Determination of the Activation Volume of PLCβ by G$\beta\gamma$-Subunits through the Use of High Hydrostatic Pressure

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ABSTRACT Activation of phospholipase Cβ (PLCβ) by G-proteins results in increased intracellular Ca^{2+} and activation of protein kinase C. We have previously found that activated PLCβ-G$\beta\gamma$ complex can be rapidly deactivated by Gα(GDP) subunits without dissociation, which led to the suggestion that Gα(GDP) binds to PLCβ-G$\beta\gamma$ and perturbs the activating interaction without significantly affecting the PLCβ-G$\beta\gamma$ binding energy. Here, we have used high pressure fluorescence spectroscopy to determine the volume change associated with this interaction. Since PLCβ and G-protein subunits associate on membrane surfaces, we worked under conditions where the membrane surface properties are not expected to change. We also determined the pressure range in which the proteins remain membrane bound: PLCβ binding was stable throughout the 1–2000 bars range, G$\beta\gamma$ binding was stable only at high membrane concentrations, whereas Gα_s(GDP) dissociated from membranes above 1 kbar. High pressure dissociated PLCβ-G$\beta\gamma$ with a $\Delta V = 34 \pm 5$ ml/mol. This same volume change is obtained for a peptide derived from Gβ which also activates PLCβ. In the presence of Gα_s(GDP), the volume change associated with PLCβ-G$\beta\gamma$ interaction is reduced to 25 ± 1 ml/mol. These results suggest that activation of PLCβ by G$\beta\gamma$ is conferred by a small (i.e., 3–15 ml/mol) volume element.

INTRODUCTION

Signal transduction through heterotrimeric G-proteins is initiated by the binding of an extracellular ligand to its corresponding G-protein coupled receptor (for background, see Alberts et al., 1994). The receptor in turn catalyzes the exchange of GTP for GDP on the α-subunit of a heterotrimeric G-protein. In the basal state, Gα(GDP) is strongly bound to G$\beta\gamma$-subunits, but GTP-bound Gα has a much weaker affinity (e.g., Runnels and Scarlata, 1999). This reduced affinity allows both Gα(GTP) and G$\beta\gamma$ to bind to and change the catalytic activity of a variety of intracellular effector proteins. There are four families of Gα-subunits, and phospholipase C-β(PLCβ) is the main effector of the Gα_q family (Rebecchi and Pentyala, 2000; Rhee, 2001). There are four known families of PLCβ enzymes and all are activated by Gα_q-subunits (for review, see Rebecchi and Pentyala, 2000; Rhee, 2001). Additionally, PLCβ_2 and β_3 can independently be activated by G$\beta\gamma$-subunits as well.

PLCβ enzymes are part of a larger family of mammalian PLCs. These enzymes catalyze the hydrolysis of a minor component in membranes, PI(4,5)P_2, to generate two second messengers which lead to a rise in intracellular calcium and activation of protein kinase C (see Rebecchi and Pentyala, 2000; Rhee, 2001). Thus, activation by an agonist through the PLCβ-G-proteins pathway affects the activity of a host of Ca^{2+}-sensitive enzymes.

Gα_q and G$\beta\gamma$ are bound to the plasma membrane through strong interactions that in part involve posttranslational modifications; Gα_q is dually palmitoylated, whereas G$\beta\gamma$ is geranylgeranylated (Kleuss and Krause, 2003; Wedegaertner et al., 1995). PLCβ does not have these modifications and is aqueous soluble; however, it binds strongly to membrane surfaces with little specifically (Runnels et al., 1996). Activation of PLCβ by G-protein subunits thus appears to occur through lateral association of the membrane-bound species.

Our laboratory has previously characterized the affinity between PLCβs and their G-protein activators on model membrane surfaces using FRET (Runnels and Scarlata, 1999). In a study that focused on the interaction between PLCβ_2- and G$\beta\gamma$-subunits, we found, not surprisingly, that the measured steady-state dissociation constant for the two proteins was equal to the ratio of the on- and off-rates (Runnels and Scarlata, 1998). However, the off-rate of the association was too slow to be physiologically relevant (~2 min), suggesting that other cellular processes might serve to reduce the off-rate. One possibility was deactivated Gα-subunits that could displace G$\beta\gamma$ from PLCβ_2, but the addition of a large molar excess of Gα_i(GDP)-subunits did not affect either the on- or off-rates. Even though Gα_i(GDP) did not significantly affect the PLCβ_2-G$\beta\gamma$ equilibrium, it unexpectedly resulted in rapid deactivation of the PLCβ-G$\beta\gamma$ complex when PLCβ activity was monitored (Runnels and Scarlata, 1998). It was then suggested that PLCβ_2 binds to G$\beta\gamma$-subunits through several strong contacts, but that activation proceeds through more subtle, weaker contacts. Gα_i(GDP) can then bind to activated PLCβ_2-G$\beta\gamma$ and perturb the weak activating

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Abbreviations used: PLC, mammalian phosphoinositide-specific phospholipase C; PI(4,5)P_2, phosphatidylinositol 4,5- bisphosphate; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; PE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; PH, pleckstrin homology; DNS-PE, dansyl phosphadtiylethanolamine; FRET, fluorescence resonance energy transfer.

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interactions without affecting the strong binding interactions as depicted in the scheme shown in Fig. 1.

The model described above implies that activation of PLCβ_2 by G$\beta\gamma$-subunits occurs through one or more small interactions whose energies are within the experimental error of the measured affinities. Here, we have used high hydrostatic pressure to determine the volume change association with these interactions. By comparing the volume change of PLCβ-G$\beta\gamma$ alone and in the presence of Gα_s(GDP), we can estimate the volume of activation of PLCβ_2 by G$\beta\gamma$-subunits.

It is notable that although pressure techniques have been used to monitor the associations between aqueous soluble proteins, pressure has not yet been applied to monitor lateral associations of membrane-bound proteins (for recent reviews, see Ernst, 2002). In general, the application of hydrostatic pressure in the range of 1–2000 bar (where 1 bar = 1.013 atm) forces solvent into intersubunit voids resulting in subunit dissociation. Higher pressures force solvent into the protein matrix, resulting in unfolding through a molten globule state. For membrane-bound proteins, we expect a similar dissociation mechanism, assuming that pressure does not alter the properties of the membrane surface or promote dissociation of the membrane-bound proteins. Therefore, we characterized the membrane binding behavior of PLCβ and G-protein subunits before protein-protein association studies. We find that pressure enables us to observe the perturbations of PLCβ-G$\beta\gamma$ association caused by Gα(GDP) subunits that were not observable by atmospheric association measurements.

MATERIALS AND METHODS

Peptides and proteins

The synthetic peptide corresponding to residues 86–105 of Gβ_1 (NH_2-TTNKVHAIPLRSSWVMTCAY-$CONH_2$) and the corresponding control peptide that does not activate PLCβ (Gβ86-105(M101N)) were gifts from Dr. Ravi Iyengar (Dept. of Pharmacology, Mt. Sinai School of Medicine).

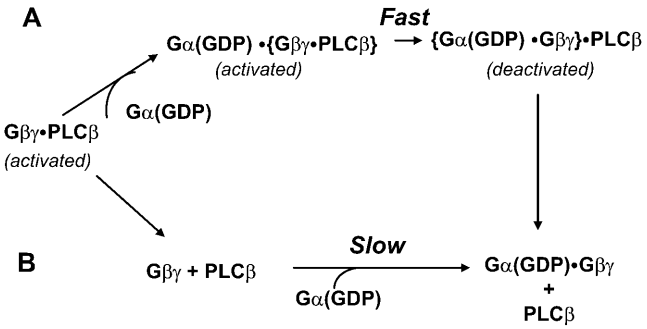

FIGURE 1 Reaction scheme of the two pathways of deactivation of PLCβ-G$\beta\gamma$. The fast one, depicted in the upper scheme (*A*) occurs when local Gα(GDP)-subunits bind to G$\beta\gamma$-subunits and disrupt the interactions that result in PLCβ activation. The slow pathway in the lower panel, *B*, occurs through a simple biomolecular dissociation of the G$\beta\gamma$-PLCβ and subsequent recombination of the Gα(GDP)-G$\beta\gamma$ heterotrimer.

G$\beta_1\gamma_2$, termed G$\beta\gamma$, was expressed in Sf9 cells using a baculovirus system and purified as previously described (see Runnels et al., 1996). Gα_s was purified from SP9 cells by the same methods used for Gα_q purification (see Runnels et al., 1996). The Sf9 expression system allows for postsynthetic modifications of the proteins. The stability of the palmitoyl chains on Gα-subunits and the geranylgeranyl chain on Gγ_2-subunits was assessed by thin layer chromatography on LK5D linear-k silica gel thin layer chromatography plates (Whatman, Florham Park, NJ) as detailed before (Philip and Scarlata, 2004). The plates were developed in chloroform/methanol/2.5M ammonium hydroxide (9:7:2, v/v) under nonreducing conditions. To remove the hydrophobic modifications, the protein was treated with 1mM dithiothreitol.

These studies employed a chimeric PLCβ in which the N-terminal PH domain, encompassing residues 1–134 of PLCβ_2 replaces residues 1–117 of PLCδ_1 (Wang et al., 2000). This enzyme lacks the long C-terminal domain of PLCβ, which is necessary for activation by Gα_q(GTP). However, the PLCβ_2 N-terminal PH domain allows for full activation by G$\beta\gamma$-subunits (Wang et al., 2000). Preliminary activity studies show that addition of Gα_s(GDP) deactivates the activated PLCβ-G$\beta\gamma$ complex similar to the behavior seen for the full length enzyme. An advantage of this PLCβ construct is that it can be bacterially expressed (see Wang et al., 2000).

Lipids

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Lipids were mixed in chloroform and dried to a thin film in a small pear shaped flask using a rotary evaporator. Dried lipids were suspended in 150mM NaCl, 20mM Hepes (pH 7.2) buffer and taken through 10 alternating freeze-thaw cycles using liquid nitrogen and a 37°C water bath. Large unilamellar vesicles (LUVs) were freshly prepared by extrusion through a 100-nm pore filter before every experiment. For DNS-PE-labeled membranes, the labeled lipid was added to the chloroform lipid solution before drying.

Fluorescent labeling and measurements

Proteins were labeled with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM). This probe becomes fluorescent when it reacts with cysteine side chains (see Molecular Probes Handbook, Eugene, OR). Proteins were labeled with CPM by removing the reducing agents by dialysis against buffer composed of 20 mM Hepes, 0.16 M KCl, 1 mM EGTA, pH 7.2 and then adding the probe at a 2:1 molar ratio. The labeling reaction was quenched after 10 min by the addition of 20 mM β-mercaptoethanol.

Fluorescence measurements and analysis

Fluorescence measurements were performed on an ISS spectrofluorometer (Champaign, IL). CPM-labeled proteins were excited at 385 nm and scanned from 420 to 540 nm. Atmospheric measurements were performed using 3-mm cuvettes. Pressure measurements were performed using a small quartz bottle with a lid that collapses under pressure. The bottle was then placed in a homemade optical pressure cell based on the design of Paladini and Weber (1981). The pressure cell was inserted into the optical module of the fluorometer allowing for fluorescence measurements under pressure.

Membrane binding for PLCβ was determined by following the increase in acceptor fluorescence from PLCβ Trp donors to model membranes doped with 0.1% DNS-PE using unlabeled protein as a control (Runnels et al., 1996). Membrane binding of the G-protein subunits was followed by changes in the fluorescence of the CPM-labeled proteins (see Philip and Scarlata, 2004). These measurements monitored the change in fluorescence of a dilute solution of labeled protein as freshly extruded LUVs were added. All results were fit to a binding isotherm after correcting for dilution and background scattering. The reported partition coefficient refers to the lipid concentration at which the amount of bound versus unbound protein ($K_p = [P_b]/[P_{un}]$) is identical.

PLCβ-G$\beta\gamma$ association was determined by the change in emission of CPM labeled on either of the two proteins as its unlabeled partner is added. We have found that the emission intensity of labeled coumarin-PLCβ and coumarin-G$\beta\gamma$ showed a substantial and reproducible increase upon the addition of its unlabeled partner, which gives apparent K_d values similar to those obtained using FRET (Guo et al., 2003; Philip and Scarlata, 2004). Similar to the results obtained for the proteins labeled with other probes, the titration curves for the proteins labeled with CPM showed the appropriate shift in midpoint when the initial concentration of CPM-labeled protein was changed, thus showing that the changes in coumarin fluorescence reflect protein-protein associations.

Data analysis

The pressure results were analyzed as follows. The volume change associated with a particular reaction at constant temperature is equal to the change in free energy as a function of pressure:

$$(\mathrm{d}\Delta G/\mathrm{dp})_{\mathrm{T}} = \Delta V, \quad \text{where} \quad \Delta G = -RT\ln K.$$

Substituting for ΔG and integrating the expression from atmospheric to pressure, p, and assuming that ΔV is constant in the pressure range, gives

$$\ln K(\mathrm{p}) - \ln K(\mathrm{atm}) = p\Delta V/RT \text{ at constant temperature.}$$

The value of K(atm) for the protein-protein or protein-membrane associations described in this study was determined from titration measurements conducted at atmospheric pressure. The value of K(p) was calculated by knowing the initial concentrations and degree of dissociation (D) of the complexes. We then calculated the change in D with pressure by comparing the fluorescence emission energy at pressure, p, to the value of fluorescence at the same pressure for the labeled sample under conditions were it is not bound to a partner (u) for fully bound (b). These latter two curves were run independently. Therefore,

$$D(\mathrm{p}) = (D(\mathrm{p}) - D(\mathrm{u}))/(D(\mathrm{b}) - D(\mathrm{u})).$$

Conversion of the apparent K(d) obtained on membrane surfaces to the K(d) that would be obtained if the proteins were freely diffusing has been previously treated by assuming that the proteins are confined to interact within a reduced surface volume (v) that is equal to the surface area of the membrane multiplied by the distance from the membrane surface into the aqueous phase where the proteins interact, which is taken to be 50 nm. For typical membrane concentrations used in these studies, the apparent K(d) should be ~200-fold stronger than the true K(d) (see Runnels and Scarlata, 1999).

RESULTS

Measurement of protein-membrane associations under pressure

Previous studies of model membranes under pressure suggest that the primary effect of pressure is in the compressible hydrocarbon region, and the application of pressure increases the gel to liquid crystal phase transition by a constant that depends primarily on the lipid chains (Li and Kay, 1977). At room temperature, membranes composed of 1-, 2-dioleoyl (DO) chains, which melt at −18°C, will undergo the gel to liquid crystal phase transition close to 2.2 kbar, whereas membranes composed of 1-palmitoyl, 2-oleoyl chains will undergo this transition around 1.5 kbar (see Sassaroli et al., 1993). In contrast to the interior, pressure has little effect on the membrane surface charge (Scarlata and Rosenberg, 1990). We have found that for membranes composed of lipids with electrically neutral or negatively charged head groups, no change in surface charge could be detected at neutral pH from 1 to 2000 bar. Additionally, little change in membrane polarity and hydration of POPC/PI(4,5)P_2 (67:33) membranes are detected in this same pressure range (Rebecchi et al., 1999). Therefore, the following studies were conducted assuming that properties of the membrane surface are largely unchanged in the 1–2000 bar range.

We have previously characterized the binding of PLC$\beta_{1\text{-}3}$ enzymes to different model membranes and found that they bind strongly to various membranes with little specificity (Runnels et al., 1996). Here, we have used fluorescence methods to follow the membrane association of a chimeric PLCβ/δ, referred to here simply as PLCβ (see Methods) under pressure. We monitored membrane binding by FRET from PLCβ tryptophan side chains to DNS-PE incorporated into POPC/POPS (2:1) LUVs. We found no changes in DNS fluorescence over 1–2000 bars, suggesting stable membrane binding even through the phase transition (Fig. 2).

In contrast to the pressure behavior seen for PLCβ membrane interactions, we find that G$\beta\gamma$-subunits are perturbed under pressure. Membrane association of G$\beta\gamma$ can be monitored by changes in emission of a covalently linked CPM probe (Fig. 3 *A*). In Fig. 3 *B*, we show results where we have monitored the change in fluorescence of CPM-G$\beta\gamma$ bound to membranes as a function of pressure. At subsaturation levels of membranes, G$\beta\gamma$ dissociates above 1000 atm, which may be due to the phase transition, but at higher lipid

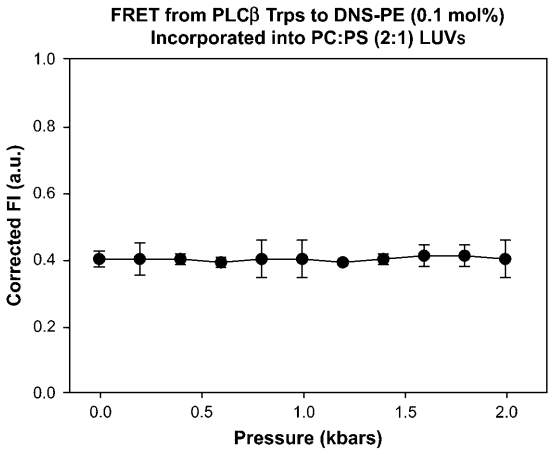

FIGURE 2 Measurement of the change in fluorescence resonance energy transfer from PLCβ Trp donors at 100 nM to DNS-PE acceptors under pressure. This study monitored the fluorescence of DNS-PE, incorporated into 50 μM POPC/POPS (2:1) large, unilamellar vesicles at 0.1 mol % where the fractional increase in acceptor was determined by the integrated DNS intensity exciting at 280 nm and scanning from 380-520 as compared to control samples where buffer was substituted for PLCβ, where $n = 3$ and standard error is shown. It is noteworthy that changes in DNS intensity may also result from dequenching when bound protein displaces water. We did not differentiate between these processes.

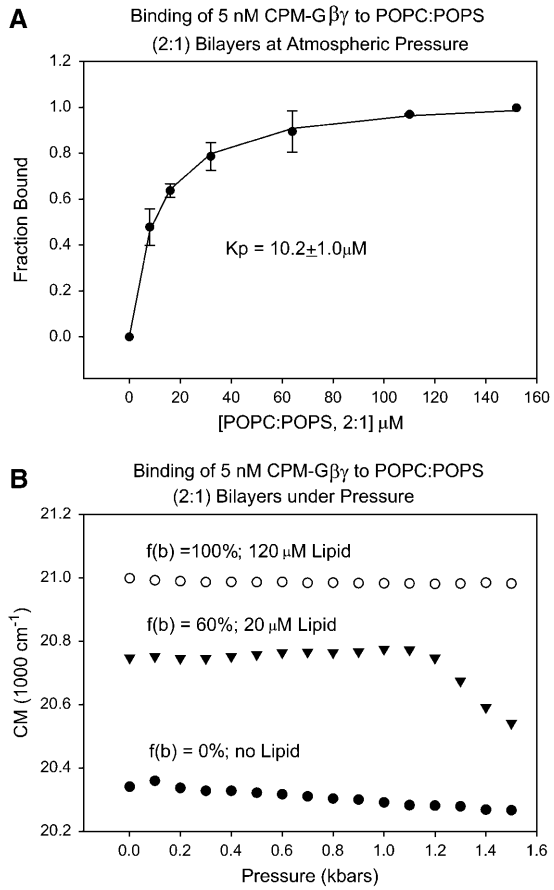

FIGURE 3 (*A*) Binding of 5 nM CPM-G$\beta\gamma$ to POPC/POPS (2:1) bilayers at atmospheric pressure as measured by the 1.8-fold increase in emission intensity as bilayers are added, after correction for dilution and background, which substituted buffer for protein, where $n = 3$ and standard error is shown. (*B*) Binding of 5 nM CPM-G$\beta\gamma$ to 0, 20, and 120 μM POPC/POPS (2:1) bilayers as a function of pressure showing that under partially bound conditions, high pressure destabilizes membrane binding ($n = 2$ and the sample-to-sample error is within 50 cm^{-1}).

concentrations, CPM-G$\beta\gamma$ membrane association is stable throughout the pressure range (Fig. 3 *B*).

We also tested the ability of Gα_s to remain membrane-bound under pressure. In Fig. 4, we show that Gα_s is destabilized under pressure even at relatively high lipid concentrations, although we note that membrane binding of Gα_s is weaker than G$\beta\gamma$ (i.e., $K_p \sim 70$ as opposed to 10 μM; Philip and Scarlata, 2004). Gα_s is modified by two palmitoyl chains (Kleuss and Krause, 2003) which are thought to contribute to its membrane stability. Palmitoyl groups are typically attached to Cys side chains, and these linkages can be reversed by the addition of reducing agents. We treated Gα_s(GDP) with dithiothreitol (see Philip and Scarlata, 2004) and found that its pressure behavior was unchanged (data not shown). Destabilization under pressure could be caused by the gel to liquid crystal phase transition. Therefore, studies using pressure studies using Gα_s were limited to a maximum of 1 kbar in pressure.

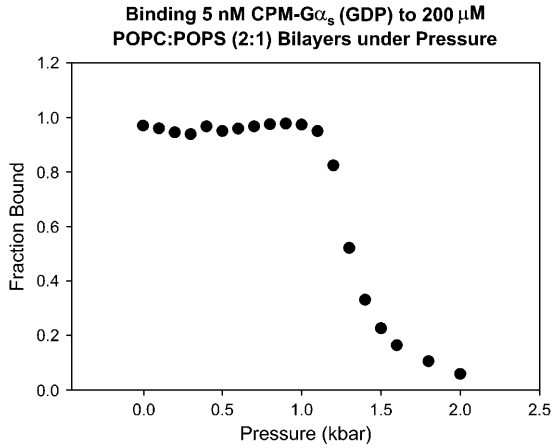

FIGURE 4 A similar binding study as in Fig. 3 *B* ($n = 2$) showing the pressure destabilization of CPM-Gα_s(GDP), where the membrane partition coefficient at atmospheric pressure is $\sim$70 μM as recently reported and was calculated by the 220 cm^{-1} change in CPM-Gα_s(GDP) emission energy (see (Philip and Scarlata, 2004).

Membrane-bound PLCβ appears stable under pressure

In previous work, we found that pressure increased the reaction rate of PLCδ (Rebecchi et al., 1999), and we suggested that pressure stabilizes an activated form of the enzyme. To determine whether this could be the case for PLCβ, we labeled the enzyme with CPM. CPM-PLCβ is sensitive to binding by G$\beta\gamma$-subunits giving an $\sim$200 cm^{-1} shift in emission energy and an $\sim$2-fold increase in intensity (G. Drin and S. Scarlata, unpublished), allowing us to measure the apparent K_d at a given membrane concentration (Fig. 5 *A*, and see below). The concentration dependence of binding closely matches the activation profile (Runnels and Scarlata, 1998).

Using CPM-PLCβ as a readout of possible conformational changes corresponding to G$\beta\gamma$ activation, we monitored CPM-PLCβ fluorescence under pressure and compared it directly to using CPM-PLCβ with supersaturating levels of G$\beta\gamma$-subunits. We find that pressure does not increase the tendency of CPM-PLCβ toward the fluorescence emission properties of the activated, G$\beta\gamma$-bound form (Fig. 5 *B*).

Characterization of CPM-PLCβ-G$\beta\gamma$ associations at atmospheric pressure

In Fig. 5 *A*, we present results showing that the affinity between CPM-PLCβ and G$\beta\gamma$ at atmospheric pressure on lipid membranes is strong and matches the K_d(app) (i.e., K_d(app) $\sim$ 7 nM) previously determined by FRET (Runnels and Scarlata, 1998). This apparent K_d, which reflects protein-protein associations on a confined membrane surface, is predicted to be $\sim$2 orders of magnitude weaker when the proteins are free to associate in solution (see Runnels and

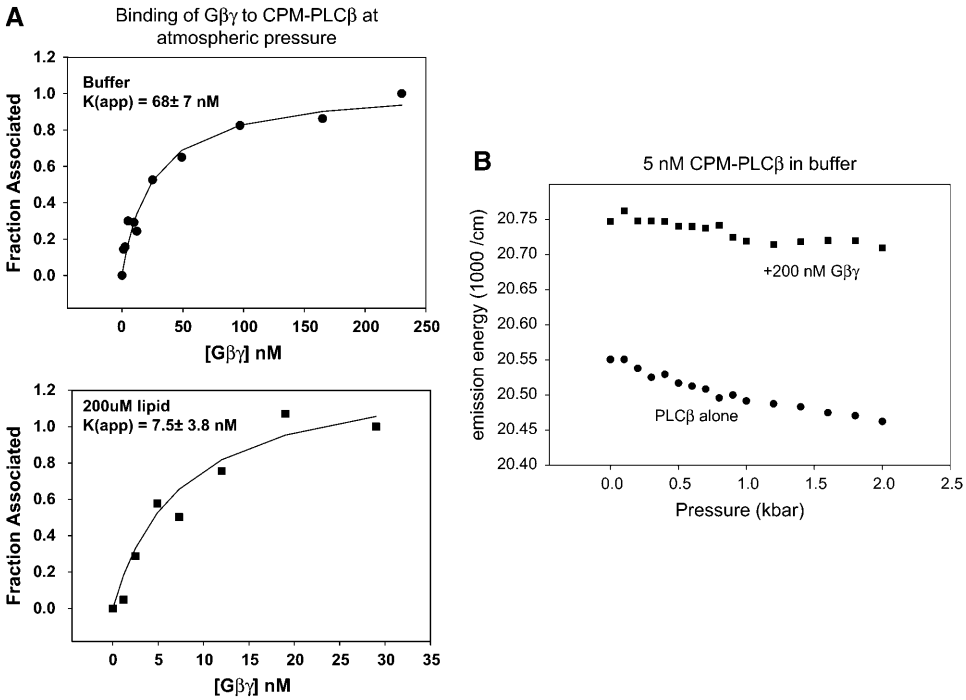

FIGURE 5 (*A*) Comparison of the association between CPM-PLCβ and G$\beta\gamma$ dispersed in buffer (*top panel*) and reconstituted on 200 μM POPC/POPS (2:1) bilayers (*lower panel*), where the fraction associated was calculated by the $\sim$200 cm^{-1} shift in emission energy in CPM-PLCβ fluorescence that occurs upon G$\beta\gamma$ association. (*B*) Changes in the emission energy of isolated CPM-PLCβ and CPM-PLCβ bound to G$\beta\gamma$ under pressure ($n = 4$).

Scarlata, 1999). However, repeating this study for solubilized proteins that were freshly dialyzed to remove detergent gave an apparent K_d only 10-fold weaker than predicted, suggesting that we may be not be viewing a simple protein-protein association ($K_d \sim 70$ nM; Fig. 5 *A*). We repeated this study in a mild detergent, β-octylglucoside, since stronger detergents such as sodium dodecyl sulfate eliminate enzyme activity. The detergent-dispersed proteins also showed a K_d(app) $\sim$ 70 nM.

Measurements of PLCβ-G$\beta\gamma$ associations under pressure

PLCβ-G$\beta\gamma$ associations were monitored as a function of pressure by either following the changes in fluorescence of CPM-PLCβ bound to G$\beta\gamma$ or by the changes in fluorescence of CPM-G$\beta\gamma$ bound to PLCβ. An example of the behavior of CPM-PLCβ at subsaturating (i.e., 60%) concentrations of G$\beta\gamma$ on lipid membrane and at supersaturating concentrations of G$\beta\gamma$ are shown in Fig. 6 *A*, where the degree of dissociation was calculated as described in the methods. These results gave a volume change of 34 $\pm$ 5 ml/mol. The pressure behavior of membrane-bound CPM-PLCβ-G$\beta\gamma$ complexes were then studied in the presence of 50 nM Gα_s(GDP) from atmospheric to 1000 kbar. This concentration of Gα_s(GDP) was able to eliminate the 2.1-fold activation of 2 nM PLCβ by 50 nM G$\beta\gamma$ (S. Scarlata, unpublished). We find that Gα(GDP) significantly decreased the volume change for the CPM-PLCβ-G$\beta\gamma$ association (Table 1). Interestingly, the association of the activating Gβ85-105 peptide (see Buck et al., 1999) to CPM-PLCβ gave a volume change similar to G$\beta\gamma$-subunits (Table 1), which suggests that the contacts made by this peptide reflect the same ones made by the whole G$\beta\gamma$-subunit (see Discussion).

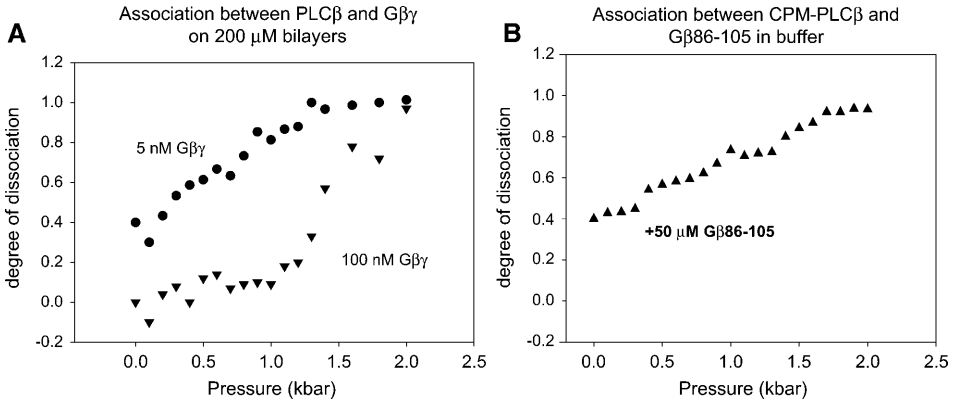

FIGURE 6 (*A*) Changes in the degree of dissociation (see Methods) with pressure between CPM-PLCβ and G$\beta\gamma$, initially bound at $\sim$30% and 100% on 200 μM POPC/POPS (2:1) bilayers. (*B*) Change in the degree of dissociation of CPM-PLCβ and activating peptide, Gβ86-105, initially bound at 40% in buffer under pressure. In these studies, $n = 3$ and the maximum error of the degree of dissociation was estimated to be $\pm$0.08.

TABLE 1 Summary of the volume changes of CPM-PLCβ and G$\beta\gamma$-subunits where ΔV is in (ml/mol)

CPM-PLCβ-G$\beta\gamma$ (solution)	78 ± 3
CPM-PLCβ-G$\beta\gamma$ (membranes)	34 ± 5
CPM-PLCβ-G$\beta\gamma$-Gα_s(GDP) (membrane)	25 ± 1

We repeated this study in the absence of membranes using freshly dialyzed CPM-PLCβ-G$\beta\gamma$ and found that in solution the volume change increases to 78 ± 3 ml/mol. In Fig. 7, we show the pressure dependence of CPM-G$\beta\gamma$-PLCβ in buffer, which is presented as the fraction associated rather than the degree of dissociation to emphasize that these data are from changes in CPM-G$\beta\gamma$ fluorescence rather than in CPM-PLCβ. These results, and those using CPM-PLCβ gave similar volume changes which were approximately twice as high as that seen for the associations on membrane surfaces.

DISCUSSION

In this study, we have applied high pressure techniques to determine the volume change associated with PLCβ-G$\beta\gamma$ interactions on membrane surfaces and in buffer. Hydrostatic pressure has been used extensively in the past to measure the volume change of protein-protein associations in solution, but it has not yet been used to monitor associations of membrane-bound proteins. Before studying the association of membrane-bound proteins, it was necessary to determine whether pressure affects the properties of the membranes themselves and whether pressure will affect the membrane association of peripherally bound proteins.

The effect of pressure on lipid membranes has been well characterized. Pressure has profound effects on the highly compressible membrane interior promoting phase transition to gel phases and, for the studies presented here, the phase transition will occur ~1.5 kbar (e.g., Li and Kay, 1977; Sassaroli et al., 1993). In contrast to the large effects of pressure on the membrane interior, pressure does not appear to affect the membrane surface charge and has little effect on surface hydration (Rebecchi et al., 1999; Scarlata and Rosenberg, 1990). Thus, we assume in this study that the surfaces of the membranes remain relatively unperturbed as pressure is applied.

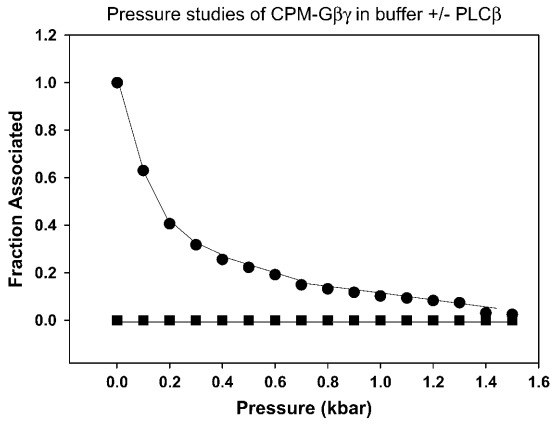

FIGURE 7 Change in the association of CPM-G$\beta\gamma$-PLCβ in buffer (●) under pressure, where the data are presented as fractions associated to differentiate CPM-G$\beta\gamma$ data from the CPM-PLCβ presented above, where $n = 3$ and the maximal error is slightly greater than the symbol size (±0.04). (■) Data for CPM-G$\beta\gamma$ in the absence of PLCβ.

The effect of high pressure on the membrane association of different types of peripherally bound and integral membrane proteins have been reported, and their pressure behavior is variable (e.g., Deckmann et al., 1986; Plager and Nelsestuen, 1992); however, some general guidelines have emerged. Protein association to membranes is stabilized by a combination of electrostatics as well as polar and hydrocarbon interactions. Since pressure does not alter the surface charge or hydration of the membrane, electrostriction effects are not expected to play a significant role, and previous studies of the electrostatic component of the small peptide melittin correlate well with this idea (Teng and Scarlata, 1993). For nonionic interactions, the dominant pressure effect resides in void volumes between the protein and the membrane surface. If a protein packs well with the membrane interface, then pressure is not expected to destabilize its membrane interactions. In contrast, when there are packing voids, the stability of membrane binding of a protein under pressure will depend on its ability to deform under pressure to fill in voids and reduce the volume. If the interface has void volume and the protein cannot easily deform, then water will instead fill the voids and promote membrane disassociation (see discussion in Teng and Scarlata, 1993).

We find that membrane binding of PLCβ, which is a chimera of PLCβ_2 and PLCδ_1, is pressure independent as expected from the pressure behavior of wild-type PLCδ_1 (Rebecchi et al., 1999). PLCβ contains the N-terminal PH domain of PLCβ_2 and the catalytic domain of PLCδ_1 (Wang et al., 2000). The PH domains of the enzymes are responsible for their different membrane binding characteristics; PLCδ_1 and its isolated PH domain binds specifically to membranes containing its substrate PI(4,5)P_2 through a series of specific hydrogen and ionic bonds (Ferguson et al., 1995). In contrast, PLCβ_2 and its isolated PH domain, and the PLCβ chimera used here, binds to different membranes with little specificity (Wang et al., 1999). Unlike PH-PLCδ_1, the membrane binding face and orientation of PH-PLCβ_2 are unknown. Pressure would be expected to stabilize the specific interactions that occur between PH-PLCδ_1 and PI(4,5)P_2 although enhancement of binding under pressure has not been observed (Rebecchi et al., 1999). This lack of pressure stabilization may be due to the inability of the PH-PLCδ_1 to further penetrate the membrane surface to allow for stronger hydrogen bonding and ionic interactions. Interestingly, a lack of enhanced membrane binding was also observed for the nonspecific PLCβ-membrane association,

suggesting that the enzyme packs efficiently with the membrane interface.

The membrane binding behavior of G$\beta\gamma$ and Gα(GDP) contrasted with that of the PLCβ. Both types of G-protein subunits were destabilized under pressure, suggesting an inefficient packing with the membrane surface. This effect mainly occurred at higher pressures where the lipid enters the gel phase. It is noteworthy that all G-protein subunits are posttranslationally modified with hydrocarbon chains (see Wedegaertner et al., 1995). The G$\beta\gamma$-subunits used in this study are geranylgeranylated, whereas Gα_s-subunits are dually palmitoyated (Kleuss and Krause, 2003; Wedegaertner et al., 1995). These modifications are thought to insert into the lipid matrix and stabilize membrane binding of the host protein. Thus, they would be expected to compress with the hydrocarbon chains of the membrane interior and be stabilized under pressure. However, not only do we find that pressure destabilizes the membrane binding of these G-protein subunits but that identical pressure behavior is seen for Gα_s even when its palmitoyl chains are removed by reducing agents. It is notable that the saturated palmitoyl chains would be expected to become more stabilized as the membranes enter the gel phase, but the opposite is observed. Taken together, our results suggest that these hydrocarbon chain modifications do not play a significant role in the membrane binding stability of these proteins in accord with previous results (Philip and Scarlata, 2004). We suggest that under pressure the hydrocarbon chains compress around or into the protein matrix to promote membrane dissociation that allows a reduction in volume.

Previous studies of the pressure behavior of PLCδ showed that pressure increased the reaction rate of substrate hydrolysis of this enzyme (Rebecchi et al., 1999), and these studies led to the suggestion that the application of pressure stabilized an activated form of the enzyme. To determine whether this could be the case for PLCβ_2, we monitored the change in emission of CPM-PLCβ_2 under pressure. CPM-PLCβ_2 emission is responsive to the binding of G$\beta\gamma$, which occurs in the nanomolar concentration range, and is also responsive to the activating peptide Gβ86-105, which occurs in the micromolar concentration range. The concentration dependence of binding of these two activators closely matches their respective concentration dependence of enzyme activation (Buck et al., 1999; Runnels and Scarlata, 1998). This close correlation of binding and activation of these two species which have such different affinities suggests that the change in emission intensity correlates to activation. If this interpretation is correct, then the data in Fig. 5 *B* indicate that pressure does not promote the transition of PLCβ to an activated conformation. Of course, activity studies must be done to verify this idea.

The use of high pressure also allowed us to determine the volume change associated with the interaction between G$\beta\gamma$ and PLCβ. The application of pressure will cause the dissociation of proteins due to penetration of solvent into the subunit interfaces, which usually occurs in the range of 1–3 kbars. Higher pressures (usually above 5 kbars) will result in penetration of solvent into the protein matrix, resulting in a molten globule (e.g., see Jonas, 2002). In principle, the protein-protein interface would be the same whether the protein is freely diffusing in solution or confined on a membrane surface, and thus we expect that for a specific association the volume change would be similar if the proteins were freely diffusing or confined to a membrane surface.

In this study, we determined the volume change for G$\beta\gamma$-PLCβ interactions in solution and bound to membranes. We note that we have found that the affinity between these proteins does not depend on the lipid phase (L. W. Runnels and S. Scarlata, unpublished). Although we predict similar volume changes for this association in the two different environments, the apparent affinities will differ because the effective concentration of membrane-bound proteins tends to be much higher due to their confinement on the reduced area of the membrane surface giving stronger apparent affinities (see Runnels and Scarlata, 1999). We directly measured the apparent K_d values of G$\beta\gamma$-PLCβ in solution and bound to membranes. We find that the K_d value for this interaction in solution is higher than expected based on a simple model where we calculate protein association on the reduced volume of the membrane. Our pressure studies show that the volume change for this association is twice as high in solution as on membranes. Taken together, these data suggest that in solution PLCβ-G$\beta\gamma$ dimers further oligomerize to a higher order species such as tetramers.

The molecular details of how PLCβ interacts with G$\beta\gamma$ is unknown. G$\beta\gamma$ has a propeller-like structure (Wall et al., 1995), and our previous work has shown that a peptide corresponding to one of the Gβ blades, Gβ86-105, binds to and activates PLCβ, whereas peptides that correspond to other blades may bind but not activate PLCβ (Buck et al., 1999). We find that the association of Gβ86-105 to PLCβ gives a volume change that is similar to the full G$\beta\gamma$-subunit. These similar volume changes may correspond to a significant portion of the interaction volume between the two proteins. Alternately, it is possible that multiple Gβ86-105 peptides bind to the enzyme giving the same overall volume change. This idea is based on studies suggesting that multiple Gβ peptides can bind to PLCβ and give different levels of activation (Buck et al., 2002). Experiments are underway to differentiate between these possibilities.

When pressure studies are carried out in the presence of Gα_s(GDP) subunits are under conditions where the protein will be completely bound (i.e., $\leq$1 kbar), we find that the volume change of PLCβ-G$\beta\gamma$ association is reduced. We have previously shown that Gα(GDP)-subunits cause deactivation of PLCβ-G$\beta\gamma$ without significantly affecting their association (Runnels and Scarlata, 1998). The simplest explanation is that GDP-bound Gα_s-subunits bind to G$\beta\gamma$, but not necessarily at its primary interaction site. This interaction perturbs the contacts that G$\beta\gamma$ makes with PLCβ,

which causes enzyme activation. If this idea is correct, then the volume associated with $G\beta\gamma$ activation of PLCβ corresponds to the difference in volume changes for PLCβ-G$\beta\gamma$ alone and in the presence of $G\alpha_s$(GDP), ranging from $\Delta V \sim 3$ to 15 ml/mol (Table 1). Thus, pressure methods allow for the estimation in the volume associated with the $G\alpha_s$(GDP) deactivation of the PLCβ-G$\beta\gamma$ complex even though the change in interaction energy between PLCb and $G\beta\gamma$ caused by $G\alpha_s$(GDP) could not be detected by spectroscopic atmospheric pressure measurements.

To our knowledge, this study represents the first application of pressure to membrane-bound proteins under conditions where the proteins are bound to the membrane surface. We find that pressure allows for determination of volume changes which give novel information about the interactions between proteins in the G-protein-PLC signaling system. Studies using mutants are underway to better identify the residues responsible for these interaction volumes.

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