Membrane-binding properties of phospholipase C- β_1 and phospholipase C- β_2 : role of the C-terminus and effects of polyphosphoinositides, G-proteins and Ca^{2+}

John M. JENCO, Kevin P. BECKER and Andrew J. MORRIS¹

Department of Pharmacological Sciences and Institute for Cell and Developmental Biology, Stony Brook Health Sciences Center, State University of New York at Stony Brook, Stony Brook, NY 11794-8651, U.S.A.

We have studied the binding of two G-protein-regulated phospholipase C (PLC) enzymes, PLCs- β_1 and - β_2 , 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_1 and PLC- β_2 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_1 and PLC- β_2 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 $\beta\gamma$ -subunit activators in the phospholipid vesicles does not increase the binding of PLC- β_1 or PLC- β_2 , 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_1 and - β_2 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 heterotrimeric 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 $\beta\gamma$ -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 $\beta\gamma$ -subunits [13–19]. Mutagenesis and proteolysis experiments implicate sequences in the C-terminus of PLC- β_1 as sites of interaction with G-protein α -subunits [17,18,20]. Similar experiments with PLC- β_2 suggest that the N-terminus and possibly the inter X–Y region are important for activation by G-protein $\beta\gamma$ -subunits [19,21].

Despite these advances, the molecular mechanism(s) by which G-protein α - and $\beta\gamma$ -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. In

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; PtdEtOH, phosphatidylethanolamine; PtdSer, phosphatidylserine; GTP[S], guanosine 5'-(γ -thio)triphosphate.

¹ To whom correspondence should be addressed.

support of this idea, mutants of α_4 and α_{11} , rendered cytosolic by mutations that prevent palmitoylation, are incapable of stimulating inositol lipid hydrolysis when expressed in COS-7 cells [23].

Biochemical and immunological experiments indicate that, upon cell fractionation, a significant portion of the PLC- β proteins expressed in several different tissues and cell lines is predominantly associated with particulate material [7–10,20]. More detailed investigations have localized PLC- β_1 to the nucleus in fibroblasts [24]. The mechanism by which PLC- β_1 and - β_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- δ_1 has been located to the N-terminal PH domain [27]. The N-termini of the PLC- β enzymes also contain PH domains and a recent study showed that, like PLC- δ_1 , a PLC- β isoform from turkey erythrocytes bound to membranes in a PtdIns(4,5) P_2 -dependent manner [28]. Other work suggests that the C-terminus of PLC- β_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- β 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 PLCs- β_1 and - β_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-termini of the proteins. G-proteins, PtdIns(4,5) P_2 and Ca^{2+} do not enhance membrane binding of PLC- β_1 and - β_2 .

EXPERIMENTAL

Expression and purification of PLC- β enzymes

cDNAs encoding bovine PLC- β_1 and human PLC- β_2 were generously provided by Dr. Sue Goo Rhee (NHLBI, 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- β proteins (PLC- $\beta_1\Delta C$ and PLC- $\beta_2\Delta C$) were constructed using PCR amplification to replace codons 829 and 832 of the PLC- β_1 and - β_2 open reading frames with stop codons. Recombinant baculoviruses, in which the wild-type and mutant PLC- β 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- β 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/HCl, 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. Bound proteins were eluted with a 200 ml gradient of 0–0.6 M NaCl in buffer A. Pooled active fractions were diluted to a concentration of 0.1 M NaCl with buffer A and applied to heparin-Sepharose CL4B (Pharmacia) (4-ml column). 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/10 mM $CaCl_2$) and applied to a BioGel HPHT (Bio-Rad) column. Bound proteins were eluted with a gradient of 0–350 mM KH_2PO_4 in buffer B. Active fractions were pooled, diluted in buffer A and applied to Source15Q resin (1-ml column). Bound protein was eluted with a 0–0.5 M NaCl gradient in buffer A. One–3 mg of purified PLC- β proteins were obtained from 500 ml of sf9 cell cultures 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- δ_1 was generously provided by Dr. Mario Rebbecchi, Department of Anesthesiology, SUNY-Stony Brook, NY, U.S.A.

Purification of G-protein α - 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% (v/v) Lubrol PX by anion-exchange chromatography (Source 15Q resin) [32].

α_{11} was prepared as described previously [33], with minor modification. In brief, α_{11} was co-expressed in sf9 cells with the G-protein β -subunit and a modified form of the γ -subunit containing a hexa-histidine tag using baculovirus vectors. The α_{11} virus has been described previously [34]. The β - and γ -subunit viruses were generously provided by Dr. A. Kosara and Dr. A. G. Gilman, Southwestern Medical Center, Dallas, TX, U.S.A. α_{11} was purified from cholate extracts of sf9 cell membranes by metal ion affinity chromatography and was eluted from the column using AlF_3 to dissociate the bound heterotrimer. Fractions containing α_{11} were pooled and concentrated to approx. 1 $\mu g/ml$. In some experiments purified α_{11} was pre-activated by incubation with GTP[S] as described previously [35].

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 [3H]phosphatidylcholine (PtdCho) as a recovery marker and, in some cases, [3H]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 KCl), recovered by centrifugation at 100000 g for 30 min and resuspended in isotonic buffer. Binding assays were performed in a total volume of 220 μl 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 Ca^{2+} was added to the isotonic buffer using a Ca^{2+} /3 mM EGTA buffer system to give the $[\text{Ca}^{2+}]$ indicated. In other studies, concentrated solutions of G-protein α - and $\beta\gamma$ -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) P_2 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) P_2 and G-protein α - and $\beta\gamma$ - 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- β_1 , - β_2 and mutant forms of the enzymes lacking the C-terminal ~ 380 amino acids were purified to homogeneity from

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

PLC- β_1 , PLC- β_2 , PLC- $\beta_1\Delta\text{C}$ and PLC- $\beta_2\Delta\text{C}$ 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.

sf9 cells (Figure 1). We measured the binding of these proteins and bacterially-expressed PLC- δ_1 to various concentrations of sucrose-loaded bilayer vesicles with different phospholipid com-

Figure 2 Binding of PLC- δ_1 , PLC- β_1 and PLC- β_2 to sucrose-loaded phospholipid vesicles of varying compositions

Binding of purified (upper panel) PLC- δ_1 , (middle panel) PLC- β_1 and (lower panel) PLC- β_2 to sucrose-loaded phospholipid vesicles of the following compositions, PtdCho (□); PtdCho/PtdSer/PtdEtOH (1:1:1, molar ratio) (○); PtdCho/PtdSer/PtdEtOH (1:1:1, molar ratio) containing 2% (w/v) PtdIns(4,5) P_2 (●), was measured as described in the Experimental section. Bound protein was quantified by measuring the enzyme activity remaining in the supernatant after sedimentation of the vesicles. The results shown are the means of triplicate determinations from a single experiment. Standard errors were within 10% of the means. This experiment was repeated several times with similar results.

Table 1 Binding of PLC-β₁, PLC-β₂, PLC-β₁ΔC and PLC-β₂ΔC to sucrose-loaded phospholipid vesicles of various compositions

Binding of purified PLC-β₁, PLC-β₂, PLC-β₁ΔC and PLC-β₂ΔC to sucrose-loaded phospholipid vesicles of the following compositions: PtdCho, PtdCho/PtdEtOH (1:1, molar ratio); PtdCho/PtdEtOH/PtdSer (1:1:1, molar ratio); PtdCho/PtdEtOH/PtdSer (1:1:1, molar ratio) containing 2% (w/v) PtdIns(4,5,6)P₂. Bound protein was quantified by measuring the enzyme activity remaining in the supernatant after sedimentation of the vesicles. The results are the means ± S.E.M. of triplicate determinations from a single experiment. This experiment was repeated twice with similar results.

Vesicle composition ...	PLC-β bound (% total)			
	PtdCho	PtdCho/PtdEtOH	PtdCho/PtdEtOH/PtdSer	PtdCho/PtdEtOH/PtdSer/PtdIns(4,5)P ₂
PLC-β ₁	1.1 ± 0.1	54.8 ± 4.8	84.0 ± 7.6	79.7 ± 3.2
PLC-β ₁ ΔC	0.3 ± 0.03	0.8 ± 0.1	2.0 ± 0.3	3.0 ± 0.2
PLC-β ₂	26.0 ± 3.1	12.7 ± 2.1	83.7 ± 9.1	82.0 ± 7.2
PLC-β ₂ ΔC	0.1 ± 0.02	7.2 ± 0.9	3.1 ± 0.9	3.9 ± 0.8

positions. As reported by others [36], PLC-δ₁ does not bind to PtdCho vesicles at concentrations of up to 10⁻³ M and binds weakly (*K*_d > 10⁻³ M) to vesicles composed of a mixture of PtdCho/phosphatidylserine (PtdSer)/phosphatidylethanolamine (PtdEtOH) (1:1:1, molar ratio). The enzyme binds strongly (approx. *K*_d 10⁻⁵ M) to vesicles containing, in addition to these phospholipids, 2 mole % PtdIns(4,5)P₂ (Figure 2, upper panel). We measured binding of PLC-β₁ and -β₂ to vesicles with these three compositions. Similarly to PLC-δ₁, neither PLC-β₁ nor PLC-β₂ bound detectably to PtdCho vesicles. However, unlike PLC-δ, PLC-β₁ and -β₂ bound to PtdCho/PtdSer/PtdEtOH vesicles with an approx. *K*_d of 10⁻⁵ M. Although, similar to PLC-δ₁, PLC-β₁ and PLC-β₂ contain an N-terminal PH domain, inclusion of 2% (w/v) PtdIns(4,5)P₂ in the vesicles did not further enhance the binding (Figure 2, middle panel and lower panel). In the experiments described, measurements of enzyme activity were used to quantify unbound PLC enzyme. We observed quantitatively similar results when greater amounts of PLC proteins were used and bound and unbound enzyme was determined by SDS/PAGE and staining with Coomassie Blue (results not shown).

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 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

Figure 3 Effect of GTP[S]-activated α₁₁ and G-protein βγ-subunits on binding of PLC-β₁ and -β₂ to sucrose-loaded phospholipid vesicles

Binding of purified (upper panel) PLC-β₁ and (lower panel) PLC-β₂ to sucrose-loaded PtdCho/PtdSer/PtdEtOH (1:1:1, molar ratio) vesicles containing 2% (w/v) PtdIns(4,5)P₂ was determined by measuring the enzyme activity remaining in the supernatant after sedimentation of the vesicles. The incubations contained, (upper panel) no G-proteins (○) or 20 ng of GTP[S]-activated α₁₁ (●); (lower panel) no G-proteins (○) or 2 μg of βγ-subunits (●). The results shown are the means ± S.E.M. of triplicate determinations.

(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%

Figure 4 Effect of Ca^{2+} on membrane binding of PLC- β_1 and PLC- β_2

Binding of purified (upper panel) PLC- β_1 or (lower panel) PLC- β_2 to sucrose-loaded PtdCho/PtdSer/PtdEtOH (1:1:1, molar ratio) vesicles containing 2% (w/v) PtdIns(4,5) P_2 was determined by measuring the enzyme activity remaining in the supernatant after sedimentation of the vesicles. The incubation mixtures contained either 3 mM EGTA (nominally Ca^{2+} -free conditions) (○) or 1 μM of free Ca^{2+} (●). The results shown are the means $\pm$ S.E.M. of triplicate determinations.

(v/v). The amounts of G-proteins added to the incubations (20 ng of α_{11} -subunit and 2 μg of $\beta\gamma$ -subunit) were sufficient to produce an approx. 5-fold activation of PLC- β_1 and PLC- β_2 respectively, determined using phospholipid vesicles of identical composition containing [^3H]PtdIns(4,5) P_2 substrate (see below). Inclusion of α_{11} - or $\beta\gamma$ -subunits produced no significant enhancement in the binding of PLC- β_1 or PLC- β_2 to the vesicles (Figure 3, upper panel and lower panel).

Activation of PLC- β enzymes by G-protein α - and $\beta\gamma$ -subunits is independent of lipid concentration

If G-protein α - and $\beta\gamma$ -subunits activated the PLC- β enzymes by facilitating the binding to substrate-containing membranes in our assays *in vitro*, activation would be expected to be acutely dependent on phospholipid concentration, so that at high lipid concentrations, where the enzymes are predominantly membrane bound, they would become less susceptible to G-protein-dependent activation. As expected, we found that the activity of

wild-type PLC- β_1 and - β_2 increased as the bulk concentration of substrate-containing phospholipid vesicles {PtdCho/PtdSer/PtdEtOH (1:1:1, molar ratio) containing 2% (w/v) [^3H]PtdIns(4,5) P_2 } was increased (results not shown). We compared activation of PLC- β_1 by GTP[S]-activated α_{11} -subunits and PLC- β_2 by $\beta\gamma$ -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- β_1 by GTP[S]-activated α_{11} at 10^{-6} M and 10^{-3} M phospholipid (5.5 ± 0.3 -fold and 6.3 ± 0.2 -fold respectively, means $\pm$ S.E.M.). Activation of PLC- β_2 by $\beta\gamma$ -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 $\pm$ S.E.M.).

Membrane binding of PLC- β_1 and PLC- β_2 is not regulated by Ca^{2+}

Like other members of the PLC family, both PLC- β_1 and PLC- β_2 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- β_1 and PLC- β_2 to sucrose-loaded phospholipid vesicles either at a [Ca^{2+}] of $< 10^{-8}$ 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 A_2 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) P_2 , Ca^{2+} and their G-protein α - and $\beta\gamma$ -subunit regulators in this process.

Although PLC- β_1 and PLC- β_2 did not bind to pure PtdCho vesicles, both enzymes bound with high affinity (approx. K_d 10^{-5} M) to PtdCho/PtdSer/PtdEtOH vesicles. PLC- β_1 , PLC- β_2 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- β_1 and PLC- β_2 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- β_1 in both activation by G-

protein α -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-termini 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 to that shown by PLC- δ_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 similarly 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 $\beta\gamma$ -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]-preactivated α_{11} or G-protein $\beta\gamma$ -subunits respectively. Using SDS/PAGE and Coomassie Blue staining to detect protein, we found that $\beta\gamma$ -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 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 do 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^{-6} 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 phos-

pholipid 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_2 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_2 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 μ M, 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 μ M). FRET was then used to measure the binding of labelled PLC- β_2 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 $\beta\gamma$ -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 $\beta\gamma$ -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 (1:1:1, molar ratio) 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

$\beta\gamma$ -subunits are potent and selective activators of the PLC- β enzymes but do not enhance membrane binding. The interaction between PLC- β_1 , PLC- β_2 , their G-protein α - 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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