We have studied the binding of two G-protein-regulated phospholipase C (PLC) enzymes, PLCs-β, and PLC-γ, to membrane surfaces using sucrose-loaded bilayer phospholipid vesicles of varying compositions. Neither enzyme binds appreciably to pure phosphatidylcholine vesicles at lipid concentrations up to $10^{-3}$ M. PLC-β and PLC-γ bind vesicles composed of phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine (molar ratio 1:1:1) with an approximate $K_d$ of $10^{-5}$ M. Inclusion of 2% PtdIns(4,5)P$_2$ in these vesicles had no effect on the affinity of this interaction. As reported by others, removal of the C-terminus of PLC-β and PLC-γ produces catalytically active fragments. The affinity of these truncated proteins for phospholipid vesicles is dramatically reduced suggesting that this region of the proteins contains residues important for membrane binding. Inclusion of G-protein α- and βγ-subunit activators in the phospholipid vesicles does not increase the binding of PLC-β, or PLC-γ, and the magnitude of G-protein-mediated PLC activation observed at low phospholipid concentrations ($10^{-6}$ M) is comparable to that observed at concentrations at which the enzymes are predominantly membrane-bound ($10^{-3}$ M). PLC-β and -γ contain C2 domains but Ca$^{2+}$ does not enhance binding to the vesicles. Our results indicate that binding of these enzymes to membranes involves the C-termini of the proteins and suggest that activation of these enzymes by G-proteins results from a regulated interaction between the membrane-bound proteins rather than G-protein-dependent recruitment of soluble enzymes to a substrate-containing phospholipid surface.

**INTRODUCTION**

Inositol lipid-specific phospholipases C (PLC) hydrolyse PtdIns(4,5)P$_2$ to generate the intracellular second messengers Ins(1,4,5)P$_3$ and 1,2-diacylglycerol [1,2]. Three families of PLC isoenzymes with distinct primary structures have been described in mammalian cells. Rhee et al. [3] have classified these as PLC-β, PLC-γ and PLC-δ. 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 respectively [3]. The PLC-γ class are distinguished by the presence of a sequence of ~400 amino acids between the X and Y regions containing SH2 and SH3 domains [4,5]. The three members of the PLC-δ class lack an extended C-terminal region immediately following the conserved Y domain. In comparison with the other classes of PLC isoenzymes, the hallmark features of the PLC-β family are a short region of ~80 amino acids between the X and Y regions which, in the case of the mammalian PLC-β isoenzymes contains up to ten consecutive negatively-charged amino acids, and an extended C-terminus of ~400 amino acids containing regions with a marked enrichment (approx. 40%) in charged amino acids [6–10].

This structural diversity among the PLC isoenzymes is paralleled by diversity in their modes of regulation. Representatives of two broad classes of cell surface receptors, heptahelical G-protein-coupled receptors and receptor tyrosine kinases, stimulate PLC-catalysed inositol lipid hydrolysis in target cells [3]. The PLC-γ enzymes mediate inositol lipid hydrolysis in response to activation of receptor tyrosine kinases [11]. The heptahelical class of receptors that couple to PLC employ G-proteins as intermediaries in the regulation of intracellular effectors. Members of a superfamily of GTPases, the G-proteins are hetero-trimeric proteins with an α-, β-, γ-subunit structure. There is structural diversity among each of the polypeptide components of a G-protein oligomer. The β- and γ-subunits are tightly associated and apparently can be exchanged between different α-subunits. Structural and functional classification of G-protein oligomers has been defined by the α-subunits although it is now clear that both the α- and βγ-subunits are directly involved in regulation of diverse effectors (see [12] for a review). The four identified PLC-β isoenzymes are activated by both members of the Gq class of G-protein α-subunits and by G-protein βγ-subunits [13–19]. Mutagenesis and proteolysis experiments implicate sequences in the C-terminus of PLC-β, as sites of interaction with G-protein α-subunits [17,18,20]. Similar experiments with PLC-γ suggest that the N-terminus and possibly the inter X–Y region are important for activation by G-protein βγ-subunits [19,21].

Despite these advances, the molecular mechanism(s) by which G-protein α- and βγ-subunits activate the PLC-β enzymes are poorly understood (see [22] for a review). Like many regulated lipolytic or lipid modifying enzymes, the PLC-β enzymes can be purified as stable soluble proteins [7–10]. However, their phospholipid substrates and G-protein activators are components of cellular membranes. As with the recruitment of PLC-γ by receptor tyrosine kinases [11], activation of the PLC-β enzymes by heterotrimeric G-proteins may involve regulated interaction of the enzymes with substrate-containing membrane surfaces.

**Abbreviations used:** C2 domain, calcium-dependent lipid-binding motif; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; PLC, inositol lipid-specific phospholipase C; PH-domain, pleckstrin homology domain; PtdCho, phosphatidylcholine; PtdEIOH, phosphatidylethanolamine; PtdSer, phosphatidylserine; GTP[S], guanosine 5’-(γ-thio)triphosphate.

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support of this idea, mutants of $\alpha_4$ and $\alpha_{11}$, rendered cytosolic by mutations that prevent palmitoylation, are incapable of stimulating inositol lipid hydrolysis when expressed in COS-7 cells [23].

Biological and immunological experiments indicate that, upon cell fractionation, a significant portion of the PLC- $\beta$ proteins expressed in several different tissues and cell lines is predominantly associated with particulate material [7–10,20]. More detailed investigations have localized PLC- $\beta$ to the nucleus in fibroblasts [24]. The mechanism by which PLC- $\beta_1$ and $\beta_2$ bind to membranes is not known. The proteins do not contain large stretches of hydrophobic amino acids but they do contain two sequence motifs, an N-terminal pleckstrin homology (PH) domain and an internal calcium-dependent lipid-binding motif (C2 domain) that are known to mediate membrane localization of other proteins [25,26]. Studies with other PLC enzymes have suggested a mechanism for membrane association that involves binding of PtdIns(4,5)$P_2$ to a non-catalytic site, anchoring the enzymes to the membrane allowing them to function in a scooting mode of catalysis. The PtdIns(4,5)$P_2$-binding site of PLC-$\delta_1$ has been located to the N-terminal PH domain [27]. The N-termini of the PLC- $\beta$ enzymes also contain PH domains and a recent study showed that, like PLC-$\delta_1$, a PLC-$\beta$ isoform from turkey erythrocytes bind to membranes in a PtdIns(4,5)$P_2$-dependent manner [28]. Other work suggests that the C-terminus of PLC-$\beta_1$ may be important in directing association of the protein with cellular membranes [17,20]. A C2 domain mediates membrane binding and activation of cytosolic phospholipase A$_2$ [29]. Ca$^{2+}$ ions activate the PLC-$\beta$ enzymes and it is plausible that they do so by promoting membrane-association mediated by this region of the proteins.

Using sucrose-loaded phospholipid vesicles in a sedimentation assay, we have investigated the binding of PLC-$\beta$-$\delta$ and PLC-$\beta_2$ to lipid surfaces. We report that the enzymes bind tightly to membranes in a manner that is dependent on acidic lipids but otherwise shows little lipid headgroup selectivity. Binding requires the C-terminus of the proteins. G-proteins, PtdIns(4,5)$P_2$ and Ca$^{2+}$ do not enhance membrane binding of PLC-$\beta_1$ and PLC-$\delta_2$.

**EXPERIMENTAL**

**Expression and purification of PLC- $\beta$ enzymes**

cDNAs encoding bovine PLC-$\beta_1$ and human PLC-$\beta_2$ were generously provided by Dr. Sue Goo Rhee (NHILBI, NIH, Bethesda, MD, U.S.A.) and Dr. John Knopf (Genetics Institute, Boston, MA, U.S.A.). The proteins were expressed in sf9 cells using baculovirus vectors. PCR amplification with specific primer- adapters was used to introduce unique restriction sites 40 bp 5\(' of the initiating methionine codon, allowing directional insertion into the PVL1392 transfer vector (Invitrogen Inc.). Vectors for the expression of truncated PLC- $\beta$ proteins (PLC- $\beta$-$\Delta$C and PLC-$\beta_2$-$\Delta$C) were constructed using PCR amplification to replace codons 829 and 832 of the PLC-$\beta_1$ and $\beta_2$ open reading frames with stop codons. Recombinant baculoviruses, in which the wild-type and mutant PLC- $\beta$ cDNAs are inserted into the viral polyhedron gene by homologous recombination with the transfer vectors, were generated, selected and amplified by standard methods [30]. Wild-type and mutant PLC- $\beta$ proteins were expressed and purified by the same procedure. PLC activity in column eluates was determined using exogenously provided substrates. Suspension cultures of SF9 cells (0.5–1 litre, 10$^6$ cells/ml) grown in complete Grace’s medium containing 10% (v/v) fetal bovine serum were infected with recombinant viruses at a multiplicity of 10. Cells were harvested after 48 h, washed in PBS, resuspended in 40 ml of PBS and subjected to N$_2$ cavitation (Parr cell disruption bomb, 7590 kPa for 30 min with intermittent agitation). Cells were discharged into 100 ml of ice-cold buffer A [25 mM Tris/Cl, pH 7.5/2 mM EDTA/2 mM EGTA/1 mM dithiothreitol (DTT)/0.1 mM benzamidine/0.1 mM PMSF] and the soluble fraction was recovered after ultracentrifugation at 50000 g for 1 h. This material was diluted to 15 ml with buffer A and applied to Source 15Q resin (Pharmacia) in a 8.9-ml column.

**Purification of G-protein $\alpha$- and $\beta\gamma$-subunits**

G-protein $\beta\gamma$-subunits were purified from detergent extracts of bovine brain membranes [31], except that octyl-Sepharose was used in the final step. The proteins were further concentrated and exchanged into 0.01 M NaCl and a high degree of purity was confirmed by SDS/PAGE (7.5% gel) (see Figure 1). Portions of the purified proteins were stored at $-80^\circ$C. Gel filtration analysis (Sephacryl S-300 HR resin eluted with buffer containing 100 mM NaCl) indicated that the proteins were predominantly monomeric. Bacterially-expressed PLC-$\delta_1$ was generously provided by Dr. Mario Rebbechi, Department of Anesthesiology, SUNY-Stony Brook, NY, U.S.A.

**Measurement of PLC enzyme binding to sucrose-loaded phospholipid vesicles**

Sucrose-loaded phospholipid vesicles were prepared by extrusion using minor modification of a method published previously [36]. In brief, lipids in chloroform which included [$H$]phosphatidyl-choline (PtdCho) as a recovery marker and, in some cases, [$H$]PtdIns(4,5)$P_2$ for measurement of PLC activity, were dried under vacuum and resuspended in sucrose loading buffer (50 mM Hepes, pH 7.2/3 mM EGTA/1 mM DTT/160 mM sucrose) by vortex mixing, subjected to five freeze–thaw cycles and extruded by ten passages through two 100-nm membrane filters. The vesicles were washed by resuspension in isotonic buffer (50 mM Hepes, pH 7.2/3 mM EGTA/1 mM DTT/80 mM KC1), recovered by centrifugation at 100000 g for 30 min and resuspended in isotonic buffer. Binding assays were performed in a total volume of 220 $\mu$l of isotonic buffer containing 0.9 mg/ml...
BSA and PLC-β proteins. The mixture was vortexed and incubated either on ice or at 25 °C for 10 min before centrifugation at 100000 g at 4 °C for 30 min. In some experiments Ca²⁺ was added to the isotonic buffer using a Ca²⁺/3 mM EGTA buffer system to give the [Ca²⁺] indicated. In other studies, concentrated solutions of G-protein α- and βγ-subunits were added to the phospholipid vesicles. In experiments in which PLC activity was measured the assays contained approx. 100 ng of protein. When bound and free protein was analysed on Coomassie Blue-stained SDS/polyacrylamide gels, the assay contained 10 μg of purified PLC protein.

**PLC assays**

PLC activity was determined using [³H]PtdIns(4,5)P₂ as substrate using methods described previously [13]. Under the conditions used (< 10% substrate hydrolysis), activity of both the wild-type and mutant PLC-β enzymes was linear with time. In some experiments PLC activity was determined using sucrose-loaded phospholipid vesicles containing PtdIns(4,5)P₂ and G-protein α- and βγ-subunits were included in the assays as described above.

**Data analysis**

Where indicated, binding data were analysed by methods described previously [36]. Note that this method assumes a single site interaction between protein in the soluble phase and phospholipids in the outer monolayer of the vesicles. We make no assumption of lipid–protein stoichiometry.

**RESULTS**

**Binding of intact PLC-β enzymes to sucrose-loaded phospholipid vesicles**

PLC-β₁, -β₂ and mutant forms of the enzymes lacking the C-terminal ~380 amino acids were purified to homogeneity from sf9 cells (Figure 1). We measured the binding of these proteins and bacterially-expressed PLC-δ₁ to various concentrations of sucrose-loaded bilayer vesicles with different phospholipid com-

![Figure 1 SDS/PAGE analysis of purified PLC-β proteins](image)

**Figure 1 SDS/PAGE analysis of purified PLC-β proteins**

PLC-β₁, PLC-β₂, PLC-β₁/AC and PLC-β₂/AC were expressed and purified as described in the Experimental section. The purified proteins were analysed by SDS/PAGE (7.5% acrylamide). Proteins were revealed by silver staining. Positions of molecular-mass markers are shown on the left.
Table 1 Binding of PLC-β₁, PLC-β₂, PLC-β₁ΔC and PLC-β₂ΔC to sucrose-loaded phospholipid vesicles of various compositions

<table>
<thead>
<tr>
<th>Vesicle composition</th>
<th>PLC-β₁ bound (% total)</th>
<th>PLC-β₁/PLDCHO</th>
<th>PLC-β₂/PLDCHO</th>
<th>PLC-β₁ΔC/PLDCHO</th>
<th>PLC-β₂ΔC/PLDCHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtdCho</td>
<td>1.1 ± 0.1</td>
<td>54.8 ± 4.8</td>
<td>84.0 ± 7.6</td>
<td>79.7 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>PtdCho/PLDCHO</td>
<td>0.3 ± 0.03</td>
<td>0.6 ± 0.1</td>
<td>2.0 ± 0.3</td>
<td>3.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>PtdCho/PLDCHO/PLDCho</td>
<td>28.0 ± 3.1</td>
<td>12.7 ± 2.1</td>
<td>83.7 ± 9.1</td>
<td>82.0 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>PtdCho/PLDCHO/PLDCho/Pls51(4,5)</td>
<td>0.1 ± 0.02</td>
<td>7.2 ± 0.9</td>
<td>31.0 ± 0.9</td>
<td>3.9 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Membrane-binding requires the C-termini of PLC-β₁ and -β₂

We compared the binding of wild-type and C-terminally-truncated forms of PLC-β₁ and PLC-β₂ to sucrose-loaded phospholipid vesicles of various compositions, but with a fixed total lipid concentration of 10⁻³ M. As shown above, neither wild-type PLC-β enzymes bound to PtdCho vesicles but bound tightly to PtdCho/PtdSer/PtdEtOH (1:1:1, molar ratio) vesicles. We also investigated binding to PtdCho/PtdEtOH vesicles (1:1, molar ratio). Both of the wild-type PLC-β enzymes bound to vesicles of this composition, with PLC-β₁ binding more tightly than PLC-β₂. In contrast, mutant forms of PLC-β₁ and PLC-β₂ lacking the C-termini did not bind to phospholipid vesicles of any of the compositions tested (Table 1).

G-protein α- and βγ-subunits do not enhance membrane binding of PLC-β₁ or PLC-β₂

Activation of PLC-β enzymes by G-protein α- and βγ-subunits has been proposed to be mediated by G-protein-dependent binding of the enzymes to substrate-containing membranes. We investigated the effects of GTP[S]-activated α₁₁ and G-protein βγ-subunits on binding of wild-type PLC-β₁ or PLC-β₂ to sucrose-loaded PtdCho/PtdSer/PtdEtOH (1:1:1, molar ratio) vesicles containing 2% (w/v) PtdIns(4,5)P₂. The vesicles were prepared as described in the Experimental section and purified G-proteins (either GTP[S]-pre-activated α₁₁- or βγ-subunits) from concentrated solutions in 0.01% (v/v) Lubrol PX were added to the vesicles so that the final detergent concentration was ≤ 0.001%
Inclusion of αPPtdCho/PtdSer/PtdEtOH (1:1:1, molar ratio) vesicles containing 2% (w/v) PtdIns(4,5)Pypendent activation. As expected, we found that the activity of bound, they would become less susceptible to G-protein-dependent on phospholipid concentration, so that at high lipid our assays facilitating the binding to substrate-containing membranes in phospholipid vesicles of identical composition containing [3H]PtdIns(4,5)P2 was increased (results not shown). We compared activation of PLC-β1 by GTP[S]-activated α1,-subunits and PLC-β2 by βγ-subunits at phospholipid concentrations of 10^{-6} M (where only 10–15% of the enzymes are bound to the vesicles) with activation at phospholipid concentrations of 10^{-3} M, at which 90–100% of the enzymes are vesicle-bound. We observed similar activation of PLC-β1 by GTP[S]-activated α1, at 10^{-6} M and 10^{-3} M phospholipid (5.5 ± 0.3-fold and 6.3 ± 0.2-fold respectively, means ± S.E.M.). Activation of PLC-β2 by βγ-subunits was equally insensitive when the phospholipid concentration was increased from 10^{-6} M to 10^{-3} M (4.2 ± 0.1-fold and 4.1 ± 0.2-fold respectively, means ± S.E.M.).

Membrane binding of PLC-β1 and PLC-β2 is not regulated by Ca^{2+}

Like other members of the PLC family, both PLC-β1 and PLC-β2 are strongly dependent on Ca^{2+} ions for activity. Since these proteins (as well as other PLC isoforms) contain C2 domains, some of which are known to bind phospholipid surfaces in a Ca^{2+}-dependent manner, it is possible that binding of the enzymes to phospholipid surfaces is Ca^{2+}-dependent. We measured the binding of PLC-β1 and PLC-β2 to sucrose-loaded phospholipid vesicles either at a [Ca^{2+}] of < 10^{-6} M (3 mM EGTA, no added Ca^{2+}) or in the presence of 1 μM buffered Ca^{2+}. The binding of the PLC enzymes to the vesicles was independent of Ca^{2+} over the range of concentrations tested (Figure 4, upper panel and lower panel).

**DISCUSSION**

Like many other phospholipases or phospholipid-modifying enzymes, activity of the PLC enzymes is highly dependent on the physical form of phospholipid substrate [3]. These enzymes are most active against substrates presented as components of a phospholipid surface and the factors influencing the initial affinity and lifetime of the surface-bound enzyme would be expected to exert considerable influence on the catalytic activity of the enzymes. Studies of other phospholipid signalling enzymes, including phosphoinositide 3-kinase, phospholipase A2, as well as PLC-δ and PLC-γ, support the idea that recruitment of these enzymes to substrate-containing membranes mediated by specific protein–protein or protein–lipid interactions plays an important role in the processes by which they are activated in cells stimulated with appropriate agonists [11,27,29,36–38]. The purpose of our study was to examine the membrane-binding properties of two PLC-β enzymes and to investigate the possible involvement of PtdIns(4,5)P2, Ca^{2+} and their G-protein α- and βγ-subunit regulators in this process.

Although PLC-β1 and PLC-β2 did not bind to pure PtdCho vesicles, both enzymes bound with high affinity (approx. K_a 10^{-3} M) to PtdCho/PtdSer/PtdEOH vesicles. PLC-β1, PLC-β2 and a related turkey erythrocyte PLC-β exhibit kinetic behaviour indicative of a mechanism in which interfacial binding of the proteins precedes processive catalysis [39]. Our results are clearly in accord with these observations. Removal of the C-termini of PLC-β1 and PLC-β2 produced catalytically active proteins that were incapable of binding to membranes of any composition tested at phospholipid concentrations of up to 10^{-3} M, suggesting that these regions of the proteins contain critical residues for membrane association. Mutational studies and experiments employing proteolytic digestion of purified proteins support a role for the C-terminus of PLC-β1 in both activation by G-
Coomassie Blue staining to detect protein, we found that βγ subunits were affected by addition of either GTP[S]-preactivated α subunits or PtdEtOH vesicles containing 2% PLC-PtdIns(4,5) P$_2$. The results obtained were consistent with the direct-binding measurements although, for reasons that are unclear at present, a greater FRET signal was detected upon binding of the labelled enzyme to vesicles containing PtdIns(4,5)P$_2$ than to vesicles containing PtdSer. The results showed also that the binding was insensitive to Ca$^{2+}$ and unaffected by G-protein α subunits [41]. A second study [38], using a combination of intrinsic fluorescence measurements and measurements made with fluorophore-labelled proteins to estimate the binding of PLC-β$_1$ and -β$_2$ to membranes, reported affinities that were approx. 10-fold lower than those determined in the present study, using sucrose-loaded phospholipid vesicles. Runnels et al. [38] did not observe any clear binding dependence on phospholipid composition of the vesicles. Most notably both PLC-β$_1$ and -β$_2$ bound to pure PtdCho vesicles as well as to vesicles containing a variety of other phospholipids (including PtdCho/PtdSer/PtdEtOH) with equal affinity. PtdIns(4,5)P$_2$ Ca$^{2+}$. G-protein α- and γ-subunits did not increase the affinity of this binding [38]. We have subsequently measured the binding of acrylodan-labelled PLC-β$_1$ and PLC-β$_2$ to sucrose-loaded phospholipid vesicles and found that the labelled protein did not bind to pure PC vesicles (J. M. Jenco, L. Runnels, S. Scarlata and A. J. Morris, unpublished work). The most likely explanation for the discrepancy between our data and those published by Runnels et al. [38] is that the fluorescence technique, which measures interactions under equilibrium conditions, is detecting a lower affinity interaction that we can measure by using centrifugation to separate membrane-bound and free PLC-β enzymes. It is also noteworthy that the concentrations of enzyme used in the experiments reported by Runnels et al. [38] (most notably the intrinsic fluorescence studies) were considerably higher than those used in our studies and, of necessity, their incubation mixtures did not contain BSA, which we and others have found to be essential for blocking non-specific adsorption of proteins to the silanised tubes in which the centrifugation measurements are made. It is possible that oligomerization of the proteins, which has been reported previously [42], may affect interactions with membranes.

In summary, our results indicate that PLC-β$_1$ and -β$_2$ bind to membranes through an interaction involving the C-terminus of the proteins. The C-terminus of each protein contains clusters of basic amino acids. PLC-β$_1$ and -β$_2$ do not bind to vesicles of pure PtdCho but do bind to vesicles composed of PtdCho/PtdSer/PtdEtOH with high affinity. It is tempting to speculate that binding of the PLC-β enzymes to the vesicles involves an electrostatic interaction between negatively-charged PtdSer and the basic regions of the proteins. In support of this idea, in mutagenesis experiments, in which charged lysine residues were substituted with alanine residues in this region of PLC-β$_1$, the association of the protein with a particulate fraction was decreased, when transiently expressed [20]. G-protein α- and βγ subunits and association with cellular membranes.

When transiently expressed in COS-7 cells, wild type PLC-β$_1$ localized to a total particulate fraction. Truncation of the C-terminus resulted in a redistribution of the protein to a soluble fraction [17]. More detailed analyses using a number of different cell lines indicated that a major fraction of the PLC-β$_1$ present in the particulate fraction was associated with the nucleus [24]. The C-terminus of PLC-β$_1$ is markedly enriched in regions of basic amino acids. Alanine substitution mutagenesis defines a role for these regions in association of PLC-β$_1$ with the nuclear and particulate fractions in transiently-transfected cells [20]. Our results suggest that both PLC-β$_1$ and -β$_2$ bind to membranes through an interaction of the C-terminus of the proteins with the phospholipid surface.

Our studies indicate that PLC-β$_1$ and PLC-β$_2$ do not bind specifically to PtdIns(4,5)P$_2$. These results in turn imply that the N-terminal PH domains of these proteins are not capable of specific high-affinity interaction with the headgroup of this phospholipid. A PLC-β enzyme from turkey erythrocytes has been reported to bind to membranes with a similar selectivity for PtdIns(4,5)P$_2$ (C-terminus of PLC-β$_1$ [28]). Sequence comparisons of the PH domains of PLC-β$_1$, -β$_2$, and -δ$_1$ reveal that the PLC-β enzymes contain non-conservative substitutions of amino acids known to contact the Ins(1,4,5)P$_3$ headgroup of PtdIns(4,5)P$_2$. The sequence of the PH domain of the turkey erythrocyte PLC-β enzyme is similar divergent from that of PLC-δ$_1$, therefore it is possible that the selective high-affinity binding of this enzyme to PtdIns(4,5)P$_2$ involves another region of the protein [40].

G-protein α- and βγ-subunits activate the PLC-β enzymes. We found that binding of PLC-β$_1$ and PLC-β$_2$ to PtdCho/PtdSer/PtdEtOH vesicles containing 2% (w/v) PtdIns(4,5)P$_2$ was unaffected by addition of either GTP[S]-pretreated x$_{α_{11}}$ or G-protein βγ-subunits respectively. Using SDS/PAGE and Coomassie Blue staining to detect protein, we found that α-subunits bound tightly (K$_d$ < 10$^{-7}$ M) to vesicles of the compositions used in our studies (results not shown). Binding of α-subunits was more difficult to quantify (due to the limited amount of protein available to us) and, although we could detect binding of x$_{α_{11}}$ to sedimented vesicles by immunoblotting, precise quantitation was not possible. Irrespective of the levels to which the α-subunits are membrane bound our results indicate that, under conditions where G-proteins increase the activity of PLC-β$_1$ and -β$_2$, they do not so by enhancing their affinities for substrate-containing lipid surfaces. In agreement with this finding, the magnitude of G-protein-mediated PLC activation observed at low phospholipid concentrations (10$^{-6}$ M) is comparable with that observed at concentrations at which the enzymes are predominantly membrane bound (10$^{-3}$ M). Our results, therefore, suggest that activation does not involve G-protein-mediated translocation of constitutively active enzymes to the membrane surface.

Both PLC-β$_1$ and -β$_2$ are strongly dependent on Ca$^{2+}$ for activity. Our results imply that the C2 domains of these proteins are not involved in Ca$^{2+}$-dependent binding of the proteins to phospholipid surfaces because binding of neither enzyme to sucrose-loaded PtdCho/PtdSer/PtdEtOH (1:1:1, molar ratio) containing 2% (w/v) PtdIns(4,5)P$_2$ vesicles was affected by raising the [Ca$^{2+}$] from > 10$^{-8}$ M to 10$^{-4}$ M. The recently-described structure of PLC-δ$_1$ suggests that the C2 domain of this PLC enzyme is involved in ‘fixing’ the catalytic domain in a productive orientation to the membrane, rather than in tethering the protein to the substrate-containing phospholipid surface [39].

Two recently published studies described different fluorescence techniques to measure the binding of PLC-β$_1$ and -β$_2$ to phospholipid surfaces [38,41]. Our results with PLC-β$_2$ are in close agreement with those obtained from experiments using fluorescence resonance energy transfer (FRET) to measure the interaction between fluorescently-labelled PLC-β$_1$ and vesicles composed of Rhodamine-labelled PtdEtOH and other phospholipids [41]. These workers used centrifugation to measure the effects of PtdIns(4,5)P$_2$ on binding of fluorescently-labelled PLC-β$_1$ to either PtdEtOH/PtdSer or PtdEtOH/PtdIns(4,5)P$_2$ vesicles. They reported that this enzyme bound to PtdEtOH/PtdSer vesicles with an apparent K$_d$ of 1.3 µM, and that substitution of PtdIns(4,5)P$_2$ for PtdSer in these vesicles produced a small increase in binding affinity (K$_d$, 0.6 µM). FRET was then used to measure the binding of labelled PLC-β$_1$ to vesicles containing Rhodamine-labelled PtdEtOH and either PtdSer or PtdIns(4,5)P$_2$. The results obtained were consistent with the direct-binding measurements although, for reasons that are unclear at present, a greater FRET signal was detected upon binding of the labelled enzyme to vesicles containing PtdIns(4,5)P$_2$ than to vesicles containing PtdSer. The results showed also that the binding was insensitive to Ca$^{2+}$ and unaffected by G-protein βγ-subunits [41]. A second study [38], using a combination of intrinsic fluorescence measurements and measurements made with fluorescently-labelled proteins to estimate the binding of PLC-β$_1$ and -β$_2$, to membranes, reported affinities that were approx. 10-fold lower than those determined in the present study, using sucrose-loaded phospholipid vesicles. Runnels et al. [38] did not observe any clear binding dependence on phospholipid composition of the vesicles. Most notably both PLC-β$_1$ and -β$_2$ bound to pure PtdCho vesicles as well as to vesicles containing a variety of other phospholipids (including PtdCho/PtdSer/PtdEtOH) with equal affinity. PtdIns(4,5)P$_2$. Ca$^{2+}$. G-protein α- and γ-subunits did not increase the affinity of this binding [38]. We have subsequently measured the binding of acrylodan-labelled PLC-β$_1$ and PLC-β$_2$ to sucrose-loaded phospholipid vesicles and found that the labelled protein did not bind to pure PC vesicles (J. M. Jenco, L. Runnels, S. Scarlata and A. J. Morris, unpublished work). The most likely explanation for the discrepancy between our data and those published by Runnels et al. [38] is that the fluorescence technique, which measures interactions under equilibrium conditions, is detecting a lower affinity interaction that we can measure by using centrifugation to separate membrane-bound and free PLC-β enzymes. It is also noteworthy that the concentrations of enzyme used in the experiments reported by Runnels et al. [38] (most notably the intrinsic fluorescence studies) were considerably higher than those used in our studies and, of necessity, their incubation mixtures did not contain BSA, which we and others have found to be essential for blocking non-specific adsorption of proteins to the silanised tubes in which the centrifugation measurements are made. It is possible that oligomerization of the proteins, which has been reported previously [42], may affect interactions with membranes.
$\beta\gamma$-subunits are potent and selective activators of the PLC-$\beta$ enzymes but do not enhance membrane binding. The interaction between PLC-$\beta_1$, PLC-$\beta_2$, their G-protein $\alpha$- and $\beta\gamma$-subunit activators and phosphoinositide substrates must, therefore, occur on the membrane surface. Future work should be directed towards measuring the affinities and lifetimes of these interactions.

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Binding of phospholipases $\mathrm{C}_2\beta_1$ and $\beta_2$ to membranes 437