Arachidonate release and prostaglandin production by group IVC phospholipase A_2 (cytosolic phospholipase $A_2\gamma$)

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While the role of the group IVA Ca^{2+}-dependent cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) in arachidonic acid (AA) metabolism has been well documented, that of its paralogue, Ca^{2+}-independent group IVC PLA_2 ($cPLA_2\gamma$), has remained uncertain. Here we show, using a transfection strategy, that $cPLA_2\gamma$ has the ability to increase the spontaneous and stimulus-induced release of cellular fatty acids. The AA released by $cPLA_2\gamma$ was metabolized further to prostaglandin E_2 via cyclo-oxygenase-1 (COX-1) in the immediate response, and via COX-2 in the delayed response. Mutation of the putative catalytic-centre residue Ser^{82} abrogated the AA-releasing function of $cPLA_2\gamma$ both *in vitro* and *in vivo*. Confocal microscopy revealed that $cPLA_2\gamma$ was distributed in the perinuclear endoplasmic reticulum membranes. Mutating the C-terminal prenylation site of $cPLA_2\gamma$ abrogated its intracellular membrane localization and cellular AA-releasing function, without reducing its enzyme activity *in vitro*. Our results indicate that $cPLA_2\gamma$ is the second $cPLA_2$ enzyme that contributes to cellular AA metabolism and phospholipid remodelling under appropriate conditions.

Key words: cyclo-oxygenase, eicosanoid, HEK293 cell, mutagenesis, prenylation, transfection.

INTRODUCTION

Phospholipase A_2 (PLA_2) is a lipolytic enzyme that cleaves the *sn*-2 position of membrane glycerophospholipids to liberate fatty acids and lysophospholipids. To date, a number of PLA_2 enzymes [three cytosolic PLA_2s ($cPLA_2$s), 10 secretory PLA_2s ($sPLA_2$s), two Ca^{2+}-independent PLA_2s ($iPLA_2$s) and four platelet-activating factor acetylhydrolases] have been identified in mammals [1,2]. Among these enzymes, group IVA $cPLA_2\alpha$ plays a central role in the stimulus-coupled release of arachidonic acid (AA), a precursor of eicosanoids such as prostaglandins and leukotrienes, and of lysophospholipid-derived lipid mediators, such as platelet-activating factor [1,2]. Current evidence suggests that group IVA $cPLA_2\alpha$ and group IIA, IID, IIF, V, X and III $sPLA_2$s have the ability to trigger AA metabolism, whereas group VIA $iPLA_2$ plays a major role in phospholipid remodelling [1,2].

Group IVA $cPLA_2\alpha$ is the only PLA_2 enzyme that shows a marked preference for *sn*-2 AA over other fatty acids [3]. In activated cells, $cPLA_2\alpha$ undergoes Ca^{2+}-directed translocation from the cytosol to the perinuclear membranes [Golgi, endoplasmic reticulum (ER) and nuclear membranes] [4,5], where downstream AA-metabolizing enzymes, such as cyclo-oxygenases (COXs) and lipoxygenases, are also located [6,7]. The N-terminal C2 domain of $cPLA_2\alpha$, which binds preferentially to phosphatidylcholine, is essential for its membrane association [8], and several cationic residues clustering on surfaces of the C2 domain and the catalytic domain further facilitate interfacial binding of this enzyme to anionic membranes containing phosphatidylinositol 4,5-bisphosphate [9]. There are several phosphorylation sites that are essential for stimulus-coupled $cPLA_2\alpha$ activation, including Ser^{505} [by mitogen-activated protein kinases (MAPKs)], Ser^{727} (by MAPK-activated protein kinases) and Ser^{515} (by Ca^{2+}/calmodulin kinase II) [10–12]. $cPLA_2\alpha$ also exhibits Ca^{2+}-independent *sn*-1 lysophospholipase [13] and weak transacylase [14] activities. Gene targeting studies have provided unequivocal evidence that $cPLA_2\alpha$ plays crucial roles in various physiological and pathological events [15–18].

Nucleic acid database searches have revealed the presence of two additional $cPLA_2$ paralogues in the human genome: group IVB $cPLA_2\beta$ and group IVC $cPLA_2\gamma$ [19–22]. $cPLA_2\beta$ also possesses the N-terminal C2 domain which confers on the enzyme Ca^{2+} dependence, although the activity of this enzyme is reported to be far lower than that of $cPLA_2\alpha$. In contrast, $cPLA_2\gamma$ is devoid of the C2 domain, and its enzymic activity is Ca^{2+}-independent. The C-terminus of $cPLA_2\gamma$ undergoes prenylation [19], although the significance of this lipid modification remains unclear. Unlike $cPLA_2\alpha$, $cPLA_2\beta$ and $cPLA_2\gamma$ do not show apparent *sn*-2 AA preference [20–22]. In addition, these two paralogues display phospholipase A_1 or *sn*-1 lysophospholipase activity that is more potent than the PLA_2 activity [19–22]. Overall, the physiological functions (including AA metabolism and membrane remodelling) of these two $cPLA_2$ paralogues remain largely unclear. To address this issue, we have herein conducted an analysis of cellular fatty acid release by $cPLA_2\gamma$.

EXPERIMENTAL

Materials

Human embryonic kidney (HEK) 293 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co.) containing 10 % (v/v) fetal calf serum (FCS; Bioserum), as described previously [23]. The cDNA for human $cPLA_2\gamma$ was kindly provided by Dr L.-L. Lin (Genetics Institute, Cambridge, MA, U.S.A.) and was subcloned in the pCDNA3.1/zeo(+) vector (Invitrogen). HEK293 cells stably transfected with mouse $cPLA_2\alpha$ were

Abbreviations used: AA, arachidonic acid; COX, cyclo-oxygenase; ER, endoplasmic reticulum; FCS, fetal calf serum; HEK, human embryonic kidney; IL-1, interleukin-1; MAFP, methyl arachidonyl fluorophosphate; MAPK, mitogen-activated protein kinase; OA, oleic acid; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, Ca^{2+}-independent PLA_2; $sPLA_2$, secretory PLA_2; TBS, Tris-buffered saline; WT, wild-type.

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described previously [23]. Human COX-1 and COX-2 cDNAs were described previously [24]. Enzyme immunoassay kits for prostaglandin E_2 (PGE_2), the COX-2 inhibitor NS-398 and the cPLA$_2\alpha$ inhibitor methyl arachidonyl fluorophosphate (MAFP) were purchased from Cayman Chemicals. Goat anti-(human COX-1) and anti-(human COX-2) antibodies were from Santa Cruz. A23187 was from CalBiochem. Human interleukin-1β (IL-1β) was from Genzyme. Opti-MEM medium, LIPOFECTAMINE 2000™ reagent, geneticin, hygromycin, zeocin, TRIZOL reagent and the mammalian expression vectors pCR3.1, pCDNA3.1/hyg(+) and pCDNA3.1/zeo(+) were from Invitrogen. FITC-conjugated anti-(mouse IgG) and horseradish peroxidase-conjugated anti-(goat IgG) were from Zymed. The mouse monoclonal anti-FLAG antibody M2 was obtained from Sigma. All primers for PCR were obtained from Greiner Japan.

Establishment of transfectants

HEK293 transformants were established as described previously [23,24]. Briefly, 1 μg of plasmid was mixed with 2 μl of LIPOFECTAMINE 2000™ in 100 μl of Opti-MEM medium for 30 min, and then added to cells that had attained 40–60 % confluence in 12-well plates (Iwaki Glass) containing 0.5 ml of Opti-MEM. After incubation for 6 h, the medium was replaced with 1 ml of fresh culture medium. After overnight culture, the medium was replaced with a further 1 ml of fresh medium, and culture was continued at 37 °C in an incubator flushed with 5 % CO_2 in humidified air. The cells were cloned by limiting dilution in 96-well plates in culture medium supplemented with 1 mg/ml geneticin (for pCR3.1) or 10 μg/ml zeocin [for pCDNA3.1/zeo(+)]. After culture for 3–4 weeks, wells containing a single colony were chosen, and the expression of proteins was assessed by RNA blotting or immunoblotting. The established clones were expanded and used for the experiments as described below.

To assess functional coupling between cPLA$_2\gamma$ and COX-1, HEK293 cells stably expressing cPLA$_2\gamma$ were transfected with COX-1 subcloned into pCDNA3.1 using LIPOFECTAMINE 2000™. At 3 days after the transfection, the cells were activated with A23187 to measure PGE_2 generation and were subjected to immunoblotting to examine COX-1 expression (see below).

Measurement of PLA_2 activity

PLA_2 activity was assayed by measuring the amounts of free radiolabelled fatty acids released from the substrate 1-palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylethanolamine (Amersham Pharmacia Biotech). Each reaction mixture (total volume 250 μl) consisted of appropriate amounts of the required sample, 100 mM Tris/HCl (pH 7.4), 4 mM $CaCl_2$ or 5 mM EDTA and 10 μM substrate. After incubation for 10–30 min at 37 °C, [^{14}C]AA was extracted and radioactivity was quantified as described previously [23].

Northern blotting

Approximately equal amounts ($\sim$5 μg) of total RNA obtained from the cells were applied to separate lanes of 1.2 % (w/v) formaldehyde/agarose gels, electrophoresed and transferred to Immobilon-N membranes (Millipore). The resulting blots were then probed with the respective cDNA probes that had been labelled with [^{32}P]dCTP (Amersham Pharmacial Biotech) by random priming (Takara Biomedicals). All hybridizations were carried out as described previously [23,24].

SDS/PAGE and immunoblotting

Lysates from 10^5 cells were subjected to SDS/PAGE using 7.5–10 % (w/v) polyacrylamide gels under reducing conditions. The separated proteins were electroblotted on to nitrocellulose membranes (Schleicher and Schuell) using a semi-dry blotter (MilliBlot-SDE system; Millipore). After blocking with 3 % (w/v) skimmed milk in Tris-buffered saline (TBS) containing 0.05 % Tween-20 (TBS/Tween), the membranes were probed with the respective antibodies (1:20000 dilution for COX-1, 1:5000 dilution for COX-2 and 1:20000 for FLAG epitope, in TBS/Tween) for 2 h, followed by incubation with horseradish peroxidase-conjugated anti-goat (for COXs) or anti-mouse (for FLAG) IgG (1:5000 dilution in TBS/Tween) for 2 h. Visualization was performed using the enhanced chemiluminescence Western blot system (NEN™ Life Science Products), as described previously [23,24].

Construction of cPLA$_2\gamma$ mutants

cPLA$_2\gamma$ mutants were produced by PCR with the Advantage cDNA polymerase mix (Clontech) using cPLA$_2\gamma$ cDNA as a template. The conditions of PCR were 25 cycles at 94 °C, 55 °C and 72 °C for 30 s each. The primers used were as follows: cPLA$_2\gamma$-N primer, 5′-ATGGGAAGCTCTGAAGTTTCC-3′ cPLA$_2\gamma$-C primer, 5′-CTATCCAAGCAGCAACTTCG-3′; S82A-S primer, 5′-GCAGGGGTCGCTGGATCCACT-3′; S82A-AS primer, 5′-AGTGGATCCAGCGACCCCTGC-3′; cPLA$_2\gamma$-CS primer, 5′-CTATGCCAAGCTGCTACTTCG-3′; FLAG-N primer, 5′-ATGGACTACAAGGACGACGATGACAAGATGGGAAGCTC TGAAGTT-3′ (FLAG sequence underlined).

To obtain the cPLA$_2\gamma$ mutant CS (see the Results section), the primer set cPLA$_2\gamma$-N and cPLA$_2\gamma$-CS was used. To obtain FLAG-tagged cPLA$_2\gamma$ or cPLA$_2\gamma$-CS, the primer sets FLAG-N and cPLA$_2\gamma$-C or cPLA$_2\gamma$-CS respectively were used. In order to construct the catalytic-site mutant S82A (see the Results section), the first PCR was conducted with cPLA$_2\gamma$-N and S82A-AS primers or with S82A-S and cPLA$_2\gamma$-C primers using cPLA$_2\gamma$ cDNA as a template. The resulting two primary PCR fragments were mixed, denatured at 94 °C for 5 min, annealed at 37 °C for 30 min and then at 55 °C for 2 min, and extended at 72 °C for 4 min during each cycle. A secondary PCR product with a specific mutation was obtained after 25 additional PCR cycles with cPLA$_2\gamma$-N and cPLA$_2\gamma$-C primers.

Each PCR product was ligated into the pCR3.1 vector and was transfected into Top10F' supercompetent cells (Invitrogen). The plasmids were isolated and sequenced using a *Taq* cycle sequencing kit (Takara Biomedicals) and an autofluorimetric DNA sequencer 310 Genetic Analyzer (Applied Biosystems) to confirm the sequences.

Preparation of cell homogenates

HEK293 cells (10^7 cells) grown in 100-mm dishes (Iwaki Glass) were trypsinized and resuspended in 1 ml of 20 mM Tris/HCl (pH 7.4) containing 150 mM NaCl, 1 mM PMSF, 1 mM *N*-benzoyl-L-arginine ethyl ester hydrochloride and 10 μg/ml leupeptin (cell lysis buffer). After sonication using a Branson Sonifier, the resulting lysates were centrifuged at 100 000 ***g*** for

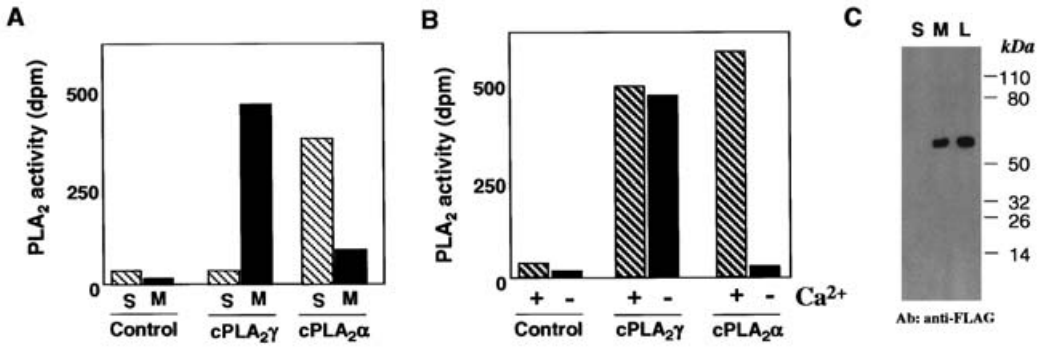

Figure 1 Enzymic properties of $cPLA_2\gamma$

(**A**) Control HEK293 cells or those transfected with $cPLA_2\gamma$ (clone #2; see Figure 2A) or $cPLA_2\alpha$ were suspended in 1 ml of cell lysis buffer at 5×10^6 cells/ml, sonicated, and centrifuged for 1 h at 100000 ***g***. The pellet was reconstituted in 1 ml of the lysis buffer. Aliquots (20 μl) of the supernatant (S) and membrane (M) fractions were taken for the PLA_2 assay using 1-palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylethanolamine as substrate. (**B**) Ca^{2+} dependence. Lysates of control and $cPLA_2\gamma$- or $cPLA_2\alpha$-transfected cells were assayed for PLA_2 activity in the presence of 1 mM Ca^{2+} (+) or 5 mM EDTA (–). (**C**) Cells transfected with FLAG-tagged $cPLA_2\gamma$ were sonicated and centrifuged at 100000 ***g***. Aliquots (10 μl) of the resulting supernatant (S) and membrane (M) fractions, as well as the total cell lysate (L), were subjected to SDS/PAGE and immunoblotting using an anti-FLAG monoclonal antibody. Control cells did not provide a visible band (not shown). Representative results of three independent experiments are shown in (**A**) and (**B**).

1 h at 4 °C. After collection of the supernatants, the pellets were reconstituted in the same volume of cell lysis buffer.

Activation of HEK293 cells

HEK293 cells (5×10^4/ml) were seeded into each well of 48-well plates. To assess fatty acid release, [^{3}H]AA or [^{3}H]oleic acid ([^{3}H]OA) (both from Amersham Pharmacial Biotech; 0.1 μCi/ml) was added to the cells in each well on day 3, when they had nearly reached confluence, and culture was continued for a further 1 day. After three washes with fresh medium, 100 μl of RPMI 1640 medium containing 1 % (v/v) FCS in the presence or absence of 10 μM A23187 or containing 10 % (v/v) FCS in the presence or absence of IL-1β was added to each well, and the amount of free [^{3}H]AA or [^{3}H]OA released into the supernatant was measured. Percentage release was calculated using the formula $[S/(S+P)]\times100$, where S and P are the radioactivity measured in the supernatant and cell pellet respectively. The supernatants from replicate cells were subjected to the PGE_2 enzyme immunoassay.

Confocal laser microscopy

Cells grown on collagen-coated coverglasses (Iwaki Glass) were fixed with 3 % paraformaldehyde for 30 min in PBS. After three washes with PBS, the fixed cells were treated sequentially with 1 % (w/v) BSA (for blocking) plus 0.2 % (v/v) Triton X-100 (for permeabilization) in PBS for 1 h, with anti-FLAG antibody (1:500 dilution) in PBS containing 1 % (w/v) BSA for 1 h, and then with FITC-conjugated goat anti-(mouse IgG) (1:500 dilution) in PBS containing 1 % (w/v) BSA for 1 h. After six washes with PBS, the cells were mounted on glass slides using Perma Fluor (Japan Tanner), and the signal was visualized using a laser scanning confocal microscope (IX70; Olympus), as described previously [25].

Statistical analysis

Data were analysed using Student's t-test. Results are expressed as means $\pm$ S.E.M., with $P = 0.05$ as the limit of significance.

RESULTS

Establishment of $cPLA_2\gamma$ transfectants

To assess the enzymic properties and cellular functions of $cPLA_2\gamma$, we transfected human $cPLA_2\gamma$ cDNA into HEK293 cells to obtain drug-resistant stable transfectants. As assessed by Northern blotting, $cPLA_2\gamma$ mRNA was detected in two stable transfectants, but not in parental cells (see Figure 2A, inset).

Sonicates of the cells were separated into soluble and membrane fractions by centrifugation at 100000 ***g***, and PLA_2 activities in both fractions were measured using 1-palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylethanolamine as substrate. As shown in Figure 1(A), the membrane fraction, but not the soluble fraction, of $cPLA_2\gamma$-transfected cells exhibited significant PLA_2 activity. This activity was Ca^{2+}-independent, as the addition of EDTA did not decrease the activity (Figure 1B). In comparison, the PLA_2 activity of $cPLA_2\alpha$-transfected cells, which we had established previously [23], was distributed preferentially in the soluble fraction (Figure 1A) and showed strict Ca^{2+}-dependence (Figure 1B), as expected.

To assess the membrane distribution of $cPLA_2\gamma$ more directly, we expressed N-terminally FLAG-tagged $cPLA_2\gamma$ in HEK293 cells, and its distribution in the supernatant and membrane fractions after centrifugation of cell lysates at 100000 ***g*** was assessed by immunoblotting with an anti-FLAG antibody. As shown in Figure 1(C), a band with an estimated molecular mass of $\sim$60 kDa (the predicted size of $cPLA_2\gamma$ [19,20]) was detected in the membrane fraction, but not in the supernatant fraction.

$cPLA_2\gamma$ induces cellular AA release and PGE_2 production

We next tested whether $cPLA_2\gamma$ is capable of affecting cellular fatty acid release. As shown in Figure 2(A), both $cPLA_2\gamma$-transfected clones released more [^{3}H]AA than control cells in response to incremental concentrations of FCS over 4 h of incubation. This $cPLA_2\gamma$-induced [^{3}H]AA release was augmented approximately 2-fold by the further addition of IL-1 (Figure 2A). Increased release of [^{3}H]OA was also observed in $cPLA_2\gamma$-transfected cells, which amounted to about half that of [^{3}H]AA (Figure 2B). In comparison, $cPLA_2\alpha$-transfected cells showed a marked increase in IL-1-stimulated [^{3}H]AA release that was not

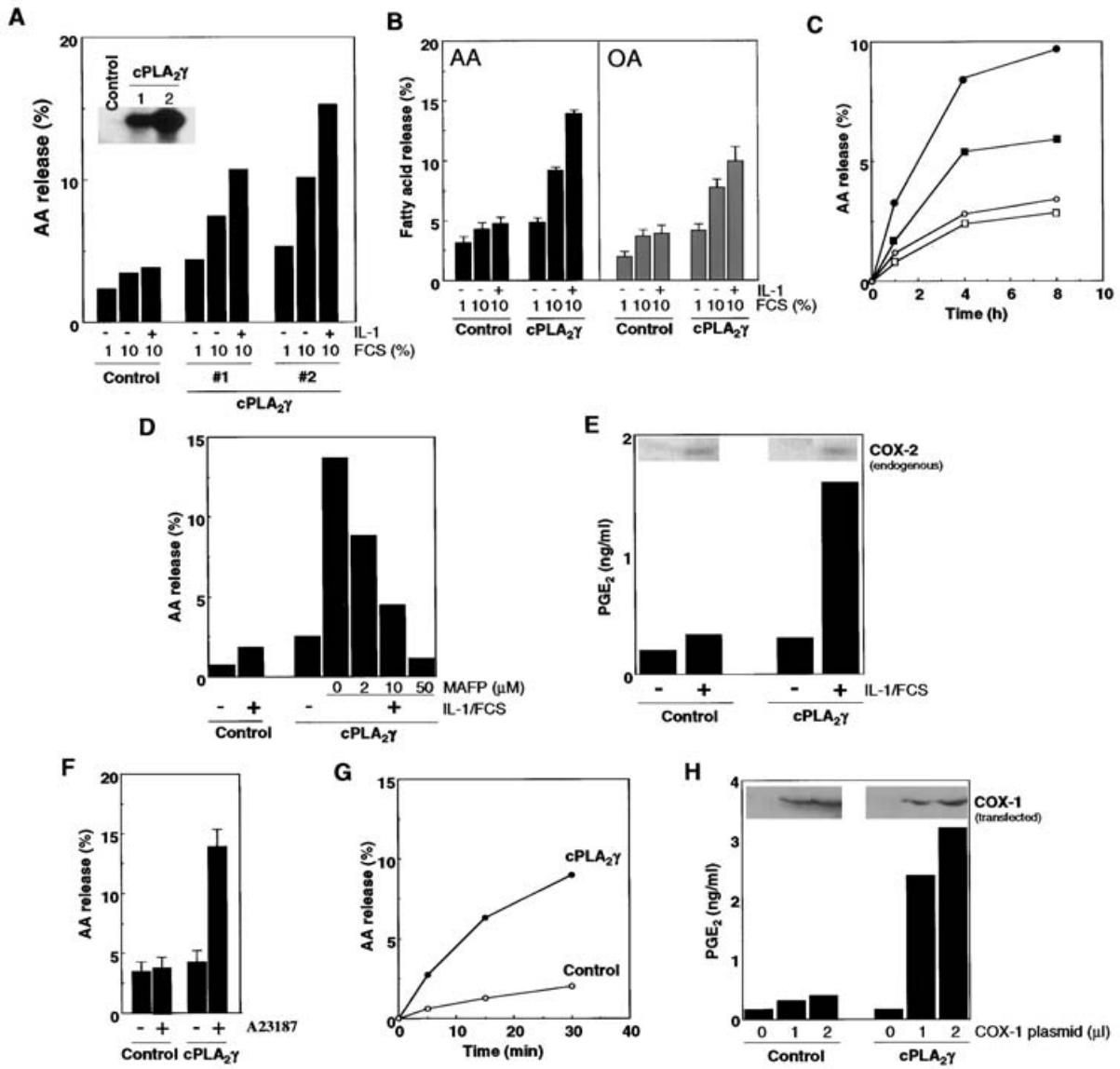

Figure 2 AA metabolism by cPLA$_2\gamma$

(**A**) FCS/IL-1-dependent AA release by cPLA$_2\gamma$. Two cPLA$_2\gamma$-expressing clones and control cells, which were prelabelled with [^{3}H]AA overnight, were incubated for 4 h in the presence of 1 % or 10 % (v/v) FCS with or without 1 ng/ml IL-1β. (**B**) Fatty acid selectivity. Control cells and cPLA$_2\gamma$-expressing cells were prelabelled with [^{3}H]AA or [^{3}H]OA, and the release of each fatty acid over 4 h under the indicated conditions was assessed. (**C**) Time course of [^{3}H]AA release by control (□, ○) and cPLA$_2\gamma$-transfected (●, ■) cells in the presence of 10 % (v/v) FCS alone (□, ■) or 10 % (v/v) FCS plus 1 ng/ml IL-1β (○, ●). (**D**) Effects of MAFP. [^{3}H]AA-prelabelled control and cPLA$_2\gamma$-transfected cells were preincubated for 4 h with the indicated concentrations of MAFP, washed, and then stimulated for 4 h with IL-1 in medium containing 10 % (v/v) FCS (IL-1/FCS) in the absence of MAFP. (**E**) COX-2 coupling. Control and cPLA$_2\gamma$-transfected cells were incubated for 4 h with or without IL-1/FCS, and PGE_2 released into the supernatants was quantified. The cells were harvested, lysed and subjected to SDS/PAGE and immunoblotting using an anti-COX-2 antibody (gels). (**F**) Immediate AA release. [^{3}H]AA-prelabelled control and cPLA$_2\gamma$-transfected cells were incubated for 30 min with or without 10 μM A23187 in medium containing 1 % (v/v) FCS. (**G**) Time course of A23187-stimulated [^{3}H]AA release by control (○) and cPLA$_2\gamma$-transfected (●) cells. (**H**) COX-1 coupling. Control and cPLA$_2\gamma$-transfected cells were transiently transfected with the indicated concentrations of the COX-1 plasmid. After 3 days, the cells were stimulated for 30 min with A23187, and PGE_2 released into the supernatants was quantified. COX-1 expression in cells was assessed by SDS/PAGE and immunoblotting using an anti-COX-1 antibody (gels). In (**B**)–(**H**), a cPLA$_2\gamma$-expressing clone #2 was used. Representative results of two to three experiments are shown in (**A**), (**C**)–(**E**), (**G**) and (**H**). Values are means $\pm$ S.E.M. of three independent experiments in (**B**) and (**F**).

accompanied by [^{3}H]OA release [23]. The extent of the increase in fatty acid release appeared to be correlated with the expression levels of cPLA$_2\gamma$ (Figure 2A). Time-course experiments showed that AA release by cPLA$_2\gamma$, in the presence or absence of IL-1, proceeded gradually over 4 h and tended to reach a plateau thereafter (Figure 2C). As shown in Figure 2(D), cPLA$_2\gamma$-mediated AA release was suppressed by MAFP, a well known cPLA$_2\alpha$ inhibitor [26], with a dose-dependence similar to that for the inhibition of cPLA$_2\alpha$-mediated AA release [23].

IL-1-stimulated AA release by cPLA$_2\gamma$ was accompanied by increased PGE_2 production (Figure 2E), which was blunted by the COX-2 inhibitor NS-398 (results not shown). Thus cPLA$_2\gamma$ is capable of supplying AA to endogenous COX-2 for delayed prostaglandin biosynthesis. As in the case of cPLA$_2\alpha$-transfected cells [27], IL-1-induced expression of endogenous COX-2 in cPLA$_2\gamma$-transfected cells was comparable with that observed in parental cells (Figure 2E, inset). This indicates that, unlike various sPLA$_2$s that enhance COX-2 expression [25,27], the cPLA$_2$

family enzymes generally fail to augment COX-2 induction in this experimental setting, even though cPLA$_2\alpha$-promoted COX-2 induction can take place in other cell systems [28].

To assess whether cPLA$_2\gamma$ also elicits an immediate response, we stimulated the [^{3}H]AA-labelled cells with A23187. After stimulation for 30 min with A23187, [^{3}H]AA release was markedly increased in cPLA$_2\gamma$-transfected cells over that in control cells (Figure 2F). Thus, in contrast with a recent report that cPLA$_2\gamma$ failed to affect calcium ionophore-stimulated AA release in cPLA$_2\alpha$-deficient human lung fibroblasts [21], cPLA$_2\gamma$ is able to augment the immediate AA-release response in our HEK293 cell system. As shown in Figure 2(G), A23187-induced AA release by cPLA$_2\gamma$-transfected cells occurred within 5 min, increasing continuously over 30 min.

Since immediate prostaglandin production can be mediated by both COX-1 and COX-2, and since the occurrence of cPLA$_2\gamma$–COX-2 coupling is already evident in Figure 2(E), we next tested if the AA released by cPLA$_2\gamma$ following stimulation by A23187 is metabolized to PGE$_2$ via COX-1 (cPLA$_2\gamma$–COX-1 coupling). To address this, we transfected COX-1 into cPLA$_2\gamma$-expressing HEK293 cells, since HEK293 cells express endogenous COX-1 only minimally [24]. As shown in Figure 2(H), there was a marked COX-1-dose-dependent increase in PGE$_2$ production in cPLA$_2\gamma$-transfected cells in response to A23187, revealing cPLA$_2\gamma$–COX-1 coupling in the immediate response.

Mutation studies

Next, we transfected HEK293 cells with cPLA$_2\gamma$ mutants that harboured amino acid replacements in the putative catalytic centre (cPLA$_2\gamma$-S82A) and in the C-terminal prenylation site (cPLA$_2\gamma$-CS; the C-terminal -CCLA was replaced by -SSLA) to assess the effects of these mutations on cPLA$_2\gamma$ function. Clones expressing cPLA$_2\gamma$-S82A or cPLA$_2\gamma$-CS, the expression levels of which were each comparable with those of wild-type cPLA$_2\gamma$ (cPLA$_2\gamma$-WT) (Figure 3A), were used in subsequent studies.

In contrast with cPLA$_2\gamma$-WT, cPLA$_2\gamma$-S82A enzymic activity was undetectable in both the 100000 ***g*** soluble and membrane fractions of cell lysates (Figure 3B), confirming that this serine residue, which is conserved among the cPLA$_2$ family enzymes [19,20], is the catalytic centre. Notably, the activity of cPLA$_2\gamma$-CS, a prenylation site mutant, was detected almost equally in both the soluble and membrane fractions, and appeared to be higher than the activity of cPLA$_2\gamma$-WT (Figure 3B) irrespective of their comparable expression levels (Figure 3A). When the membrane fraction of cPLA$_2\gamma$-CS-transfected cells was suspended in the same buffer and recentrifuged at 100000 ***g***, the enzyme activity was again distributed evenly in the soluble and membrane-bound fractions (results not shown). These results suggest that the affinity of the CS mutant for endogenous membranes is weaker than that of the WT enzyme. Like the WT enzyme, the activity of the CS mutant showed no Ca^{2+}-dependence (results not shown). As illustrated in Figure 3(C), the enzyme activity of cPLA$_2\gamma$-CS increased almost linearly in proportion to the amount of enzyme preparations added to the PLA$_2$ assay mixture, whereas the activity of cPLA$_2\gamma$-WT was readily saturated at higher doses. A likely interpretation of this observation is that tight association of cPLA$_2\gamma$-WT with endogenous membranes through its C-terminal prenylation site prevents its efficient interaction with exogenous phospholipid substrates, whereas the cPLA$_2\gamma$-CS mutant can gain access to exogenous substrate due to its reduced affinity for endogenous membranes. A higher efficacy of cPLA$_2\gamma$-WT towards endogenous membrane than exogenous substrate has been also reported by Stewart et al. [21].

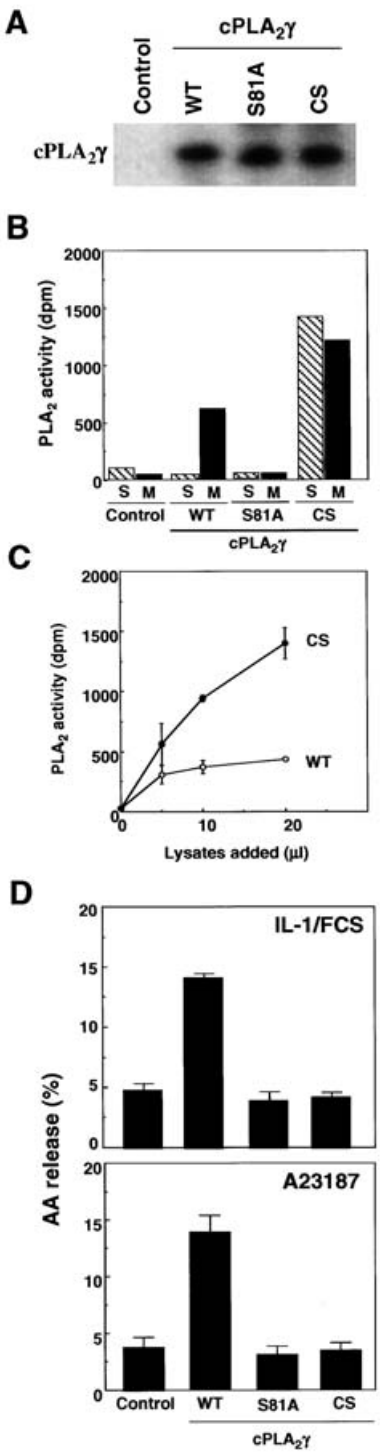

Figure 3 Mutation analysis of cPLA$_2\gamma$

(**A**) Expression of cPLA$_2\gamma$-WT, cPLA$_2\gamma$-S82A and cPLA$_2\gamma$-CS in their respective transfectants. (**B**) PLA$_2$ activities in the 100000 ***g*** supernatant (S) and membrane (M) fractions of the lysates of control cells and cPLA$_2\gamma$-WT-, cPLA$_2\gamma$-S82A- and cPLA$_2\gamma$-CS-transfected cells. A representative result of three independent experiments is shown. (**C**) PLA$_2$ activities of cPLA$_2\gamma$-WT and cPLA$_2\gamma$-CS were measured as a function of the amount of cell lysate. Values are means ± S.D. of duplicate determinations, and are representative of three independent experiments. (**D**) Control cells and cPLA$_2\gamma$-WT-, cPLA$_2\gamma$-S82A- and cPLA$_2\gamma$-CS-transfected cells, which were prelabelled with [^{3}H]AA, were incubated for 4 h with IL-1/FCS (upper panel) or for 30 min with A23187 (lower panel) to assess cellular [^{3}H]AA release. Values are means ± S.E.M. of three independent experiments.

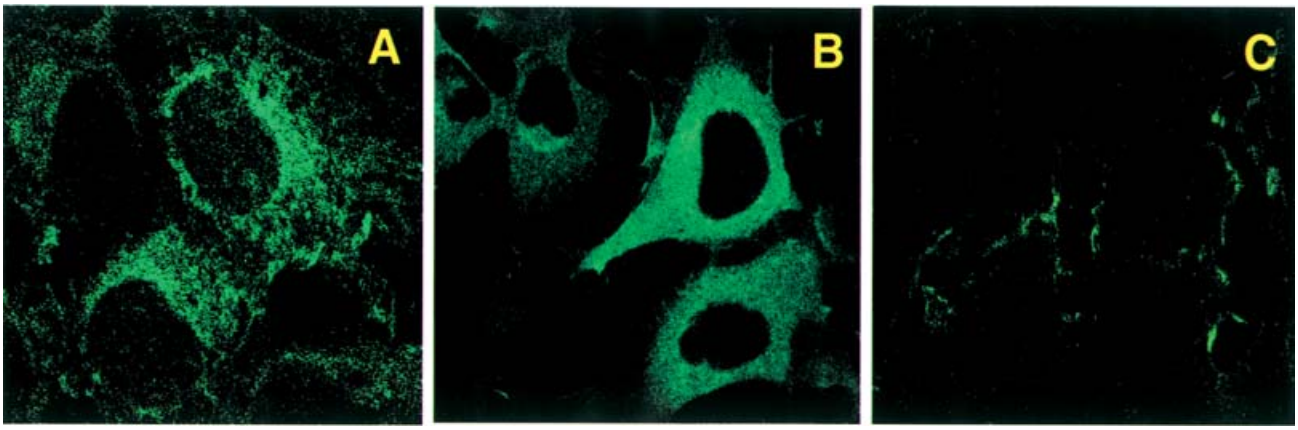

Figure 4 Subcellular localization of cPLA$_2\gamma$

N-terminally FLAG-tagged cPLA$_2\gamma$-WT (**A**) or cPLA$_2\gamma$-CS (**B**) was transfected into HEK293 cells and visualized by confocal laser microscopy. Panel (**C**) shows control staining (no transfection).

Importantly, not only the cPLA$_2\gamma$-S82A mutant, which was catalytically inactive, but also the cPLA$_2\gamma$-CS mutant, which retained catalytic activity *in vitro* but showed reduced association with endogenous membranes, failed to increase cellular [^{3}H]AA release in response to 10% (v/v) FCS in the presence (Figure 3D, upper panel) or absence (results not shown) of IL-1, or in response to A23187 (Figure 3D, lower panel) under conditions where the WT enzyme responded to both stimuli. These results suggest that the stable association of cPLA$_2\gamma$ with endogenous membranes appears to be essential for its appropriate cellular function.

Subcellular localization

To determine the intracellular sites of cPLA$_2\gamma$ action, N-terminally FLAG-tagged cPLA$_2\gamma$ was transfected into HEK293 cells and confocal immunofluorescent microscopic analyses were conducted. cPLA$_2\gamma$-WT exhibited a cytoplasmic reticular staining that was enriched in the perinuclear region, suggesting its localization in the ER membrane (Figure 4A). No signal for cPLA$_2\gamma$-WT was detected on the nuclear envelope. Neither IL-1 nor A23187 stimulation altered the localization of cPLA$_2\gamma$-WT (results not shown). In contrast, the signal for the cPLA$_2\gamma$-CS mutant did not show such a clear perinuclear/ER staining, but was dispersed in the cytoplasm throughout the cells (Figure 4B). No appreciable positive signals were detected on control staining with anti-FLAG antibody (Figure 4C). These results suggest that the constitutive sorting of cPLA$_2\gamma$ into the perinuclear/ER membranes is crucial for its cellular function.

DISCUSSION

Although amino acid residues required for enzymic catalysis are well conserved among the group IV cPLA$_2$ enzymes (α, β and γ), cPLA$_2\gamma$ differs from the other two enzymes in several aspects [19–21]. cPLA$_2\gamma$ lacks the C2 domain, which is required for the Ca^{2+}-dependent membrane association of cPLA$_2\alpha$ and (probably) cPLA$_2\beta$, and its enzymic activity is Ca^{2+}-independent. cPLA$_2\gamma$ is C-terminally prenylated and is constitutively associated with the membrane [19–21]. We herein provide the first line of evidence that this prenylation site is indeed critical for the membrane localization, and thereby the cellular function, of cPLA$_2\gamma$. Although understanding of the true physiological functions of cPLA$_2\gamma$ has to await future studies, including gene targeting, our present data nonetheless shed light on the potential role of this unique cPLA$_2$ family enzyme in AA metabolism and membrane remodelling.

We demonstrate here that cPLA$_2\gamma$ has the capacity to mobilize cellular fatty acids, increasing the spontaneous (serum-dependent) release of both AA and OA in HEK293 transfectants (Figure 2). This property is reminiscent of group VIA iPLA$_2$ [23], and is likely to reflect a phospholipid remodelling function of the enzyme. In comparison, cPLA$_2\alpha$, a signalling PLA$_2$, elicits AA-selective release only after cell activation [23]. Recently, Stewart et al. [21] also reported that cPLA$_2\gamma$ is capable of increasing cellular fatty acid release in the presence of serum, an observation consistent with our present results. The conclusion that cPLA$_2\gamma$ possesses *sn*-1 lysophospholipase activity [21] may also support a membrane remodelling role. An approx. 2-fold preference for AA over OA observed in the present study (Figure 2B) is in line with the fact that cPLA$_2\gamma$ hydrolyses *sn*-2 AA with $\sim$2-fold greater efficiency than *sn*-2 OA [19]; alternatively, it may indicate that cPLA$_2\gamma$ acts on an AA-rich phospholipid pool, as has been proposed for several sPLA$_2$s [23–25]. Asai et al. [22] have provided additional evidence that cPLA$_2\gamma$ is engaged in membrane phospholipid remodelling, particularly of phosphatidylethanolamine species containing polyunsaturated fatty acids.

At present, it is unclear how serum facilitates the cellular actions of PLA$_2$s (not only cPLA$_2\gamma$, but also several other PLA$_2$s) [23–25,27]. This might simply be because cells are more healthy in the presence of serum (due to a nutritional effect) than in its absence during the prolonged response. Alternatively, some specific factors in serum might sensitize cell membranes to be more susceptible to PLA$_2$s. With regard to cPLA$_2$ enzymes, it is well established that cPLA$_2\alpha$ is activated by phosphorylation by MAPKs, an event that can be activated by serum [10,11]. It is interesting to note that the function of cPLA$_2\gamma$ is suppressed by a tyrosine kinase inhibitor [22], suggesting the involvement of a phosphorylation event in the cPLA$_2\gamma$ activation process as well. The presence of several potential phosphorylation sites on cPLA$_2\gamma$ [19,20] supports this hypothesis. Whether or not cPLA$_2\gamma$ undergoes phosphorylation in cells under particular conditions, and, if so, which residues on cPLA$_2\gamma$ are phosphorylated by which types of protein kinase, are now under investigation.

Stimulation by IL-1 of cPLA$_2\gamma$-transfected cells leads to increased AA release, which is linked to PGE$_2$ production by endogenous COX-2 (Figure 2). Although the *in vitro* enzymic

activity of $cPLA_2\gamma$ is independent of Ca^{2+}, stimulation of $cPLA_2\gamma$-transfected cells with the Ca^{2+} ionophore A23187 also results in marked increases in AA release and attendant COX-1-mediated PGE_2 production. This A23187-elicited event is also observed with group VIA $iPLA_2$ [23,24], suggesting that the Ca^{2+}-independent types of PLA_2 can be activated by Ca^{2+}-provoked intracellular signalling, probably indirectly. In a recent study, Stewart et al. [21] failed to observe A23187-coupled AA release by $cPLA_2\gamma$ because of high background AA release by endogenous $cPLA_2\alpha$ in the cells that they used. However, our data clearly suggest that $cPLA_2\gamma$ can act as a signalling PLA_2 under appropriate conditions. In view of this, studies employing MAFP need to be interpreted with caution, as this widely used $cPLA_2\alpha$ inhibitor also blocks $cPLA_2\gamma$ as well as group VIA $iPLA_2$ [31].

$cPLA_2\gamma$ is distributed in the ER membrane, being enriched in the perinuclear area (Figure 4). Asai et al. [22] have reported a similar intracellular distribution of $cPLA_2\gamma$. Considering that the perinuclear/ER membrane is the major site of eicosanoid production [6,7] and phospholipid biosynthesis [32,33], it makes sense that $cPLA_2\gamma$, which can participate in both processes as indicated above, is located in this membrane compartment. $cPLA_2\gamma$ contains a C-terminal prenylation site (-CCLA) [19], which resembles that found in the Rab family of small G-proteins [34–37]. This motif signals the addition of geranylgeranyl to the protein. Although a previous study failed to demonstrate the importance of this lipid modification in the membrane association of $cPLA_2\gamma$ in COS cells [19], we now show that mutation of this site leads to reduced membrane binding of the enzyme in HEK293 cells (Figure 3). Importantly, this mutation abrogated the ER localization of $cPLA_2\gamma$ (Figure 4), which was accompanied by complete loss of its cellular AA-releasing function, despite no decrease in its *in vitro* enzymic activity (Figure 3). This suggests that the constitutive association of $cPLA_2\gamma$ with the ER membrane is essential for its appropriate function in cells. Due to the lack of a C2 domain, the $cPLA_2\gamma$ prenylation site mutant may not get access to the ER membrane, thereby being unable to exert its enzyme function.

Although the mechanisms whereby $cPLA_2\gamma$ is sorted preferentially into the ER membrane are unclear at present, the Rab small G-proteins, which undergo C-terminal geranylgeralylation, can be distributed in the intracellular membranous compartments and play a role in membrane trafficking and vesicular transport [37–41]. Several studies using a wide range of PLA_2 inhibitors have suggested the possible participation of certain Ca^{2+}-independent type(s) of PLA_2 in intracellular membrane fusion and trafficking [42–44]. Whether $cPLA_2\gamma$ is involved in these critical cellular processes by altering the phospholipid composition of fusing membranes is an attractive question to examine in the future.

We thank Dr L.-L. Lin (Genetics Institute, Cambridge, MA, U.S.A.) for providing human $cPLA_2\gamma$ cDNA, and Dr K. Asai, Dr N. Uozumi and Dr T. Shimizu (University of Tokyo, Japan) for helpful discussions. This work was supported by a Grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Technology of Japan.

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Received 6 January 2003/26 February 2003; accepted 3 March 2003
Published as BJ Immediate Publication 3 March 2003, DOI 10.1042/BJ20030061