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Evidence for and Characterization of Ca^{2+} Binding to the Catalytic Region of *Arabidopsis thaliana* Phospholipase Dβ*

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Most types of plant phospholipase D (PLD) require Ca^{2+} for activity, but how Ca^{2+} affects PLD activity is not well understood. We reported previously that Ca^{2+} binds to the regulatory C2 domain that occurs in the N terminus of the Ca^{2+}-requiring PLDs. Using *Arabidopsis thaliana* PLDβ and C2-deleted PLDβ (PLDβcat), we now show that Ca^{2+} also interacts with the catalytic regions of PLD. PLDβcat exhibited Ca^{2+}-dependent activity, was much less active, and required a higher level of Ca^{2+} than the full-length PLDβ. Ca^{2+} binding of the proteins was stimulated by phospholipids; phosphatidylserine was the most effective among those tested. Scatchard plot analysis of Ca^{2+} binding data yielded an estimate of 3.6 high affinity (K_d = 29 μM) binding sites on PLDβ. The Ca^{2+}-PLDβcat interaction increased the affinity of the protein for the activator, phosphatidylinositol 4,5-bisphosphate, but not for the substrate, phosphatidylcholine. This is in contrast to the effect of Ca^{2+} binding to the C2 domain, which stimulates phosphatidylcholine binding but inhibits phosphatidylinositol 4,5-bisphosphate binding of the domain. These results demonstrate the contrasting and complementary effects of the Ca^{2+}- and lipid-binding properties of the C2 and catalytic domains of plant PLD and provide insight into the mechanism by which Ca^{2+} regulates PLD activity.

It has been long recognized that Ca^{2+} is a stimulator of plant PLD[1] activity. However, regulation of plant PLD by Ca^{2+} has been a source of debate because the common plant PLD requires millimolar amounts of Ca^{2+} for activity *in vitro* (1, 2). Recent characterization of multiple plant PLDs has made it clear that most plant PLDs are capable of significant enzymatic activity at micromolar levels of Ca^{2+} near those encountered in the cell (3–7). *Arabidopsis thaliana* has 12 PLDs that are grouped into PLDα, -β, -γ, -δ, and -ζ according to the sequence similarities, gene architectures, and domain structures (8). Except for PLDζ, all other PLDs characterized to date in *A. thaliana* require Ca^{2+} for activity. PLDβ, -γ, and -δ are active in micromolar ranges of Ca^{2+} (6, 7), and PLDα, which gives rise to the common plant PLD activity, is active at micromolar levels of Ca^{2+} under acidic conditions with mixed lipid vesicles (5). Ca^{2+} increases the membrane association of PLD, which has been suggested as a mechanism for rapid activation of PLD in plant wound response (9). A positive correlation between increased cytoplasmic Ca^{2+} levels and increased PLD activity was indicated when the Ca^{2+} levels of carnation petals were perturbed using various Ca^{2+}-ATPase inhibitors and calmodulin antagonists (10).

Recent studies have provided more insight into the mechanism of Ca^{2+} regulation of PLD activity. Amino acid sequence analysis indicates that most PLDs contain a C2 domain in their N-terminal regulatory regions, except for PLDζ, which has the pleckstrin homology (PH) and phox homology (PX) domains (8). C2 domains are Ca^{2+}/phospholipid binding folds that consist of ~130 amino acid residues (11, 12). C2 domains have been identified in a number of proteins involved in signal transduction or membrane trafficking, and these domains often mediate a Ca^{2+}-dependent binding of proteins to phospholipids (13, 14). The binding of Ca^{2+} to plant PLD C2 domains has been demonstrated with isolated C2 domains from *A. thaliana* PLDβ and PLDα (15). That study also showed that the Ca^{2+} binding induced conformational changes of the C2 domain and promoted the binding of the C2 domain to PC. Thus, Ca^{2+}-binding of the C2 domain underlies, at least in part, the biochemical basis of Ca^{2+}-dependent PLD activity.

PLDβ requires both Ca^{2+} and phosphatidylinositol 4,5-bisphosphate (PIP_2) for activity (3, 6). PLD C2 domains also bind PIP_2; however, Ca^{2+} weakens the PIP_2-C2 interaction (15). Thus, the inverse relationship between Ca^{2+}- and PIP_2-binding of the C2 domain suggests a complex, multi-step process of PLD activation. Further work has identified another PIP_2-binding region in the PLD catalytic fold (16), which consists of two duplicated HxKxxxxD motifs and which lies in the C-terminal two-thirds of the protein (6, 17, 18). The PIP_2-bound catalytic domain increases the enzyme's affinity for its substrate PC, and Ca^{2+} stimulates the PLDβ-PIP_2 interaction (15, 16). These observations suggest that Ca^{2+} probably interacts with the catalytic region.

The present study explores the potential interaction of Ca^{2+} with the catalytic regions. We have determined that the C2-deleted PLDβ (PLDβcat) binds Ca^{2+} but is less activated than the full-length enzyme. Furthermore, Ca^{2+} stimulates PIP_2- but not PC-binding of PLDβcat, properties that both complement and contrast with the lipid binding properties of the C2 domain.

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[1] The abbreviations used are: PLD, phospholipase D; PH, pleckstrin homology; PX, phox-homolgy; PIP_2, phosphatidyl 4,5-bisphosphate; GST, glutathione-*S*-transferase; STE, sodium chloride/Tris/EDTA; MES, 2-(*N*-morpholino)ethanesulfonic acid; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

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MATERIALS AND METHODS

Construction of GST-PLDβ and GST-PLDβcat and GST-βC2 Proteins—Three GST fusion proteins GST-PLDβ, GST-PLDβcat, and GST-PLDβC2 (Fig. 1) were used in this study. To construct the C2-deleted PLDβcat, a DNA fragment encompassing the catalytic domain of PLDβ (amino acid residues 158–829) was generated by PCR using the PLDβ cDNA in pBluescript SK as a DNA template, T7 primer as 3′ primer, and a synthetic olignucleotide as 5′ primer, which included an EcoRI restriction site at its 5′ end. The PCR-amplified DNA fragment was digested with EcoRI restriction enzyme and ligated directly into the pGEX-2T vector (Pharmacia). The creation of the GST-PLDβC2 construct has been described previously (15). All of the constructs were transformed into *Escherichia coli* BL21 for protein expression.

Expression and Purification of Fusion Proteins in E. coli—Protein expression for each of the constructs was induced by adding 0.4 mM isopropyl-1-thio-β-D-galactopyranoside to log phase (A_{600} = 0.6–1.0) bacterial cultures and then incubating overnight at 25 °C. For activity assay, cells were collected by centrifugation at 6,000*g* for 5 min and then rinsed three times with buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM EDTA). The final rinsed pellet was resuspended in buffer A that contained 2 μg/ml antipain, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 0.2 mg/ml lysozyme and was incubated on ice for 15 min. Dithiothreitol was added to 5 mM and the cells were briefly sonicated. Cellular debris were cleared by centrifugation at 12,000*g* for 5 min. The supernatant was either used immediately or stored at −80 °C.

The purification of GST fusion proteins was performed using a modified version of a previously reported procedure (19). The bacteria pellet was resuspended in STE buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) containing 200 μg/ml lysozyme. The suspension was left on ice for 30 min. Dithiothreitol and *N*-laurylsarcosine (Sarkosyl) were then added to a final concentration of 5 mM and 1.5% (w/v), respectively. The samples were vortexed, sonicated on ice for 1 min, and then centrifuged at 27,000*g* for 15 min. The supernatant was transferred to a new tube, and Triton X-100 was added to a final concentration of 4% (v/v). The solution containing GST fusion proteins was mixed with glutathione-agarose beads (50%, w/v) at 25 °C for 1 h. The GST fusion proteins bound to agarose beads were washed with 20 bed volumes of STE buffer. At every stage, GST activity was measured and was expressed as ΔA_{340}/min/ml. The amount of purified GST fusion protein bound to the glutathione-agarose was estimated by eluting the GST fusion protein with 8 M urea in STE buffer followed by measuring the protein concentration of the eluate with a protein assay kit (Bio-Rad).

$^{45}Ca^{2+}$ Gel Overlay—Affinity-purified GST-PLDβ and GST-PLDβcat were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and allowed to renature overnight at 4 °C in 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl as described previously (20). The blots were incubated with 10 μCi of $^{45}Ca^{2+}$ and 50 mM Tris-HCl, pH 7.5, in the presence or absence of 1 mM PS that was added from a concentrated stock solution that was sonicated before use. The membrane was incubated at room temperature for 2 h with gentle rocking, after which it was rinsed three times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2 mM EDTA. The dried blot was exposed to film.

$^{45}Ca^{2+}$ Binding Assay—$^{45}Ca^{2+}$ binding of the engineered GST-PLDβ proteins and GST was evaluated using a described method with some modifications (21, 22). Twenty microliters (wet volume) of purified GST-PLDβ, GST-PLDβcat, GST-PLDβC2, or GST attached to glutathione agarose beads was incubated with 50 mM Tris-HCl, pH 7.5, and 100 μM unlabeled Ca^{2+} and 1 μCi of $^{45}Ca^{2+}$ (specific activity, 5 μCi/μg Ca^{2+}) as tracer in a final volume of 100 μl. To test the effect of phospholipid type and concentration on Ca^{2+} binding, various phospholipids were sonicated just before use and added to the incubations as specified in the text.

To obtain quantitative Ca^{2+} binding data, GST-PLDβ was incubated in the presence of 1 mM PS, 1 μCi of $^{45}Ca^{2+}$, and varying amounts of unlabeled Ca^{2+}, and $^{45}Ca^{2+}$ binding after rinsing was measured. Total calcium binding was calculated by multiplying the ratio of $^{45}Ca^{2+}$ $CPM_{recovered}$/$^{45}Ca^{2+}$ CPM_{total} by the concentration of non-radiolabeled calcium present. Before performing competitive binding experiments, it was established that 1 μCi of $^{45}Ca^{2+}$ was sufficient to saturate all GST-PLDβ Ca^{2+} sites (data not shown).

Binding experiments typically contained 0.5–5 μg of purified GST fusion proteins. After incubation at 25 °C for 15 min with moderate shaking, $^{45}Ca^{2+}$ bound to fusion proteins was pelleted with the affinity beads by centrifugation at 2,000*g* for 1 min. The pellet was washed three times with 1 ml of a rinsing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). All buffer solutions were prepared with Chelex-100-treated H_2O. $^{45}Ca^{2+}$ bound to the fusion proteins was measured by scintillation counting, and the counts were normalized to the picomolar amount of GST fusion proteins used on the basis of the following molecular masses: 103 kDa (GST-PLDβcat), 119 kDa (GST-PLDβ), 42 kDa (GST-PLDβC2), and 26 kDa (GST).

Analysis of Ca^{2+} Binding Data—The competitive binding experimental data were fit to two ligand binding models (23): a single class of independent, non-interacting sites or two classes of independent, non-interacting sites, according to the following equations: for a single class of sites, $\nu = nK[L]/(1+K[L])$; or for two classes of sites, $\nu = n_1K_1[L]/(1+K_1[L]) + n_2K_2[L]/(1+K_2[L])$. In these equations, ν is the ratio of Ca^{2+} bound (micromoles) to micromoles of fusion protein, n refers to the number of binding sites, K is the equilibrium association constant, and $[L]$ is the free Ca^{2+} concentration. The data were analyzed by both equations using a non-linear least squares fitting program in PSI Plot (Poly Software International, Pearl River, NY), which yielded estimates for n and K. The equilibrium dissociation constant, K_d, was calculated as $1/K$ and values for n and K_d were reported as the value ± S.D. of the estimate.

PLD Activity Assay—PLD activity was determined as described previously (2). The reaction mixture included 100 mM MES, pH 7.0, 100 μM $CaCl_2$, 0.5 mM $MgCl_2$, 80 mM KCl, 0.4 mM lipid vesicles, and 2–10 μg of protein in a total volume of 100 μl. The lipid vesicles were made of PE/PIP_2/PC in a ratio of 87.5:7.5:5 mol %. The PLD-mediated hydrolysis of PC was measured using dipalmitoylglycerol-3-phospho[*methyl*-^{3}H]choline. Release of [^{3}H]choline into the aqueous phase was quantified by scintillation counting.

Phospholipid Binding Assays—The same procedure employed previously for assaying phospholipid binding to PLD C2s (15, 16) was used to quantify phospholipid binding to PLDβ and its deletion mutants. In brief, in the PC binding assay, 50 μl of phospholipid stock consisting of 250 μg/ml PC (egg yolk), 100 μg/ml PS (egg yolk), and 2 μCi/ml of ^{3}H-labeled PC (dipalmitoyl-glycero-3-P-[*methyl*-^{3}H]-choline) were mixed with GST fusion proteins bound to glutathione-agarose beads (20 μl wet volume) suspended in a binding buffer containing 50 mM Tris-HCl, 200 mM NaCl, and varying concentrations of Ca^{2+} in a final volume of 100 μl. The buffered Ca^{2+} solutions were made by appropriate dilution of the standard Ca^{2+} solution (Orion Ca^{2+}-sensitive electrode standard solution, Fisher) with Chelex 100-treated buffer. The mixture was then incubated at 23 °C for 30 min with shaking. The beads were washed three times with 1 ml of the binding buffer containing the test concentration of Ca^{2+}. PC bound to the protein-agarose beads was quantified by scintillation counting. 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 counts per minute per picomole of protein. To determine PC (or PIP_2) binding as a function of Ca^{2+} concentration, a similar procedure was employed using lipid dispersions made up of 400 μg of PC (or PIP_2) mixed with 0.4 μCi of ^{3}H-labeled PC (or PIP_2 using dipalmitoyl-glycero-3-P-[*inositol*-2-^{3}H]inositol 4,5-bisphosphate) in a final volume of 100 μl.

RESULTS

C2-deleted PLDβ Is Catalytically Active and Requires High Levels of Ca^{2+}—The *A. thaliana* full-length PLDβ and the C2-deleted mutant PLDβcat were expressed as GST fusion proteins (Fig. 1*A*). The purified GST-PLDβ and GST-PLDβcat had molecular masses of ~119 and 103 kDa, respectively (Fig. 1*B*). The GST-fused PLDβ catalyzed PC hydrolysis. Its activity was stimulated by Ca^{2+} and reached a plateau at 50 μM (Fig. 2), a Ca^{2+} level similar to that required for PLDβ in plants and for PLDβ expressed without the GST fusion tag (4, 6). The GST-fused PLDβcat also hydrolyzed PC, but at a significantly reduced rate. The maximal activation of PLDβcat represented only approximately 10% of the maximal activity of PLDβ. Furthermore, optimal activity of PLDβcat occurred at millimolar levels of Ca^{2+} (Fig. 2). Finally, the failure of PLDβC2 to hydrolyze PC under any conditions indicates that the low level of activity associated with PLDβcat is bona fide PLD activity. These results establish that PLDβcat, which lacks the C2 domain, contains necessary and sufficient amino acid residues to perform PC hydrolysis but requires much higher levels of Ca^{2+} for activity.

Ca^{2+} Binds to C2-deleted PLDβ—Ca^{2+}-binding of PLDβ and PLDβcat were initially determined by a $^{45}Ca^{2+}$ gel overlay

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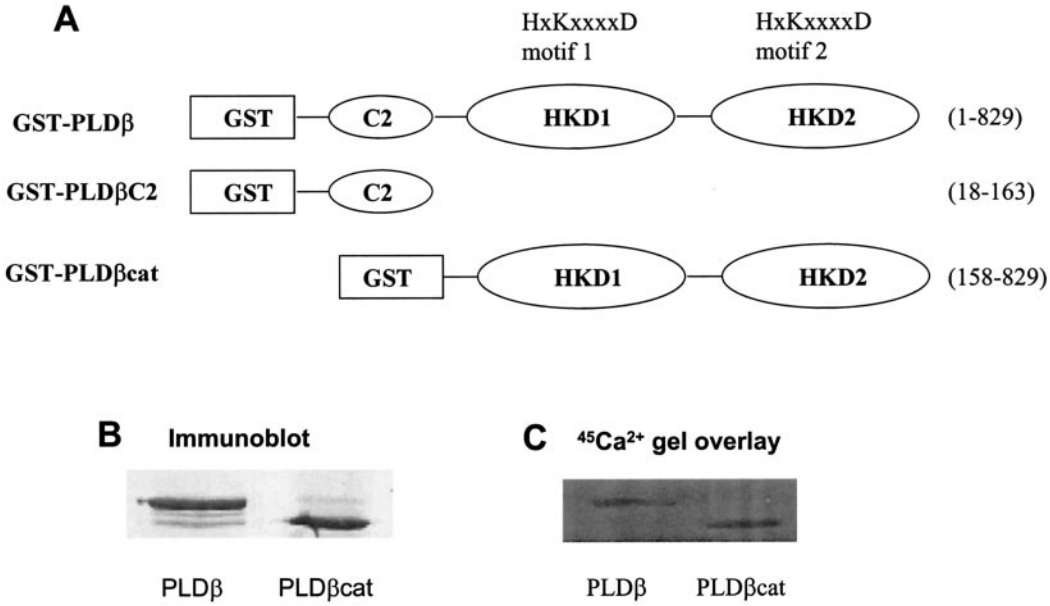

FIG. 1. **Construction, expression, and $^{45}Ca^{2+}$ binding of PLDβ and PLDβcat.** *A*, schematic representation of the domain structure of PLDβ and its GST fusion proteins. Full-length or different regions of PLDβ were fused to the C-terminal end of GST. The *numbers in parentheses* specify the starting and ending amino acid residues of each region. *B*, immunoblotting of GST-PLDβ and GST-PLDβcat. The recombinant proteins were affinity-purified using glutathione-agarose beads and applied to an 8% SDS-PAGE gel. After electrophoresis, proteins in the gel were transferred to a polyvinylidene difluoride membrane and immunoblotted with polyclonal antisera generated against the C-terminal amino acid residues of PLDβ (3). *C*, $^{45}Ca^{2+}$ binding by PLDβ and PLDβcat on a gel overlay. Affinity-purified fusion proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and incubated in the presence of 1 mM PS and 10 μCi of $^{45}Ca^{2+}$. After rinsing, the membranes were dried and exposed to film.

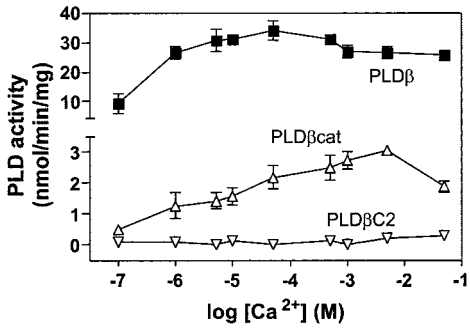

FIG. 2. **PLD activities of GST-PLDβ and GST-PLDβcat and their dependence on Ca^{2+}.** The PC-hydrolyzing activity of affinity-purified GST-PLDβ, GST-PLDβcat, and GST-βC2 were measured in response to various free Ca^{2+} concentrations using 0.4 mM vesicles composed of 87.5 mol % of PE, 7.5 mol % of PIP_2, and 5 mol % of PC. GST-PLDβ, GST-PLDβcat, and GST-βC2 were affinity-purified using glutathione-agarose, and these proteins (0.5–5.0 μg) were used in the activity assays. Values are means ± S.E. of three experiments. In the absence of Ca^{2+}, neither GST-PLDβ nor GST-PLDβcat shows any activity.

technique. PLDβ and PLDβcat demonstrated a marked ability to bind Ca^{2+} in the presence of phosphatidylserine (Fig. 1*C*) but not in its absence (data not shown). To obtain quantitative information about the Ca^{2+} binding exhibited by PLDβ, affinity pull-down binding experiments were performed in the presence of various concentrations of unlabeled Ca^{2+} and PLDβ fusion protein bound to glutathione-agarose beads. Ca^{2+} binding by PLDβ was saturable (Fig. 3, *inset*), and Scatchard analysis (Fig. 3) revealed an upward curvature that was suggestive of two classes of independent and non-interacting binding sites (23). Of the two modeling equations (*i.e.* one class of binding sites or two), the PLDβ Ca^{2+} binding data fit best in the two classes model with 3.6 ± 0.6 high affinity (K_d = 29 ± 6 μM) and 20 ± 4 low affinity (K_d = 1.4 ± 0.7 mM) binding sites.

Phospholipids Affect Ca^{2+} Binding by PLDβ—Ca^{2+} binding by some proteins, such as protein kinase C, is stimulated by acidic phospholipids (21, 22, 24, 25), and our $^{45}Ca^{2+}$ gel overlay experiments demonstrated a similar phospholipid dependence for Ca^{2+} binding. To characterize the effect of lipids on Ca^{2+} binding, glutathione-agarose bound GST-PLDβ fusion proteins were incubated with 100 μM Ca^{2+} and various concentrations of PS (Fig. 4). In the absence of PS, PLDβcat barely bound Ca^{2+}. The presence of PS increased the affinity of PLDβcat for Ca^{2+} in a concentration-dependent fashion. Ca^{2+} bound to PLDβ, PLDβcat, and PLDβC2 in a saturable manner, and half-saturation occurred at about 200 μM PS for each of these fusion proteins (Fig. 4). The Ca^{2+}-binding process seemed to be most cooperative for PLDβcat. In addition, in the presence of PS, PLDβcat bound more than twice as much Ca^{2+} as did PLDβC2. PLDβ was found to have the highest Ca^{2+}-binding capacity in the presence of PS.

The fusion proteins were incubated with several different phospholipids to determine whether or not other phospholipids besides PS could stimulate Ca^{2+} binding (Fig. 5). The GST-PLDβ fusion proteins exhibited less Ca^{2+} binding when PC or PG were substituted for PS, at a 1 mM concentration, in the binding assays. PG stimulated PLDβcat binding to Ca^{2+} to about 25% of the level observed using PS, whereas PC stimulated much less Ca^{2+} binding. Various phospholipid mixtures were also used to evaluate the effect of vesicle composition on Ca^{2+} binding by the GST-PLDβ fusion proteins. Although the phospholipid mixtures had no effect on Ca^{2+} binding to PLDβC2, they did display considerable stimulatory effects on Ca^{2+} binding by PLDβ and PLDβcat. For PLDβcat, this represents about 50% of the Ca^{2+} binding level observed using PS alone. The Ca^{2+} binding to PLDβ promoted by these phospholipids mixtures is of great interest because they resemble the lipid composition required for PLDβ activity whereas PS is a minor substrate of this enzyme (26). The finding that different phospholipid mixtures enhance Ca^{2+} binding by PLDβcat suggests that biological membranes might influence Ca^{2+} interaction with PLDβ.

Ca^{2+} Binding Differentially Modulates the Interaction of PLDβcat and PLDβC2 with Phospholipids—One possible func-

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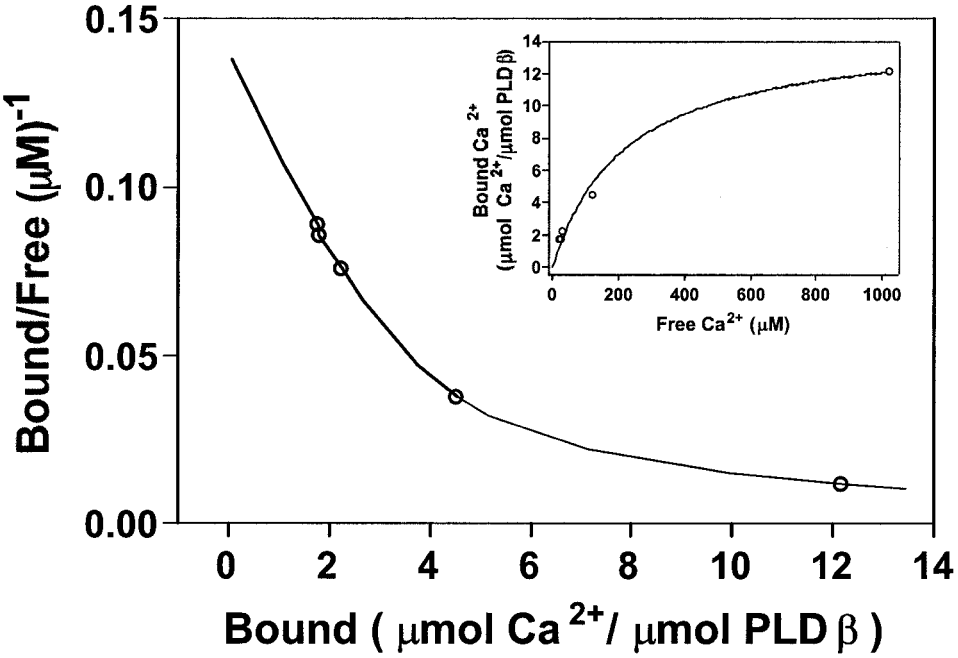

FIG. 3. **Effect of unlabeled Ca^{2+} on $^{45}Ca^{2+}$ binding to PLDβ.** $^{45}Ca^{2+}$ binding by glutathione-agarose-bound GST-PLDβ was determined in the presence of 1 mM PS, 1 μCi of $^{45}Ca^{2+}$, and various amounts of unlabeled Ca^{2+}. Binding data (*open circles*, *main panel*) were represented using a Scatchard plot and were analyzed using a non-linear least squares analysis for two classes of binding sites (see "Materials and Methods"). A theoretical curve based on the estimated n_1, n_2, K_1, and K_2 values (see "Results") and the two classes of binding site equations was plotted (*line, main panel*). Ca^{2+} binding was saturable with respect to the concentration of unlabeled Ca^{2+} added (*inset*). GST-PLDβ and GST were affinity-purified on glutathione-agarose beads. Background $^{45}Ca^{2+}$ binding by GST was small and was subtracted from the $^{45}Ca^{2+}$ binding values of the GST-PLDβ. $^{45}Ca^{2+}$ binding was determined by scintillation counting after extensive rinsing of the beads.

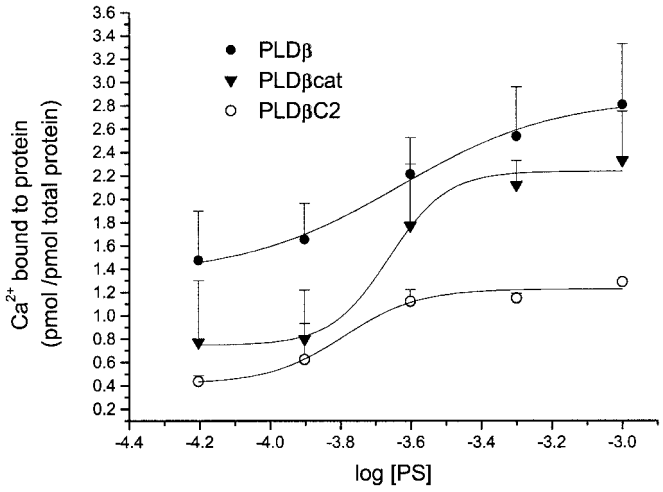

FIG. 4. **Effect of PS on Ca^{2+} binding by GST-PLDβ and GST-PLDβcat.** Ca^{2+} binding by bead-bound GST-PLDβ, GST-PLDβcat, GST-PLDβC2, and GST was measured using 1 μCi of $^{45}Ca^{2+}$ in the presence of varying amounts of PS. Background $^{45}Ca^{2+}$ binding by GST was small and was subtracted from the $^{45}Ca^{2+}$ binding values of the GST-PLD fusion proteins. The GST fusion proteins and GST were affinity-purified on glutathione-agarose beads. $^{45}Ca^{2+}$ binding was determined by scintillation counting after extensive rinsing of the beads. Values are means ± S.E. of three experiments. The lines represent the non-linear least-squares curves calculated to best fit the data points.

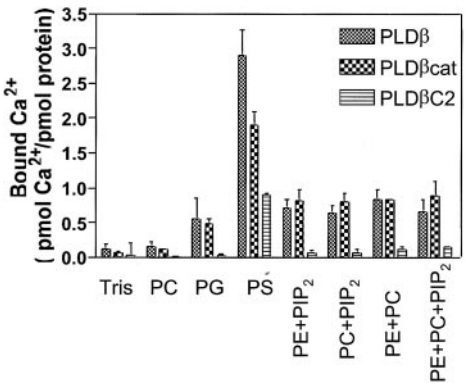

FIG. 5. **Effect of PS and other phospholipids on Ca^{2+} binding by GST-PLDβ and GST-PLDβcat.** Ca^{2+} binding by bead-bound GST-PLDβ, GST-PLDβcat, GST-PLDβC2, and GST was measured using 1 μCi of $^{45}Ca^{2+}$ in the absence (Tris) or presence of 1 mM PC, PG, PS, or in the presence of 1 mM lipid vesicles composed of: 85 mol % PE and 15 mol % PIP_2 (*PE+PIP$_2$*); 85 mol % PC and 15 mol % PIP_2 (*PC+PIP$_2$*); 85 mol % PE and 15 mol % PC (*PE+PC*); or 85 mol % PE, 10 mol % PIP_2, and 5 mol % PC (*PE+PIP$_2$+PC*). Background $^{45}Ca^{2+}$ binding by GST was low and was subtracted from the $^{45}Ca^{2+}$ values of the GST-PLD fusion proteins. The GST fusion proteins and GST were affinity-purified using glutathione-agarose. $^{45}Ca^{2+}$ binding was determined by scintillation counting after extensive rinsing of the beads. Values are means ± S.E. of three experiments.

tion for Ca^{2+} binding to membrane proteins is to modulate the interaction of such proteins and phospholipids. Previous studies have shown that association of PLDβC2 with Ca^{2+} enhances PC binding of the C2 domain but inhibits binding of PIP_2, a critical activator of PLDβ (15). In this study, the effect of Ca^{2+} on the interaction of PC and PIP_2 with PLDβ, PLDβcat, and PLDβC2 were compared directly (Fig. 6). In the absence of Ca^{2+}, PLDβ, PLDβcat, and PLDβC2 bound PC at a low level. As the Ca^{2+} concentration reached 100 μM, the amount of PC bound to PLDβ and PLDβC2 increased considerably, but no increase was noted for PLDβcat. Only at millimolar levels did Ca^{2+} slightly stimulate PLDβcat binding to PC (Fig. 6*A*). The decreased PC binding might partly account for the much reduced catalytic activity and the increased Ca^{2+} requirement of C2-deficient PLDβcat.

PLDβ, PLDβcat, and PLDβC2 all bound PIP_2 at comparable levels in the presence of 1 μM Ca^{2+} (Fig. 6*B*). Increases in Ca^{2+} concentration above 10 μM inhibited PIP_2 binding of PLDβC2.

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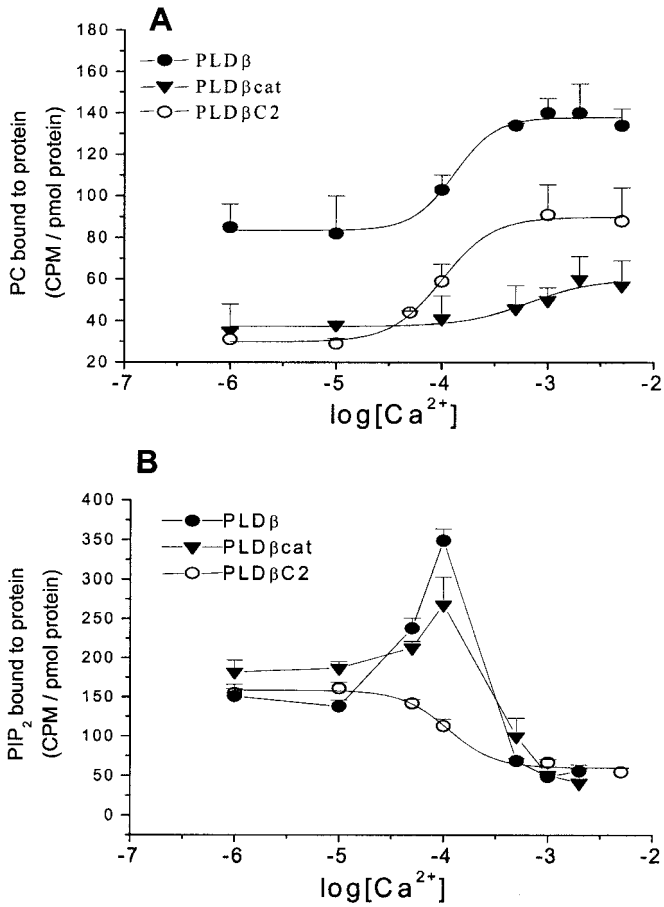

FIG. 6. **Effect of Ca^{2+} on the binding of PC and PIP_2 to GST-fusion proteins of PLDβ, PLDβcat, and the C2 domain.** *A*, stimulation of PC-binding of the PLDβ proteins as a function of Ca^{2+} concentration. ^{3}H-labeled PC in PC/PS (2.5:1 molar ratio) vesicles was used as tracer. *B*, effect of Ca^{2+} on PIP_2 binding of the PLDβ proteins. ^{3}H-labeled PIP_2 was used as tracer. In both cases, the beads were washed three times with 1 ml of the binding buffer containing the test concentration of Ca^{2+}. Phospholipid bound to the protein-agarose beads was quantified by scintillation counting. GST bound to glutathione-agarose beads was used to determine background lipid binding. Values are means ± S.D. of one representative experiment, and all the experiments were repeated at least three times.

In contrast, PIP_2 binding by PLDβ increased with Ca^{2+} levels up to 100 μM concentrations of the cation (Fig. 6*B*). Further increases in Ca^{2+} concentration sharply diminished the amount of PIP_2 bound to PLDβ. PLDβcat displayed a similar pattern but with a smaller magnitude (Fig. 6*B*). These results indicate that the catalytic region is primarily responsible for the enhanced binding of PIP_2 by PLD, and Ca^{2+} regulates this binding in a concentration dependent manner.

DISCUSSION

PLD plays important, multifaceted roles in cellular metabolism and regulation (27–29). The PLD family in plants is much more diverse than that in other organisms. The *A. thaliana* PLD family has 12 PLD genes (8, 27), whereas only two PLD genes are known in mammals and one in yeast (29, 30). Furthermore, 10 of the 12 *A. thaliana* PLDs contain the C2 domain, which are unique to plant PLDs. The remaining two *A. thaliana* PLDs have PH/PX domains that are also found in mammalian PLDs. All of the C2-containing plant PLDs studied required Ca^{2+} for activity, whereas the activity of PH/PX-containing *A. thaliana* PLDζ1 is independent of Ca^{2+}. The present study shows that *A. thaliana* PLDβ has multiple Ca^{2+} binding regions, the C2 domain and the C-terminal catalytic region. Ca^{2+} binding to the catalytic region improves interaction of PLD with the activator, PIP_2, which is required for PLDβ activity (3). Our previous work has revealed that PIP_2 binding to the catalytic region enhances the enzyme's affinity for the substrate, PC (16, 31). Thus, Ca^{2+} interaction with the catalytic region is likely to be coupled with enzyme-substrate binding and lipid hydrolysis.

The finding that Ca^{2+} is needed to promote PIP_2 binding explains, at least in part, why Ca^{2+} is required for the activity of the C2-containing PLDs but not the PX/PH-containing PLDs. PIP_2 stimulates the activity of all other *A. thaliana* PLDs examined (6–8) and also mammalian and yeast PLDs (29, 30). Both PH and PX domains are known to interact with polyphosphoinositides. In addition, a PIP_2 binding motif has been identified in the catalytic region of the mammalian and yeast PLDs (30). This motif is required for the animal PLD activity (30) and is conserved in the PH/PX-containing *A. thaliana* PLDζ (8). However, PLDβ and other Ca^{2+}-dependent PLDs miss two of the core basic amino acid residues involved in PIP_2 binding (16). Instead, these PLDs use Ca^{2+} to augment the binding of PIP_2 to the PLD catalytic fold, as shown in our previous (16) and present results.

The C2-deficient PLDβcat exhibited a much lower catalytic activity and needed a much higher concentration of Ca^{2+} to reach maximal activation compared with the whole enzyme. In addition, PC binding by PLDβcat was diminished. Thus, the reduced catalytic activity of PLDβcat probably results from an inefficient binding of substrate vesicles caused by the absence of the C2 domain. The present results also suggest that Ca^{2+} binding by the C2 domain facilitates the binding of PLDβ to its substrate in membranes, as proposed for cytosolic phospholipase A_2 (32). A truncated cytosolic phospholipase A_2 that lacked the C2 domain failed to associate with membranes and had no hydrolytic activity toward PC presented in liposomes; however, it could hydrolyze monomeric PC as efficiently as the full-length cytosolic phospholipase A_2 (32).

Ca^{2+} binds to PLDβ in a phospholipid-dependent manner; in the absence of phospholipids, Ca^{2+} binding is much reduced. Different phospholipids promote Ca^{2+} binding to different extents. By far, PS seems to be most effective, but PG and phospholipid mixtures also seem to influence Ca^{2+} binding. This phenomenon may be explained by the ability of these acidic phospholipids, via their net negative charge at physiological pH, to attract Ca^{2+} ions. Similar phospholipid-dependent Ca^{2+} binding has been reported for several other proteins (24, 25, 33, 34). For example, protein kinase C and annexin each bind 8–12 Ca^{2+} ions in the presence of acidic phospholipids but almost none in their absence (24, 33). Association of these proteins with membranes depends on membrane composition, which has an effect on the amount of Ca^{2+} required for optimal lipid-protein interaction (24). A binding model has been proposed to describe the Ca^{2+}-dependent binding of protein kinase C βII to membranes and its subsequent Ca^{2+}-dependent activation (25). According to this model, low levels of Ca^{2+} promote a weak interaction between protein kinase C and membranes, whereas higher Ca^{2+} concentrations activate the enzyme. Both high and low affinity Ca^{2+} interactions trigger conformational changes in protein kinase C βII. Findings reported in this and other studies (15, 16) suggest a similar two-stage membrane-binding and enzyme activation model for PLDβ.

The present study establishes that Ca^{2+} has differential effects on phospholipid binding to the C2 domain and the catalytic region. At elevated levels, Ca^{2+} inhibits PLDβC2 binding to PIP_2. At near physiological levels, Ca^{2+} stimulates PLDβcat binding to PIP_2 but, as it approaches millimolar levels, Ca^{2+} dramatically reduces PIP_2 binding (Fig. 6*B*). Ca^{2+}

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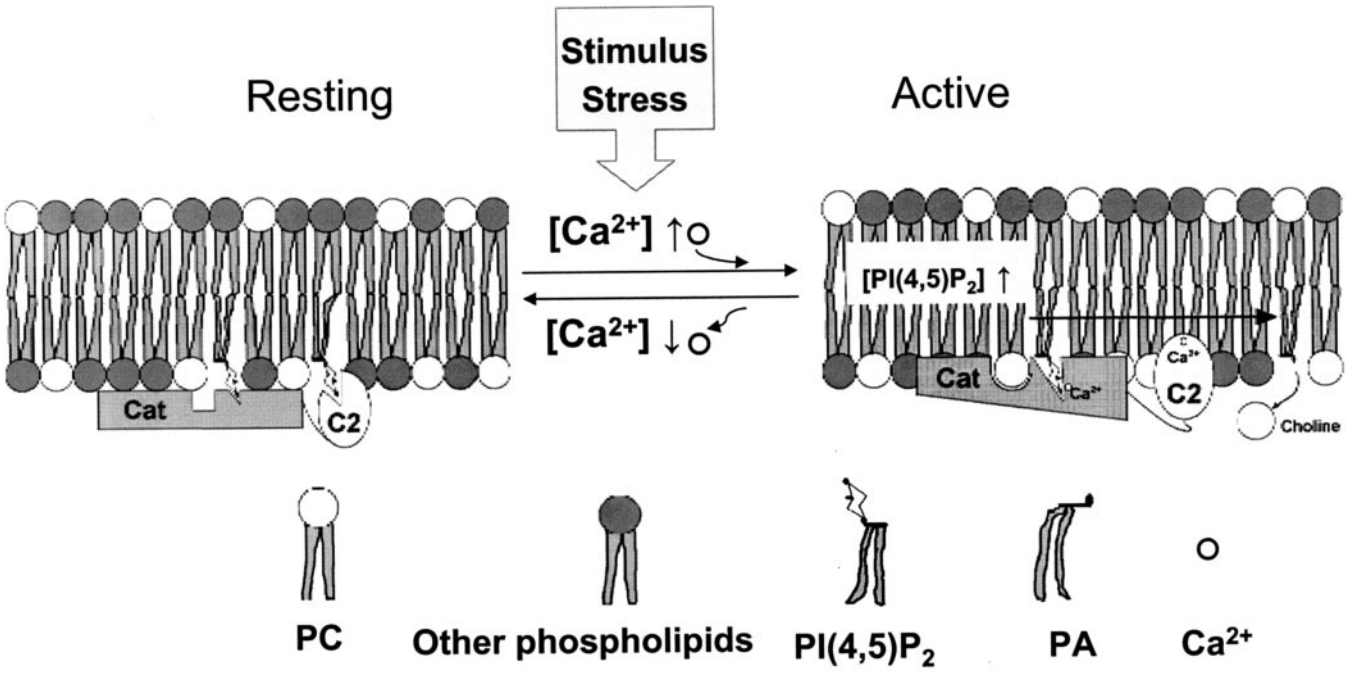

FIG. 7. **Membrane-scooting model depicting the regulation of PLDβ activity by changing cellular Ca^{2+} concentrations.** The C2 domain binds the membrane in a different orientation with and without Ca^{2+}. At a resting, low [Ca^{2+}] state (↓), the C2 domain and/or the PIP_2 binding region of PLDβ binds the membrane with its cationic residues interacting with anionic lipids, including scarce PIP_2. When membrane PIP_2 and cytosolic [Ca^{2+}] increase (↑), Ca^{2+} binds to the C2 domain and induces a conformational change that leads the C2 domain to bind the membrane with its calcium binding loop partially penetrating the membrane. Therefore, the calcium-C2 interaction alters the relative affinity of the C2 domain in favor of PC binding over PIP_2 (15). As a result, the C2 domain releases PIP_2 and binds to PC. Meanwhile, Ca^{2+} binds to the catalytic region and increases the affinity of this region to now more abundant PIP_2. The PIP_2 binding induces a conformational change in the catalytic region (16), which increases PLD activity by increasing its affinity to its substrate, PC.

has a similar but greater effect on PIP_2 binding to the whole enzyme (Fig. 6*B*), with the maximal value being attained at about 100 μM Ca^{2+}, a pattern resembling the Ca^{2+}-dependence of PLDβ activity (Fig. 2). Millimolar level Ca^{2+} inhibited PIP_2 binding by PLDβ but caused only a mild reduction PLDβ activity. On the other hand, millimolar Ca^{2+} inhibited both the activity and PIP_2 binding of PLDβcat. Ca^{2+} binding by the C2-domain of PLDβ causes a conformational change (15) that may lead to the optimal positioning of basic amino acid residues of the PIP_2 binding region flanking the active site (16). However, the significant stimulation of PIP_2-PLDβcat binding by Ca^{2+} suggests that this cation acts directly within the catalytic domain to promote PIP_2 binding. Inhibition of this protein-phosphoinositide interaction by high concentrations of Ca^{2+} probably reflects competition between the Ca^{2+} and the acidic residues of the PIP_2 binding region for binding to negatively charged PIP_2. Because PLDβ, but not PLDβcat, can efficiently bind PC through its C2-domain, its activity toward mixed PE/PC/PIP_2 vesicles is less reduced at millimolar Ca^{2+} levels.

Although there are clear limitations to extrapolating the data from these *in vitro* studies to the ligand binding and regulation of PLDβ *in vivo*, there is a reason to believe that these studies are relevant to the activation and regulation of plant PLDβ *in vivo*. For instance, it is known that steady state cytosolic concentrations of Ca^{2+} up to 1 μM can be elicited after applying an external stimulus, such as cold stress or wounding. Even so, the capacity of the plant cytosol to buffer Ca^{2+} is substantially greater (estimates range from 0.1 to 1 mM), strongly suggesting that the influx of Ca^{2+} after stimulation leads to transient local Ca^{2+} elevations in the range over which Ca^{2+}-mediated increases in PIP_2 binding and PLDβ activity are observed. These local dramatic increases of Ca^{2+} are expected to activate enzymes associated with Ca^{2+} channels or those tethered nearby on the membrane (35) and this is in agreement with our model of Ca^{2+}-induced PLDβ activity (Fig. 7).

In this model (Fig. 7), the C2 domain binds the membrane in a different orientation with and without Ca^{2+}, and this is in agreement with our previous observation that Ca^{2+} alters the conformation of the C2 domain (15). Without Ca^{2+}, it binds the membrane with its cationic residues interacting with anionic lipids, including PIP_2, whereas with Ca^{2+}, it binds the membrane with its calcium binding loop partially penetrating the membrane (Fig. 7). The binding and release of membrane phospholipids by the C2 and catalytic regions occur alternately to produce enzyme movement over the membrane surface without complete detachment. At resting Ca^{2+}, PLDβ interacts with PIP_2 through the C2 domain and/or the PIP_2 binding region to remain attached to the membrane in an inactive state. With increased Ca^{2+} concentration (and PIP_2 concentration; see below), PIP_2 binding affinity of the C2 domain decreases, whereas that of the catalytic region increases.

Although Fig. 2 suggests that PLDβ is active at resting Ca^{2+} levels, this is unlikely to be the case *in vivo*, where PIP_2 levels are lower than those present in the substrate vesicles used for our *in vitro* activity assay (36). Our previous studies clearly demonstrate the concentration-dependent activation of PLDβ by PIP or PIP_2 (3, 4, 16) and, like Ca^{2+}, cellular levels of these signaling molecules increase in response to external stimuli and stress (37). In addition, although increased PIP_2 levels increase the binding of PLDβcat to PC, PLDβ-C2 domain binding to PC is not stimulated by PIP_2 at any concentration (16). Thus, dynamic changes in the concentrations of Ca^{2+} and PIP_2 in response to external stimuli and stress can coordinately increase the binding of both activator (*i.e.* PIP_2) and substrate (*i.e.* PC) to the catalytic domain of PLDβ and increase its activity (Fig. 7). This mechanism underlies a basis for post-translational regulation of PLD activity, which is suggested to occur under different conditions, such as oxidative assaults

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(38), temperature stress (39), and in response to a plant hormone (40). Both Ca^{2+} and PIP_2 function as cellular messengers in various cellular processes, and characterization of their direct interaction with PLD thus provides insights into the *in vivo* activation and function of Ca^{2+}-dependent PLDs.

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