Determination of the Affinities between Heterotrimeric G Protein Subunits and Their Phospholipase C-β Effectors†

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ABSTRACT: Phosphatidylinositide-specific phospholipase C-βs play a key role in Ca^{2+} signaling and are specifically activated by the α_q family of heterotrimeric G proteins and as well as $\beta\gamma$ subunits. We have determined the affinity between G$\beta\gamma$ subunits and GTPγS and GDP-liganded Gα_q subunits on membrane surfaces, and their respective affinities to PLC-β_1, -β_2 and -β_3 effectors by fluorescence spectroscopy. We find that activation of Gα_q by GTPγS decreases its affinity for G$\beta\gamma$ subunits at least 36-fold compared to the GDP-liganded form, but increases its affinity for PLC-βs at least 40−200-fold depending on the PLC-β isoform. The affinity of Gα_q(GTPγS) is similar for PLC-β_1 and -β_3 and 10-fold stronger for PLC-β_2, which corresponds to the reported relationship between the concentration of Gα_q(GTPγS) and PLC-β activation on lipid bilayers. We find that a large portion of the PLC-β-Gα_q association energy lies within the 400 residue C-terminal region of PLC-β_1 since truncating this region reduces its Gα_q affinity. In contrast, the isolated N-terminal region does not interact with Gα_q. G$\beta\gamma$ subunits interact with all three PLC-β isotypes, but only showed strong binding to PLC-β_2, and activation of the three PLC-βs by G$\beta\gamma$ subunits parallels this behavior. We also tested the possibility that both Gα_q and G$\beta\gamma$ can simultaneously bind PLC-β_2. Our data argue against simultaneous binding and show that Gα_q and G$\beta\gamma$ independently regulate this effector.

Phosphatidylinoside-specific phospholipase C (PLC)[1] catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtsIns(4,5)P_2] to generate two second messengers, diacylglycerol (DAG) and inositol, 1,4,5-trisphosphate [Ins(1,4,5)-P_3], which mediate the activation of protein kinase C and intracellular Ca^{2+} ion release, respectively (*1*, *2*). There are three known mammalian families of PLC, and one of these, the PLC-β family, is activated by the Gα_q family of heterotrimeric G proteins. The PLC-β signaling pathway is initiated upon activation of a seven-transmembrane receptor which acts as a guanine nucleotide exchanger, promoting the exchange of GDP for GTP on the α_q subunits of membrane-resident heterotrimeric G proteins. The GTP-liganded α_q has reduced affinity for G$\beta\gamma$ subunits, and it is thought that the heterotrimer dissociates on the membrane surface into α_q monomers and G$\beta\gamma$ heterodimers. Both Gα_q and G$\beta\gamma$ subunits can independently activate various effector proteins, one of which is PLC-β (see *3* for review).

The mammalian PLC-β family has four known members, PLC-β_1, PLC-β_2, PLC-β_3, and PLC-β_4,which are all activated by Gα_q. Here, we have focused on the first three of these, PLC-β_1, PLC-β_2, and PLC-β_3. These isozymes vary in their tissue distribution as well as their ability to be activated by α_q and $\beta\gamma$ subunits. PLC-β_1 and -β_2 have been shown to bind strongly to membranes where they can laterally associate to membrane-bound G protein subunits (*4*, *5*). PLC-β_1 and PLC-β_3 have been shown to be more strongly activated by α_q subunits than PLC-β_2 in *in vitro* reconstitution assays (*6*−*8*), but reports differ on whether this difference is due to the potency of activation or simply a difference in interaction energies. In contrast, G$\beta\gamma$ activation appears to follow the order PLC-β_3 > PLC-β_2 > PLC-β_1 (*8*−*11*).

Biochemical and molecular biology studies of PLC-β isozymes indicate that Gα_q and G$\beta\gamma$ subunits may bind to the PLC-βs at separate sites. Deletion of a ∼400 residue region on the C-terminus of PLC-β_1 abolishes its ability to be activated by Gα_q subunits, indicating that this region plays a role in Gα_q association (*12*−*14*). On the other hand, G$\beta\gamma$ may interact with the N-terminus of PLC-β_1 and -β_2 and also a region within the catalytic domain, (*3*, *15*−*17*).

Since Gα_q and $\beta\gamma$ subunits interact at distinct sites on PLC-β, it has been suggested that these proteins could simultaneously regulate PLC-βs (*7*). Precident for simultaneous regulation comes from two G$\beta\gamma$-sensitive isoforms of adenylyl cylase, AC II and IV, which require concurrent activation by Gα_s and G$\beta\gamma$ (for review, see *18*). Simultaneous binding of α_q and $\beta\gamma$ subunits to PLC-β cannot be easily assessed by standard radiometric-based activity studies because these assays are sensitive to the concentration and

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[1] Abbreviations: PLC, phosphoinositide-specific phospholipase C; PtsIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; A, 6-acryloyl-2-dimethylaminonaphthalene (acrylodan); C, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin or 7-diethylamino-3-(4′-isothiocyanatophenyl)-4-methylcoumarin; DAB, (maleimide), 4-dimethylaminophenylazophenyl-4′-maleimide or (succinimidyl ester) 4-(4-((4-(dimethylamino)phenylazophenyl)azo)benzoic acid, succinimidyl ester.

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type of divalent cations, the presence of detergent which the G protein subunits are solubilized in, the nature of the substrates used, and the linearity of assay conditions (see *19*). Simultaneous binding is also difficult to detect by physical methods since there are few techniques available to view the formation of complexes on membrane surfaces.

One technique that allows direct measurement of protein associations on membrane surfaces is fluorescence resonance energy transfer (FRET). In this method, two proteins are individually labeled with either a donor or an acceptor fluorescent probe. Transfer of energy from the donor to the acceptor will only occur when the two proteins come within a certain distance, typically between 10 and 50 Å (see *20*). Thus, when two proteins labeled with an energy transfer pair laterally associate on a membrane surface, energy transfer will be observed.

Although the regulation of PLC activity by G proteins has been extensively studied biochemically, the relationship between G protein activation and the physical interaction between these proteins is unknown, and this relationship is necessary to understand the mechanism through which G protein−effector activation occurs. The purpose of this study is 2-fold. The first is to measure the affinities of activated and deactivated α_q subunits for G$\beta\gamma$ and for PLC-βs. These values will enable us to evaluate the possible interactions that may be involved in PLC-β activation by G proteins, the level of local G protein subunits needed for productive interactions with PLC-β isozymes, the PLC-β isotypes that are likely to be the best G protein effectors, and whether inactive α subunits could remain bound to PLC-β and be poised for activation. The second purpose of this study is to evaluate the hypothesis that PLC-βs can be regulated by α_q and $\beta\gamma$ subunits simultaneously. If both G protein activators bind simultaneously to PLC-β, then it would suggest that PLC-βs can be synergistically regulated by both G protein subunits released from one or several types of G protein coupled receptors. Also, it would keep the G protein subunits in close proximity to each other and promote their reassociation. Our results strongly argue that α_q and $\beta\gamma$ subunits independently regulate PLC-βs.

METHODS

Proteins. The purification of recombinant PLC-β_1 and -β_2 and PLC-$\beta_1\Delta C$ from Sf9 cells has been described (*4*, *5*). PLC-β_3 was expressed in Sf9 cells using a baculovirus vector provided by Dr. Sue Goo Rhee (NHLBI, NIH, Bethesda, MD). The recombinant baculovirus was selected and amplified by standard methods (*21*). Suspension cultures of Sf9 cells (0.5−1 L, 1×10^6 cells/mL) grown in complete Grace's medium containing 10% fetal calf serum were infected with PLC-β_3 recombinant baculovirus at a multiplicity of 10. Cells were harvested after 48 h, washed in phosphate-buffered saline (PBS), pH 6.2, resuspended, and subjected to nitrogen cavitation (Parr cell disruption bomb, >500 psi for 30 min with intermittent agitation). All buffers were degassed, and the pH was adjusted with KOH or phosphoric acid. Cells were discharged into 100 mL of ice-cold buffer A1 (25 mM Tris, pH 7.5, 5 mM EDTA, 5 mM EGTA, 1 mM DTT with 10 μg/mL PMSF, 5 μg/mL pepstatin A, 10 μg/mL leupeptin, and 5 μg/mL aprotinin), and the soluble fraction was recovered after ultracentrifugation at 100000*g*. This material was diluted to 150 mL with buffer A1 and applied to 9 mL column of Source 15Q resin (Pharmacia, Uppsala, Sweden). Bound proteins were eluted with a 160 mL gradient of 0−0.6 M NaCl and a 40 mL gradient of 0.6−1.0 M NaCl. Four milliliter fractions were collected, and their PLC activity was determined using exogenously added substrates (*4*). Fractions containing PLC activity were pooled, diluted to a NaCl concentration of 0.1 M with buffer A1, and applied to a 4 mL column of heparin−Sepharose CL4B (Pharmacia) and eluted in 1 mL fractions with a 40 mL gradient of 0−1 M NaCl in buffer A1. Fractions containing PLC activity were pooled and exchanged into buffer A2 (10 mM KH_2PO_4, pH 7.5, 1 mM DTT) and applied to a 3 mL Biogel HPHT (BioRad, Hercules, CA) column, eluted with a 40 mL gradient of 10−650 mM KH_2PO_4 in buffer A2, collecting 1 mL fractions. Active fractions were pooled, diluted 3-fold into buffer A1, and applied to a 2 mL Q2 column (Biorad). Bound protein was eluted with a 25 mL gradient of 0−1.0 M NaCl in buffer A3 (25 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM EGTA, 1 mM DTT), collecting 0.5 mL fractions.

The final yield is typically 1 mg of purified PLC-β_3 from 500 mL of Sf9 cell culture. SDS−PAGE and Coomassie staining showed the protein to be pure. Glycerol was added to purified protein to 10% (v/v), and the protein was aliquoted, flash-frozen with liquid nitrogen, and stored at −80 °C. If PLC-β_3 was to be labeled using thiol-reactive reagents, DTT was not included in the final buffer for the Q2 column. After the enzyme was labeled (see labeling procedure below), DTT was added to 1 mM, and the labeled protein was then flash-frozen.

G protein $\beta\gamma$ subunits were purified from cholate detergent extracts of bovine brain membranes using the method of Sternweis and Robishaw (*22*), substituting octyl-Sepharose (Pharmacia) for the final step, with further purification. α_q subunits were expressed and purified using the method of Kozasa and Gilman (*23*) which involves coexpressing α_q with viruses for Gβ_1 and His_6-Gγ_2 in Sf9 cells, batch-loading onto a Ni−NTA column, washing, and eluting at 30 °C after activation with aluminum fluoride. α_q subunits were identified by Western blotting, and we estimated our preparations to be >90% pure as judged by silver staining.

The rates describing the kinetics of GDP and GTPγS dissociation and α_q denaturation have been recently delineated (*24*). Specifically, Ross and co-workers demonstrated that the presence of $(NH_4)_2SO_4$ allowed activation of α_q to occur in a short incubation time and give high levels of GTPγS binding. Based on this study, α_q was activated by incubating the protein for 1 h at 30 °C in 50 mM Hepes, 100 μM GTPγS, 100 mM $(NH_4)_2SO_4$, 150 mM $MgSO_4$, 100 mM EDTA, and 0.7% CHAPS. These conditions are reported to give a level of 80 ± 24% of active α_q with the remainder being primarily denatured protein (*24*), and our estimates fall in this range. The activated protein was able to increase the activity of PLC-β_3 at least 5-fold above control on sonicated bilayers composed of PC:PE:PS 1:1:1 with 2% PtsIns(4,5)P_2.

Preparation of Lipid Vesicles and Protein Reconstitution. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (PS) were purchased from Avanti Polar Lipid, Inc. (Alabaster, AL). [^{3}H]PtdIns(4,5)P_2 from [^{3}H]-*myo*-inositol-labeled turkey

erythrocytes and PtdIns(4,5)P_2 from Type I:Folch Fraction source (Sigma, St. Louis, MO) were a generous gift from Dr. Andrew Morris (SUNY Stony Brook, Stony Brook, NY). Lipid bilayers were prepared by extrusion (*25*) to produce uniform large unilamellar vesicles (LUVS) of 100 nm diameter. Lipid concentrations were determined by phosphate analysis.

G proteins were reconstituted into bilayers either by adding a concentrated lipid solution (2−5 mM) to the detergent-solubilized G protein subunits and removing the detergent by dialysis, or by the direct addition of the proteins to preformed vesicles. This latter method was used only at total lipid concentrations above 75 μM, which is well above the partition coefficient of the proteins as determined by fluorescence methods (data not shown). The surfaces used in this study [POPC:POPS:POPE 1:1:1 for fluorescence studies and with 2% PtdIns(4,5)P_2 for activity studies] were chosen as substrates because their surface charge and PE/PC concentrations are similar to the plasma membrane from rat liver (see *26*).

Fluorescent Labeling of Phospholipase C-β Isozymes. All probes were purchased from Molecular Probes, Eugene, OR. Thiol-reactive coumarin, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin, and amine-reactive coumarin, 4-dimethylaminophenylazophenyl-4′-maleimide, are abbreviated as C. Thiol-reactive phenylazophenyl probe, 4-dimethylaminophenylazophenyl-4′-maleimide, and amine-reactive phenylazophenyl probes, 4(4-((4-(dimethylamino)phenylazophenyl)azo)benzoic acid, succinimidyl ester, are abbreviated as DAB. 6-Acryloyl-2-dimethylaminonaphthalene is referred to as acrylodan, and fluorescein isothiocyanate as fluorescein.

Phospholipase C-β isozymes were labeled with the thiol-reactive probes by adding the probe from a concentrated DMF solution at a 4:1 to 3:1 probe:protein ratio on ice for 1 h in the final column buffer from its purification (25 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM EGTA). Excess probe was removed by dialysis using Slide-A-Lyzer 10 000 MWCO, 0.1−0.5 mL Cassette (Pierce Chemical Co., Rockford, IL) at 4 °C in a 1000-fold excess of buffer for two 2−3 h dialysis steps and then overnight (∼12 h) using buffer supplemented with 1 mM DTT. Phospholipase C-β isozymes were typically modified by this reaction with a labeling ratio of 2−3 mol of probes/mol of enzyme.

G protein $\beta\gamma$ subunits were labeled with thiol-reactive probes at a 2:1 probe:protein ratio for 2 h on ice. Excess probe was removed by three dialysis changes using a 1000-fold excess of 20 mM Hepes, pH 7.0, 160 mM KCl, 1% octyl glucoside, 40 mM β-mercaptoethanol at 4 °C.

α_q subunits were labeled with amine-reactive reagents in the presence of reducing agents by first increasing the pH through the addition of a 50-fold molar excess of phosphate buffer, pH 8.5, adding a 4−5-fold molar excess of probe from a concentrated stock in DMF, and allowing the material to react on ice for 20−30 min. Afterward, the sample was subjected to extensive dialysis with buffer (50 mM Hepes, 100 mM ammonium sulfate, 150 mM magnesium sulfate, and 100 mM EDTA) at pH 7.0. For deactivated α_q, the buffer included 100 μM GDP. For activated α_q, 100 μM GTPγS was added after dialysis.

After labeling, the concentrations of all proteins were determined by BCA (Pierce) analysis. The proteins were then mixed with 10% glycerol, aliquoted, flash-frozen with liquid nitrogen, and stored at −70 °C until further use. Labeled α_q and PLC-βs were stable, as determined by activity and spectral characteristics, 3−4 days after thawing whereas G$\beta\gamma$ samples were stable for at least 7 days.

To verify that labeling of the proteins is not affecting the activity of the PLC-β enzymes, or their ability to be activated by G protein subunits, two types of studies were done. We first verified that the activities of the amine- or thiol-modified PLC-βs were the same within error as their unlabeled counterparts. We also verified that they were activated to the same extent at three concentrations of G protein subunits. In these latter studies we substituted labeled G protein for unlabeled and observed the same level of activation.

The second series were fluorescence-based where we found that the emission intensity and center of spectral mass of acrylodan-G$\beta\gamma$ are sensitive to the association of unlabeled PLC-β as well as dabcyl-labeled. Measurements of the association of the three unlabeled PLC-β isozymes gave identical results as the FRET studies, indicating that labeling the PLC-βs does not interfere with G$\beta\gamma$ association. Similar studies were attempted with C-α_q, and we found that the emission intensity of the coumarin probe increases ∼10% with association although this increase was more variable than the acrylodan-G$\beta\gamma$ studies. Nevertheless, unlabeled PLC-β_2 and PLC-β_3 gave identical binding C-α_q affinities, within error, as dabcyl-labeled protein, indicating that α_q is not affected by the dabcyl label (PLC-β_1 was not done due to lack of material).

Fluorescence Measurements. Steady-state measurements were taken on an I.S.S. K2 (Champaign, IL) time-resolved spectrofluorometer using a 3 mm × 3 mm × 40 mm quartz micro cell (NSG Precision Cells Inc., Farmingdale, NY). For acrylodan, where the scattering contribution sometimes exceeded 1% of the initial signal, emission data were corrected for background using a solution of vesicles at identical concentrations.

Affinities of Protein Complexes on Membrane Surfaces. The associations that we are monitoring in this study all occur between proteins confined to membrane surfaces. Thus, each affinity is highly dependent on the concentration of lipids used in each experiment. To compare affinities that were measured at different lipid concentrations, we wished to normalize the data for the amount of lipid used and to keep the affinities in familiar terms that are comparable to other nonmembrane associations. We thus adapted a simple model where we assume that the membrane-bound proteins associate in a given surface volume and translate the membrane-bound concentrations to their three-dimensional counterparts [see (*29*)].

Here, protein association curves are obtained by fluorescence resonance energy transfer in which we follow the decrease in donor emission (coumarin or acrylodan) as a nonfluorescent energy transfer acceptor (DAB) is added. From the titration data, the degree of association (*a*) is calculated using

$$a(x) = \frac{\mathrm{FI}(x) - \mathrm{FI}(x{=}\mathrm{initial})}{\mathrm{FI}(x{=}\mathrm{final}) - \mathrm{FI}(x{=}\mathrm{initial})} \qquad (1)$$

FI(x=initial) is the initial fluorescence intensity before the addition of species x. FI(x=final) is the final fluorescence intensity once saturating concentrations of species x have

been added. $a(x)$ may then be fit to a simple bimolecular association curve for two proteins interacting in the bulk solution.

To analyze the affinity of the protein complexes on membrane surfaces, we assume that the proteins interact within a surface phase of volume v which is equal to the surface area of the bilayer with an outer radius r multiplied by the thickness of the membrane/solvent interface d where d is chosen to reflect the diameter of the protein [$\sim$50 nm for the proteins used here; see (*30*, *31*)]. The relationship between the bulk phase concentration of the protein to the membrane surface concentration, $[P_m]$, is simply the ratio to the volume of the bulk phase solution (V_b) to the surface phase volume (*32*), i.e., $[P_m] = (V_b/v) = \epsilon$. Thus, the apparent dissociation constant (K_{d-app}) can be translated to a corresponding three-dimensional of "bulk" value (K_b) by

$$K_b = \epsilon[G_o]\frac{(x-a)(1-a)}{a}; K_b = \epsilon K_{d-app} \qquad (2)$$

where $[G_o]$ is the initial concentration of G protein.

For proteins reconstituted into large, unilamellar vesicles, ϵ can be calculated from the amount of lipid used:

$$\epsilon = \frac{2 \times 10^{24}}{[\text{lipid}](\text{mol/L})N_A d(\text{nm})\rho(\text{nm}^2)} \qquad (3)$$

where N_A is Avogadro's number, ρ is the average surface area of a lipid headgroup, and d is the width of the surface phase which is arbitrarily chosen to match the dimensions of the protein. If we let d = 50 nm, then for a lipid concentration of 350 $\times$ 10^{-6} M, ρ = 0.75 nm^2 [see (*26*)], and ϵ = 632.

RESULTS

Affinities between α_q and $\beta\gamma$ Subunits on Membrane Surfaces. (A) α_q(GDP)·$\beta\gamma$ Complexes. We observed the lateral association between GDP-liganded Gα_q and G$\beta\gamma$ subunits using fluorescence resonance energy transfer. In these studies, one of the subunits was labeled with a donor probe (coumarin or C) and reconstituted into bilayers. The other was labeled with an acceptor probe (DAB) and titrated into the donor−lipid solution at high enough lipid concentrations (i.e., above 20 μM PC:PE:PS 1:1:1) to ensure complete membrane binding (*29* and data not shown). Since the DAB is not fluorescent, energy transfer is monitored by the loss of the integrated fluorescence intensity of courmarin as the two proteins interact on the membrane surface. Control studies consisted of monitoring the emission intensity of the coumarin-labeled protein when dialysis buffer, the unlabeled protein partner, or the denatured DAB-labeled protein partner was added. The overlap spectra for these two types of probes have been reported (*29*).

In Figure 1, we show the change in dilution-corrected emission intensity of C-α_q(GDP) reconstituted on lipid bilayers when DAB-G$\beta\gamma$ in a 0.7% CHAPS solution is added, relative to DAB-G$\beta\gamma$ that had been boiled for $\sim$20 min. We find a consistent and reproducible 20−30% decrease in fluorescence intensity. From the change in integrated emission intensity of C-α_q(GDP) with increasing DAB-G$\beta\gamma$, we can calculate the molecular association curve (eq 1).

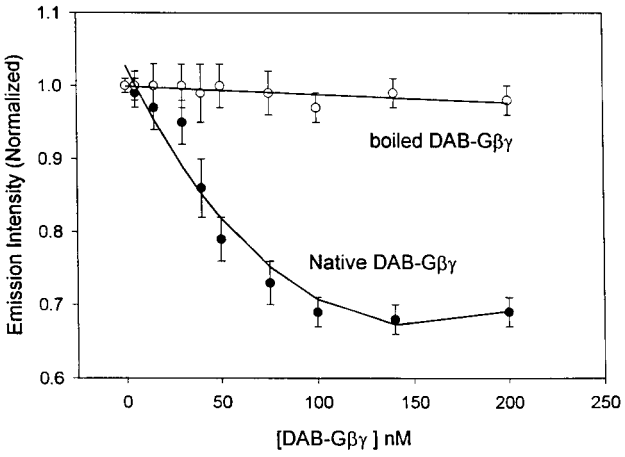

FIGURE 1: Lateral association of α_q(GDP) and $\beta\gamma$ subunits on membrane surfaces as seen by energy transfer. Decrease in the normalized emission intensity of 28 nM CM-α_q(GDP) reconstituted on 75 μM PC/PS/PE membranes as DAB-$\beta\gamma$ subunits are added (●) under conditions of complete membrane binding, relative to DAB-$\beta\gamma$ that was boiled for 20 min (○). The decrease in emission intensity is interpreted to be due to fluorescence resonance energy transfer from the coumarin donor attached to α_q(GDP) to the DAB acceptor on G$\beta\gamma$ as the two subunits associate on the membrane surface. Sample-to-sample errors are shown.

The apparent dissociation constant for the studies presented here will depend on the concentration of the proteins on the membrane surface and thus the concentration of lipid used. To compare two sets of data taken at different lipid concentrations, we relate these apparent dissociation constants to a three-dimensional or bulk value (K_b, see eq 2 in Methods). Values of K_b will always be higher than the apparent dissociation constant since the membrane-bound proteins are more concentrated on the two-dimensional surface volume of the bilayer. For example, varying the lipid concentration from 20 to 112 μM lipid caused the apparent α_q(GDP)-$\beta\gamma$ dissociation constant to change from 0.2 to 1.2 nM but left the transformed, three-dimensional dissociation constant, K_b, unchanged.

Identical dissociation constants were obtained by labeling $\beta\gamma$ subunits with coumarin, reconstituting them on bilayers, and following energy transfer as DAB-α_q(GDP) was added. From the five separate studies using three different labeled preparations of G$\beta\gamma$ and two different α preparations, an average value of 3.2 $\pm$ 0.8 μM was obtained.

(B) α_q(GTPγS)·$\beta\gamma$ Complexes. To determine the change in affinity between α_q and $\beta\gamma$ subunits that occurs upon α activation, we repeated the above the studies using activated α_q(GTPγS) (see Methods). In Figure 2 we show an example of the association of DAB-α_q(GTPγS) to membrane-bound C-$\beta\gamma$. In several studies such as the one shown here, we find a high-affinity association at low concentrations of α_q-(GTPγS) which varied from 0 to 15% of the total change, followed by a low-affinity association at higher concentrations. The large decrease in C-$\beta\gamma$ emission at higher α_q concentrations (Figure 2) was always seen in each preparation and is interpreted to be due to the α_q(GTPγS)−$\beta\gamma$ association. We interpret the low-affinity association as corresponding to interaction between G$\beta\gamma$ and α_q(GDP) due to incomplete exchange of GTPγS for GDP. This behavior is in accord with previous studies showing incomplete activation of α_q (*24*). While these latter studies and ours indicate that only $\sim$80% of α_q becomes activated, in the

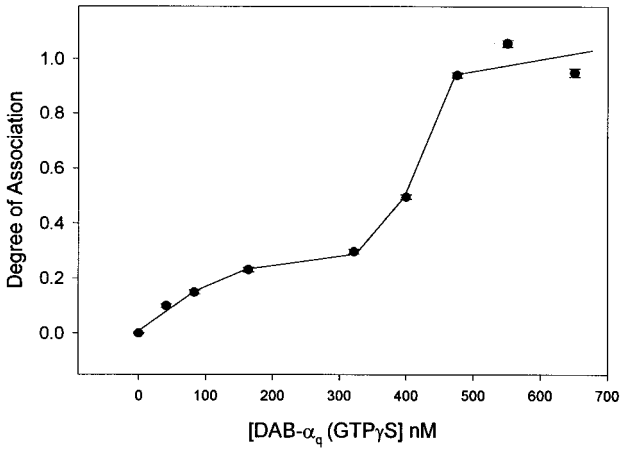

FIGURE 2: Lateral association of α_q(GTPγS) and $\beta\gamma$ subunits on membrane surfaces as seen by energy transfer. Association between activated α_q(GTPγS) and $\beta\gamma$ subunits was followed by measuring the change in the normalized fluorescence intensity of 4 nM CM-$\beta\gamma$ reconstituted on 75 μM PC/PS/PE membranes when DAB-α_q(GTPγS) is added and the corresponding degree of association was determined (see eq 1). The initial association is interpreted to be due to nonactivated α_q (see text and *24*). Sample-to-sample errors are shown.

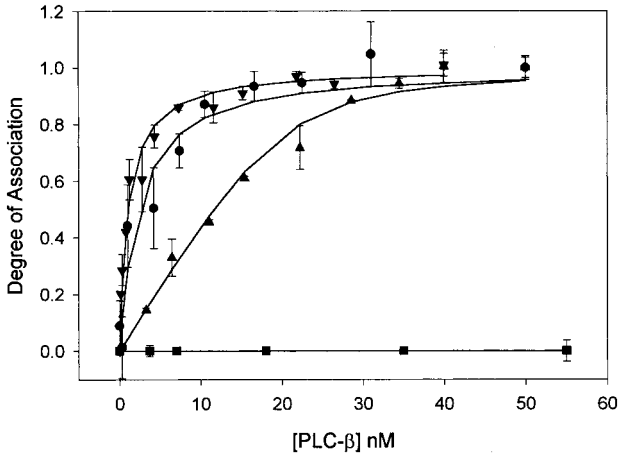

FIGURE 3: Differential association of PLC-β isotypes with Gα_q(GTPγS) on membrane surfaces. Degree of association, as determined by the normalized change in fluorescence intensity (eq 1), for DAB-PLC-βs binding to reconstituted 1.2 nM CM-α_q-(GTPγS) reconstituted on 205 μM PC/PS/PE; DAB-PLC-β_3 (▼), DAB-PLC-β_1 (●), DAB-PLC-β_2 (▲), and DAB-PH−PLC-β_2 which consists of residues 1−177 (■). The K(0.5) values for activation are estimated at ~1 nM for PLC-β_1 and PLC-β_3 and ~10 nM for PLC-β_2. Sample-to-sample errors are shown

results presented here we have calculated K values assuming 100% activation.

Interaction of PLC-β_1, -β_2, and -β_3 with α_q Subunits. The affinities of three PLC-β isotypes for activated α_q(GTPγS) were determined by monitoring fluorescence energy transfer from coumarin-labeled α_q subunits (C-α_q) on membrane surfaces as DAB-labeled PLC-β isozymes were added. These studies were performed at lipid concentrations where PLC-βs are completely membrane-bound which enables us to view lateral associations of the proteins on the membrane surface without contributions from PLC-β membrane binding.

All three PLC isozymes bound tightly to α_q(GTPγS) subunits (see Figure 3) as seen by the decrease in C-α_q-(GTPγS) fluorescence as DAB-PLC-βs were added, but not when dialysis buffer or boiled DAB-PLC-β was added. The

Table 1: Bulk Value Dissociation Constants (μM)[a]

	α(GDP)	
$\beta\gamma$	K(b) = 1.0 ± 0.4	n = 6
PLC-β_2	K(b) = 17 ± 8	n = 3
PLC-β_3	K(b) = 11 ± 2	n = 2
	α(GTPγS)	
$\beta\gamma$	K(b) ~ 36 ± 10	n = 2
PLC-β_1	K(b) = 0.04 ± 0.02	n = 4
PLC-β_2	K(b) = 0.4 ± 0.2	n = 3
PLC-β_3	K(b) = 0.06 ± 0.02	n = 4
PLC-$\beta_1\Delta C$	K(b) = 1.0 ± 0.6	n = 2
PH-PLC-β_1	K(b) > 300	n = 2
	$\beta\gamma$	
PLC-β_1	K(b) > 300	n = 3
PLC-β_2	K(b) = 3.2 ± 0.8	n = 3
PLC-β_3	K(b) ~ 60	n = 1

[a] Bulk value dissociation constants were calculated from K_{app} as determined by FRET (see text) and are independent of lipid concentration. Here, n = number of experiments, and the sample-to-sample errors are reported. Values were determined for at least two different lipid concentrations, ranging from 50 to 350 μM. Values for Gα_q(GTPγS) assume 100% concentration and did not take into account that our method only activates ~80% of the material.

apparent affinities followed the hierarchy PLC-$\beta_1 \approx$ PLC-β_3 > PLC-β_2. Changing the initial concentration of α_q-(GTPγS) from 3 to 75 nM while keeping the lipid concentration constant gave the same bulk dissociation constant. The data in Figure 3 were fit to an apparent bimolecular association constant, K_{d-app}, and then translated to bulk values, K_b, which are given in Table 1. These data represent results for two different α_q(GTPγS) preparations and three different lipid preparations.

Deletion of the C-terminus of PLC-β_1 has been shown to abolish activation by α_q(GTPγS), implying that this region may play a key role in α_q(GTPγS) association (*12*, *13*, *33*). To test this idea, we first expressed and purified the C-terminal truncated protein, PLC-$\beta_1\Delta C$. In accord with these previous studies, the protein product has a high basal activity that was completely unchanged by the addition of 1−100 nM α_q(GTPγS) in contrast to the ~8-fold activation we observed for the full-length protein in this concentration range (data not shown). Previous sedimentation studies indicated that the truncated protein does not bind strongly to lipid bilayers (*5*). However, when we labeled the protein with acrylodan and measured its change in emission when PC/PS/PE bilayers are added, we find that the protein binds strongly to membranes (K < 100 μM, see *4*). We speculate that the difference in membrane binding affinities seen in the two techniques may be due to technical difficulties in achieving complete pelleting of the bilayers in the sedimentation studies (*5*).

We then measured the affinity between DAB-PLC-$\beta_1\Delta C$ for C-α_q(GTPγS) reconstituted on 250 μM bilayers using fluorescence resonance energy transfer. We found a 25-fold reduction in affinity relative to the full-length enzyme. However, this residual affinity is still strong and on the order of that obtained for PLC-β_2 (Table 1). Thus, the residual binding of the truncated mutant indicates that other regions in PLC-β_1 are involved in association, but that the contribution of the C-terminal region is necessary for activation by α_q(GTPγS). This idea is supported by the observation that no increase in PLC-$\beta_1\Delta C$ activity was seen even when the α_q concentration was increased more than 25-fold (i.e., 100

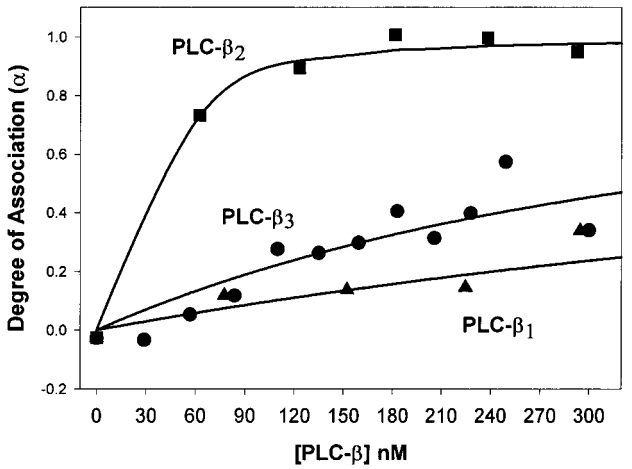

FIGURE 4: Differential association of PLC-β isotypes with G$\beta\gamma$ from bovine brain. Degree of association (see eq 1) between acrylodan-labeled G$\beta\gamma$ and DAB-labeled PLC-β_1 (▲), PLC-β_2 (■), and PLC-β_3 (●) on 350 μM PC/PS/PE surfaces as determined from the decrease in donor (acylodan)-integrated fluorescence intensity as acceptor (DAB-) is added.

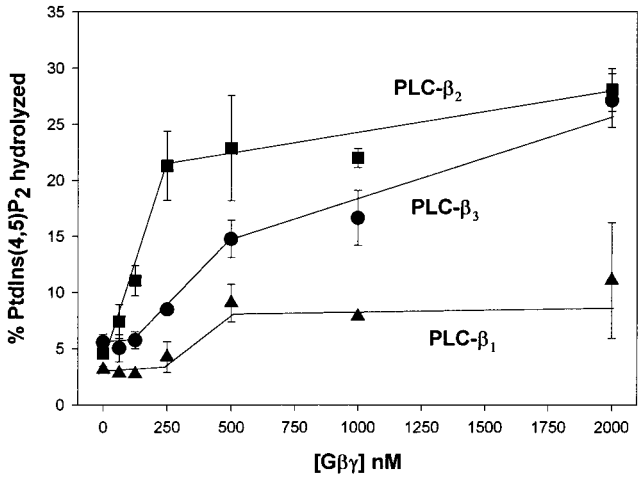

FIGURE 5: Activation of PLC-β isozymes with G$\beta\gamma$ purified from bovine brain. Activation of PLC-β_1 (▲) (150 nM), PLC-β_2 (■) (100 nM), and PLC-β_3 (●) (150 nM) as G$\beta\gamma$ is added. Since the basal activity of PLC-β_3 is much lower than PLC-β_1 and -β_2, we increased the assay time for PLC-β_3 ~10-fold. Substrate vesicles are extruded large unilamellar vesicles (LUVs) composed of 1:1:1 PC/PS/PE with 2 mol % PtdIns(4,5)P_2, and were used at a concentration of 1 mM. Sampling errors are shown.

nM) above the threshold needed to observe activation.

To further localize the interaction region between PLC-βs and α_q(GTPγS), we measured the affinity of the isolated N-terminal pleckstrin homology domain (residues 1−177) of PLC-β_2 (PH-β_2) for α_q(GTPγS). This region has been shown to strongly associate to G$\beta\gamma$ subunits (*39*). We found that the addition of up to 200 nM DAB-labeled PH-β_2 did not result in measurable energy transfer from C-α_q(GTPγS) (Figure 3). This result supports the idea that the N-terminus of PLC-β isozymes does not interact with α_q(GTPγS) (see *3*).

A final series of studies were done to characterize the ability of PLC-β to interact with nonactivated α_q(GDP). This characterization will not only help determine the importance of GTP hydrolysis in the dissociation of the α_q(GTPγS)−PLC-β complexes and subsequent reassociation of α_q(GDP) to $\beta\gamma$ subunits, but will also help determine whether α_q(GDP) remains bound to PLC-β and poised for reactivation. We find that deactivated α_q(GDP) has a ~50−100 fold weaker affinity for PLC-β_2 and -β_3 than α_q(GTPγS) (Table 1).

Association of PLC-β_1, -β_2, and -β_3 to G$\beta\gamma$ Subunits. The affinities between the three PLC-β isozymes and $\beta\gamma$ on membrane surfaces were determined by modifying PLC-βs with DAB and G$\beta\gamma$ with acrylodan (A-$\beta\gamma$). Acrylodan has similar emission properties as coumarin, and energy transfer to DAB reduces its integrated intensity 10−15%.

The lateral association between A-G$\beta\gamma$ and DAB-PLC-βs is shown in Figure 4. The data compiled in Table 1 show that the affinity of PLC-β_2 for G$\beta\gamma$ is at least 30-fold greater than that of PLC-β_3 for G$\beta\gamma$, and at least 200-fold greater than that of PLC-β_1 for G$\beta\gamma$.

The results in Figure 4 are in contrast to previous biochemical studies reporting that PLC-β_3 is more responsive to stimulation by $\beta\gamma$ subunits than PLC-β_2 or -β_1. However, these previous studies were done at different concentrations of each enzyme and used micelles rather than bilayer substrates. To determine whether the activation of the three PLC isoforms by $\beta\gamma$ reflects their strength of interaction, we measured activation under identical conditions as the fluorescence study. The results, presented in Figure 5, show that PLC-β activation by $\beta\gamma$ subunits parallels the strength of their $\beta\gamma$ interaction with PLC-β_2 > PLC-β_3 > PLC-β_1.

Simultaneous Binding of α and $\beta\gamma$ Subunits to PLC-β_2. Since α_q and $\beta\gamma$ appear to bind to different regions of PLC-β, we tested the idea of simultaneous binding of the two G protein subunits to PLC-β_2 since this isoform shows significant binding to G$\beta\gamma$ subunits at relatively low G$\beta\gamma$ concentrations. Two types of fluorescence methods were used. The first, fluorescence homotransfer, is a specialized type of energy transfer that occurs between identical probes. Transfer in this case is detected by the loss in polarization or anisotropy of the emitted light as the light is transferred between the probe molecules in the complex. As the number of probes that can participate in homotransfer increases, the anisotropy decreases (see *20* for background).

We carried out the homotransfer studies by first labeling the three proteins, α_q(GTPS), $\beta\gamma$, and PLC-β_2, with fluorescein isothiocynate at a 1:1 stoichiometry. We used fluorescein for these studies because its absorption and emission spectra have substantial overlap which greatly increases the probability of homotransfer. The results (Table 2) show that transfer between F-PLC-β_2 and F-G$\beta\gamma$ was low but measurable (~10%) while transfer between PLC-β_2 and α_q(GTPS) was ~28%. When the F-PLC-β_2−F-G$\beta\gamma$ pair was mixed with unlabeled α_q or when F-PLC-β_2−F-α_q(GTPS) was mixed with unlabeled G$\beta\gamma$, the values for homotransfer did not significantly change, indicating that the addition of an unlabeled component did not change the interaction between the transferring species. When the three labeled components were mixed at equimolar concentrations on bilayers, we found that no further transfer occurs, and the resulting anisotropy corresponded to the value of the PLC-$\beta\gamma$ complex, arguing against the formation of a ternary complex (Table 2). Supporting this idea, we could not detect transfer between F-$\beta\gamma$ and F-α_q(GTPγS) at 75 nM in the presence of excess PLC-β_2. We note that these results indicate that, under our conditions, F-PLC-β_2 appears to prefer F-$\beta\gamma$ subunits over F-α_q(GTPγS). This preference of

Table 2: % Fluorescence Homotransfer between PLC-β_2 and G Protein Subunits

complex	% transfer	
F-PLC-β_2−*F*-G$\beta\gamma$	10 ± 6	$n = 5$
U-PLC-β_2−*F*-G$\beta\gamma$	0 ± 2	$n = 2$
F-PLC-β_2−*U*-G$\beta\gamma$	0 + 2	$n = 2$
F-PLC-β_2−*F*-α_q(GTPγS)	28 ± 9	$n = 4$
U-PLC-β_2−*F*-α_q(GTPγS)	0 ± 2	$n = 2$
F-PLC-β_2−*U*-α_q(GTPγS)	0 + 2	$n = 2$
F-α_q(GTPγS)−*U*-G$\beta\gamma$	0 ± 1	$n = 4$
F-α_q(GTPγS)−*F*-G$\beta\gamma$	0 ± 1	$n = 4$
U-α_q(GTPγS)−*F*-G$\beta\gamma$	0 + 1	$n = 4$
F-G$\beta\gamma$-*F*-PLC-β_2−*F*-α_q(GTPγS)	5 ± 4	$n = 2$
F-G$\beta\gamma$- *F*-PLC-β_2−*U*-α_q(GTPγS)	10 ± 6	$n = 2$
U-G$\beta\gamma$- *F*-PLC-β_2−*F*-α_q(GTPγS)	25 ± 9	$n = 2$
F-G$\beta\gamma$- *U*-PLC-β_2−*F*-α_q(GTPγS)	0 + 1	$n = 5$

[a] The percent homotransfer was calculated by comparing the measured anisotropy of the fluorescein-labeled proteins using exciting and emitting wavelengths of 480 and 525 nm, respectively, to their theoretical values for the complex (A_{theor}) where $\{A_{theor} = 1/I_{complex}\sum IA_i \cdot I_i\}$. *F*- refers to the fluorescein-labeled protein; *U*- refers to unlabeled protein. All the concentrations of all of the proteins were identical at 75 nM reconstituted on 167 μM PC/PS (2:1). The value of *n* refers to the number of sets of samples, and the reported error refers to the sampling error. Each sample was measured 4 times each, consisting of 20−50 anisotropy measurements.

PLC-β_2 affinity for $\beta\gamma$ may be due to incomplete activation of F-α_q, or a change in the properties of one of the proteins by the fluorescein label since the affinity of DAB-PLC-β_2 for C-$\beta\gamma$ relative to C-α_q(GTPγS) is ~8-fold weaker. Similar affinity measurements were not possible using fluorescein-labeled proteins due to the 70% light loss from the polarizers and the low protein concentrations necessary to observe association. Nevertheless, it is clear from the homotransfer studies that under our experimental conditions, F-PLC-β_2−F-α_q(GTPγS) complexes form.

We also assessed the possibility of simultaneous binding of α_q(GTPγS) and $\beta\gamma$ subunits to PLC-β_2 by determining whether the presence of PLC-β_2 would shift the association curve between C-α_q(GTPγS) and DAB-$\beta\gamma$ subunits. If both G protein subunits bind to PLC-β_2, then the addition of unlabeled PLC-β_2 to membrane-bound G protein subunits labeled with an energy transfer pair could increase the amount of energy transfer occurring in them. If PLC-β_2 shifts the concentration at which $\beta\gamma$ associates to α_q(GTPγS) subunits to lower values, then this would be indicative of simultaneous binding.

In Figure 6 we present the association of 24 nM C-α_q-(GTPγS) to DAB-G$\beta\gamma$. Without PLC-β_2 these proteins associate with an approximate midpoint of 350 nM DAB-$\beta\gamma$. When this study was repeated in the presence of 72 nM PLC-β_2, the midpoint shifts to 500 nM DAB-$\beta\gamma$. Thus, PLC-β_2 shifts the curve by an amount expected if it bound free G-$\beta\gamma$, and more G$\beta\gamma$ is required to overcome PLC-β_2−α_q-(GTPγS) association. Addition of higher amounts of PLC-β_2 (292 nM) shifts the midpoint out of the experimental range and essentially prevents α_q(GTPγS)−$\beta\gamma$ association. It is also possible that a ternary complex forms but that the two G protein subunits are out of energy transfer range. In this case, the curves in Figure 6 represent the association of DAB-$\beta\gamma$ to the PLC-β_2−C-α_q(GTPγS) complex which is inconsistent with the homotransfer results. Taken together, these results argue that PLC-β_2 binds G protein subunits individually.

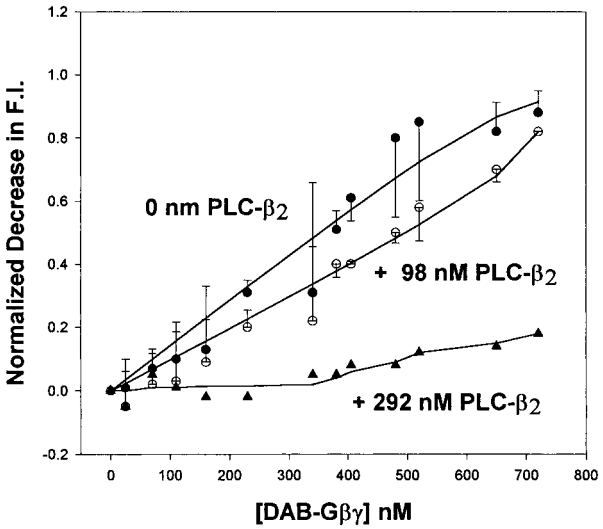

FIGURE 6: Shift in the association of CM-α_q(GTPγS) and DABMI-G$\beta\gamma$ at varying concentrations of PLC-β_2. DAB-$\beta\gamma$ was reconstituted on PC/PS/PS (1:200 protein:lipid ratio) and added to a solution of 24 nM CM-α_q(GTPγS), and the normalized decrease in fluorescence emission due to energy transfer was followed (●). Titration curves were repeated at 98 (○) and 292 nM (▲) unlabeled PLC-β_2, resulting in a shift in the approximate midpoint. Sample-to-sample errors are shown.

DISCUSSION

In this study we have characterized the relative affinities between G protein subunits and their PLC-β effectors which may allow us to predict the probable interactions and activation pathways that occur in the cell. Our results show that the G protein effector affinities directly reflect effector activation. This finding indicates that activation of the effector proceeds through simple association of the PLC-β effector with the G protein subunit and more complicated models do not need to be invoked.

The affinities that we report have three caveats that should be considered. None of these are expected to greatly change the values listed in Table 1 or their interrelationships. First, we measured the affinities using proteins with covalently attached fluorescent probes, and while control studies show that these probes do not affect PLC-β activities, or their ability to be activated by G protein subunits (see Methods), the resolution of the activation data may not detect small, subtle changes in affinity. Second, activation of α_q by GTPγS is only 80 ± 20% (*24*), and our α_q(GTPγS) affinities may be underestimated. Third, the apparent affinities that we measure for these membrane-bound proteins depend on the lipid concentration, and we have translated them into lipid-independent bulk values which enable us to compare the affinities measured at different lipid concentrations. While there are several methods to convert apparent dissociation constants of membrane-bound proteins to ones that are independent of lipid concentration, we chose a method that relates the volume in which the proteins can interact on the membrane surface to the bulk volume. In this method, we must define the distance from the membrane surface in which the proteins interact. The value we have used, 50 nm, is based on the dimensions of the proteins (*30*, *31*). This distance causes the translated K_b values to be ~630-fold greater than the apparent dissociation constants. If we choose a 10-fold larger dimension of (i.e., ~500 nm), then the K_b values in Table 1 will be reduced 10-fold. We are presently trying to

better define the value chosen for the surface volume by comparing the association of model proteins in solution and anchored to the membrane surface.

This study is the first direct measurement of the association between G protein subunits on membrane surfaces. In general, our measured apparent affinities fall in the same range as other types of Gα(GDP) subunits and $\beta\gamma$ subunits in detergent solutions (*34*, *35*) or reconstituted on an immobilized phase (*36*). Also, the 40-fold decrease in affinity that we observe upon α_q activation is in accord with previous solution studies and with crystallographic studies showing a dramatic decrease in $\alpha_{i1}-\beta\gamma$ subunit contacts that occurs upon α activation (*30*). It is important to note that activation of α_q is not always complete and thus the affinity between α_q(GTPγS) and $\beta\gamma$ and also between α_q(GTPγS) and the PLC-βs represents an upper and lower estimation, respectively.

This study also presents the first direct measurement between these G protein subunits and their PLC-β effectors. We find that all three PLC isoforms bind strongly to activated α_q(GTPγS) with PLC-$\beta_1 \approx$ PLC-β_3 and PLC-β_2 being 10-fold lower. This result agrees with the concentration dependence of PLC-β activation by α_q(GTPγS) on lipid bilayers seen by Harden and co-workers (*37*), who showed that PLC-β_1 was maximally activated at a 10-fold lower concentration of α_{11} than PLC-β_2. However, studies using micelle substrates do not detect differences in the concentration dependence of α_q(GTPγS) activation between PLC-β_1 and -β_2, but rather find differences in the extent of activation (*6*, *7*). Since the presence of detergents affects the basal activity of the PLC-βs, as well as their extent of G protein activation, we suggest that detergents affect the conformation of the activated PLC-$\beta-$G$\beta\gamma$ complex, its interaction with substrate, or both.

Activation of α_q decreases its affinity for $\beta\gamma$ at least ~40-fold and increases its affinity for PLC-βs at least 40−100-fold. The extent of these changes in affinities should ensure a complete shift in interaction to the stronger complex. Once the activated α_q(GTPγS)−effector complex forms, the GTPase activity of the α subunit, along with the GAP activity of PLC-β (*38*), will increase the deactivation rate of α, and in turn increase the rate at which the transfer of α subunits from PLC-β to $\beta\gamma$ occurs.

Although the affinities between α_q subunits and PLC-βs fit well with previous biochemical studies, our results of the PLC-$\beta-\beta\gamma$ affinities were unexpected because previous in vitro studies report the hierarchy of $\beta\gamma$ activation to be PLC-β_3 > PLC-β_2 > PLC-β_1 (*7*, *8*). However, the strong activation of PLC-β_3 *in vitro* is not observed when $\beta\gamma$ subunits are cotransfected into COS7 cells or when receptors such as C5a and fMet-Leu-Phe are activated (*39*). The stronger activation of PLC-β_3 relative to -β_1 and -β_2 *in vitro* was observed in studies where the activation profiles were made at identical activity levels of the three isozymes. Since the basal activity of PLC-β_3 is much lower than PLC-β_2 or PLC-β_1, these studies used higher PLC-β_3 concentrations, shifting the equilibrium toward PLC-β_3-G$\beta\gamma$ complex formation. We find that when studies were done at the same concentrations of PLC-β, the activation profile matches the affinity profile well (see Figure 5). The results presented here resolve the conundrum between the apparent differences seen in the *in vivo* and *in vitro* studies.

The idea that PLC-β_2, whose expression in various cell types is more limited than PLC-β_3, is a strong effector of G$\beta\gamma$ indicates that this relatively nonspecific pathway to increase intracellular levels of Ca^{2+} may only occur in a few specific cell lines, or under particular circumstances (see below). The amount of PLC-βs in the cell is thought to be much lower than heterotrimeric G proteins (1), and barring alterations in the relative protein concentrations due to formation of protein domains, we can speculate about the activation pathways that may occur. PLC-β_1 is expected to only be activated by members of the α_q family of G proteins and not $\beta\gamma$ subunits. Given PLC-β_3's strong affinity for Gα_q and its relatively weak affinity for G$\beta\gamma$, PLC-β_3 is more likely to be regulated through α_q rather than $\beta\gamma$ subunits, although if a sufficient amount of $\beta\gamma$ is released by various α subunits, then PLC-β_3 could be stimulated. A similar argument can be extended to PLC-β_2, whose affinity for $\beta\gamma$ is also weaker than for α_q(GTPγS). Of course, these *in vitro* studies have been done using $\beta\gamma$ subunits that were purified from bovine brain and are composed of a distribution of β and γ isotypes. It is possible that different $\beta\gamma$ dimers interact differently with α family members and PLC-βs.

Deletion and mutational studies all suggest that PLC-βs contain physically separate interaction sites for α_q and $\beta\gamma$ subunits (see *3*). Our observation that deletion of the 400 residues on the C terminus of PLC-β_1 results in a large reduction in α_q(GTPγS) affinity and loss of activation by α_q is consistent with previous activation studies that have also shown that these deletion mutants can still be activated by $\beta\gamma$ (*12*, *13*, *33*). Other mutational studies suggest that the N-terminal region and a region in the catalytic domain of PLC-β_2 are the major sites of G$\beta\gamma$ association and activation (see *3*). We have found that the N-terminal pleckstrin homology domain of PLC-β_1 and -β_2 serve as docking sites for G$\beta\gamma$ subunits (*39*) but not α_q subunits as shown here (Figure 3). Since the G protein α and $\beta\gamma$ subunits may interact at distinct sites, it has been suggested by activity studies that they may bind simultaneously to PLC-βs to synergistically activate these effectors. However, our homo- and heterotransfer studies argue against the formation of a ternary α_q(GTPγS)−PLC-$\beta_2-\beta\gamma$ complex and show independent binding. It is possible that even though α_q and $\beta\gamma$ subunits bind to different regions of PLC-β_2, there may be some overlap of these binding sites which may sterically prevent multiple binding, perhaps in the vicinity of the catalytic core.

The studies done here show which complexes will form *in vitro*, and would only reflect the situation *in vivo* if the proteins were freely diffusing on the plasma membrane. However, it is possible that these proteins are localized in domains. Even in protein aggregates, the relative affinities listed in Table 1 would still dictate the prevalent interactions. However, the formation of protein domains would promote the interactions of weakly association species, and it is likely that higher order complexes between two strongly associating species, such as α(GDP) and $\beta\gamma$, and a weakly associating species, such as PLC-β_2, may occur if the subunit association sites are not entirely occluded. These higher order aggregates would allow for rapid switching of association depending on the activation state of α. Studies directed at defining these complexes are underway.

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