Regulation of the Lateral Association of Phospholipase Cβ_2 and G Protein Subunits by Lipid Rafts[†]

Suzanne Scarlata*

Department of Physiology and Biophysics, State University of New York at Stony Brook, Stony Brook, New York 11794-8661

Received February 4, 2002; Revised Manuscript Received March 26, 2002

ABSTRACT: One function of membrane domains of liquid-ordered lipids or "rafts" may be to stabilize complexes of signaling proteins, thereby playing a role in the transduction of cellular signals. Here, we have used fluorescence methods to directly test this idea by assessing the ability of phospholipase Cβ_2 (PLCβ_2) to associate with G protein subunits on model membranes in the fluid phase and on membranes that contain domains of lipids in the liquid-ordered phase (rafts). We find that the apparent dissociation constant for the equilibrium between PLCβ_2 and Gα_q(GTPγS) was identical on both types of membrane surfaces. However, the degree of association between PLCβ_2 and G$\beta\gamma$ subunits was significantly reduced on the surfaces containing rafts. Time studies indicate that this phenomenon is a dynamic process. Incorporating the lipid substrate of PLCβ_2 into membranes that forms rafts, we find that its basal activity is unaffected. However, its activation by G$\beta\gamma$ subunits is inhibited, supporting a reduced degree of interaction between these two proteins when rafts are present. Since lipid rafts affected PLCβ_2−G$\beta\gamma$ association and not PLCβ_2−Gα_q(GTPγS) association, we explored the possibility that the membrane interaction of G$\beta\gamma$ differed when rafts are present. We find that although the membrane partition coefficient of G$\beta\gamma$ is not significantly changed in the presence of rafts, proteolysis of G$\beta\gamma$ by trypsin increases and the ability of G$\beta\gamma$ Tyr/Trp fluorescence to be quenched by iodide ions decreases when rafts are present. These results suggest a model in which lipid rafts occlude the PLCβ_2 interaction site on G$\beta\gamma$ subunits by localizing these subunits at the domain interface.

The activation of phosphoinositide-specific phospholipase Cβ (PLCβ)[1] by the α or $\beta\gamma$ subunits of heterotrimeric G proteins is initiated by the binding of an agonist to its cognate seven-transmembrane receptor which catalyzes the exchange of GDP for GTP on Gα subunits. The GTP-bound Gα subunit has a weakened affinity for G$\beta\gamma$ subunits and allows the two to interact with intercellular effectors, one of which is PLCβ. PLCβ isoenzymes are a family of four known species, PLCβ_{1-4}, that differ in their tissue distribution and their ability to be activated by G protein subunits (for recent reviews, see refs *1* and *2*). The isoform used here, PLCβ_2, is activated by both GTP-bound Gα_q subunits and G$\beta\gamma$ subunits. PLCβs catalyze the hydrolysis of a minor lipid component in membranes, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2]. This reaction releases two second messengers in the cell that result in an increased level of intracellular calcium and a host of mitogenic and proliferative changes.

Both PLCβ and G protein subunits can be considered peripheral membrane proteins that laterally associate to produce activated complexes. PLCβ is soluble in aqueous solution and binds strongly and largely nonspecifically to membrane surfaces (*3*). Gα_q and G$\beta\gamma$ subunits also bind strongly to membranes, and this binding may be stabilized by attachment of two saturated acyl groups on Gα_q and a geranylgeranyl group on G$\beta\gamma$ (*4*, *5*). The lateral association of PLCβ_2 and G protein subunits on membrane surfaces can be assessed in real time using fluorescence resonance energy transfer (FRET). Characterization of these association energies has been reported using uniform model membranes where the proteins can freely diffuse (*6*, *7*). However, in recent years, it has become clear that natural membranes contain specialized domains, the most notable of which are the domains that remain after disruption of cell membranes by detergents (*8*−*10*). These detergent-insoluble domains have been found to be aggregates of glycosphingolipids/sphingomylein (SPM) and cholesterol (CH) that exist in the liquid-ordered phase (L_o) rather than the fluid, liquid-disordered phase (L_d) which is characteristic of most cell membranes (*11*). The L_o phase is characterized by tight chain packing, reduced fluidity, and extended lipid chains, although the lipid mobility is still high. Model membranes containing regions of lipids in the L_o phase, termed "rafts", can be formed by using high concentrations of cholesterol or cholesterol/sphingomylein, and lipids that contain at least one unsaturated acyl chain (*12*), which includes most membrane phospholipids. Phase diagrams of these domains have been characterized (*13*).

[†] This work was supported by a Grant-in-Aid from the American Heart Association (015051N) and by National Institutes of Health General Medicine Grant GM 53132.

* To whom correspondence should be addressed. Phone: (631) 444-3071. Fax: (631) 444-3432. E-mail: suzanne@dualphy.pnb.sunysb.edu.

[1] Abbreviations: PLC, mammalian phosphoinositide-specific phospholipase C; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Laurdan, 6-lauroyl-2-(dimethylamino)naphthalene; Dabcyl SE, 4-(dimethylamino)phenylazophenyl-4-sulfonyl chloride succinyl ester; coumarin, coumarin-3-carboxylic acid, succinyl ester; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; SM, egg sphingomyelin; Ch, cholesterol.

10.1021/bi025625j CCC: $22.00
Published on Web 05/10/2002

Several studies have shown that some membrane-associated proteins will preferentially localize to lipid rafts, whereas others prefer the more fluid phase lipids. This partitioning may be determined by the nature of the acyl modification (*14*), as well as interactions with other surface-associated proteins (*15*). Saturated hydrocarbon chains such as myristoyl and palmitoyl will pack well in gel-like rafts. Alternatively, kinked, unsaturated chains such as geranylgeranyl would be more soluble in fluid phase regions. This picture agrees with model system studies suggesting that $G\alpha_q$ subunits will partition into rafts whereas $G\beta\gamma$ subunits remain in fluid phases (*14*), although cellular studies indicate that in the absence of caveolin both $G\alpha_q$ and $G\beta\gamma$ segregate into rafts (*16*).

While the physical properties of lipid rafts have been characterized, their role in cell function is not clear. These domains may serve to localize particular protein isoforms (*17*), organize the actin cytoskeleton (*18*), or serve as nucleation sites for the assembly of signaling proteins through the immobilization of one or more key proteins (*19*). Indeed, there are several lines of evidence that suggest that the components involved in the inositol signaling pathway exist in signaling complexes (*6, 20*−*23*).

In this study, we address the question of whether lipid rafts may promote the formation of these complexes, and stabilize them once they have formed. Using a cellular system, it has been found that disruption of rafts through depletion of cholesterol or sphingomylein did not alter signaling by G_s (*20*). Since many factors contribute to protein localization in cells, we took a minimalist approach to investigating the role that lipid rafts may play in cell signaling. Specifically, we measured the effect that lipid rafts may have on the lateral association of G protein subunits and PLCβ_2. We conducted these studies using purified proteins reconstituted on model membranes that either contain lipid rafts or have uniform surfaces. We find that the presence of lipid rafts greatly inhibits PLCβ_2−G$\beta\gamma$ association and activation, but not PLCβ_2−Gα_q association and activation. Our data suggest that inhibition of the PLCβ_2−G$\beta\gamma$ interaction is due to changes in the membrane orientation of G$\beta\gamma$ in the presence of raft domains.

MATERIALS AND METHODS

Lipids. All lipids were from Avanti Polar Lipids, Inc. The sphingomyelin was a natural product from egg extract and had entirely saturated acyl chains of 83.9% 16:0, 6.3% 18:0, and other minor, longer chains. Lipid bilayers were prepared by extrusion through a 0.1 μm filter.

Proteins. Expression and purification of recombinant PLCβ_2, $G\alpha_q$, and $G\beta_1\gamma_2$ through baculovirus infection of Sf9 cells and the radiometric procedures for determining PLCβ activity and its activation by G protein subunits have been described previously (*3*, *7*).

Fluorescent Probes. All probes were purchased from Molecular Probes, Inc. Concentrated stocks of the probes were made in DMF and stored at −20 °C under nitrogen in bottles wrapped with aluminum foil.

Proteins were labeled with the amine-reactive probe dabcyl SE or coumarin SE by initially increasing the pH of the protein solution to 8.0 and adding a small aliquot of probe dissolved in DMF at a 4:1 probe:protein molar ratio. Unreacted probe was removed by extensive dialysis in 20 mM Hepes, 0.16 M KCl, 1 mM EGTA, and 1 mM DTT (pH 7.0) for PLCβ_2 and G$\beta\gamma$. Gα subunits were dialyzed against 150 mM Hepes, 40 mM β-mercaptoethanol, 100 mM $(NH_4)_2SO_4$, 150 mM $MgSO_4$, and 100 mM EDTA. GTPγS (100 μM) was added immediately after dialysis. The protein:probe labeling ratio was determined by absorption spectroscopy using the calculated extinction coefficients for the protein and the coefficients provided by the probe manufacturer and was ∼1:1 (moles per mole) for all preparations.

Methyl-β-cyclodextrin Treatment. Partial cholesterol removal by treatment with methyl-β-cyclodextrin was accomplished by adding 15 μL of a 1 mM methyl-β-cyclodextrin solution to 150 μL of 200 μM lipids, incubating at 37 °C for 1 h, and spinning at 24K rpm to remove any precipitated material.

Fluorescence Measurements and Analysis. Fluorescence measurements were performed on an ISS (Champaign, IL) spectrofluorometer using 3 mm cuvettes. Coumarin-labeled proteins were excited at 340 nm and scanned from 380 to 500 nm. Laurodan probes were excited at 350 nm and scanned from 380 to 560 nm. The intrinsic fluorescence was measured using 280 nm excitation and scanning from 300 to 420 nm. The emission intensity was taken from the integrated area of the spectrum. In the lipid titration curves, background spectra of lipid alone were subtracted from each spectrum along the titration curve. Protein association spectra were corrected for lipid background by subtracting a control spectrum using all components except labeled proteins from the titration spectra. In all cases, the control spectra gave intensity values of less than 5% of the sample. All spectra were corrected for the 10−12% dilution that occurred during the titration.

Initial measurements of the degree of lateral association of PLCβ_2 and G$\beta\gamma$ on membrane surfaces were accomplished by fluorescence resonance energy transfer from coumarin-labeled G protein subunits to dabcyl-labeled PLCβ_2 as previously described (*7*). However, we found that the emission intensity of coumarin-labeled Gq(GTPγS) and G$\beta\gamma$ showed a substantial and reproducible decrease (22−28%) upon addition of unlabeled PLCβ_2 and gave apparent K_d values identical to those using FRET. Therefore, in these studies, we determined the degree of lateral association by this latter method which minimizes the handling of these proteins.

We analyzed the titration curves assuming that all proteins can form complexes and that they do so at a 1:1 stoichiometry. Fitting the curves to a bimolecular association gives apparent K_d values in moles per liter that are dependent on the total available membrane surface area. We have previously translated the apparent K_d that we experimentally observe, which depends on the lipid concentration, to the dissociation constant that would be observed if the proteins were not bound to membranes (*7*). However, since all studies here were done at an identical lipid concentration, this analysis is not required. We have only reported the experimental values of K_d to be used for comparative purposes.

RESULTS

Effect of Rafts on Membrane Binding. Before monitoring the association of the proteins on membrane surfaces, we

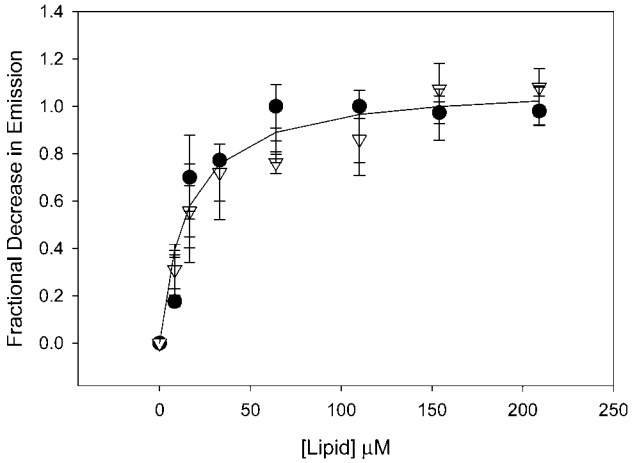

FIGURE 1: Binding of 50 nM PLCβ_2 to membranes composed of PC and PS (●) or PC, SM, and Ch (▽) as determined by the 10% decrease in intrinsic fluorescence measured by exciting at 280 nm and scanning from 300 to 420 nm.

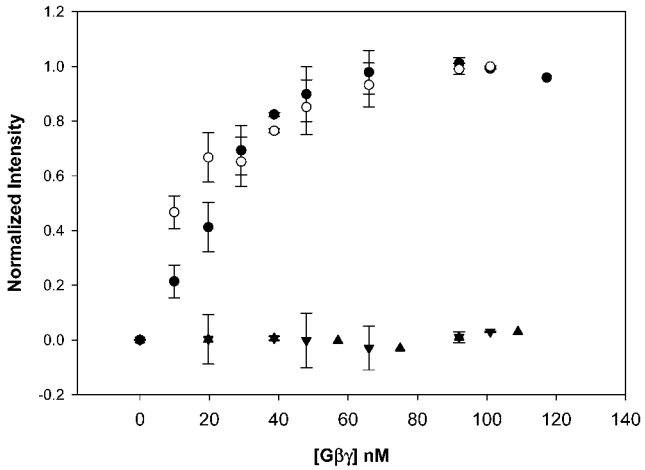

FIGURE 2: Binding of 80 nM G$\beta\gamma$ subunits to PC/PS (2:1) (●) or PC/SM/Ch (○) bilayers doped with 0.1% Laurdan as determined by the 12% increase in Laurdan emission intensity as scanned from 380 to 580 nm using a λ_{ex} of 340 nm. Data for the buffer control are shown with ▲ and ▼ for PC/PS and PC/SM/Ch bilayers, respectively.

characterized the ability of these proteins to bind to membranes containing lipid rafts. The membrane binding properties for the partitioning of PLCβ_2 and G$\beta\gamma$ subunits to model membranes have been previously determined using fluorescence techniques, and here, we have extended this study to compare the binding of these proteins to uniform and rafted membranes. To best mimic the surface charge of the plasma membrane, we used POPC/POPS (2:1) membranes for the uniform membrane surfaces. This negative surface allows for strong G$\beta\gamma$ binding (*21*). Since lipid rafts will form in the presence of a high (33 mol %) level of cholesterol, and this formation may be promoted by sphingomylelin, we used POPS/SM/Ch (1:1:1) membranes for the rafted lipid membranes. While this composition is similar to those that have been shown to form rafts, we verified that rafts form in these bilayers by solubilizing 2 mM membranes with 5% (v/v) Triton X-100 and measuring the loss in the percent optical density (% OD) relative to nondetergent controls, based on the method described by Xu and London (*22*). We find that while light scattering from the PC/PS membranes shows complete solublity by Triton giving a % OD value close to 1% (0.91 ± 0.04%) (see ref *25*), the PS/SM/Ch membranes give a % OD value of 50.5 ± 0.3%, indicating that Triton can only solubilize half of the material. This behavior is consistent with lipid raft formation.

We initially measured the membrane binding of PLCβ_2 to lipids by the decrease in intrinsic fluorescence of the enzyme when it binds to the membrane surface. This quenching has been suggested to be due to quenching of interfacial Tyr or Trp residues by the ionic groups on the membrane surface. Previous studies using this method show that PLC isoenzymes bind strongly and nonspecifically [i.e., $K(p) \sim 5-50$ μM] to lipid bilayers with only a minor dependence on lipid composition (*3*). In Figure 1, we show a comparison of the binding of PLCβ_2 to large, unilamellar vesicles composed of PC and PS (2:1), which form uniform surfaces to PS, SM, and Ch (1:1:1) which contain lipid rafts. As can be seen, PLCβ_2 binds to each surface with a similar affinity within experimental error.

To assess the relative binding of G$\beta\gamma$ subunits to membranes, we found that the most reliable results were those that monitored the changes in the emission properties of the membrane probe Laurdan (see refs *3* and *26*). The fluorescent headgroup of Laurdan is very sensitive to the local dielectric environment, and replacement of the protein for water upon binding causes the emission intensity of Laurdan to increase and the energy of its spectra to shift to higher energies. Using membranes doped with 0.1% Laurdan, we followed the 1.5-fold increase in Laurdan intensity when G$\beta\gamma$, solubilized in octyl glucoside, was added to uniform bilayers and those containing rafts. The results depicted in Figure 2 show that G$\beta\gamma$ binds to both uniform and rafted bilayers with comparable affinities.

PLCβ_2−G$\beta\gamma$ Association on Membrane Surfaces. We directly measured the binding of PLCβ_2 to G$\beta\gamma$ on membrane surfaces using fluorescence methods. For these studies, we labeled G$\beta\gamma$ at a 1:1 molar ratio with the highly fluorescent amine-reactive coumarin probe (see Materials and Methods). As noted in Materials and Methods, we found that the emission intensity of all our preparations of coumarin-labeled Gα and G$\beta\gamma$ showed a consistent (i.e., 22−28%) decrease upon addition of unlabeled PLCβ_2. Titration curves based on this increase gave dissociation constants identical to those obtained by fluorescence resonance energy transfer and showed an appropriate concentration shift when the initial G protein concentration was changed. The studies presented here use this method.

Titrations were conducted by labeling G$\beta\gamma$ with coumarin, reconstituting it into bilayers by simple addition, and titrating PLCβ_2 into the solution at lipid concentrations such that all of the PLCβ_2 will be membrane-bound. Since the apparent affinities in this system are directly dependent on lipid concentration, we have compared the affinities at identical amounts of lipid, but we note that these affinities can be easily translated to values independent of membrane concentration (*7*). In Figure 3a, we show a comparison of the lateral association between the two proteins on freshly prepared uniform bilayers and ones containing lipid rafts. We find that the apparent affinity of the two proteins is greatly inhibited on rafted bilayers.

In reproducing these studies, we found an interesting time dependence in the PLCβ_2−G$\beta\gamma$ association over 3 days

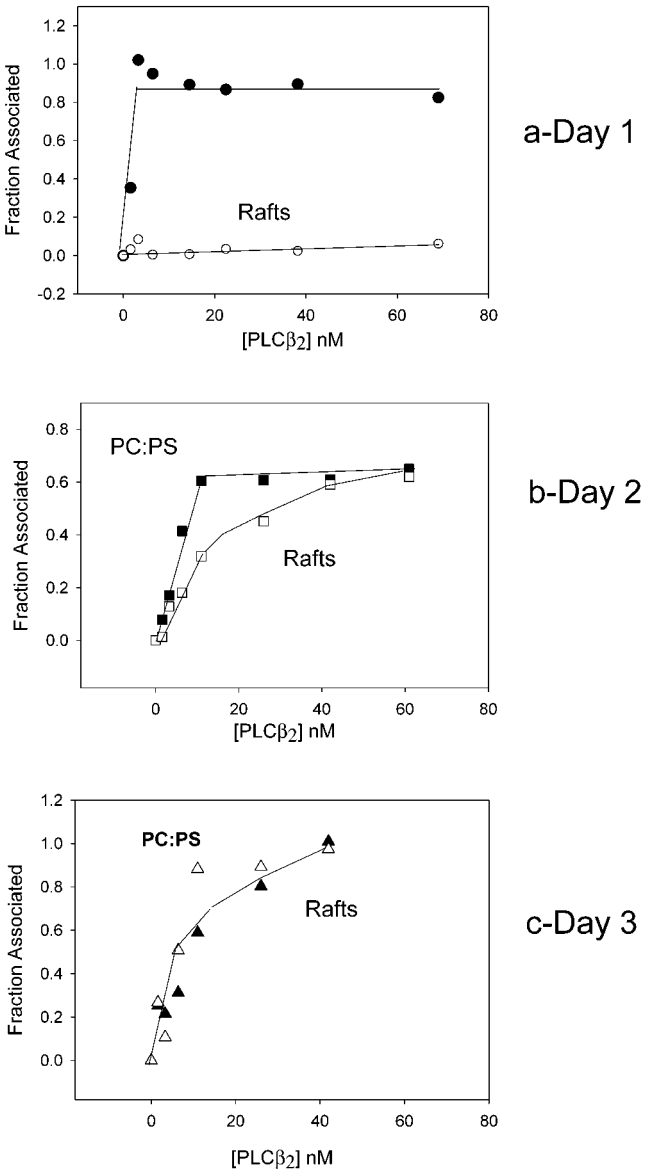

FIGURE 3: Fraction of the PLCβ_2−G$\beta\gamma$ complex formed on 120 μM PC/PS (2:1) (●) or 120 μM PC/SM/Ch (○) bilayer surfaces. Fraction associated was determined by the 25% loss of donor (coumarin−G$\beta\gamma$) fluorescence due to the addition of PLCβ_2. Fluorescence was determined by the integrated area of the peak scanned from 380 to 500 nm by exciting at 340 nm. (a, day 1) Titration curves obtained using freshly labeled proteins and freshly extruded membranes. (b, day 2) Titration curves obtained 24 h after preparation. (c, day 3) Titration curves obtained 48 h after preparation.

(Figure 3a−c). When freshly prepared lipids and labeled proteins were used, the affinity between the two proteins on PC/PS (2:1) surfaces was too strong to be quantified under these conditions, whereas the associations on the surface containing rafts were too weak. Storing the proteins and membranes separately at 4 °C and repeating the experiment over the next 3 days resulted in a dramatic change in the titration behavior. We found that differences in affinities on these two types of surfaces diminished until the third day in which the two became indistinguishable. The decreased affinity on the membranes with uniform surfaces can be attributed to aging of the proteins and is seen in both activity and physical measurements even when using freshly prepared lipids (L. W. Runnels and S. Scarlata, unpublished results).

Table 1: Changes in Anisotropy of 2- and 12-AS in Uniform and Rafted Bilayers upon Treatment with Methyl-β-cyclodextrin[a]

probe	PC/PS (2:1)	PS/SM/Ch (1:1:1)	ratio (rafts/uniform)
2-AS, control	0.120 ± 0.003	0.208 ± 0.005	1.733
2-AS, mβcyclo	0.108 ± 0.003	0.175 ± 0.003	1.62
12-AS, control	0.076 ± 0.003	0.180 ± 0.004	2.37
12-AS, mβcyclo	0.074 ± 0.003	0.137 ± 0.003	1.85

[a] Where control samples were given the identical treatment as the samples containing methyl-β-cyclodextrin (mβcyclo). Anisotropy values were measured using an excitation wavelength of 380 nm and an emission wavelength of 460 nm, and averaged 25 times for two samples. Anisotropy is calculated from the difference between light emitted parallel vs perpendicular to the exciting light over the total light. The anisotropy is related to the microviscosity by the Perrin−Weber equation ($A_o/A - 1 = RT\tau/\eta V$ where A_o is the anisotropy in the absence of rotational motion, R the gas constant, T the absolute temperature, τ the fluorescence lifetime, and η the local viscosity). Values for all of these parameters using these probes have been reported previously (*34*).

Alternatively, the increase in affinity on the bilayers containing lipid rafts may be attributed to aggregation of lipids which is visibly seen by the settling of the isolated PS/SM/Ch membranes within 24 h after preparation, although the extent of this process decreased in the presence of G$\beta\gamma$. We note that visible aggregation of PC/PS (2:1) membranes is only seen at least 10 days after preparation. If the raft domains aggregated and their surfaces were no longer available for protein interaction, then the effective concentration of the proteins on the remaining surfaces would be higher and give a higher apparent K_d. The distinct aging processes of the protein and the lipid and their relative importance result in very different titration behaviors on the two types of surfaces as seen in Figure 3. Thus, only freshly prepared samples were used for further studies.

The lipid rafts used here are formed by high concentrations of cholesterol and sphingomylein. If rafts are causing the decreased level of association of PLCβ_2 and G$\beta\gamma$ on membrane surfaces, then removal of cholesterol should reverse this effect. To test this idea, we treated both sets of membranes with a cholesterol chelator, methyl-β-cyclodextrin. The effectiveness of methyl-β-cyclodextrin treatment was assessed by doping the bilayers with a small amount of either 2- or 12-anthroyloxystearic acid (AS). These probes are incorporated into lipid bilayers either close to the surface (2-AS) or at deeper locations in the membrane (12-AS). Their values of fluorescence anisotropy, which is a unitless quantity, report on the local fluidity of the membranes in which they are embedded (*27*). Incorporation of cholesterol into membranes causes a large reduction in membrane fluidity which can be assessed by an increase in the fluorescence anisotropy of 2-AS and 12-AS due to the decrease in the extent of rotational motion (*25*). Doping PC/PS membranes with either 2-AS or 12-AS and treating them with methyl-β-cyclodextrin had only minor effects on the fluorescence anisotropy (Table 1). The anisotropy values of PS/SM/Ch (1:1:1) samples were initially 45 ± 1 and 59 ± 1% higher than the PC/PS samples for 2-AS and 12-AS probes, respectively. These higher values are due to the decrease in membrane fluidity due to cholesterol. When these rafted membranes were treated with methyl-β-cyclodextrin, these values decreased to 31 ± 1 and 45 ± 1% higher than the PC/PS values for 2-AS and 12-AS, respectively. Thus,

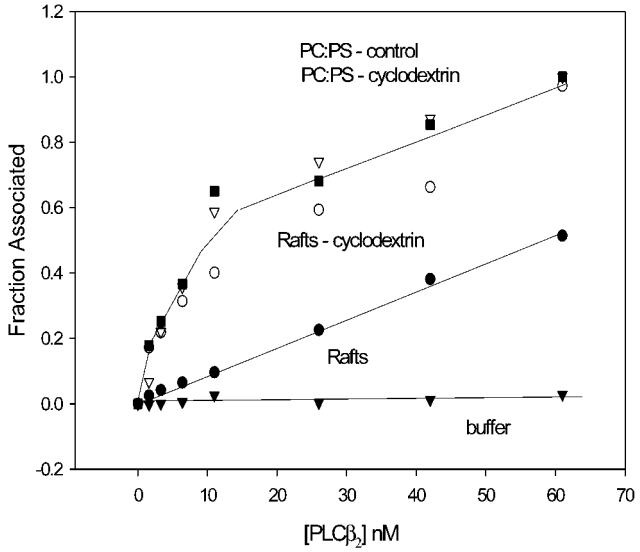

FIGURE 4: Effect of methyl-β-cyclodextrin treatment on G$\beta\gamma$−PLCβ_2 association with PC/PS (2:1) or PS/SM/Ch (1:1:1) membranes. Treatment of PC/PS (2:1) membranes with (▽) and without (■) methyl-β-cyclodextrin. Treatment of PC/SM/Ch (1:1:1) membranes with (○) and without (●) methyl-β-cyclodextrin. Titration where buffer was substituted for PLCβ_2 (▼). Coumarin G$\beta\gamma$ subunits were reconstituted by simple addition onto 400 μM treated lipid immediately prior to fluorescence measurements.

treatment with methyl-β-cyclodextrin results in removal of a significant amount of cholesterol from membranes, consistent with other reports (*26*).

In Figure 4, we show the effect of methyl-β-cyclodextrin treatment on PLCβ_2−G$\beta\gamma$ associations. Larger amounts of lipids were used for these studies to decrease the effective protein concentration on the membrane surface and allow quantification of the dissociation constant. The apparent affinities [K_d(app)] for the protein association on control membranes containing lipid rafts were 10-fold weaker than on PC/PS membranes [K_d(app) = 80.8 ± 15.5 vs 8.4 ± 2.4 nM]. After methyl-β-cyclodextrin treatment, the apparent affinity between these proteins on membranes containing rafts became closer to that measured for the PC/PS bilayers [K_d(app) = 15.6 ± 3.8 vs 8.8 ± 2.2 nM]. These data show that dissolution of a portion of lipid rafts from the membrane through partial removal of cholesterol reverses the diminutive effect on PLCβ_2−G$\beta\gamma$ associations.

The decrease in the level of lateral association between PLCβ_2 and G$\beta\gamma$ as seen by fluorescence methods was verified functionally by the stimulation of PLCβ_2 enzymatic activity by G$\beta\gamma$ subunits. We reasoned that if the association between PLCβ_2 and G$\beta\gamma$ was unchanged in the presence of rafts but could not be detected by fluorescence, then the stimulation of PLCβ_2 activity by G$\beta\gamma$ should be similar in both types of membranes. We tested the G$\beta\gamma$ stimulation of PLCβ_2 hydrolysis using 5% PI(4,5)P_2 incorporated into membrane suspensions containing either PC/PS (2:1) or PS/SM/Ch (1:1:1) membranes that were sonicated briefly at low power. The data in Figure 5 show that larger amounts of G$\beta\gamma$ subunits are required for PLCβ_2 stimulation when its substrate, PI(4,5)P_2, is embedded in membranes containing lipid rafts. Although the membranes used in these studies differed somewhat from the ones used in the fluorescence studies, the data support a diminished affinity between these proteins due to rafts.

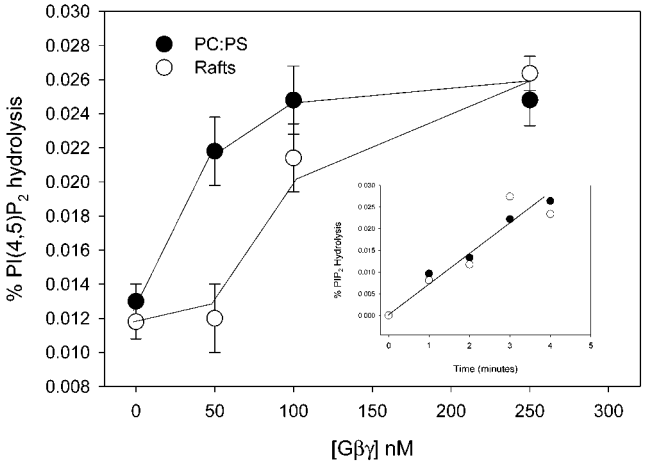

FIGURE 5: Comparison of the activation of 10 nM PLCβ_2 by G$\beta\gamma$ when 5% substrate, PI(4,5)P_2, is incorporated into sonicated PC/PS (2:1) (●) or PC/SM/Ch (○) membrane suspensions. PLCβ_2 has the same basal activity on either PC/PS (2:1) (●) or PC/SM/Ch (○) bilayers (inset).

Inhibition of PLCβ_2−G$\beta\gamma$ Association Due to Lipid Rafts Is Not Due to Differential Partitioning of the Proteins. A simple explanation for the results in Figures 3−5 is that PLCβ_2 and G$\beta\gamma$ partition differently into the fluid and rafted regions of the membranes. Since G$\beta\gamma$ has been shown to favor fluid phase membranes (*14*), then for this explanation to be valid, PLCβ_2 must partition into lipid rafts. We conducted two series of experiments where we studied the interaction of PLCβ_2 with a membrane component that partitions into the fluid phase [i.e., PI(4,5)P_2] and one that partitions into lipid rafts (i.e., Gα_q) to test this hypothesis. We note that the size of lipid rafts has been estimated to be 10−300 nm (*27*), which is large enough to sequester both protein and lipid components into domains. If the actual sizes of rafts under our conditions are 1 order of magnitude smaller, then PI(4,5)P_2 but not Gα_q would be adequately sequestered.

At first, we monitored the effect of rafted surfaces on the enzymatic activity of PLCβ_2. The PI(4,5)P_2 used in these studies was from brain extract, and the majority of acyl chains are unsaturated which cause them to be excluded from rafts and reside only in the fluid regions of model membranes (A. Gambhir and S. McLaughlin, manuscript in preparation). Thus, if PLCβ_2 preferentially partitioned into rafts as opposed to the fluid regions where its substrate resides, we would expect a decrease in basal activity relative to uniform membranes. However, we find that the specific activity is identical when substrate is incorporated into either type of membrane (Figure 5 inset), suggesting that PLCβ_2 does not preferentially partition into rafts.

The ability of PLCβ_2 to partition similarly onto fluid phase domains and lipid rafts was also assessed by measuring its association with a protein that will localize primarily in rafts (i.e., Gα_q−GTPγS subunits). Gα_q subunits will sequester into ordered phases due to favorable packing interactions between its saturated acyl chain modifications and the gel-like rafted lipids (*14*, *16*). We thus carried out a series of studies designed to assess the association between PLCβ_2 and Gα on rafted and uniform surfaces similar to those conducted using G$\beta\gamma$. Unlike G$\beta\gamma$ studies, we find that the lateral associations between PLCβ_2 and Gα are identical on both types of surfaces (Figure 6). If PLCβ_2 preferentialy parti-

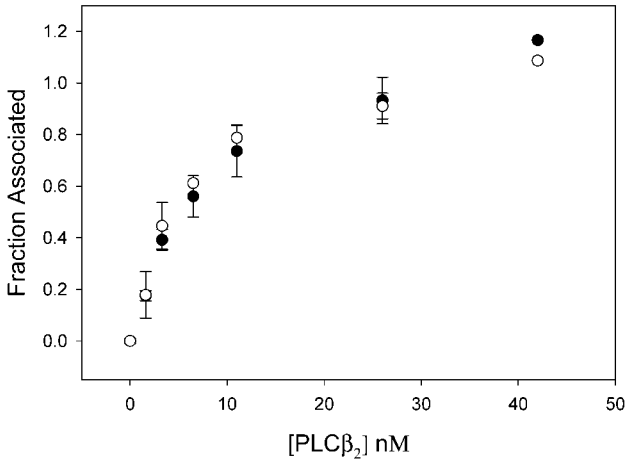

FIGURE 6: Comparison of the lateral association between PLCβ_2 and 10 nM C-Gα_q(GTPγS) on 400 μM PC/PS (2:1) bilayers (●) and 400 μM PS/SM/Ch bilayers (○) as determined by fluorescence methods.

tioned onto the surfaces of rafts, then we would expect its apparent affinity with Gα_q to be greater on the PS/SM/Ch surfaces than on the PC/PS surfaces due to a higher effective concentration of the two proteins corralled in the raft domains. This is not the case. Rather, we find strong association occurs on the surfaces of both types of membranes, indicating that PLCβ_2 moves freely between fluid and raft domains.

Regions of G$\beta\gamma$ Subunits Are Occluded on Rafted Surfaces. Since the interaction between PLCβ_2 and G$\beta\gamma$ is perturbed in the presence of rafts but PLCβ_2−Gα association is not, we explored the possibility that lipid rafts affect the membrane orientation or structure of bound G$\beta\gamma$ subunits.

We first assessed whether lipid rafts may promote aggregation of G$\beta\gamma$ subunits. The basis for this idea is that since G$\beta\gamma$ is excluded from rafts, its effective concentration is higher in the nonraft domains, and this higher concentration may promote self-association of the protein. To test this idea, we monitored the fluorescence homotransfer of G$\beta\gamma$ subunits labeled with the probe Oregon Green (Molecular Probes, Inc.). Fluorescence homotransfer refers to fluorescence resonance energy transfer between identical probes and is detected by the amount of depolarization of the emitted light due to the excited state transfer of energy from donors to acceptors (*30*). Efficient homotransfer occurs when there is a good overlap between the excitation and emission spectra, and probes such as fluorescein and Oregon Green have this characteristic. The value is that at which 50% of the light is transferred (i.e., $R_o \sim 50$ Å).

We labeled G$\beta\gamma$ at a 1:1 probe:protein molar ratio with amine reactive probes as described in Materials and Methods and reconstituted it onto 133 μM PC/PS or PS/SM/Ch surfaces. The anisotropy of OR-labeled G$\beta\gamma$ remained fairly constant at 0.018 ± 0.005 and 0.021 ± 0.006 on the uniform and rafted membranes, respectively, from 2.5 to 110 nM G$\beta\gamma$ which is close to its value in detergent (0.017 ± 0.007). If the protein tended to oligomerize, then a decrease in anisotropy would be seen due to homotransfer. These values remained the same within error over a 3 day period, suggesting that oligomerization does not occur. Thus, lipid rafts do not inhibit PLCβ_2-G$\beta\gamma$ association by promoting G$\beta\gamma$ aggregation.

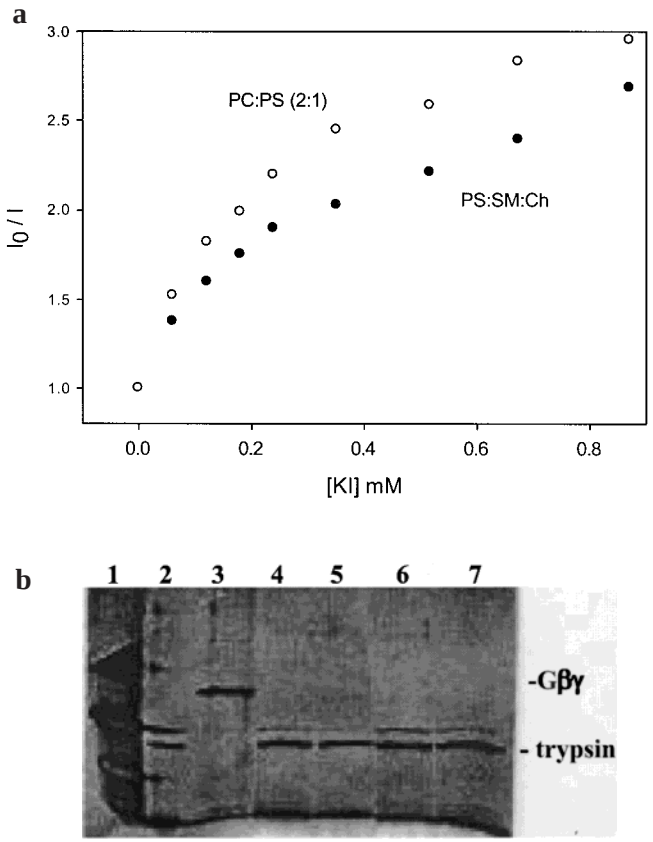

FIGURE 7: (a) Quenching of 100 nM G$\beta\gamma$ Tyr and Trp residues, where G$\beta\gamma$ is reconstituted on PC/PS (2:1) (○) or PC/SM/Ch (●) bilayers, by the addition of KI as shown in Stern−Volmer form, i.e., I_0/I, where I_0 is the intensity before the addition of quencher. (b) SDS electrophoresis pattern visualized by silver staining: lane 1, low-molecular mass markers; lane 2, trypsin; lane 3, Gβ; lane 4, Gβ reconstituted on PC/PS (2:1) bilayers after trypsin digestion for 5 min; lane 5, Gβ reconstituted on PS/SM/Ch (1:1:1) bilayers after trypsin digestion for 5 min; lane 6, Gβ reconstituted on PC/PS (2:1) bilayers after trypsin digestion for 20 min; and lane 7, Gβ reconstituted on PS/SM/Ch (1:1:1) bilayers after trypsin digestion for 5 min. Note that Gγ, with a molecular mass of 7 kDa, is too small to be resolved in these gels.

To assess the possibility that the presence of rafts alters the orientation of G$\beta\gamma$ on the membrane surface so as to occlude PLCβ_2 interaction sites, we measured the ability of the fluorescence emanating from seven Trp and five Tyr residues of G$\beta\gamma$ to be quenched by iodide ions. The most accessible residues are quenched at low iodide concentrations, while high iodide concentrations are needed to quench the more shielded residues. Curvature in the plots indicates a transition from the more to less accessible residues. We find that when G$\beta\gamma$ is bound to PS/SM/Ch membranes, its Trp and Tyr residues are less accessible to quenching by iodide ions over a large concentration range than when it is bound to PC/PS membranes (Figure 7a) as seen by the lower I_0/I values across the titration curve. Thus, the presence of lipid rafts protects a portion of the Trp and Try population from anionic quenchers.

We also compared the trypsin digestion pattern of G$\beta\gamma$ bound to rafts as opposed to uniform membranes. On both membrane surfaces, trypsin cleaves Gβ from a band whose electrophoretic migration corresponds to a 35 kDa band to one at 26 kDa, or ~75 residues. However, when we find that when G$\beta\gamma$ is bound to PS/SM/Ch membranes, it is more

susceptible to trypsin digestion (Figure 7b) than when it is bound to PC/PS membranes.

DISCUSSION

In this study, we have found that the presence of lipid rafts inhibits the interactions between G$\beta\gamma$ and PLCβ_2. Inhibition was observed in both the physical association of these proteins (Figure 3) and the ability of PLCβ_2 to be activated by G$\beta\gamma$ (Figure 5). In contrast, the presence of rafts did not alter the ability of PLCβ_2 to access its substrate in the fluid phase (Figure 5 inset), or affect its association with activated Gα_q (Figure 6). Our studies indicate that inhibition of PLCβ_2−G$\beta\gamma$ subunits was not due to differential partitioning of the proteins into the fluid and raft domains. Rather, inhibition appears to be caused by a change in the membrane orientation of G$\beta\gamma$ when rafts are present.

Since G$\beta\gamma$ binds strongly to membranes containing negatively charged lipids (*21*), we performed our experiments on model membranes with negative surfaces and used large amounts of cholesterol to promote raft formation (*11*) which was verified by the resistance to detergent solubilization. Cholesterol is a planar, rigid molecule, and it packs well with lipids which have saturated hydrocarbon chains. Also, cholesterol will form tightly packed stoichiometric complexes with sphingolipids (*29*), and sphingomylein may promote raft formation. These model systems correlate well to the composition of detergent-resistant membranes isolated from cells (*8*).

Previous work has shown that the two G protein subunits tested here, Gα_q and G$\beta\gamma$, will preferentially partition into different lipid phases (*14*). The saturated acyl chains on Gα_q would pack well into rafted domains allowing strong van der Waals interactions and kinetically trap the subunits into the domains. Alternatively, the unsaturated, kinked geranylgeranyl chain of G$\beta\gamma$ will sequester into fluid phases, increasing the retention time of the subunit in these regions of the membrane. The observation that Gα_q−PLCβ_2 association is the same in the presence or absence of rafts, and that the PLCβ_2-catalyzed hydrolysis of the fluid phase PI(4,5)P_2 is unchanged, indicates that PLCβ_2 can diffuse freely between the two phases. We note that since PLCβ_2−G protein complexes are long-lived [~120 s, much longer than the lateral diffusion rate (*37*, *38*)], then differences in the retention time of PLCβ_2 on uniform and raft surfaces may not be apparent. These kinetic studies are now being planned.

The ordered nature of lipid rafts due to extended dispersion interactions confers topological features on the membrane surface. It is this distinct surface structure that may be responsible for the inhibition of PLCβ_2−G$\beta\gamma$ association. Atomic force microscopy studies of POPC/SM mixtures show SM domains that are substantially higher than POPC (C. LeGrimellec, personal communication; *33*). If a comparable situation exists in the membranes used here, then the interface between PS and the SM/Ch-rich aggregates may be a significant portion of the height of G$\beta\gamma$ (*31*). If G$\beta\gamma$ localizes close to the raft domain interface, then some of its residues may be occluded by the domain interface. Since the interaction between G$\beta\gamma$ and PLCβ_2 is inhibited by rafts, we speculate that the region of G$\beta\gamma$ that localizes at the raft domain interface may be common with the PLCβ2 binding site.

The G$\beta_1\gamma_2$ subunits used in this study bind more strongly to negatively charged lipids than electrically neutral lipids (S. Scarlata and L. W. Runnels, unpublished results), consistent with other reports showing an electrostatic component in $G_t\beta\gamma$- membrane association (*4*, *5*). Recent computational studies show a lobe of strong positive potential in $G_t\beta\gamma$ that would favor binding to anionic lipids (*21*). Interestingly, above this positive surface is an anionic region that could destabilize membrane binding if the surface potential was too high. In these studies, we controlled for overall membrane charge by using 33% negatively charged POPS in both the uniform and rafted membranes. However, in the uniform membranes, POPS is expected to be evenly dispersed with POPC, whereas in the presence of electrically neutral SM/Ch rafts, POPS will phase separate, creating fluid, highly anionic regions with which G$\beta\gamma$ can associate. This highly electronegative environment may cause the protein to favor the anionic−neutral raft domain interface. In their analysis, Murray and co-workers note that the residues that form the basic patch in $G_t\beta$ are conserved in all mammalian Gβ subunits, and it is possible that other electrical characteristics are conserved as well. Thus, the electronegativity of the surface may modulate the membrane orientation of G$\beta\gamma$ subunits.

Our studies indicate an increase in the level of trypsin cleavage and a decrease in the level of Trp and Tyr quenching by iodide ions when G$\beta\gamma$ is bound to PS/SM/Ch surfaces. While the decreased level of fluorescence quenching may be caused by the charged repulsion from the PS headgroups in the fluid regions where G$\beta\gamma$ resides, the trypsin digestion studies can only be rationalized by a difference in the disposition of G$\beta\gamma$ on the surface of membranes containing rafts. In recent work using peptides corresponding to various G$\beta\gamma$ regions, Buck and Iyengar (*32*) show that G$\beta\gamma$ has multiple PLCβ_2 binding regions and that other distinct regions confer PLCβ_2 activation (*32*). The three major binding regions span residues 115−135, 228−249, and 321−340. Noting that the N-terminal region of G$\beta_1\gamma_2$ is rich in positively charged residues that are substrates for trypsin, whereas the C-terminal region contains more Trp and Tyr residues, we speculate that the PLCβ_2 binding domains in the C-terminal regions of G$\beta\gamma$ are occluded by the interface region of the raft domains. Structure-based studies designed to confirm this hypothesis are currently underway.

We can only speculate about the biological relevance of these observations. The acyl modifications on the G protein subunits may allow these proteins to localize in different membrane compartments for stable heterotrimers to form. Saturated acyl groups are stable in rafts, but clearly may partition into fluid phase lipid regions. Also, recent studies show that under conditions where all species are membrane-bound, the gerenylgerenyl group on G$\beta\gamma$ is required for PLCβ_2 association and activation (*33*), suggesting that the gerenylgerenyl group is not deeply embedded in the lipid matrix and may possibly be associated with the protein itself. Thus, the localization of G$\alpha\beta\gamma$ in rafts may be a dynamic process in which activation of an initial raft-bound heterotrimer releases G$\beta\gamma$ to the fluid or to the interface of the raft domain if the local surface charge was very high. As our data suggest, the proposed association of G$\beta\gamma$ with the domain interface would inhibit activation of PLCβ_2, but may leave other binding sites available to alternate protein partners.

ACKNOWLEDGMENT

I am indebted to Dr. Yuanjian Gao and Louisa Dowal for providing the proteins and to Dr. Deborah Brown, Dr. Yuanjian Gao, and Vijaya Narayanan for critical reading of the manuscript.

REFERENCES

1. Rhee, S. G. (2001) *Annu. Rev. Biochem. 70*, 281−312.
2. Rebecchi, M., and Pentylana, S. (2000) *Physiol. Rev. 80*, 1291−1335.
3. Runnels, L. W., Jenco, J., Morris, A., and Scarlata, S. (1996) *Biochemistry 35*, 16824−16832.
4. Matsuda, T., Takao, T., Shimonishi, Y., Murata, M., Asano, T., Yoshizawa, T., and Fukada, Y. (1994) *J. Biol. Chem. 269*, 30358−30363.
5. Seitz, H., Heck, M., Hofmann, K., Alt, T., Pelland, J., and Seelig, A. (1999) *Biochemistry 38*, 7950−7960.
6. Runnels, L. W., and Scarlata, S. F. (1998) *Biochemistry 37*, 15563−15574.
7. Runnels, L. W., and Scarlata, S. (1999) *Biochemistry 38*, 1488−1496.
8. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell Dev. Biol. 14*, 111−136.
9. Anderson, R. G. (1998) *Annu. Rev. Biochem. 67*, 199−225.
10. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) *J. Biol. Chem. 273*, 5419−5422.
11. Ahmed, S. N., Brown, D. A., and London, E. (1997) *Biochemistry 36*, 10944−10953.
12. Sankaram, M. B., and Thompson, T. E. (1990) *Biochemistry 29*, 10670−10675.
13. Silvius, J., del Giudice, D., and LaFleur, M. (1996) *Biochemistry 35*, 15198−15208.
14. Moffett, S., Brown, D. A., and Linder, M. E. (2000) *J. Biol. Chem. 275*, 2191−2198.
15. McCabe, J. B., and Berthiaume, L. G. (2001) *Mol. Biol. Cell 12*, 3601−3617.
16. Oh, P., and Schnitzer, J. E. (2001) *Mol. Biol. Cell 12*, 685−698.
17. Prior, I. A., Harding, A., Yan, J., Sluimer, J., Parton, R. G., and Hancock, J. F. (2001) *Nat. Cell Biol. 3*, 368−375.
18. Gomez-Mouton, C., Abad, J. L., Mira, E., Lacalle, R. A., Gallardo, E., Jimenez-Baranda, S., Illa, I., Bernad, A., Manes, S., and Martinez-A., C. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 9642−9647.
19. Xiao, Z., and Devreotes, P. N. (1997) *Mol. Biol. Cell 8*, 855−869.
20. Miura, Y., Hanada, K., and Jones, T. L. Z. (2001) *Biochemistry 40*, 15418−15423.
21. Murray, D., McLaughlin, S., and Honig, B. (2001) *J. Biol. Chem. 276*, 45153−45159.
22. Xu, X., and London, E. (2000) *Biochemistry 39*, 843−849.
23. Wang, D. S., Miller, R., Shaw, R., and Shaw, G. (1996) *Biochem. Biophys. Res. Commun. 225*, 420−426.
24. Scarlata, S. F. (1996) in *High Pressure Effects in Molecular Biophysics and Enzymology*, Oxford Unversity Press, New York.
25. Sankaram, M. B., and Thompson, T. E. (1990) *Biochemistry 29*, 10676−10684.
26. Gidwani, A., and Baird, B. (2001) *Biochemistry 40*, 12422−12429.
27. Pierini, L. M., and Maxfield, F. R. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 9471−9473.
28. Runnels, L. W., and Scarlata, S. F. (1995) *Biophys. J. 69*, 1569−1583.
29. Smaby, J. M., Brockman, H. L., and Brown, R. E. (1994) *Biochemistry 33*, 9135−9142.
30. Giocondi, M. C., Vie, V., Lesniewska, E., Goudonnet, J. P., and LeGrimellec, C. (2000) *J. Struct. Biol. 131*, 38−43.
31. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell 83*, 1047−1058.
32. Buck, E., and Iyengar, R. (2001) *J. Biol. Chem. 276*, 36014−36019.
33. Fogg, V. C., Azpiazu, I., Linder, M. E., Smrcka, A., Scarlata, S., and Gautam, N. (2001) *J. Biol. Chem. 276*, 41797−41802.
34. Scarlata, S. (1989) *Biophys. J. 55*, 1215−1223.

BI025625J