially occur through activation by βγ-subunits. It also is possible, as has been suggested by the results of Blank *et al.* (22), that phospholipase C isoenzymes exist that are activated by βγ-subunits but not by G-protein α-subunits.

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is more clear-cut for the reasons discussed earlier. Additionally, the amount of G_i and G_o in target tissues is relatively high with the extreme case that for G_o in brain where this G-protein accounts for up to 2% of total membrane protein. Thus, the potential exists for receptor-promoted releases of large amounts of βγ-subunit from these toxin-sensitive G-proteins. Release of βγ-subunit from G-proteins that are not pertussis toxin substrates also might have important signaling consequences. For example, G_s, in which no phospholipase C-stimulating activity is known to reside in the α-subunit, could conceivably activate phospholipase C through release of βγ-subunit. In this regard, it is of interest that activation of β-adrenergic receptors in turkey erythrocyte membranes results in an increase in inositol lipid hydrolysis (42, 43).

One of the principle hypotheses tested in the current work was the idea that βγ-subunits of differing composition would have differing activities for stimulation of phospholipase C. This was the case for only one of the subunits tested here, $\beta_2\gamma_3$, which was clearly less active for stimulation of phospholipase C than it was in other tests of activity. This result potentially could be a harbinger of even larger differences in activity of yet to be tested βγ-dimers, or in tests of $\beta_2\gamma_3$-subunit with other phospholipase C isoenzymes. It should be noted that activation of phospholipase C by transducin βγ-subunit only occurs at 100 to 1000-fold higher concentrations of transducin βγ-subunit (20) than the activating concentrations of βγ-subunit in the current study.

In summary, the results reported here with purified components strongly favor the idea that receptor-promoted dissociation of pertussis toxin-sensitive G-proteins results in activation of phospholipase C through the release of βγ- rather than α-subunits. Our results do not indicate major differences in activity of βγ-subunits of differing composition. However, lower activity of the $\beta_2\gamma_3$-subunit together with the fact that phospholipase C-β1 is markedly activated by $G\alpha_{11}$, but not by βγ-subunits, suggests that it will be crucial to delineate the relative activity of all possible βγ-dimers and to establish their existence as authentic dimers in mammalian cells. The properties of the inositol lipid response of an individual cell to extracellular ligands will not only depend on the complement of cell surface receptors, but also on the G-protein α-, β-, and γ-subunits and phospholipase C isoenzymes that are expressed. An important step in molecularly defining receptor-regulated inositol lipid signaling pathways will be to identify all of the G-proteins and phospholipase C isoenzymes that exist in a given homogeneous cell and to define the specificity of interaction of these components during activation of receptors that signal through pertussis toxin-sensitive and insensitive components.

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