Calcium-Dependent and -Independent Interfacial Binding and Catalysis of Cytosolic Group IV Phospholipase A_2[†]

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ABSTRACT: Cytosolic group IV phospholipase A_2 (cPLA2) plays a role in liberating arachidonic acid from the *sn*-2 position of mammalian cellular phospholipids. The enzyme consists of a catalytic domain joined to an N-terminal calcium-dependent, membrane binding domain (C2 domain). The interfacial binding properties of the full-length, nonphosphorylated enzyme and its C2 domain to phospholipid vesicles were studied as a function of vesicle phospholipid composition and calcium concentration. The binding of cPLA2 to phosphatidylcholine vesicles is mostly governed by its C2 domain; binding is relatively weak, and calcium enhances binding and interfacial catalysis by about 10-fold. Catalytically productive interfacial binding was measured by monitoring the increase in the rate of cPLA2-catalyzed hydrolysis of a fluorimetric substrate present in vesicles as a function of bulk vesicle concentration. Enzyme−vesicle binding was also measured by fluorescence as was enzyme−calcium binding. Compared to zwitterionic vesicles, cPLA2 binding to anionic phosphatidylmethanol vesicles is of higher affinity and calcium-independent, although calcium is required for the binding of the C2 domain to these anionic vesicles. cPLA2 is fully catalytically active on phosphatidylmethanol vesicles in the absence of calcium. Phosphatidylserine is not a good replacement for phosphatidylmethanol for inducing high-affinity, calcium-independent binding of cPLA2. These results reveal two modes of catalytically productive interfacial binding of cPLA2: calcium-dependent anchoring via the C2 domain and a calcium-independent component involving a phosphatidylmethanol recognition element in the catalytic domain. They also show that membrane binding of cPLA2 is not, in general, predicted by the interfacial binding properties of its C2 domain.

Evidence is accumulating that the 87 kDa cytosolic phospholipase A_2 (cPLA2)[1] (also known as group IV phospholipase A_2) is directly responsible for releasing arachidonic acid from the *sn*-2 position of phospholipids for the biosynthesis of eicosanoids (*1*). In unstimulated cells such as platelets, mast cells, and macrophages the enzyme resides in the cytosol. Following stimulation with agonists that induce arachidonate release, cPLA2 translocates to the nuclear envelope and probably the endoplasmic reticulum and/or the golgi (*2*, *3*). The molecular basis for this targeting is not known. A rise in intracellular calcium and cPLA2 phosphorylation seems to activate this enzyme (*1*, *4*, *5*). Sequence analysis reveals that cPLA2 contains an N-terminal calcium-dependent phospholipid binding domain, termed a C2 domain, which is found in a number of proteins that bind to membranes in the presence of calcium (*6*−*8*). Recombinant C2 domain, when expressed as a separate entity, binds to phospholipid vesicles when the concentration of calcium is raised to about 5 μM. The remaining portion of cPLA2 contains the lipolytic domain (*9*). The crystal structure of the cPLA2 C2 domain has recently been reported (*10*), and this should aid in the understanding of cPLA2 interfacial binding at the molecular level.

Besides calcium, the other cPLA2 regulatory element is phosphorylation. cPLA2 is phosphorylated on four serines (Ser-437, -454, -505, and -727) when expressed in a baculovirus/Sf9 cell system (*11*). In human platelets, thrombin and collagen stimulate the phosphorylation of cPLA2 at serines 505 and 727 (*12*). Ser-505 is part of a consensus sequence recognized by a group of kinases of the mitogen-activated protein kinase family. In some cells, p42/44^{MAPK} may be responsible for cPLA2 phosphorylaton (*1*). The stress-activated protein kinase p38^{MAPK} or a closely related kinase is responsible for cPLA2 phosphorylation in human plateletes (*13*). The kinases responsible for the three other phosphorylations have not yet been identified.

The relative importance of calcium versus phosphorylation in regulating lipolysis by cPLA2 seems to depend on the cell type. For example, in a Chinese hamster ovary cell/

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[1] Abbreviations: C2, residues 17−141 of human cPLA2 with N-terminal extension MRGSHHHHHHGLVPRGS; cPLA2, 87 kDa human cytosolic phospholipase A_2 (group IV); cPLA2-A4, mutant of cPLA2 in which serines 437, 454, 505, and 727 have been changed to alanine and containing YHHHHHH fused to the C-terminal residue (alanine-749); *N*-dansyl-DHPE, *N*-dansyl-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine; $DO_{et}PC$, 1,2-dioleyl-*sn*-glycero-3-phosphocholine; $DO_{et}PE$, 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine; DOPM, 1,2-dioleoyl-*sn*-glycero-3-phosphomethanol; $DO_{et}PM$, 1,2-dioleyl-*sn*-glycero-3-phosphomethanol; $DO_{et}PS$, 1,2-dioleyl-*sn*-glycero-3-phosphoserine; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GLU, ester formed between γ-linolenoic acid and 7-hydroxycoumarin; LUV, large unilamellar vesicle; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine.

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cPLA2 gene transfection system, mutation of Ser-505 to alanine leads to a dramatic reduction in the level of agonist-induced arachidonate liberation, and an increase in cytosolic calcium is also required for cPLA2 activity (*4*). In contrast, calcium seems to be the major regulator of cPLA2 activity in thrombin-stimulated platelets (*13*). One of the difficulties in understanding the role of phosphorylation for cPLA2 regulation is that this protein modification only modestly increases the enzymatic activity of cPLA2, when assayed in cell extracts with synthetic phospholipids (*14*). The possible role of putative intracellular cPLA2 binding proteins such as p11 in the regulation of cPLA2 is notable (*15*). In addition, anionic phospholipids have been shown to activate cPLA2 in vitro (*16, 17*).

Since the C2 domain of cPLA2 as a separate entity undergoes calcium-promoted binding to membranes, it is tempting to speculate that interfacial binding of the entire protein is driven soley by its C2 domain. However, we have found that interfacial binding and catalysis is calcium-independent when cPLA2 substrate is incorporated into anionic phosphatidylmethanol vesicles (*18*). Here, we report studies of the binding of full-length nonphosphorylated cPLA2 and its C2 domain to vesicles composed of synthetic phospholipids. The calcium-dependence of interfacial binding was studied in detail. Interestingly, whereas the C2 domain is strictly dependent on micromolar amounts of calcium for binding to vesicles, full-length cPLA2 displays calcium-dependent and -independent interfacial binding and catalysis modes.

MATERIALS AND METHODS

Materials. Diether phospholipids were synthesized from 1,2-dioleyl-*sn*-glycerol, which was prepared from selachyl alcohol as described (*19*). Selachyl alcohol was obtained from Pfaltz and Bauer and shown to have the published specific rotation. $DO_{et}PC$ was prepared from the diglyceride using 2-chloro-2-oxo-1,3,2-dioxaphospholane (Fluka) as described (*20*). $DO_{et}PM$ was prepared by treating 1,2-dioleyl-*sn*-glycerol with $POCl_3$ (*21*) followed by methanol. The phosphate triester was treated with LiBr to yield DO_{et}-PM as described (*22*). $DO_{et}PS$ and $DO_{et}PE$ were prepared from 1,2-dioleyl-*sn*-glycerol as described (*21*). All phospholipids were purified to homogeneity by flash chromatography on silica gel. After the column, the phospholipid was dissolved in $CHCl_3$/MeOH, and the organic layer was washed once with an equal volume of water to remove trace amounts of silica. After solvent removal, the phospholipids were lyophilized from benzene and stored under Ar either as solids or as solutions in $CHCl_3$ in vials at −20 °C. All phospholipids were judged to be pure by thin-layer chromatographic analysis on silica plates (*23*). GLU was prepared as described (*24*). DOPM, POPC, and POPS are from Avanti. *N*-Dansyl-1-[*O*-(11-undecylamino)]-2-decanoyl-*sn*-glycero-3-phosphocholine was a gift from Prof. H. S. Hendrickson (*25*), and *N*-dansyl-DHPE was prepared as described (*26, 27*).

N_α-(7-Nitro-2,1,3-benzoxadiazol-4-yl)-*N*,*N*-dihexadecyl-L-glutamic acid diamide was prepared by treating *N*,*N*-dihexadecyl-L-glutamic acid diamide (*28*) with 1.5 equiv of NBD-fluoride (Molecular Probes) in ethyl acetate/methanol (5/1) containing 1.5 equiv of triethyamine for 1 h at room temperature. The product was purified by flash chromatography on silica using 5% methanol in $CHCl_3$. The product showed a single spot on a silica thin-layer plate, and its structure was verified by ^{1}H NMR.

Proteins. cPLA2 containing its native N- and C-termini was purified from an Sf9 cell/baculovirus expression system as described (*17, 18*). cPLA2-A4 contains the fusion peptide YHHHHHH attached to the C-terminal residue of native cPLA2 (Ala-749). This peptide was added by using site-directed mutagenesis to create a unique restriction site, *Stu*I, in front of the termination codon and ligating a linker between this *Stu*I site and the *Bgl*II site of pVLCP (*29*). This mutagenesis and the serine-to-alanine changes were carried out with the Altered Sites mutagenesis system (Promega) as described (*30*).

Sf9 cells were grown in Graces medium supplemented with 3.33 g/L lactalbumin, 4 g/L yeastolate, 0.35 g/L $NaHCO_3$, 10% fetal calf serum, and the antibiotic-antimycotics penicillin G, streptomycin, amphotericin (Gibco BRL 15240-062). Cells were grown at 27 °C in spinner flasks turning at 120 rpm. Cells in log-phase growth at a cell density of 10^6 mL^{-1} were infected with baculovirus at an MOI of 5. After 3 days post infection, the cells were harvested by centrifugation at 5000*g* for 5 min.

Unless otherwise specified, all procedures were performed at 4 °C. Centrifuged cell paste from 1−2 L of cell culture was resuspended with 1.5 volumes of lysis buffer containing 25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Pefablac, 1 mM benzamidine, 50 μg/mL leupeptin, 10 μM pepstatin A, and 5 μg/mL aprotinin. The above solution was placed in a nitrogen cavitation bomb and incubated for 30 min at 600 psi. Next, the cell lysate was centrifuged at 91000*g* for 40 min. The supernatant was loaded onto a 50 mL Q-Sepharose (Pharmacia) column equilibrated with 25 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, and 0.5 mM 2-mercaptoethanol. After washing with the above buffer until the eluant achieved a stable absorbance at 280 nm (∼500 mL), a 1 L linear NaCl gradient was run from 0.1 to 0.7 M. cPLA2 elution was determined by SDS−PAGE (7.5% gel). Fractions containing cPLA2 were pooled and concentrated to 20 mL in an ultrafiltration stirred cell (Amicon). The concentrate was dialyzed against 25 mM $NaHPO_4$, pH 7.9, 1 mM imidazole, and 0.75 M NaCl. At room temperature, the concentrate was added to 1 mL of nickel-chelated TSK gel AF Chelate 650M (TosoHaas) and mixed for 30 min. The suspension was poured into a 10 mL column, and the column was washed with phosphate dialysis buffer until the A_{280} achieved a stable base line (∼40 mL). Next, 20 mL of a 1−200 mM linear imidazole gradient was run. Two milliliter fractions were collected into tubes containing 1 mL of the above phosphate buffer with 6 mM 2-mercaptoethanol. cPLA2 elutes approximately halfway through the gradient and is greater than 97% homogeneous based on SDS−PAGE. Collected fractions were pooled and dialyzed against 25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 2 mM dithiothreitol. Glycerol (30% by volume) was added to the dialyzed cPLA2, and the enzyme was stored at −25 °C. It appears to be indefinitely stable under these conditions when activity is monitored with the DOPM/GLU assay (*24*). Concentrations of cPLA2 were determined using $\epsilon_{280} = 0.827$ mg^{-1} mL^{-1} cm^{-1} (*17*).

Recombinant C2 (cPLA2 residues 17−141 with N-terminal tail MRGSHHHHHHGLVPRGS) was prepared by modification of the published procedure (*10*). The bacterial expression system harboring the expression plasmid has been described (*10*), and it was obtained as a gift from R. L. Williams (University of Cambridge, U.K.). Cells were grown (0.5 L culture), protein expression was induced, inclusion body pellet was obtained, and protein was solubilized in urea buffer as described (*10*). Metal ion affinity chromatography at room temperature was carried out as described (*10*) except that TSK gel AF Chelate 650M was used (3 mL gel). Protein was concentrated and submitted to in vitro refolding and dialysis as described (*10*). After dialysis at 4 °C, the protein solution was concentrated to ∼1 mL by ultrafiltration in a stirred cell (Amicon, YM10 membrane) at 4 °C. After centrifugation to remove particulates the protein solution was loaded onto a column of Poros 20 HQ (PerSeptive Biosystems, 1.3 mL of gel packed as described by the manufacturer) that had been equilibrated with 50 mM Tris, pH 8.0, at room temperature and at 5 mL/min. After the column was loaded, it was washed with equilibration buffer until the absorbance at 280 nm reached a minimum. Then a 5 min linear gradient of 0−0.7 M NaCl in equilibration buffer was run. The peak of folded C2 eluted between ∼0.1 and 0.3 M NaCl. The peak fractions were pooled, and the solution was concentrated to ∼3 mL (0.6 mg/mL protein) in the Amicon stirred cell at 4 °C. This protein solution was flash-frozen in liquid nitrogen and stored at −20 °C. The concentration of C2 was determined using $\epsilon_{280} = 0.824$ mg^{-1} mL^{-1} cm^{-1}, which was calculated from its amino acid sequence (*31*).

Calcium-Containing Buffers. Two different ranges of calcium concentrations were prepared as follows. (1) Method I: buffered calcium (0−2.0 μM calcium) was made by adding various amounts of 0.4 M Ca^{2+}•EGTA (*32*) to buffer I [50 mM Tris, 150 mM NaCl, 30% (v/v) glycerol, pH 7.5] containing 100 μM EGTA to give the desired concentration of free calcium. The latter was calibrated with the indicator dye Fluo-3 as described (*33*). The use of buffered calcium allays any concern about a drop in free calcium concentration due to binding of calcium to vesicles. (2) Method II: calcium solutions (2−100 μM, unbuffered) were prepared by adding the appropriate amount of $CaCl_2$ in excess of EGTA present in buffer I containing 10 μM EGTA. To determine the amount of contaminating calcium present in the buffer, 50 nM Calcium Green 5N indicator (Molecular Probes) was added to buffer I. EGTA was added in 1 μM increments, and the amount needed to reduce the indicator fluorescence to a constant minimum was taken as the concentration of calcium present in buffer I (typically 5 μM) (the dissociation constant for the calcium−indicator complex is ∼50 μM). Thus, the concentration of calcium after addition of various amounts of calcium is the amount added plus the amount present as a contaminant minus 10 μM. Using buffer containing 2 μM free calcium (method II) and 50 nM Calcium Green 5N indicator (Molecular Probes), it was found that the indicator fluorescence did not change when 10 μM $DO_{et}PC$, $DO_{et}PS$, or $DO_{et}PM$ vesicles were added. This indicates that the presence of 10 μM vesicles does not decrease the concentration of free calcium present at 2 μM (the indicator emission is sensitive to small changes in free calcium near 2 μM since the dissociation constant for the indicator−calcium complex is ≈30 μM). This was expected since the dissociation constant for the complex of anionic vesicles with calcium is ≫2 μM (*34*).

Vesicle Binding and Kinetic Studies. All vesicle binding and kinetic studies were carried out in 1 mL of buffer containing various amounts of free calcium and vesicles in a quartz fluorescence cuvette thermostated at 30 °C (solution compositions are given in the figure legends). Vesicles containing mixtures of lipids were prepared by mixing $CHCl_3$ stock solutions of components in a glass tube, and removing the solvent with a stream of N_2 and then in vacuo for at least 1 h prior to making vesicles. LUVs were made by extrusion in buffer I without glycerol as described (*17*). Aqueous suspensions of vesicles were stored for up to 2 days under Ar in vials at room temperature.

Assays with GLU were carried out by adding this substrate from a stock solution in DMSO to the reaction mixture containing buffer and vesicles such that the DMSO concentration was never more than 0.4%. cPLA2-A4 was added last to initiate the reaction. GLU hydrolysis was followed fluorimetrically, and product was calibrated as described (*24*). All kinetic reactions were carried out with 10.8 nM cPLA2-A4 at 30 °C.

Fluorescence resonance energy transfer was carried out with excitation at 284 nm (5 nm slit width) and emission at 530 nm (10 nm slit width) for dansyl probes or at 540 nm for N_α-(7-nitro-2,1,3-benzoxadiazol-4-yl)-(*N*,*N*-dihexadecyl-L-glutamic acid diamide probe. When the dansyl probes were used, a 460 nm, long-pass, cutoff interference filter was placed on the emission side; this is critical for preventing artifacts due to second-order scattering. Vesicles containing dansyl probes could also be used to monitor GLU hydrolysis. For all binding studies, it was shown that the energy transfer emission intensity occurs only when fluorophores are present in vesicles. Furthermore, the energy transfer intensity was reduced 2-fold when enzyme was added to an equimolar mixture of vesicles without fluorophore and vesicles with fluorophore compared to adding enzyme to fluorophore-containing vesicles alone. These controls show that the energy transfer signal is due to binding of protein to vesicles.

All kinetic and binding experiments were carried out in duplicate, and many were carried out in triplicate. Estimated errors for GLU initial hydrolysis rates and energy transfer emission intensities are <10% and <3%, respectively.

RESULTS

Characterization of cPLA2-A4 and C2 Proteins. The baculovirus/Sf9 cell protein expression system provides multi-milligram amounts of cPLA2 for mechanistic studies, but the protein obtained is a mixture of phosphorylated molecular species due to partial phosphorylation on four serine residues (Ser-437, -454, -505, and -727) (*11*). Although treatment of cPLA2 from this expression system with phosphatase quantitatively removes the phosphates (*17*, *18*, *35*), overall yields can be low after removal of phosphatase. In this study, we focus on a single molecular species of cPLA2, the nonphosphorylated form cPLA2-A4. Mutation of all four serine phosphorylation sites to alanine was expected to prevent in vivo phosphorylation. Tens of milligrams of this protein (bearing a short C-terminal 6-histidine tag, YHHHHHH) are readily obtained from the

cytosol of baculovirus-infected Sf9 cells after anion exchange chromatography and metal ion affinity chromatography as described under Materials and Methods. The specific activity of cPLA2-A4, measured with a fluorimetric assay using GLU dispersed in DOPM LUVs, was within 10% of the previously reported value for wild type, nonphosphorylated cPLA2 that does not have a fusion peptide (*17*). To test whether the 6-histidine tag is affecting interfacial binding and catalysis, as noted below key experimental findings were investigated with both cPLA2-A4 and native, dephosphorylated cPLA2.

To verify that cPLA2-A4 is poorly phosphorylated, a 10 mL culture of baculovirus-infected Sf9 cells was incubated with 0.5 mCi of [^{32}P]orthophosphate, and cPLA2-A4 was isolated by immunoprecipiation (*12*). The protein was submitted to SDS−PAGE alongside wild-type cPLA2 obtained from an identical cell culture that was labeled and processed in the same way. Staining of the gel revealed the same amount of cPLA2 in each lane. cPLA2 bands were excised from the gel, dissolved in 30% H_2O_2, and submitted to scintillation counting. This analysis revealed that the phosphorylation of cPLA2-A4 was only 5−8% of that for wild-type cPLA2. This result further confirms our phosphorylation site assignments (*11*).

C2 prepared for this study is the same protein that was subjected to X-ray structural analysis (*10*). Based on the fact that the X-ray structure of C2 shows that the N-terminus lies at the end of the domain opposite from the calcium and phospholipid binding face (*10*), it was considered unnecessary to remove the 6-histidine tag for these membrane binding studies. The tryptophan fluorescence emission spectrum of C2 contains a single peak at 320 nm. Denaturation of C2 with 8 M urea leads to broadening of the emission peak, and the emission maximum shifts to 340 nm (not shown). This behavior of the intrinsic tryptophan fluorescence of native and denatured protein is virtually the same as that reported by Nalefski et al. for the C2 domain studied by these investigators (fusion protein of the C-terminus of glutathione *S*-transferase fused to the N-terminus of residues 1−155 of cPLA2) (*36*). When tryptophan emission at 330 nm (excitation at 280 nm) was monitored for C2 in buffer without vesicles as a function of added calcium, the emission intensity decreased by 40% (not shown). The data well fit a hyperbola to give a dissociation constant for the C2·Ca^{2+} complex of $K_{Ca} = 16 \pm 0.6$ μM, which agrees well with the value of 24 μM reported for the C2 domain studied by Nalefski et al. (*36*).

Choice of Vesicles for Binding and Kinetic Studies. All vesicle binding studies with cPLA2-A4 were carried out initially with diether phospholipids. The use of diether phospholipids avoids possible problems due to hydrolysis of vesicles by cPLA2-A4 during binding experiments. It cannot be argued a priori based on the preference of cPLA2 for *sn*-2-arachidonyl chains that phospholipids composed of ester-linked saturated or monounsaturated fatty acyl chains will not undergo significant hydrolysis. This is because binding experiments typically involve a high density of enzymes on vesicles, and cPLA2 displays finite activity toward phospholipids that have *sn*-2 saturated or monounsaturated fatty acyl chains. It has been shown with vesicles containing diether and diester phospholipids as the main component and a good cPLA2 substrate as a minor component that the specific activities of cPLA2 acting on these vesicles are similar (*17*). Thus, it appears that the glycerol backbone carbonyl groups are not required for high-affinity binding of cPLA2 to the interface. Results of GLU hydrolysis and fluorescence energy transfer studies with diether phospholipids versus diester phospholipids are virtually identical, and data from only one set are shown as indicated below. Since cPLA2 is chiral, it might show different behavior on vesicles composed of *sn*-3 versus *sn*-1 phospholipids. Thus, only the naturally occurring *sn*-3 phospholipids were used. Phospholipids with two saturated fatty chains were avoided in order to minimize possible complications due to phase separation of phospholipids in mixed phospholipid vesicles (*17*).

Binding of cPLA2-A4 to LUVs. Two methods were used to monitor binding of cPLA2-A4 to vesicles. Fluorescence resonance energy transfer from tryptophans on the enzyme to a dansylated phospholipid probe present as a minor component (5 mol %) in vesicles occurs when 14 kDa secreted phospholipases A_2 and cPLA2 bind to vesicles (*9*, *37*). This method was used to measure interfacial binding to low concentrations of vesicles (<50 μM). Energy transfer was also used to monitor binding of C2 to vesicles (*38*). With higher concentrations of vesicles, high concentrations of fluorophore cause detector saturation. Thus, cPLA2-A4 binding to vesicles was also monitored by measuring the rate of GLU hydrolysis as a function of the bulk concentration of vesicles. In these experiments, the mole percent GLU in the vesicles is held constant so that the kinetics are not altered by surface dilution of substrate. We have previously shown by two independent methods that 10 μM or greater concentrations of vesicles are sufficient to ensure that all of the GLU is partitioned into the interface (*17*). Thus, an increase in the observed initial rate of GLU hydrolysis as the concentration of vesicles is increased above 10 μM is due to an increased fraction of cPLA2 at the interface. The advantage of using GLU rather than radiolabeled phospholipids as substrate for cPLA2 is that the GLU assay is continuous, and the reaction time course is linear for several minutes. Also, GLU exchanges between vesicles much faster than the time taken for enzymatic depletion of a significant fraction of the GLU in a vesicle, and thus the reaction is not limited by intervesicle GLU exchange (*17*, *18*).

Binding of cPLA2-A4 to Phosphatidylcholine LUVs. Figure 1 shows that the rate of cPLA2-A4-catalyzed GLU hydrolysis increases as the concentration of POPC/GLU vesicles is increased from 10 μM to 1 mM, and the rates are higher in the presence of 10 μM free calcium compared to the rates without calcium (100 μM EGTA). The rates in the presence and absence of calcium approach each other as the lipid concentration reaches 1 mM. Since the number of enzyme binding sites on the vesicle is much larger than the amount of enzyme, the fraction of total enzyme bound to the vesicles is given by $[L]_T/([L]_T + K_d)$, where $[L]_T$ is the total concentration of phospholipid in the enzyme-facing layer of the vesicle (i.e., 50% of the total lipid) and K_d is the enzyme−vesicle dissociation equilibrium constant (in units of concentration of vesicle lipid molecules). From the data in Figure 1, it is concluded that cPLA2-A4 binds to POPC/GLU vesicles in the presence of 10 μM calcium with a $K_d = 90 \pm 18$ μM. Binding in the absence of calcium is weaker ($K_d = 700 \pm 200$ μM). All binding and kinetic data for the entire study are summarized in Table 1.

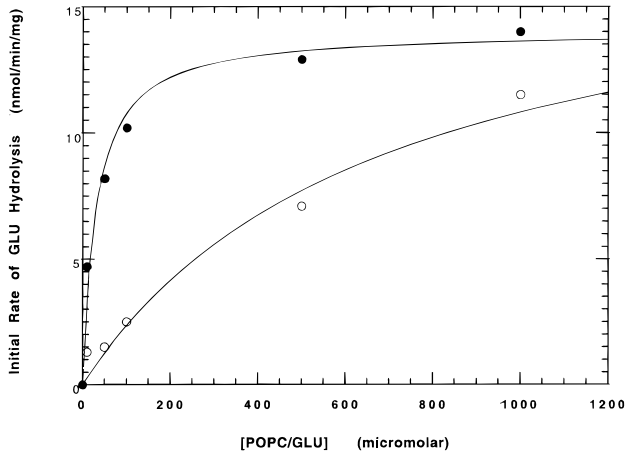

FIGURE 1: Binding of cPLA2-A4 to POPC vesicles as monitored by GLU hydrolysis. Reaction mixtures contained the indicated concentration of POPC LUVs plus the appropriate amount of GLU such that its mole percent in the vesicles is constant at 2.5% in 1 mL of buffer I at 30 °C without calcium (100 μM EGTA, ○) or with 10 μM calcium (●).

The results in Figure 2A show that the rate of GLU hydrolysis in POPC vesicles (30 μM) increases as the concentration of free calcium is increased from 0 to 23 μM. The apparent dissociation constant for the enzyme−calcium complex $^{a}K_{Ca}$ is 10 ± 2 μM. Calcium binding appears somewhat cooperative in that the data in Figure 2A were better fit to the Hill equation with Hill coefficient $n = 1.6 \pm 0.3$ than to the hyperbolic equation. Measurable GLU hydrolysis occurs in the absence of calcium (100 μM EGTA), and the presence of calcium increases the rate only slightly (~4-fold), which is consistent with the results of Figure 1.

Calcium promoted binding of cPLA2-A4 to zwitterionic vesicles was also examined by fluorescence energy transfer. As shown in Figure 2B, addition of increasing amounts of calcium to a fixed amount of cPLA2-A4 (80 nM) in the presence of a fixed amount of $DO_{et}PC$/dansyl-DHPE vesicles (30 μM) led to an increase in energy transfer. Fitting the data to the Hill equation gave $^{a}K_{Ca} = 15 \pm 3$ μM and $n = 1.8 \pm 0.3$, which agree with the binding values obtained by monitoring GLU hydrolysis. Virtually identical results were obtained when dansyl-DHPE was replaced with 5 mol % of the charge-neutral probes *N*-dansyl-1-[*O*-(11-undecylamino)]-2-decanoyl-*sn*-glycero-3-phosphocholine or 5 mol % N_{α}-(7-nitro-2,1,3-benzoxadiazol-4-yl)-(*N*,*N*-dihexadecyl-L-glutamic acid diamide (not shown). This indicates that the results with dansyl-DHPE are not influenced by the fact that this probe has a charge of −1.

Binding of cPLA2-A4 to zwitterionic vesicles was confirmed by fluorescence energy transfer studies (Figure 3A). When increasing amounts of cPLA2-A4 were added to a fixed amount of $DO_{et}PC$ vesicles (containing 5 mol % dansyl-DHPE) in the presence of saturating amounts of calcium (30 μM), dansyl emission intensity increased in a linear fashion. Since sufficient cPLA2-A4 was added to cover all of the interface (see below), the fact that no downward curving of the energy transfer signal was seen as more enzyme was added implies that $K_d > 30$ μM. This is consistent with $K_d = 90$ μM obtained from Figure 1.

Binding of cPLA2-A4 to Phosphatidylmethanol LUVs. Compared to phosphatidylcholine vesicles, dramatically different results were found when the binding of cPLA2-A4 to vesicles of the anionic phospholipids DOPM and DO_{et}-PM was studied. As shown in Figure 3B, addition of increasing amounts of cPLA2-A4 to 10 μM $DO_{et}PM$/dansyl-DHPE vesicles leads to an increase in energy transfer until the vesicles are maximally covered with enzyme. This saturation behavior is in marked contrast to the linear curve seen in the binding of enzyme to $DO_{et}PC$ vesicles (Figure 3A). The data show that enzyme binds tighter to $DO_{et}PM$ vesicles than to zwitterionic vesicles. Interfacial binding is calcium-independent as very similar binding curves are seen in the presence 22 μM calcium or in its absence (100 μM EGTA) (Figure 3B). Roughly 50 nM of cPLA2-A4 is required to saturate the vesicles. Since the concentration of phospholipid in the outer monolayer of vesicles (i.e., that which is accessible to enzyme) is 5 μM, it may be concluded that maximum vesicle coverage occurs when each enzyme covers about 100 phospholipids. This is a reasonable number given the corresponding numbers for the smaller 14 kDa secreted phospholipases A_2 of 30−50 lipids (39, 40). These results imply that the value of K_d is less than the concentration of vesicles used; i.e., $K_d \ll 10$ μM for the interaction of cPLA2-A4 with $DO_{et}PM$ in the presence or absence of calcium. Due to limited signal-to-noise, it is problematic to carry out energy transfer studies with significantly lower lipid concentrations in order to derive values of K_d.

Remarkably, the rate of hydrolysis of GLU present in DO_{et}-PM and DOPM vesicles in the presence of 25 μM calcium is 85 ± 5% of the rate measured in the absence of calcium (100 μM EGTA) (over a dozen trials with six independent preparations of vesicles). The rate of GLU hydrolysis in the absence of calcium (100 μM EGTA) is independent of the concentration of DOPM/GLU or $DO_{et}PM$/GLU vesicles in the range 10−100 μM. This shows that $K_d < 10$ μM for the cPLA2-A4·DOPM complex in the presence and absence of calcium. Thus, by multiple techniques, it is concluded that cPLA2-A4 binds tightly and in a catalytically productive manner to phosphatidylmethanol vesicles in the absence of calcium. This calcium-independent binding of cPLA2-A4 to phosphatidylmethanol vesicles is not due to electrostatic binding of the C-terminal 6-histidine tag with the interface since the action of native cPLA2 on GLU/DOPM vesicles occurs with the same specific activity as that of cPLA2-A4 in the presence and absence of calcium (not shown).

The specific activity of cPLA2-A4 on $DO_{et}PM$ and DOPM vesicles containing GLU is comparable to that measured with saturating amounts of POPC vesicles and calcium (Table 1). It is not easy to compare these specific activities since phosphatidylcholine and phosphatidylmethanol may compete differentially with GLU for the binding to the active site of vesicle-bound cPLA2-A4. Nevertheless, the data suggest that cPLA2-A4 is highly active on phosphatidylmethanol vesicles in the absence of calcium.

Binding of cPLA2-A4 to Phosphatidylserine LUVs. As shown in Figure 4A, the rate of hydrolysis of GLU present at a fixed mole percent in POPS vesicles increases as the concentration of vesicles increases in the presence of 15 μM calcium. A value of K_d of 60 ± 10 μM in the presence of 15 μM calcium was obtained from the fit of the data to the binding equation. Thus, cPLA2-A4 seems to bind much weaker to phosphatidylserine vesicles than to phosphatidylmethanol vesicles despite the fact that both LUVs are anionic.

Table 1: Binding of cPLA2-A4 and C2 to LUVs[a]

protein	LUV	$K_d(+Ca^{2+})$ (μM)	$K_d(-Ca^{2+})$ (μM)	$^aK_{Ca}$, (μM), n	specific activity[d] (pmol min^{-1} mg^{-1})
cPLA2-A4	PC	90 ± 18[b]	700 ± 200	10 ± 2,[b] 1.6 ± 0.3[b] 15 ± 3,[c] 1.8 ± 0.3[c]	13
C2	PC	11 ± 3	no binding	9.5 ± 1,[c] 1.1 ± 0.01[c]	–
C2	no lipid	–	–	16 ± 1	–
cPLA2-A4	PM	≪10	≪10	calcium-independent	34
C2	PM	10 ± 2		5 ± 0.4,[c] 1.4 ± 0.1[c]	–
cPLA2-A4	PS	60 ± 10[b]	nd	14 ± 2,[b] 3 ± 0.9[b]	16
C2	PS	no binding	no binding	–	–

[a] Parameter symbols are defined in the text. [b] Measured by GLU kinetics. [c] Measured by fluorescence energy transfer. [d] Measured with saturating amounts of calcium and LUVs at 30 °C.

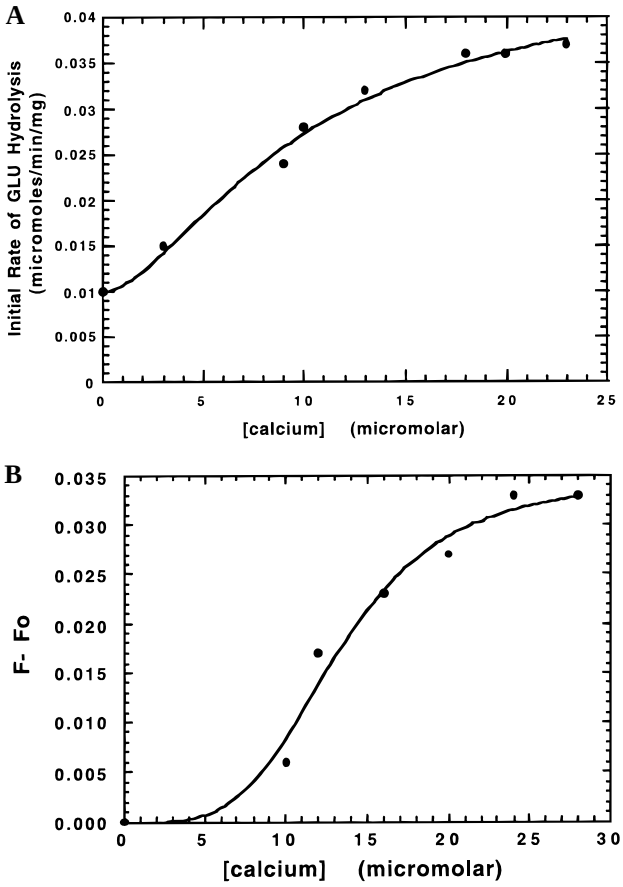

FIGURE 2: (A) Initial rate of GLU hydrolysis in POPC LUVs as a function of calcium concentration. Reaction mixtures contained 30 μM POPC LUVs containing 2.5% GLU in 1 mL of buffer I at 30 °C. (B) Binding of cPLA2-A4 to $DO_{et}PC$ vesicles measured by energy transfer. Binding mixtures (1 mL) contained 80 nM cPLA2-A4, 30 μM $DO_{et}PC$ vesicles containing 5 mol % dansyl-DHPE, and increasing amounts of calcium. *F* is the energy transfer emission intensity (monitored at 530 nm) measured in the presence of enzyme and vesicles, and F_o is the intensity in the presence of vesicles only.

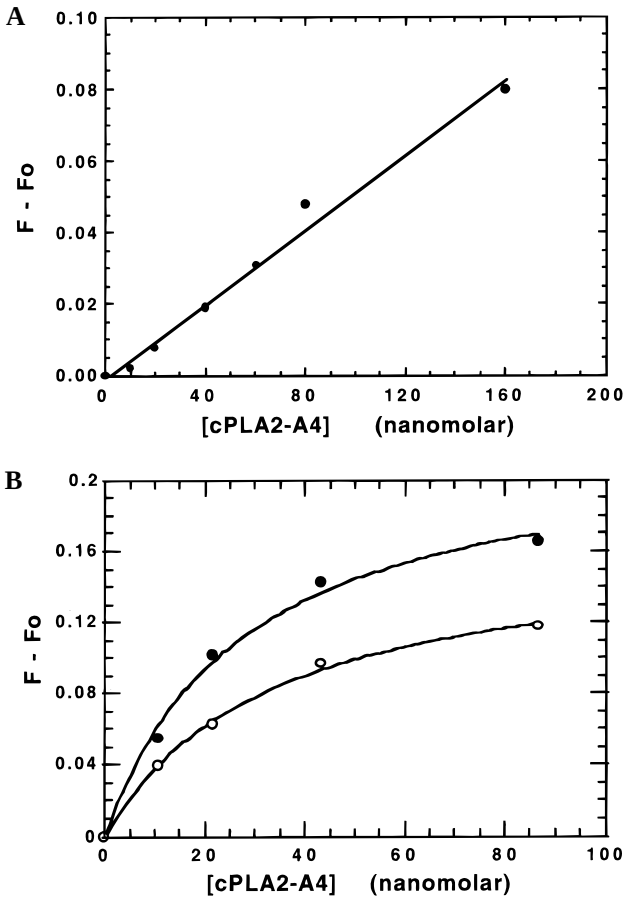

FIGURE 3: (A) Binding of cPLA2-A4 to $DO_{et}PC$ LUVs monitored by energy transfer. Binding mixtures (1 mL) contained 30 μM DO_{et}-PC vesicles containing 5 mol % dansyl-DHPE and 30 μM calcium in buffer I and the indicated concentration of cPLA2-A4. (B) Binding of cPLA2-A4 to $DO_{et}PM$ vesicles monitored by energy transfer. Binding mixtures (1 mL) contained 10 μM $DO_{et}PM$ vesicles, which contained 5 mol % dansyl-DHPE, in buffer I without calcium (100 μM EGTA, ○) or with 22 μM calcium (●) and the indicated concentration of cPLA2-A4.

cPLA2-A4 binds slightly tighter to POPS vesicles than to zwitterionic vesicles (Table 1).

As shown in Figure 4B, cPLA2-A4 binding to 10 μM $DO_{et}PS$/dansyl-DHPE vesicles was observed by monitoring energy transfer in the presence of 15 μM calcium. Note that significantly higher amounts of enzyme are required to saturate the vesicles compared to the analogous study with DOPM (Figure 3B). However, the tendency toward saturation in the binding of cPLA2-A4 to phosphatidylserine vesicles is distinctly different than the linear energy transfer response seen with phosphatidylcholine vesicles (Figure 3A).

These results are consistent with the GLU kinetic studies. The calcium dependence for the hydrolysis of GLU in POPS vesicles is shown in Figure 5. In the presence of 25 μM vesicles, catalytic activity is seen in the absence of calcium (100 μM EGTA), and the activity increase ~4-fold upon addition of saturating calcium ($^aK_{Ca} = 14 \pm 2$ μM). With saturating calcium, the specific activity of GLU hydrolysis is comparable to that measured with phosphatidylmethanol and phosphatidylcholine vesicles (Table 1).

Binding of C2 to Phosphatidylcholine LUVs. When increasing amounts of C2 were added to 10 μM POPC/

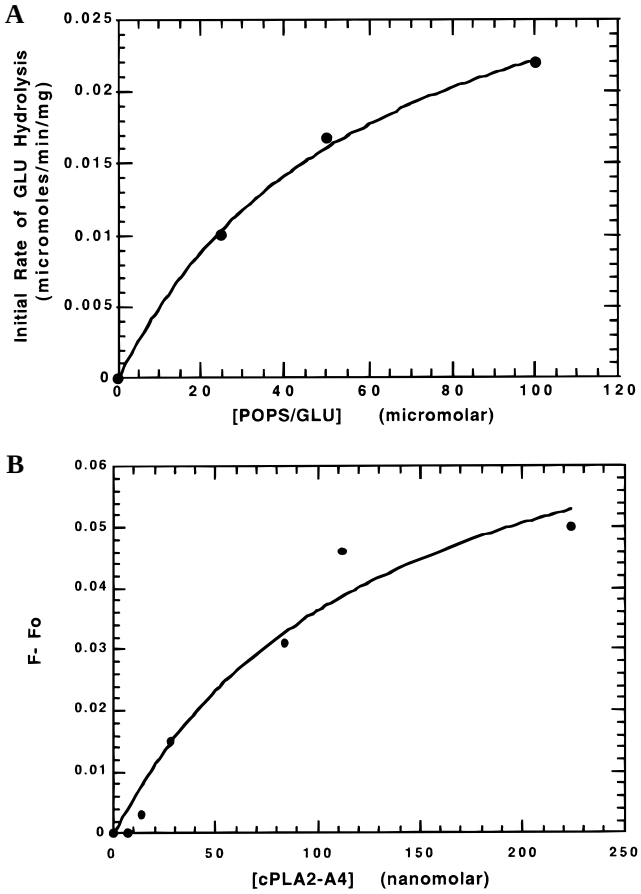

FIGURE 4: (A) Initial rate of GLU hydrolysis in POPS LUVs as a function of LUV concentration. Reaction mixtures contained 15 μM calcium in 1 mL of buffer I at 30 °C. Vesicles contained 2.5 mol % GLU. (B) Binding of cPLA2-A4 to $DO_{et}PS$ vesicles monitored by energy transfer. Binding mixtures contained 10 μM POPS/dansyl-DHPE LUVs in 1 mL of buffer I with 15 μM calcium at 30 °C.

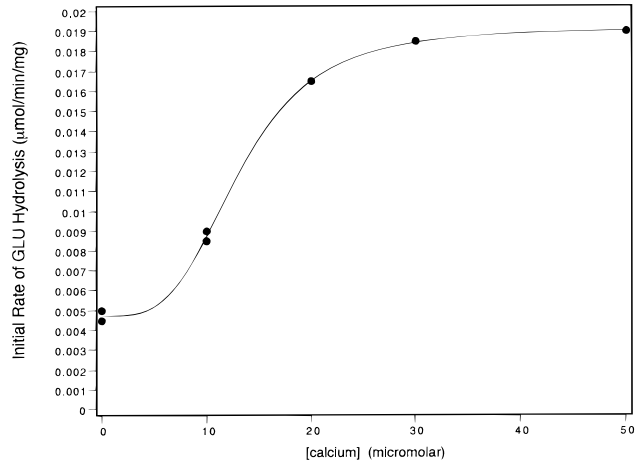

FIGURE 5: Calcium-induced increase in the rate of cPLA2-A4-catalyzed hydrolysis of GLU (2.5 mol %) in 25 μM POPS LUVs in 1 mL of buffer I at 30 °C.

dansyl-DHPE vesicles in the presence of 25 μM calcium, the energy transfer emission increased in a linear fashion up to 1 μM C2 (Figure 6A). This indicates that K_d is not much less than 10 μM. As shown in Figure 6B, when increasing concentrations of POPC/dansyl-DHPE were added to 400 nM C2 in the presence of 25 μM calcium, the energy transfer increased in a hyperbolic manner, and $K_d = 11 \pm 3$

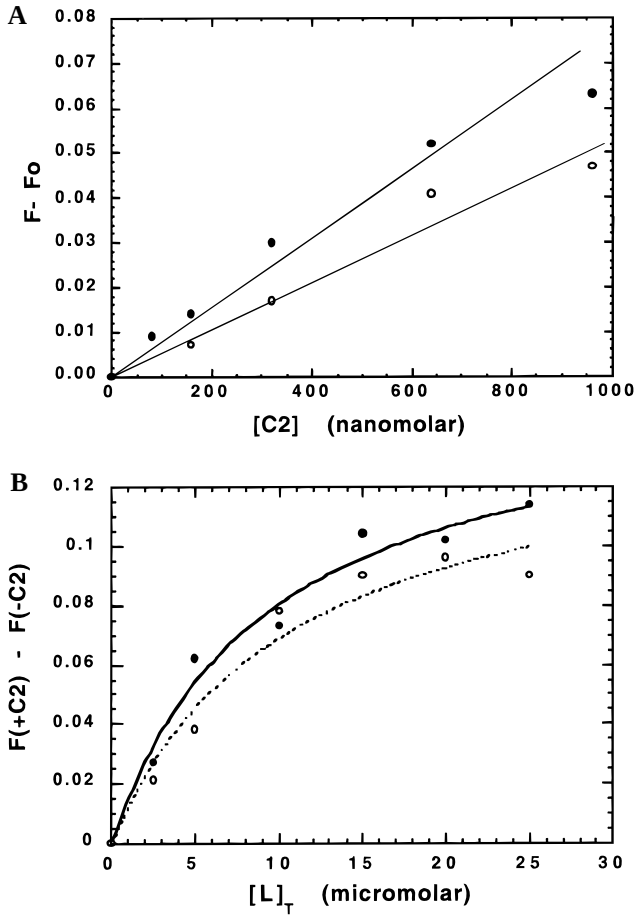

FIGURE 6: (A) Binding of C2 to POPC/dansyl-DHPE (●) or DOPM/dansyl-DHPE (○) LUVs as detected by fluorescence energy transfer. Binding mixtures (1 mL) contained 10 μM phospholipid in buffer I containing 25 μM calcium (method II) at 30 °C. (B) Binding of C2 to LUVs monitored by energy transfer. Binding mixtures contained 400 nM C2 in 1 mL of buffer I with 25 μM calcium at 30 °C and the indicated concentrations of DOPM/dansyl-DHPE vesicles (●, solid line) or POPC/dansyl-DHPE vesicles (○, dashed line). For each concentration of total lipid $[L]_T$, the energy transfer emission in the presence of LUVs alone, $F(-C2)$, was subtracted from the emission measured in the presence of LUVs and C2, $F(+C2)$.

μM was obtained from the fit to the binding equation. No binding to 10 μM POPC/dansyl-DHPE LUVs was detected in the absence of calcium (100 μM EGTA) when up to 600 nM C2 was added (not shown).

The calcium dependence of binding of C2 to $DO_{et}PC$/dansyl-DHPE LUVs is shown in Figure 7A. In the presence of 10 μM LUVs, binding is seen as reported previously for POPC vesicles (*36*), and values of $^{a}K_{Ca}$ and n are given in Table 1.

Binding of C2 to Anionic LUVs. When increasing amounts of C2 were added to 10 μM DOPM/dansyl-DHPE vesicles in the presence of 25 μM calcium, the energy transfer emission increased in a linear fashion up to 1 μM C2 (Figure 6A). Thus, compared to cPLA2-A4 (Figure 3B), C2 binds to phosphatidylmethanol LUVs much more weakly. As shown in Figure 6B, when increasing concentrations of DOPM/dansyl-DHPE were added to 400 nM C2 in the presence of 25 μM calcium, the energy transfer increased in a hyperbolic manner, and $K_d = 10 \pm 2$ μM was obtained from the fit to the binding equation. No binding was detected in the absence of calcium (100 μM EGTA) when up to 600

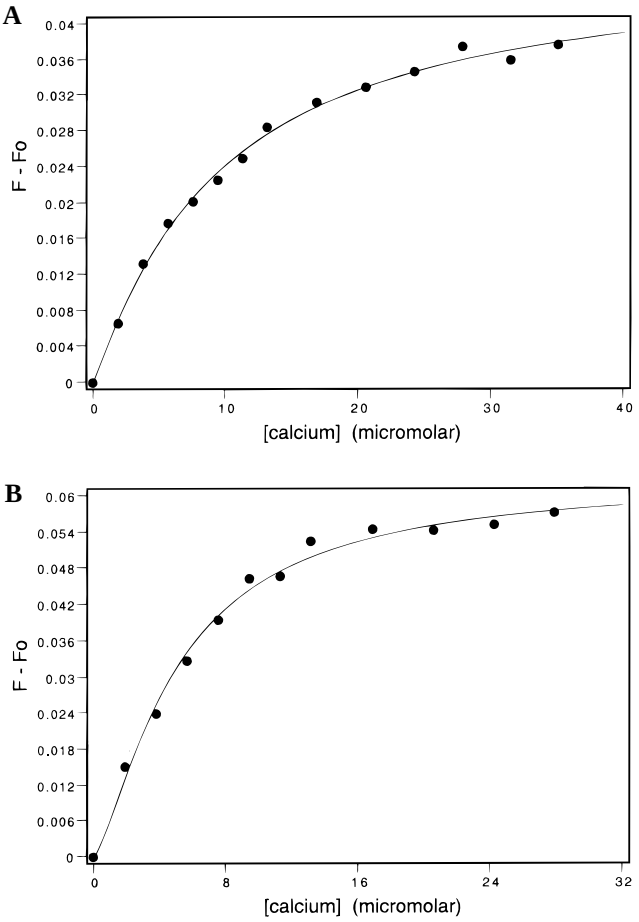

FIGURE 7: (A) Calcium dependence of binding of C2 to $DO_{et}PC$/dansyl-DHPE vesicles in 1 mL of 50 mM Tris, 150 mM NaCl, pH 7.5, containing 10 μM vesicles and 160 nM C2. (B) Same as (A) but with 10 μM $DO_{et}PM$/dansyl-DHPE vesicles.

nM C2 was added to 10 μM DOPM/dansyl-DHPE LUVs (not shown). This is in marked contrast to the results with cPLA2-A4 showing calcium-independent binding to phosphatidylmethanol vesicles.

The calcium dependence of binding of C2 to $DO_{et}PM$/dansyl-DHPE LUVs is shown in Figure 7B. In the presence of 10 μM LUVs, slightly cooperative binding is seen, and values of $^aK_{Ca}$ and n are given in Table 1.

In marked contrast to the full-length enzyme, C2, up to 1.3 μM, did not bind to POPS or $DO_{et}PS$ vesicles containing 5 mol % dansyl-DHPE when present at concentrations up to 50 μM and in the absence of calcium (100 μM EGTA) or in the presence of 25 μM calcium (not shown). C2 binding was also undetectable when two other fluorescent lipid probes were used, 5 mol % *N*-dansyl-1-[*O*-(11-undecylamino)]-2-decanoyl-*sn*-glycero-3-phosphocholine or 5 mol % N_α-(7-nitro-2,1,3-benzoxadiazol-4-yl)-(*N*,*N*-dihexadecyl-L-glutamic acid diamide in POPS vesicles (not shown).

DISCUSSION

This is the first study of the properties of a single molecular species of cPLA2, the nonphosphorylated form cPLA2-A4. The main conclusions of this study are that the interfacial binding properties of cPLA2-A4 cannot be predicted in general from analogous studies of its C2 domain, and calcium-dependent and -independent interfacial binding and catalysis modes are possible depending on the phospholipid composition of the vesicles. The simplest explanation for our observations is that both the C2 domain and the catalytic domain of cPLA2 contact the membrane. Another possibility, although unlikely, is that the catalytic domain does not contact the membrane but somehow influences the interfacial binding properties of the attached C2 domain. The C2 domain as a separate entity binds to vesicles in a calcium-dependent way, but the catalytic domain of cPLA2 (cPLA2 lacking residues 1−134) does not seem to bind to vesicles since it hydrolyzes soluble lipids in the aqueous phase but not when they are present in vesicles (*9*). This should not be interpreted to mean that the binding of cPLA2 to membranes is driven entirely by its C2 domain. Based on consideration of entropy (*41*), the receptor binding affinity of a ligand with two domains (cPLA2 in this case) that interact with the receptor (membrane in this case) cannot be deduced solely from the receptor binding affinities of the separate domains. For example, the two-domain ligand compactin binds extremely tightly to its enzyme target hydroxymethylglutaryl-coenzyme A reductase ($K_d \sim 10^{-10}$ M), yet one domain binds extremely weakly and the other does not detectably bind (*42*). To conclude that the nonbinding domain does not contribute to the binding of compactin would be obviously incorrect. Additionally, it seems very likely that the catalytic domain of cPLA2 binds to membranes. Presumably the active site of the catalytic domain must sit close to the membrane so that the phospholipid can access the active site, as is the case for all lipases of known three-dimensional structure (*43*−*46*). This is because of the extremely slow rate of desorption of naturally occurring long-chain phospholipids from the membrane into the aqueous phase. It may be mentioned that the X-ray structure of phosphatidylinositol-specific phospholipase Cδ1 shows that its C2 and catalytic domains are positioned such that they can bind simultaneously to the same interface (*47*).

cPLA2-A4 binds relatively weakly to phosphatidylcholine vesicles in the presence of saturating calcium (K_d of 90 μM). This conclusion seems inescapable since it is supported by GLU hydrolysis and energy transfer studies (Figures 1 and 2). Binding of cPLA2-A4 to POPC vesicles is about 10-fold weaker in the absence of calcium (Table 1).

Given that many investigators detect cPLA2 activity with 0.5−2 μM amounts of *sn*-2-arachidonyl-containing phosphatidylcholine, it was surprising to find weak binding to phosphatidylcholine vesicles. However, as shown previously, cPLA2 reaction products promote high-affinity binding of enzyme to phosphatidylcholine vesicles (*33*). Thus, when vesicles of pure *sn*-2-arachidonyl-containing phosphatidylcholine are used, the vesicle lipid will undergo significant hydrolysis since it is a good substrate for cPLA2, and high rates are obtained with low micromolar amounts of lipid as enzyme binds tightly to product-containing vesicles.

Weak calcium-promoted binding of cPLA2-A4 to phosphatidylcholine vesicles can also be inferred from the data in Figure 2. The affinity of cPLA2-A4 in the aqueous phase for calcium ($K_{Ca} = 16$ μM, if it is assumed that cPLA2 binds calcium with the same affinity as its C2 domain) is only slightly larger than the affinity in the presence of 30 μM POPC or $DO_{et}PC$ vesicles ($^aK_{Ca} = 10$ μM). According to the laws of thermodynamics, if calcium binding to the C2 domain enhances its affinity for vesicles (it does since K_d is 700 μM in the absence of calcium and is 90 μM in the

presence of 10 μM calcium), then the presence of vesicles must enhance the affinity of the enzyme for calcium. If the process is strictly ordered, i.e., only cPLA2·calcium binds to membranes (it is almost ordered), and if calcium binding to enzyme and enzyme binding to vesicles are at equilibrium, then the fraction of protein bound to the membrane is given by eq 1.

$$\frac{[E^*Ca]}{[E] + [ECa] + [E^*Ca]} = \frac{[L]_T[Ca]/(K_d + [L]_T)}{[Ca] + K_d K_{Ca}/(K_d + [L]_T)} \quad (1)$$

Here, the asterisk designates enzyme at the interface. ${}^aK_{Ca}$ is the concentration of calcium that causes 50% of the enzyme to bind to vesicles. When most of the enzyme is in the aqueous phase ($[L]_T \ll K_d$), the affinity of the enzyme for calcium will be about the same as in the absence of vesicles (${}^aK_{Ca} \approx K_{Ca}$). ${}^aK_{Ca}$ decreases as the concentration of vesicles increases. For example, when $[L]_T \approx K_d$, ${}^aK_{Ca} = K_{Ca}/2$. For our studies, 50% of the enzyme is bound to 30 μM POPC or $DO_{et}PC$ LUVs in the presence of 10 μM calcium (${}^aK_{Ca} = 10$ μM). This agrees well with the value ${}^aK_{Ca} = 12$ μM obtained using eq 1 with $K_d = 90$ μM and $K_{Ca} = 16$ μM. Consideration of eq 1 and the observations of enhanced binding of cPLA2 to vesicles containing reaction products (*33*) may explain why values of ${}^aK_{Ca}$ determined using vesicles of *sn*-2-arachidonylphosphatidylcholine are typically 0.5−1 μM (*16*, *33*).

The binding behaviors of cPLA2-A4 and its C2 domain to phosphatidylcholine vesicles are similar (Table 1). ${}^aK_{Ca}$ for C2 in the presence of POPC vesicles is an order of magnitude smaller than in the absence of lipid. Nalefski and co-workers reported $K_{Ca} = 24$ μM and ${}^aK_{Ca} = 3$ μM for their C2 construct in the presence of 100 μM POPC vesicles (*38*, which is in good agreement with our data). Interestingly, in the presence of saturating calcium, C2 binds about 9-fold tighter to phosphatidylcholine vesicles than does cPLA2-A4. The molecular basis for this remains to be understood.

C2 binds to phosphatidylmethanol and phosphatidylcholine LUVs with similar affinities, and both are calcium-dependent (we could not test if C2 binds to high concentrations of vesicles, i.e., >100 μM lipid, in the absence of calcium because of fluorescence detector saturation, and we cannot measure C2 interfacial binding by GLU hydrolysis). It is not clear how calcium binding to C2 triggers membrane attachment. The X-ray structure of C2 in the presence of calcium reveals two protein-bound metals, but there is no major conformational change in the protein structure upon calcium binding, at least for crystallized C2 (*10*). Calcium-enhanced binding of C2 to anionic vesicles may be due to removal of electrostatic repulsion between anionic phospholipids and anionic calcium-coordinating ligands as the two dicationic metals bind. However, this explanation seems inconsistent with the observation that calcium promotes the binding of C2 to charge-neutral phosphatidylcholine vesicles. In addition, the fact that values of ${}^aK_{Ca}$ measured with C2 in the presence of DOetPC and DOetPM vesicles are similar (Table 1) argues against this electrostatic repulsion model.

Remarkably, cPLA2-A4 binds ≫9-fold tighter to anionic phosphatidylmethanol vesicles compared to zwitterionic phosphatidylcholine vesicles, and such interfacial binding is completely calcium-independent. Thus, it seems certain that the catalytic domain has a calcium-independent interfacial binding motif that is able to interact with the phosphatidylmethanol headgroup. We have previously shown that whereas the half-time for cPLA2 dissociating from phosphatidylcholine vesicles is <10 s, it remains bound to phosphatidylmethanol vesicles for several minutes (*17*). The binding data presented here are fully consistent with these previous results.

It is not clear if the C2 domain of cPLA2-A4 is bound at the interface of phosphatidylmethanol vesicles in the absence of calcium. Although the C2 domain as a separate entity requires calcium for binding to phosphatidylmethanol vesicles, the binding of the catalytic domain to the interface could force the C2 domain to attach to the interface in the absence of calcium. As noted above, we have not determined if C2 binds to high concentrations of vesicles in the absence of calcium. As shown in Figure 3B, the efficiency of fluorescence energy transfer for cPLA2-A4 bound to phosphatidylmethanol vesicles is ∼25% lower than in the presence of calcium. This might be expected if the C2 domain is dangling away from the interface in the absence of calcium, but other explanations cannot be ruled out.

The potential ability of specific anionic phospholipids to drive cPLA2 onto cellular membranes in the absence of calcium may be the molecular basis for activation of cPLA2 by anionic phospholipids and the lowering of its calcium requirement first reported by Leslie and co-workers (*48*). After completion of our studies, Buckland and Wilton showed that small amounts of phosphatidylinositol 4-phosphate and phosphatidylinositol-4,5 bisphosphate (∼1 mol %) in phosphatidylcholine vesicles lead to 3−4-fold activation of cPLA2 (*49*), and Mosior et al. showed that this latter anionic lipid, when added to phosphatidylcholine vesicles, enhances interfacial binding and activation of cPLA2 in the presence and absence of calcium (*50*). It is becoming apparent that a number of proteins such as phospholipase Cδ1, pleckstrin, and spectrin, which contain pleckstrin homology domains, bind tightly to membranes containing phosphatidylinositol phosphates via specific ligation of the inositol phosphate with basic amino acids of the pleckstrin homology domain (*51*−*54*). Mosior et al. point out that residues 263−354 of cPLA2 display intriguing homology to the pleckstrin homology domains of these other proteins. This would place the putative pleckstrin homology domain in the portion of cPLA2 that does not include the C2 domain, possibly looping out of the catalytic domain (*50*). Phospholipase Cδ1 has an N-terminal pleckstrin homology domain, followed by a C2 domain, and then the catalytic domain (*45*). Since phosphatidylmethanol induces high-affinity calcium-independent binding of cPLA2-A4 to vesicles, it is tempting to speculate that this anionic phospholipid acts like phosphatidylinositol 4,5-bisphosphate, but clearly the latter is a more potent inducer of cPLA2 interfacial binding and may be physiologically relevant to the regulation of cPLA2.

cPLA2-A4 binds to phosphatidylserine vesicles despite the fact that its C2 domain, as a separate identity, fails to bind to these vesicles in the presence or absence of calcium. The latter result is consistent with the recent study of Nalefski et al. showing the failure of their C2 construct to bind to phosphatidylserine vesicles (*36*). The fact that cPLA2-A4 shows modestly enhanced binding to POPS vesicles in the presence of calcium argues that in the context of the full-

length protein, the C2 domain is able to interact with the bilayer. These results again underscore the fact that the interfacial binding properties of cPLA2 are not reliably predicted by the membrane affinity of the C2 domain alone. It has already been mentioned above that because of entropic reasons, the lack of binding of C2 to phosphatidylserine does not imply that this domain does not promote binding of full-length protein to vesicles. Binding of cPLA2-A4 to POPS vesicles is significantly weaker than the binding of this enzyme to DOPM vesicles (Table 1), which suggests that phosphatidylserine is inferior to phosphatidylmethanol for supporting calcium-independent binding to the catalytic domain. Phosphatidylserine cannot function like phosphatidylinositol phosphates to bind tightly to pleckstrin homologue domains found on other proteins (*53*).

The data in Table 1 show that the specific activities of cPLA2-A4 on phosphatidylcholine, phosphatidylmethanol, and phosphatidylserine LUVs are very similar, less than a 3-fold variation when one accounts for the fact that these numbers were measured in the presence of 50 μM phospholipid, and not all of the enzyme is bound to phosphatidylcholine. Thus, one can conclude that activation of the enzyme by these anionic phospholipids is mainly due to an increase in the fraction of enzyme bound to membranes.

It must be stressed that the values of K_d obtained in this study for cPLA2-A4 interacting with synthetic phospholipids in vitro provide only a beginning for understanding the interaction of this structurally complex enzyme with compositionally complex cellular membranes. The physiological K_d values are likely to be different in magnitude, but it may be noted that the magnitude of the K_ds determined in these in vitro studies are lower than the concentration of phospholipid that intracellular cPLA2 "sees", and thus are in the range for supporting translocation of cPLA2 from the cytosol to internal cellular membranes. Finally, the effect of phosphorylation on the interfacial kinetic and binding properties of cPLA2 can now be better addressed using calcium concentration and vesicle composition as experimental variables.

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