Role of Phosphorylation Sites and the C2 Domain in Regulation of Cytosolic Phospholipase A_2

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Abstract. Cytosolic phospholipase A_2 (cPLA$_2$) mediates agonist-induced arachidonic acid release, the first step in eicosanoid production. cPLA$_2$ is regulated by phosphorylation and by calcium, which binds to a C2 domain and induces its translocation to membrane. The functional roles of phosphorylation sites and the C2 domain of cPLA$_2$ were investigated. In Sf9 insect cells expressing cPLA$_2$, okadaic acid, and the calcium-mobilizing agonists A23187 and CryIC toxin induce arachidonic acid release and translocation of green fluorescent protein (GFP)-cPLA$_2$ to the nuclear envelope. cPLA$_2$ is phosphorylated on multiple sites in Sf9 cells; however, only S505 phosphorylation partially contributes to cPLA$_2$ activation. Although okadaic acid does not increase calcium, mutating the calcium-binding residues D43 and D93 prevents arachidonic acid release and translocation of cPLA$_2$, demonstrating the requirement for a functional C2 domain. However, the D93N mutant is fully functional with A23187, whereas the D43N mutant is nearly inactive. The C2 domain of cPLA$_2$ linked to GFP translocates to the nuclear envelope with calcium-mobilizing agonists but not with okadaic acid. Consequently, the C2 domain is necessary and sufficient for translocation of cPLA$_2$ to the nuclear envelope when calcium is increased; however, it is required but not sufficient with okadaic acid.

Key words: phospholipase A_2 • calcium • phosphorylation • C2 domain • Sf9 cells

THE 85-kD cytosolic phospholipase A_2 (cPLA$_2$)[1] plays an important role in agonist-induced arachidonic acid release (Clark et al., 1995; Leslie, 1997). This is a tightly regulated process since the availability of free arachidonic acid controls the level of eicosanoid production. The oxygenated metabolites of arachidonic acid, including leukotrienes, prostaglandins, and thromboxanes, have important roles in normal homeostasis and acute inflammation. An essential role for cPLA$_2$ in reproductive function and inflammation has been confirmed recently from studies using cPLA$_2$-deficient mice (Bonventre et al., 1997; Uozumi et al., 1997). cPLA$_2$ is regulated posttranslationally by phosphorylation and by calcium (Channon and Leslie, 1990; Clark et al., 1991, 1995; Lin et al., 1993; Qiu et al., 1993; Leslie, 1997). Phosphorylation of cPLA$_2$ on S505 by mitogen-activated protein kinases has been shown

to be required for agonist-induced arachidonic acid release in some cell models but not in others (Lin et al., 1993; Kramer et al., 1996; Qiu et al., 1998). A critical step in the regulation of cPLA$_2$ is its translocation from cytosol to membrane for access to phospholipid (Channon and Leslie, 1990; Clark et al., 1990). In several cell models cPLA$_2$ has been shown to translocate primarily to the nuclear envelope and endoplasmic reticulum, where 5-lipoxygenase and cyclooxygenase also localize (Glover et al., 1995; Schievella et al., 1995; Sierra-Honigmann et al., 1996; Hirabayashi et al., 1999). The amino terminus of cPLA$_2$ contains a C2 domain, which mediates the calcium-dependent binding of cPLA$_2$ to membrane and phospholipid vesicles (Nalefski et al., 1994). C2 domains were originally described in protein kinase C (PKC) and now have been identified in >60 proteins (Nalefski and Falke, 1996; Rizo and Südhof, 1998). They are involved in the calcium-dependent association of proteins to membrane or in membrane trafficking, although the function of all C2 domains is not known. The C2 domain of cPLA$_2$ is a β-sandwich structure that binds two ions of calcium and preferentially interacts with phosphocholine headgroups (Hixon et al., 1998; Nalefski et al., 1998; Perisic et al., 1998; Xu et al., 1998; Bittova et al., 1999). An increase in intracellular calcium promotes translocation of cPLA$_2$ to membrane,

1. *Abbreviations used in this paper:* cPLA$_2$, cytosolic phospholipase A_2; GFP, green fluorescent protein; HSA, human serum albumin; PKC, protein kinase C; Sf9, *Spodoptera frugiperda*.

which is an important regulatory step necessary for association of the catalytic domain of $cPLA_2$ with phospholipid substrate.

In macrophages, we have demonstrated that a transient increase in calcium and phosphorylation of $cPLA_2$ on S505 by mitogen-activated protein kinase can act together to fully activate $cPLA_2$ leading to arachidonic acid release (Qiu et al., 1998). However, neither of these events alone is sufficient for activation. There are also alternative pathways for regulating $cPLA_2$ activation in the macrophage model since both PMA and okadaic acid induce arachidonic acid release without increasing intracellular calcium (Qiu et al., 1998). We have used the baculovirus expression system in insect cells to study regulation of $cPLA_2$ (de Carvalho et al., 1996). This model is now being used not only as a source of recombinant proteins but also to study the function of proteins and their role in signal transduction (Stancato et al., 1993, 1997; Chefalo et al., 1994). Similar to macrophages, *Spodoptera frugiperda* (Sf9) cells expressing $cPLA_2$ release arachidonic acid in response to A23187 and okadaic acid (de Carvalho et al., 1996). $cPLA_2$ is phosphorylated on multiple sites when expressed in Sf9 cells, and phosphorylation on S727 is preferentially induced by okadaic acid (de Carvalho et al., 1996). Okadaic acid also induces phosphorylation of the S727-containing tryptic peptide in mouse peritoneal macrophages and human monocytes (de Carvalho et al., 1996; Qiu et al., 1998). Phosphorylation of $cPLA_2$ on S505 and S727 also occurs in thrombin- and collagen-stimulated platelets and cytokine-stimulated HeLa cells (Börsch-Haubold et al., 1998). The mechanisms involved in regulating the translocation of $cPLA_2$ to membrane in response to agonists that do not increase intracellular calcium, and the preferential targeting of $cPLA_2$ to the nuclear envelope are not understood. The role of these novel phosphorylation sites and the C2 domain in regulating $cPLA_2$ translocation and arachidonic acid release in Sf9 cells in response to okadaic acid and the calcium-mobilizing agonists was investigated.

Materials and Methods

Reagents

Sf9 cells, the transfer vector pVL1393, and the TA cloning kit were obtained from Invitrogen. Grace's insect cell culture medium was obtained from JRH Biosciences. The transfer vectors BioGreen-His and pAc-GHLT-A, as well as baculovirus linearized DNA (BaculoGold), were from PharMingen. Yeastolate was obtained from Becton Dickinson. Lactalbumin hydrolysate, penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml)/L-glutamine (29.2 mg/ml) solution, and a human spleen SuperScript cDNA library were from Life Technologies. FBS was purchased from Gemini Bioproducts. Human serum albumin (HSA) was from Intergen Co. [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (sp act 100 Ci/mmol), 1-*O*-hexadecyl-2-[^{3}H]arachidonyl-phosphatidylcholine (200 Ci/mmol), and L-1-[1-^{14}C]palmitoyl-2-lysophosphatidylcholine (55 mCi/mmol) were from DuPont/NEN. Anti–rabbit horseradish peroxidase–linked F(ab′)$_2$ IgG fragment, reagents for enhanced chemiluminescence detection on immunoblots, and glutathione–Sepharose beads were purchased from Amersham Pharmacia Biotech. Calcium ionophore A23187 and pluronic acid F-68 (10% solution) were obtained from Sigma Chemical Co. Ionomycin was from Calbiochem-Novabiochem. Okadaic acid (ammonium salt) was from Alexis Biochemicals. Fura-2 acetoxymethyl ester (AM) and the nuclear dye Hoechst 33342 were obtained from Molecular Probes. A donkey anti–rabbit IgG antibody conjugated to Cy3 was obtained from Jackson ImmunoResearch. Reagents for protein determination by the bicinchoninic acid method were from Pierce Chemical Co. *Bacillus thuringiensis*

δ-endotoxin CryIC was generously provided by Dr. Jean-Louis Schwarz (National Research Council, Montreal, Canada) and Dr. Marianne Carey (Case Western Reserve University, Cleveland, OH).

Preparation of DNA Constructs

The DNA encoding for human $cPLA_2$ (provided by Dr. James Clark, Genetics Institute, Cambridge, MA) was cloned into the transfer vector pVL1393 as previously described (de Carvalho et al., 1993). For fusion-protein construction, the human $cPLA_2$ coding region was obtained by PCR from a human spleen cDNA library and cloned into the pCR2.1 vector following the TA cloning method. The PCR primers used were 5′-GGATCCTGACTGAAAGCTAGAGGC-3′ (bases 22–45; GenBank accession number M72393) and 5′-CAGCCAGTCTCTCATGATCAG-TACGAC-3′ (bases 2475–2501). Two in-frame codons present in the 5′ untranslated region were removed by PCR amplification using the primers 5′-GAGAGCGGGTACCCCGGTTTGAAGTGTGAAAACATTT-CCTG-3′ (which contains a KpnI restriction site) and 5′-CCTGAT-TAGGATCCAAAATAAATTCAAAGGTCTC-3′ (which straddles a BamHI site). The PCR product was cut with KpnI and BamHI and cloned into pCR2.1 containing the $cPLA_2$ gene. The modified $cPLA_2$ sequence was cloned into the transfer vectors pAcGHLT (for expression of GST-$cPLA_2$) and BioGreen-His (for expression of green fluorescent protein [GFP]-$cPLA_2$) using the SacI and PstI sites. Different mutants of $cPLA_2$ were obtained by site-directed mutagenesis using a kit from 5Prime→3Prime Inc., following the manufacturer's instructions. The mutagenic oligonucleotides used were 5′-CTTGATACTCCAAATCCC-TATGTG-3′ (D43N), 5′-CGTTAATGAATGCCAATTATG-3′ (D93N), 5′-GATAGCTCGGCACGCTGATGATGAATCAC-3′ (S437A), 5′-GAA-GATGCTGGAGCTGACTATCAAAGTG-3′ (S454A), 5′-CTTATCC-ACTGGCTCCTTTGAG-3′ (S505A), and 5′-CCATCTCGTTGCGCTG-TTTCCC-3′ (S727A). A truncated version of $cPLA_2$ (Δ721–749) was generated by introducing a stop codon after amino acid 720 using the mutagenic oligonucleotide 5′-GAATATAGAAGATAGAATCCATC-TCG-3′. A GFP-C2 construct was generated by using the oligonucleotide 5′-GCCCAGACCTATGATTTAGTATGGC-3′ to insert a stop codon after amino acid 144. All mutations were confirmed by sequencing of DNA from several bacterial colonies selected on agar plates.

Cell Culture and Generation of Recombinant Baculovirus Stocks

Sf9 cells were routinely cultured in 100-ml vented spinner flasks, at 27°C, in TNM-FH medium (Grace's medium supplemented with 3.3 g/liter yeast-olate, 3.3 g/liter lactalbumin hydrolysate, and 1% penicillin/streptomycin/glutamine solution) containing 10% FBS and 0.1% pluronic acid. Cell density was maintained at $0.2–1.5 \times 10^6$ cells/ml. Recombinant baculovirus was generated by cotransfection of Sf9 cells with the transfer vector pVL1393 containing the gene for $cPLA_2$ and linearized baculovirus DNA (BaculoGold; PharMingen) following the manufacturer's instructions. Viral stocks derived from single virus particles were obtained by plaque purification and amplified (two cycles) following standard procedures. The titer of the working viral stocks was determined by infecting cells with serial dilutions of the stocks, overlaying them with agarose-containing TNM-FH medium, and counting the plaques after 4–6 d of incubation.

Measurement of Intracellular Calcium Concentration

Sf9 cells were plated on glass coverslips (13 mm diam) in 24-well plates (4×10^5 cells/well) and infected with baculovirus containing wild-type $cPLA_2$. After infection (50–54 h), Sf9 cells were washed three times in buffer A (10 mM 2-[*N*-morpholino]ethanesulfonic acid, 10 mM NaCl, 60 mM KCl, 25 mM MgCl$_2$, 4 mM D-glucose, 110 mM sucrose, adjusted to pH 6.2 with Trizma base) and incubated with 2 μM Fura-2 AM (in buffer A) for 60 min at room temperature protected from light. The cells were rinsed twice with buffer A, positioned in a fluorometer cuvette, and maintained at 27°C with continuous stirring. Calcium chloride or EGTA was added to buffer A to reach the final concentrations indicated in the figures. The effect of agonists on the intracellular concentration of calcium was determined by spectrofluorometry as previously described (Qiu et al., 1998).

Arachidonic Acid Release

Sf9 cells were plated in 24-well tissue culture plates (2.5×10^5 cells/well) and infected with baculovirus. After infection (30–35 h), they were labeled

with 0.2 μCi/well [³H]arachidonic acid and incubated overnight (16–18 h). The cells were washed three times with TNM-FH medium containing 0.1% HSA to remove unincorporated arachidonic acid and then treated with agonists. After stimulation, the medium was centrifuged at 500 *g* for 10 min, and the amount of radioactivity in the supernatant was determined by scintillation counting. Cells were scraped in 0.5 ml of 0.1% Triton X-100 for determining the total cellular radioactivity. For immunoblot analysis of cPLA₂, Sf9 cells were scraped in 100 μl of Laemmli buffer and the protein concentration was determined. DTT was added to a final concentration of 25 mM, and samples were boiled for 5 min before loading (0.5 μg/lane) on a SDS-polyacrylamide gel (Qiu et al., 1998). After electrophoresis, proteins were transferred to nitrocellulose and cPLA₂ was detected by chemiluminescence using a 1:5,000 dilution of polyclonal antiserum to cPLA₂ (de Carvalho et al., 1993; Qiu et al., 1998).

Phospholipase A₂ Assay

Sf9 cells (1.25×10^6 cells/35 mm dish) were infected with recombinant baculovirus for 48–50 h. The monolayers were rinsed with 2 ml of PBS and scraped into 100 μl of ice-cold homogenization buffer (10 mM Hepes, pH 7.4, 1 mM EGTA, 0.34 M sucrose, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Cells were lysed with a probe sonicator (3 times for 5 s, then 2 times for 10 s) on ice, and the homogenates centrifuged at 100,000 *g* for 1 h. The protein concentration in the cytosols was determined and then DTT was added to a final concentration of 1 mM. The cytosols were kept on ice and assayed within 24 h, or stored at −20°C and thawed just before the assay. The PLA₂ and lysophospholipase activities of cPLA₂ were determined by using 1-*O*-hexadecyl-2-[³H]arachidonylphosphatidylcholine (final concentration 30 μM), or [1-¹⁴C]palmitoyl-2-lysophosphatidylcholine (50 μM), respectively, as previously described

(de Carvalho et al., 1995). Reactions were started with the addition of cytosolic protein (10 μg for PLA₂ assays, 5 μg for lysophospholipase assays), and proceeded for 5 min at 37°C. Equal expression of the mutant enzymes was verified by immunoblotting and quantitation of the cPLA₂ bands using a Storm 840 system from Molecular Dynamics.

Affinity Purification of cPLA₂ Antibody

Sf9 cells grown in suspension (6×10^8 cells in 600 ml medium) were infected with baculovirus encoding the fusion protein GST-cPLA₂. Cells were lysed in 20 mM Hepes, pH 7.4, containing 2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates were incubated with 200 μl glutathione–Sepharose beads for 2 h at 4°C. The beads were washed sequentially with 2 ml each of 10 mM Tris (pH 7.5), 10 mM Tris (pH 8.8), 100 mM triethylamine (pH 11.5), and 10 mM Tris (pH 7.5). The beads were mixed with rabbit anti-cPLA₂ antiserum (500 μl, diluted 1:9 in 10 mM Tris, pH 7.5), and then washed with 2 ml each of 10 mM Tris (pH 7.5), 0.5 M NaCl in 10 mM Tris (pH 7.5), and 10 mM Tris (pH 8.8). Antibody was eluted with 2 ml of 100 mM triethylamine (pH 11.5) into tubes containing 1 M Tris (pH 8.0) to adjust the pH.

Microscopy

For microscopy experiments, 1.25×10^6 cells were plated on 35-mm tissue culture dishes and infected with baculovirus containing the genes for the different GFP-containing fusion constructs at multiplicities of infection ranging from 2 to 10 colony forming units per cell. After infection (40–50 h), cells were washed twice with TNM-FH medium and observed at room temperature using an Olympus Vanox-T Microscope equipped with an Achroplan 63X Zeiss lens and a FITC filter. Digital confocal images were
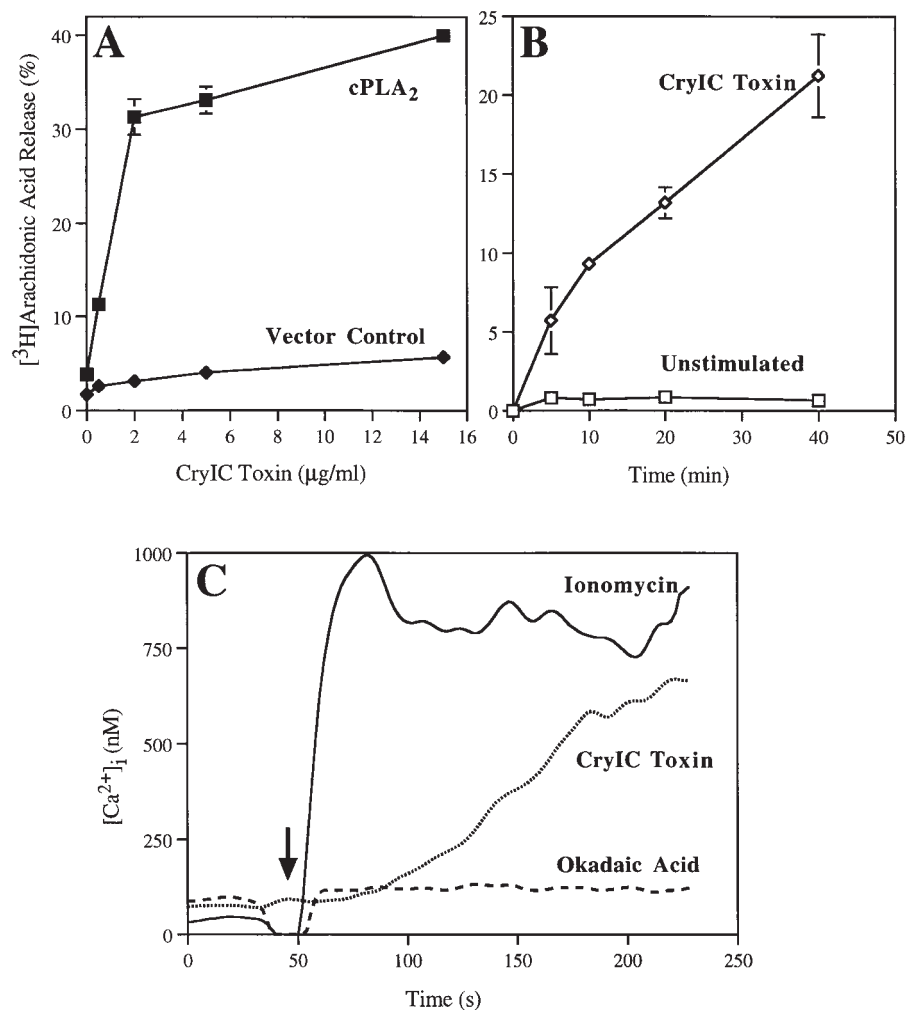

Figure 1. CryIC toxin–induced arachidonic acid release and calcium mobilization in Sf9 cells expressing cPLA₂. Sf9 cells were infected with recombinant baculovirus containing transfer vector pVL1393 alone (Vector Control) or the gene for human cPLA₂. After infection (50–54 h), cells were prelabeled with [³H]arachidonic acid and then incubated for 20 min with the indicated concentrations of CryIC toxin (A) or with 2 μg/ml CryIC toxin or vehicle (Unstimulated), for the times indicated (B). The amount of [³H]arachidonic acid released into the medium is expressed as a percentage of the total radioactivity incorporated (cell-associated plus medium). Data represent the average ± SD of a representative experiment and were verified in at least three independent experiments performed in triplicate. (C) Calcium mobilization was measured in Fura-2 AM–loaded Sf9 cells expressing cPLA₂ after addition (indicated by the arrow) of ionomycin (1 μM), okadaic acid (1 μM), or CryIC toxin (2 μg/ml). The intracellular calcium concentration was monitored over time by recording the excitation spectra at 340 and 380 nm. The extracellular calcium concentration was 9 mM. Results shown are from a representative experiment and were verified in at least three independent experiments.

obtained with a SpectraSource Orbis 16 camera, using Slidebook software from Intelligent Imaging Innovations, Inc. Images shown in the figures are representative of at least three independent experiments.

For immunofluorescence, Sf9 cells (2.5×10^5) were plated on 13-mm glass coverslips in 24-well culture plates and infected with baculovirus containing cPLA$_2$. After infection (40–50 h), cells were rinsed twice with 0.5 ml PBS, pH 7.5, and then fixed for 15 min with 0.5 ml 3% paraformaldehyde in PBS containing 3% sucrose. After rinsing with PBS, cells were permeabilized with 0.2% Triton X-100 for 15 min, and then incubated for 30 min with HBSS containing 10% FBS (blocking solution). Cells were incubated for 2 h with affinity-purified anti-cPLA$_2$ antibody diluted 1:20 in blocking solution. After extensive rinsing with PBS, cells were incubated for 1 h with donkey anti–rabbit IgG antibody conjugated to Cy3 (10 μg/ml in blocking solution). Coverslips were mounted on microscope slides in 0.1 M Tris-HCl, pH 8.5, containing 90% glycerol and 2 mg/ml orthophenylenediamine. Cells were observed by digital confocal microscopy using a Cy3 filter.

Results

Arachidonic Acid Release and Calcium Mobilization in Sf9 Cells Expressing cPLA$_2$

Sf9 cells expressing cPLA$_2$ release arachidonic acid in response to A23187 and okadaic acid (de Carvalho et al., 1996). We have found that CryIC toxin from *Bacillus thuringiensis* also stimulates arachidonic acid release from Sf9 cells expressing cPLA$_2$, but not from cells infected with baculovirus containing the vector alone (Fig. 1 A). The crystal (Cry) protein toxins are inclusions formed by the gram-positive soil bacterium during sporulation. CryIC toxin has been shown to induce an increase in intracellular calcium in Sf9 cells, probably in a receptor-mediated fashion (Schwartz et al., 1991). A 40-kD binding protein for CryIC toxin has been identified recently (Kwa et al., 1998). Arachidonic acid release in response to CryIC toxin was near maximal at 2 μg/ml, a concentration well below

the ED$_{50}$ for toxicity (15 μg/ml) for Sf9 cells (Monette et al., 1997). Rapid and prolonged release of arachidonic acid was induced by CryIC toxin (Fig. 1 B).

An increase in intracellular calcium is an important mechanism for regulating cPLA$_2$ by promoting its translocation and binding to membrane. However, we demonstrated previously that okadaic acid induces arachidonic acid release in macrophages without increasing intracellular calcium (Qiu et al., 1998). Experiments were carried out to determine the effect of okadaic acid on intracellular calcium levels in the Sf9 model in comparison to ionomycin and CryIC, which are known to increase intracellular calcium. As shown in Fig. 1 C, okadaic acid did not promote an increase in intracellular calcium in Sf9 cells expressing cPLA$_2$, and no effect was seen when the incubation was prolonged for as long as 30 min. In contrast, CryIC toxin induced an increase in intracellular calcium that did not occur as rapidly as with ionomycin, but did reach a similar level. As previously reported, the increase in intracellular calcium induced by CryIC was sustained for at least 15 min (not shown) (Schwartz et al., 1991).

Agonist-induced Translocation of GFP-cPLA$_2$ to the Nuclear Envelope in Sf9 Cells

GFP was linked to the amino terminus of cPLA$_2$ and its subcellular localization was visualized in living Sf9 cells. GFP-cPLA$_2$, like GFP alone, was uniformly distributed throughout unstimulated cells when observed in different planes of the cell by confocal analysis (data not shown). In Sf9 cells treated with the calcium-mobilizing agonists A23187 and CryIC toxin, as well as okadaic acid, the GFP-cPLA$_2$ fusion protein exhibited time-dependent translocation to the nuclear envelope (Fig. 2). Translocation was evident in at least 80–85% of the cells observed in each ex-
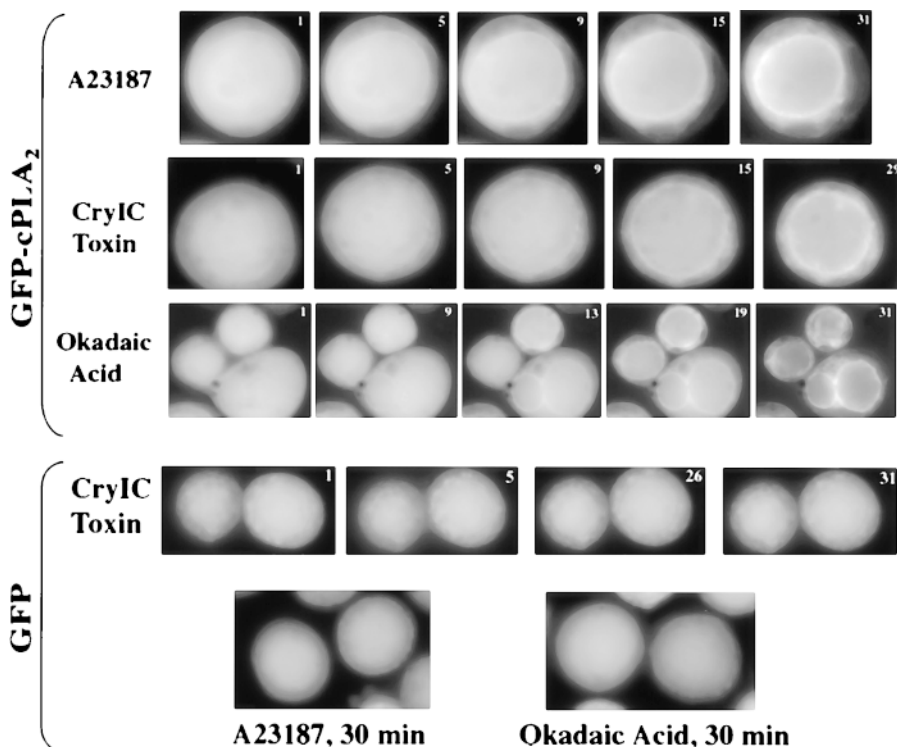

Figure 2. Agonist-induced translocation of GFP-cPLA$_2$, but not GFP alone, to the nuclear envelope in living Sf9 cells. Sf9 cells expressing GFP-cPLA$_2$ were incubated with 2 μg/ml A23187, 2 μg/ml CryIC toxin, or 1 μM okadaic acid. Digital confocal images were obtained at the times indicated (in minutes). The bottom two series show images of Sf9 cells expressing GFP alone at different times after addition of CryIC toxin (in minutes), or at 30 min after addition of A23187 (bottom left) or okadaic acid (bottom right).

periment. Diffuse fluorescence emanating from the nuclear envelope could also be observed and may represent cPLA$_2$ bound to endoplasmic reticulum. cPLA$_2$ that is present both inside the nucleus and in the cytosol in unstimulated cells translocated to the nuclear envelope. Importantly, GFP alone did not translocate in response to agonist treatment and remained uniformly distributed. Translocation of GFP-cPLA$_2$ to the nuclear envelope was evident by 5–10 min after treatment with the agonists and was nearly complete by 20–30 min (Fig. 2). The time course of translocation was not identical from cell to cell in the population and could vary by minutes. As previously reported the agonists induce significant arachidonic acid release by 15 min, which is consistent with the time course of translocation (Fig. 1 B) (de Carvalho et al., 1996).

To further substantiate that GFP-cPLA$_2$ localizes at the nuclear envelope, Sf9 cells expressing GFP-cPLA$_2$ were treated with the nuclear dye Hoechst 33342. As shown in Fig. 3 A, GFP-cPLA$_2$ was uniformly distributed throughout the cell, whereas the Hoechst localized to the nucleus. When GFP-cPLA$_2$ and Hoechst colocalized, as seen inside the nucleus of the unstimulated cell, the purple color was diminished and the fluorescence appears white. As shown in the cell treated with A23187, GFP-cPLA$_2$ localized to the nuclear envelope and the perinuclear region, whereas the purple Hoechst stain remained distributed throughout the nucleus. When the GFP-cPLA$_2$ and Hoechst fluorescence were visualized together a bright ring of GFP-cPLA$_2$ was seen surrounding the purple nucleus in the stimulated cell.

To verify that the translocation of cPLA$_2$ was not influenced by the GFP moiety, cPLA$_2$ was expressed alone and as a GFP fusion protein in Sf9 cells and visualized by immunofluorescence using affinity-purified polyclonal anti-cPLA$_2$ antibody. Localization of cPLA$_2$ was similar to GFP-cPLA$_2$ in fixed Sf9 cells (Fig. 3 B). In unstimulated cells, the enzyme was uniformly distributed throughout the cell. In response to A23187, cPLA$_2$ and GFP-cPLA$_2$ similarly translocated to the nuclear envelope and perinuclear region. Experiments were also carried out to verify that the GFP-cPLA$_2$ fusion protein was catalytically active in Sf9 cells and could mediate agonist-induced arachidonic acid release. As shown in Fig. 3 C, cells expressing either cPLA$_2$ or GFP-cPLA$_2$ released arachidonic acid to a similar extent in response to A23187 and okadaic acid. GFP-cPLA$_2$ exhibited a decrease in electrophoretic mobility (gel shift) in response to okadaic acid, as occurs with the native enzyme (Fig. 3 C) (de Carvalho et al., 1996).
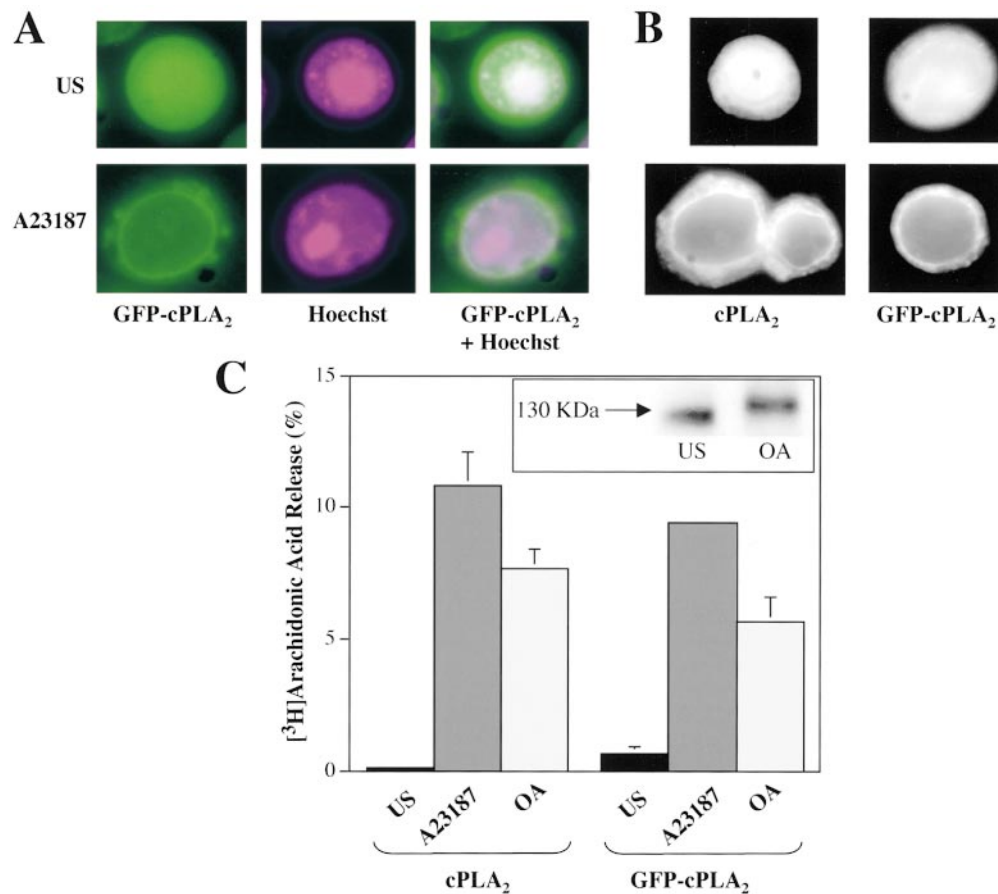

Figure 3. Translocation of cPLA$_2$ and GFP-cPLA$_2$ to the nuclear envelope in living and fixed Sf9 cells. (A) Sf9 cells expressing GFP-cPLA$_2$ were incubated for 20 min with the nuclear dye Hoechst 33342 (10 μg/ml), and then treated with vehicle (0.1% DMSO; US) or 2 μg/ml A23187 for 15 min. Digital confocal images of living Sf9 cells were obtained with a FITC filter to visualize the fluorescence from GFP (shown in green, left) or a UV filter for Hoechst (shown in purple, middle). A combined image showing the signals from both fluorophores is shown on the right. (B) Sf9 cells expressing cPLA$_2$ or GFP-cPLA$_2$ were treated with vehicle or 2 μg/ml A23187 for 20 min. Proteins were visualized by immunofluorescence in fixed cells using an affinity-purified anti-cPLA$_2$ antibody, and images were obtained by digital confocal microscopy. (C) [^{3}H]Arachidonic acid release from Sf9 cells expressing cPLA$_2$ or GFP-cPLA$_2$ was measured after treatment with vehicle, 2 μg/ml A23187, or 1 μM okadaic acid for 90 min at room temperature. The data shown are average ± SD from a representative experiment of at least three performed in triplicate. The inset shows an immunoblot of cPLA$_2$ from unstimulated and okadaic acid–treated Sf9 cells.

Arachidonic Acid Release in Sf9 Cells Expressing Phosphorylation Site Mutants of cPLA₂

We reported previously that treatment of Sf9 cells with okadaic acid increases phosphorylation of cPLA$_2$ on S727 and to a lesser extent on S437, S454, and S505 (de Carvalho et al., 1996). To determine if phosphorylation of these sites was functionally important for okadaic acid–induced arachidonic acid release, the serines were mutated to alanines and the mutant cPLA$_2$ constructs expressed in Sf9 cells. A double mutant construct, S505A/S727A, was also generated since both sites may act cooperatively in regulating cPLA$_2$. In addition, a carboxy-terminal deletion (Δ721–749) was produced to avoid the possibility that serines near S727 (S724, S729, and S731) may become phosphorylated when S727 is converted to an alanine. Sf9 cells expressing the S727A, S437A, or S454A mutant constructs exhibited the same magnitude of arachidonic acid release in response to A23187, CryIC toxin, and okadaic acid as Sf9 cells expressing wild-type cPLA$_2$ (Fig. 4). These results demonstrate that phosphorylation of these sites does not play a functional role in cPLA$_2$ activation in this model. cPLA$_2$ containing the S505A substitution was the only phosphorylation site mutant that was less effective (by 50%) at mediating arachidonic acid release. The results with cells expressing cPLA$_2$ containing either the S505A/S727A double mutation or the S505A single mutation were not significantly different. Arachidonic acid release from Sf9 cells expressing the carboxy-terminal dele-

tion mutant was no different from the response of Sf9 cells expressing wild-type cPLA$_2$, confirming that phosphorylation of S727 plays no functional role in this model and that the extreme carboxy terminus of cPLA$_2$ is not essential. Importantly, the level of expression of wild-type and mutant forms of cPLA$_2$ was similar (Fig. 4). All the mutant cPLA$_2$ enzymes except S505A were able to gel-shift in response to okadaic acid, demonstrating that the characteristic retardation of the electrophoretic mobility is only due to phosphorylation of S505, and not the other sites. A23187 and CryIC toxin have no effect on cPLA$_2$ electrophoretic mobility in Sf9 cells (de Carvalho et al., 1996).

Arachidonic Acid Release in Sf9 Cells Expressing C2 Domain Mutants of cPLA₂

Although okadaic acid induces arachidonic acid release without increasing intracellular calcium, our previous work with macrophages has shown that resting levels of calcium are required (Qiu et al., 1998). This suggested that the C2 domain of cPLA$_2$ is functionally important for regulation of the enzyme in response to okadaic acid. The crystal structure of the cPLA$_2$ C2 domain has revealed several amino acid residues (D40, T41, D43, N65, D93, A94, and N95) that participate in binding calcium, and it has been shown recently that mutation of these residues impairs PLA$_2$ activity and binding of cPLA$_2$ to phospholipids (Perisic et al., 1998; Bittova et al., 1999). To determine if a functional C2 domain was required for cPLA$_2$ to
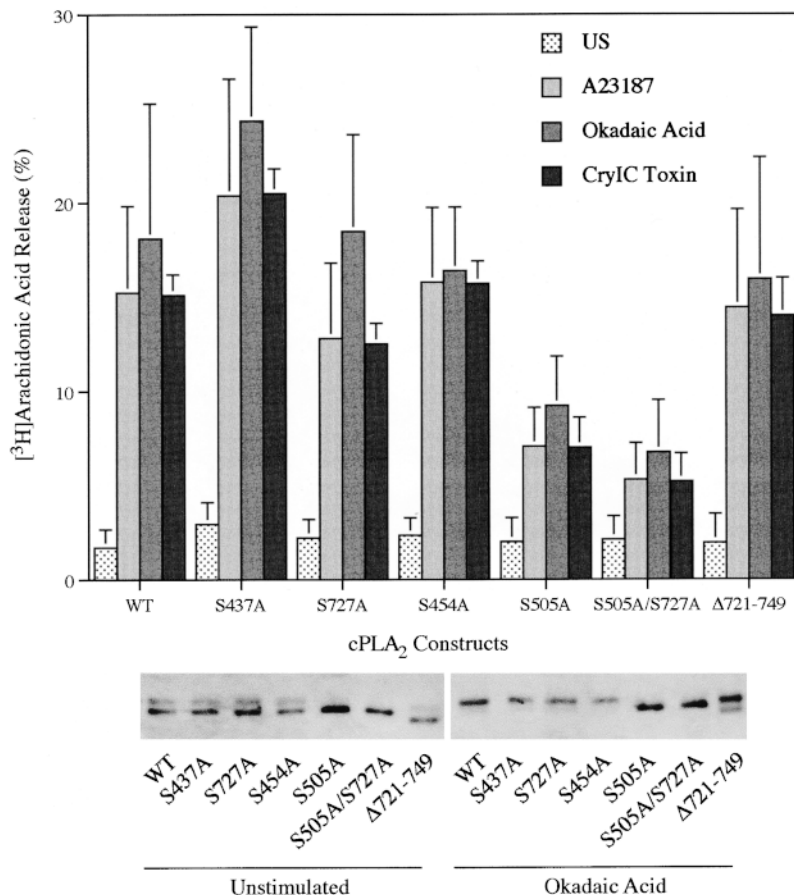

Figure 4. Agonist-induced arachidonic acid release from Sf9 cells expressing wild-type or phosphorylation-site mutants of cPLA$_2$. [^{3}H]Arachidonic acid release from Sf9 cells was measured after treatment with A23187 (2 µg/ml) or okadaic acid (1 µM) for 90 min, or with CryIC toxin (2 µg/ml) for 20 min. The amount of label released into the medium is expressed as a percentage of the total radioactivity incorporated (cell-associated plus medium). Data represent the mean ± SEM of three experiments performed in triplicate. The bottom panel shows a representative immunoblot of cPLA$_2$.
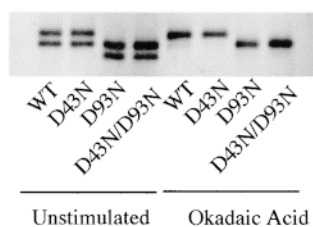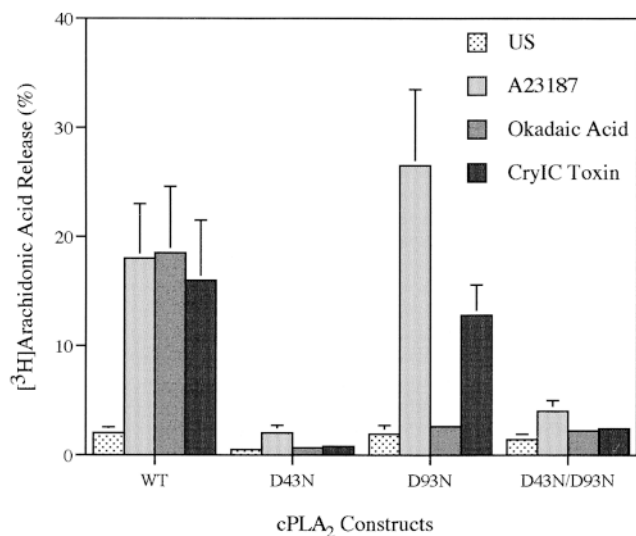
Figure 5. Arachidonic acid release from Sf9 cells expressing wild-type or C2 domain mutants of cPLA$_2$. [^{3}H]Arachidonic acid release from Sf9 cells was measured after treatment with A23187 (2 μg/ml) or okadaic acid (1 μM) for 90 min, or with CryIC toxin for 20 min. Data represent the mean $\pm$ SEM of three experiments performed in triplicate. The bottom panel is a representative immunoblot of cPLA$_2$ and the results were verified in at least three independent experiments.

mediate arachidonic acid release in response to okadaic acid compared with the calcium-mobilizing agonists, two calcium binding residues (D43 and D93) were mutated to asparagine residues. cPLA$_2$ containing the D43N, D93N, or D43N/D93N mutations was unable to mediate okadaic acid–induced arachidonic acid release in Sf9 cells (Fig. 5). Arachidonic acid release induced by A23187 or CryIC toxin was also dramatically suppressed in Sf9 cells expressing D43N or D43N/D93N cPLA$_2$, whereas the D93N mutant was fully functional. In fact, the D93N mutant exhibited a modest but reproducible increased response to A23187, as compared with wild-type cPLA$_2$. As shown in the Western blot, the C2 domain mutants of cPLA$_2$ are expressed at similar levels and gel-shifted in response to okadaic acid. Mutation of D93N to an asparagine resulted in an increased electrophoretic mobility of cPLA$_2$ (Fig. 5).

The effect of the C2 domain mutations on translocation of GFP-cPLA$_2$ in response to okadaic acid and the calcium-mobilizing agonists was evaluated. The D43N or D43N/D93N mutant enzymes were unable to translocate to the nuclear envelope in living Sf9 cells treated with

A23187, whereas GFP-cPLA$_2$ containing the D93N mutation was fully functional and translocated in response to the calcium ionophore (Fig. 6). Translocation was evident in at least 85% of the cells observed in each experiment. Similar results were also obtained with CryIC toxin (not shown). In contrast, the D93N mutant did not translocate to the nuclear envelope in response to okadaic acid (Fig. 6). The D43N and D43N/D93N constructs were also unable to translocate in response to okadaic acid (not shown).

Role of Extracellular Calcium in Regulating Arachidonic Acid Release by Wild-Type and C2 Mutants of cPLA$_2$

The implication of the above results is that arachidonic acid release induced by okadaic acid in Sf9 cells would show less dependency on extracellular calcium than the response to A23187 or CryIC toxin, but would be dependent on resting levels of intracellular calcium. As shown in Fig. 7, depleting extracellular calcium dramatically reduced A23187-induced arachidonic acid release from Sf9 cells expressing wild-type cPLA$_2$ but had less effect on cells treated with okadaic acid. The significant effect of depleting extracellular calcium on okadaic acid–induced arachidonic acid release may be due to the observed decrease in basal levels of intracellular calcium that occurs when extracellular calcium levels are decreased. In normal Sf9 medium containing 9 mM calcium the intracellular calcium concentration was 68 ± 16 nM (mean $\pm$ SEM, $n = 4$), and the level decreased to 17 ± 8 nM when no calcium was
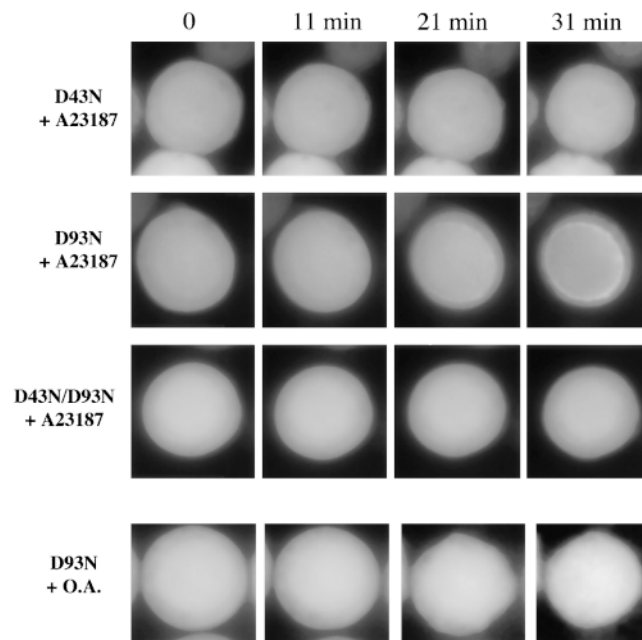

Figure 6. Effect of D43N and D93N mutations on A23187- and okadaic acid–induced translocation of GFP-cPLA$_2$. Sf9 cells expressing the indicated mutant constructs of GFP-cPLA$_2$ were treated with A23187 or okadaic acid, and the intracellular distribution of GFP-cPLA$_2$ was visualized by digital confocal microscopy.
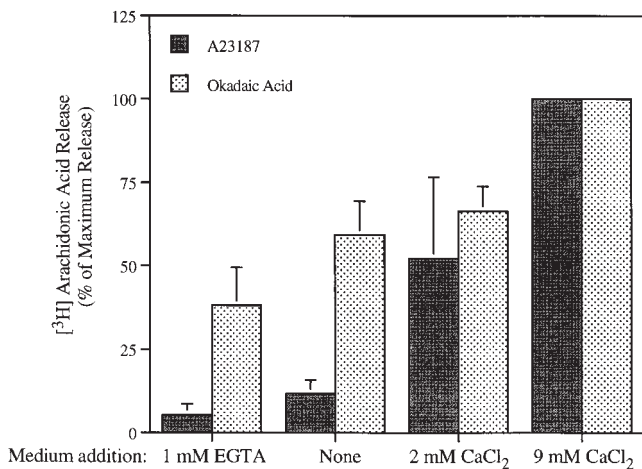
Figure 7. Role of extracellular calcium in regulating A23187- and okadaic acid–induced arachidonic acid release. Sf9 cells expressing cPLA$_2$ were labeled with [^{3}H]arachidonic acid and then rinsed in calcium-free TNM-FH medium containing 0.1% HSA. The cells were incubated in TNM-FH medium containing 0.1% HSA with the indicated concentrations of EGTA or calcium chloride and then treated with vehicle, A23187 (2 μg/ml), or okadaic acid (1 μM) for 90 min. The data (mean $\pm$ SEM of four experiments in triplicate) are expressed as a percentage of the maximum [^{3}H]arachidonic acid release, which was 19.5 $\pm$ 8.2% for A23187-treated cells and 15.0 $\pm$ 2.9% for okadaic acid–treated cells. The measured percent release from unstimulated cells was 3.0%.

added to the medium. With EGTA in the medium, intracellular calcium was below the level of detection ($\leq$5 nM).

The agonist-induced level of intracellular calcium can be manipulated by incubating Sf9 cells in medium containing varying levels of extracellular calcium. The effect of extracellular calcium concentration on the ability of CryIC toxin to induce arachidonic acid release in Sf9 cells expressing the C2 mutant constructs of cPLA$_2$ was evaluated and correlated with the intracellular levels of calcium (Fig. 8 A). Arachidonic acid release induced by CryIC toxin was dependent on extracellular calcium and decreased to near baseline levels when it was chelated with EGTA. Sf9 cells expressing cPLA$_2$ containing the D43N or D43N/D93N mutations exhibited little arachidonic acid release above baseline levels in response to CryIC toxin. The ability of cPLA$_2$ containing the D93N mutation to mediate arachidonic acid release when extracellular calcium was decreased from 9 to 2 mM paralleled the response observed with wild-type cPLA$_2$, both exhibiting an $\sim$30–35% decrease in responsiveness. In contrast, when no calcium was added to the medium, the D93N mutant enzyme was unable to mediate arachidonic acid release, whereas there was still a significant response in cells expressing wild-type cPLA$_2$. These results demonstrate that the D43N and D43N/D93N mutations dramatically reduce the ability of the enzyme to mediate arachidonic acid release even at the highest levels of intracellular calcium achieved (630 nM) with CryIC toxin–treated Sf9 cells in medium containing 9 mM calcium. However, cPLA$_2$ containing the D93N mutation is functional at concentrations of intracellular cal-

cium in the 0.2–0.6 μM range but not at 36 nM intracellular calcium.

Calcium Differentially Affects Enzymatic Activity of C2 Domain Mutants of cPLA$_2$

The results of expressing the C2 domain mutants of cPLA$_2$ in Sf9 cells suggested that mutating D43 and D93 differentially affected the affinity of the enzyme for calcium. This was verified in vitro by measuring the effect of calcium concentration on cPLA$_2$ activity of the mutant enzymes in the cytosolic fraction of Sf9 cells in which equal expression of cPLA$_2$ was confirmed by densitometry (Fig. 8 B). Wild-type cPLA$_2$ exhibited significant activity at nanomolar calcium that continued to increase substantially up to 1 μM calcium with little additional increase in the millimolar range. Consistent with the results of expressing the C2 mutants in Sf9 cells, mutating D43 had a more profound effect on the calcium sensitivity of cPLA$_2$ than mutating D93. cPLA$_2$ containing the D43N or D43N/D93N mutations exhibited very little activity at concentrations of calcium $\leq$1 μM but exhibited significant activity at millimolar calcium. Unlike wild-type cPLA$_2$, the D93N mutant did not exhibit an increase in activity at 50 nM calcium, but did have significant activity in the 0.25–1 μM range. At 1 mM calcium the wild-type and D93N mutant enzymes exhibited similar activities. As previously reported, cPLA$_2$ exhibits lysophospholipase activity, which is calcium independent (Leslie, 1991; de Carvalho et al., 1995). The lysophospholipase activity of cPLA$_2$ containing the mutations in the C2 domain was not significantly different from wild-type cPLA$_2$ (data not shown).

Translocation of the C2 Domain of cPLA$_2$ to the Nuclear Envelope

Experiments were undertaken to determine if the C2 domain of cPLA$_2$ played a role in the preferential targeting of cPLA$_2$ to the nuclear envelope. GFP was linked to the amino terminus of the C2 domain and expressed in Sf9 cells. GFP-C2 was uniformly distributed throughout unstimulated cells as observed for GFP-cPLA$_2$ (Fig. 9). A23187 induced translocation of the C2 domain to the nuclear envelope in Sf9 cells with a similar time course as the full-length enzyme. The same results were observed in response to the CryIC toxin (not shown). In contrast to the calcium-mobilizing agonists, okadaic acid did not induce translocation of GFP-C2. Translocation of GFP-C2 to the nuclear envelope in response to A23187 and CryIC toxin required extracellular calcium as observed for full-length cPLA$_2$ (Fig. 10). The calcium-mobilizing agonists did not induce translocation of GFP-C2 in Sf9 cells incubated in medium containing EGTA. However, the subsequent addition of calcium to the medium resulted in translocation of GFP-C2 to the nuclear envelope.

Discussion

Sf9 Cells as a Model for cPLA$_2$ Structure–Function Studies

The baculovirus expression system in Sf9 insect cells is a useful model to study the regulation of cPLA$_2$. This system

A

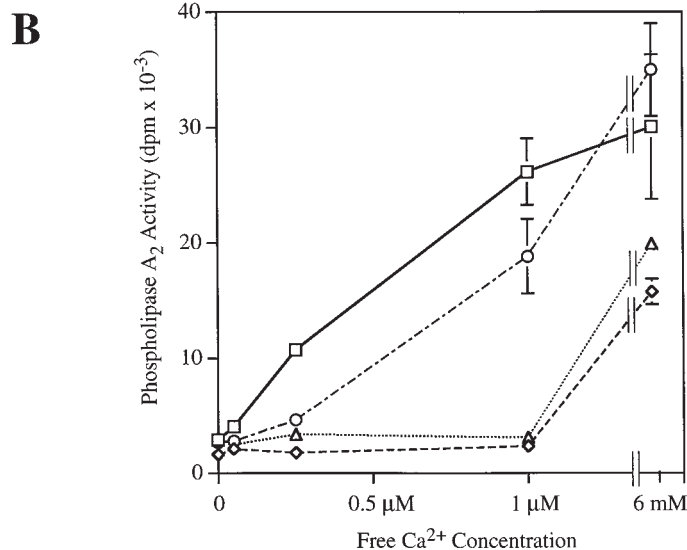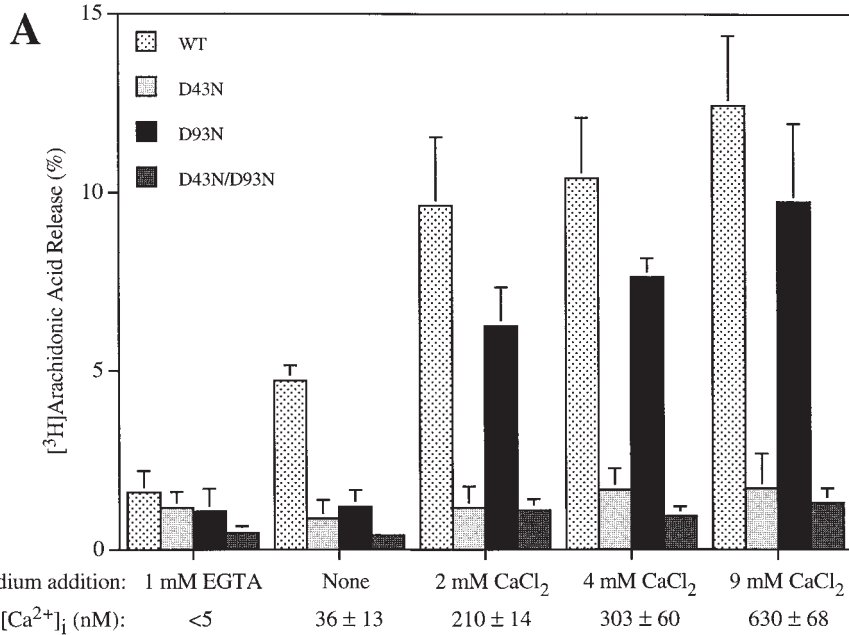| Medium addition:	1 mM EGTA	None	2 mM CaCl$_2$	4 mM CaCl$_2$	9 mM CaCl$_2$
[Ca^{2+}]$_i$ (nM):	<5	36 ± 13	210 ± 14	303 ± 60	630 ± 68

B

Figure 8. Differential sensitivity of D43N and D93N C2 domain mutant cPLA$_2$ enzymes to calcium. (A) Correlation of CryIC toxin–induced arachidonic acid release from Sf9 cells by C2 domain mutants of cPLA$_2$ and intracellular calcium levels. [^{3}H]Arachidonic acid–labeled Sf9 cells expressing wild-type and C2 domain mutants of cPLA$_2$ were incubated in TNM-FH medium containing 0.1% HSA with the indicated concentrations of EGTA or calcium chloride, and then treated with vehicle or 2 μg/ml CryIC toxin for 20 min. The amount of [^{3}H]arachidonic acid released into the medium is expressed as a percentage of the total incorporated (cell-associated plus medium). Results shown are average ± SD of a representative experiment and were verified in at least three independent experiments. The levels of intracellular calcium in Sf9 cells treated with CryIC toxin are shown below the abscissa. Fura-2 AM–loaded Sf9 cells expressing cPLA$_2$ were incubated in medium containing the indicated concentrations of EGTA or calcium and then treated with 2 μg/ml CryIC toxin for 5 min. Data shown are the mean ± SEM of three independent experiments. (B) Effect of calcium concentration on the enzymatic activity of wild-type and C2 domain mutants of cPLA$_2$. Cytosols were prepared from Sf9 cells expressing wild-type (□), D43N (◇), D93N (○), or D43N/D93N (△) cPLA$_2$ enzymes and assayed for PLA$_2$ activity at various calcium concentrations. Results shown are average ± SD of a representative experiment and were verified in three independent experiments in triplicate. Background radioactivity (typically <1,000 dpm) was determined by incubating the substrates in the absence of enzyme, and was subtracted from the data.

has been used extensively to study the function of expressed proteins and their roles in signal transduction (Mancini and Evans, 1993; Stancato et al., 1993, 1997; Chefalo et al., 1994). The effect of cPLA$_2$ mutations on arachidonic acid release and translocation can be directly compared in intact, living cells. Importantly, the stimulated arachidonic acid release is due to cPLA$_2$ since there is little contribution of endogenous PLA$_2$ enzymes. The responses of Sf9 cells expressing cPLA$_2$, such as arachidonic acid release and phosphorylation, are similar to responses that we have observed in murine macrophages and human monocytes (Qiu et al., 1993; de Carvalho et al., 1996; Qiu et al., 1998). In addition, GFP-cPLA$_2$ is targeted to the nuclear envelope in response to the calcium-mobilizing agonists and okadaic acid. Using the GFP fusion protein allows translocation to be followed in real time in living cells, and the GFP moiety attached to the amino terminus

of cPLA$_2$ does not appear to influence the function of the enzyme. It should be noted that the mechanisms involved in the regulation of cPLA$_2$ such as the importance of phosphorylation, calcium, and even the site of subcellular localization, appear cell type– and agonist-dependent. Sf9 cells are ovarian cells and may lack regulatory components present in certain mammalian cells. However, there are also considerable differences in the regulation of cPLA$_2$ activation among different mammalian cell types. For example, phorbol esters or calcium ionophores alone are poor inducers of cPLA$_2$ activation (and arachidonic acid release) in many cell types, but can act synergistically. However, in macrophages and certain hemopoietic cells these are potent agonists. Similar to macrophages, Sf9 cells expressing cPLA$_2$ respond to A23187 and okadaic acid but, unlike macrophages, not to PMA.

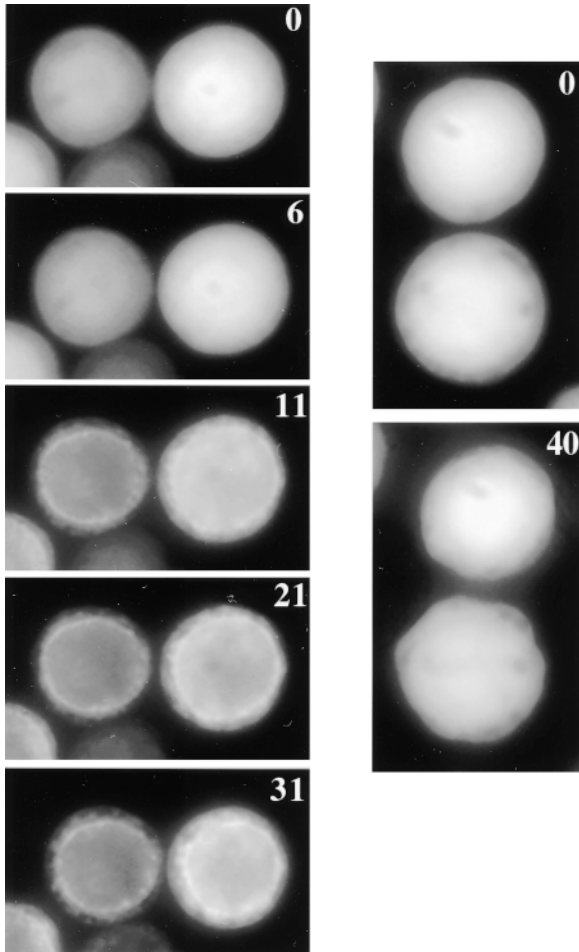In unstimulated Sf9 cells, the GFP-cPLA$_2$ fusion protein

Figure 9. GFP-C2 translocates to the nuclear envelope in Sf9 cells treated with A23187 but not okadaic acid. Sf9 cells expressing a GFP-C2 domain fusion protein were incubated with 2 μg/ml A23187 (left) or 1 μM okadaic acid (right) for the times indicated (in minutes). Images were obtained with a digital confocal microscopy system.

was uniformly distributed both inside the nucleus and in the cytosol. This distribution was not influenced by the level of GFP-cPLA$_2$ expression, which can be varied by decreasing the multiplicity of infection and by decreasing the infection time. In several unstimulated mammalian cells, cPLA$_2$ has been observed inside the nucleus, in the cytosol, and in both locations depending on the cell type and growth conditions (Glover et al., 1995; Schievella et al., 1995; Peters-Golden et al., 1996; Sierra-Honigmann et al., 1996; Hirabayashi et al., 1999). In many of these cell models cPLA$_2$ translocates to the nuclear envelope and endoplasmic reticulum in response to cell stimulation, although exceptions have been observed. In confluent endothelial cells treated with histamine, cPLA$_2$ translocates to both the nuclear envelope and to the plasma membrane at intercellular junctions (Sierra-Honigmann et al., 1996). A punctate clustering of cPLA$_2$ has been observed in the cytoplasm of mouse 3T3 fibroblasts overexpressing the epidermal growth factor receptor, and this distribution is not influenced by epidermal growth factor or calcium iono-

phore (Bunt et al., 1997). A punctate cytoplasmic localization of cPLA$_2$ occurs in U937 cells and appears to be due to association with cytoplasmic lipid bodies (Yu et al., 1998).

The Role of Phosphorylation in cPLA$_2$ Activation in Sf9 Cells

Using a variety of agonists that act on cells by diverse mechanisms, it has become clear that there are alternative regulatory pathways that can lead to activation of cPLA$_2$ and arachidonic acid release (Leslie, 1997). The ability of okadaic acid to activate cPLA$_2$ and induce arachidonic acid release in macrophages and Sf9 cells without increasing intracellular calcium provides a useful tool to elucidate novel mechanisms involved in cPLA$_2$ regulation. Previous work in macrophages and other cell models has demonstrated that phosphorylation on S505 is not sufficient for activation of cPLA$_2$ and arachidonic acid release in the absence of an increase in intracellular calcium (Lin et al., 1992; Qiu et al., 1998). This is also evident in Sf9 cells since a significant portion of cPLA$_2$ is constitutively phosphorylated on S505 when expressed in Sf9 cells, yet there is no arachidonic acid release unless the cells are treated with okadaic acid or agonists that increase intracellular calcium (Abdullah et al., 1995; de Carvalho et al., 1996). This implicates an alternative mechanism in the regulation of cPLA$_2$ in response to okadaic acid. One possibility was a role for S727 phosphorylation which is preferentially induced in okadaic acid–treated Sf9 cells (de Carvalho et al., 1996). S505, S437, and S454 are constitutively phosphorylated on cPLA$_2$ in unstimulated Sf9 cells, and their phosphorylation state is increased only modestly in response to agonist treatment. These four residues represent the only major phosphorylation sites on cPLA$_2$ in okadaic acid–treated Sf9 cells. Whether phosphorylation of these novel sites, particularly S727, plays a functional role in cPLA$_2$ activation has been an important question. The mutagenesis data demonstrate that okadaic acid–induced phosphorylation of cPLA$_2$ on S727, S437, or S454 plays no functional role in cPLA$_2$-mediated arachidonic acid release in this model. Whether phosphorylation of S727 plays a functional role in cPLA$_2$ regulation in other cells where it has been identified remains to be established (Börsch-Haubold et al., 1998). Phosphorylation of S505 does play a role in augmenting arachidonic acid release by both okadaic acid and the calcium-mobilizing agonists. However, it is not absolutely essential since Sf9 cells expressing cPLA$_2$ containing the S505A mutation still release arachidonic acid to ~50% of the level of Sf9 cells expressing wild-type cPLA$_2$. The role played by S505 phosphorylation in the regulation of cPLA$_2$ appears to be cell type– and agonist-dependent. In macrophages, S505 phosphorylation is not essential for arachidonic acid release in response to agonists that induce a sustained increase in intracellular calcium but does appear to be required when there is only a transient increase in calcium (Qiu et al., 1998). In CHO cells overexpressing cPLA$_2$, S505 phosphorylation is essential for agonist-induced arachidonic acid release (Schievella et al., 1995). However, S505 phosphorylation of cPLA$_2$ is not required for arachidonic acid release in thrombin-stimulated platelets (Kramer et al., 1996).
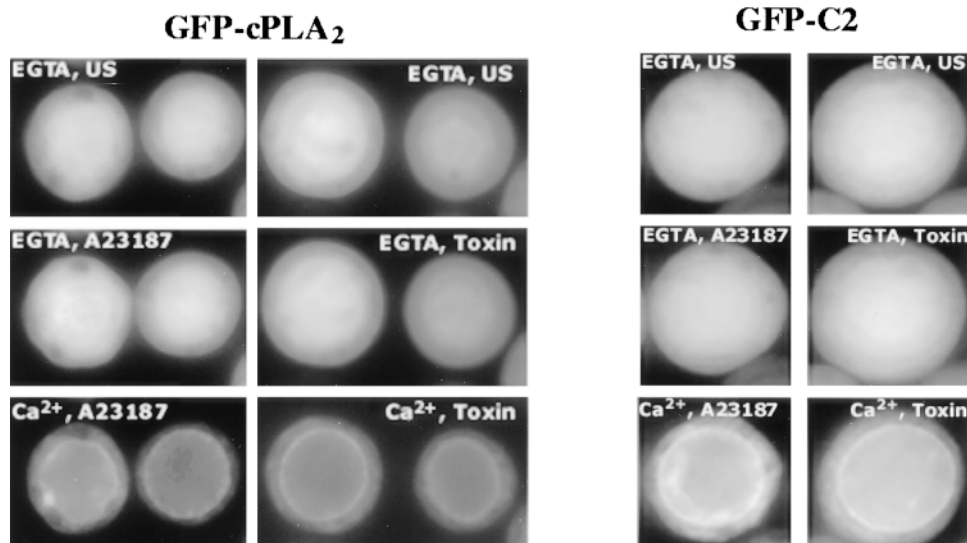
GFP-cPLA$_2$

GFP-C2

Figure 10. Extracellular calcium is required for translocation of GFP-C2 and GFP-cPLA$_2$ in Sf9 cells. Sf9 cells expressing GFP-cPLA$_2$ (left series) or GFP-C2 (right series) were treated with 2 μg/ml A23187 or 2 μg/ml CryIC toxin in TNM-FH medium containing 10 mM EGTA. After a 15-min incubation, calcium chloride was added (final concentration of 9 mM), and cells were incubated for an additional 10 min. Images were obtained with a digital confocal microscopy system.

The Role of the C2 Domain in cPLA$_2$ Regulation

Our translocation results show a steady accumulation of GFP-cPLA$_2$ in the perinuclear region of Sf9 cells after addition of A23187 or CryIC toxin. This observation is consistent with recent data showing that a sustained increase in the intracellular calcium concentration is required for the stable association of cPLA$_2$-GFP to the nuclear envelope of CHO cells, which correlates with agonist-induced arachidonic acid release (Hirabayashi et al., 1999). Although okadaic acid does not increase intracellular calcium, our results demonstrate a requirement for a functional C2 domain. Mutagenesis of calcium-binding residues D43 and D93 in the C2 domain quantitatively suppress okadaic acid–induced arachidonic acid release and translocation of cPLA$_2$ to the nuclear envelope. In addition, lowering the levels of intracellular calcium by including EGTA in the medium diminished okadaic acid–induced arachidonic acid release, suggesting a requirement for the resting levels of calcium. Attempts to completely chelate intracellular calcium by also including BAPTA or Quin-2 were unsuccessful due to adverse effects on Sf9 cells. The differences in the functional responses of Sf9 cells expressing the D43N and D93N mutant enzymes suggest that these mutations differentially affect the affinity of the enzyme for calcium. This is consistent with the structural analysis of the cPLA$_2$ C2 domain, which binds two calcium ions with different affinities (Nalefski et al., 1997; Perisic et al., 1998; Xu et al., 1998; Bittova et al., 1999). D43 forms one interaction with each of the two calcium ions, whereas both side chain oxygens of D93 participate in binding only one of the calcium ions at site II. It has been shown recently that purified cPLA$_2$ containing D43N and D93N mutations exhibits greatly diminished PLA$_2$ activity and binding to phospholipids as compared with the wild-type enzyme (Bittova et al., 1999). The calcium requirements are higher for cPLA$_2$ containing the D43N mutation than D93N, consistent with the functional differences we observe in the Sf9 model. Differential roles for calcium binding residues in the C2 domain of PKCα

have also been reported (Medkova and Cho, 1998). D246 plays the most critical role for PKCα activation and calcium-dependent binding to lipid vesicles in vitro, and is thought to be due to its ability to coordinate two Ca^{2+} ions tightly. Our results demonstrate a pivotal role for D43 in mediating cPLA$_2$ translocation in intact cells. cPLA$_2$ containing the D43N mutation is unable to translocate to the nuclear envelope or to mediate arachidonic acid release in response to okadaic acid, which acts at resting levels of calcium (60–70 nM), but also in response to calcium-mobilizing agonists A23187 and CryIC toxin, which increase intracellular calcium to ~0.6 μM calcium. In contrast, cPLA$_2$ containing the D93N mutation translocates and releases arachidonic acid at intracellular calcium in the 0.2–0.6 μM range but not at resting calcium. This difference in affinity of the mutant cPLA$_2$ enzymes is also evident when assayed in vitro. However, the mutations do not adversely affect the conformation of the active site since they all have significant enzymatic activity when assayed in vitro at millimolar calcium, and they exhibit similar lysophospholipase activity as the wild-type enzyme.

The Role of the C2 Domain in Nuclear Localization of cPLA$_2$

The mechanisms involved in the preferential targeting of cPLA$_2$ to the nuclear envelope are not known. However, an important observation from this study is that the C2 domain of cPLA$_2$ translocates to the nuclear envelope in response to the calcium-mobilizing agonists. This suggests that the sequence determinants for this preferential localization reside in the C2 domain. The C2 domain of cPLA$_2$ has been shown to preferentially bind to phosphatidylcholine headgroups (Nalefski et al., 1998). However, this would not be expected to be responsible for nuclear targeting since phosphatidylcholine is found in all cellular membranes. The C2 domain of Nedd4 also exhibits preferential binding to phosphatidylcholine but has been shown recently to translocate to the plasma membrane in cells treated with calcium ionophore (Plant et al., 1997). Al-

though the C2 domains of Nedd4 and cPLA$_2$ have similar properties including the same topology (topology II), similar affinities for calcium, and preference for binding amphipathic phospholipid, they exhibit considerable sequence diversity that may be responsible for their differences in subcellular localization. The C2 domain of PKCα, which binds phosphatidylserine, has been shown recently to bind to the plasma membrane when expressed in COS cells (Corbalán-García et al., 1999). The membrane binding of the PKCα C2 domain occurs at resting levels of calcium, consistent with its higher affinity for calcium for binding to phospholipid vesicles as compared with the cPLA$_2$ or the Nedd4 C2 domains (Nalefski et al., 1997; Hixon et al., 1998; Corbalán-García et al., 1999).

Results of several recent in vitro studies have begun to shed light on the mechanisms involved in interaction of the C2 domain and full-length cPLA$_2$ with phospholipid vesicles. Experiments have shown that the three calcium-binding loops are involved in the binding of the isolated C2 domain to phosphatidylcholine vesicles (Nalefski and Falke, 1998). These results are consistent with the observation that the two Ca^{2+} ions bound to the C2 domain become occluded, and dissociate more slowly upon binding of the C2 domain to phospholipid vesicles (Nalefski et al., 1997). Regions of the cPLA$_2$ C2 domain that interact with phospholipid micelles have also been identified by ^{15}N-HSQC spectroscopy revealing a role for the calcium-binding loops, proximal ends of attached β strands, and portions of β strands 2 and 3 (Xu et al., 1998). It has been shown recently that calcium induces not only binding but penetration of cPLA$_2$ and the C2 domain into artificial lipid membranes (Davletov et al., 1998; Lichtenbergova et al., 1998). An important role for hydrophobic residues in the interaction of the cPLA$_2$ C2 domain with phospholipid has been implicated from several studies (Davletov et al., 1998; Lichtenbergova et al., 1998; Nalefski and Falke, 1998; Nalefski et al., 1998; Bittova et al., 1999). The residues F35, L39, Y96, and V97 in the calcium binding loops 1 and 3 appear to be particularly important for phospholipid binding and membrane penetration of the C2 domain of cPLA$_2$ (Nalefski and Falke, 1998; Bittova et al., 1999). Two tryptophan residues in the calcium-binding region 3 of PKCα C2 domain also have been shown recently to be essential for binding phospholipid vesicles and membrane penetration in vitro (Medkova and Cho, 1998). Mutagenesis studies of the C2 domain of PKCβII have shown that the calcium-induced interaction of PKC with membrane is not due to charge neutralization of the acidic residues (Edwards and Newton, 1997). However, unlike cPLA$_2$ and PKC in which hydrophobic interaction with phospholipid plays a predominant role, the binding of the C2A domain of synaptotagmin I to phospholipid is largely electrostatic (Davletov et al., 1998; Zhang et al., 1998).

Contribution of Regions Other Than the C2 Domain of cPLA$_2$ in Membrane Binding

Recent studies have demonstrated that the binding properties of the isolated C2 domain of cPLA$_2$ do not necessarily predict the binding properties of the full-length enzyme (Hixon et al., 1998). Binding of the C2 domain to anionic and zwitterionic phospholipid vesicles occurs with simi-lar affinities and requires calcium. However, full-length cPLA$_2$ binds much tighter to anionic (phosphatidylmethanol) than zwitterionic (phosphatidylcholine) vesicles and, surprisingly, the binding to anionic vesicles does not require calcium. Consequently, this implicates regions downstream of the C2 domain in interacting with membranes containing anionic phospholipids. There is evidence that anionic phospholipids, particularly phosphatidylinositol-4,5-bisphosphate (PIP$_2$), enhance enzymatic activity and promote high affinity binding of cPLA$_2$ to phospholipid vesicles (Leslie and Channon, 1990; Buckland and Wilton, 1997; Mosior et al., 1998). Interestingly, PIP$_2$ enhances both calcium-dependent and -independent binding and activity of cPLA$_2$ and may represent a physiological equivalent of phosphatidylmethanol (Hixon et al., 1998; Mosior et al., 1998).

Our results demonstrate that the C2 domain of cPLA$_2$ can translocate to the nuclear envelope in response to calcium-mobilizing agonists but not in response to okadaic acid. This provides evidence in the intact cell that another region of cPLA$_2$ may also be involved in membrane targeting induced by agonists that do not increase intracellular calcium. However, mutating calcium-binding residues in the C2 domain of cPLA$_2$ and depletion of intracellular calcium suppress okadaic acid–induced arachidonic acid release indicating a requirement for the C2 domain and resting levels of calcium for translocation. These data suggest that cPLA$_2$ translocation to membrane induced by okadaic acid may be coordinately regulated by the C2 domain and another region of the enzyme. The cooperative regulation of membrane association by two domains has been observed in numerous proteins including cytohesin-1, Tiam1, Ras-GRF, and phospholipase C, to name a few (Buchsbaum et al., 1996; Williams and Katan, 1996; Stam et al., 1997; Nagel et al., 1998). Membrane binding of phospholipase Cδ1 has been studied extensively, and appears to involve a cooperative role for the PH, C2, and catalytic domains (Williams and Katan, 1996).

Our results demonstrate that the effects of the C2 domain mutations on the ability of cPLA$_2$ to mediate arachidonic acid release correlate directly with their ability to translocate to the nuclear envelope. In addition, a differential functional role is shown for specific calcium-binding residues in the C2 domain in cPLA$_2$ regulation in intact cells. A functional C2 domain is required for agonist-induced translocation of cPLA$_2$ and arachidonic acid release in the Sf9 model even with okadaic acid, which activates cPLA$_2$ without increasing intracellular calcium. This suggests that binding of the C2 domain to calcium, even at resting levels, may be required for stable association of the enzyme with the membrane. Interestingly, okadaic acid induces translocation of the full-length cPLA$_2$, but not the C2 domain. Our results indicate that the C2 domain is necessary and sufficient for cPLA$_2$ translocation to the nuclear envelope in response to calcium-mobilizing agonists. However, a functional C2 domain is necessary but not sufficient for translocation in response to okadaic acid, suggesting that additional regions of the enzyme are also involved.

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