Dissection of the Steps of Phospholipase Cβ_2 Activity That Are Enhanced by G$\beta\gamma$ Subunits†

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ABSTRACT: Phosphatidylinositol-specific phospholipase C (PLC) enzymes catalyze the hydrolysis of phosphatidylinositol 4,5 bisphosphate in a two step reaction that involves a cyclic intermediate. The PLCβ family are activated by both the α and $\beta\gamma$ subunits of heterotrimeric G proteins. To determine which catalytic step is affected by G$\beta\gamma$ subunits, we compared the change in PLCβ_2 activity catalysis toward monomeric short-chain phosphatidylinositol (PI) substrates and monomeric water-soluble cyclic inositol phosphates as well as long-chain PI in bilayer and micellar interfaces in the absence and presence of G$\beta\gamma$ subunits. Unlike other PLC enzymes, no cyclic products were detected for either wild-type PLCβ_2 or a chimeric protein composed of the PH domain of PLCβ_2 and the catalytic domain of PLCδ_1. Using cIP as a substrate to examine the second step of the reaction, we found that the presence of G$\beta\gamma$ subunits stimulated this step by a higher level than that for the overall reaction (k_{cat} 1.5-fold (cIP) as opposed to 1.20-fold for soluble diC$_4$PI). Detergents above their CMC can generate the same kinetic activation of PLCβ_2 as G$\beta\gamma$, suggesting that hydrophobic compounds stabilize the activated state of the enzyme. The most pronounced effect of G$\beta\gamma$ is that it relieves competitive product inhibition. Taken together, our results show that activation of PLCβ_2 occurs through enhancement in the catalytic rate of hydrolysis of the cyclic intermediate and increased product release, and that hydrophobic interactions play a key role.

Inositol-specific mammalian phospholipase C (PLC)[1] enzymes are critical components of the phosphoinositide signaling pathway (for reviews see refs *1* and *2*). These enzymes catalyze the hydrolysis of a minor component in membranes, phosphatidylinositol 4,5 bisphosphate (PI(4,5)P$_2$) to generate diacylglyerol (DAG), an activator of protein kinase C, and inositol (1,4,5) trisphosphate (Ins(1,4,5)P$_3$) which binds to receptors in the endoplasmic reticulum that release of Ca^{2+} into the cytosol. The increase in intracellular Ca^{2+} in turn affects the activity of a large number of signaling enzymes.

PI(4,5)P$_2$ hydrolysis by PLC involves a sequential two step mechanism with an initial intramolecular attack of the inositol 2-OH on the phosphodiester phosphorus of PI(4,5)P$_2$ to generate a cyclic intermediate (a phosphorylated analogue of inositol 1,2-(cyclic)-phosphate, cIP), followed by attack of an appropriately activated water molecule on the cyclic inositol phosphate to generate Ins(1,4,5)P$_3$ (*3*). The reaction scheme for PI is given in Figure 1.

There are now five family members of PLCs, β, δ, γ, ϵ, and ζ, which have different cellular and tissue distributions and different modes of regulation (*1*, *2*). While bacterial PLCs release only cyclic inositol phosphophosphates, the mammalian PLCs investigated thus far (i.e. PLCδ_1 and PLCγ_1), have the ability to release both cyclic and noncyclic products at a ratio that depends on assay conditions (*4*−*6*).

PLCβ family members are regulated by heterotrimeric G proteins. Of the four known PLCβ isozymes (PLCβ_{1-4}), all are regulated by Gα_q subunits, and PLCβ_{2-3} are also regulated by G$\beta\gamma$ subunits. Regulation by G$\beta\gamma$ has been shown to involve the N-terminal pleckstrin homology (PH) domain; replacement of the PH domain of the G protein insensitive enzyme PLCδ with the PH domain of PLCβ_2 confers G$\beta\gamma$ activation to the chimeric enzyme (*7*).

G$\beta\gamma$ binds PLCβ_2 tightly in 1:1 complex with an apparent nanomolar binding constant on model membrane surfaces (*8*−*12*), and this association yields a 2−7-fold increase in PLCβ_2 catalytic activity depending on the nature of the substrates and the presence of detergents. Most kinetic assays utilize PI(4,5)P$_2$ substrate dispersed in a vesicle matrix, and changes in the membrane surface may be part of the mechanism for the observed activation by G$\beta\gamma$. Since G$\beta\gamma$ and PLCβ proteins can partition onto the vesicle surface independently as well as in a complex (11), and since catalysis occurs on the membrane surface, it is difficult to assess the role of the membrane surface in the activation process. Here, we separate the role of the membrane surface

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[1] Abbreviations: PLC, phosphatidylinositol-specific phospholipase C; diC$_7$PC, diheptanoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PI, phosphatidylinositol; PI(4,5)P$_2$, phosphatidylinositol-4,5-bisphosphate; diC$_4$PI, dibutyroylphosphatidylinositol; diC$_4$PI(4,5)P$_2$, dibutyroylphosphatidylinositol-4,5-bisphosphate; cIP, inositol 1, 2-(cyclic)-phosphate; 1-I-P, inositol-1-phosphate; IP$_3$, inositol-1,4,5-trisphosphate; DAG, diacylglycerol; SM, egg sphingomyelin; CMC, critical micelle concentration; TX-100, Triton X-100; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles.

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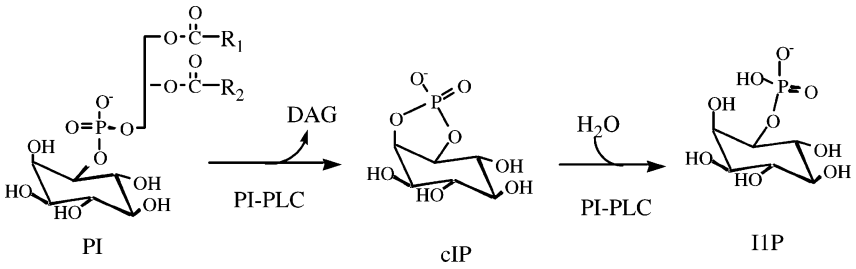

FIGURE 1: Reaction scheme showing the phosphotransferase and phosphodiesterase steps of PI hydrolysis by PLC.

in G$\beta\gamma$ activation of PLCβ_2 by comparing the hydrolysis of substrates embedded in lipid matrixes of varying surface properties, and membrane-bound versus soluble substrates.

Since the hydrolysis of PI(4,5)P_2 catalyzed by mammalian PLCs occurs through a two step mechanism (Figure 1), we have separated the influence of G$\beta\gamma$ on the phosphotransferase versus cyclic phosphodiesterase steps by employing soluble synthetic short-chain soluble phosphatidylinositols and cyclic inositol phosphate (cIP), and determined the appearance or disappearance of cyclic intermediate or water-soluble products by ^{31}P NMR spectroscopy. These measurements have been augmented by radiometric activity assays to monitor the effect of G$\beta\gamma$ on Ins(1,4,5)P_3 inhibition of PLC. Our data show that G$\beta\gamma$ increases the catalytic rate of cIP hydrolysis and relieves Ins(1,4,5)P_3 inhibition of PI(4,5)-P_2 cleavage. Thus, a major effect of G$\beta\gamma$ binding to PLCβ_2 is to increase the rate for the second step of the reaction by enhancing product release.

MATERIALS AND METHODS

Chemicals. PI, diC_7PC, egg sphingomyelin and POPC were obtained from Avanti Polar Lipids; diC_4PI and diC_4-PIP_2 were purchased from Echelon Biochemicals. [^{3}H]PI-(4,5)P_3 was purchased from New England Nuclear. Triton X-100 was obtained from Sigma Chemical Co. All other chemicals were reagent grade.

Protein Expression and Purification. PLCβ_2 was expressed in Sf9 cells by a baculovirus system and purified as previously described (see ref *12*). Some of the studies used His_6−PLCβ_2 which was also expressed in Sf9 cells and purified on a Ni^{2+}-NTA column following the procedure of Fogg (*13*). We also used a chimeric protein PHβ_2−PLCδ_1 which is comprised of the N-terminal pleckstrin homology domain of PLCβ_2 and the catalytic domain of PLCδ_1. This construct, which also contains a His_6 tag on the N-terminus, is expressed in *E. coli* BL-21 cells and shows identical membrane binding, G$\beta\gamma$ binding, and G$\beta\gamma$ activation as wild-type PLCβ_2.

The G$\beta\gamma$ subunits used in these studies were prepared by coexpression of Gα_q, Gβ_1 and His_6-Gγ_2 in Sf9 cells and purification of the complex on a Ni^{2+}-NTA column (*13*). The His_6-G$\beta_1\gamma_2$ construct has an identical PLCβ_2 activation profile as its non-His tagged counterpart.

^{31}P NMR Assays. PLC activities toward PI (dispersed in diC_7PC or Triton X-100 micelles, or in SUVs in the absence and presence of POPC) and cIP were measured by ^{31}P NMR (202.3 MHz) spectroscopy looking at either fixed time points as described for other PLC enzymes (*14*), or following the reaction over a 5 h time course acquiring free induction decays every 5−10 min. All reactions were carried out in a reaction buffer of 50 mM HEPES, pH 7.5, with 0.5 mM Ca^{2+} unless otherwise noted. Assays were run at 34 °C unless otherwise specified. Enzyme (covering a range of 0.1 to 0.6 μg depending on whether the phosphotransferase or cyclic phosphodiesterase activity was examined) was added to the 400 μL sample and incubation times were chosen so that less than 20% substrate cleavage occurred. cIP and/or I-1-P content in the aqueous phase was quantified in the ^{31}P spectrum by adding glucose-6-phosphate as an internal standard. A final concentration of 0.61 μM G$\beta\gamma$ protein, which was dialyzed to remove detergent prior to use in these assays, was used in these assays.

Dried films of PI and PI/POPC were rehydrated with reaction buffer, then sonicated to prepare SUVs as described elsewhere (*6*). Powdered diC_4PI and diC_4PI(4,5)P_2 were dissolved directly in the reaction buffer. cIP was prepared enzymatically from PI and purified by anion exchange chromatography (*5*), and then an aliquot of the aqueous cIP stock (∼200 mM) was diluted to the desired concentration in reaction buffer.

Radiometric Measurements of PI(4,5)P_2 Hydrolysis in Phospholipid Vesicles. For studies that monitored the dependence of G$\beta\gamma$ activation with vesicle composition, PLCβ_2-catalyzed hydrolysis of^3[H]PI(4,5)P_2 was carried out using 30 nM PLCβ_2 and SUVs composed of varying amounts of POPE, POPS, POPC and eggSM as described in the text. All vesicles contained 2% PI(4,5)P_2 and were at a total phospholipid concentration of 1 mM. The G$\beta\gamma$ concentration was varied from 0 to 125 nM.

For studies that determined the dependence of G$\beta\gamma$ activation on PI(4,5)P_2 concentration, vesicles were composed of POPE−POPS−PI(4,5)P_2 (66:33:1) incorporating 1−20 mol % PI(4,5)P_2. These studies employed 4−6 nM of the PHβ_2-PLCδ_1 chimera.

Assays were carried out by doping PI(4,5)P_2 with [^{3}H] PI(4,5)P_2 to obtain approximately 6000−7000 cpm/assay. The purified enzymes were diluted in Ca^{2+} free-MAIN buffer (50 mM Hepes, 160 mM KCl, 3 mM EGTA, 1 mM DTT, pH 7.2). Prior to assays, G$\beta\gamma$ or storage buffer was dialyzed 3 times for 20 min at 4 °C against 50 mM Hepes, 150 mM NaCl, 1 mM DTT, pH 7.4. For the product inhibition studies, Ins(1,4,5)P_3 was solubilized in Ca^{2+}-free MAIN buffer and added to the protein to a final assay concentration of 3 mM.

For the activity assays, the PLC-containing solutions were mixed on ice with 10 μL of freshly sonicated 3 mM SUVS and 5 μL of 12 mM Ca^{2+}−containing MAIN buffer such that the final free Ca^{2+} concentration was 1000 nM. Samples were incubated for 30 s at 37 °C and the reaction was immediately terminated by placing the samples in an ice water bath and adding 0.2 mL of cold 10% trichloroacetic acid, followed by 0.1 mL of cold 1% bovine serum albumin. The aqueous and organic phases were separated by centrifugation (5 min

Table 1: Effect of G$\beta\gamma$ on the Specific Activity of PLCβ_2[a]

sample	specific activity (nmol min^{-1} mg^{-1})[b]		SA(+G$\beta\gamma$)/ SA(−G$\beta\gamma$)
	−G$\beta\gamma$	+G$\beta\gamma$	
4 mM PI SUV	26.9	62.0	2.30
4 mM PI SUV/16 mM POPC	52.9[c]	86.9[c]	1.64
8 mM PI/16 mM TX-100	44.3	41.2	0.93
8 mM PI/32 mM diC$_7$PC	100	106	1.06
0.5 mM diC$_4$PI	2.0[c]	2.4[c]	1.20
1 mM diC$_4$PI	1.1	1.3	1.18
1 mM diC$_4$PI(4,5)P$_2$	141.9	176.2	1.24

[a] Assay conditions: 50 mM HEPES buffer, pH 7.5, with 0.5 mM Ca^{2+}, 34 °C. [b] The error in specific activities, determined by comparing activities for several duplicate samples, was usually less than 10%. [c] The batch of PLCβ_2 used in these assays was ~50% more active than the other batches used for the rest of the experiments in this table.

at 72000 g) and 0.27 mL of the upper aqueous phase containing ^{3}[H]Ins(1,4,5)P$_3$, mixed with 2 mL of scintillation liquid, was counted. To determine the maximum amount of radioactivity in the samples, 10 μL of each vesicle stock was mixed with 260 μL of cold 10% trichloroacetic acid and counted in triplicate. For G$\beta\gamma$ activation assays, control samples substituted G$\beta\gamma$ storage buffer for G$\beta\gamma$.

Fluorescence Measurements. Spectra were recording on an ISS photon counting spectrofluorometer by placing the samples in a 3 mm cuvette. Purified PLCβ2 was labeled with acrylodan (Molecular Probes, Inc.) by first dialyzing against 20 mM Hepes, 160 mM KCL, pH 7.2 to remove reducing agents. PLCβ2 was then incubated with a 4-fold molar excess of acryodan from a concentrated stock solution in DMF for 1 h on ice. The reaction was quenched by the addition of 1 mM DTT and excess probe was removed by dialysis against buffer containing 1 mM DTT.

NBD-labeled PC and PI(4,5)P2 were from Echleon Research Laboratories, Inc. (Salt Lake City, UT) and bilayers containing these lipids were prepared by extrusion through a 0.1 μm filter.

RESULTS

Substrate Hydrolysis by PLCβ_2 Is Interface Dependent. Although mammalian PLC enzymes are optimized for PI(4,5)P$_2$ cleavage to DAG and IP$_3$, they will also process PI. ^{31}P NMR assays of PLC activity toward this substrate in different interfaces (e.g., vesicles and detergent mixed micelles) can monitor the relative concentration of cyclic and acyclic water-soluble products (*5*, *6*) and whether this product distribution is affected by the interface.

We found that pure PI SUVs were not well hydrolyzed by PLCβ_2 although the enzyme did cleave it to I-1-P with a specific activity of 26.9 nmol min^{-1} mg^{-1} (Table 1). Well above 90% of product generated under these assay conditions was I-1-P; in general, no detectable cIP accumulated over the time course of the reaction. When PLCβ_2 was complexed with G$\beta\gamma$ subunits, the enzyme specific activity increased 2.3-fold toward PI SUVs. This enhancement in PLCβ_2 catalytic rate caused by binding of G$\beta\gamma$ was comparable to the value previously observed with PI(4,5)P$_2$ as a substrate in PC−PS bilayers (*11*). If the PI (4 mM) was presented in vesicles with 16 mM POPC, G$\beta\gamma$ activation of PLCβ_2 was reduced to 1.6-fold.

The rate for PLCβ_2 cleavage of PI solubilized in detergent mixed micelles (either Triton X-100 or diC$_7$PC) was faster than for PI SUVs, but at most 2-fold. This is in contrast to many phospholipases including the bacterial PI−PLC (*6*), where the activity of the enzyme toward PI SUVs is often much lower (<10%) than what is observed when the substrate is presented in a detergent matrix. Solubilizing PI in a diC$_7$PC matrix provided a better substrate for the enzyme than presenting the substrate in a Triton X-100 matrix (Table 1) which may be due to ionic nature of the micellular surface, although the basis is unclear. While G$\beta\gamma$ clearly enhanced the PLCβ_2 specific activity toward PI SUVs, the addition of the G$\beta\gamma$ to the enzyme had no effect on the activity toward PI/detergent mixed micelles (either Triton X-100 or diC$_7$PC (Table 1)). We note that these studies were done under conditions were the two proteins are completely bound as determined by fluorescence resonance energy transfer (*12*) (*results not shown*). Again, I-1-P represented >90% of the water-soluble product detected in these assays.

While both PLCδ_1 and PLCγ_1 catalyzed-cleavage of PI(4,5)P$_2$ yielded only Ins(1,4,5)P$_3$, their interaction with PI produced both cIP and I-1-P at a fixed ratio at a given temperature (*5*, *15*). With both of these PLC isozymes, increasing the assay temperature enhanced release of cIP so that it became the major product at higher temperatures. In contrast, increasing the assay temperature from 25 to 40 °C for PLCβ_2 did not enhance accumulation of cIP. Under all the conditions we examined, I-1-P was the major product (>90%) observed. This suggests that the cIP generated at the active site of PLCβ_2 is held long enough for attack by a water molecule so that I-1-P becomes the only observed water-soluble product of the reaction. This is a pronounced difference from all the other characterized PLC isozymes.

Effect of Varying Surfaces on G$\beta\gamma$ Activation of PLCβ_2. Activation of PLCβ_2 by G$\beta\gamma$ subunits only enhanced hydrolysis of PI when this substrate was presented in vesicles and not in detergent mixed micelles. There are several ways through which G$\beta\gamma$ could activate the reaction for a substrate presented in a bilayer. One possibility is that G$\beta\gamma$ may increase access of the enzyme substrate. Access to substrate is limited by the tension of the membrane surface (*16*−*18*), and thus complexation of PLCβ_2 with G$\beta\gamma$ may reduce the amount of work needed to penetrate the surface. If this were the case, then we would expect reduced G$\beta\gamma$ activation on surfaces with a low surface tension.

We determined whether G$\beta\gamma$ activation of PLCβ_2 was dependent on the nature of the membrane surface by incorporating PI(4,5)P$_2$ substrate in vesicles containing egg SM lipids, which have a tightly compact surface, or POPE lipids, which have a less dense, more hydrophobic surface (Figure 2). EggSM is primarily composed of palmitoyl chains (see the Avanti Polar Lipid handbook), and we do not expect phase separation. However, SM lipids form interlipid hydrogen bonding networks that PLC must disrupt to access substrate (*19*, *20*), and we expect a reduction in activity when these lipids are present in fluid phase membranes independent of the nature of the acyl chains. In contrast, the smaller and more hydrophobic PE lipid head, which are less hydrated, allow for better access of substrate (see refs *21* and *22*)).

We compared G$\beta\gamma$ activation of PLCβ_2 on different membrane surfaces using PI(4,5)P$_2$ as a substrate since it can be used in low concentrations and allow the overall

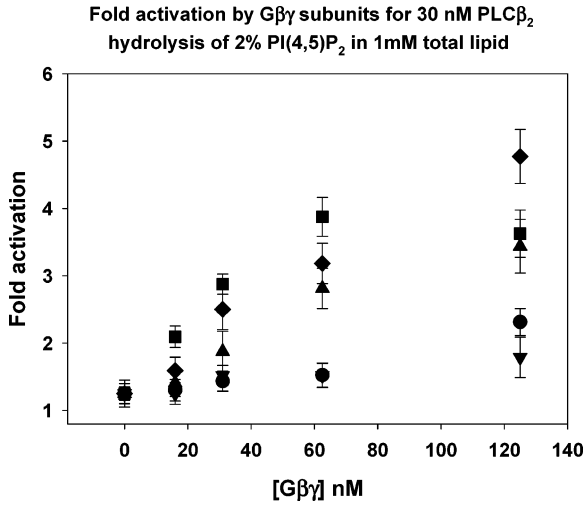

FIGURE 2: Differences in the G$\beta\gamma$ activation profile of PI(4,5)P_2 hydrolysis by 30 mM PLCβ_2 where substrate, at 2 mol %, is presented in SUVs of differing compositions at a total lipid concentration of 1 mM for all samples: ◆, PE−PS (66:31); ■, SM−PS (66:31); ▲, PC−PE−PS (33:33:31); ●, PC−PS (66:31); ▼, PC (98). Each point is an average of two sets of three independent samples, and standard errors are shown. Activation assays are described in the Materials and Methods.

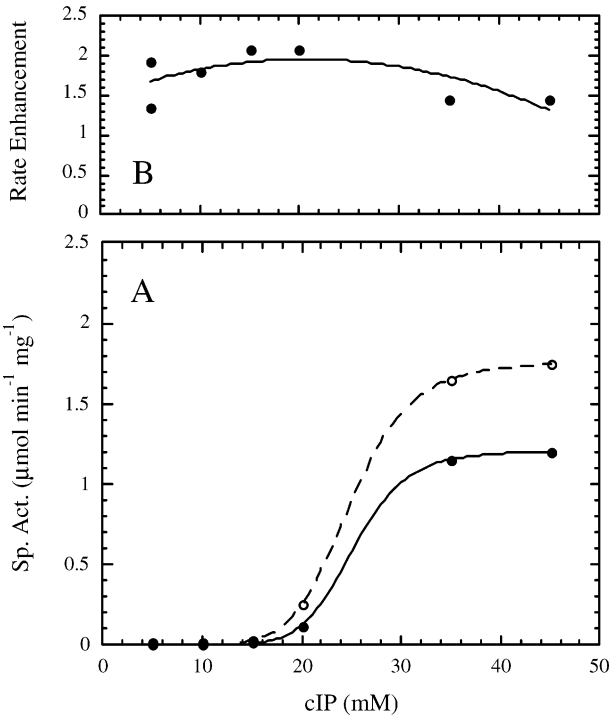

FIGURE 3: Panel A shows the change in the specific activity toward cIP as a function of substrate concentration in the presence and absence of G$\beta\gamma$. Panel B shows the ratio of the specific activity in the presence and absence of G$\beta\gamma$.

vesicle properties to be dominated by the other phospholipids. In the absence of G$\beta\gamma$, PLCβ_2 was 25% more active when the bilayers contained PE lipid and 20% less active when the bilayers contained SM, as compared to PC lipids. However, G$\beta\gamma$ activated PLCβ_2 to similar extents in both PE and SM, arguing against the idea that activation of PLCβ_2 by G$\beta\gamma$ occurs by allowing better access to substrate and suggesting that characteristics other than surface tension must play a role in G$\beta\gamma$ activation.

Effect of G$\beta\gamma$ on the Phosphodiesterase Activity of PLCβ_2. We did not observe the release of cyclic product cIP from PLCβ_2 hydrolysis of PI suggesting that the cyclic intermediate remains in the PLCβ_2 active site for a sufficient time to allow attack by water. With this in mind, we carried out a series of kinetic studies to determine whether the association of G$\beta\gamma$ with PLCβ_2 affects either K_m or k_{cat} of the cIP hydrolysis reaction. For all phosphatidylinositol-specific PLCs examined thus far, the cyclic phosphodiesterase reaction is far less efficient with a lower V_{max} and much higher K_m than the phosphotransferase reaction (*5*, *6*, *15*). In line with this, the activity of PLCβ_2 toward cIP was very low with 5 mM substrate but increased dramatically as the substrate concentration increased (Figure 3). The dependence of rate on cIP concentration was sigmoidal with a V_{max} of 1.20 ± 0.01 μmol min^{-1} mg^{-1} and a K_m of 25.3 ± 0.3 mM. The K_m for this reaction was very similar to values observed for other mammalian PLCs (*5*, *15*). cIP does not partition into interfaces and the extreme cooperativity may reflect cIP binding weakly to multiple sites on the enzyme and inducing a critical conformational change.

When the same substrate concentration dependence of activity toward cIP was examined with G$\beta\gamma$ bound to PLCβ_2, all specific activities were increased roughly by 1.5- to 2.0-fold (Figure 3). The same analysis of the kinetics indicated K_m was essentially unaltered (25.0 ± 0.2 mM) while V_{max} increased to 1.76 ± 0.01 μmol min^{-1} mg^{-1}. Taken together, our results suggest that the bound G$\beta\gamma$ must alter the conformation of the enzyme in a way that enhances its ability to hydrolyze this soluble substrate.

Table 2: Effect of diC_7PC on the Ability of G$\beta\gamma$ To Activate PLCβ_2 for cIP Hydrolysis

substrates		specific activity[a] (μmol min^{-1} mg^{-1})		SA(+G$\beta\gamma$)/ SA(−G$\beta\gamma$)
cIP (mM)	diC_7PC (mM)	−G$\beta\gamma$	+G$\beta\gamma$	
5	0.5	5.2	10.0	1.92
5	2.5	11.8	12.3	1.04
10	5.0	*b*	*b*	1.08

[a] The specific activity of the PLCβ_2 showed slight variations between different preparations; the enzyme used in these assays was roughly 1.3 times as active as previous preparations in comparing cIP hydrolysis in the absence of G$\beta\gamma$ or detergents. [b] These assays used an earlier preparation of PLCβ_2 with lower specific activity. However, the presence of the micellar diC_7PC activated the enzyme and made it insensitive to G$\beta\gamma$.

If the activating interaction between G$\beta\gamma$ and PLCβ_2 is hydrophobic, then it is possible that amphiphiles such as detergents would enhance cIP cleavage similar to G$\beta\gamma$ subunits and could explain why no activation was seen with PI presented in detergent mixed micelles. To test this, we examined the effect of diC_7PC on cIP cleavage in the absence and presence of G$\beta\gamma$ (Table 2). Above 1.5 mM, diC_7PC forms micelles (*23*), while at 0.5 mM it is primarily monomeric. At this latter concentration, addition of G$\beta\gamma$ enhanced the activity 1.9-fold. When the diC_7PC concentration was increased above its critical micelle concentration (i.e. to 2.5 mM) the addition of G$\beta\gamma$ had no effect on the enzyme activity. Furthermore, the specific activity with only diC_7PC was comparable to that achieved by adding G$\beta\gamma$ to the sample in the absence of the detergent. Thus, like G$\beta\gamma$, these zwitterionic micelles are able to induce an activated conformation of PLCβ_2.

Effect of G$\beta\gamma$ on the Phosphotransferase Step. G$\beta\gamma$ may also affect the phosphotransferase step of catalysis as well the phosphodiesterase step. We isolated this step by using soluble diC$_4$PI and diC$_4$PI(4,5)P$_2$ substrates, where interfacial surface affects would not contribute, and compared the activity toward diC$_4$PI to cIP to isolate the first step in overall catalysis. The CMC for both these short-chain phospholipids is likely to be well over 150 mM (*23*) so that in solution they will exist as monomers. As seen in Table 1, diC$_4$PI was a very poor substrate for the enzyme at 1 mM. The very low activity for these substrates as compared to long-chain PI strongly suggests that the interactions of PLCβ_2 with the interface provided by the acyl chain are important, perhaps in orienting the substrate for better attack by the enzyme. Again, no cIP was observed and only I-1-P was detected. Two different concentrations of this substrate were used, 0.5 and 1 mM, with the thought that if the K_m for this soluble substrate is high, the extent of G$\beta\gamma$ activation might vary if it reduced K_m. The addition of G$\beta\gamma$ led to at most a 20% increase in specific activity (the error in these assays is <10% if the same batch of enzyme is used and if the assays are done at the same time) for both these concentrations. While the extent of G$\beta\gamma$ activation is significant, it is much smaller than the G$\beta\gamma$ activation of cIP hydrolysis showing that kinetic activation is toward the phosphodiesterase rather than the phosphotransferase reaction.

PLCβ_2 showed specific activities for diC$_4$PI(4,5)P$_2$ which were higher than those observed for cleavage of PI solubilized in diC$_7$PC (Table 1). Again, the addition of G$\beta\gamma$ to PLCβ_2 had a small, but significant effect (24% increase) on diC$_4$PI(4,5)P$_2$ cleavage.

G$\beta\gamma$-Induced Changes in Substrate Binding and Product Release. It is possible that the reason that activation of PLCβ_2 by G$\beta\gamma$ is larger on bilayers as opposed to soluble substrates is that it may increase the number of productive encounters that the enzyme has with substrate. We thus attempted to measure directly the steady-state binding of soluble PI(4,5)P$_2$ and Ins(1,4,5)P$_3$ to PLCβ_2 in the presence and absence of G$\beta\gamma$ using several types of fluorescence measurements using a short-chain NBD-PI(4,5)P$_2$. However, the binding of this substrate was too weak to yield interpretable results. We then attempted to measure substrate binding on membranes by determining the amount of fluorescence resonance energy transfer from acrylodan-PLCβ_2 to long-chained NBD-PI(4,5)P$_2$ incorporated into POPC large, unilamellar vesicles (99.5:0.5). We find this binding to be unchanged in the presence of G$\beta\gamma$ subunits (Figure 4) suggesting that activation by G$\beta\gamma$ is not due to an increase in K_m.

G$\beta\gamma$ activation of cIP hydrolysis by PLCβ_2 may instead occur through an increase of the catalytic rate and an increase in the release of the inositol phosphate. Direct binding of soluble Ins(1,4,5)P$_3$ to the enzyme was too weak (i.e. >1 mM) to be directly measured by changes in intrinsic fluorescence of PLCβ_2 or changes in coumarin-labeled PLCβ_2 (i.e. linear changes in fluorescence were observed up to 2 mM Ins(1,4,5)P$_3$ without saturation), and the concentrations of labeled substrate required for displacement assays were prohibitively high. Therefore, we indirectly assessed the association of Ins(1,4,5)P$_3$ product with the PLCβ_2 through product inhibition. By this method, the effect of G$\beta\gamma$ on the ability of Ins(1,4,5)P$_3$ to inhibit PLCβ_2 could then be determined.

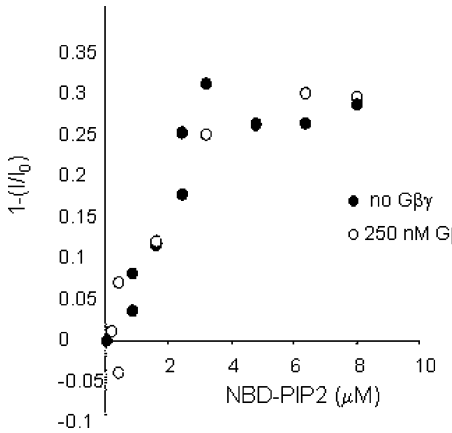

FIGURE 4: Change in the extent of fluorescence resonance energy transfer (FRET) from acrylodan-PLCβ_2 to NBD-PI(4,5)P$_2$ incorporated into large, unilamellar vesicles composed of POPC−POPS (2:1) at increasing amounts where the total lipid concentration was constant at 200 μM and the POPC concentration was held constant while the POPS was varied. The studies monitored the decrease in acylodan total fluorescence, presented as $1 - I/I_0$, where I is the acrylodan intensity at a given NBD concentration divided by the intensity in the absence of NBD (i.e. I_0), and show similar curves in the absence (●) and presence (○) of 250 nM G$\beta\gamma$. Spectra were corrected for the change in acrylodan-PLCβ_2 fluorescence upon membrane binding using membranes with identical unlabeled PI(4,5)P$_2$. We note that the membrane binding curves could be fit to a model that assumes random distributions of acrylodan-PLCβ_2 and NBD-PI(4,5)P$_2$ fluorophores mixed with lateral association between the fluorescent-tagged species. The contribution of FRET due to a random distribution was determined using NBD-PC. These results gave an apparent association between acrylodan-PLCβ_2 and NBD-PI(4,5)P of ~4.2 μM.

For these kinetic studies, we employed a chimeric enzyme containing the PH domain of PLCβ_2 and the catalytic domain of PLCδ_1, PHβ_2-PLCδ_1. This enzyme has the same membrane and G$\beta\gamma$ binding and G$\beta\gamma$ activation properties as the wild-type enzyme, and does not have the C-terminal tail which has been proposed to cause dimerization in PLCβ_1 (*24*, *25*). The chimeric enzyme also exhibited a 2-fold increase in the specific activity for 5 mM cIP hydrolysis in the presence of G$\beta\gamma$.

The activity of PHβ_2-PLCδ_1 was measured by monitoring the generation of ^{3}[H]Ins(1,4,5)P$_3$ from hydrolysis of ^{3}[H]-PI(4,5)P$_2$ in the presence of various concentrations of PI(4,5)P$_2$, and in the presence or absence of added Ins(1,4,5)P$_3$ and G$\beta\gamma$. Assays were carried out at high lipid concentration (1 mM which is 10-fold higher than the K_p of ~100 μM (*26*)) so that we could consider the PHβ_2-PLCδ_1 to be completely membrane bound. PHβ_2-PLCδ_1 activities toward vesicles containing 1 to 20 mol % PI(4,5)P$_2$ were measured at 3 mM Ins(1,4,5)P$_3$, a concentration of product chosen based on previous studies (*27*, *28*).

As shown in the Figure 5, the specific activity of the enzyme increased with the mole fraction of PI(4,5)P$_2$ and was higher in the presence of G$\beta\gamma$. The data were fitted with the equation $v = V_{max}([PI(4,5)P_2]^n/([PI(4,5)P_2]^n + K_m^n)$. Without G$\beta\gamma$, the activity studies gave a Hill coefficient of $n = 2$ which was unchanged in the presence of G$\beta\gamma$ subunits (see Discussion). Interestingly, a comparison of the experimental ratio $v_{+\beta\gamma}/v_{-\beta\gamma}$ (i.e., PLC activation) suggests a much higher level of activation at low PI(4,5)P$_2$ levels.

The V_{max} of PHβ_2-PLCδ_1 alone was equal to 101 μmol min^{-1} mg^{-1}. When Ins(1,4,5)P$_3$ was added, a significant

increase in K_m from 84 to190 μM was observed without a detectable change in V_{max}. Since Ins(1,4,5)P_3 acts as a competitive inhibitor, we can estimate the K_i for Ins(1,4,5)-P_3 as equal to 2.4 mM.

When G$\beta\gamma$ was incubated with PHβ_2-PLCδ_1 prior to the reaction, a 3-fold increase in reaction velocity was observed without a concomitant change in K_m. These results correlate well with the studies described above suggesting that G$\beta\gamma$ does not increase the apparent affinity for PI(4,5)P_2 but causes some change in the catalytic site that increases the reaction rate. Also, since the phosphodiesterase reaction appears to be less efficient with a lower V_{max} and higher K_m than the phosphotransferase reaction, then our results suggests that this second step is rate limiting. Importantly, the presence of G$\beta\gamma$ relieves the ability of Ins(1,4,5)P_3 to inhibit PHβ_2-PLCδ_1 activity suggesting that activation by G$\beta\gamma$ subunits is through an increase in the release of product.

DISCUSSION

Activation of PLCβ_2 by G$\beta\gamma$ may occur at specific points in the course of PI lipid hydrolysis pathway. Cleavage of PI(4,5)P_2 by PLCβ_2 occurs in two steps either of which can also be regulated by G protein subunits either through increases in the catalytic rate or promotion of substrate binding or product/intermediate dissociation. The mechanistic steps for PI hydrolysis that G$\beta\gamma$ may affect can be written as follows:

$$\underset{\text{substrate binding}}{\text{PLC}\beta_2 + \text{PI}} \leftrightarrow \underset{\text{phosphotransferase}}{\text{PLC}\beta_2\cdot\text{PI}} \leftrightarrow \underset{\text{phosphodiesterase}}{\text{PLC}\beta_2\cdot\text{cIP} + \text{DAG}} \leftrightarrow \underset{\text{product release}}{\text{PLC}\beta_2\cdot\text{I-1-P}} \leftrightarrow \text{PLC}\beta_2 + \text{I-1-P}$$

Before discussing the effect of G$\beta\gamma$ on individual mechanistic steps of PLCβ_2 substrate hydrolysis, we first comment on the general aspects of substrate hydrolysis by PLCβ_2. The activity profiles reported here in both the absence and presence of G$\beta\gamma$ fit best to a Hill coefficient greater than 1.0 even though the enzyme appears to be a monomer (*29* and Guo et al., JBC, in press*)*. It is noteworthy that a Hill coefficient greater than one has been observed for other interfacial enzymes as well as PLC enzymes including the small, monomeric *B. cerus* PI−PLC and a comprehensive discussion of its kinetic properties has been presented (*32*). Elevated Hill coefficients have been previously reported for PLCβ_2 (*17*) and from our results we speculate that this apparent cooperativity arises from binding of a hydrophobic component, such as fatty acid (*30*) or the geranylgeranyl group of G$\beta\gamma$ subunits (*31*) to a subsite on the enzyme.

A simple model to explain this suggested cooperativity is that PLCβ_2 exists in an activated and nonactivated conformation and the extent of activation can be modulated by the membrane surface, detergents or G$\beta\gamma$ subunits. Apparent cooperativity could then arise from a concerted conformational change from the inactivated form of the enzyme to the activated conformation. It is tempting to speculate that for the activation of PLCβ_2, a lipid component, which could be the prenyl group of Gγ and protein−protein interactions of G$\beta\gamma$, work together to achieve full activation of the enzyme.

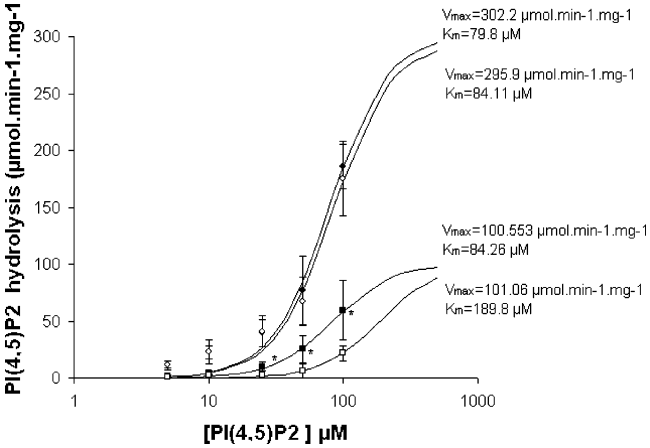

FIGURE 5: Dependence of the PLC δ_1/β_2 chimera activity (μmol min^{-1} mg^{-1}) on PI(4,5)P_2 concentration in the absence (■) or in the presence of 350 nM G$\beta\gamma$ (◆). These curves are compared to studies that were repeated in the absence of G$\beta\gamma$ and in the presence of 3 mM Ins(1,4,5)P_3 (□), and in the presence of 350 nM G$\beta\gamma$ and 3 mM Ins(1,4,5)P_3 (◇). The PLC $\delta 1/\beta 2$ chimera (2−3nM) was incubated with 1 mM vesicles containing various amounts of PE, PS, and PI(4,5)P_2 (molar ratio 66:33:1, 66:32:2, 66:29:5, 66:24:10, 66:14:20, respectively). The reaction was carried out at 37 °C for 30 s. The figure shows the mean of three independent experiments performed at least in duplicate. The difference in PLC activity in the absence and the presence of Ins(1,4,5)P_3 is significant when indicated (ANOVA test; ∗; $p < 0.01$). The data fit was obtained with $R > 0.99$.

Effect of G$\beta\gamma$ on Substrate Binding and the Role of Substrate Presentation. PLCβ_2 has been shown to bind to lipid membranes with low specificity unlike PLCδ_1 whose membrane association is only strong when PI(4,5)P_2 or a very high level of anionic lipid is present (*26*). Furthermore, activating concentrations of G$\beta\gamma$ do not significantly affect the binding of PLCβ_2 to membranes and thus activation is not due to membrane recruitment (*26, 29, 33*). We show here that the magnitude of G$\beta\gamma$ activation of PLCβ_2 toward interfacial substrates is highly dependent on the nature of the lipid surface. High levels (i.e., 8 to 10-fold) of G$\beta\gamma$ activation are seen when membranes composed of PE−PI-(4,5)P_2 (7:3) are used, whereas much lower levels (2 to 5-fold) are seen using bilayers containing 33 mol % negatively charged lipids and 2−5 mol % PI(4,5)P_2 which are thought to more closely resemble the plasma membrane lipid surface. G$\beta\gamma$ activation (∼2-fold) also occurs for PI vesicles, and addition of a 4-fold excess of POPC reduces activation as predicted from simple surface dilution effects (*34*).

The presence of hexagonal phase promoting PE lipids enhanced G$\beta\gamma$ activation of PLCβ_2 for PI(4,5)P_2 hydrolysis. There are numerous factors that may underlie this effect. PE lipids may promote and/or stabilize PLCβ_2/G$\beta\gamma$ complexes and also provide a more hydrophobic surface. PLCβ_2 shows a slightly stronger binding to PE-containing surfaces, and the longer off-rate may allow for longer G$\beta\gamma$/PLCβ_2 complex lifetimes. However, we have found the lateral association between the proteins to be constant when PE lipids are present (Guo and Scarlata, unpublished results). Perhaps the presence of PE lipid combined with the hexagonal phase forming properties of DAG promotes DAG release. PE lipids may also better poise the protein to switch to the active conformation, or gain better access of hydro-

phobic moieties for binding to a putative subsite. Whatever the case, it is clear that the manner in which an interfacial substrate is presented alters the activation properties of PLCβ_2 by G$\beta\gamma$ subunits.

PLCβ_2 is also capable of hydrolyzing monomeric PI and monomeric PI(4,5)P_2. Enzyme specific activity was much higher toward diC_4PI(4,5)P_2 than toward diC_4PI reflecting the preference of the enzyme for phosphorylated inositols. Yet for both substrates, enzyme specific activities for the monomeric compounds were much lower than toward a long-chain PI in different aggregates. These lower rates for monomeric substrates correlate well with the idea that the strong and nonspecific membrane binding of PLCβ_2 allows for a reduction of dimensionality in accessing substrate. That G$\beta\gamma$ was also able to activate PLCβ_2 hydrolysis of monomeric substrates 20−25% supports the idea that activation of PLCβ_2 by G$\beta\gamma$ proceeds through a change in the catalytic core. The catalytic step affected by G$\beta\gamma$ is less clear from kinetics with these substrates since both sequential steps of PI cleavage occurred and only linear I-1-P (or IP_3) could be detected.

The specificity of G$\beta\gamma$ as an activator is unclear. The small G$\beta\gamma$ activation that occurs for monomeric substrates as well as SUVs is not detected when PI is presented in TX-100 or diC_7PC micelles. While the presence of detergents at concentrations much higher compared to enzyme or G$\beta\gamma$ may disrupt G$\beta\gamma$/PLCβ_2 specific interactions, these detergents could also directly interact with PLCβ_2. The nonsubstrate amphiphile may bind to PLCβ_2 and promote conversion of the enzyme to an active conformation or occupy a hydrophobic subsite (see above). Various detergents could have different affinities for the protein that lead to different specific activities which would explain why PI presented in diC_7PC micelles is a better substrate than PI solubilized in Triton X-100. This would imply that G$\beta\gamma$ is an effective activator in vivo because it has a very high affinity for PLCβ_2, but other amphiphiles could regulate the enzyme under certain conditions.

Effect of G$\beta\gamma$ on the Phosphotransferase Step of the Reaction. PLCβ_2 does not produce measurable amounts of cyclic intermediates and thus the rate of phosphotransferase activity can only be indirectly assessed. The G$\beta\gamma$ activation of PLCβ_2 toward diC_4PI and diC_4P(4,5)P_2 soluble substrates in the absence of micelles was relatively minor (20−25%). Since G$\beta\gamma$ activation of PLCβ_2 for hydrolysis of soluble cIP was higher than for the diC_4PI and diC_4PI(4,5)P_2 hydrolysis, it is likely that G$\beta\gamma$ does not contribute significantly to the chemistry of the phosphotransferase step. Higher G$\beta\gamma$ activation for cIP hydrolysis could suggest that when cIP is generated in situ, the DAG generated from the reaction has an effect on the distribution between activated and non-activated forms of the enzyme. We base this idea on the observation that the only factor that may account for the reduced effect of G$\beta\gamma$ toward PI as compared to cIP must be due to some component that effects a lipid bilayer as opposed to micelles and DAG is a likely factor. More studies need to be done to begin to characterize the conformational states of the enzyme and the factors which stabilize them.

G$\beta\gamma$ Activates the Phosphodiesterase Activity. All PLC hydrolysis of PI involves a cyclic inositol phosphate intermediate (e.g., cIP_3 from PI(4,5)P_2 or cIP from PI). cIP is the major product of PI cleavage by bacterial PLC enzymes, but it is produced along with I-1-P by mammalian PLCδ and PLCγ enzymes (*5*, *15*). It has been proposed that electrostatic interactions at the active site of the Ca^{2+}-dependent mammalian PLC enzymes between the inositol phosphates and cationic side chains keep the cyclic intermediate bound to the enzyme long enough so that attack by water to generate I-1-P effectively competes with release of the intermediate. cIP was not detected as a product in any of the PLCβ_2 reactions, even at higher temperatures where the other two mammalian isozymes exhibited enhanced cIP release (*5*, *15*). This observation suggests that the cyclic intermediate is held in the catalytic site of PLCβ_2 longer than in the other isozymes. The catalytic cores of PLCβ_2 and PLCδ_1 are highly homologous (52% identical, 85% homologous) and threading models of the catalytic core of PLCβ_2 based on PLCδ_1 show that catalytic region that interacts with the inositol ring is more hydrophobic than PLCδ_1 (*35*, *36*). While it is possible that this altered polarity may underlie the higher stabilization of the cyclic intermediate in the catalytic site, we did not observe cIP products using the PHβ_2-PLCδ_1 chimera which has the more polar PLCδ_1 catalytic core. Thus, the differences in retention of the cyclic intermediate must lie in the structural changes that the PH domain confers on the catalytic core (*36*).

Enhancement of phosphodiesterase activity toward cIP by G$\beta\gamma$ binding to PLCβ_2 was 1.5-fold. In the presence of diC_7-PC micelles, PLCβ_2 showed the same elevated catalytic rate for cIP hydrolysis as when G$\beta\gamma$ was present in the absence of micelles. Not surprisingly, in the presence of micelles G$\beta\gamma$ activation no longer occurs suggesting that the activation process of PLCβ_2 that occurs in the presence of a hydrophobic component, or in the presence G$\beta\gamma$, occurs for this step of the reaction.

Effect of G$\beta\gamma$ on Substrate Access and Product Release. How does G$\beta\gamma$ affect the phosphodiesterase step? We have indirectly assessed the contribution of substrate binding and product release to the observed phosphoinositide cleavage rate byproduct inhibition studies. The presence of G$\beta\gamma$ does not affect the K_m for substrate (PI(4,5)P_2) indicating that activation is not due to substrate access. Product inhibition by soluble IP_3 is clearly seen in the absence of G$\beta\gamma$. However, no inhibition can be detected when G$\beta\gamma$ is present. These data suggest that activation of PLCβ_2 by G$\beta\gamma$ is through enhanced release of product.

Summary of PLCβ_2 Activation. Taken together, the results here suggest that PLCβ_2 activation is caused by a combination of either hydrophobic substances and/or G$\beta\gamma$ and that this activation proceeds through changes in the catalytic core to allow for enhanced cyclic phosphodiesterase activity and increased product release. The molecular factors that allow for this activation process are presently being explored.

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