Ultrastructural localization of cPLA2 in unstimulated and EGF/A23187-stimulated fibroblasts

Gertrude Bunt1,*, Joris de Wit1, Henk van den Bosch2, Arie J. Verkleij1 and Johannes Boonstra1

1Department of Molecular Cell Biology, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands
2Centre for Biomembranes and Lipid Enzymology, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands
*Author for correspondence (e-mail: gertrude@emsaserv.biol.ruu.nl)

SUMMARY

The 85 kDa cytosolic phospholipase A2 is the key enzyme in the release of arachidonic acid. To gain insight into cytosolic phospholipase A2 action in mitogen-activated cells, the localization of the phospholipase was investigated in fibroblasts upon stimulation with epidermal growth factor and the calcium ionophore A23187. By the use of indirect immunofluorescence microscopy, staining of endogenous cytosolic phospholipase A2 resulted in a punctate labeling pattern randomly distributed throughout the cytoplasm of the cell. Immunogold electron microscopy revealed that this punctate labeling pattern exhibited the presence of the 85 kDa phospholipase A2 in small clusters. These clusters were found in the cytosol in the vicinity of all organellar membranes, except for the Golgi system. The enzyme showed no preference for the nuclear envelope, the endoplasmic reticulum or the plasma membrane. Stimulation of cells with epidermal growth factor or A23187 or both did not change the punctate immunofluorescence labeling pattern. Furthermore, a similar labeling pattern was observed by the artificial introduction of extremely low or high intracellular calcium concentrations. Even by electron microscopy, translocation of cytosolic phospholipase A2 to membranes was not observed after stimulation of cells with epidermal growth factor and A23187. From these results it is concluded that cytosolic phospholipase A2 is localized in clusters close to membranes in stimulated as well as unstimulated fibroblasts, without preference for a specific organellar membrane.

Key words: cPLA2, Cluster, Epidermal growth factor, Cryoultramicrotomy

INTRODUCTION

Cytosolic phospholipase A2 (cPLA2), a high molecular mass (85 kDa) form of the phospholipase A2 family, preferentially hydrolyses arachidonic acid at the sn-2 position of phospholipids (Clark et al., 1991, 1995). Arachidonic acid release is the rate-limiting step in the biosynthesis of prostaglandins and leukotrienes. Because of its selectivity for arachidonic acid an important role has been ascribed to cPLA2 in the production of the eicosanoids and thus also in many biological processes mediated by the eicosanoids, such as cell migration (Tertoolen et al., 1994) and mitogenic signalling (Handler et al., 1990).

The activation of cPLA2 is dependent on submicromolar to micromolar concentrations of Ca\(^{2+}\) (Clark et al., 1991). Ca\(^{2+}\) induces translocation of the enzyme from the cytosol to membranes (Channon and Leslie, 1990; Yoshihara and Watanabe, 1990; Clark et al., 1991; Rehfeldt et al., 1993; Schalkwijk et al., 1995). The Ca\(^{2+}\)-dependent phospholipid binding domain of the enzyme is essential for this translocation process (Nalefski et al., 1994). Besides the Ca\(^{2+}\)-induced membrane translocation, cPLA2 can be activated by phosphorylation. It has been shown in vitro that the serine/threonine-specific mitogen activated protein (MAP) kinase is able to phosphorylate cPLA2 on residue Ser-505 (Lin et al., 1993; Nemenoff et al., 1993). Phosphorylation of this residue was also found in vivo after stimulation of cells by various extracellular stimuli (Lin et al., 1993; Nemenoff et al., 1993; Qiu et al., 1993). Using site-directed mutagenesis it was shown that MAP kinase-mediated phosphorylation of Ser-505 is required for agonist-induced arachidonate release (Lin et al., 1993).

cPLA2 can be activated by several mitogens, including platelet-derived growth factor (PDGF) (Domin and Rozengurt, 1993), transforming growth factor-\(\alpha\) (TGF-\(\alpha\)) (Kast et al., 1993) and epidermal growth factor (EGF) (Bonventre et al., 1990; Spaargaren et al., 1992; Schalkwijk et al., 1995) suggesting a role in regulation of cell proliferation. Insight into the processes involved in cell proliferation requires an understanding of the molecular components involved.

In earlier studies it was shown that EGF induces phosphorylation of cPLA2 and that the EGF-induced rise in [Ca\(^{2+}\)]\(_i\) results in a translocation of cPLA2 from the cytosol to the membranes (Schalkwijk et al., 1995). Recently, we investigated the role of the two EGF-induced pathways involved in the activation of cPLA2 in vivo using two fibroblast cell lines, Her14 and Herc13 (Schalkwijk et al., 1996). Upon sequential stimulation of the cells with EGF and A23187 and vice versa, it was possible to discriminate between the effect of Ca\(^{2+}\) and MAP kinase in EGF-dependent activation of cPLA2. It was shown that phosphorylation has to precede the calcium-
induced translocation to membranes for maximal activation of cPLA2.

However, an understanding of cPLA2 action in mitogen-activated cells also requires knowledge of the cellular localization of cPLA2. In this study, we therefore have extended these investigations by localizing cPLA2. Using indirect immunofluorescence microscopy it is shown that endogenous cPLA2 is confined to punctate structures in the cytoplasm of EGF and A23187-stimulated fibroblasts as well as in unstimulated fibroblasts. The localization of cPLA2 is investigated in detail using immunogold electron microscopy. At the ultrastructural level, the punctate pattern, as seen by immunofluorescence microscopy, is expressed by the cytoplasmatic localization of cPLA2 in small clusters in the vicinity of membranes. These clusters do not prefer a specific organelar membrane. The labeling is independent of stimulation with EGF and A23187 indicating that the membrane translocation of cPLA2 apparently occurs only over a small distance not even visible at the ultrastructural level. The observation that cPLA2 is localized in clusters may have important implications for the regulation and function of cPLA2.

MATERIALS AND METHODS

Cell culture and stimulation

All studies were carried out with Her14 and Herc13 fibroblasts, unless indicated otherwise. These cells are mouse 3T3(0) fibroblasts overexpressing the human EGF-receptor cDNA. Cells were cultured in DMEM supplemented with 7.5% FCS in a humidified atmosphere of 7% CO2 for at least 48 hours at 37°C. Subconfluent cultures were used in all experiments.

Prior to activation, cells were serum-starved by overnight exposure to serum-free medium. Cells were activated with 200 ng/ml EGF or 1 μM A23187 at 37°C for the indicated time periods. In translocation studies with artificially induced calcium levels, cells were exposed to either a medium containing 2 mM Ca2+ or a Ca2+-free medium supplemented with 1 mM EGTA for 15 minutes. Next, 1 μM A23187 was added to the medium and cells were incubated for 10 minutes.

Cell lysates and cell fractionation

The medium was discarded and the cells were washed twice with ice-cold PBS. Cells were scraped in homogenization buffer (50 mM Hepes, pH 7.4, 250 mM sucrose, 50 mM NaF, 10 mM leupeptin, 1 mM pepstatin, 250 mM Na3VO4, 0.1 mM PMSE). The scraped cells were sonicated three times at 60 W for 5 seconds using a Branson sonicator equipped with a micro tip to obtain a cell lysate. Alternatively, the scraped cells were sheared by ten passages through a 23 gauge needle when needed for fractionation studies. The resulting homogenate was centrifuged at 1,500 g for 5 minutes and the ensuing supernatant was centrifuged at 200,000 g for 30 minutes to obtain the particulate fraction and the cytosolic fraction.

Extraction of the cytoskeleton

Cells were washed twice with PBS and extracted with 0.5% Triton X-100 in CSK-buffer (10 mM Pipes, pH 6.8, 250 mM sucrose, 3 mM MgCl2, 150 mM KCl, 10 μM leupeptin, 5 μM aprotinin, 250 μM Na3VO4, 50 mM NaF, and 1 mM PMSE) for 5 minutes. The extraction was performed at room temperature to preserve the tubulin network. The Triton X-100 solubilized fraction was removed and the remnants of the lysed cells, the cytoskeleton and nuclei, were washed briefly with 0.5% Triton X-100 in CSK-buffer, scraped off the dish in CSK-buffer, transferred to microcentrifugation vials and used for immunoblotting.

Immunoprecipitation

Cells were washed three times with ice-cold PBS and lysed in 750 μl radioimmunoassay (RIPA) buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM Na2HPO4, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 50 mM NaF, 250 μM Na3VO4). Lysates were incubated on ice for 5 minutes and clarified by centrifugation at 14,000 g for 5 minutes at 4°C. Protein A-Sepharose beads (Pharmacia, Upssala, Sweden) were gently mixed with the lysates for 1 hour at 4°C, to precipitate the lysates. Next, 1 μg of the monoclonal antibody raised against cPLA2 (Santa Cruz, sc-454) was added to the lysates and mixed by gentle rotation overnight at 4°C. Protein A-Sepharose beads were added for 1 hour to precipitate the immune complexes. Finally, the immune complexes were washed once with RIPA buffer and twice with 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Triton X-100 and subjected to electrophoresis and immunoblotting.

Protein determination, SDS-PAGE, immunoblotting

Protein concentrations were determined using the Bio-Rad Coomassie-based or BCA protein determination kit according to the instructions provided by the manufacturer. Proteins were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose by semi-dry blotting onto a Nova blot II apparatus (Pharmacia). cPLA2 was detected with a monoclonal or polyclonal antibody obtained from Santa Cruz (cat # sc-454, sc-438) which are both raised against the amino-terminal domain (amino acids 1-216) of cPLA2. The antibodies are used at a concentration of 0.08 μg/ml. Tubulin was detected with a rat monoclonal antibody from Sera-lab (MAS 77p) and actin with a mouse monoclonal from Amersham (N350). Subsequently, primary antibodies were detected with goat anti-rabbit, goat anti-rat or goat anti-mouse IgG conjugated to horseradish peroxidase and the signal was visualized by chemiluminescence (Amersham).

Immunofluorescence microscopy

Cells were grown on gelatin coated glass coverslips. Serum-exposed cells were briefly washed with PBS and fixed with 4% formaldehyde in PBS for 30 minutes at room temperature. Serum-starved and stimulated cells were fixed for 60 minutes on ice. After fixation, cells were washed twice with 100 mM glycine in PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, washed twice with 2% gelatin in PBS and incubated with the primary antibody raised against cPLA2 (Santa Cruz; sc-454, sc-438) at a concentration of 0.4 μg/ml for 1 hour at room temperature. As a control, the antibody sc-454 was pre-blocked with a 10-fold molar excess of cPLA2 (kind gift of Dr. R. M. Kramer, Lilly Research Laboratories, Indianapolis) for one hour at room temperature. Next, cells were washed four times with PBS-gelatin and subsequently incubated with GAM-FITC or GAR-FITC for 1 hour at room temperature. Labeled cells were washed five times with PBS-gelatin, twice with PBS and once with distilled water and finally mounted in Mowiol-PPD (Mowiol-4-88, 25% glycerol, 1 mg/ml para-phenylenediamine).

For staining of the cytoskeletal components, cells were fixed with 3% formaldehyde, 0.25% glutaraldehyde and 0.25% Triton X-100 in PBS for 15 minutes at room temperature, washed twice with PBS and subsequently incubated with 1 mg/ml NaBH4 in PBS for 5 minutes. After washing with PBS-gelatin, cells were incubated as described above. Tubulin was labeled with a rat monoclonal antibody from Seralab. As secondary antibodies GAM-Cy3 and GAR-FITC were used. Actin was directly labeled using phallolidin-FITC.

Cells were viewed on a Zeiss Axioplan fluorescence microscope with fluorescein or rhodamine filter settings. Within experiments, photographs were taken with identical exposure times. Confocal laser scanning microscopy was performed with a Bio-Rad 500 confocal unit on a Zeiss Axioplan microscope.

Electron microscopy

For electron microscopy, serum-exposed cells were fixed with 2% formaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1.5 hours at room temperature. An equal volume of twice-concentrated fixative was added to the culture medium and was replaced...
Cells were briefly washed in PB-50 mM glycine and transferred to 10% gelatin-PB at 37°C. After pelleting the cells, the gelatin was solidified gently scraped from the bottom of the dish with a rubber policeman.

Sections were contrasted and subsequently embedded by incubation with 1% GA in PBS for 5 minutes and washed with distilled water. Coated grids were placed on 2% gelatin-PB at 4°C overnight.

For immunogold labeling, the gelatin was melted at 37°C and the grids were placed on 100 mM glycine in PBS for at least 5 minutes. After blocking twice with PBG (0.5% BSA Fr V, 0.1% (w/v) cold water fish gelatin 45% in PBS) for 10 minutes, sections were incubated with the cPLA2 antibody sc-454 at a concentration of 0.15 μg/ml for 1 hour at room temperature. The antibody was centrifuged at 14,000 g for 2 minutes prior to incubation. The sections were extensively washed on drops of PBG and incubated with the 10 nm gold-precipitation, the sections were washed with PBG and PBS, post-fixed with 1% GA in PBS for 5 minutes and washed with distilled water. Sections were contrasted and subsequently embedded by incubation with 0.3-0.5% uranylacetate (UA) in 1.6% methylcellulose for 5 minutes and air-dried for 30 minutes. Sections were viewed on a CM10H Philips electron microscope at 100 kV.

RESULTS

Immunodetection of endogenous cPLA2: specificity of the antibodies

The specificities of the antibodies against cPLA2, the monoclonal sc-454 and polyclonal sc-438, were determined by western blot analysis of a total Her14 cell lysate (Fig. 1). Using increasing amounts of cell lysate, both antibodies revealed a 110 kDa band which is characteristic for cPLA2. The antibodies, however, differ in their specificity. Since the monoclonal antibody sc-454 detected only the 110 kDa band which comigrated with purified cPLA2, it appears to be very specific (Fig. 1A). The polyclonal antibody sc-438 is less specific, because in addition to the 110 kDa band it also recognized a prominent 60 kDa band and some minor bands (Fig. 1B).

Because of its high specificity, the monoclonal antibody sc-454 is the most appropriate antibody to study the localization of cPLA2 in cells. Besides a high specificity, this monoclonal antibody also exhibits a high affinity for cPLA2. This is stressed by the fact that it immunoprecipitates cPLA2 from a crude cell homogenate under stringent conditions (Fig. 1C, lane 3). cPLA2 did not precipitate when the antibody was omitted (Fig. 1C, lane 2). The extra bands at the height of approximately 50 kDa result from the antibody and Protein A.

These results demonstrate that the levels of endogenous cPLA2 in Her14 fibroblasts are sufficiently high to be detected and that the monoclonal antibody sc-454 is able to recognize the endogenous cPLA2 specifically and with a high affinity.

Localization of endogenous cPLA2 by indirect immunofluorescence

Since the antibody sc-454 was shown to specifically recognize cPLA2, the localization of endogenous cPLA2 was studied with this antibody by immunofluorescence microscopy. Visualization of cPLA2 in serum-exposed Her14 cells by conventional immunofluorescence microscopy revealed a punctate pattern randomly distributed throughout the cytoplasm of the cell (Fig. 2A). Label was found up to the distal parts of cellular extensions. No preference was observed for a particular cellular membrane, such as the plasma membrane or the nuclear envelope. The random nature of the distribution was supported by the fact that confocal optical sections revealed a punctate staining at all depths in the cell (results not shown).

Although the antibody sc-454 is highly specific in western blot analysis, additional control experiments were carried out to assess its specificity in immunofluorescence microscopy. When the primary antibody was omitted no fluorescence was detected (Fig. 2B), indicating that the punctate pattern results from primary antibody recognition. Furthermore, when the primary antibody was pre-blocked with purified cPLA2 (one hour incubation with 10-fold molar excess of cPLA2) the punctate pattern was eliminated, leaving only a faint nuclear staining (Fig. 2C).

This nuclear staining was not present when the primary antibody was omitted, indicating a faint aspecific background labeling of the nucleus by the primary antibody. When viewed by confocal microscopy the nucleus was unlabeled.

To exclude the possibility that the punctate labeling is caused by aggregation of the monoclonal antibody, cPLA2 was also detected using the polyclonal antibody. As shown in Fig. 2D a punctate pattern is also observed, demonstrating that the punctate structures are not caused by the antibodies used. However, staining with the polyclonal antibody resulted in a labeling which is denser and of a finer structure (Fig. 2D). The reason for this difference in staining is unknown. It might be explained by the lower specificity of this antibody and by less sensitive epitope recognition due to fixation as compared to the monoclonal. No residual staining is present when the polyclonal antibody is omitted (Fig. 2E).

Finally, to exclude the possibility that only membrane-bound
cPLA₂ is detected in these studies, the supernatant of the fixative step was analyzed for the presence of cPLA₂ on western blot. No cPLA₂ was recovered in the supernatant (result not shown), indicating that the punctate pattern reflects total cellular cPLA₂.

In conclusion, these results show that endogenous cPLA₂ is localized in punctate structures randomly distributed throughout the cytoplasm of the cell. Interestingly, the punctate labeling pattern of endogenous cPLA₂ appeared not to be restricted to fibroblasts but is a feature which can be observed in different cell types. Human epidermal carcinoma cells (A431) (Fig. 3A), rat mesangial cells (RM) (Fig. 3B), Chinese hamster ovary cells (CHO) (Fig. 3C) and neuroblastoma cells (N2A) (Fig. 3D) showed a comparable punctate labeling pattern. In CHO cells, however, labeling was concentrated around the nucleus.

**EGF and A23187 stimulation and the introduction of extreme intracellular Ca²⁺-concentrations have no effect on the labeling pattern**

Fractionation studies showed that the calcium ionophore A23187 and EGF induce a translocation of cPLA₂ from the cytosol to the particulate fraction (Schalkwijk et al., 1995).

EGF and A23187 stimulation are therefore expected to change the localization of cPLA₂ as compared to quiescent cells. For this reason, immunofluorescence studies were performed on EGF/A23187-stimulated and unstimulated Her14 and Herc13 fibroblasts. As shown by Schalkwijk et al. (1996), Herc13 fibroblasts failed to release arachidonic acid in response to EGF stimulation because of the inability to increase the intracellular calcium concentration. cPLA₂ present in Herc13 cells is presumed to be incapable of membrane translocation and for this reason these cells were used as controls in EGF-induced translocation experiments.

Serum-starved cells were stimulated with 200 ng/ml EGF or 1 μM A23187 for 5 minutes and the immunofluorescence staining of cPLA₂ was compared to that of unstimulated cells. In unstimulated quiescent Her14 fibroblasts, the labeling of cPLA₂ reveals a similar punctate pattern as found in serum-exposed fibroblasts (Fig. 4A). Upon stimulation with EGF (Fig. 4B) or A23187 (Fig. 4C) no difference in the labeling pattern was observed. No translocation to a particular cellular membrane was detected and using confocal microscopy the punctate labeling pattern remained randomly distributed (result not shown).

Since maximal cPLA₂ activation requires a Ser-phosphory-
Ultrastuctural localization of cPLA₂ by MAP kinase which precedes the Ca²⁺-induced membrane translocation (Schalkwijk et al., 1996), cells were stimulated with EGF followed by A23187 (Fig. 4D). The order of stimulation was reversed as a control for a possible altered translocation behavior of the phosphoprotein (Fig. 4E). Surprisingly, even maximal activation of cPLA₂ had no effect on the labeling pattern. Also, the reversed order of incubation had no influence on the labeling.

In Herc13 fibroblasts, the punctate pattern was comparable to that found in Her14 fibroblasts (Fig. 4F) and also independent of the stimulation of the cells.

The absence of a detectable translocation upon all stimulation conditions might be explained by the translocation of a small fraction of the cPLA₂ pool. In the next experiment, therefore, the amount of translocated cPLA₂ was increased by artificially introducing extreme intracellular Ca²⁺ conditions. Cells were placed in a Ca²⁺-rich medium (2 mM Ca²⁺) or in a Ca²⁺-free medium supplemented with 1 mM EGTA. Subsequently, cells were incubated with 1 μM A23187 for 10 minutes. Cells were homogenized and subjected to ultracentrifugation to separate the cytosolic from the particulate fraction. Sixty to seventy percent of cPLA₂ translocated to the particulate fraction under the high calcium condition as judged by western blotting (Fig. 5A). Under the calcium-free condition most of the cPLA₂ was recovered in the cytosolic fraction. These results show that endogenous cPLA₂ is capable of a substantial Ca²⁺-mediated membrane translocation in these cells. This translocation, however, is not shown at the immunofluorescence level (Fig. 5B-E). Cells were fixed immediately after stimulation and stained for cPLA₂. The punctate labeling pattern was observed under both conditions and remained randomly distributed throughout the cell (Fig. 5B,C). Apparent differences in intensity of the labeling are due to an altered morphology of the cells (Fig. 5D,E). Cells placed on Ca²⁺-free medium exhibited rounding up of the cells whereas cells placed in high Ca²⁺ medium showed ruffling of the plasma membrane and formation of extrusions such as filopodia.

**Immunoelectron microscopy reveals that cPLA₂ is localized in as yet unidentified particles near membranes, without preference for a particular membrane**

The lack of a detectable translocation in our immunofluorescence studies may be due to translocation over a small distance, not visible by light microscopy. Therefore, electron microscopy studies were performed to visualize the localization of cPLA₂ at the ultrastructural level. First, immunogold staining of cPLA₂ on cryosections of serum-exposed Her14 fibroblasts was performed (Fig. 6). Labeling of the sections with sc-454 displayed small clusters of gold label distributed throughout the cytosol of the cell. Labeling was not found within a specific organelar structure and no membrane was found enclosing these aggregates. These clusters, however, are not randomly distributed in the cytosol but are located in the proximity of organellar membranes without preference for a
particular membrane. In fact, labeling was found near all organellar membranes, except for the Golgi system. Minor amounts of label were found at the plasma membrane. Furthermore, labeling is not enriched at the nuclear envelope and the endoplasmic reticulum, membranes which have been indicated as target membranes of cPLA2 in other cell types.

The clusters of gold label reflect the punctate pattern found with immunofluorescence microscopy. Ultrathin cryosections of serum-exposed Herc13 fibroblasts and A431 cells revealed the same labeling pattern as found for Her14 fibroblasts (results not shown). Labeling of the cryo-sections without the primary antibody revealed no gold label.

To positively allocate the clusters of gold label as cPLA2-containing particles, gold-labeled IgG (Fig. 7A) was replaced by gold-labeled Protein A. Protein A, which binds at a 1:1 ratio to the primary antibody, still revealed small clusters of gold label (Fig. 7B). Coating of the antibody sc-454 directly onto grids and subsequent detection with Protein A shows separate isolated gold particles (Fig. 7C). This confirms that Protein A binds in a 1:1 ratio and additionally demonstrates that the aggregates are not the result of aggregation of sc-454 itself.

Together, these observations indicate that cPLA2 itself is localized in particles and that the clusters of label are not a consequence of the antibodies used.

To detect a possible translocation of cPLA2 at the ultrastructural level, the localization of cPLA2 in ultrathin cryosections of unstimulated quiescent Her14 fibroblasts was compared to the localization in fibroblasts stimulated with both EGF and A23187. No apparent difference in the labeling pattern was observed. cPLA2 was localized in clusters close to membranes under both conditions. After stimulation, no organellar membrane was preferred by cPLA2 as compared to unstimulated cells. Interestingly, as shown in Fig. 8, the nuclear membrane, the plasma membrane and the endoplasmic reticulum reveal the same amount of label in stimulated as in unstimulated cells. From these results it is concluded that in stimulated as well as in unstimulated cells, cPLA2 is localized in small particles in the vicinity of membranes without preferring a particular membrane.

cPLA2 is not associated with the cytoskeleton

The only cellular organelle not detectable in the cryosections is the cytoskeleton. A relationship between cPLA2 and this cellular component has been suggested by several authors.
Ultrastructural localization of cPLA₂ (Nakano et al., 1989; Akiba et al., 1993). Collagen-induced arachidonic acid release in human platelets was inhibited by cytochalasin B, an inhibitor of actin polymerization, suggesting involvement of the actin skeleton in cPLA₂ activation (Nakano et al., 1989). Furthermore, in thrombin-stimulated rabbit platelets an increased cPLA₂ activity was found in the cytoskeletal fraction, which could be inhibited by the PLA₂ inhibitor ובromo-phenacyl bromide (Akiba et al., 1993).

The particles of cPLA₂ might be scaffolded by the cytoskeleton, thereby explaining their permanent position near membranes. Therefore, we investigated a possible association of cPLA₂ with the cytoskeleton in serum-exposed, EGF-stimulated and unstimulated cells.

Cytoskeletons were isolated by extracting cells with 0.5% Triton X-100. The obtained Triton X-100 insoluble fractions as well as the corresponding soluble fractions were subjected to western blot analysis (Fig. 9A). Actin present in the Triton X-100 insoluble fractions represents the polymerized form of actin known as F-actin, and thereby represents the actin network. Actin which remained in the corresponding Triton X-100 soluble fraction represents the soluble form or G-actin. cPLA₂ was exclusively detected in the Triton X-100 soluble fraction irrespective of the stimulation of the cells.

The possible association of cPLA₂ with the cytoskeleton was also investigated using immunofluorescence microscopy.

Double immunolabeling of cPLA₂ with actin and tubulin was performed on cells fixed according to a protocol optimized for fixation of the cytoskeleton (Van Bergen en Henegouwen et al., 1992). This protocol is based on simultaneous fixation and permeabilization.

The overlaid fluorescence staining of actin and cPLA₂ in serum-exposed fibroblasts, obtained by confocal microscopy, is shown in Fig. 9B. The stress fibers of the actin network (green) and the punctate staining of cPLA₂ (red) are visible. No overlap of cPLA₂ with the actin staining, which would have resulted in a yellow signal, could be detected. In this projection of the total cell, however, some co-localization of cPLA₂ with actin might be suggested at the plasma membrane. Confocal sections, on the other hand, showed that no co-localization was present at this site. Double labeling of tubulin and cPLA₂ (Fig. 9C) also revealed no colocalization. The results obtained were independent of the stimulation of the cells. These data demonstrate that cPLA₂ is not associated with the cytoskeleton.

**DISCUSSION**

Using indirect immunofluorescence microscopy, endogenous cPLA₂ was shown to be confined to punctate structures in the

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Fig. 5. Maximal cPLA₂ translocation by artificial introduction of extreme intracellular Ca²⁺ conditions. Cells were exposed to either a 2 mM Ca²⁺-containing medium (+) or a Ca²⁺-free medium supplemented with 1 mM EGTA (−). Afterwards, cells were stimulated with 1 μM A23187 for 10 minutes and immediately homogenized for fractionation studies or fixed for immunofluorescence.

(A) Western blot analysis of cPLA₂ in particulate fractions (P), cytosolic fractions (C) and nuclei containing fraction (N).

(B-E) Immunofluorescence detection of cPLA₂ by sc-454 in (B) cells exposed to medium free of Ca²⁺, (C) cells exposed to medium rich in Ca²⁺, (D,E) corresponding phase contrast photographs. Bar, 15 μm.
cytoplasm of the cell. At the ultrastructural level, the punctate labeling was reflected by the localization of cPLA$_2$ in small clusters. These clusters of cPLA$_2$ were found in the cytosol in the vicinity of all organellar membranes, except for the Golgi system. The enzyme showed no preference for the nuclear envelope, the endoplasmic reticulum or the plasma membrane. Stimulation of the cells with EGF and A23187 did not change the punctate immunofluorescence labeling pattern. The same labeling pattern was observed in cells exposed to medium essentially free of Ca$^{2+}$ or to medium rich in Ca$^{2+}$ and subsequently stimulated with A23187. Even with electron microscopy, a translocation of cPLA$_2$ to membranes was not observed after stimulation of cells with EGF and A23187. From these results it is concluded that cPLA$_2$ is localized in clusters close to membranes in stimulated as well as unstimulated cells, without preference for a specific organellar membrane. Finally, in immunofluorescence double-labeling studies it was shown that the cPLA$_2$ clusters are not scaffolded by the cytoskeleton.

cPLA$_2$ is localized in small clusters rather than being aligned along membranes. Control labeling with Au-Protein A indicated that cPLA$_2$ itself is localized in particles and that the clusters of label are not caused by an amplification effect of the IgG molecules. The concept of cPLA$_2$-clustering finds support in in vitro observations from a continuous fluorescence displacement study in which purified cPLA$_2$ binds in clusters to highly curved substrate vesicles (Creaney et al., 1995). The preferred size of these vesicles is within the range of the size of our aggregates, 25-50 nm diameter.

The punctate labeling of cPLA$_2$ appears not to be restricted to fibroblasts. As we showed in this study, other cell types like the human epidermal carcinoma A431 cells, neuroblastoma cells N2A, rat mesangial cells and Chinese hamster ovary cells all exhibited the punctate cPLA$_2$ labeling. Furthermore, in rat

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**Fig. 6.** Electron microscopic localization of cPLA$_2$ in ultrathin cryosection of a serum-exposed Her14 fibroblasts. cPLA$_2$ immunoreactivity is detected by the antibody sc-454/IgG-10 nm Au. cPLA$_2$ is present in particles in proximity of membranes with no specific preference for a particular membrane. g, Golgi; n, nucleus; ne, nuclear envelope; lys, lysosome; m, mitochondria. Bar, 300 nm.
Basophilic leukemia cells aggregates of cPLA$_2$ were occasionally found in stimulated cells at the electron microscope level by Glover et al. (1995). The punctate labeling of cPLA$_2$ therefore appears to be a common feature and represents an intriguing finding.

Furthermore, the localization of a phosphatidylethanolamine $N$-methyltransferase (Cui et al., 1993; Rusiñol et al., 1994) displays some similarity with the present observation, namely, a punctate fluorescence pattern which is reflected by the presence of protein clusters associated with a unique mitochondria-associated membrane fraction at the ultrastructural level. This finding illustrates that enzyme clustering is a feature occurring on a wider scale.

Our findings are surprising in the light of the fractionation

Fig. 7. Gold clusters represent clusters of cPLA$_2$. (A) cPLA$_2$ detected by sc-454/IgG-10 nm Au (B) cPLA$_2$ detected by sc-454/Protein A-10 nm Au. (C) the monoclonal antibody coated to grids and detected by Protein A-10 nm Au. Bars, 100 nm.

Fig. 8. cPLA$_2$ immunogold labeling at the nuclear membrane (A,B), plasma membrane (C,D) and endoplasmic reticulum (E,F) of unstimulated (left:A.C.E) and EGF-A23187 stimulated Her14 fibroblasts (right: B.D.F). cPLA$_2$ is detected by sc-454/IgG-10 nm Au. n, nucleus; ne, nuclear envelope; pm, plasma membrane; er, endoplasmic reticulum. Bars, 300 nm.
studies performed by Schalkwijk et al. (1995), showing a cPLA₂ membrane translocation upon stimulation with A23187 and EGF. In our studies, however, this membrane translocation was not observed at the microscope level. Moreover, the introduction of extreme high or low Ca²⁺ levels revealed no difference in labeling whereas western blotting showed a marked translocation to the particulate fraction. These apparent contradictory results underline the importance of a combined biochemical and microscopic approach in order to gain a better insight in cellular processes. From the biochemical data, it follows that cPLA₂ translocates from the cytosol to membranes. In cells, however, this translocation appears to take place over a small distance not even visible at the ultrastructural level. The translocation of cPLA₂ to membranes, might therefore be considered as a tighter interaction of cPLA₂ with membranes upon stimulation of cells. The membrane translocation of cPLA₂ as found in fractionation studies can be explained by the homogenization conditions used. Upon homogenization, membrane-attachment of cPLA₂ is easily lost in unstimulated cells as compared to stimulated cells and therefore appears to be translocated.

Endogenous cPLA₂ was found near all organellar membranes, except for the Golgi, with no preference for a particular membrane. This non-selective recognition of membranes is consistent with results obtained in binding studies of cPLA₂ to natural membranes (Diez et al., 1994) in which no selectivity of cPLA₂ was found for phospholipids with PI, PC or PE as headgroup or acyl ether and plasmencyl linkages at the sn-1 position. This excludes organellar preference caused by any difference in phospholipid composition or membrane asymmetry. Additionally, the Ca²⁺-dependent association of cPLA₂ to membranes has been shown to occur without interaction of an accessory membrane protein in vitro studies in which cPLA₂ binds to liposomes (Schalkwijk et al., 1995), also arguing against selectivity for a membrane as a result of recognition by a binding protein.

In several cell types, the nuclear envelope and the endoplasmic reticulum have been suggested as the target membrane of cPLA₂. Fractionation studies using Ca²⁺-ionophore stimulated macrophages (Peters-Golden and McNish, 1993) and microscopy studies of A23187 or IgE/Antigen-stimulated rat basophilic leukemia cells (Glover et al., 1995), A23187-stimulated Chinese hamster ovary cells (CHO) stably overexpressing cPLA₂ (Schievella et al., 1995), and histamine treatment of confluent and subconfluent endothelial cells (Rocio Sierra-Honigmann et al., 1996) all showed a translocation of cPLA₂ from the cytosol to the nuclear membrane. In the case of the CHO cells, cPLA₂ also translocated to the endoplasmic reticulum. In our study, label was detected in the perinuclear region, nuclear envelope and ER, indicating that cPLA₂ is localized in this region, although in fibroblasts it is not a region preferred by cPLA₂. Most of the studies that report on the translocation of cPLA₂ to the perinuclear region were performed on cells involved in inflammatory responses. The role of cPLA₂ in inflammatory responses might be quite different from the role in cellular proliferation and mitogenicity, explaining the differences in the localization of cPLA₂ in the cell types.

Current knowledge about the role of cPLA₂ is based on its function in prostaglandin and leukotriene synthesis. Since the arachidonic acid metabolizing enzymes, the cyclooxygenase-1/2 and 5-lipoxygenase, have been shown to be associated with the nuclear envelope in several cell types (Regier et al., 1993; Morita et al., 1995; Peters-Golden and McNish, 1993; Woods et al., 1993, 1995; Pouliot et al., 1996), the perinuclear translocation of cPLA₂ agreed with a role of cPLA₂ in the prostaglandin and leukotriene synthesis. In cells involved in inflammatory responses, cPLA₂ may have a more prominent role in prostaglandin and leukotriene metabolism, since these are inflammatory mediators. Although arachidonic acid metabolites affect cellular proliferation and cell migration (Nolan et al., 1988; Handler et al., 1990; Terstoelen et al., 1994; Eling and Glasgow, 1994), the synthesis of these products does not necessarily have to occur at the nuclear envelope. In fibroblasts, as well as in the other cell types exhibiting the punctate labeling of cPLA₂, little is known about the localization of the eicosanoid synthesizing enzymes in general and even less is known about these enzymes upon EGF-stimulation. Furthermore, the general membrane recognition of cPLA₂ might indicate another role for this enzyme besides its role in eicosanoid synthesis.

Why cPLA₂ is localized in aggregates and whether other proteins are also present in these particles has to be investigated in further detail to provide insight in the role of cPLA₂ in cellular proliferation and mitogenic signaling.

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(Understood.)