Phospholipase C β2 Association with Phospholipid Interfaces Assessed by Fluorescence Resonance Energy Transfer

G PROTEIN βγ SUBUNIT-MEDIATED TRANSLOCATION IS NOT REQUIRED FOR ENZYME ACTIVATION*

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Phospholipase C β2 (PLC β2) is activated by G protein βγ subunits and calcium. The enzyme is soluble and its substrate, phosphatidylinositol 4,5-bisphosphate (PIP$_2$), is present in phospholipid membranes. A potential mechanism for regulation of this enzyme is through influencing the equilibrium association of the enzyme with membrane surfaces. In this paper we describe a fluorescence resonance energy transfer (FRET) method for measuring the association of PLC β2 with phospholipid bilayers. The method allows equilibrium measurements to be made under a variety of conditions, including those that support enzymatic activity and ability to be regulated by G proteins. Using this method it was found that PLC β2 bound to vesicles containing anionic lipids and demonstrated a selective and unique interaction with PIP$_2$-containing vesicles. The FRET data were corroborated with a centrifugation based method for estimating the affinity of PLC β2 for vesicles. Apparently different modes of association of PLC β2 with vesicles of different composition can be distinguished based on alterations in resonance energy transfer efficiency. Association of PLC β2 with PIP$_2$ vesicles requires an intact lipid bilayer, is blocked by neomycin, and is not affected by d-myoinositol 1,4,5-trisphosphate (d-IP$_3$). G protein βγ subunits do not alter the affinity of PLC β2 for lipid bilayers and at the PIP$_2$ concentrations used to measure βγ-dependent stimulation of PLC activity, the majority of the PLC β2 is already associated with the vesicle surface. Furthermore, under conditions where βγ subunits strongly activate PLC activity, the extent of association with vesicles is unaffected by βγ subunits or calcium. These results indicate that activation of PLC β2 by G protein βγ subunits or Ca$^{2+}$ in vitro does not involve translocation to the vesicle surface.

Enzymes of the phospholipase C β (PLC β)$^{1}$ class hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP$_2$) in response to activation of G protein-coupled receptors (1). Many receptor-coupled responses that are insensitive to treatment with the toxin Bordatella pertussis are mediated by α subunits of the G$_i$ family members (2–4), while responses inhibited by this toxin are thought to be via the βγ subunits from G$_i$ proteins. Both of these G proteins can activate PLC β through direct protein-protein interactions as has been demonstrated in reconstitution assays with purified proteins (1, 5–8).

G protein βγ subunits have been implicated as primary signal transducers in wide variety of signal transduction pathways. βγ subunits have been shown to directly activate some isoforms of adenyl cyclase and inhibit others (9), activate β-adrenergic receptor kinase (10), muscarinic receptor kinase (11), cardiac K$^+$ channels (12), and phosphatidylinositol 3-kinase (13, 14). βγ subunits mediate cell cycle arrest via the mating pathway in the yeast Saccharomyces cerevisiae (15), but the effector involved has not been identified. Other work suggests that βγ subunits can lead to activation of Ras and mitogen-activated protein kinase (MAP kinase) in COS-7 (16–18) cells, implicating βγ subunits in the regulation of cell growth. The list of potential effectors continues to grow, but the molecular mechanisms and interactions involved have yet to be understood.

Phospholipase C β is a soluble enzyme whose subcellular localization has not been clearly elucidated. A large proportion of the enzyme is found associated with both the soluble and particulate fractions from cell and tissue lysates (8, 19, 20). In turkey erythrocytes 98% of the PLC β is in the cytosol, while the PLC β found in the particulate fraction is associated with the cytoskeleton, not the plasma membrane (21). Immunocytochemical studies demonstrate a large proportion of the PLC β associated with the nucleus (22) but have failed to demonstrate a plasma membrane or cytosolic location. Thus, there is no conclusive direct evidence demonstrating PLC β association with the plasma membrane. A potential mechanism for activation of the PLC β2 by βγ subunits is through translocation of the PLC from the cytosol by binding to plasma membrane bound βγ subunits. A translocation mechanism has been proposed for activation of cytosolic β-adrenergic receptor kinase by G protein βγ subunits (10). Translocation is also one suggested mechanism for activation of cytosolic PLC β by the epidermal growth factor receptor (23).

Reconstitution of PLC β2 and β3 with βγ subunits in vitro in the presence of phospholipid vesicles containing the substrate results in activation of the phospholipases (8, 24). A potential mechanism for this activation is through βγ subunit-mediated association of PLC β2 with the vesicle surface. We have developed a method based on fluorescence resonance energy transfer transfer, C$_4$E$_{10}$ polyoxethylene-10-lauryl ether; PH domain, pleckstrin homology domain; SBTI, soybean trypsin inhibitor.
for measurement of the association of fluorescently-labeled PLC β2 with vesicles. This method allows us to monitor association of PLC β2 with vesicles under conditions where βγ stimulates PLC β2 activity. In this paper we have examined the properties of lipid vesicles and PLC β2 that contribute to association with lipid bilayer surfaces and examine the influence of G protein βγ subunits on this association. The results presented here could help in understanding the role of βγ subunit-dependent translocation in mediating PLC β activation in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**

Phosphatidylethanolamine (bovine liver) (PE), phosphatidylserine (bovine brain) (PS), phosphatidylinositol (bovine liver) (PI), phosphatidyld choline (bovine brain) (PC), phosphatidic acid (dilinoleoyl), and rhodamine-conjugated phosphatidylethanolamine (egg) (PE-rho) were from Avanti Polar Lipids. PIP2 was prepared from bovine brain lipids by an ammonium sulfate precipitation procedure. Inositol trisphosphate (IP3) was obtained from Calbiochem. S-100 was obtained from Sigma according to the method of Schacht or obtained from Sigma. Phospholipase C isoform (FITC) was obtained from Molecular Probes.

**Methods**

**Plasmid Construction and Cloning of Recombinant Baculovirus—**To obtain the large quantities of PLC that are required for these experiments, recombinant PLC β2 was expressed using a baculovirus expression system. The 3.7-kilobase fragment extending from the 5'-end of the full-length cDNA fragment to a SpeI site in pVL1393 containing the complete coding sequence for PLC β2 and with AcpP3-LacZ baculovirus DNA that had been linearized with BsaI was ligated into pBluescript KS at the SpeI and XbaI sites as above. The modified cDNA fragment and the XbaI fragment were ligated into pBluescript KS as above. The modified cDNA fragment was excised from pBluescript KS with EcoRI and NotI, and this fragment was ligated into pBluescript KS at the EcoRI and NotI sites in pVL1393.

Recombinant baculoviruses were generated by cotransfection (Lipofectin, Life Technologies, Inc.) of fall armyworm cells (SF9 cells) with pVL1393 containing modified PLC β2 cDNA and with AcpP3-LacZ baculovirus DNA that had been linearized with BsaI. Positive clones were isolated by plaque assay and were identified by their ability to directly express the PLC β2 as determined by immunoblotting and by PLC activity assays (described below).

**SF9 Cell Culture and Purification of Phospholipase C—**SF9 cells were grown at 27°C in IPL-41 medium containing 10% fetal bovine serum, 0.1% pluronic acid, and 50 μg/ml gentamicin. For large scale cultures (1 liter above) the cells were switched into medium containing 1% fetal bovine serum and 1% lipid concentrate (Life Technologies, Inc.). Baculoviruses directing expression of recombinant His6 PLC β2 were used to infect SF9 cells at a density of 1.5 × 10^7 cells/ml. Cells were incubated at 27°C and shaken at 125 rpm for 48 h. Cells were collected by centrifugation at 2500 rpm in a Beckman JA10 rotor for 20 min, washed with 1 mL of 50 mM Tris-Cl, pH 8.0. The supernatant was removed, and the pellet was frozen in liquid N2 and stored at −70°C until further processing could be performed.

**Cells were lysed by thawing the frozen pellet in 25 mL of lysis buffer (50 mM NaHepes, pH 7.4, 0.1 mM EGTA, 0.1 mM EDTA, 0.1 mM DTT, 100 mM NaCl, and protease inhibitors) and loaded onto a heparin column.** The column was washed with wash buffer lacking C12E10 and SBTI and with six successive 4-ml applications of 10 mM NaHepes, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 100 mM NaCl, and protease inhibitors without SBTI. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue.

**Expression and Purification of G Protein βγ Subunits—**Baculoviruses encoding βγ subunits, His6-tagged α1, were obtained from Alfred Gilman’s laboratory. 1 liter of SF9 cells at 1 × 10^7 cells/ml were simultaneously infected with the three baculovirus constructs, and the βγ subunits were purified according to the published procedure (27). Briefly, cells were lysed by repeated freeze thawing (four times) and the membranes were centrifuged at 100,000 × g for 1 h. The membranes were suspended and extracted with 1% cholate. Insoluble material was removed by centrifugation, and the detergent extract was applied to a 4-mL column of nickel-nitrilotriacetic acid-agarose. Under these conditions the membrane bound to the resin via the His6 tag on the α subunit. βγ subunits were selectively eluted by activation of the G protein with αIP3. The final preparation was concentrated and gel filtered into βγ vehicle (50 mM NaHepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1% cholate). The yield was approximately 1 mg of βγ-filament of clathrin.

**Fluorescent Labeling of Phospholipase C—**0.5–1.5 mg of PLC (1 mL) was incubated with 20 mM NaHepes, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 800 mM NaCl, and protease inhibitors without SBTI. The reaction was quenched by addition of 200 μl of 1 X Tris-Cl, pH 8.0. The solution was left an additional 15 min on ice before proceeding. Fluorescently labeled PLC β2 was separated from free FITC by chromatography on heparin-Sepharose and hydroxyapatite. A 1-mL column of heparin-Sepharose CL-6B (Pharmacia Biotech Inc.) was equilibrated with 6 mL of buffer A (50 mM NaHepes, pH 8.0, 200 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, and protease inhibitors). The FITC-PLC-Tris sample was diluted to 5 mL in a salt-free NaHepes buffer (50 mM NaHepes, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA plus protease inhibitors) and loaded on the heparin column. The column was washed with 50 mL of equilibration buffer, and labeled protein was eluted with five successive 1-mL volumes of a high salt buffer (50 mM NaHepes, pH 8.0, 800 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA plus protease inhibitors). All washes and elutions were collected individually and kept on ice. At this stage the preparations contain between 20 and 40% of the total fluorescence as free FITC (see below for methods).

To remove the remaining free dye, fractions containing the highest protein concentration were processed by chromatography on hydroxyapatite. A 2-mL column of Macro-Prep ceramic hydroxyapatite (Bio-Rad) was equilibrated with 50 mM NaHepes, pH 8.0, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 100 mM potassium phosphate, pH 8.0, plus protease inhibitors). Pooled, FITC-labeled protein from the heparin column was loaded onto the column followed by a wash with equilibration buffer until the absorbance at 280 nm returned to baseline. The protein was eluted with a linear gradient in equilibration buffer from 100 mM to 700 mM potassium phosphate in 20 mL. 1-mL fractions were collected and assayed for protein. Fractions containing the highest concentrations of protein were pooled and concentrated if necessary. After this step less than 1% of the total fluorescence was due to free FITC. The yield of protein starting with 1 mg of purified PLC β2 was generally about 10–25%. Labeled protein was quantitated by Amido Black protein assay, and fractions having the highest protein concentration were stored at −80°C until later use.

**Characterization of Labeled Protein—**The stoichiometry of protein labeling was assessed by measurement of the absorbance of the modified protein at 495 nm to determine the number of moles of dye present based an extinction coefficient for FITC of 82,000 cm⁻¹ mol⁻¹ (Molecular Probes catalog). This was divided by the molar amount of labeled PLC as determined by the Amido Black protein assay. 

Assessment of relative amounts of bound and free dye was done by...
acetyl precipitation and SDS-polyacrylamide gel electrophoresis (28, 29). After acetyl precipitation, 500 μl of acetyl was added to a 20-μl FITC-labeled PLCβ (F-PLCβ) sample (6–20 μg of protein), incubated on ice 20 min to facilitate protein precipitation, and centrifuged at 13,000 × g for 15 min. The acetyl supernatant containing free FITC was transferred to a new tube and dried under a gentle nitrogen stream. The dried acetyl supernatant was resuspended in 20 μl of elution buffer. The fluorescence of this sample was determined by dilution into 900 μl of FRET buffer (50 mM NaHepes, pH 8.0, 67 mM KCl, 17 mM NaCl, 0.83 mM MgCl₂, 3 mM EGTA, 0.17 mM EDTA, 1 mM DTT, 1 mg/ml BSA). The fluorescence was measured with 495 nm excitation and 520 nm emission wavelengths on a Perkin-Elmer spectrofluorimeter. The fluorescence of an equal volume of unprecipitated sample was also determined and the amount of free FITC based on the percentage of original fluorescence remaining in the dried acetyl extract after acetyl precipitation.

Labeled protein samples were also run on a 9% polyacrylamide SDS gel to separate bound from free dye. Free dye was visible in the dye front under ultraviolet light in samples that had not been subjected to hydroxyapatite chromatography. After the hydroxyapatite, no dye was visible in the ion front of the gel. Protein-associated dye migrated at the position of unlabeled PLCβ (data not shown).

Determination of Lipid Vesicle Binding by Fluorescence Resonance Energy Transfer (FRET)—Experiments were carried out in 0.5–1 ml of FRET buffer (50 mM NaHepes, pH 8.0, 67 mM KCl, 17 mM NaCl, 0.83 mM MgCl₂, 3 mM EGTA, 0.17 mM EDTA, 1 mM DTT, 1 mg/ml BSA). F-PLCβ was added in 2–5 μl and the fluorescence determined in a Perkin-Elmer LS-5B spectrofluorimeter at 30°C with an excitation wavelength of 495 nm and emission at 520 nm. Fluorescence quenching was found to be independent of protein concentration between 0.2 and 2 μg of F-PLCβ. Lipid vesicles were prepared by drying the appropriate amount of lipid in chloroform under a stream of nitrogen. Sonication buffer (50 mM NaHepes, pH 8.0, 3 mM EGTA, 80 mM KCl, 1 mM DTT) was added, and the lipids sonicated for 5 min in a bath sonicator. Vesicles were prepared with 500 μM PIP₂ in various ratios with other lipids as described in the figure legends. In some experiments other anionic lipids were substituted for PIP₂. In most of the experiments PE was used in a 4:1 ratio with PIP₂. Whatever the lipid composition, PE-rhodamine was included at 1% of the total lipid. To measure association of F-PLCβ with vesicles, rhodamine-containing lipid vesicles were added at the appropriate concentration after determination of F-PLCβ fluorescence. Non-specific fluorescence quenching was determined by addition of 0.1% C₁₀E₅₉ prior to addition of lipids.

Calculations for energy transfer were done by division of fluorescence obtained after addition of lipid (F) by original fluorescence due to F-PLCβ alone (F₀). The data are expressed as a percentage of the original fluorescence, calculated according to the formula: (100 – ([F]/[F₀] × 100)). Where appropriate, data were normalized by subtraction of the amount of quenching in the presence of C₁₀E₅₉ from the values obtained in the absence of detergent (as explained below).

Assay of F-PLCβ Vesicle Binding by Centrifugation—F-PLCβ was incubated with vesicles of various lipid composition as described in the figure legends. The incubation was performed in FRET buffer in a total volume of 150 μl for 5 min at 25°C. 100 μl was transferred to centrifuge tubes and centrifuged in a Beckman TL100.3 rotor at 80,000 × g for 30 min at 25°C. The fluorescence of rhodamine in the vesicles was monitored before and after centrifugation at an excitation wavelength of 570 nm and emission wavelength of 590 nm to determine the efficiency of vesicle pelleting by centrifugation at all vesicle concentrations. In all of the experiments, 90% or greater of the vesicles were removed from the supernatant by centrifugation. The extent of F-PLCβ binding was determined by measuring the amount of fluorescein fluorescence (measured at an excitation wavelength of 495 and emission wavelength of 520 nm) in the supernatant of samples containing vesicles and comparing with samples centrifuged in the absence of vesicles (taken as 0% removed). In experiments where βγ subunits were added in a volume of 5 μl, an equal volume of βγ vehicle was added to controls without βγ subunits. Fluorescence was measured by adding 25 μl of supernatant to 1 ml of NaHepes, pH 8.0, 0.1% C₁₀E₅₉.

Phospholipase C Assay—Phospholipase C assays were conducted as described previously (8). Briefly, phospholipid vesicles containing 50 μM PIP₂, 200 μM PE, and 100 μM PIP₃ ([HIP]₃ (8000 cpm/assay) were prepared by sonication in 50 mM NaHepes, pH 8.0, 3 mM EGTA, 80 mM KCl, and 1 mM DTT. βγ subunits were diluted in 50 mM NaHepes, pH 8.0, 3 mM EGTA, 1 mM EDTA, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.14% cholate. Vesicles, PLC, and βγ subunits were mixed on ice in a total volume of 50 μl. 10 μl of a solution containing CuCl₂ was added to start the reaction, and the samples were transferred to a 30°C water bath.
saturation but binding occurred with an apparent affinity similar to that seen for PIP2 vesicles (Fig. 2C). Very little FRET was observed with PC vesicles, suggesting that negatively charged lipids are required in the bilayer to observe significant vesicle association.

In Table I the results from several treatments are shown that are designed as controls to demonstrate that the fluorescence quenching is due to F-PLC β2-phospholipid bilayer interactions and not other trivial causes of fluorescence quenching. First it is shown that quenching is strictly dependent on interactions and not other trivial causes of fluorescence quenching. Fluorescence quenching is due to F-PLC β2 and phospholipid vesicles. First it is shown that quenching is strictly dependent on interactions and not other trivial causes of fluorescence quenching. Fluorescence quenching is due to F-PLC β2 and phospholipid vesicles. First it is shown that quenching is strictly dependent on interactions and not other trivial causes of fluorescence quenching. Fluorescence quenching is due to F-PLC β2 and phospholipid vesicles.
tion to separate bound from unbound F-PLC β2 (data not shown). These data all indicate that binding to phospholipid vesicles and association with PIP2 are required for high efficiency resonance energy transfer.

Since FRET preferentially detects interactions between F-PLC β2 and PIP2-containing vesicles, we tested whether d-IP3 could disrupt FRET. It has been reported previously that d-IP3 blocks binding of PLC δ to PIP2-containing vesicles by up to 90% at low μM concentrations of d-IP3 (30). This is thought to occur through disruption of a specific interaction between a pleckstrin homology (PH) domain at the N terminus of PLC δ and PIP2 (31). In our experiments, 1 mM d-IP3 has no effect on the association of PLC β2 with PIP2-containing vesicles at any PIP2 concentration tested (10 nM to 30 μM data not shown).

Vesicle Association of F-PLC β2 Assessed by Centrifugation—To corroborate the data obtained by FRET we examined the association with of F-PLC β2 with vesicles by centrifugation to separate F-PLC β2 bound to vesicles from free F-PLC β2 (Fig. 3). Using this method we estimate that F-PLC β2 associates with vesicles containing an apparent Kd of 0.6 μM and the binding saturates above 10 μM. If PE-rho is omitted from the vesicles the apparent Kd is 1.3 μM. These data are similar to the data obtained by FRET and indicate that the presence of PE-rhodamine in the vesicles does not significantly affect the apparent affinity of the F-PLC-vesicle interaction. Interestingly there is also interaction between F-PLC and PS containing vesicles with an apparent Kd of 1.3 μM that is saturable above 10 μM PIP2. When compared with the results obtained by FRET, these data suggest that the nature of the interaction between F-PLC β2 and vesicles containing PIP2 and vesicles containing PS is different, since the affinities measured by centrifugation and FRET are similar yet there are large differences in FRET efficiency. It could be that the specific interaction with PIP2 orients the F-PLC such that the fluorescein label is positioned for better resonance energy transfer. Alternatively, the PIP2 and PS could orient the PE-rho differently. It should be noted less of the F-PLC β2 is removed from the supernatant by PS vesicles at saturation. This is not due to a difference in the ability to centrifuge PS-containing vesicles versus PIP2-containing vesicles, because in both cases, greater than 90% of the vesicles were pelleted by centrifugation. The reason for this difference in binding at saturation is unclear, but it may contribute to some of the observed differences in FRET efficiency.

Requirement for a Phospholipid Bilayer for Efficient FRET—The dramatic inhibition of FRET following addition of detergent could be the result of conversion of vesicles to micelles thereby disrupting the interaction between PLC and PIP2. This suggests that a lipid bilayer is required for association of PLC β2 with lipids. An alternate explanation is that the detergent is simply diluting the mole fraction of PE-rho on the surface of the vesicle, thereby reducing the efficiency of FRET. To distinguish between these possibilities, the concentration dependence of detergent effects on FRET were examined with vesicles containing either 1% PE-rho or 10% PE-rho. The results from this experiment are shown in Fig. 4. C12E10 inhibits FRET with an IC50 of ~100 μM for vesicles containing 1% PE-rho and ~200 μM for vesicles containing 10% PE-rho. If the inhibition were truly a dilution phenomenon, then there should be a 10-fold difference in the IC50 for C12E10.

The sharp dependence on concentration for inhibition of FRET (Fig. 4) is suggestive of a cooperative process that probably corresponds to the formation of micelles. The critical micellar concentration (CMC) for C12E10 is approximately 100 μM, which corresponds to the IC50 for inhibition of FRET. If octyl glucoside is used for the same experiment, inhibition is again independent of PE-rho mole fraction and occurs between 10 and 30 mM octyl glucoside, which corresponds to the CMC for octyl glucoside (data not shown). These data suggests that it is the formation of micelles that disrupts FRET, not simply dilution on the vesicle surface.

Another possible explanation for the observations is that significant levels of detergent do not partition in to the phospholipid vesicles below the CMC and only when micelles are formed is significant dilution on the surface observed. In the experiments shown in Fig. 4, 50 μM total lipid is present at each detergent concentration (10 μM PIP2, 40 μM PE). At the IC50 values, the lipids are diluted 3-fold in the case of 1% PE-rho vesicles or 5-fold in the case of 10% PE-rho vesicles (assuming all the C12E10 partitions into the micelles). Thus, at the respective IC50 values, the 10% PE-rho vesicles have a 6-fold higher effective concentration of PE-rho on the surface of the micelle than 1% PE-rho vesicles. This indicates that dilution is not responsible for the inhibition and the disruption of FRET must be due to the physical conversion of vesicles to micelles.

Effects of βγ Subunits and Ca2+ on Affinity of PLC β2 for Vesicles—We measured the effects of G protein βγ subunits on the affinity and final extent of binding of F-PLC β2 to substrate vesicles using FRET. This binding is assessed under conditions that are known to support the activation of PLC β2 by βγ subunits (8). The results presented in Fig. 5 demonstrate association of F-PLC β2 with PE:PIP2:PE-rho (4:1:0.05) vesicles (Fig. 5A) or PE:PS:PE-rho (4:1:0.05) vesicles (Fig. 5B) as a function of lipid concentration in the absence or presence of βγ subunits (300 nM). Neither βγ subunits nor Ca2+ has a significant effect on the interaction of F-PLC β2 with either PIP2- or PS-containing vesicles. Binding to PIP2-containing vesicles was slightly inhibited in the presence of Ca2+, which may have been due to substrate hydrolysis during the course of the measurements. To support the results from FRET, the effects of βγ were
examined in an experiment where vesicles were centrifuged to separate bound and free F-PLC β2 at various vesicle concentrations. The results in Fig. 6 show that βγ subunits do not alter the binding of F-PLC β2 to these vesicles in agreement with the data obtained by FRET.

Significant substrate hydrolysis occurs at low PIP2 concentrations in the presence of both βγ and Ca2+, making affinity measurements difficult. For this reason we tested binding at a fixed, relatively high concentration (50 μM) of PIP2 in the presence of both βγ subunits and Ca2+ and measured the fluorescence immediately after addition of lipids. This concentration of PIP2 is the same as that used in our enzyme assays and the conditions exactly mimic conditions known to support significant PLC β2 activation by βγ subunits. Since the interaction between F-PLC β2 and vesicles, as assessed by FRET, approaches saturation above 10 μM (a hypothesis supported by the centrifugation experiments in Figs. 3 and 6), it suggests that the majority of the F-PLC β2 is bound to vesicles at 50 μM and therefore that it would not be possible for βγ subunits to activate the PLC by translocation. The results presented in Fig. 7A demonstrate that βγ and Ca2+ together do not significantly alter the association of PLC β2 with the vesicle surface. In Fig. 7B is shown an experiment where activation of PLC β2 was measured under identical conditions to the FRET assay, demonstrating a strong activation of PLC β2 by βγ subunits.

**DISCUSSION**

Soluble enzymes whose lipid substrates are localized to membrane surfaces can potentially be regulated by controlling the equilibrium association of the enzyme with the surface of the lipid bilayer. The paradigm for this type of enzyme is secretory phospholipase A2 (reviewed in Ref. 34). A number of G protein βγ subunit-regulated effectors are also likely to be regulated by the equilibrium association of the enzyme with the cell membrane, including PLC β2 and β3, β-adrenergic receptor kinase (10), Brutons tyrosine kinase, and phosphati-
dylinositol 3-kinase (13). To determine if altering equilibrium vesicle association of effectors by βγ subunits is involved in their activation mechanism, it is critical that measurements of vesicle binding are performed under conditions where regulation of enzymatic activity by G protein βγ subunits is known to occur. The goal of the experiments presented here was to monitor the association of PLC β2 with phospholipid bilayers under conditions that are known to support the activation of PLC β2 by βγ subunits. We chose to use fluorescence energy transfer to monitor vesicle association, because it allows equilibrium measurements to be made under virtually any reaction condition. Thus, it can be determined whether factors known to influence PIP2 hydrolysis under certain conditions do so by influencing PLC β2-membrane bilayer association under that same condition.

The results presented here demonstrate that resonance energy transfer can be used to monitor the association of PLC β2 with phospholipid vesicles. Data obtained by centrifugation analysis strongly support and add to the results obtained by FRET. In these assays several factors can affect FRET: 1) association of F-PLC β2 with the bilayer; 2) the orientation of the protein and fluorophore on the vesicle surface; 3) the concentration of PE-rho on the vesicle surface. Since the PE-rho mole fraction on the vesicle surface is constant in all of our experiments, this factor is not influencing the data presented here. Changes in affinity for the vesicle surface will be reflected in changes in the concentration dependence for binding, while orientation factors will influence the maximum transfer effi-
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ciency that occurs at saturation. Changes in both of these parameters can be observed in the data presented. For example, while a greater resonance energy transfer is observed for the interaction of F-PLC β2 with PIP2, vesicles relative to PE vesicles containing other anionic lipids, the binding affinities for these vesicles are similar. We do, however, detect a specific interaction with PIP2 that is probably the result of positioning of F-PLC β2 on the membrane surface such that a relatively high resonance energy transfer efficiency is observed.

Vesicle association can be disrupted by low concentrations of C12E10 in a manner that is insensitive to the PE-cholesterol fraction. That detergent disrupts the interaction suggests that association is not solely based on the presence of PIP2 or other anionic lipids. The phospholipid head groups are still present in the mixed micelles that result after detergent addition, yet no F-PLC β2 binding to these micelles is detected. This suggests that the PIP2 must be presented in the context of a phospholipid bilayer to permit a high affinity association of PLC β2.

PIP2-dependent association of PLC isoforms with vesicles has been reported previously. PLC β and δ isoforms were shown to bind to PIP2 containing vesicles in a centrifugation-based assay (35, 36). In these experiments it was reported that vesicle binding was critically dependent on the presence of PIP2, which could not be duplicated by the addition of PS. We observe a specific and unique interaction of PLC with PIP2 detected as an increase in FRET efficiency, but in our hands PIP2 does not alter the affinity of F-PLC β2 for vesicles. Several factors distinguish the experiments here from those presented previously. In the experiments by the other investigators, PIP2 or PS were included in the vesicles at a small mole fraction (% of PC vesicles). It is possible that PLC β2 does not bind well to PS in the context of PC lipid or that the low mole fraction of PS did not allow association because of insufficient charge on the vesicle. This could explain our apparent lack of an absolute requirement for PIP2 for lipid association. The conditions used for their experiments were unlikely to support enzyme activity and certainly would not support activation by βγ subunits. Thus, while specific conditions may be found where PIP2 is required for PLC-vesicle association, our conditions are particularly relevant to measurable enzyme activity and regulation by G proteins.

The binding of PLC δ to PIP2 containing vesicles is thought to occur via a pleckstrin homology (PH) domain at the N terminus. Binding to vesicles is blocked by addition of 15–60 μM d-IP3, but not l-IP3 (30). It has been shown recently that the isolated PH domain from PLC δ binds d-IP3 with high affinity (1.7 μM) (31). PLC β isoforms have been proposed to contain PH domains at their N termini (37). In our experiments 1 mM d-IP3 has no effect on PLC β2 binding to vesicles, measured either as a change in affinity or FRET efficiency (data not shown). Additionally the binding is mimicked by inclusion of other anionic lipids as has been discussed. This suggests that either PLC β2 PH domain is not required for vesicle association or that the PH domain on this protein is relatively nonselective. It is possible, however, that binding of the PH domain to IP3 is required for the orientation of the PLC on the membrane surface that yields high efficiency FRET and that IP3 does not interact with the PH domain on PLC β isoforms. A role for putative PH domains in PLC β isoforms remains to be demonstrated.

Inclusion of βγ subunits in the vesicle binding assays has no effect on the affinity of F-PLC β2 for PIP2 vesicles or on the orientation of the PLC on the vesicle surface. In our in vitro assays we normally monitor βγ-mediated increases in PLC β activity using 50 μM PIP2 in the presence of 0.001–1.0 μM Ca2+. The fact that binding of PLC β2 to vesicles occurs at low lipid concentrations, and that this binding approaches saturation at concentrations below those that have been used to assay βγ-stimulated PLC β2 activity, indicate that under standard assay conditions (50 μM PIP2), most of the PLC is bound to the vesicle surface. Enhancing this interaction, therefore, is not a viable mechanism for activation of this enzyme by βγ subunits, Ca2+, or any other factors at this PIP2 concentration. This is further emphasized by the direct demonstration that neither βγ nor Ca2+ influences the extent of PLC β2 binding to these vesicles, while under identical conditions, βγ significantly increases the activation of PLC β2 enzymatic activity (Fig. 7). Also of significance is that neither βγ or Ca2+ alters the FRET efficiency on the surface of PIP2 vesicles, suggesting that βγ subunits are not significantly altering the orientation of the PLC on the vesicle surface and the specific interaction with PIP2 that we observe.

Demonstration that βγ subunits do not translocate PLC β2 in vitro does not preclude a role for this process in vivo. These results show that translocation is not required to observe enzyme activation by βγ subunits and that other kinetic mechanisms such as altering Kd or Vmax must be involved in the activation of the enzyme. If one considers that PS, PIs, and PA are restricted to the inner surface of the plasma membrane, there is considerable anionic character to this surface. This suggests that PLC β isoforms could bind to the plasma membrane surface by binding to PIP2 and/or other anionic lipids to bring the enzyme in proximity to the G protein and the substrate.

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