Translocation of cytosolic phospholipase A₂ to the nuclear envelope elicits topographically localized phospholipid hydrolysis

Marc PETERS-GOLDEN*, Keli SONG, Teresa MARSHALL and Thomas BROCK

Division of Pulmonary and Critical Care Medicine, University of Michigan Medical Center, Ann Arbor, MI 48109-0360, U.S.A.

Cytosolic phospholipase A₂ (cPLA₂) is a good candidate for mediating the agonist-stimulated release of arachidonic acid (AA) from membrane phospholipids. This enzyme undergoes a Ca²⁺-dependent translocation from the cytosol to a membrane site in a variety of cell types, and this site has recently been identified as the nuclear envelope in leucocytes. The functional correlate of this finding has not yet been established. The present study was therefore undertaken to determine whether translocation of cPLA₂ to the nuclear envelope was associated with localized phospholipid hydrolysis at this site. Rat alveolar epithelial cells, previously shown to contain cPLA₂, were prelabelled with [³H]AA and stimulated with the model agonist, ionophore A23187. Ionophore-induced AA release exhibited characteristics typical of a cPLA₂-mediated response, in that it was Ca²⁺-dependent, *sn*-2 AA-selective, and inhibited by arachidonyl trifluoromethyl ketone. As determined by indirect immuno-

fluorescence microscopic analysis as well as subcellular fractionation with immunoblotting, ionophore treatment resulted in a translocation of cPLA₂ protein from the cytoplasm to the nuclear envelope. To determine whether the nuclear membrane was indeed the source of released AA, prelabelled cells were incubated in the presence or absence of A23187, after which the phospholipid radioactivity was quantified in nuclear and non-nuclear membrane fractions. [³H]AA was distributed in both nuclear and non-nuclear membrane phospholipids. Following A23187 stimulation, the loss of [³H]AA from nuclear membrane phospholipids accounted for 88.1 ± 5.8 % of the total loss from phospholipids and for 92.9 ± 2.3 % of the total [³H]AA released into the medium. These results demonstrate for the first time that agonist-stimulated translocation of cPLA₂ to the nuclear envelope is associated with phospholipid hydrolysis which is preferentially localized to that site.

INTRODUCTION

The hydrolysis of arachidonic acid (AA) and other unsaturated fatty acids from the *sn*-2 position of membrane phospholipids initiates a cascade of metabolic events which mediate both intracellular signal transduction and intercellular communication [1]. Thus, AA is a precursor for a family of oxygenated metabolites synthesized via the cyclo-oxygenase (prostanoids) and lipoxygenase (leukotrienes) pathways [2]. These bioactive eicosanoids exert pleiotropic effects on cellular functions which implicate them as important mediators of both normal homeostasis and pathophysiological states. Alternatively, free unsaturated fatty acids can themselves function as second messengers, directly modulating such processes as release of intracellular Ca²⁺ [3], activation of protein kinase C [4] and mitogen-activated protein kinases [5], generation of superoxide anion [6], polypeptide synthesis [7], and transcription of heat shock genes [8]. Finally, the other products of phospholipid hydrolysis, namely lysophospholipids, can themselves be metabolized to platelet-activating factor as well as modulate the transduction of cellular signals [9].

The key enzyme responsible for *sn*-2 deacylation is phospholipase A₂ (PLA₂). A substantial body of work carried out in recent years has identified at least five separate PLA₂ isoforms, each being a distinct gene product with its own distinct biochemical characteristics [10,11]. Of these, cytosolic or cPLA₂ is one isoform which has been suggested to be a good candidate to

mediate agonist-stimulated phospholipid hydrolysis because of (i) its preference for AA over other fatty acids, (ii) its activity at physiological (nM) intracellular Ca²⁺ concentrations, and (iii) its phosphorylation-dependent regulation by hormonal and other relevant stimuli. As its name implies, this 85 kDa protein is located in the cytosol of resting cells, but undergoes a Ca²⁺-dependent translocation to a membrane site following cell activation [12]. This site was initially assumed to be the plasma membrane; however, recent studies employing immunofluorescence microscopic analysis [13] and subcellular fractionation/immunoblot analysis [14] in rat basophilic leukaemia cells and peritoneal macrophages, respectively, have instead demonstrated it to be the nuclear envelope. As surprising as this finding was, it remains to be established whether this translocation event is associated with functional evidence of phospholipid hydrolysis at the nuclear envelope. The present study was undertaken to address precisely this question.

Herein, we have utilized primary cultures of rat type-II alveolar epithelial cells (AECs), or pneumocytes. These cells line the alveolar surface of the lung, and therefore comprise the immense interface between the lung and the inspired air. They are capable of releasing AA and metabolizing it to large quantities of prostaglandins and to lesser amounts of hydroxyeicosatetraenoic acids [15]. We have previously reported that AECs contain cPLA₂ as well as a Ca²⁺-independent PLA₂ activity [16]. Our results demonstrate that the model agonist, ionophore A23187, promotes AA release in AECs via a cPLA₂-mediated mechanism,

Abbreviations used: AA, arachidonic acid; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; AEC, alveolar epithelial cell; DMEM, Dulbecco's modified Eagle's medium; ETYA, eicosatetraenoic acid; BAPTA, 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; AACOCF₃, arachidonyl trifluoromethyl ketone; ICAM-1, intracellular adhesion molecule-1; COX-1, cyclo-oxygenase-1.

* To whom correspondence should be addressed.

that it causes translocation of cPLA₂ protein from the cytosol to the nuclear envelope, and that it causes phospholipid hydrolysis primarily at the nuclear membrane.

EXPERIMENTAL

Materials

Male specific pathogen-free Sprague–Dawley rats (180–200 g) were obtained from Charles River. Dulbecco's modified Eagle's medium (DMEM) and fetal-calf serum were obtained from Gibco. [5,6,8,9,11,12,14,15-³H]AA (76 Ci/mmol) and [9,10-³H]oleic acid (9.2 Ci/mmol) were purchased from DuPont–New England Nuclear. Fatty acid-free BSA, eicosatetraenoic acid (ETYA), and rhodamine-conjugated goat anti-rabbit IgG secondary antibody were from Sigma. A23187 and 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra-(acetoxymethyl) ester (BAPTA/AM) were from Calbiochem. The trifluoromethyl ketone derivative of AA (AACOCF₃) was generously provided by Dr. Michael Gelb, University of Washington. The rabbit anti-cPLA₂ IgG was generously provided by Dr. Ruth Kramer, Lily Research Laboratories, and rabbit anti-sheep seminal vesicle cyclo-oxygenase-1 (COX-1) was generously provided by Dr. William Smith, Department of Biochemistry, Michigan State University. A monoclonal antibody (clone 1-A29) against intracellular adhesion molecule-1 (ICAM-1) was obtained from Seikagaku America, International. Aluminium-backed silica gel TLC plates were from Merck. All solvents were reagent grade from Aldrich.

Cell isolation and culture

Rat type-II AECs were isolated by standard techniques [17] and cultured in DMEM containing 10% (v/v) fetal-calf serum (serum-containing medium). They were studied at culture day 7 because it is at this time that AA release is maximal [18], reflecting increased expression of cPLA₂ protein and activity as compared with earlier time points [19]. AEC purity, assessed by cytokeratin staining, was ~95% [18]. For AA release studies, 0.5 × 10⁶ AECs were cultured in 1 ml in each well of a 24-well plate; for phospholipid analysis of subcellular fractions, 10⁷ cells were cultured in 10 ml in a 100-mm-diam. dish.

Prelabelling of cellular lipids

AEC lipids were prelabelled to equilibrium by adding 0.3 µCi of [³H]AA or [³H]oleic acid per ml of serum-containing medium [19] at day 6 in culture. After a 24 h labelling interval, the labelling medium was removed, cells were washed three times with Ca²⁺- and Mg²⁺-free PBS, and cellular uptake of radiolabel was determined by scraping cells in methanol and scintillation counting. Incorporation was routinely ~60% of added radioactivity for AA and ~50% for oleic acid.

Determination of fatty acid release in intact cells

[³H]Fatty acid release was ordinarily determined by stimulating prelabelled cells in DMEM containing 0.1% BSA [19] and counting total radioactivity in the medium. BSA serves to trap all the released fatty acid, thereby preventing its re-acylation back into phospholipids as well as its metabolism to eicosanoids. Under these conditions, radioactivity measured in the medium reflects only deacylation. In experiments testing the effects of AACOCF₃, stimulation was performed in the absence of BSA and free [³H]AA levels were instead determined by extracting lipids from cells plus medium and separating neutral lipids by TLC [19]. The spots on the chromatogram which correspond to

free fatty acid were then cut out and subjected to scintillation counting. In all instances, radioactivity was expressed as a percentage of the radiolabel incorporated into cells.

Experimental incubations

Cells were stimulated for 60 min in serum-free DMEM alone (with or without BSA) or in the same medium containing 5 µM A23187. The effects of the intracellular Ca²⁺ chelator BAPTA were examined by preloading cells with the cell-permeant acetoxymethyl ester derivative BAPTA/AM for 60 min prior to stimulation. A 2-min preincubation with the inhibitor AACOCF₃ was preceded by a 10-min pretreatment with 10 µM ETYA to inhibit re-acylation [20]; A23187 was then added.

Indirect immunofluorescence microscopy

AECs were plated on sterilized glass coverslips placed into 4-well plates and cultured as described above. At culture day 7, serum-containing medium was removed, cells were washed twice with PBS, and incubated with DMEM in the absence or presence of A23187 at 37 °C for 30 min. After agonist stimulation, they were fixed in methanol at –20 °C for 30 min, permeabilized in acetone at –20 °C, and air-dried. They were then rehydrated with PBS–3% BSA for 5 min at room temperature, and blocked by the addition of 10% non-immune goat serum for 30 min. Cells were incubated for 1 h at 37 °C with anti-cPLA₂ IgG at a titre of 1:20 or with an identical amount of normal rabbit IgG. They were then washed six times with PBS–3% BSA and incubated for 30 min at 37 °C with a 1:200 titre of rhodamine-conjugated goat anti-rabbit IgG. The coverslip was mounted on a slide and fluorescence visualized with a Nikon Labophot 2 microscope equipped for epifluorescence.

Subcellular fractionation and immunoblot analysis

AECs were washed twice with ice-cold PBS containing 1 mM EGTA, detached by incubation at 37 °C for 5 min with 1 ml of 0.25% trypsin, and immediately diluted into medium. They were then pelleted, washed once with medium, and resuspended in medium at 2 × 10⁶ cells/ml. Cells were treated for 30 min with an equal volume of medium alone or medium containing A23187 (final concentration, 5 µM), washed with PBS, and resuspended at 10⁷ cells/ml in ice-cold TKM buffer (50 mM Tris/HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 1 mM EGTA) containing 1 mM each of PMSF, leupeptin, NaF, and sodium orthovanadate, as described [21]. They were permitted to swell by incubation on ice for 10 min, and were then disrupted by dounce homogenization. The relative numbers of unbroken cells and intact nuclei were determined by microscopic examination of cytospin preparations of these suspensions which had been mixed with Trypan Blue. On the basis of preliminary experiments, a protocol employing 250 strokes was selected because it consistently resulted in a minimal number of unbroken cells (<2%) along with minimal disruption of the nuclear membrane; fewer strokes yielded more unbroken cells, and more strokes yielded fewer intact nuclei. For subcellular fractionation, 0.6 ml of 0.25 M sucrose–TKM was added to the homogenate and this was centrifuged at 1000 *g* for 5 min. This low-speed pellet was designated the 'nuclear' fraction. The low-speed supernatant was centrifuged at 100 000 *g* for 60 min; the resulting high-speed supernatant was designated the 'cytosol' and the high-speed pellet was designated the 'non-nuclear membrane' fraction, consisting of endoplasmic reticulum, plasma membrane and other organelles. It should be noted that in this protocol, any unbroken cells would contaminate the nuclear fraction and any membrane from disrupted nuclei would

contaminate the non-nuclear membrane fraction. COX-1 was utilized as a marker protein distributed in the endoplasmic reticulum and nuclear envelope, and ICAM-1 was utilized as a plasma membrane marker. Isolated nuclei were disrupted by sonication (10 bursts at 20% duty cycle) with a Branson sonicator, and soluble and pelletable fractions separated by ultracentrifugation as described above. Aliquots of subcellular fractions were sonicated (10 bursts at 20% duty cycle) and protein concentrations were determined by a modified Coomassie dye-binding assay (Pierce Chemical), using BSA as standard. Samples containing 10 μg of protein were separated by 7.5% SDS/PAGE under reducing (for cPLA₂ and COX-1) or non-reducing (for ICAM-1) conditions and transferred to nitrocellulose membranes for immunoblot analysis as described previously [21]. Membranes were washed and probed with antibodies against cPLA₂ (1:1000 titre), COX-1 (1:200 titre), and ICAM-1 (1:1000 titre), washed, and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (1:5000). Bands were visualized using the ECL chemiluminescence system (Amersham). Multiple exposure times were taken for all blots to ensure that band densities were within the linear range of exposure.

Analysis of phospholipid hydrolysis

To determine the membrane site(s) at which phospholipids were hydrolysed in response to agonist stimulation, 10⁷ prelabelled AECs were washed and incubated for 60 min in DMEM containing 0.1% BSA in the presence or absence of 5 μM A23187. Total release of [³H]AA in each plate was determined by scintillation counting of an aliquot of medium. Cells were trypsinized, after which the trypsin was neutralized by the addition of 5 ml of DMEM plus 10% (v/v) fetal-calf serum. Cells were pelleted, disrupted, and separated into subcellular fractions as described above. Lipids in both the nuclear and non-nuclear membrane fractions were extracted in chloroform/methanol (2:1, v/v) as previously described [19], evaporated to dryness under N₂, and stored under N₂ at -20 °C. Neutral lipids were separated by TLC [19], and radioactivity in the spot corresponding to phospholipids was quantified by scintillation counting.

RESULTS

Effect of chelation of intracellular Ca²⁺ on deacylation in response to A23187

In order to determine whether deacylation induced by A23187 was dependent on intracellular Ca²⁺, cells were preloaded for 60 min with the intracellular Ca²⁺ chelator, BAPTA, prior to stimulation. The effects of BAPTA on basal and agonist-stimulated AA release are shown in Figure 1. Since constitutive deacylation of AA is due to the actions of a Ca²⁺-independent PLA₂ [22], it is not surprising that basal AA release was uninhibited by BAPTA. In fact, BAPTA pretreatment itself elicited a rise in basal AA release; this has been reported previously in endothelial cells [23]. Nevertheless, BAPTA dose-dependently inhibited the incremental net release elicited by ionophore. For example, the control level of net agonist-stimulated [³H]AA release (~14.9% of incorporated radioactivity) was reduced to ~3.7% and ~2.3% by pretreatment with 200 μM and 500 μM BAPTA, respectively. Ionophore-stimulated AA release could also be inhibited by carrying out incubations in medium containing the Ca²⁺ chelator EGTA (net A23187-induced [³H]AA release without EGTA, 11.2% of incorporated radioactivity; net A23187-induced release with

Figure 1 Effects of the intracellular Ca²⁺ chelator BAPTA on [³H]AA release by prelabelled AECs

Cultures of AECs were prelabelled, and preincubated with BAPTA/AM for 60 min at the concentration indicated, prior to incubation for an additional 60 min with DMEM–0.1% BSA alone (control) or DMEM–0.1% BSA containing 5 μM A23187. Radioactivity in the medium was determined, and expressed as a percentage of d.p.m. incorporated into cell lipids. Each value represents the mean $\pm$ S.E.M. from triplicate determinations in one experiment. Similar results were obtained in two other experiments.

Figure 2 Effect of AACOCF₃ on [³H]AA release stimulated by A23187

Prelabelled AECs were incubated for 60 min with 5 μM A23187 in the absence or presence of pretreatment with the indicated concentration of AACOCF₃. Lipids in cells plus medium were extracted, separated by TLC, and [³H]AA determined by scintillation counting. For each concentration of AACOCF₃, the net release attributable to agonist is expressed as a percentage of the net agonist-induced [³H]AA release in the absence of inhibitor (control). Each value represents the mean $\pm$ S.E.M. from $n = 4$ independent experiments. Control net release for A23187 was 7.8 $\pm$ 2.1%.

3 mM EGTA, 1.3%). Finally, the combination of pretreatment with 200 μM BAPTA plus stimulation in medium containing 3 mM EGTA completely eliminated the increment in AA release attributable to ionophore. Taken together, these results strongly suggest that AA release stimulated by A23187 in AECs is indeed Ca²⁺-dependent, and that this increment in Ca²⁺ most likely results from an influx of extracellular cation, as has been demonstrated in other cell types [24].

sn-2 Fatty acid preference for phospholipid hydrolysis in response to A23187

A characteristic of cPLA₂ is its preference for hydrolysing phospholipids containing AA over other fatty acids in the *sn*-2

Figure 3 Indirect immunofluorescence microscopic analysis of cPLA₂ localization in AECs

AECs were cultured on glass coverslips and at culture day 7, were incubated for 30 min with DMEM alone (**A**, **B**) or DMEM containing 5 μ M A23187 (**C**). Cells were washed, fixed, permeabilized, and stained with either non-immune rabbit IgG (**A**) or rabbit anti-cPLA₂ IgG (**B**, **C**), prior to incubation with a rhodamine-conjugated secondary antibody.

position [25,26], which we have also verified to be true for the AEC enzyme [16]. As another approach to investigating the role of cPLA₂ in mediating phospholipid hydrolysis stimulated by A23187, we compared the ability of this agonist to release [³H]AA versus [³H]oleic acid in prelabelled cells. [³H]AA release into the medium was $3.02 \pm 0.20\%$ of incorporated radioactivity in control cells and $24.15 \pm 5.68\%$ in A23187-stimulated cells. [³H]Oleic acid release was $3.92 \pm 0.74\%$ and $9.65 \pm 1.21\%$ in control and stimulated cells, respectively. Thus the net release (A23187 minus basal) of [³H]AA was severalfold greater than the net release of [³H]oleic acid.

Effect of AACOCF₃ on AA release in response to A23187

Among Ca²⁺-dependent PLA₂s, cPLA₂ is selectively inhibited by the trifluoromethyl ketone derivative of AA in cell-free systems [27], and its inhibition by this agent has previously been demonstrated in intact cells as well [20,28]. The effects of AACOCF₃ on AA deacylation stimulated by A23187 were therefore examined. This drug binds avidly to albumin. As an alternate means to minimizing the possible contributions of both re-acylation and metabolism, cells were therefore pretreated with 10 μ M ETYA prior to addition of the inhibitor and, subsequently, the agonist. As shown in Figure 2, AACOCF₃ dose-dependently inhibited AA deacylation in response to A23187 over the same concentration range reported to inhibit AA release and metabolism in other cell types [28]; peak inhibition of $\sim 80\%$ occurred at 75 μ M. These results provide further support for the involvement of cPLA₂ in mediating ionophore-induced AA release.

Effects of A23187 on translocation of cPLA₂ in rat AECs: immunofluorescence microscopy

We utilized indirect immunofluorescence microscopy with a rabbit anti-cPLA₂ antibody to examine the intracellular distribution of cPLA₂ in resting and stimulated rat AECs. Monolayers of resting cells incubated for 30 min with medium alone showed diffuse fluorescence across the entire cell (Figure 3B). That this staining specifically represented cPLA₂ was demonstrated by the negative staining obtained with normal non-immune rabbit IgG (Figure 3A). Cells incubated with 5 μ M A23187 for 30 min (Figure 3C) exhibited a dramatic change in their staining pattern from that seen in resting cells. The diffuse staining across the entire cell was diminished, and a concentration of fluorescence was instead observed at the nucleus, particularly in a rim around the nuclear margin. This shift in immunofluorescence pattern in AECs was dependent on the concentration of A23187 added, as doses less than 1–2 μ M caused no or little change, and 3 μ M caused an intermediate degree of redistribution (not shown); these results correspond to the dose-dependency of ionophore-induced AA release [15] in these cells. A similar pattern of fluorescence around the nuclear margin was observed when AECs were stimulated with the protein kinase C activator phorbol myristate acetate (results not shown).

Effects of A23187 on translocation of cPLA₂ in rat AECs: immunoblot analysis

To complement the immunofluorescence microscopy data presented above, translocation of cPLA₂ was also assessed by immunoblot analysis of subcellular fractions obtained from unstimulated and A23187-stimulated AECs. As shown in Figure 4, cPLA₂ was distributed almost exclusively in the cytosolic fraction of unstimulated cells. Upon ionophore activation, there was a loss of cPLA₂ protein from the cytosol and a corresponding increase of this band in the nuclear pelletable fraction, consistent with redistribution to the nuclear envelope. Importantly, no discernible increase was detected in the non-nuclear membrane fraction of activated cells. Distribution of ICAM-1 was also examined in the subcellular fractions; as an exclusive plasma membrane marker, its abundance in the nuclear pellet provides an indication of the extent to which the nuclear fraction was contaminated with unbroken intact cells. As demonstrated in Figure 4, a very low level of ICAM-1 in the nuclear pellet indicates a very low level of contamination with intact cells. Given the relative abundance of both ICAM-1 and COX-1 in the non-nuclear membrane fraction, an appreciable cPLA₂ band would also be expected in this fraction if the cPLA₂ in the

Figure 4 Immunoblot analysis of cPLA₂ and marker proteins in subcellular fractions of AECs

AECs in suspension were incubated for 30 min in the absence or presence of 5 μ M A23187, then washed, disrupted, and fractionated. Aliquots of subcellular fractions (C, cytosol; M, non-nuclear membrane; S, nuclear soluble; P, nuclear pelletable) containing 10 μ g of protein were subjected to immunoblot analysis for cPLA₂, COX-1 and ICAM-1. Blots are representative of three independent experiments.

nuclear pelletable fraction of activated cells represented contamination from either plasma membrane or endoplasmic reticulum. The finding that cPLA₂ protein was redistributed exclusively to the nuclear pellet is consistent with the immunofluorescence data suggesting that translocation of this enzyme is targeted primarily to the nuclear envelope.

Intracellular site of phospholipid hydrolysis in AECs stimulated with A23187

It is assumed that translocation of cPLA₂ to a membrane site is required in order for the enzyme to gain access to its phospholipid substrates. However, it remains to be established whether hydrolysis of AA from nuclear membrane phospholipids occurs subsequent to cPLA₂ translocation to the nuclear envelope. We therefore prelabelled AECs for 24 h with [³H]AA, incubated them with or without A23187 in the presence of BSA, and examined the loss of radioactivity from phospholipids extracted separately from nuclear and non-nuclear membranes. In three independent experiments, 63.7 $\pm$ 2.6 % (mean $\pm$ S.E.M.) of the total [³H]AA d.p.m. incorporated into cellular phospholipids of unstimulated control cells was distributed in the nuclear membrane phospholipids. As shown in Table 1, all of the increase in

radioactivity measured in the medium of A23187-stimulated versus control cells could be accounted for by the loss of radioactivity measured in total phospholipids from both fractions of stimulated cells. The loss of [³H]AA from nuclear membrane phospholipids accounted for 88.1 $\pm$ 5.8 % of the total loss from phospholipids in both fractions, and for 92.9 $\pm$ 2.3 % of the total [³H]AA released into the medium. These results clearly demonstrate that translocation of cPLA₂ to the nuclear envelope of ionophore-stimulated rat AECs, as seen in Figures 3 and 4, is associated with deacylation of AA primarily from nuclear membrane phospholipids.

DISCUSSION

A hallmark of cPLA₂ activation is thought to be its ability to undergo Ca²⁺-dependent translocation from the cytosol to intracellular membranes [12,26]. It is assumed that this translocation event serves to bring the enzyme in proximity to its phospholipid substrates. Previous studies with peritoneal macrophages [14] and rat basophilic leukaemia cells [13] have led to the surprising conclusion that it is the nuclear envelope, rather than the plasma membrane or endoplasmic reticulum, that is the target for redistribution of cPLA₂ in activated cells. However, it has not previously been established that the translocation of cPLA₂ protein results in the enzymic function of cPLA₂, namely, phospholipid hydrolysis, at this intracellular site. In this study, we have utilized an experimental system (ionophore A23187 stimulation of rat AECs) in which cPLA₂ was shown to mediate deacylation of AA. Using both indirect immunofluorescence microscopy and subcellular fractionation with immunoblot analysis, we have confirmed, for the first time in a non-bone-marrow-derived cell type, that cPLA₂ protein translocates to the nuclear envelope upon cell activation. Most importantly, using subcellular fractionation techniques, we have demonstrated for the first time in any cell type that phospholipid hydrolysis which occurs following agonist stimulation is localized predominantly to the nuclear membrane. These results therefore provide the functional information, previously lacking, which links the enzymic action of cPLA₂ with its presence at the nuclear envelope in intact cells.

We have previously demonstrated that rat AECs contain cPLA₂ [16]. Moreover, we showed that expression and activity of cPLA₂ increase as these cells are cultured for 7 days following isolation [18], with corresponding increases in ionophore-stimulated AA release [19]. In fact, Ca²⁺-dependent PLA₂ activity in lysates of day 7 AECs was largely abolished by incubation of lysates with a neutralizing monoclonal antibody against cPLA₂ [19]. We chose to carry out the present experiments in day 7

Table 1 Contribution of nuclear membrane phospholipids to AA hydrolysis in response to A23187

Prelabelled AECs (10⁷ cells) were incubated for 60 min with DMEM containing 0.1% BSA in the presence or absence of A23187. [³H]AA release into the medium was determined, and the cells were then trypsinized, disrupted by dounce homogenization, and nuclei and non-nuclear membranes obtained by differential centrifugation. Extracted lipids from both of these compartments were subjected to TLC in order to separate phospholipids, and the radioactivity in the phospholipid spots quantified. Differences in d.p.m. between ionophore-stimulated and unstimulated dishes are presented as positive values for AA release into the medium, and as negative values for nuclear, non-nuclear, and total (nuclear plus non-nuclear) phospholipids. Each value represents the mean $\pm$ S.E.M. from three independent experiments.

	Distribution of label (d.p.m.)			
	Free AA in medium	Nuclear phospholipids	Non-nuclear phospholipids	Total phospholipids
Control	56424 $\pm$ 13777	1278564 $\pm$ 168430	762761 $\pm$ 180437	2041326 $\pm$ 348046
A23187	580702 $\pm$ 100465	793979 $\pm$ 100360	686534 $\pm$ 164654	1480492 $\pm$ 258894
Difference	(+) 524278 $\pm$ 110728	(-) 484585 $\pm$ 96209	(-) 76228 $\pm$ 41441	(-) 560834 $\pm$ 131556

AECs precisely because of their high level of cPLA₂ expression and the aforementioned evidence indicating the functional significance of this enzyme in mediating ionophore-stimulated deacylation. In this study, we confirmed that the A23187-induced release of [³H]AA from lipids of these day 7 cells exhibited a variety of characteristics which would be expected if it were indeed mediated by cPLA₂. These included (i) dependence on intracellular Ca²⁺, as indicated by inhibition by the intracellular chelator BAPTA; (ii) a definite preference for hydrolysis of *sn*-2 AA over oleic acid; and (iii) inhibition by the cPLA₂ inhibitor AACOCF₃, which, although it also inhibits Ca²⁺-independent PLA₂s [29], is selective for cPLA₂ among known Ca²⁺-dependent enzymes [27]. For all of these reasons, it is highly likely that the experimental system employed is one in which AA release is dependent on the actions of cPLA₂.

Using immunofluorescence microscopic analysis of cells incubated with an anti-cPLA₂ antibody, we were able to visualize a redistribution of fluorescence from the cytoplasm of resting cells to the perinuclear region of ionophore-stimulated cells. Moreover, cPLA₂ translocation exhibited a dependence on A23187 dose which corresponded closely to that which we have observed previously for AA release in these cells [15]. The immunofluorescence microscopy data were further supported by sub-cellular fractionation and immunoblot analysis data demonstrating that cPLA₂ protein redistributed from the cytosolic fraction to the nuclear pelletable fraction (which contains the nuclear envelope), but not to the non-nuclear membrane fraction, upon stimulation. It is recognized that ionophore A23187 is a non-physiological agonist which may result in increases in cytosolic Ca²⁺ concentration which are of greater magnitude and duration than those observed with physiological agonists. However, this characteristic may also facilitate the detection of cPLA₂ translocation to the nuclear envelope. Glover and co-workers [13] noted in their studies of rat basophilic leukaemia cells that the translocation of cPLA₂ to the nuclear envelope observed with antigen stimulation was both of lesser magnitude as well as more transient than that seen with A23187. Thus, while the potency of the ionophore may facilitate investigations such as those presented herein, it will be important to extend such studies to more physiological agonists in the future. The association of cPLA₂ with membrane lipids is thought to be mediated by its Ca²⁺-dependent lipid binding domain [26], similar to those present in other proteins such as protein kinase C. However, it is remarkable that in all of the cell types in which cPLA₂ translocation has been studied thus far, the nuclear envelope has been the exclusive destination for activated enzyme. The molecular mechanisms responsible for targeting cPLA₂ specifically to the nuclear envelope, to the exclusion of other intracellular membranes, are not clear.

Electron microscopic autoradiography studies with a mouse fibrosarcoma cell line have demonstrated that isotopic AA rapidly accumulates at the nuclear membrane, but redistributes to other cellular membranes at later time points [30]. Using this same cell line, Capriotti and associates [31] observed that agonist-stimulated release of pre-incorporated radiolabelled AA was favoured at an early time point when the isotope was preferentially distributed in nuclei, and declined over time in parallel with the loss of isotope from nuclei. These results suggested that releasability of AA was uniquely dependent on its presence in nuclear membrane phospholipids. However, no direct examination of phospholipid hydrolysis at any membrane site was attempted in those studies. In the present report, we have measured the [³H]AA content of phospholipids isolated from both nuclear and non-nuclear membranes of cells incubated with and without the agonist A23187. We did not investigate the

kinetics of AA incorporation into different membranes of rat AECs; however, a substantial proportion of isotopic fatty acid was found incorporated in nuclear membrane phospholipids 24 h after its addition. By measuring the loss of phospholipid radioactivity from both nuclear and non-nuclear compartments upon agonist stimulation, we were able to quantitatively account for all of the radioactivity accumulated in the medium. Our results demonstrate clearly that most (~90%) of the loss of phospholipid radioactivity derived from the nuclear membrane, as opposed to non-nuclear membranes. It is recognized that the accuracy of data derived from studying nuclei isolated from homogenates by low-speed centrifugation is greatly dependent on the intensity with which plasma membranes are disrupted [21]. If cell disruption is sufficiently harsh as to lyse nuclear membranes, these membranes will distribute in the high-speed pellet rather than the low-speed pellet. On the other hand, if it is excessively gentle, the low-speed pellet will be contaminated with unbroken cells. Therefore, the method and precise protocol of cell disruption must be optimized for a given cell type and culture conditions. The relative absence of cPLA₂ in the high-speed pellet (non-nuclear membrane fraction) and of the plasma membrane marker ICAM-1 in the low-speed pellet (nuclear fraction) of activated cells argues against a significant problem of this type with our disruption and fractionation protocol.

Our data establishing that cPLA₂ activation evokes AA release at the nuclear envelope must be considered in the context of a growing body of information indicating that the nuclear envelope is a centrally important locale where other proteins involved in phospholipid synthesis and metabolism are clustered. These include CTP:phosphocholine cytidyltransferase [32], the inducible isoform of cyclo-oxygenase [33], 5-lipoxygenase [14,34,35], 5-lipoxygenase-activating protein [14,34,35] and LTC₄ synthase [36]. Most of these proteins are constitutively present at the nuclear envelope, but one, 5-lipoxygenase, is translocated to that site in response to cell activation [14,34,35], as is the case for cPLA₂. In any event, selectively targeting the enzymic action of cPLA₂ to the nuclear envelope results in the release of AA in close proximity to those proteins which effect its oxygenation to bioactive eicosanoids. The teleologic rationale for clustering these processes near the nucleus is not clear. However, it probably reflects the importance of the nucleus as an intracellular site for the actions of eicosanoids and/or free fatty acids. The intracellular membrane(s) at which PLA₂ species other than cPLA₂ act have not been conclusively identified. Accomplishing this may provide important insights into the specific biological roles subserved by distinct PLA₂s.

M.P.-G. was the recipient of a Career Investigator Award from the American Lung Association. This work was supported by National Heart Lung and Blood Institute Grant R01 HL47391 and Specialized Center of Research Grant P50 HL46487. We thank Dr. Ruth Kramer for providing anti-cPLA₂ IgG, Dr. William Smith for providing anti-COX-1 serum, and Dr. Michael Gelb for providing AACOCF₃ and advice about its use. We also thank Dr. Robert Paine for assistance with immunofluorescence microscopy.

REFERENCES

- Dennis, E., Rhee, S., Billah, M. and Hannun, Y. (1991) FASEB J. **5**, 2068–2077
- Needleman, P., Turk, J., Jakisch, B. A., Morrison, A. R. and Lefkowitz, J. B. (1986) Annu. Rev. Biochem. **55**, 69–102
- Kolesnick, R., Musacchio, I., Thaw, C. and Gershengorn, M. (1984) Am. J. Physiol. (Endocrinol. Metab. 9) **246**, E458–E462
- Murakami, K. and Routtenberg, A. (1985) FEBS Lett. **192**, 189–193
- Rao, G., Baas, A., Glasgow, W., Eling, T., Runge, M. and Alexander, R. (1994) J. Biol. Chem. **269**, 32586–32591
- Badwey, J., Curnutte, J. and Karnovsky, M. (1981) J. Biol. Chem. **256**, 12640–12643
- Rotman, E., Brostrom, M. and Brostrom, C. (1992) Biochem. J. **282**, 487–494

- 8 Jurivich, D., Sistonen, L., Sarge, K. and Morimoto, R. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2280–2284
- 9 Asaoka, Y., Oka, M., Yoshida, K., Sasaki, Y. and Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6447–6451
- 10 Mayer, R. and Marshall, L. (1993) *FASEB J.* **7**, 339–348
- 11 Dennis, E. (1994) *J. Biol. Chem.* **269**, 13057–13060
- 12 Channon, J. and Leslie, C. (1990) *J. Biol. Chem.* **265**, 5409–5413
- 13 Glover, S., Bayburt, T., Jonas, M., Chi, E. and Gelb, M. (1995) *J. Biol. Chem.* **270**, 15359–15367
- 14 Peters-Golden, M. and McNish, R. (1993) *Biochem. Biophys. Res. Commun.* **196**, 147–153
- 15 Chauncey, J., Peters-Golden, M. and Simon, R. (1988) *Lab. Invest.* **58**, 133–140
- 16 Neagos, G., Feyssa, A. and Peters-Golden, M. (1993) *Am. J. Physiol. (Lung Cell. Mol. Physiol. 8)* **264**, L261–L268
- 17 Dobbs, L., Gonzalez, R. and Williams, M. (1986) *Am. Rev. Respir. Dis.* **134**, 141–145
- 18 Lipchik, R. J., Chauncey, J. B., Paine, R., Simon, R. H. and Peters-Golden, M. (1990) *Am. J. Physiol. (Lung Cell. Mol. Physiol. 3)* **259**, L73–L80
- 19 Peters-Golden, M. and Feyssa, A. (1994) *Am. J. Physiol. (Cell Physiol. 35)* **266**, C382–C390
- 20 Riendeau, D., Guay, J., Weech, P. K., Laliberte, F., Yergey, J., Li, C., Desmarais, S., Perrier, H., Liu, S., Nicoll-Griffith, D. and Street, I. P. (1994) *J. Biol. Chem.* **269**, 15619–15624
- 21 Brock, T. G., Paine, R. and Peters-Golden, M. (1994) *J. Biol. Chem.* **269**, 22059–22066
- 22 Balsinde, J., Bianco, I., Ackermann, E., Conde-Frieboes, K. and Dennis, E. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8527–8531
- 23 Boeynaems, J., Heilporn, S., Broeders, F. and Braekman, J. (1993) *Eur. J. Pharmacol.* **233**, 13–20
- 24 Schatz-Munding, M., Hatzelmann, A. and Ullrich, V. (1991) *Eur. J. Biochem.* **197**, 487–493
- 25 Wijkander, J. and Sundler, R. (1989) *FEBS Lett.* **244**, 51–56
- 26 Clark, J., Lin, L., Kriz, R., Ramesha, C., Sultzman, L., Lin, A., Milona, N. and Knopf, J. (1991) *Cell* **65**, 1043–1051
- 27 Street, I., Lin, H.-K., Laliberte, F., Ghomaschi, F., Wang, Z., Perrier, H., Tremblay, N., Huang, Z., Weech, P. and Gelb, M. (1993) *Biochemistry* **32**, 5935–5940
- 28 Bartoli, F., Lin, H.-K., Ghomashchi, F., Gelb, M., Jain, M. and Apitz-Castro, R. (1994) *J. Biol. Chem.* **269**, 1560–1563
- 29 Ackermann, E., Conde-Frieboes, K. and Dennis, E. (1995) *J. Biol. Chem.* **270**, 445–450
- 30 Neufeld, E., Majerus, P., Krueger, C. and Saffitz, J. (1985) *J. Cell Biol.* **101**, 573–581
- 31 Capriotti, A., Furth, E., Arrasmith, M. and Laposata, M. (1988) *J. Biol. Chem.* **263**, 10029–10034
- 32 Watkins, J. and Kent, C. (1992) *J. Biol. Chem.* **267**, 5686–5692
- 33 Morita, I., Schindler, M., Regier, M., Otto, J., Hori, T., DeWitt, D. and Smith, W. (1995) *J. Biol. Chem.* **270**, 10902–10908
- 34 Woods, J., Evans, J., Ethier, D., Scott, S., Vickers, P., Hearn, L., Charleson, S., Heibein, J. and Singer, I. (1993) *J. Exp. Med.* **178**, 1935–1946
- 35 Woods, J., Coffey, M., Brock, T., Singer, I. and Peters-Golden, M. (1995) *J. Clin. Invest.* **95**, 2035–2040
- 36 Penrose, J., Spector, J., Lam, B., Friend, D., Xu, K., Jack, R. and Austen, K. (1995) *Am. J. Respir. Crit. Care Med.* **152**, 283–289