Membrane Binding of Phospholipases C-β_1 and C-β_2 Is Independent of Phosphatidylinositol 4,5-Bisphosphate and the α and $\beta\gamma$ Subunits of G Proteins†

Loren W. Runnels,‡ John Jenco,§ Andrew Morris,§ and Suzanne Scarlata*,‡

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

Received July 2, 1996; Revised Manuscript Received October 3, 1996⊗

ABSTRACT: We have measured the membrane binding affinities of purified phosphatidylinositol-specific phospholipases C-β_1 and C-β_2 to membranes of varying lipid composition using fluorescence methods. Our studies show that these proteins bind with affinities of $10^{-5}-10^{-4}$ M, with a small dependence on lipid type. Binding was relatively insensitive to the presence of phosphatidylinositol-specific phospholipases C-βs' major physiological substrate, phosphatidylinositiol 4,5-bisphosphate, as well as the presence of Ca^{2+}, which is required for activity. The presence of purified GTPγS-activated α_{11} subunits of heterotrimeric guanine nucleotide binding proteins (G proteins) did not alter the membrane binding affinity of phosphatidylinositol-specific phospholipases C-β_1, even though α_{11} is a potent activator of this protein. Similarly, the presence of purified $\beta\gamma$ subunits of G proteins did not alter the membrane association of phosphatidylinositol-specific phospholipases C-β_2 even though these subunits strongly activate this isoform. These results argue against a recruitment model for PLC-β activation by G proteins, negatively charged lipids, Ca^{2+}, or substrate, and suggest that activation occurs through association of the membrane-bound species.

Inositol lipid-specific phospholipases C (PLC)[1] hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the intracellular second messengers 1,2-diacylglycerol and inositol 1,4,5-triphosphate (IP_3) [see Berridge and Irvine (1984) and Exton (1994)]. Three families of mammalian PLC isoenzymes with distinct primary structures have been described. Rhee and co-workers have classified these as PLC-γ, -β, and -δ with approximate molecular masses of 148, 140, and 85 kDa, respectively (Suh et al., 1988b). Multiple PLC isoenzymes exist within each class. Overall sequence similarity between these proteins is low, but there is considerable homology between members of all three classes within two regions of ~170 and 260 amino acids which have been termed the X and Y regions. The PLC-γ class is distinguished by the presence of a sequence of ~400 amino acids between the X and Y regions containing Src-homology 2 (SH2) and Src-homology 3 (SH3) domains (Stahl et al., 1988; Emori et al., 1989; Suh et al., 1988a,b). The three members of the PLC-δ class lack an extended C-terminal region immediately following the conserved Y region (Kriz et al., 1989; Meldrum et al., 1991; Park et al., 1993). In comparison to the other classes of PLC isoenzymes, the PLC-β family has a short region of ~80 amino acids between the X and Y regions, which in the case of the mammalian PLC-βs contains up to 10 consecutive negatively charged residues, and an extended C-terminus of ~400 residues with a marked enrichment (~40%) of charged amino acids [see Rhee and Choi (1992)].

PLC isoenzymes also have distinct modes of regulation. Representative of two broad classes of cell surface receptors, heptahelical G protein-coupled receptors and receptor tyrosine kinases activate PLC-catalyzed inositol lipid hydrolysis in target cells. The PLC-γ enzymes mediate inositol lipid hydrolysis in response to activation of receptor tyrosine kinases. When cells expressing these receptors are challenged with appropriate growth factors, stimulated inositol lipid hydrolysis is accompanied by tyrosine phosphorylation of PLC-γ enzymes [see Berridge and Irvine (1984), Exton (1994), and Suh et al. (1988a)]. Regulation of PLC-γ_1 by EGF (epidermal growth factor) receptors has been studied in the most detail (Suh et al., 1988a,b; Wahl et al., 1989; Meisenhelder et al., 1989). Both *in vitro* and *in vivo*, PLC-γ_1 is phosphorylated on four tyrosine residues by the EGF receptor, two of which are essential for PLC-γ_1 activation. This activation and phosphorylation is preceded by an SH2 domain-mediated association of PLC-γ_1 with autophosphorylated EGF receptor, and translocation of cytosolic PLC-γ_1 to the plasma membrane.

The majority of receptors that couple to PLC belong to the family of cell surface proteins typified by the retinal light receptor rhodopsin. These receptors are single polypeptides composed of seven transmembrane-spanning α-helices linked by intracellular and extracellular loops and employ guanine nucleotide-dependent regulatory proteins (G proteins) as intermediaries in the regulation of intracellular effectors by extracellular signals [see Dohlman et al. (1991) and Neer (1994, 1995)]. Members of a superfamily of GTPases, the G proteins are heterotrimeric proteins with an α, β, and γ subunit structure. There is structural diversity among each

† This work was supported by NIH GM53132 and GM50388. An abstract of this work has previously appeared (Runnels et al., 1996).

* Corresponding author. Telephone: 516-444-3071. FAX: 516-444-3432. Email: suzanne@physiology.pnb.sunysb.edu.

‡ Department of Physiology & Biophysics.

§ Department of Pharmacology.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

[1] Abbreviations: PLC, phosphatidylinositol-specific phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; TEPLC, turkey erythrocyte PLC; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; luvs, large, unilamellar vesicles; CH, cholesterol.

S0006-2960(96)01606-6 CCC: $12.00

of the polypeptide components of a G protein oligomer. To date, cDNA cloning has identified 21 α, 4 β, and 6 γ subunits in mammalian cells. The β and γ subunits are tightly associated and can apparently be interchanged between different α subunits. Structural and functional classification of G protein has been defined by the α subunits, although it is now clear that both the α and the $\beta\gamma$ subunits are directly involved in regulation of diverse effectors, including adenylyl cyclases, Ca^{2+}, Na^+, and K^+ channels, phosphoinositide-3-kinase, and certain members of the G protein-coupled receptor kinase family (Iniguez-Iluhi et al., 1993; Clapham & Neer, 1993; Taylor et al., 1990, 1991; Simon et al., 1991; Smrcka et al., 1991; Waldo et al., 1991; Camps et al., 1992; Boyer et al., 1992; Smrcka & Sternweis, 1993; Helper et al., 1993). The four identified PLC-β enzymes are activated by both members of the G_q class of G protein α subunits and by G protein $\beta\gamma$ subunits. Proteolysis and mutagenesis experiments implicate sequences in the C-terminus of PLC-β_1 in activation by G protein α subunits (Park et al., 1993; Wu et al., 1993), and suggest that the N-terminus of PLC-β_2 is important for activation by G protein $\beta\gamma$ subunits.

Despite these advances, the molecular mechanism(s) through which G protein subunits activate PLC-β enzymes is (are) poorly understood. Like many regulated lipolytic or lipid-modifying enzymes, the PLC-β enzymes appear to be predominantly cytosolic and can be purified as stable soluble proteins (Exton, 1994). However, their phospholipid substrates and G protein activators are components of cellular membranes. In a scenario similar to the recruitment of PLC-γ proteins by receptor tyrosine kinases, activation of PLC-β by the α and $\beta\gamma$ subunits of heterotrimerc G proteins may involve regulated interaction of the enzymes with substrate-containing membrane surfaces.

The membrane association of mammalian PLC-β_1 and -β_2 has not yet been studied. Membrane binding studies of turkey erythrocyte PLC (TEPLC) and PLC-δ_1 enzymes (James et al., 1995; Rebecchi et al., 1992) have suggested a mechanism for membrane association that involves binding of PIP_2 to a noncatalytic site which anchors the enzymes to the membrane and allows them to function in a scooting mode of catalysis. The PIP_2 binding site of PLC-δ_1 appears to lie in its N-terminal pleckstrin-homology (PH) domain (Garcia et al., 1995). Both PLC-β_1 and PLC-β_2 possess PH domains at their NH_2 termini, and it is possible that these domains could also bind PIP_2. PLC-β_1 and -β_2 also contain regions homologous to the calcium-dependent lipid binding motif (calB or C2 domain) that mediates membrane binding and activation of cytosolic phospholipase A_2 (Sutton et al., 1995). Ca^{2+} ions activate PLC-β, and it is possible that they do so by promoting the enzyme's membrane association through its C2 domain. As observed for other lipid-associating proteins, the membrane binding affinity of PLC-β may also be mediated by components that alter the bulk properties of the membrane surface, such as anionic lipid, phosphatidylethanolamine lipids, cholesterol, and integral membrane proteins (e.g., Bazzi et al., 1992; Epand & Leon, 1992).

Here, we have measured the membrane binding affinity of the β_1 and β_2 isoforms of PLC using fluorescence spectroscopy. We have measured the extent that membrane binding depends on the presence of activated G protein subunits, Ca^{2+}, negatively charged lipids, PIP_2, and other membrane components. We find that activation of PLC-β_1 and -β_2 does not occur by its recruitment to the membrane by G protein subunits, Ca^{2+}, or other membrane components. Our results also show that, unlike PLC-δ_1 and TEPLC, PLC-β_1 and -β_2 bind to bilayers of varying composition with relatively high affinity and show little dependence on the presence of PIP_2.

MATERIALS AND METHODS

Recombinant human PLC-δ_1 was a generous gift from Dr. Mario Rebecchi (SUNY Stony Brook, Stony Brook, NY). Fluorophores were purchased from Molecular Probes, Inc. (Eugene, OR). All lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), and used without further purification. Lipid bilayers were prepared by extrusion using the method of Hope et al. (1985), to produce uniform large, unilamellar vesicles (luvs) of 100 nm diameter. Lipid loss during vesicle purification was monitored either by phosphate analysis or by doping the vesicles with [^{14}C]phosphatidylcholine during preparation. For the studies involving Laurdan-labeled membranes, membranes were prepared by first doping them with a small aliquot from a concentrated solution of Laurdan in ethanol, and then sonicating at low energy in a bath sonicator for 5−10 min. Ca^{2+} concentrations were estimated fluorimetrically using Mg^{2+}-FURA-2 (Molecular Probes, Eugene, OR).

Expression and Purification of PLC-β_1 and PLC-β_2. cDNAs encoding rat PLC-β_1 and human PLC-β_2 were generously provided by Drs. Sue Goo Rhee (NHLBI, NIH, Bethesda, MD) and John Knopf (Genetics Institute, Boston, MA). The proteins were expressed in Sf9 cells using baculovirus vectors. We used PCR amplification with specific primer-adapters to introduce a unique restriction site 40 base pairs 5′ of the initiating methionine codon allowing directional insertion into the PVL1392 transfer vector (Invitrogen Inc.). Recombinant baculoviruses in which the PLC-β cDNAs were inserted into the viral polyhedron gene by homologous recombination with the transfer vector were generated, selected, and amplified by standard methods (Lucklow & Sumner, 1988). Suspension cultures of Sf9 cells (0.5−1 L, 10^6 cells/mL) grown in complete Grace's medium containing 10% fetal bovine serum were infected with PLC-β_1 or PLC-β_2 recombinant baculovirus at a multiplicity of 10. Cells were harvested after 48 h, washed in phosphate-buffered saline (PBS), resuspended in 40 mL of PBS, and subjected to nitrogen cavitation (Parr cell disruption bomb, 1100 psi for 30 min with intermittent agitation). Cells were discharged into 100 mL of ice-cold buffer A (25 mM Hepes, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.1 mM benzamidine, and 0.1 mM PMSF), and the soluble fraction was recovered after ultracentrifugation at 100000*g*. This material was diluted to 150 mL with buffer A and applied to an 8.9 mL column of Source 15Q resin (Pharmacia, Uppsala, Sweden). Bound proteins were eluted with a 200 mL gradient of 0−1.0 M NaCl in buffer A. Fractions were collected, and their PLC activity was determined using exogenously-added substrates (Morris et al., 1990). Fractions containing PLC activity were pooled, diluted to an NaCl concentration of 0.1 M with buffer A, and applied to a 4 mL column of heparin−Sepharose CL4B (Pharmacia). Bound protein was eluted with a 50 mL gradient of 0−1 M NaCl in buffer A. Fractions containing PLC activity were pooled and exchanged into buffer B (10 mM KH_2PO_4, 1 mM DTT) and applied to a 3 mL BioGel HPHT (BioRad,

Hercules, CA) column. Bound proteins were eluted with a 40 mL gradient of 10−650 mM KH_2PO_4 in buffer B. Active fractions were pooled, diluted into buffer A, and applied to a 1 mL column of Source 15Q resin (Pharmacia). Bound protein was eluted with a 25 mL gradient of 0−1.0 M NaCl in buffer A. The final yield was typically 1−3 mg of purified PLC-β proteins from 500 mL of Sf9 cell culture. PLC-β_1 and PLC-β_2 were purified to apparent homogeneity as determined by SDS−PAGE and Coomassie staining. Glycerol was added to purified protein to 10% (v/v), and the protein was aliquoted, flash-frozen with liquid nitrogen, and stored at −80 °C. Gel filtration analysis [Sephacryl S-300HR resin (Pharmacia), eluted in buffer A containing 100 mM NaCl] indicated that the purified proteins were predominantly monomeric. We note that PLC-β_1 and PLC-β_2 expressed in Sf9 cells have similar catalytic properties to those derived from native sources (Paterson et al., 1992).

Purification of G Protein α *and* $\beta\gamma$ *Subunits.* G protein $\beta\gamma$ subunits were purified from detergent extracts of bovine brain membranes using the method of Sternweis and Robishaw (1984), substituting octyl-Sepharose for the final step. The proteins were further concentrated and exchanged into 0.01% lubrol PX by anion-exchange chromatography on a 1 mL Source 15Q column (Pharmacia). Glycerol was added to purified protein to 10% (v/v), and the protein was aliquoted, flash-frozen with liquid nitrogen, and stored at −80 °C.

α_{11} was prepared by the procedure reported by Biddlecome et al. (1996). The α_{11} recombinant baculovirus has been described previously (Maurice et al., 1992). The β_1 and His_6-γ_2 subunit recombinant baculoviruses were generously provided by Drs. A. Kozasa and A. G. Gilman at Southwestern Medical Center, Dallas, TX. Glycerol was added to purified protein to 10% (v/v), and the protein was aliquoted, flash-frozen with liquid nitrogen, and stored at −80 °C.

Preparation of Inside-Out Erythrocyte Membranes. Red blood cells were isolated from outdated whole blood from the University at Stony Brook hospital blood bank. Cells were homogenized either with $MgSO_4$, which causes them to vesiculate into the extracellular space and produce right-side-out vesicles, or in the absence of $MgSO_4$, which causes them to vesiculate into the cell interior and form inside-out membranes (Steck et al., 1971). These two types of membranes were then purified by ultracentrifugation in a sucrose density gradient, as described (Steck et al., 1971). The orientation of the membranes (i.e., inside-out or right-side-out) was verified using fluorescein-labeled concanavalin A (F-ConA) which binds tightly to carbohydrates such as glucose-6-*N*-acetylglucosamine (GlcNAG). The two sets of membranes were incubated at room temperature with F-ConA for 20 min and washed 3 times with buffer. Strong fluorescence was observed from the right-side-out samples, but only background signals were obtained for the inside-out samples. The specificity of F-ConA for carbohydrate labeling was confirmed by monitoring the reduction in fluorescent labeling of the cells upon addition of GlcNAG to the samples. Also, addition of the detergent saponin to the inside-out vesicles allowed concanavalin to diffuse into the interior of the vesicles, and labeling was observed.

Binding Assessment Using Concentrator Tubes. Millipore Ultrafree-MC 0.1 μm concentrator tubes were used consisting of a 1.5 mL centrifuge tube housing and carrying a 0.5 mL filter cup. The filters were first equilibrated with buffer F (25 mM Hepes, pH 7.0, 100 mM NaCl, 2 mM EGTA, and 2 mM EDTA) using slow-speed centrifugation on a table-top centrifuge at 1000*g*. For each binding experiment, the tested enzyme was diluted into buffer F to a total volume of 1.5 mL. Filters were then equilibrated in the presence of the enzyme solution by placing 450 μL of the solution in the filter cup and spinning at 1000*g* for 30 s, allowing roughly 50 μL of the solution to pass through. A sample of the filtrate was taken at this point, and the residual filtrate was discarded. The filter cup was then refilled to 450 μL with enzyme solution and mixed, and the above process was repeated 4 more times, each time taking a sample of the filtrate and refilling the cup to 450 μL with the enzyme solution and mixing. Following the fifth spin, freshly prepared large, unilamellar vesicles, doped with [^{14}C]PC and extruded with two 0.6 μm filters, were added to the filter cup, and the total volume was brought up to 450 μL with enzyme solution and mixed. The lipid−enzyme mixture was spun for 30 s at 1000*g*, and a sample was taken of the filtrate. Leakage of the vesicles through the pore was assessed either by radioactivity or by 90° light scattering and was typically less than 10%. To titrate the enzyme with lipid, more lipid vesicles were added to the filter cup, and the total volume was brought up to 450 μL with enzyme solution, mixed, and then spun again at 1000*g*. Qualitative binding of enzyme to lipid vesicles was assessed by a loss of activity in the samples from the filtrates. We note that this assay is best suited for low lipid concentrations, where interference of the lipid with filtration is low.

Protein Labeling. All proteins were labeled with the probe acrylodan which is highly reactive with thiol groups and has a very low reactivity toward other functional groups (Molecular Probes). Labeling was done by adding a 4-fold stoichiometric amount of probe to the protein from a concentrated solution in DMF, mixing at 5 °C for 3 h, and removing the unreacted probe either by microdialysis or by absorbing the free label onto Source 15Q (Pharmacia) under conditions where the protein does not absorb. This method removes greater than 95% of the unreacted probe. The probe-to-protein labeling ratios were determined by BCA analysis (Pierce, Rockford, IL) and absorption of the probe ($\epsilon_{340} = 20\,000\ M^{-1}\ cm^{-1}$), and were estimated to be 1:1 for PLC-β_2 and 0.3:1 for PLC-β_1. The labeling ratios varied little with sample preparation, and studies to investigate which residues were modified on the proteins were not conducted. Other fluorescent labels were attached using similar methodology. The labeled proteins had comparable activity to their unlabeled counterparts, and could be activated by G protein α and $\beta\gamma$ subunits under appropriate conditions.

Activity Assays. PLC activity was determined by reconstituting the enzyme with extruded phospholipid vesicles composed of a mixture of 1:1:1 PC/PS/PE with 2 mol % PIP_2. The total concentration of lipid in the assay was 1 mM, with 10 000−20 000 cpm of [^{3}H]PIP_2 per assay. Typically, PLC was diluted into 20 μL of MAIN buffer, consisting of 50 mM Hepes, pH 7.0, 160 mM KCl, 3 mM $MgCl_2$, 3 mM EGTA, and 1 mM DTT, and was added to 20 μL of substrate vesicles, also in MAIN buffer. The mixtures were kept in 1.5 mL Siliconized Flat Top Microtubes (Fisher Scientific, Pittsburgh, PA) on ice, and the assays were initiated by the addition of 10 μL of MAIN buffer plus 12 mM $CaCl_2$ and transfer of the tubes to a 37 °C water bath.

The free Ca^{2+} concentration in the assay was kept between 500 and 1000 nM, as estimated using a $K_{Ca^{2+}}$ for EGTA of 2.45×10^6 M^{-1} (Bers, 1982), or by FURA-2 (Molecular Probes) measurements. Activity assays were allowed to proceed for 5−15 min, and were terminated by the addition of 200 μL of ice-cold 10% (w/v) TCA and 100 μL of ice-cold 10 mg/mL BSA. Tubes were centrifuged for 5 min at 7200*g* to pellet precipitated protein and nonhydrolyzed PIP_2, and 300 μL of the supernatant was transferred to a scintillation vial for counting.

For G protein activation studies, the G protein subunits were diluted into 5 μL of MAIN buffer plus 0.2 mM *n*-dodecyl β-D-maltoside (Calbiochem, La Jolla, CA), and added to the PLC/lipid mixture. $G\alpha_{11}$ subunits were first activated before dilution into MAIN plus 0.2 mM *n*-dodecyl β-D-maltoside either by incubating the protein with 1 mM GTPγS and 50 mM $MgCl_2$ at 30 °C for 1 h or by incubating with 10 μM GDP, 50 mM $MgCl_2$, 10 mM NaF, and 20 μM $AlCl_3$ at room temperature for 15 min. Using these vesicle substrates, G$\beta\gamma$ was able to activate 50 nM PLC-β_2 3.5−5.6-fold with a half-maximal concentration of 150 nM. Due to the limited availability of α_{11} subunits, only 2−3 activation points were done per experiment, but maximal activation (5-fold) was observed at 10 nM.

Fluorescence Measurements. Fluorescence measurements were taken on an I.S.S. K2 (Champaign, IL) time-resolved spectrofluorometer. Silonized microcuvettes were used to prevent adhesion of the protein to cuvette walls. Samples were diluted in filtered buffers containing 20 mM Hepes, 0.1 M NaCl, 1 mM EGTA, and 1 mM DTT, pH 7. All samples and buffers were purged with nitrogen to prevent photobleaching of the fluorophores. Samples were stirred immediately before taking data. For the intrinsic fluorescence studies, 310 cut-on filters were placed before the emission monochromator to eliminate any contribution from scattered light. Sample signals were corrected for light fluctuations by simultaneously monitoring the exciting light on a reference photomultiplier. Emission was corrected for any background signal from control cuvettes under identical optical conditions.

Intrinsic fluorescence data were taken by exciting at 280 nm and either scanning the emission from 310 to 450 nm or monitoring the intensity at 335 nm (both gave identical results). Laurdan-doped membranes and acrylodan-labeled proteins were excited at 350 nm, and the emission was scanned from 400 to 600 nm.

We wanted to determine whether the addition of protein promoted vesicle aggregation and light scattering. We found that the amount of light scattering in the presence or absence of PLC-β was constant throughout the time course of the binding titrations. This behavior is in sharp contrast to the greater than 50-fold initial increase in scattering intensity, which decays to a 5-fold increase over 5−10 min, when vesicle aggregation is induced by the addition of 1 mM calcium to PIP_2-containing vesicles at identical concentrations. These observations indicate that PLC-β does not induce vesicle aggregation under our conditions.

We report the shift in the emission energy in terms of the center of spectral mass (CM) in kilokaisers (kk), which is a unit of energy (1 kk = 10 000 cm^{-1}). The center of spectral mass takes into account changes in the skewness of the peak and is calculated from the emission energy (EE) and intensity (INT) at each wavelength (λ_i) by

$$CM = \sum_i EE_i INT_i / \sum_i INT_i$$

Data Analysis. Binding constants from intrinsic fluorescence studies were analyzed by first correcting the data for dilution and background signal, normalizing the observed fluorescence parameters measured at the particular lipid concentration to its value in the absence of lipid, and then fitting the adsorption isotherm to a hyperbolic function using Sigmaplot (Jandel Scientific). Binding constants from acrylodan-labeled proteins were analyzed by first making the appropriate corrections for background and dilution, and then analyzing the data by the normalized increase in fluorescence emission intensity.

The energy transfer efficiency between the acrylodan label of PLC-β_2 and its intrinsic tryptophan residues was estimated by comparing the emission spectrum from 400−600 nm, when exciting both Trp donors and the acrylodan acceptor at 280 nm, to the spectrum obtained when exciting only the acrylodan acceptor at 340 nm, as described by Kleinfeld (1988). Extinction coefficients of 20 000 M^{-1} cm^{-1} at 340 nm for acrylodan and 97 200 M^{-1} cm^{-1} at 280 nm for PLC-β_2, assuming random orientions between energy transferring species, were used for this calculation. Similar studies for Cascade-blue and fluorescein-labeled PLC-β_2 compared the spectral emission of the Cascade-blue and fluorescein-labeled species to that of only the fluorescein-labeled species, and used extinction coefficients of 27 000 M^{-1} cm^{-1} at 340 nm for Cascade blue and 49 000 M^{-1} cm^{-1} at 490 nm for fluorescein. We note that because of the uncertainty in the fluorophore's labeling distribution, the efficiency reported should only be regarded as a crude estimate.

RESULTS

Binding of the two PLC-β isoforms to membranes was monitored by three types of fluorescence methods: (1) Changes in the intrinsic fluorescence from the tryptophan and tyrosine residues of the proteins as lipid is titrated into the protein solutions. (2) Changes in the fluorescence properties of a covalently-linked probe as lipid is titrated into the protein solutions. The probe used for these studies, acrylodan, selectively attaches to cysteine side chains. This probe is highly sensitive to the polarity of its environment and, depending upon the position of the probe on the protein, is expected to be sensitive to environmental changes that occur with membrane binding. (3) Changes in the fluorescence properties of a probe located on the membrane surface as protein is added to the lipid solution. The membrane probe used in these studies, Laurdan, undergoes a shift in emission energy and an increase in intensity as the protein replaces water on the membrane surface. We conducted a full study using all three methods with PLC-β_2, and then used the second method to determine the membrane binding affinities of PLC-β_1.

We observed that PLC-β_2 undergoes a large (~35%) decrease in intrinsic fluorescence in the presence of lipids (Figure 1) which appears to be caused by membrane binding (see below). The decrease in intensity is due either to quenching of interfacial tryptophan residues by charged groups on the lipid surface or to conformational changes of the protein upon binding. Since the extent of this decrease does not significantly depend on the charge of the lipid surface, we postulate that the decrease is caused by confor-

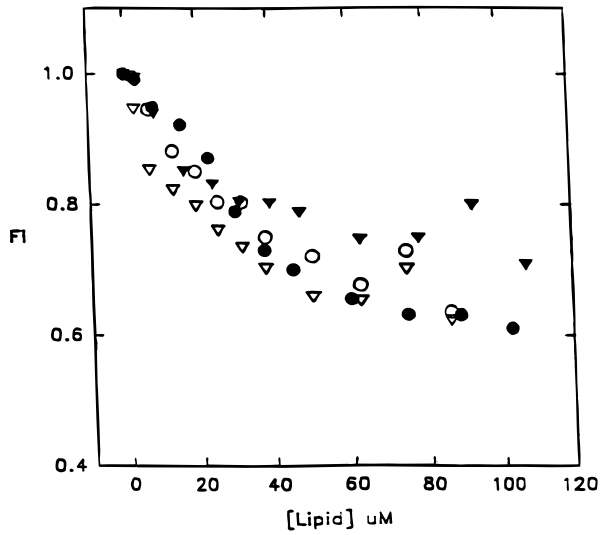

FIGURE 1: Decrease in the intrinsic fluorescence intensity (FI) of PLC-β_2, monitored at $\lambda_{ex} = 280$ nm and $\lambda_{em} = 335$ nm, with the addition of POPC (●), POPC/POPS (2:1) (○), POPC/POPS/cholesterol (1:1:1) (▼), and POPC/POPS/POPE (1:1:1) (▽). Data were corrected for background scatter and dilution, and were normalized to the initial point (i.e., without added lipid). The maximum error is ±0.03.

mational changes. For all the lipid systems studied, the titration curves showed complete binding by 100 μM lipid (see discussion below) with no further changes up to the last point taken, 500 μM, where light scattering from the vesicles precluded reliable measurements. It is worth noting that both PLC-β_1 and PLC-δ_1 show similar decreases in intrinsic fluorescence upon membrane binding.

The next series of studies were conducted to assess the extent of the conformational changes that may accompany binding. First, we monitored changes in the tryptophan and tyrosine accessibility to two aqueous soluble quenchers, acrylamide and I^-, upon membrane binding. Quenching by acrylamide was linear in the 0−150 mM range tested and decreased the emission of both the membrane-bound and free proteins equally, causing a 75% decrease at the highest acrylamide concentration. KI also quenched the membrane-bound and free protein equally. These results indicate that binding is not accompanied by a change in the accessibility of tryptophan or tyrosine residues to either of these quenchers, and that the tryptophan/tyrosine population whose emission decreases upon membrane binding is not accessible to quencher. Second, we monitored changes in energy transfer from PLC-β tryptophans to an acrylodan label covalently attached to the protein upon membrane binding. Changes in energy transfer efficiency would indicate alterations in the average distance between the covalent probe and the PLC's tryptophan residues. We found that the transfer efficiency remained constant at ~12% upon binding. Third, we labeled the protein with two probes that will occupy external sites on the protein: the thiol-reactive Cascade blue and the amine-reactive fluorescein isothiocynate. We found that the extent of energy transfer between these probes remained unchanged upon membrane binding. These studies indicate that any conformational changes that PLC-β_2 undergoes upon binding are not large scale and probably only involve localized regions of the protein.

Binding of PLC-β_2 was also monitored by adding protein to membranes doped with a small amount (1 mol %) of

Table 1: Membrane Binding Affinities of PLC-β_2

species[a]	K_d (μM)
PLC-β_2-POPC	49 ± 12
PLC-β_2-POPC + Ca^{2+}	42 ± 15
PLC-β_2-POPC/POPS (2:1)	33 ± 6
PLC-β_2-POPC/POPS/CH (1:1:1)	18 ± 3
PLC-β_2-POPC/POPS/ POPE (1:1:1)	24 ± 8
PLC-β_2-POPC/PIP_2 (98:2)	65 ± 13
PLC-β_2-POPC/POPG/ PIP_2 (68:30:2)	20 ± 7
PLC-β_2-LR-POPC/POPS/ PIP_2 (68:30:2)	25 ± 9

[a] LR denotes membranes labeled with 1% Laurdan; $n = 2-8$ independent samples.

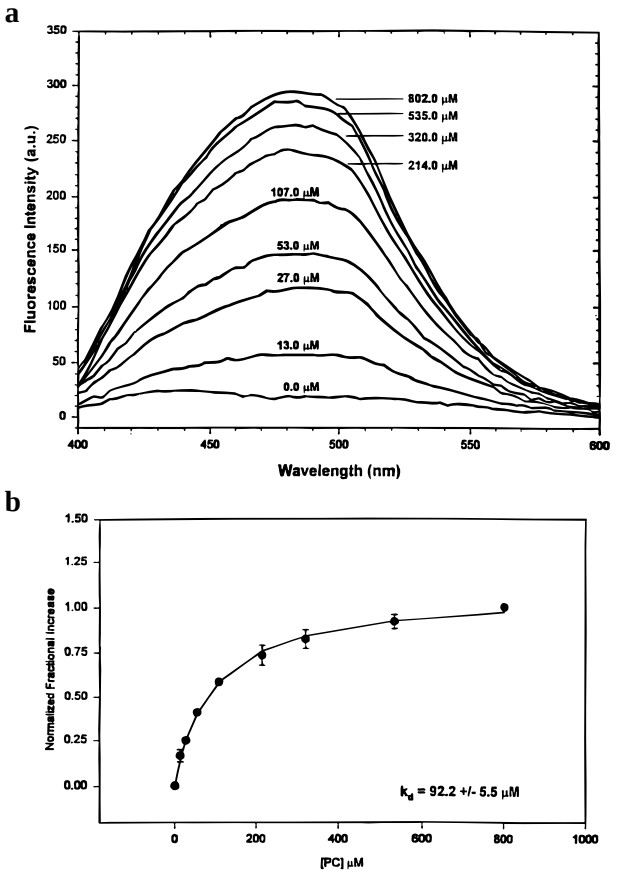

FIGURE 2: (a) Changes in the emission spectrum of acrylodan-PLC-β_2 (130 nM) upon the addition of POPC bilayers. (b) The corresponding binding curve using the fractional increase in emission intensity.

Laurdan. The addition of protein resulted in a shift of the Laurdan emission to higher energies and an increase in fluorescence intensity. These changes correspond to a decrease in the polarity of the Laurdan environment, and are consistent with protein binding to the membrane surface. The binding affinities determined by this method closely match those determined by intrinsic fluorescence (Table 1).

PLC-β_2 was covalently labeled with acrylodan, and the changes in acrylodan emission properties were monitored with lipid addition. This probe proved to be very responsive to membrane binding, showing a significant increase in emission intensity (Figure 2a,b). Binding affinities were determined by the change in intensity, assuming that the extinction coefficient of the probe does not change significantly upon binding. The binding affinities of acrylodan−

Table 2: Binding Affinites (K_d, μM) of Acrylodan-Labeled Proteins

membrane	PLC-β_1	PLC-β_2
PC	200 ± 41	92 ± 8
PC + Ca^{2+}	−	86 ± 8
PC/PIP_2 (98:2)	83 ± 21	72 ± 9
PC/PS/PE (34:33:33)	53 ± 12	82 ± 8
+0.5 M NaCl	76 ± 18	
PC/PS/PE/PIP_2 (32:33:33:2)	43 ± 5	−
PC/PS (2:1)	−	52 ± 7

PLC-β_2 are somewhat lower, but on the order of those obtained by intrinsic fluorescence. We note that the binding constants determined by energy transfer of unlabeled protein to Laurdan probes on the membrane surface gave affinities similar to those following acrylodan-labeled protein, indicating that the acrylodan label does not alter binding. Also, sedimentation studies of the labeled protein with sucrose-encapsulated vesicles [see Rebecchi et al. (1992)] gave qualitatively similar binding affinities.

Binding results, as shown in Table 1, indicate that PLC-β_2 binds to PC membranes with an affinity that is lower than vesicles with other compositions. Incorporation of PS at a 2:1 PC:PS molar ratio causes a minor enhancement in binding. This dependence on ionic lipid head groups indicates an electrostatic component to membrane binding; however, increasing the ionic strength from 0.1 to 0.5 M did not greatly affect the observed K_d. Incorporation of lipids with PE head groups causes an additional small increase in affinity.

PIP_2 is only a minor component of natural membranes, comprising only ~3% of the total lipid in the inner monolayer of human erythrocyte plasma membranes (Christensen, 1986; Mitchell et al., 1986). Thus, we conducted our studies at low PIP_2 concentrations. It is important to note that this small amount of PIP_2 is sufficient to recruit PLC-δ_1 to the membrane surface (Rebecchi et al., 1992), and is low enough such that changes to the physical properties of the membrane would not be expected. We find that in each lipid system, incorporation of 2% PIP_2 produces only a small, but not significant, change in binding affinity.

The enzymatic activity of PLC-β is Ca^{2+}-dependent, and PLC-β has been reported to contain a C2 or calB (Ca^{2+}-activated lipid binding) domain. To determine whether the presence of Ca^{2+} enhances binding, we measured binding in the presence and absence of activating amounts of free Ca^{2+} (see Materials and Methods). Although the addition of Ca^{2+} alters the fluorescence properties of the protein, no difference in binding affinities was observed. Also, the binding affinities to PIP_2-containing membranes were also unchanged in the presence of activating amounts of Ca^{2+} (data not shown).

We then repeated this series of studies on acrylodan-labeled PLC-β_1, and the results are presented in Table 2. The PLC-β_1 binding affinities closely match those observed for the β_2 isoform for all lipid systems investigated. We verified the fluorescence studies of selected samples by a binding assay using concentrator tubes in which the proteins can pass freely through the pores but vesicles cannot (see Materials and Methods). This method gives binding affinities of PLC-β_1 to PC/PS/PE and PC vesicles that are in good agreement with the fluorescence results. The above studies involved model membranes which should have uniform size and surfaces. To compare the binding affinities of PLC-β_1 to model membranes with a natural membrane system, we prepared purified inside-out erythrocyte plasma membranes (see Materials and Methods). Unlike model membranes, these bilayers contain significant amounts of integral proteins along with a large number of membrane components such as cholesterol, PE lipids, phosphatidylinositol lipids, and lysolipids. In Figure 3, we show the binding of protein to these membranes where the concentration of membranes is expressed in terms of the lipid concentration. We find that the binding affinity of PLC-β_1 to these natural membranes is similar to those for model membranes.

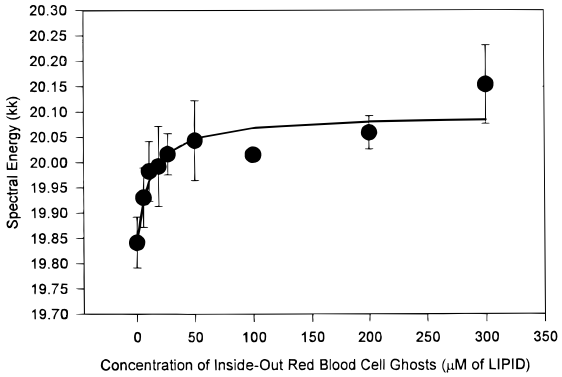

FIGURE 3: Binding results for the association of acrylodan-PLC-β_1 (200 nM) to inside-out red blood cell membranes as seen by the increase in emission energy where $n = 3$.

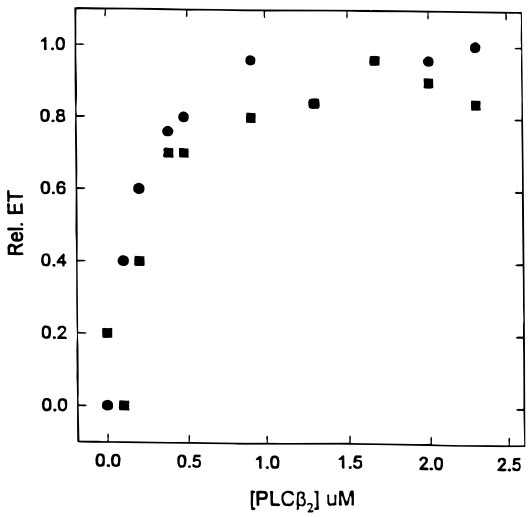

FIGURE 4: Increase in the relative energy transfer (Rel. ET) from PLC-β_2 tryptophan residues to Laurdan probes on the surface of POPC/POPS (2:1) bilayers in the absence (●) and presence (■) of 1.73 μM G$\beta\gamma$ subunits. The relative energy transfer was determined by the ratio of the spectral areas, taken between 380 and 545 nm, when only Laurdan is excited (i.e., $\lambda = 340$ nm) to when both PLC Trp residues and Laurdan are excited (i.e., $\lambda = 280$ nm). Maximum error is ± 0.015.

The presence of G protein subunits does not alter the binding affinities of PLC-β_1 and PLC-β_2: If G protein subunits activate PLC-βs by recruiting or increasing their affinity for membrane surfaces, then the PLC binding affinity should increase in the presence of G proteins. We first investigated the possibility of PLC-β_2 recruitment by $\beta\gamma$ subunits using the Laurdan-based assay. In Figure 4, we show the increase in energy transfer (normalized) as PLC-β_2 is added to POPC/POPS luvs. These data give an association constant similar to those found in intrinsic fluorescence measurements (Table 2). When $\beta\gamma$ subunits are incorporated into the membranes at concentrations

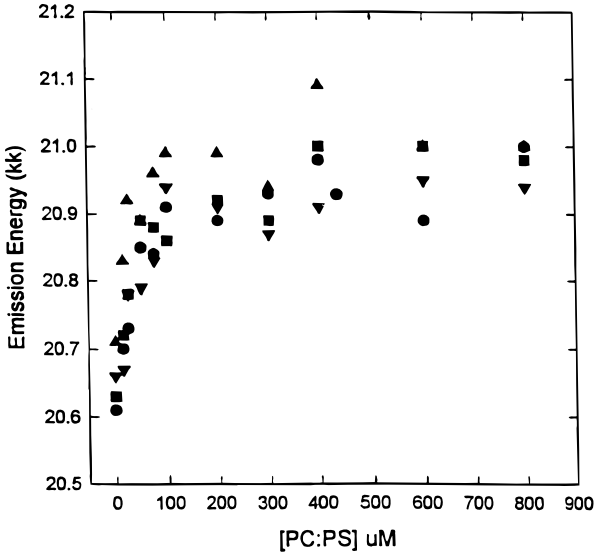

FIGURE 5: Increase in the emission energy of acrylodan-PLC-β_2 (150 nM) upon binding to POPC/POPS (2:1) bilayers (▼) without G$\beta\gamma$ subunits, (●) with 0.75 μM G$\beta\gamma$ subunits, (■) with 1.5 μM G$\beta\gamma$ subunits, and (▲) with 2 μM G$\beta\gamma$ subunits.

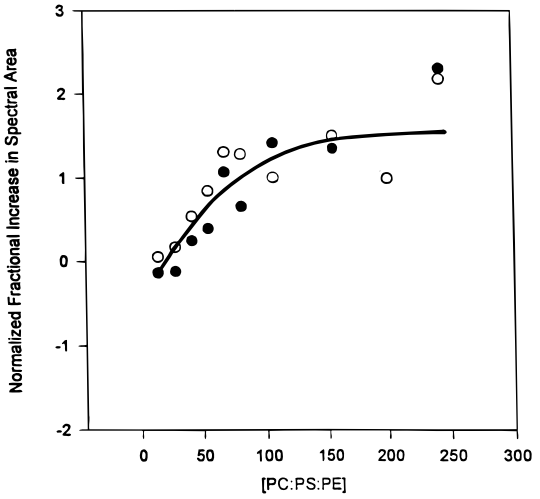

FIGURE 6: Binding 20 nM acrylodan-PLC-β_1 to POPC/POPS/POPE (1:1:1) bilayers in the presence of 10 nM Gα_{11} (○) and 10 nM heat-inactivated Gα_{11} (●).

sufficient to produce activation, the binding affinity of PLC-β_2 is unaltered. Reducing the surface concentration of bound $\beta\gamma$ subunits by half gives identical results.

Changes in the binding affinity of PLC-β_2 due to $\beta\gamma$ subunits were also assessed by labeling the protein with acrylodan and determining membrane association at various concentrations of membrane-bound $\beta\gamma$ subunits. When $\beta\gamma$ subunits are bound to the membrane surface at activating concentrations, identical PLC-β_2 binding curves are obtained as for membranes without $\beta\gamma$ subunits. The binding affinity remains relatively constant, showing only a small increase when an over 13-fold stoichiometric amount of $\beta\gamma$ is bound to the membrane (Figure 5). Thus, regardless of the order in which the titrations are carried out, our results show that the presence of $\beta\gamma$ subunits does not greatly influence the binding affinity of PLC-β_2 to membranes. These data clearly show that PLC-β_2 is not recruited to the membrane surface by $\beta\gamma$ subunits.

Similar studies were carried out using purified GTPγS-activated α_{11} and acrylodan-PLC-β_1 (see Materials and Methods). The concentrations of α_{11} used were sufficient to give a 2−3-fold activation of acrylodan-labeled PLC-β_1. The results are presented in Figure 6. These data clearly show that PLC-β_1 is not recruited to the membrane surface by α_{11} subunits.

These data, coupled with our observation of $\beta\gamma$ activation of PLCβ_2 and α_{11} activation of PLC-β_1 under conditions where most of PLCβ should be membrane-bound (see Materials and Methods), clearly rule out PLC-βs' recruitment to the membrane surface as the mechanism for G protein activation of PLC-β.

DISCUSSION

The studies presented here clearly demonstrate that PLC-β_1 and PLC-β_2 bind strongly to various lipid membranes under many conditions. We have found that binding does not require the presence of PIP_2, anionic lipids, Ca^{2+}, or G protein subunits. This behavior contrasts that of other signaling proteins that require either PIP_2 (e.g., PLC-δ and TEPLC), Ca^{2+} (e.g., PKC, annexin, etc.), or G protein subunits (e.g., βark) (James et al., 1995; Rebecchi et al., 1992; Garcia et al., 1995; Sutton et al., 1995; Bazzi et al., 1992; Epand & Leon, 1992; Pitcher et al., 1992). Our data indicate that activation of these PLC-β isoforms occurs by the encounter of these proteins with G protein subunits on the membrane surface.

PLC-δ_1 and TEPLC bind very weakly ($<10^{-3}$ M) to membranes that do not contain PIP_2 (Rebecchi et al., 1992; James et al., 1995). The higher membrane binding affinities of PLC-β_1 and -β_2 in the absence of PIP_2 were unexpected for several reasons. First, these isoforms, as well as PLC-δ and TEPLC, each contain a pleckstrin homology (PH) domain [see Ferguson et al. (1995a,b)]. These domains are roughly 100 residues long and have been identified in over 70 proteins, including all mammalian PLC isoforms, guanine nucleotide exchange factors, GTPase activating proteins, tyrosine or serine/threonine kinases, kinase substrates, and structural and regulatory elements of the cytoskeleton. Although the sequence homology of these domains is very weak, the tertiary structure is well conserved and consists of two pairs of nearly orthogonal β sheets in sandwich arrangement closed off at one end by a C-terminal α helix (Ferguson et al., 1995a,b; Yoon et al., 1994; Marias et al., 1994). PH domains are thought to function as modular units. Some of these (the N and C terminals of pleckstrin, T-cell specific tyrosine kinase, ras GTPase activating protein, and the β adrenergic receptor kinase) have been shown to bind specifically to PIP_2, and the crystal structure of the PH domain of PLC-δ with bound product has recently been reported (Ferguson et al., 1995a,b). It has been demonstrated that the PH domain of PLC-δ_1 is responsible for the enzyme's preference for PIP_2 and anionic lipids (Garcia et al., 1995). Specificity for PIP_2 in the N-terminal PH domain of pleckstrin appears to be conferred by the residues in the loop between the first two β strands as demonstrated by NMR (Yoon et al., 1994). Structural studies of β-spectrin also implicate this region (Macias et al., 1994). In Figure 7, we compare the sequences of the PH domains of PLC-β_1 and PLC-β_2 to PLC-δ_1 and the N-terminal domain of pleckstrin. From this figure, it is clear that pleckstrin and PLC-δ contain three positively charged residues in this region that are missing in the β isoforms. More importantly, many of the residues that have been found to be critical for PIP_2

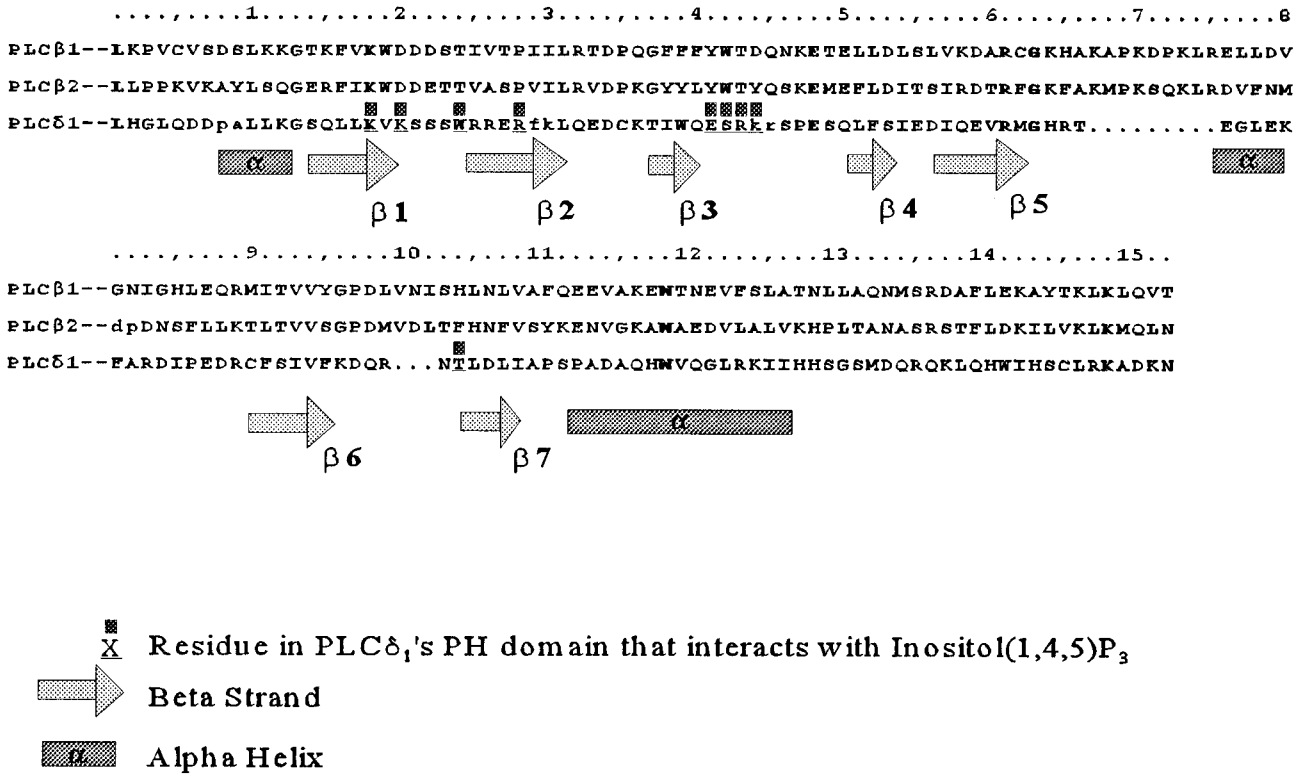

FIGURE 7: Multiple sequence alignment of the PH domain of PLC proteins using the program MaxHom (Sander & Schneider, 1991) where small letters designate insertions. Initially, the PH domain of human recombinant PLC-δ_1 was aligned to the sequences of rat β-spectrin and the N-terminal PH domain of pleckstrin, and then used to align the other PLCs. The resulting alignment was identical to that obtained by Ferguson and co-workers (Ferguson et al., 1995a,b).

specificity in PLC-δ (Ferguson et al., 1995a,b) are not found in the β isoforms. It is highly likely that the differences in the sequence of the PH domains of PLC-δ and PLC-β account for the differences in PIP_2 specificity. We do note, however, that the sequence of the PH domain of TEPLC, which has been shown to have PIP_2-specific membrane binding, is highly homologous to that of PLC-β_2. We can only conclude that the PIP_2 specificity of this protein is derived from regions other than the PH domain.

While the PH domains of the β isoforms do not function as PIP_2 binding domains, not all proteins with PH domains show specificity for PIP_2. The C-terminal region of the PH domain in some proteins appears to confer affinity for the $\beta\gamma$ subunits of G proteins (Inglese et al., 1995). Thus, it is possible that the PH domains of PLC-βs serve as G$\beta\gamma$ interaction sites, since both isoforms are activated to some extent by these subunits. It is tempting to speculate that the differences in binding affinity and specificity of PLC-β and PLC-δ underlie their different mechanisms of activation. For the δ isoform, the membrane interface serves as an allosteric effector, and catalysis is driven by the binding of the protein to the membrane surface via its PH domain. On the other hand, for the β_1 and β_2 isoforms, which possess relatively high membrane binding affinities, catalysis is driven by their interaction with G protein subunits on the membrane surface.

Aside from PH domains, PLC-βs also contain C2 or CalB (calcium-activated lipid binding) domains, and Ca^{2+} is needed for enzyme activity. However, in their CalB domains, PLC-β_1 and -β_2 are missing the putative critical Ca^{2+} binding residues in the loop preceding the third β sheet and the loop preceding the seventh β sheet (Sutton et al., 1995). Based on these amino acid substitutions, the lack of Ca^{2+} dependence on membrane binding is not surprising. The independence of binding with Ca^{2+} supports the idea that binding is independent of any conformational changes caused by Ca^{2+} binding, and that membrane binding and activation are independent events. The observation that membrane binding is unchanged in the presence of both calcium and PIP_2 negates the possibility of synergistic effects between the putative calcium binding region and the possible substrate-bound conformation of the enzyme. A lack of Ca^{2+} dependence was also reported for TEPLC (James et al., 1995). We do note that we observe changes in the intrinsic fluorescence of PLC-β_2 and changes in the acrylodan fluorescence of labeled PLC-β_2 that appear to be specific for calcium (Runnels et al., unpublished observations), implying that the protein does associate with calcium in solution, but any structural or electrostatic changes incurred from binding do not alter membrane association.

Besides differing from PLC-δ and turkey erythrocyte PLC, the trends in PLC-β membrane affinities sharply contrast other cytosolic proteins that bind to lipid membranes. Usually, these proteins contain clusters of basic residues that promote binding, and the addition of acidic phospholipids enhances the affinity by orders of magnitude depending on the size of the cluster (Kim et al., 1991). The observation that PLC-β isoforms lack a strong dependence on membrane composition indicates that electrostatic interactions play only a minor role in association. The presence of cholesterol and PE lipids also tends to increase the affinity of surface-associating peptides and molecules [e.g., see Bazzi et al. (1992) and Epand and Leon (1992)]. Not only do the binding affinities of β_1 and β_2 change little with electrostatic charge or small amounts of substrate, our results show that binding changes little in the presence of hexagonal-phase-forming lipids (PE), which alters the curvature of membrane

surfaces. Also, only minor changes are observed when the surface is made more heterogeneous by the presence of cholesterol. The observation that PLC-β_1 binds to inside-out erythrocyte membranes with the same lipid specificity as model membranes leads to the conclusion that the presence of integral membrane proteins and nonlipid components in the membranes does not alter binding and suggests that model membranes are suitable models for studying membrane binding events.

ACKNOWLEDGMENT

We thank Drs. Sue Goo Rhee, John Knopf, and Drs. Kozasa Alfred Gilman for sharing reagents. We also thank Heather McBath, Jiyun Kim, and Kevin Becker for their technical help, and Drs.Mario Rebecchi and Stuart McLaughlin for their helpful discussions.

REFERENCES

Bazzi, M., Youakim, A., & Nelsestuen, G. (1992) *Biochemistry 31*, 1125−1134.
Berridge, M. J., & Irvine, R. F. (1984) *Nature 312*, 315−321.
Bers, D. M. (1982) *Am. J. Physiol.* C404−C408.
Biddlecome, G. H., Berstein, G., & Ross, E. M. (1996) *J. Biol. Chem. 271*, 7999−8007.
Boyer, J. L., Waldo, G. L., & Harden, T. K. (1992) *J. Biol.Chem. 267*, 25451−25456.
Boyer, J. L., Graber, S. G., Waldo, G. L., Harden, T. K., & Garrison, J. C. (1994) *J. Biol. Chem. 269*, 2814−2819.
Camps, M., Hou, C., Sidiropoulos, D., Stock, J. B., Jakobs, K. H., & Gierschik, P. (1992) *Eur. J. Biochem 206*, 821−831.
Christensen, S. (1986) *Biochem. J. 233*, 921−924.
Clapham, D., & Neer, E. (1993) *Nature 365*, 403−406.
Dohlman, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653−688.
Emori, Y., et al. (1989) *J. Biol. Chem. 264*, 21885−21890.
Epand, R., & Leon, B. (1992) *Biochemistry 31*, 1550−1554.
Exton, J. H. (1994) *Annu. Rev. Physiol. 56*, 349−369.
Ferguson, K., Lemmon, M., Sigler, P., & Schlessinger, J. (1995a) *Struct. Biol. 2*, 715−718.
Ferguson, K., Lemmon, M., Schlessinger, J., & Sigler, P. (1995b) *Cell 79*, 199−209.
Garcia, P., Shah, S., Morris, A., Rudge, S., Scarlata, S., Petrov, V., McLaughlin, S., & Rebecchi, M. (1995) *Biochemistry 34*, 16228−16232.
Hepler, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternwies, P. C., & Gilman, A. G. (1993) *J. Biol. Chem. 268*, 14367−14375.
Hope, M., Bally, M., Webb, G., & Cullis, P. (1985) *Biochim. Biophys. Acta 812*, 55−65.
Inglese, J., Koch, W. J., Touhara, K., & Lefkowitz, R. J. (1995) *Trends Biochem. Sci. 20*, 151−156.
Iniguez-Iluhi, J., Kluess, C., & Gilman, A. G. (1993) *Trends Cell. Biol. 3*, 230−236.
James, S. R., Paterson, A., Harden, T. K., & Downes, C. P. (1995) *J. Biol. Chem. 270*, 11872−11881.
Kim, J., Mosior, M., Chung, L., Wu, H., & McLaughlin, S. (1991) *Biophys. J. 60*, 135−148.
Kleinfeld, A. (1988) *Spectroscopic Membrane Probes*, pp 64−92, CRC Press, Inc., Boca Raton, FL.
Kriz, R., Lin, L.-L., Sultzman, L., Eliis, C., Heldin, C.-H., Pawson, T., & Knopf, J. (1989) *CIBA Found. Symp. 150*, 112−127.
Lucklow, V. A., & Sumner, M. D. (1988) *Bio/Technology 6*, 47−55.
Macias, M. J., Musacchio, A., Ponstingl, H., Nilges, M., Saraste, M., & Oschkinat, H. (1994) *Nature 369*, 675−677.
Maurice, D. H., Waldo, G. L., Morris, A. J., & Harden, T. K. (1992) *Biochem. J. 290*, 765−770.
Meisenhelder, J., Suh, P.-G., Rhee, S. G., & Hunter, T. (1989) *Cell 57*, 1099−1122.
Meldrum, E., Kriz, R., Totty, N., & Parker, P. J. (1991) *Eur. J. Biochem. 196*, 159−165.
Mitchell, K. T., Ferrell, J. E., Jr., & Huestis, W. H. (1986) *Anal. Biochem. 158*, 447−453.
Morris, A., Waldo, G., Downes, C. P., & Harden, T. K. (1990) *J. Biol. Chem. 265*, 13508−13514.
Neer, E. (1994) *Protein Sci. 3*, 3−14.
Neer, E. (1995) *Cell 80*, 249−257.
Park, D., Jhon, D. Y., Lee, C.-W., Ryu, S. H., & Rhee, S. G. (1993) *J. Biol. Chem. 268*, 3710−3714.
Paterson, A., Boyer, J. L., Watts, A. J., Morris, A. J., Price, E. M., & Harden, K. T. (1992) *Cell Signalling 7*, 709−720.
Pitcher, J., Inglese, J., Higgins, J., Arriza, J., Casey, P. J., Kim, C., Benovic, J., Kwatra, M., Caron, M., & Lefkowitz, R. (1992) *Science 257*, 1264−1267.
Rebecchi, M., Peterson, A., & McLaughlin, S. (1992) *Biochemistry 31*, 12742−12747.
Rhee, S. G., & Choi, K. D. (1992) *J. Biol. Chem. 267*, 12393−12396.
Runnels, L. W., et al. (1996) *Biophys. J. 70*, A107 and A423.
Sander, C., & Schneider, R. (1991) *Proteins 9*, 56−68.
Simon, M. I., Strathmann, M. P., & Gautam, N. (1991) *Science 252*, 802−808.
Smrcka, A., & Sternweis, P. C. (1993) *J. Biol. Chem. 268*, 9667−9674.
Smrcka, A., Hepler, J., Brown, K., & Sterweis, P. (1991) *Science 251*, 804−807.
Stahl, M. L. Ferenz, C. R., Kelleher, K. L., Kriz, R., & Knopf, J. L. (1988) *Nature 332*, 269−272.
Steck, T., Fairbanks, G., & Wallach, D. (1971) *Biochemistry 10*, 2617−2624.
Sternweis, P. C., & Robishaw, J. D. (1984) *J. Biol. Chem. 259*, 13806−13813.
Suh, P.-G., Ryu, S. H., Moon, K. H., Suh, H. W., & Rhee, S. G. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5419−5423.
Suh, P. G., Ryu, S. H., Moon, K. H., Suh, W., & Rhee, S. G. (1988b) *Cell 54*, 161−169.
Sutton, R. B., Davletov, B. A., Berghuis, A. M., Sudhof, T. C., & Sprang, S. R. (1995) *Cell 80*, 929−938.
Taylor, S. J., Smith, J. A., & Exton, J. H. (1990) *J. Biol. Chem. 265*, 17150−17156.
Taylor, S. J., Chae, H. Z., Rhee, S. G., & Exton, J. (1991) *Nature 350*, 516−518.
Wahl, M. I., Nishibe, S., Suh, P. G. Rhee, S. G., & Carpenter, G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1568−1572.
Waldo, G., Boyer, J. L., Morris, A. J., & Harden, T. K. (1991) *J. Biol. Chem. 266*, 14217−14225.
Wu, D., Jiang, H., Katz, A., & Simon, M. I. (1993) *J. Biol. Chem. 268*, 3704−3709.
Yoon, H. S., Hajduk, P. J., Petros, A. M., Olejniczak, E., Meadows, R. P., & Fesik, S. (1994) *Nature 369*, 672−675.

BI961606W