

Distinct Ca^{2+} Binding Properties of Novel C2 Domains of Plant Phospholipase D α and β *

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Li Zheng, Ramaswamy Krishnamoorthi‡, Michal Zolkiewski, and Xuemin Wang§

From the Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506

Of the isoforms of plant phospholipase D (PLD) that have been cloned and characterized, PLD α requires millimolar levels of Ca^{2+} for optimal activity, whereas PLD β is most active at micromolar concentrations of Ca^{2+} . Multiple amino acid sequence alignments suggest that PLD α and PLD β both contain a Ca^{2+} -dependent phospholipid-binding C2 domain near their N termini. In the present study, we expressed and characterized the putative C2 domains of PLD α and PLD β , designated PLD α C2 and PLD β C2, by CD spectroscopy, isothermal titration calorimetry, and phospholipid binding assay. Both PLD C2 domains displayed CD spectra consistent with anticipated major β -sheet structures but underwent spectral changes upon binding Ca^{2+} ; the magnitude was larger for PLD β C2. These conformational changes, not shown by any of the previously characterized C2 domains of animal origin, occurred at micromolar Ca^{2+} concentrations for PLD β C2 but at millimolar levels of the cation for PLD α C2. PLD β C2 exhibited three Ca^{2+} -binding sites: one with a dissociation constant (K_d) of 0.8 μM and the other two with a K_d of 24 μM . In contrast, isothermal titration calorimetry data of PLD α C2 were consistent with 1–3 low affinity Ca^{2+} -binding sites with K_d in the range of 590–470 μM . The thermodynamics of Ca^{2+} binding markedly differed for the two C2 domains. Likewise, PLD β C2 bound phosphatidylcholine (PC), the substrate of PLD, in the presence of submillimolar Ca^{2+} concentrations, whereas PLD α C2 did so only in the presence of millimolar levels of the metal ion. Both C2 domains bound phosphatidylinositol 4,5-bisphosphate, a regulator of PC hydrolysis by PLD. However, added Ca^{2+} displaced the bound phosphatidylinositol 4,5-bisphosphate. Ca^{2+} and PC binding properties of PLD α C2 and PLD β C2 follow a trend similar to the Ca^{2+} requirements of the whole enzymes, PLD α and PLD β , for PC hydrolysis. Taken together, the results suggest that the C2 domains of PLD α and PLD β have novel structural features and serve as handles by which Ca^{2+} differentially regulates the activities of the isoforms.

Phospholipase D (PLD)¹ (EC 3.1.4.4) catalyzes the hydrolysis of phospholipids at the terminal phosphodiester bond, producing a free head group and phosphatidic acid. Activation of PLD in the cell generates signaling messengers and is involved in a wide range of cellular processes, including phytohormone action (1, 2), meiosis (3), defense response (4), and vesicular trafficking (5). The activity of PLD is tightly regulated, and its cellular regulation is often coordinated with the networks of cellular signaling machinery (6, 7). In plants, Ca^{2+} has been proposed to be an important regulator for PLD (7). All PLDs cloned from plants require Ca^{2+} for activity. A positive correlation between increased cytoplasmic Ca^{2+} levels and increased PLD activity was indicated in plant tissues in which Ca^{2+} levels were perturbed, using various Ca^{2+} -ATPase inhibitors and calmodulin antagonists (8). In addition, plant PLDs display two distinct types of Ca^{2+} dependence. PLD α represents the conventional PLD that requires Ca^{2+} in the millimolar range and can be active toward vesicles composed of phosphatidylcholine (PC) only (9, 10). This PLD has been purified, cloned, and characterized from several plant species (7). PLD β represents the newly identified PLD that is polyphosphoinositide-dependent and most active at micromolar ranges of Ca^{2+} (11, 12).

Little is known, however, about the mechanism by which Ca^{2+} modulates PLD activity. Potentially, Ca^{2+} may associate directly with PLD, and such a binding could induce a conformational change in the enzyme to facilitate its binding to a membrane surface and/or its activation for catalysis. Alternatively, Ca^{2+} may change the surface charge, potential, and/or shape of a membrane. In this case, a specific interaction between Ca^{2+} and PLD is not envisaged.

Sequence analyses of several cloned plant PLDs have suggested possible ways by which Ca^{2+} can differentially control the activities of the isoforms. Each of the PLDs is inferred to have a Ca^{2+} /phospholipid-binding C2 domain of approximately 130 amino acid residues near its N terminus (9, 13). C2 domains have been identified in more than 100 proteins, most of which are involved in lipid metabolism, signal transduction, or membrane trafficking (13–17). These domains often mediate Ca^{2+} -dependent phospholipid binding and thus play an important role in associating C2-bearing proteins with substrates and membranes (18–20).

Three-dimensional structures determined for the C2 domains in snaptagmin 1A (21–23), phospholipase C δ_1 (24), cPLA₂ (25, 26), and protein kinase C β (27) reveal in each case an anti-parallel eight-stranded β -sandwich structure. Two or three Ca^{2+} ions bind to the loops that connect the strands at

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, Kansas State University, Willard Hall, Manhattan, KS 66506. Tel.: 785-532-6262; Fax: 785-532-7278; E-mail: krish@ksu.edu.

§ To whom correspondence should be addressed: Dept. of Biochemistry, Kansas State University, Willard Hall, Manhattan, KS 66506. Tel.: 785-532-6422; Fax: 785-532-7278; E-mail: wangs@ksu.edu.

¹ The abbreviations used are: PLD, phospholipase D; PC, phosphatidylcholine; PIP₂, phosphatidylinositol 4,5-bisphosphate; cPLA₂, cytoplasmic phospholipase A₂; GST, glutathione S-transferase; PS, phosphatidylserine; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

	← β1 →	Ca ²⁺ Binding Loop1 (CBL1)	← β2 →	←β3→	CBL2	← β4 →	
PLD α	GTLHATYIYHVDALHGGVVMQGFGLGKILANVBEET	. IGVGKGETRLYASIDLQK	. . .	ARVSDPRKIKNEPKNPKWYSEFQVSV			(8-84)
PLD β	GNLDIWIYHAKNLPNMDMFHKTLDGDMFGLPGKIGDQLTSKITSDPVVSVSVAG	. . .	AVIGRTYVMSNSE	NPVVMQHFFYVPV			(156-233)
PLD $\beta_{\Delta 173-190}$	GNLDIWIYHAKNLPNMD	ELTSKITSDPVVSVSVAG	. . .	AVIGRTYVMSNSE	NPVVMQHFFYVPV	(156-233)
cPLA ₂	HKFTVVVLRATKVKTKGA	FGDMLDTPDPYVELFIS	. . .	TPDSRKRTRHFND	INPVWNETFEPILD	(18-80)
PLC δ_1	ERLRVRIISGQQLPK	. V	NKNKNSIVDPKVIIVEIHGVGRD	TGSRQTAVITNNGFNPRWDMEFEFVET			(629-689)
	← β5 →	CBL3	← β6 →	← β7 →	← β8 →		
PLD α	. . . TWLLISSSLSKDDIPI	. GATLIGRAYIPVDQVING	. . .	EVDQWVEILDNDRNPIQGSKIHVKLQYFVHE			(85-151)
PLD β	. . AHHAAEVHFVVKSDV	. VGSQILGLVTIPVEQIYSGAKIEGTYPIILTSNGK	. . .	PCKPGANLSLSIQYTPM			(234-302)
PLD $\beta_{\Delta 173-190}$. . AHHAAEVHFVVKSDV	. VGSQILGLVTIPVEQIYSGAKIEGTYPIILTSNGK	. . .	PCKPGANLSLSIQYTPM			(234-302)
cPLA ₂	PNQE . NVLEITLMDANY	. VMDETLGTATFTVSSMKVGEKKEVFFIFNQV	TEMVLEMSLEVCS			(81-141)
PLC δ_1	VDDL . ALVRFMVEDYSSSKNDFIGQSTIPWNSLKQG	. . .	YRHVHLLSKNGDQHPSATLVFKISIQD				(690-756)

FIG. 1. Alignment of amino acid sequences of PLD α C2 and PLD β C2 with cPLA₂ C2 and phospholipase C δ_1 C2. Ca²⁺ ligands are identified by boldface letters (amino acid codes). In the case of PLD α C2, nonacidic amino acid residues that occur in positions corresponding to Ca²⁺ ligands in cPLA₂ C2 and phospholipase C δ_1 C2 are *underlined*. The eight β -sheet strands and three Ca²⁺ binding loops (CBL), as defined previously (13), are identified.

one end (21–27). Alignment and molecular modeling of the N-terminal sequences of PLD α and PLD β with known C2 structures (Figs. 1 and 2) suggest that PLD β C2 has all the conserved Ca²⁺-binding residues, whereas PLD α C2 lacks at least two of these potential Ca²⁺ ligands due to substitution. This predicts a lower affinity toward Ca²⁺ for PLD α C2. Thus, the observed requirements of different levels of Ca²⁺ for catalysis by the whole enzymes, PLD α and PLD β (9–12), may arise out of different Ca²⁺ affinities of the C2 domains.

Herein we present results that provide the first evidence that the isolated domains PLD α C2 and PLD β C2 bind Ca²⁺ with different affinities and exhibit differences in the levels of Ca²⁺ required for PC binding, a pattern analogous to the Ca²⁺ demands of catalysis by the whole enzymes. Furthermore, Ca²⁺ binding triggers unprecedented conformational changes in PLD α C2 and PLD β C2, thus suggesting that these plant C2 domains are novel structural variants.

EXPERIMENTAL PROCEDURES

Construction of PLD α C2 and PLD β C2 Expression Plasmids—The putative C2 domain of *Arabidopsis* PLD α corresponds to the amino acid sequence 8–151. A DNA fragment coding for this region was amplified by the polymerase chain reaction (Perkin-Elmer Cetus) and inserted into vectors pET 15b (Novagen) and pGEX-2T (Amersham Pharmacia Biotech). The pET vector was used for producing the C2 proteins with 6 histidine residues fused at the N terminus, and the pGEX vector was used for those fused with glutathione S-transferase (GST) at the N terminus. For fragments inserted into the pET vector, an *Nde*I restriction site was added to the 5'-end, and a stop codon followed by a *Bam*HI site was added to the 3'-end. To clone the DNA fragments into the pGEX vector, a *Bam*HI site was incorporated into the 5'-end, and a stop codon followed by an *Eco*RI site was incorporated into the 3'-end. Similarly, the DNA region coding for *Arabidopsis* PLD β C2 (amino acid residues 156–302) was generated by the polymerase chain reaction and cloned into pET and pGEX vectors. To increase the solubility of PLD β C2, a loop region consisting of amino acid residues 173–190 was deleted from PLD β C2 (Fig. 1), and the deletion was generated by a sequential polymerase chain reaction, as described previously (28). The single cysteine residue, Cys-285, near the C-terminal end of the PLD β C2 constructs was substituted with serine to minimize possible complications in solubility and refolding. The constructs were verified by DNA sequencing and transformed into *Escherichia coli* BL21 or *E. coli* BL21 (DE3) for protein expression.

Expression and Purification of Recombinant PLD C2 Proteins—The poly-His-fused C2 proteins were expressed in *E. coli* BL21(DE3) (Novagen), and the GST fusion proteins were expressed in *E. coli* BL21. The cells were grown at 37 °C to an absorbance of ~1.0 at 600 nm and induced at 25 °C with isopropyl-1-thio- β -galactopyranoside at a final concentration of 0.2 mM for the GST fusion proteins and 1 mM for the poly-His fusion proteins. After overnight induction, the cells were harvested and lysed by sonication. After centrifugation, both the poly-His and GST fusion proteins were found in particulate fractions. To purify the His-tagged proteins, the particulate pellet isolated from 1 liter of culture was washed with a binding buffer (20 mM Tris-HCl, 0.5 M NaCl, and 5 mM imidazole at pH 8.0) and solubilized in the same binding

buffer containing 8 M urea. After centrifugation, the supernatant was passed through a 10-ml Ni²⁺-charged His-resin column equilibrated with the binding buffer. The column was thoroughly washed with a washing buffer containing 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 8 M urea at pH 8.0. Proteins bound to the resin were eluted with a 1 M imidazole elution buffer. Urea in the denatured recombinant protein was diluted by adding the solution dropwise into a stirring buffer containing 50 mM Tris-HCl (pH 8.8) and 2.0 M urea at 4 °C. The solution was kept at 4 °C for 1 h and incubated at room temperature for 1 h. The protein solution was dialyzed against 50 mM Tris-HCl, pH 8.4, overnight at 4 °C and then concentrated by means of a negative pressure dialysis system (Spectrum).

To remove the His tag, the solubilized protein was incubated with highly pure thrombin at a ratio of 1 unit of thrombin to 1 mg of fusion protein at room temperature for 8 h. To remove undigested fusion proteins, urea was added to the solution to a final concentration of 8 M, and the solution was passed through a Ni²⁺-charged His-resin column. The C2 proteins without the tag did not bind to the resin and were refolded by passing through a Sephacryl S-100 gel filtration column (29). The monomeric, refolded proteins were collected and concentrated by dialysis under a negative pressure. Purity of the proteins was tested by SDS-polyacrylamide gel electrophoresis (30) and reverse-phase high pressure liquid chromatography. The monomeric form of the proteins was confirmed by size-exclusion chromatography. The refolding of the fusion proteins and the isolated C2 domains was monitored by CD spectroscopy (31). Protein molar concentrations were determined using ϵ_{280} values of 24,300 M⁻¹ cm⁻¹ for PLD α C2 and 20,300 M⁻¹ cm⁻¹ for PLD β C2, on the basis of their amino acid sequences and calculated molecular masses of 16,673 and 13,500 Da, respectively (32).

The GST fusion proteins were purified according to the procedure described previously (33). Bacteria were pelleted from a 50 ml culture, washed twice with a buffer (STE buffer: 10 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, pH 8.0) containing 10 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA at pH 8.0, and suspended in 5 ml of STE buffer containing 200 μ g/ml lysozyme. The suspension was incubated on ice for 30 min, and dithiothreitol and Sarkosyl (9% (w/v); Sigma) were added to the suspension to final concentrations of 5 mM and 1.5%, respectively. After mixing, the cell suspension was sonicated for 2 min and centrifuged at 27,200 \times g for 10 min at 4 °C. Triton X-100 (20% v/v) was added to the supernatant to a final concentration of 4%. The solution was then incubated with 600 μ l of 50% (w/v) glutathione-agarose for 30 min at room temperature. The fusion protein-agarose beads in a column were washed with 20 volumes of STE buffer to remove unbound proteins, stored at 4 °C, and used within 2 weeks.

CD Spectroscopy of PLD C2 Domains—CD spectra of PLD α C2 and PLD β C2 were recorded with a Jasco J-720 spectropolarimeter. Each sample was scanned 64 times to improve the signal-to-noise ratio. PLD C2 in 10 mM Tris-HCl buffer, pH 7.5, was placed in a 1-cm cell, and Ca²⁺ or EGTA was added using a microsyringe (Hamilton). Reference CD spectra were recorded with the same series of Ca²⁺ solutions, but without the protein, and were subtracted from the corresponding protein spectra. The molar ellipticity was calculated using an analysis program supplied with the instrument. For Ca²⁺ titration, the molar ellipticity was determined at a specific UV wavelength as a function of the added metal ion concentration.

Microcalorimetric Titration Studies of PLD C2 Domains—Thermodynamic properties of Ca²⁺ binding by PLD C2 domains were measured by isothermal titration calorimetry using a MicroCal OMEGA calorim-

eter (34). PLD α C2 (0.05 mM) or PLD β C2 (0.25 mM) in 10 mM Tris-HCl, pH 7.5 (Chelex-100 treated) was placed in a 1.38-ml sample cell. A syringe (100 or 250 μ l) loaded with 10 mM standard CaCl₂ solution (Fisher Scientific) was used for a series of automatic injections of 10 μ l each into a protein solution. After each injection, a 5-min pause was allowed for reaching the baseline. Heat produced due to dilution was measured by injecting the Ca²⁺ solution into the sample cells from which PLD C2 proteins were omitted. For each titration step, the heat of dilution was subtracted from the corresponding Ca²⁺ binding data of the C2 domains. Data were fit to appropriate binding models and thermodynamic parameters determined from nonlinear least-squares fits, using the ORIGINTM software.

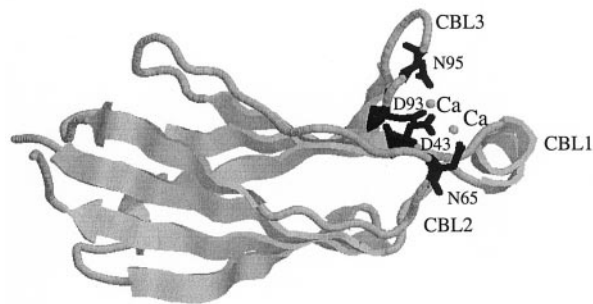
Phospholipid Binding Assays—A modified version of a previously described method (35) was employed to determine the amounts of phospholipids bound to PLD C2 domains. A phospholipid mixture consisting of 250 μ g of PC (egg yolk), 100 μ g of phosphatidylserine (PS) (egg yolk), and 2 μ Ci of ³H-labeled PC (dipalmitoyl-glycero-3-P-[methyl-³H]choline, DuPont) in chloroform was dried under a stream of nitrogen. The dried lipids were resuspended in 1 ml of water by vigorous vortexing for 2 min, followed by sonication for 30 s on an ice bath. The lipid vesicles were centrifuged briefly to remove large aggregates. GST fusion proteins bound to glutathione-agarose beads were suspended in 9 volumes of a binding buffer containing 50 mM Tris-HCl, 200 mM NaCl, and the various concentrations of Ca²⁺ to be tested. The buffered Ca²⁺ solutions were prepared by appropriate dilution of the standard Ca²⁺ solution with Chelex 100-treated buffer. Equal volumes of the agarose beads and PC/PS vesicles (50 μ l each) were mixed and incubated at room temperature for 30 min with vigorous shaking. The beads were pelleted by centrifugation and washed three times with 1 ml of the binding buffer containing the test concentration of Ca²⁺. Lipids bound to the protein-agarose beads were quantitated by scintillation counts. GST bound to glutathione-agarose beads was used to determine background phospholipid binding. All experiments were repeated at least three times. Binding activity was expressed as cpm per unit of GST activity. A similar procedure was followed to determine phosphatidylinositol 4,5-bisphosphate (PIP₂) binding by PLD C₂ domains as a function of Ca²⁺ concentration, using lipid vesicles made up of 400 μ g of PIP₂ mixed with 0.4 μ Ci of ³H-labeled PIP₂ (dipalmitoyl-glycero-3-P-[inositol-2-³H]inositol 4,5-bisphosphate, DuPont).

RESULTS

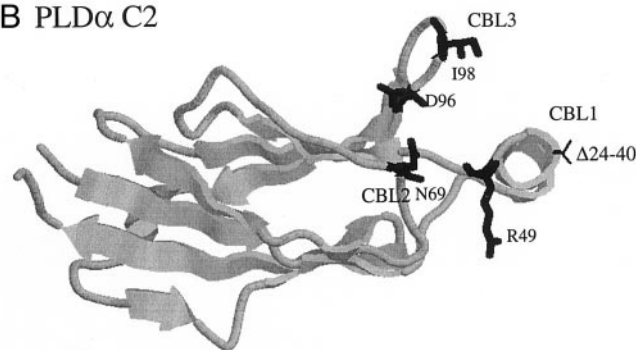
Solubility and Refolding of PLD α C2 and PLD β C2—The His-tagged PLD α C2 and PLD β C2 were solubilized with urea, purified using a Ni²⁺-affinity column, refolded by step dilutions of urea, and subjected to size exclusion chromatography. PLD α C2 was found to exist as a monomer up to a concentration of 1 mg/ml. A workable concentration of refolded PLD β C2 was difficult to attain, as the protein, in the absence of urea, precipitated at much lower concentrations (<100 μ g/ml) than did PLD α C2. Addition of agents such as 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (Chaps), ammonium sulfate, and arginine did not increase solubility of the protein. In the presence of 4 M proline, which has been found to increase protein solubility (36), PLD β C2 remained in solution after urea concentration was decreased to 0.1 M by dilution with 6 M proline. However, PLD β C2 aggregated when proline was removed by dialysis. Therefore, a deletion mutant, PLD β C2 Δ _{173–190}, was constructed (Fig. 1) with the hope of improved solubility. Deletion of residues 173–190 made loop 1 of the mutant similar in length to that of cPLA₂ C2 and was anticipated to have a minimal effect on the overall folding and function of PLD β C2 on the basis of amino acid sequence alignment (Fig. 1) and molecular modeling (Fig. 2). Indeed, a dominant β -sheet structure of PLD β C2 Δ _{173–190} was indicated by CD spectroscopy (31), thus suggesting that it was correctly refolded (Fig. 3). The deletion mutant possessed significantly improved solubility. PLD β C2 Δ _{173–190} was used in subsequent studies, as it remained a soluble monomer up to a concentration of 3 mg/ml. For brevity, the deletion mutant is referred to as PLD β C2 throughout the text.

Ca²⁺-induced Conformational Changes of PLD C2 Domains—The CD spectra of PLD α C2 and PLD β C2 are consist-

A cPLA₂ C2



B PLD α C2



C PLD β C2

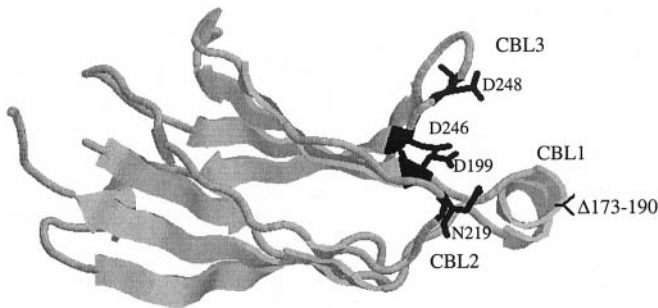


FIG. 2. Structure modeling of PLD α C2 and PLD β C2. A, ribbon model of the x-ray crystal structure of cPLA₂ C2 (Protein Data Bank code 1rlw; 25). B, a modeled structure of PLD α C2 generated with the amino acid sequence shown as PLD α in Fig. 1 except that the fragment 24–40 was deleted. C, a modeled structure of PLD β C2 generated with the amino acid sequence shown as PLD β Δ _{173–190} in Fig. 1. The amino acid residues serving as Ca²⁺ ligands in cPLA₂ C2 (25) and the corresponding residues of PLD C₂s are labeled and appear in **boldface**. The molecular models were generated with the Swiss-Model program (43–45), using the crystal structure of cPLA₂ C2 (25) as a template. The amino acid residues omitted for the purpose of construction of the models are identified in B and C. All the structures were viewed and manipulated using the RasMol program (University of Massachusetts).

ent with proteins having dominant β -sheet structures (31). Both PLD α C2 and PLD β C2 exhibited spectral changes in response to titration of Ca²⁺ (Fig. 3). The CD-detected conformational changes occurred at millimolar levels of Ca²⁺ for PLD α C2 (Fig. 3A) but at micromolar levels of the metal ion for PLD β C2 (Fig. 3C). The negative peak in the 203–210 nm range and its shoulder in the 215–222 nm range both showed decreases in ellipticity with the addition of Ca²⁺, and the magnitude was greater for PLD β C2. The CD spectral changes were specific to Ca²⁺, as Mg²⁺ had almost no effect (Fig. 3, A and C). The Ca²⁺-triggered conformational changes were reversible; addition of the chelator EGTA to Ca²⁺-bound PLD α C2 or

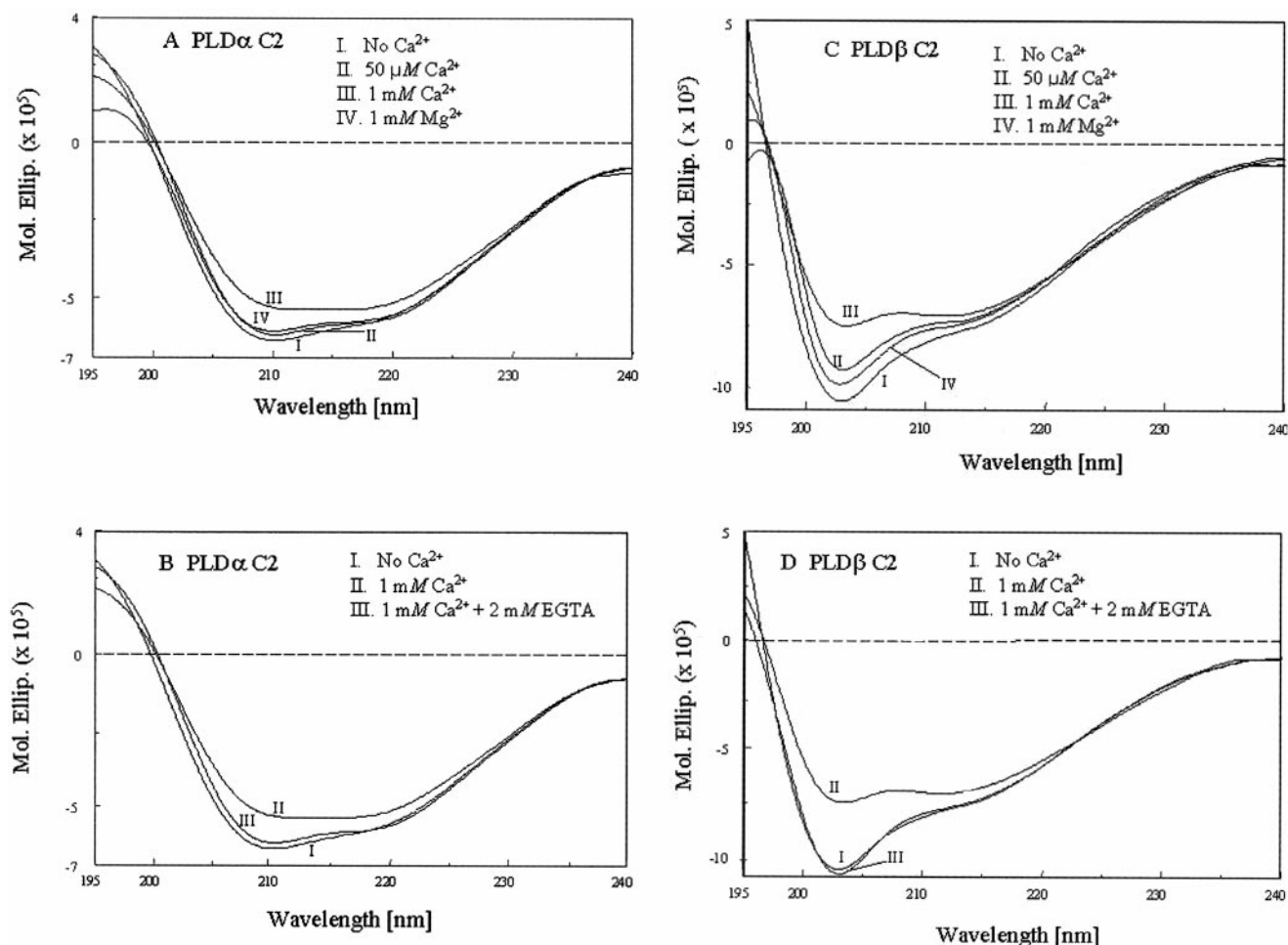


FIG. 3. Far UV CD spectra of PLD α C2 and PLD β C2 in the presence and absence of Ca²⁺. A, Ca²⁺ binding by PLD α C2 at different concentrations of Ca²⁺ and Mg²⁺ as indicated. B, reversibility of Ca²⁺-induced conformational changes of PLD α C2 following the addition of 2 mM EGTA to PLD α C2 in 1 mM Ca²⁺. C, Ca²⁺ binding by PLD β C2 at different concentrations of Ca²⁺ and Mg²⁺ as indicated. D, reversibility of Ca²⁺-induced conformational changes of PLD β C2 following the addition of 2 mM EGTA to PLD β C2 in 1 mM Ca²⁺. The protein concentrations used were 3 μ M for PLD α C2 and 2 μ M for PLD β C2. The C2 samples were dissolved in Chelex 100-treated 10 mM Tris-HCl, pH 7.5, for all the measurements.

PLD β C2 reproduced the CD spectrum obtained of the Ca²⁺-free C2 domain (Fig. 3, B and D). These results suggest that the expressed PLD α C2 and PLD β C2 are properly folded and undergo reversible conformational changes upon binding Ca²⁺. Irreversible spectral changes might imply trapping of metastable structures such as misfolded and/or partly folded conformer(s).

Ca²⁺ binding by the two PLD C2s was monitored by measuring negative ellipticity at 208 nm for PLD α C2 and at 203 nm for PLD β C2 as a function of the metal ion concentration (Fig. 4). From the CD data, apparent dissociation constants of \sim 1 mM and \sim 80 μ M are estimated for Ca²⁺ complexes with PLD α C2 and PLD β C2, respectively.

Thermodynamics of Ca²⁺ Binding by PLD α C2 and PLD β C2—Thermodynamic properties of Ca²⁺ binding to PLD α C2 and PLD β C2 were determined by isothermal titration calorimetry experiments (Fig. 5). In the case of PLD β C2, the binding data (Fig. 5A) are consistent with one high affinity site ($K_d = 0.8 \mu$ M) and two low affinity sites ($K_d = 24 \mu$ M). For PLD α C2, the microcalorimetric titration (Fig. 5B) could be carried out only with micromolar, rather than millimolar, protein solutions due to low solubility (1 mg/ml). The CD data yield an apparent dissociation constant of \sim 1 mM for Ca²⁺-PLD α C2 complex (Fig. 4). Therefore, microcalorimetric titrations are best carried out with millimolar protein solutions in order to determine with certainty all the three binding parameters: number of

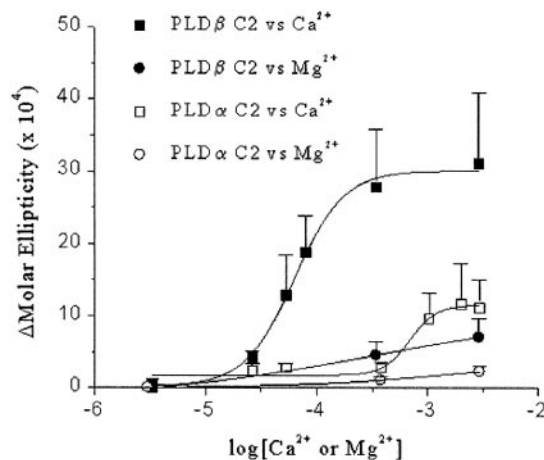


FIG. 4. Ca²⁺ titration of PLD α C2 and PLD β C2 as monitored by CD spectroscopy. CD spectra were recorded at different concentrations of Ca²⁺ or Mg²⁺ added to PLD α C2 or PLD β C2 in Chelex 100-treated 10 mM Tris-HCl, pH 7.5, at 25 °C. Changes in ellipticity at 208 and 203 nm were plotted as a function of Ca²⁺ concentration for PLD α C2 and PLD β C2, respectively. Values were means \pm S.E. of three independent experiments. Lines drawn through the data points represent nonlinear least-squares fits. From the graphs, apparent dissociation constants of \sim 1 mM and \sim 80 μ M are estimated for Ca²⁺ complexes with PLD α C2 and PLD β C2, respectively.

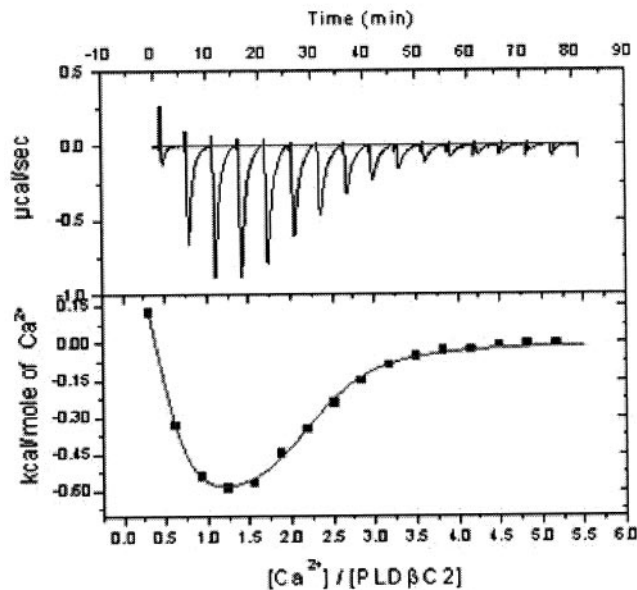
binding sites (n), dissociation constants, and enthalpy change (34). In the present case, the titration data (Fig. 5B) were fit to binding equations by assuming $n = 1, 2,$ or 3 .² All the three cases appear equally feasible, with K_d ranging from 580 μM for $n = 1$ to 470 μM for $n = 3$. Thus, it has not been possible to determine the number of binding sites on PLD α C2 unambiguously. However, the dissociation constants calculated are all in the millimolar range, irrespective of the number of binding sites assumed. This permits us to draw the valid conclusion that PLD α C2 has a lower affinity for Ca²⁺, relative to PLD β C2.

A striking difference was also observed between PLD α C2 and PLD β C2 in the thermodynamics of Ca²⁺ binding (Table I). PLD β C2 binds endothermically one Ca²⁺ at the high affinity site and exothermically two Ca²⁺ at the low affinity sites (Fig. 5A). Thus, the binding process is entropy-driven at the first site, whereas at the second and third sites, it is aided by both enthalpy and entropy changes. On the other hand, Ca²⁺ binding to PLD α C2 involves a positive enthalpy change and is consequently entropy-driven (Fig. 5B; Table I). This thermodynamic conclusion is unaffected by the number of binding sites assumed.² The results suggest that PLD α C2 and PLD β C2 each have distinct structural features that affect the thermodynamics of Ca²⁺ binding. Interestingly, cPLA₂ C2 (25) and PLD β C2 show drastic differences in thermodynamics of Ca²⁺ binding (Table I), despite having similar binding loops (Figs. 1 and 2).

Ca²⁺-dependent Phospholipid Binding of PLD α C2 and PLD β C2—One of the potential consequences of Ca²⁺ binding to a C2 domain is to promote phospholipid binding. To facilitate measurements of phospholipid binding, PLD α C2 and PLD β C2 domains were each fused to GST, and the effects of Ca²⁺ on the PC- and PIP₂ binding capabilities of the fusion proteins were assessed. Phospholipid binding by GST alone was determined and used as a reference. In the absence of Ca²⁺, both PLD α C2 and PLD β C2 bound low levels of PC/PS vesicles, and the binding ability increased with Ca²⁺ concentration (Fig. 6A). PLD β C2 exhibited enhanced PC binding at submillimolar Ca²⁺ and reached a plateau at ~ 1 mM Ca²⁺. The apparent dissociation constant for the Ca²⁺·PLD β C2 complex is estimated to be ~ 100 μM . In contrast, PLD α C2 bound PC only in the presence of millimolar Ca²⁺, with an apparent dissociation constant of >1 mM for the Ca²⁺·PLD α C2 complex. The different levels of Ca²⁺ requirements by PLD α C2 and PLD β C2 for binding PC are analogous to the different Ca²⁺ affinities of the two C2 domains as determined from CD and isothermal titration calorimetry data. The amounts of phospholipids bound to PLD α C2 and PLD β C2 decreased when EGTA was added to the lipid-bound proteins (data not shown), thus indicating that formation of Ca²⁺·EGTA complex led to dissociation of the C2 domains from PC/PS vesicles. Mg²⁺, on the other hand, had a negligible effect on PC binding by the two PLD C2 domains (Fig. 6A).

In the case of PIP₂, an acidic phospholipid, a negative effect on binding due to Ca²⁺ was observed for both PLD α C2 and PLD β C2 (Fig. 6B): maximal amounts of PIP₂ bound to the C₂ domains in the absence of Ca²⁺, and the added metal ion displaced the bound phospholipid. The Ca²⁺ titration data are consistent with an apparent dissociation constant of ~ 100 μM for the Ca²⁺·PLD β C2 complex and >0.6 mM for the Ca²⁺·PLD α C2 complex. The PIP₂ binding characteristics of the PLD C2

A. PLD β C2



B. PLD α C2

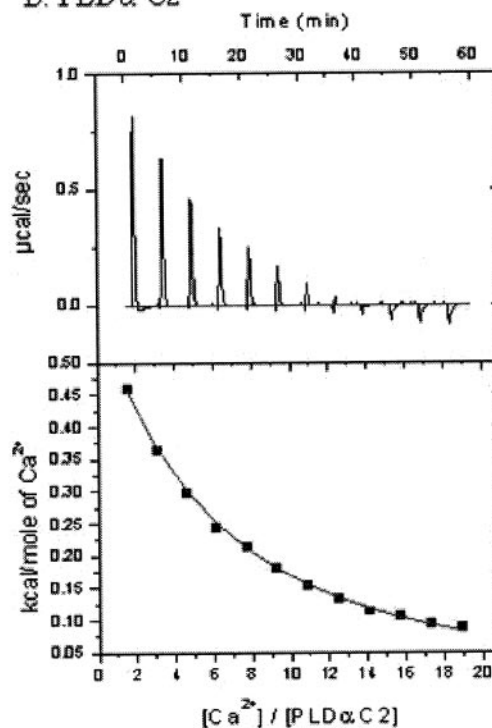


FIG. 5. Isothermal calorimetric titration of Ca²⁺ with PLD α C2 and PLD β C2. A, Ca²⁺ titration of PLD β C2; B, Ca²⁺ titration of PLD α C2. Proteins were dissolved in Chelex-100 treated, 10 mM Tris-HCl, pH 7.5. A 250- or 100- μl syringe loaded with the stock CaCl₂ solution provided a series of automatic injections of 10 μl each into the protein solution and the heat absorbed or liberated was recorded for each injection (upper panels in A and B). Identical injections were carried out using a solution without protein in the sample cell at 30 °C. In this case, the heat of dilution was measured for each injection (data not shown) and subtracted from the respective data obtained with the C2 domains to determine heat changes due solely to Ca²⁺ binding. These results are shown in the bottom panels in A and B. Lines drawn through the data points represent nonlinear least-squares fits, and the calculated thermodynamic parameters are given in Table I. The line in B corresponds to the case of one binding site.

² The following thermodynamic parameters were obtained from the least-squares fits of the experimental data with three different binding models: for $n = 1$, $K_d = 590 \pm 10$ μM ; $\Delta H = 6.52 \pm 0.07$ kcal/mol; for $n = 2$, $K_d = 530 \pm 10$ μM ; $\Delta H = 3.16 \pm 0.04$ kcal/mol; for $n = 3$, $K_d = 470 \pm 20$ μM ; $\Delta H = 2.03 \pm 0.04$ kcal/mol.

TABLE I
Thermodynamics of Ca²⁺ binding to PLD α C2 and PLD β C2 at 30 °C, pH 7.5

Protein	Number of binding sites	K_d	ΔG°	ΔH°	ΔS°
	n	μM	kcal/mol	kcal/mol	cal/mol-deg
PLD β C2 ^a	$1.0 \pm 0.1 (n_1)$	0.8 ± 0.7	-8.5	0.14 ± 0.04	28.4
	$1.8 \pm 0.1 (n_2)$	24.0 ± 3.4	-6.4	-0.7 ± 0.05	18.7
PLD α C2 ^b	1-3	590-470	~ -4.6	6.52-2.03	36-22
cPLA2 C2 ^c	2	1.7	-7.9	-6.2	5.7

^a PLD β C2 has two classes of binding sites.

^b See Footnote 2.

^c Taken from Ref. 26.

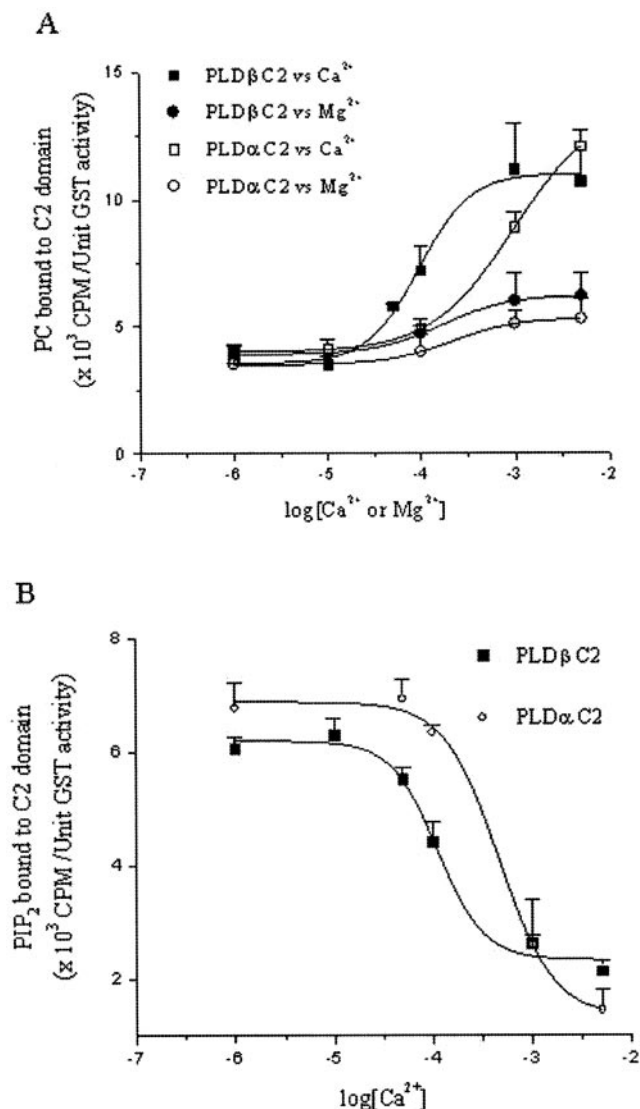


FIG. 6. Ca²⁺ dependence of phospholipid binding by PLD α C2 and PLD β C2. Glutathione-agarose beads bound with PLD α C2 or PLD β C2 were mixed with ³H-labeled phospholipid vesicles at 25 °C. The beads were washed with buffer three times, and scintillation counts were determined. Bound phospholipids were quantified as counts per min per unit of GST activity. Values are means \pm S.E. of three independent experiments. A, binding of vesicles made up of PC/PS (2.5:1) and ³H-labeled PC. B, binding of vesicles made up of unlabeled and ³H-labeled PIP₂. Lines drawn through the data points represent nonlinear least-squares fits.

domains are in contrast to their Ca²⁺-dependent PC binding properties.

DISCUSSION

Although plant PLD has long been known to require Ca²⁺ for activity, the mechanism by which Ca²⁺ influences PLD activity

is yet to be understood. The present study has provided the first evidence that the putative C2 domain of plant PLD is indeed an independent Ca²⁺-binding unit. Furthermore, PLD α C2 and PLD β C2 exhibit distinct affinities for Ca²⁺; Ca²⁺-dependent conformational changes and substrate binding both occur at millimolar ranges of Ca²⁺ for PLD α C2 but at submillimolar levels of the cation for PLD β C2. These different Ca²⁺ requirements by PLD α C2 and PLD β C2 have been demonstrated by three independent methods: CD spectroscopy, microcalorimetric titration, and an affinity pull-down assay. The difference in Ca²⁺ affinities between the two C2 domains is consistent with the multiple amino acid sequence alignments (Fig. 1) and molecular models (Fig. 2); a lower affinity toward Ca²⁺ is anticipated for PLD α C2 due to the absence of two potential Ca²⁺ ligands. The Ca²⁺ affinities and Ca²⁺-dependent PC binding behavior of PLD α C2 and PLD β C2 parallel the Ca²⁺ requirements of the whole enzymes, PLD α and PLD β , for phospholipid hydrolysis (11, 12). This correspondence suggests that Ca²⁺ modulates PLD α and PLD β activities by differentially interacting with the C2 domains of the isoforms.

Both PLD α C2 and PLD β C2 also bind PIP₂. Unlike PC binding, PIP₂ binding occurs without Ca²⁺ and is inhibited by the metal ion in a concentration-dependent manner, in the submillimolar range for PLD β C2 and in the millimolar range for PLD α C2. This suggests that the C2 domains bind PC and PIP₂ by different mechanisms, as discussed below. Interestingly, the whole enzymes exhibit a different behavior; PIP₂ is an effector required for the activity of PLD β , but not PLD α , in the presence of millimolar concentrations of Ca²⁺ (9, 12). Also, PLD β binds PIP₂ twice as much as does PLD α (9). It is, therefore, inferred that PLD contains an additional PIP₂-binding site(s) in a region other than the C2 domain.

The present study shows that both PLD α C2 and PLD β C2 associate with PC vesicles in a Ca²⁺-dependent manner. This Ca²⁺-promoted phospholipid binding renders C2-bearing proteins able to be associated with membranes (13), which can be important for PLD targeting and activation for catalysis. Two models have been advanced to explain how Ca²⁺ binding increases the affinity of C2 domains for membrane phospholipids. According to the model based on NMR studies of snaptotagmin 1A C2 (13, 21, 23), charge neutralization of Asp residues by Ca²⁺ allows positively charged side-chains in the loops to interact with negatively charged phospholipids, and the ternary structure thus formed is mainly supported by electrostatic interactions. An alternative electrostatic-hydrophobic switch model has been proposed on the basis of x-ray crystallographic studies of cPLA₂ C2 (25). In this case, Ca²⁺ binding not only brings about charge neutralization but also exposes hydrophobic residues in the Ca²⁺-binding loops for subsequent insertion into neutral membrane lipids. Both of these models do not predicate any significant Ca²⁺-conferred conformational changes. The structurally characterized Ca²⁺-free and Ca²⁺-bound C2 domains from snaptotagmin 1A, cPLA₂, phospholipase C δ_1 , and protein kinase C β do not reveal any well defined conformational transitions either for the flexible bind-

ing loops or for the rigid the β -sheet scaffold region (21–27).

The present work, however, shows that PLD α C2 and PLD β C2 undergo significant conformational changes due to Ca²⁺ binding and that Ca²⁺ removal reverses these changes. This may present a variation of the electrostatic-hydrophobic switch model; Ca²⁺ binding to a PLD C2 domain perturbs the secondary structure of the protein to expose a hydrophobic surface that binds to neutral phospholipids, such as PC, and to membranes. Conversely, withdrawal of Ca²⁺ dissociates the complex by restoring the original conformation of the C2 domain. The PIP₂ binding data of PLD α C2 and PLD β C2 also appear to be consistent with the proposed model; the anionic phospholipid binds to the C2 domain, at a site different from the PC-binding site, by electrostatic attractions, and the Ca²⁺-exposed hydrophobic surface hinders this binding process. The envisioned model is supported by the CD-sensitive conformational changes and thermodynamics of Ca²⁺ binding by PLD α C2 and PLD β C2. Positive entropy changes, as determined for the plant PLD C2 domains, have been observed for Ca²⁺ binding by EF-hand proteins, such as calmodulin (37) and bullfrog troponin C (38) and are thought to arise out of exposure of hydrophobic groups to water (39). In these and other Ca²⁺-binding EF-hand proteins, such as recoverin (40) and S100B (41), the metal ion binding results in conformational changes that expose otherwise hidden hydrophobic groups to facilitate protein binding. In the case of annexins, Ca²⁺ binding causes a major conformational shift that favors interaction with phospholipid membranes (42). The significant conformational changes that accompany Ca²⁺ binding by the plant PLD C2 domains, however, have no precedence among all the C2 domains studied thus far. This suggests the presence of unique variations of the C2 domain structure in plant PLDs. Structural studies of Ca²⁺-free and Ca²⁺-bound PLD C2s will provide insight into the functional diversity of this class of C2 domains and the differential regulation of plant PLD isoforms by Ca²⁺.

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