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N-terminal and C-terminal plasma membrane anchoring modulate differently agonist-induced activation of cytosolic phospholipase A_2

Elsa Klapisz[1], Mouloud Ziari[1], Dominique Wendum[1], Kamen Koumanov[1], Corinne Brachet-Ducos[1], Jean-Luc Olivier[1], Gilbert Béréziat[1], Germain Trugnan[2] and Joëlle Masliah[1]

[1]*UPRES-A CNRS 7079 and* [2]*CJF INSERM 9607, CHU Saint-Antoine, Paris, France*

The 85 kDa cytosolic phospholipase A_2 (cPLA$_2$) plays a key role in liberating arachidonic acid from the *sn*-2 position of membrane phospholipids. When activated by extracellular stimuli, cPLA$_2$ undergoes calcium-dependent translocation from cytosol to membrane sites which are still a matter of debate. In order to evaluate the effect of plasma membrane association on cPLA$_2$ activation, we constructed chimeras of cPLA$_2$ constitutively targeted to the plasma membrane by the N-terminal targeting sequence of the protein tyrosine kinase Lck (Lck-cPLA$_2$) or the C-terminal targeting signal of K-Ras4B (cPLA$_2$-Ras). Constitutive expression of these chimeras in Chinese hamster ovary cells overproducing the α_{2B} adrenergic receptor (CHO-2B cells) did not affect the basal release of [^{3}H]arachidonic acid, indicating that constitutive association of cPLA$_2$ with cellular membranes did not ensure the hydrolysis of membrane phospholipids. However, Lck-cPLA$_2$ increased [^{3}H]arachidonic acid release in response to receptor stimulation and to increased intracellular calcium, whereas cPLA$_2$-Ras inhibited it, compared with parental CHO-2B cells and CHO-2B cells producing comparable amounts of recombinant wild-type cPLA$_2$. The lack of stimulation of cPLA$_2$-Ras was not due to a decreased enzymatic activity as measured using an exogenous substrate, or to a decreased phosphorylation of the protein. These results show that the plasma membrane is a suitable site for cPLA2 activation when orientated correctly.

Keywords: cytosolic phospholipase A_2; phosphorylation; plasma membrane; translocation.

Phospholipases A_2 (PLA$_2$) belong to a growing superfamily of enzymes which hydrolyze membrane phospholipids into fatty acids and lysophospholipids, providing the precursors for many of the lipid mediators involved in regulating physiological and pathological processes [1]. There are at least three groups of PLA$_2$s in mammalian cells: Ca^{2+}-dependent secretory PLA$_2$s (sPLA$_2$s), intracellular Ca^{2+}-independent PLA$_2$s (iPLA$_2$s) and the cytosolic 85-kDa Ca^{2+}-dependent PLA$_2$ (cPLA$_2$). Recent evidence indicates that cPLA$_2$ plays a major role in the overproduction of lipid mediators during inflammation [2,3]. cPLA$_2$ activated by extracellular stimuli triggers the rapid hydrolysis of membrane phospholipids to give free arachidonic acid (Δ_4Ach), which is the rate-limiting step of eicosanoid production [4,5]. This activation is dependent on at least two mechanisms: the phosphorylation of cPLA$_2$ by various kinases and its translocation to membrane phospholipids by a Ca^{2+}-dependent lipid-binding domain (CaLB domain) [6], following an increase in intracellular Ca^{2+}. This gives this cytosolic enzyme access to its membrane substrate [7,8]. However, the way in which cPLA$_2$ is activated by extracellular stimuli, and whether this activation occurs at specific sites are not yet known. Various intracellular sites of translocation have been found for cPLA$_2$, depending on the cell type and the stimulus used. They include nuclear and endoplasmic reticulum membranes [9–13], where cPLA$_2$ could colocalize with the enzymes transforming Δ_4Ach into eicosanoids, the cyclooxygenases and lipoxygenases [12,14]. However, little is known about the relative importance of these different sites for cPLA$_2$ activation. The plasma membrane may also be a possible site for translocation of cPLA$_2$, but early studies suggesting the direct interaction of PLA$_2$ with heterotrimeric G proteins have not been confirmed [15]. Recent data indicating that cPLA$_2$ interacts with Jak kinases [16] or is activated by phosphatidylinositol 4,5-bisphosphate [17] have raised the possibility that translocation of cPLA$_2$ to the plasma membrane might be part of its activation by membrane receptors. Such a translocation of cPLA$_2$ to the plasma membrane was recently observed in confluent endothelial cells [18].

In order to evaluate the influence of plasma membrane anchoring on cPLA$_2$ activation, we have constructed chimeras of cPLA$_2$ by adding C-terminal or N-terminal plasma membrane targeting signals to the molecule. We added the plasma membrane targeting signal of the Lck protein kinase to the N-terminus of the cPLA$_2$ [19] and the plasma membrane localizing sequence of K-Ras4B to the C-terminus [20]. These chimeras were expressed in Chinese hamster ovary (CHO) cells overproducing the G-protein-coupled α_{2B} adrenergic receptor (CHO-2B cells). CHO cells contain three forms of PLA$_2$s, like many other cell types, and they seem to play different roles in Δ_4Ach release: an sPLA$_2$, which is not involved in rapid receptor-mediated Δ_4Ach release [4,21], an iPLA$_2$ [22], which

Correspondence to J. Masliah, UPRES-A CNRS 7079, Dept de Biochimie, CHU Saint-Antoine, 27 rue Chaligny, 75012 Paris, France. Fax: + 33 1 4001 1495, Tel.: + 33 1 4001 1341, E-mail: masliah@ccr.jussieu.fr

Abbreviations: CaLB, Ca^{2+}-dependent lipid binding domain; CHO, Chinese hamster ovary; CHO-2B, CHO cells overproducing rat α_{2B} adrenergic receptor; cPLA$_2$, cytosolic PLA$_2$; Δ_4Ach, arachidonic acid; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; iPLA$_2$, Ca^{2+}-independent PLA$_2$; MAP kinase, mitogen-activated protein kinase; PLA$_2$, phospholipase A_2; Sf9, *Spodoptera frugiperda*; sPLA$_2$, secreted PLA$_2$.

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is mainly involved in the remodeling of membrane phospholipids [23], and $cPLA_2$, which is responsible for Δ_4Ach release after stimulation by extracellular agonists or an increase in intracellular Ca^{2+} [4,5]. In CHO-2B cells it was possible to separately induce receptor-mediated phosphorylation and activation of $cPLA_2$ by Ca^{2+} [24]. Stimulation of the receptor with epinephrine causes pertussis toxin-sensitive, Ras-independent activation of mitogen-activated protein kinase (MAP kinase; [25]), leading to $cPLA_2$ phosphorylation [24,26], but has only a modest effect on the release of Δ_4Ach [24]. Free Δ_4Ach is maximally produced by stimulating the cells with both epinephrine and the Ca^{2+} ionophore A23187, which results in a massive entry of extracellular Ca^{2+} [24]. We used this cell model to determine whether targeting $cPLA_2$ to the plasma membrane led to the constitutive hydrolysis of membrane phospholipids or could promote an increase in $cPLA_2$ activation in response to receptor stimulation, increased intracellular Ca^{2+}, or both. Our results show that the N-terminal and C-terminal lipid-modified anchoring sequences used promote a constitutive targeting of $cPLA_2$ to the plasma membrane. In contrast with wild-type $cPLA_2$, the two chimeras have a significant but opposite effect on Δ_4Ach release by receptor stimulation or by Ca^{2+}. These results suggest a new mechanism involving plasma membrane components in the regulation of $cPLA_2$ activation.

EXPERIMENTAL PROCEDURES

Plasmids and recombinant viruses construction

The $cPLA_2$ cDNA was obtained from the wild-type $cPLA_2$/PVL1393 plasmid [27] by PCR. A Kozak consensus sequence was added to the forward primer and a *Nhe*I site was placed just before the stop codon in the reverse primer. The digested PCR product was inserted into the *Kpn*I and *Bgl*II sites of the pcDNA3 to obtain the wild-type $cPLA_2$/pcDNA3. Three successive PCR were performed to epitope-tag $cPLA_2$ at the N-terminus. A Kozak consensus sequence and the epitope tag sequence of human c-Myc (MEQKLISEEDL) were introduced at the N-terminus of wild-type $cPLA_2$ through three successive rounds of PCR by using three overlapping forward primers and a common reverse primer encompassing the unique *Eco*RI site of $cPLA_2$ cDNA. The digested PCR product was then ligated into the *Kpn*I and *Eco*RI sites of the wild-type $cPLA_2$/pcDNA3 to obtain the myc-$cPLA_2$/pcDNA3. A similar procedure was used to fuse the N-terminal 10 amino acids of human Lck (MGCGCSSHPE) to the N-terminal end of $cPLA_2$. The PCR product was ligated into the wild-type $cPLA_2$/pcDNA3 to obtain the Lck-$cPLA_2$/pcDNA3. To obtain the sequence encoding the 18 C-terminal amino acids of mouse K-Ras4B (SKDGKKKKKKSRTRCTVM), a sense oligonucleotide containing a *Nhe*I site and the coding sequence of the first 15 amino acids of the K-Ras4B C-terminus was hybridized to an antisense oligonucleotide encoding the last 12 amino acids of K-Ras4B, a stop codon and a *Xho*I site. The double-stranded DNA fragment was blunt-ended by Vent Polymerase, digested and ligated into the *Nhe*I and *Xho*I sites of the wild-type $cPLA_2$ expression plasmid to obtain the $cPLA_2$-Ras/pcDNA3. The Lck-$cPLA_2$ and $cPLA_2$-Ras cDNA were amplified by using the respective pcDNA3 constructs as template and inserted into the PVL 1393 plasmid. The corresponding PVL 1393 constructs and the linear transfection module from Pharmingen were used to obtain recombinant viruses from *Spodoptera frugiperda* (Sf9) cells cultures. All constructs were verified by DNA sequencing.

Cell culture, stable transfection of CHO-2B cells, infection of Sf9 cells

The CHO cells stably expressing the rat α_{2B} adrenergic receptor (CHO-2B cells) were maintained as described previously [25]. CHO-2B cells were stably transfected with either wild-type $cPLA_2$/pcDNA3, myc-$cPLA_2$/pcDNA3, Lck-$cPLA_2$/pcDNA3 or $cPLA_2$-Ras/pcDNA3 using the calcium phosphate precipitation method. Resistant clones were selected in medium supplemented with 0.8 $mg \cdot mL^{-1}$ G418 and picked out by trypsinization using cloning rings. Individual clones were screened by immunoblot analysis. Stably transfected cells were maintained in complete medium supplemented with 0.65 $mg \cdot mL^{-1}$ G418. All cells were maintained for up to 10 passages after thawing at 37 °C in a 5% CO_2 incubator.

Sf9 insect cells were grown at 27 °C in Grace's medium supplemented with 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, 10% fetal bovine serum. Sf9 cells were allowed to adhere to tissue culture dishes for 1 h and then were infected at a multiplicity of infection of 10–20 for 1 h with the appropriate recombinant virus (wild-type $cPLA_2$, Lck-$cPLA_2$ or $cPLA_2$-Ras recombinant baculovirus). All the experiments were performed at 68–70 h postinfection.

Transient transfection and immunofluorescence analysis

CHO-2B cells were plated on glass coverslips at 3×10^5 cells per well in six-well plates and grown for 18 h. Cells were transfected with 0.5 μg of each indicated plasmid using Lipofectamine-PLUS reagent (Life Technologies). Immunostaining experiments were all performed 48 h after the start of transfection, at room temperature. Transiently transfected cells were washed three times with NaCl/P_i, (140 mM NaCl, 10 mM P_i) fixed with 2% paraformaldehyde in NaCl/P_i for 10 min, washed three times with NaCl/P_i, incubated for 10 min with 50 mM NH_4Cl, again washed three times in NaCl/P_i and then permeabilized with 0.075% saponin in NaCl/P_i for 10 min. Cells were washed three times and incubated for 1 h with primary anti-$cPLA_2$ (1 : 200 dilution of rabbit polyclonal $cPLA_2$ antibody [28], a gift from R. M. Kramer, Lilly Research Laboratories, Indianapolis) and washed three times. They were then incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-(rabbit IgG) (diluted 1 : 100; Jackson ImmunoResearch Laboratories) for 1 h, washed and incubated for 10 min with 1 $mg \cdot mL^{-1}$ RNAse A. Nuclei were stained with 2.5 $\mu g \cdot mL^{-1}$ propidium iodide for 10 min. Cells were examined under a Leica fluorescence microscope and a laser scanning confocal imaging system [29].

Cell fractionation and immunoblot analysis

Confluent CHO cells or infected Sf9 cells were rinsed twice with ice-cold NaCl/P_i, scraped into buffer B (40 mM Tris/HCl pH 7.4, 0.25 M sucrose, 1 mM EDTA, 5 mM dithiothreitol, 100 $\mu g \cdot mL^{-1}$ 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 10 $\mu g \cdot mL^{-1}$ leupeptin, 1 $\mu g \cdot mL^{-1}$ pepstatin A, 1 $\mu g \cdot mL^{-1}$ antipain, 200 μM sodium orthovanadate, 10 mM sodium fluoride) and disrupted by sonication [20 kHz with an MSE (Crawley, Surrey, UK) tip probe at $\approx$ 100 W for 15 s]. Cell lysates were centrifuged at 100 000 ***g*** for 60 min at 4 °C. Supernatant (cytosolic fraction) and pellet (membrane fraction, resuspended in buffer B) were stored at −80 °C at protein concentrations of 1–2 $\mu g \cdot \mu L^{-1}$. Cell lysates (50 μg), cytosolic fractions (25 μg) or membrane fractions (100 μg) from CHO cells and cell lysates from infected Sf9 cells (300 ng) were resolved by 7.5% or 10% SDS/PAGE under reducing

conditions, transferred to nitrocellulose membranes for immunoblot analysis [27]. Blots were probed with 1 $\mu g \cdot mL^{-1}$ mouse monoclonal anti-$cPLA_2$ (Santa-Cruz, sc-454) followed by horseradish peroxidase-conjugated anti-(mouse IgG) (Biosys, France), and detected with the Amersham ECL chemiluminescence system.

Release of [^{3}H]arachidonic acid from CHO and Sf9 cells

Stable transfectants and parental CHO-2B cells (5×10^4 cells per well in 12-well plates) were labeled for 18 h with [^{3}H]Δ_4Ach (0.5 $\mu Ci \cdot mL^{-1}$, Amersham). Under these conditions, the total incorporation of [^{3}H]Δ_4Ach into confluent cells was similar in the parental CHO-2B cells and in the stable transfectants wild-type $cPLA_2$ CHO, myc-$cPLA_2$ CHO, Lck-$cPLA_2$ CHO and $cPLA_2$-Ras CHO cells (respectively 63.39 ± 1.03, 62.77 ± 2.14, 63.67 ± 3.72, 63.02 ± 1.51, 62.75 ± 1.45% of the total radioactivity, mean ± SEM of five independent experiments performed in triplicate). Unincorporated [^{3}H]Δ_4Ach was removed, confluent cells were washed with NaCl/P_i containing 0.2% fatty acid-free BSA, and incubated for 15 min in fresh medium alone (unstimulated cells) or in medium containing 1 μM epinephrine, 1 μM calcium ionophore A23187, or both. The medium was removed and centrifuged at 2000 ***g*** to remove cell debris. The cells were washed and scraped off. The radioactivity in the cells and media was measured by scintillation counting. The release of [^{3}H]Δ_4Ach was expressed as the percentage of the total radioactivity incorporated using the formula [radioactivity in medium/(radioactivity in medium + radioactivity in cells)] × 100. The stimulation of [^{3}H]Δ_4Ach release in each stable transfectant was compared with the stimulation in parental cells performed in the same experiment (Fig. 4C).

Infected Sf9 cells (5×10^5 cells per well in 12-well plates) were labeled at 50 h postinfection with [^{3}H]Δ_4Ach (0.5 $\mu Ci \cdot mL^{-1}$) for 18 h. At 68 h postinfection, cells were incubated with vehicle (unstimulated cells), 1 μM A23187 or 1 μM okadaic acid for 2 h [30]. The stimulation of [^{3}H]Δ_4Ach release was monitored as described above.

Phospholipase A_2 activity

Cell lysates from Sf9 cells producing wild-type $cPLA_2$, $cPLA_2$-Ras or Lck-$cPLA_2$ were isolated as described above. The phospholipase A_2 activity was determined using 4 μM of L-α-1-stearoyl-2-[^{14}C]arachidonyl-phosphatidylcholine (Amersham) [27]. The assay mixture contained 100 mM Tris/HCl pH 8.5, 30% glycerol, 50 μM Triton X-100, 5 mM $CaCl_2$, 0.1% (w/v) fatty acid-free BSA and 300 ng of cell lysates, in a final volume of 250 μL. The mixture was incubated at 37 °C for 10 min and the lipids were extracted using the Bligh and Dyer procedure [27]. After separation by TLC, radioactivity of free fatty acid and phosphatidylcholine was quantified by liquid scintillation counting. The percentage of hydrolysis of the substrate was used to calculate the specific activity of the enzyme in the cell lysates.

Immunoprecipitation

Immunoprecipitation of $cPLA_2$-Ras from $cPLA_2$-Ras CHO cells using an anti-KRas4B and of [^{32}P]-labeled wild-type $cPLA_2$ or $cPLA_2$-Ras from infected Sf9 cells using an anti-$cPLA_2$ antibody was performed as follows. Infected Sf9 cells (68 h postinfection) or confluent CHO cells were washed three times with ice-cold NaCl/P_i containing 1 mM sodium orthovanadate and lysed in 1 mL ice-cold lysis buffer (10 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium orthovanadate, 10 mM sodium fluoride, 30 mM sodium pyrophosphate, 100 $\mu g \cdot mL^{-1}$ AEBSF, 1 $\mu g \cdot mL^{-1}$ leupeptin, 1 $\mu g \cdot mL^{-1}$ pepstatin A, 1 $\mu g \cdot mL^{-1}$ antipain, 1 $\mu g \cdot mL^{-1}$ aprotinin). After sonication (Ultrasonic Cleaner Branson-1200 for 1 min), lysates were clarified by centrifugation at 15 000 ***g*** for 15 min at 4 °C and precleared by incubation with 50 μL mouse or rabbit IgG–agarose beads (Jackson ImmunoResearch Laboratories) for 1 h at 4 °C. The resulting supernatants were transferred to a microfuge tube containing 1.5 μg mouse monoclonal anti-$cPLA_2$ (Santa-Cruz, sc-454), or 1 μg rabbit polyclonal anti-KRas4B (Santa-Cruz, sc-521) and mixed by gentle rotation overnight at 4 °C. Protein A/G PLUS–agarose beads (Santa Cruz) were added for 2 h at 4 °C. The immunoprecipitates were recovered by centrifugation, washed five times with 1 mL ice-cold lysis buffer, boiled for 5 min in 1× Laemmli buffer containing 0.5 mM sodium orthovanadate in the case of [^{32}P]-labeled immunoprecipitates, separated by 7.5% SDS/PAGE, and transferred to a nitrocellulose membrane. The [^{32}P]-labeled immunoprecipitates were vizualized by exposing the nitrocellulose membrane at −80 °C to Kodak XAR films. Immunoblot analysis was performed as described above with anti-$cPLA_2$ (Santa Cruz, sc-454).

Phosphorylation studies in Sf9 cells

Sf9 cells (4×10^6 cells per p60 dish) were labeled at 20 h postinfection with [^{32}P]-orthophosphoric acid (0.5 mCi in 1.5 mL Grace's supplemented medium containing 10% fetal bovine serum) for 48 h. At 68 h postinfection, cells were incubated with vehicle or 1 μM okadaic acid for 2 h, and [^{32}P]-labeled wild-type $cPLA_2$ or $cPLA_2$-Ras were immunoprecipitated overnight, separated by SDS/PAGE and visualized by autoradiography as described above. In each experiment, the amount of immunoprecipitated [^{32}P]-labeled wild-type $cPLA_2$ and $cPLA_2$-Ras was then assessed by anti-$cPLA_2$ immunoblotting.

RESULTS

Targeting of $cPLA_2$ to the plasma membrane by Lck and Ras sequences

$cPLA_2$ was targeted to the plasma membrane by the addition of lipid-modified anchoring sequences. The 10 first amino acid-targeting signal of the Lck protein kinase in which Gly2 is myristoylated and Cys3 and Cys5 are palmitoylated was added at the N-terminus of the $cPLA_2$ (Fig. 1, Lck-$cPLA_2$). This dual acylation motif has been used previously to target cytosolic proteins to the plasma membrane [19]. The C-terminal 18 amino acids of K-Ras4B containing a polybasic domain of six lysine residues and ended by a CAAX box (CTVM) was added to the C-terminus of $cPLA_2$. This CAAX box undergoes postranslational modifications, including farnesylation of the cysteine, proteolysis and carboxymethylation (Fig. 1, $cPLA_2$-Ras). This sequence ensures the plasma membrane localization of K-Ras4B [31], and has been used to anchor cytosolic proteins to the plasma membrane [20,32]. Plasmids containing cDNAs encoding human wild-type $cPLA_2$ (Fig. 1, WT $cPLA_2$) or c-myc epitope-tagged $cPLA_2$ (Fig. 1, myc-$cPLA_2$) were used as controls to compare the effects of cytosolic overproduction of $cPLA_2$ with that of targeting $cPLA_2$ to membranes.

Indirect immunofluorescence microscopy was used to determine the location of endogenous $cPLA_2$ in parental CHO-2B

Fig. 1. Schematic representation of the wild-type cPLA$_2$, myccPLA$_2$, LckcPLA$_2$ and cPLA$_2$Ras constructs. Wild-type cPLA$_2$ (WT cPLA$_2$) was epitope-tagged at its N-terminus with the c-myc epitope tag (myc-cPLA$_2$). cPLA$_2$ was targeted to the plasma membrane by adding N-terminal or C-terminal plasma membrane targeting signal to wild-type cPLA$_2$. The 10 first amino acids of the Lck protein kinase were added to its N-terminus (Lck-cPLA$_2$). This motif is myristoylated on Gly2 (G), and palmitoylated on Cys3 and 5 (C). The 18 last amino acids of K-Ras4B were added to its C-terminus (cPLA$_2$-Ras). This motif undergoes post-translational modifications, including farnesylation of the cysteine (C).

cells. Endogenous cPLA$_2$ was barely detected in CHO-2B cells as punctate staining distributed throughout the cytoplasm (Fig. 2A). Treatment of cells with epinephrine and/or calcium ionophore A23187 caused a significant release of [^{3}H]Δ_4Ach from prelabeled cells [24]. However, stimulation with these agonists produced no detectable translocation of endogenous cPLA$_2$ from cytosol to membranes in agreement with a recent report [33].

The CHO-2B cells were transiently transfected with either wild-type cPLA$_2$, myc-cPLA$_2$, Lck-cPLA$_2$ or cPLA$_2$-Ras expression plasmids. Wild-type cPLA$_2$ and myc-cPLA$_2$ had a similar pattern of distribution within the cytoplasm (Fig. 2B, C, respectively), but with more intense staining than endogenous cPLA$_2$ (Fig. 2A), suggesting that the very weak signal in parental cells was not due to a loss of soluble cPLA$_2$ during the staining procedure. By contrast, Lck-cPLA$_2$ and cPLA$_2$-Ras which contained an N-terminal or C-terminal plasma membrane targeting sequence showed a distribution very different to wild-type cPLA$_2$ with distinct staining in the plasma membrane (Fig. 2D,E, respectively). A vesicular structure adjacent to the nucleus was also stained in cells producing Lck-cPLA$_2$ (Fig. 2D). No signal was detected within the nucleus or the nuclear membrane. These results clearly demonstrated that addition of an N-terminal or C-terminal plasma membrane targeting sequence to cPLA$_2$ promoted plasma membrane association.

Anchoring of cPLA$_2$ to the plasma membrane has no effect on the basal release of [^{3}H]arachidonic acid, but opposite effects on its stimulation by agonists

The effect of the constitutive location of cPLA$_2$ at the plasma membrane on its activation was examined using CHO cell lines stably producing either Lck-cPLA$_2$, cPLA$_2$-Ras, myc-cPLA$_2$ or wild-type cPLA$_2$. The cells were generated by stably transfecting CHO cells overproducing rat α_{2B} adrenergic receptor (CHO-2B cells). Immunofluorescence showed the same pattern of distribution as that of transiently transfected cells, although the staining was less intense (not shown). Cytosol and crude membranes of each cell line were separated and analysed by anti-cPLA$_2$ immunoblotting. Lck-cPLA$_2$, cPLA$_2$-Ras and

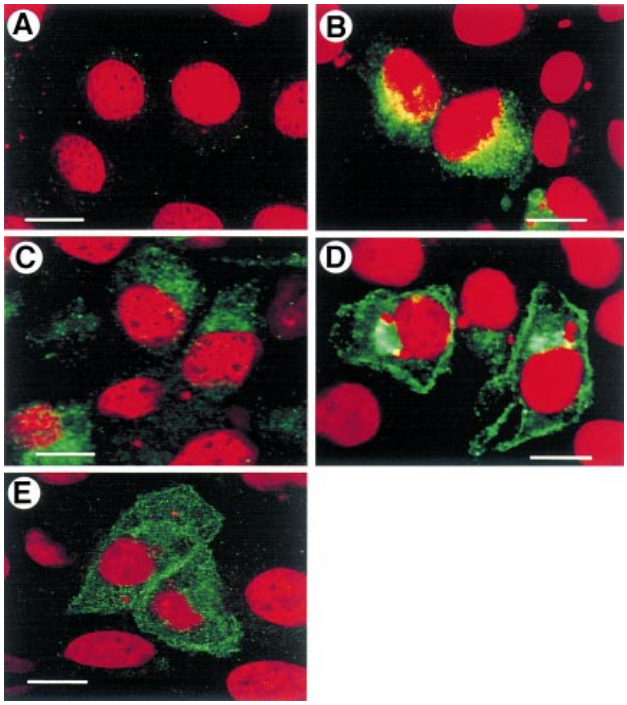

Fig. 2. Subcellular distribution of wild-type cPLA$_2$, myc-cPLA$_2$, Lck-cPLA$_2$ and cPLA$_2$-Ras in CHO-2B cells. Parental CHO-2B cells (A) and cells transiently transfected with either wild-type cPLA$_2$ (B), myc-cPLA$_2$ (C), Lck-cPLA$_2$ (D) or cPLA$_2$-Ras (E) constructs were stained with a polyclonal anti-cPLA$_2$ and a FITC-conjugated second antibody (green). Nuclei were stained with propidium iodide (red). Immunofluorescent staining was visualized by confocal microscopy. Note the plasma membrane distribution of Lck-cPLA$_2$ (D) and cPLA$_2$-Ras (E) as opposed to the punctate cytoplasmic distribution of wild-type cPLA$_2$ (B) and myc-cPLA$_2$ (C). Immunofluorescence analysis was also performed on stable transfectants producing the various forms of cPLA$_2$. The patterns of staining were similar to those of the transiently transfected cells. Bar, 10 μm.

myc-cPLA$_2$ all had reduced electrophoretic mobility, probably due to the addition of 9–18 amino acids, but wild-type cPLA$_2$ did not (Fig. 3). Parental CHO-2B cells contained endogenous cPLA$_2$ mainly in the cytosol, with a small amount in membranes (Fig. 3, CHO-2B). There was increased wild-type cPLA$_2$

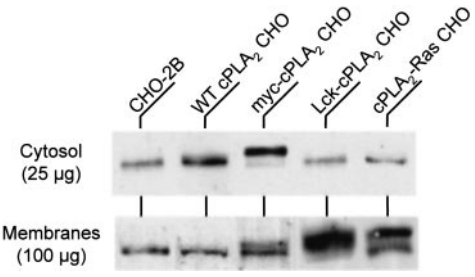

Fig. 3. Distribution of wild-type cPLA$_2$, myc-cPLA$_2$, Lck-cPLA$_2$ and cPLA$_2$-Ras in the cytosolic and membrane fractions of CHO cells. Cytosolic fractions (25 μg) and crude membrane fractions (100 μg) of parental CHO-2B cells and stable transfectants producing wild-type cPLA$_2$, myc-cPLA$_2$, Lck-cPLA$_2$ or cPLA$_2$-Ras were resolved by 7.5% SDS/PAGE (overnight) and detected with a monoclonal anti-cPLA$_2$. Under these conditions, myc-cPLA$_2$, Lck-cPLA$_2$ and cPLA$_2$-Ras have reduced electrophoretic mobility compared with endogenous cPLA$_2$ and wild-type cPLA$_2$.

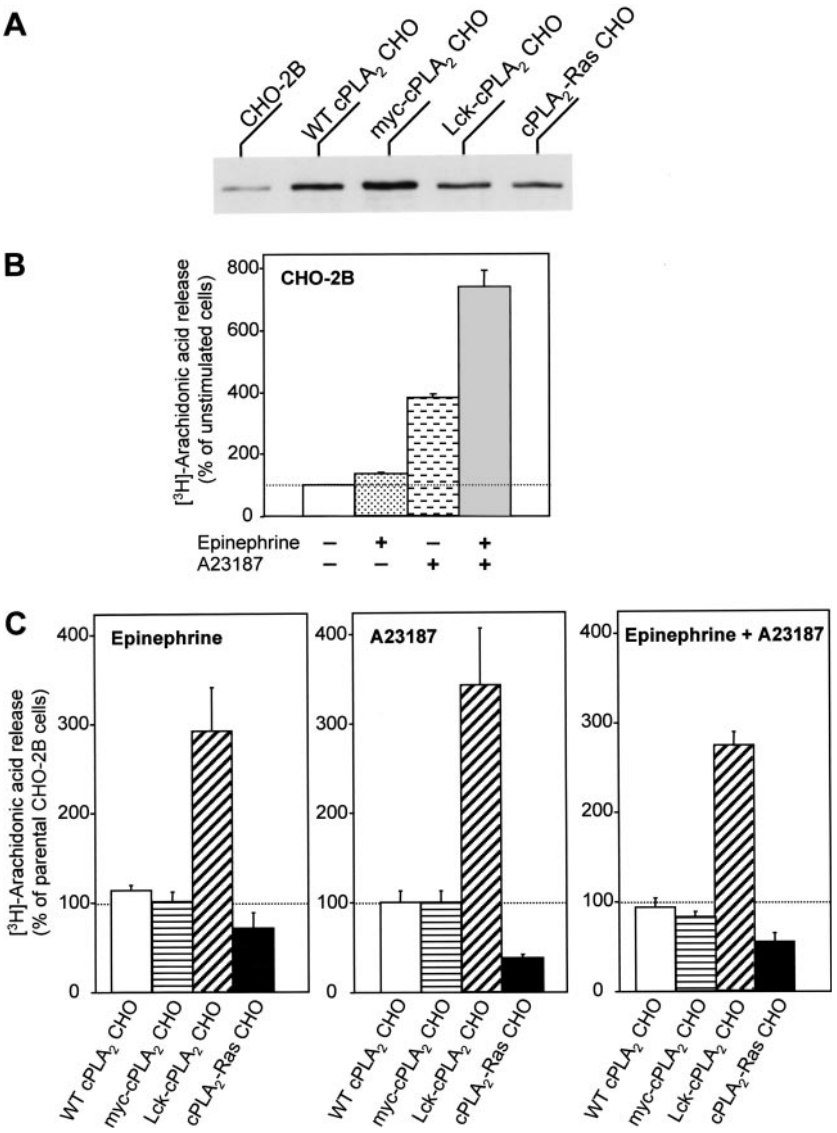

Fig. 4. N-terminal and C-terminal targeting of cPLA$_2$ to the plasma membrane have opposite effects on agonist-induced [^{3}H]arachidonic acid release. (A) Comparison of the amounts of wild-type cPLA$_2$, myc-cPLA$_2$, Lck-cPLA$_2$ and cPLA$_2$-Ras produced in the stable CHO transfectants. Fifty micrograms of total cell lysates were resolved by 10% SDS/PAGE (3 h electrophoresis) and detected with a monoclonal anti-cPLA$_2$ (B) Release of [^{3}H]Δ_4Ach from prelabeled parental CHO-2B cells stimulated with 1 μM epinephrine, 1 μM Ca^{2+} ionophore A23187 or both for 15 min. The stimulation of [^{3}H]Δ_4Ach release is expressed relative to the release from unstimulated cells which was taken as 100% (dotted line). Data are means ± SEM of three independent experiments performed in triplicate. (C) Comparison of [^{3}H]Δ_4Ach release from prelabeled stable transfectants producing wild-type cPLA$_2$ (empty bars), epitope-tagged myc-cPLA$_2$ (horizontal bars), Lck-cPLA$_2$ (hatched bars), or cPLA$_2$-Ras (solid bars) stimulated with 1 μM epinephrine, 1 μM Ca^{2+} ionophore A23187 or both for 15 min. For each stimulus, results are expressed as the net release of [^{3}H]Δ_4Ach from stable transfected cells relative to the net release from parental cells measured in the same experiment and which was taken as 100% (dotted line). Data are means ± SEM of 3–4 independent experiments performed in triplicate.

and myc-cPLA$_2$ in the cytosolic fraction of the corresponding stable transfectants (Fig. 3, WT cPLA$_2$ CHO and myc-cPLA$_2$ CHO), giving between three and five times the amount of endogenous cPLA$_2$ in cell lysates (Fig. 4A), and to a lesser extent there was increased myc-cPLA$_2$ in membranes. In contrast, there was increased Lck-cPLA$_2$ and cPLA$_2$-Ras in the membrane fraction but not in the cytosol of the corresponding stable transfectants (Fig. 3, Lck-cPLA$_2$ CHO and cPLA$_2$-Ras CHO), giving 2.5 and 2 times the amount of endogenous cPLA$_2$ in cell lysates (Fig. 4A).

We then tested the ability of chimeric Lck-cPLA$_2$ and cPLA$_2$-Ras to modify the release of [^{3}H]Δ_4Ach from prelabeled cells. In unstimulated cells, the basal release of [^{3}H]Δ_4Ach was not affected by the overproduction of any of the recombinant cPLA$_2$s (Table 1). This result was expected for wild-type cPLA$_2$ and myc-cPLA$_2$, which are overproduced in the cytosol where they do not have access to their phospholipid substrate. The lack of increase of basal Δ_4Ach release in Lck-cPLA$_2$ and cPLA$_2$-Ras CHO cells was more surprising, showing that constitutive association of cPLA$_2$ with membranes by means other than the CaLB domain does not ensure the hydrolysis of membrane phospholipids in unstimulated cells.

We have previously shown that stimulation of the α_{2B} adrenergic receptor in CHO-2B cells with epinephrine produces a small but significant increase in Δ_4Ach release [24]. The maximal stimulation of Δ_4Ach release was obtained by stimulating the cells with 1 μM epinephrine plus the calcium ionophore A23187, which potentiates the stimulation induced

Table 1. Influence of wild-type cPLA$_2$, myc-cPLA$_2$, Lck-cPLA$_2$ or cPLA$_2$-Ras production on the basal release of [^{3}H]arachidonic acid by CHO-2B cells. Cells were labeled overnight with [^{3}H]Δ_4Ach. Unincorporated [^{3}H]Δ_4Ach was removed, cells were washed with NaCl/P_i containing 0.2% fatty acid-free BSA, and incubated for 15 min in fresh medium. The radioactivity in the cells and media was measured by scintillation counting. Δ_4Ach release is expressed as the percentage of the total radioactivity incorporated. Data are means ± SEM of (*n*) independent experiments performed in triplicate.

Cell line	*n*	Δ_4Ach release (% of total radioactivity)
CHO-2B	21	2.57 ± 0.17
WT cPLA$_2$ CHO	3	2.70 ± 0.15
myc-cPLA$_2$ CHO	4	2.32 ± 0.56
Lck-cPLA$_2$ CHO	5	2.29 ± 0.45
cPLA$_2$-Ras CHO	5	2.25 ± 0.14

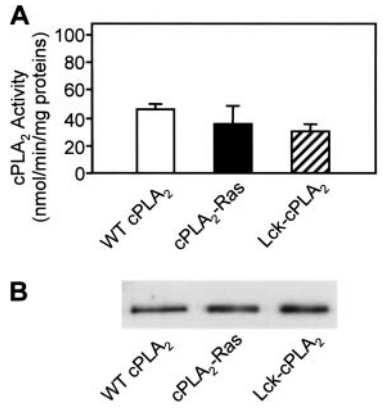

Fig. 5. Specific activity of wild-type $cPLA_2$, $cPLA_2$-Ras and Lck-$cPLA_2$ produced in Sf 9 cells. Sf 9 cells were infected with each indicated recombinant baculovirus and lysates were isolated at 68 h postinfection. (A) $cPLA_2$ activity was measured in 300 ng of total cell lysates using L-α1-stearoyl-2-[^{14}C]arachidonyl-phosphatidylcholine as substrate, as described under Experimental procedures. Data are expressed in nmol substrate hydrolysed per min per mg of total proteins (mean ± SEM of four independent experiments performed in duplicate). The specific activity of lysates from uninfected Sf 9 cells was less than 1 nmol·min^{-1}·mg^{-1} proteins. (B) Comparison of the amounts of each recombinant protein produced in Sf 9 cells. Total cell lysates (300 ng) were resolved by 10% SDS/PAGE and detected with a monoclonal anti-$cPLA_2$.

by each agonist (Fig. 4B). This stimulation was insensitive to specific inhibitors of calcium-independent PLA_2, but was strongly inhibited by specific $cPLA_2$ inhibitors [24]. The stimulation of Δ_4Ach release by these agonists was measured in the transfectants overproducing wild-type $cPLA_2$, myc-$cPLA_2$, Lck-$cPLA_2$ or $cPLA_2$-Ras, and compared with the stimulation in parental CHO-2B cells measured in parallel in the same experiment. In contrast with previous data obtained in CHO cells overproducing high amounts of wild-type $cPLA_2$ [4,5], the

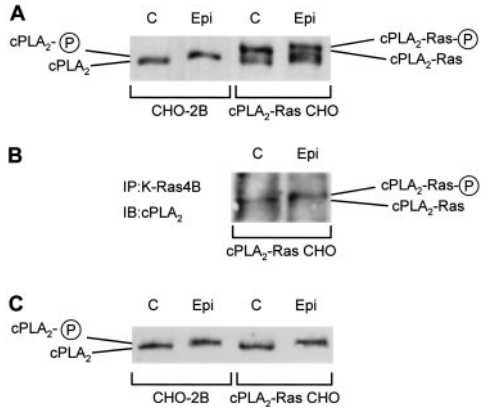

Fig. 6. Agonist-induced mobility shift of $cPLA_2$-Ras in CHO cells. Parental CHO-2B cells and $cPLA_2$-Ras CHO cells were incubated with 1 μM epinephrine (Epi) or vehicle (C) for 15 min. Cytosolic and membrane fractions were isolated as described under Experimental procedures, resolved by 7.5% SDS/PAGE (overnight) and detected with a monoclonal anti-$cPLA_2$. (A) Immunoblot analysis of membrane fractions (100 μg). There were agonist-induced mobility shifts in $cPLA_2$-Ras CHO cells for both endogenous $cPLA_2$ and $cPLA_2$-Ras. (B) anti-$cPLA_2$ immunoblot analysis (IB: $cPLA_2$) of $cPLA_2$-Ras immunoprecipitated from $cPLA_2$-Ras CHO cells with a polyclonal anti-KRas4B (IP: K-Ras4B). (C) Immunoblot analysis of cytosolic fractions (25 μg).

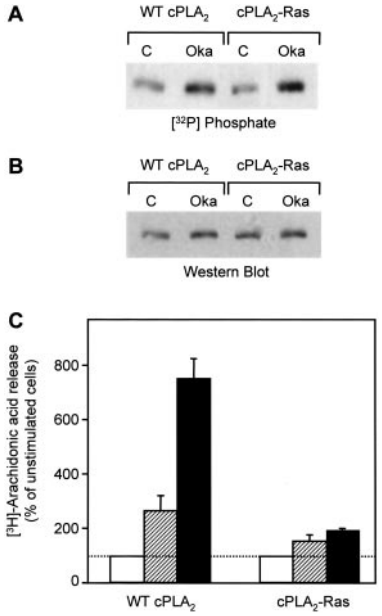

Fig. 7. Agonist-induced phosphorylation and [^{3}H]arachidonic acid release from prelabeled Sf 9 cells producing wild-type $cPLA_2$ or $cPLA_2$-Ras. (A) Sf 9 cells were infected with wild-type $cPLA_2$ or $cPLA_2$-Ras recombinant baculovirus. At 20 h postinfection, cells were labeled with [^{32}P]phosphate for 48 h. At 68 h postinfection, cells were incubated with vehicle (C) or 1 μM okadaic acid (Oka) for 2 h. [^{32}P]-labeled wild-type $cPLA_2$ or $cPLA_2$-Ras were immunoprecipitated from cell lysates with a monoclonal anti-$cPLA_2$, separated by 7.5% SDS/PAGE and visualized by autoradiography. Results are from one representative experiment out of three. (B) The amount of immunoprecipitated wild-type $cPLA_2$ or $cPLA_2$-Ras was assessed by anti-$cPLA_2$ immunoblotting. (C) Infected Sf 9 cells were prelabeled with [^{3}H]Δ_4Ach and incubated at 68 h postinfection with vehicle (empty bars), 1 μM A23187 (hatched bars) or 1 μM okadaic acid (solid bars) for 2 h. The stimulation of [^{3}H]Δ_4Ach release is expressed relative to the release from unstimulated cells which was taken as 100% (dotted line). Results are means ± SD of triplicate values from one representative experiment out of four. Basal levels of [^{3}H]Δ_4Ach release from Sf 9 cells producing wild-type $cPLA_2$ or $cPLA_2$-Ras were comparable.

moderate overproduction of $cPLA_2$ in wild-type $cPLA_2$ and myc-$cPLA_2$ CHO cells did not affect agonist-induced Δ_4Ach release (Fig. 4C, empty and horizontal bars, respectively). In contrast, the constitutive production of Lck-$cPLA_2$ increased the release of [^{3}H]Δ_4Ach by threefold compared with parental and wild-type $cPLA_2$ CHO cells in response to epinephrine, calcium ionophore A23187, or both, showing that N-terminal anchoring of $cPLA_2$ to the plasma membrane had a positive effect on its stimulation (Fig. 4C, hatched bars), but was still sensitive to calcium. However, $cPLA_2$-Ras decreased the release of Δ_4Ach induced by the same agonists twofold compared with parental cells (Fig. 4C, solid bars). This effect of $cPLA_2$-Ras suggests that the C-terminal anchoring of $cPLA_2$ not only inhibits its activation, but also interferes with the activation of endogenous $cPLA_2$.

The membrane-bound $cPLA_2$-Ras is enzymatically active, is phosphorylated but is weakly activated in response to stimuli

The first explanation for the lack of activation of $cPLA_2$-Ras was that C-terminal anchoring decreased or abolished the enzymatic activity of the protein. To verify this hypothesis, we first measured the $cPLA_2$ enzymatic activity in membranes

isolated from parental CHO-2B cells and cPLA$_2$-Ras CHO cells using an exogenous radiolabeled substrate [27]. The very low activity in both cell lines prevented evaluation of any significant difference between endogenous cPLA$_2$ and cPLA$_2$-Ras (not shown). We then used a more sensitive assay to measure the ability of cPLA$_2$-Ras to hydrolyze membrane phospholipids *in vitro*. Crude membranes were incubated with calcium. The release of free fatty acids was quantified by GC coupled to MS [34], showing that cPLA$_2$-Ras released fatty acids as efficiently as endogenous cPLA$_2$ (not shown). In order to compare more accurately the activity of cPLA$_2$-Ras with that of wild-type cPLA$_2$, we used the baculovirus–Sf9 insect cell system which produces high levels of recombinant proteins. Moreover, uninfected Sf9 cells can be considered to be free of cPLA$_2$ as they contain no immunoreactive cPLA$_2$ and have no cytosolic PLA$_2$ activity when measured using an exogenous radiolabeled arachidonyl-phosphatidylcholine (not shown). Figure 5 shows that wild-type cPLA$_2$ and cPLA$_2$-Ras as well as Lck-cPLA$_2$, produced at the same level in Sf9 cells (Fig. 5B), exhibit similar specific activities (Fig. 5A).

Next we examined the possibility that the lack of cPLA$_2$-Ras activation was because of a lack of phosphorylation in response to agonists. Several studies have shown that agonist-induced phosphorylation of cPLA$_2$ on Ser505 reduces its electrophoretic mobility on SDS/PAGE [26]. Incubation of CHO-2B cells with epinephrine induced a gel shift of endogenous cPLA$_2$ in membrane fractions (Fig. 6A, CHO-2B). The same gel retardation was observed in membrane fractions of cPLA$_2$-Ras CHO cells for both endogenous cPLA$_2$ and cPLA$_2$-Ras (Fig. 6A, cPLA$_2$-Ras CHO). The mobility shift of cPLA$_2$-Ras was confirmed by immunoprecipitation of the chimera using an anti-KRas4B (Fig. 6B). In addition, expression of cPLA$_2$-Ras did not seem to prevent the phosphorylation of endogenous cPLA$_2$ as epinephrine caused a mobility shift of endogenous cPLA$_2$ in the cytosol of both CHO-2B and cPLA$_2$-Ras CHO cells (Fig. 6C). We then compared the phosphorylation level of cPLA$_2$-Ras and wild-type cPLA$_2$ by producing the recombinant proteins in Sf9 cells which perform the same post-translational modifications of recombinant proteins as do mammalian cells, including phosphorylation of human recombinant wild-type cPLA$_2$ [30]. The phosphorylation of wild-type cPLA$_2$ and cPLA$_2$-Ras was examined using Sf9 cells metabolically labeled with [^{32}P]phosphate for 48 h. The amounts of [^{32}P] incorporated into wild-type cPLA$_2$ and cPLA$_2$-Ras were identical in unstimulated cells (Fig. 7A) producing the same amounts of recombinant cPLA$_2$s (Fig. 7B). Stimulation of the cells with the phosphatase inhibitor okadaic acid caused similar increases in [^{32}P] incorporation into both wild-type cPLA$_2$ and cPLA$_2$-Ras (Fig. 7A).

We therefore used this cell system to evaluate the contributions of wild-type cPLA$_2$ and cPLA$_2$-Ras to Δ_4Ach release after stimulation with agonists. Sf9 cells infected with wild-type cPLA$_2$ or cPLA$_2$-Ras recombinant baculovirus were labeled with [^{3}H]Δ_4Ach and stimulated with 1 μM okadaic acid or 1 μM calcium ionophore A23187 for 2 h. The basal release of [^{3}H]Δ_4Ach was not affected by the production of wild-type cPLA$_2$ or cPLA$_2$-Ras (not shown). In agreement with a previous study [30], the production of wild-type cPLA$_2$ in Sf9 cells increased [^{3}H]Δ_4Ach release by 2.5-fold and sevenfold over the control values in response to calcium ionophore and okadaic acid, respectively (Fig. 7C). In contrast, a similar content of cPLA$_2$-Ras as wild-type cPLA$_2$ produced little increase in [^{3}H]Δ_4Ach release by the same agonists, reaching 1.5-fold the control values for calcium ionophore and 1.8-fold for okadaic acid.

Caveolin binding motif	Φ X Φ X X X X Φ
Human cPLA$_2$	**F Q Y P N Q A F**
Mouse cPLA$_2$	**F Q Y P N Q A F**
Chicken cPLA$_2$	**F Q Y P N E A F**
Fish cPLA$_2$	**F K Y N N Q A F**
PKC-α	**F S Y V N P Q F**

Fig. 8. A putative caveolin-binding motif, located at the C-terminal end of cPLA$_2$ (residues 683–690) is conserved from human to zebrafish and is identical to the caveolin binding motif of PKCα, a known caveolin-interacting protein. Φ represents aromatic residues and X denotes any amino acid.

DISCUSSION

We have used CHO cells overproducing the rat α_{2B} adrenergic receptor (CHO-2B cells) as cells that discriminate between receptor-specific phosphorylation and activation of cPLA$_2$ by calcium. These two key events leading to the activation of cPLA$_2$ can be induced separately in these cells, phosphorylation by epinephrine and activation by calcium ionophore [24]. Endogenous cPLA$_2$ is poorly expressed in CHO-2B cells and is found mainly in the cytosol, as shown by indirect immunofluorescence microscopy and by immunoblotting of cytosolic and membrane fractions. Using these techniques we detected no translocation to membranes in response to epinephrine, calcium ionophore or both. This contrasts with the findings of other studies, in which increasing intracellular calcium or stimulating G-protein-coupled receptors caused the translocation of cPLA$_2$ to the endoplasmic reticulum [10], to the nuclear membrane [9,10,13] or to the plasma membrane [18]. But this agrees with other recent results that found no detectable translocation in EGF/A23187-stimulated Her 14 fibroblasts [33]. However, in studies where cPLA$_2$ was shown to translocate to a definite membrane, it is possible that small amounts of cPLA$_2$ were translocated to other membrane sites but were not detected. In addition, even when cPLA$_2$ has been demonstrated to translocate to a definite cell compartment, there is still no clear evidence that cPLA$_2$ is activated at these sites.

The present study uses another approach to evaluate a possible regulatory role of plasma membrane association on cPLA$_2$ activation. We constructed chimeras of cPLA$_2$, which were constitutively targeted to the plasma membrane by N-terminal or C-terminal lipid-modified anchoring sequences (Lck-cPLA$_2$ and cPLA$_2$-Ras), whereas wild-type cPLA$_2$ and epitope-tagged cPLA$_2$ (myc-cPLA$_2$) remained in the cytosol. Four cell lines overproducing these recombinant cPLA$_2$s to the same extent were established to evaluate the influence of plasma membrane targeting on cPLA$_2$ activation.

The release of [^{3}H]Δ_4Ach from prelabeled cells was used to assay cPLA$_2$ activation in whole cells. The basal release of Δ_4Ach was unaffected by the overproduction of cPLA$_2$ in cytosol (wild-type cPLA$_2$ and myc-cPLA$_2$). This is in agreement with previous results showing that iPLA$_2$ is involved in the basal release of Δ_4Ach, while cPLA$_2$ is not [23]. More surprisingly, the basal release of Δ_4Ach was also unchanged by the constitutive presence of Lck-cPLA$_2$ and cPLA$_2$-Ras at the plasma membrane (Table 1). This shows that constitutive association of cPLA$_2$ with membranes is not sufficient to cause the hydrolysis of membrane phospholipids.

In contrast, the N-terminal anchoring of cPLA$_2$ enhanced its ability to be activated by stimuli. The constitutive production of Lck-cPLA$_2$ increased the release of [^{3}H]Δ_4Ach stimulated by epinephrine threefold (Fig. 4C), showing its responsiveness to G-protein-coupled receptor transduction pathway. Surprisingly,

stimulation with calcium ionophore was also enhanced in Lck-cPLA$_2$ CHO cells over that of wild-type cPLA$_2$ CHO cells (Fig. 4C), showing that N-terminal plasma membrane anchoring of cPLA$_2$ does not render it insensitive to calcium. This confirms that the CaLB domain of cPLA$_2$ is not only necessary for cPLA$_2$ translocation to membranes, but may also take part in activation by promoting specific interaction with phospholipids [35,36], thus allowing the orientation of the catalytic domain of the enzyme.

cPLA$_2$-Ras produced at the same level as Lck-cPLA$_2$ cells did not enhance the release of Δ_4Ach induced by the agonists, and even inhibited it significantly (Fig. 4C). This lack of activation was confirmed by the weak stimulation of Δ_4Ach release by Sf9 cells producing cPLA$_2$-Ras, but devoid of endogenous cPLA$_2$ (Fig. 7C). The three-dimensional structure of cPLA$_2$ has been elucidated recently [37] confirming that the amino acids of the active site lie within a large C-terminal domain of the enzyme [6,38]. We could not rule out that the addition of a C-terminal sequence to cPLA2 might have disturbed the conformation of the enzyme resulting in decreased activity. But this did not seem to be the case, because cPLA$_2$-Ras produced in Sf9 cells exhibited a specific PLA$_2$ activity in the same range than that of wild-type cPLA$_2$ and Lck-cPLA$_2$ (Fig. 5A).

We also examined the possibility that cPLA$_2$-Ras was unphosphorylated in response to agonists and/or prevented the phosphorylation of endogenous cPLA$_2$. Phosphorylation of cPLA$_2$ by various kinases, including MAP kinases, is presumed to occur in the cytosol and to be required for its translocation [39] and activation [26]. Hence, the membrane-bound cPLA$_2$-Ras might not be accessible to phosphorylation by kinases. Our results show that both the recombinant cPLA$_2$-Ras and the endogenous cPLA$_2$ were shifted in epinephrine-stimulated cPLA$_2$-Ras CHO cells, showing that the membrane-bound cPLA$_2$-Ras was phosphorylated and did not prevent the phosphorylation of endogenous cPLA$_2$ (Fig. 6). Moreover, the phosphorylation level of wild-type cPLA$_2$ and cPLA$_2$-Ras was identical in unstimulated Sf9 cells and was increased to the same extent by stimulating the cells with the phosphatase inhibitor okadaic acid (Fig. 7A). Hence, the lack of activation of cPLA$_2$-Ras is not due to either defective enzymatic activity or to a lack of phosphorylation, but perhaps to the inappropriate orientation of the active site towards membrane phospholipids *in situ* [37].

Our results show that plasma membrane anchoring of cPLA$_2$ by a N-terminus or a C-terminus lipid-modified targeting sequence has a major influence on its activation. The anchoring might modify the configuration of the enzyme or its accessibility to the substrate, altering its ability to be activated *in situ*. However, this does not explain why Lck-cPLA$_2$ is activated more efficiently than wild-type cPLA$_2$.

The two plasma membrane targeting sequences used to construct Lck-cPLA$_2$ and cPLA$_2$-Ras belong to the Src family of protein tyrosine kinases and to the family of p21 Ras monomeric G proteins. These two classes of proteins associate with specialized domains of the plasma membrane, the caveolae, where a variety of signaling molecules are concentrated. Therefore, another hypothesis to explain our results is that the chimeras Lck-cPLA$_2$ and cPLA$_2$-Ras could be targeted to the caveolae. The major structural component of caveolae is caveolin, a 21 to 24-kDa integral membrane protein. Caveolin 1 interacts directly with a variety of signaling molecules [Ha-Ras, Src family tyrosine kinases, G protein α subunits, endothelial NOS, epidermal growth factor (EGF) receptor and protein kinase C (PKC) isoforms]. This interaction is mediated by a scaffolding domain within caveolin, which recognizes a common sequence motif within caveolin-binding proteins (ΦXΦXXXXΦ and ΦXXXXΦXXΦ, where Φ is an aromatic amino acid and X denotes any amino acid; 40). We found a putative caveolin binding motif (FQYPNQAF) in the C-terminal end of the cPLA$_2$ sequence (residues 683–690; Fig. 8). This motif is present in all species examined to date, from human to zebrafish [6], and is identical to the caveolin-binding motif of PKC-α, a known caveolin-interacting protein [40].

Furthermore, preliminary results of co-immunoprecipitation show that Lck-cPLA$_2$, but not cPLA2-Ras or endogenous cPLA2, interacts with caveolin 1 (unpublished results). This interaction might occur through the caveolin-binding motif of cPLA$_2$ as the 10 first amino acids of Lck do not contain any caveolin-binding motif. Therefore, it seems likely that the N-terminal targeting of Lck-cPLA$_2$ allows its interaction with caveolin 1, which might account for its enhanced activation. This hypothesis is consistent with the caveolar location of molecular components involved in the regulation of cPLA$_2$ activation. For example, the MAP kinases ERK-1/2 are concentrated in caveolae membranes [41] and caveolin regulates the p42/44 MAP kinase cascade [42] involved in the agonist-induced phosphorylation of cPLA$_2$. The protein p11, a member of S100 family proteins, forms a heterotetrameric annexin II$_2$p11$_2$ complex that is concentrated in caveolae [43]. This protein interacts with a large C-terminal domain of cPLA$_2$, inhibiting its enzymatic activity [44]. Also, phosphatidylinositol 4,5-bisphosphate, which is concentrated in caveolae [45], interacts with a putative pleckstrin homology domain of cPLA$_2$, resulting in increased enzymatic activity [17]. Similarly, diacylglycerol, which is also concentrated in caveolae [46] promotes cPLA$_2$ activity [47]. Consistent with this hypothesis, preliminary experiments on an epithelial cell line show that endogenous cPLA2 is located at the plasma membrane in unstimulated cells and that cPLA2 cofractionates with caveolin 1 in detergent-resistant membranes (unpublished results).

Taken together, our results show that anchoring cPLA$_2$ to the plasma membrane has no effect on phospholipids hydrolysis in unstimulated cells, but modulates its activation. Moreover, the way cPLA$_2$ is anchored determines its ability to release arachidonic acid in response to stimuli. Finally, these results show that plasma membrane is a suitable site for cPLA$_2$ activation when correctly orientated. Moreover, interaction of cPLA$_2$ with plasma membrane microdomains such as caveolae should be explored further as a possible regulatory mechanism in cPLA$_2$ activation.

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