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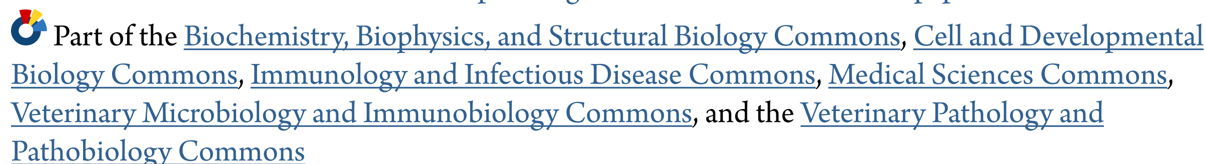

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Regulation of Cytosolic Phospholipase A2 (cPLA$_2\alpha$) and Its Association with Cell Proliferation in Human Lens Epithelial Cells

Yin Wang,[1,2] *Kui-Yi Xing,*[1,2] *and Marjorie F. Lou*[1,2,3]

Purpose. To investigate the molecular mechanism for cytosolic phospholipase A2 (cPLA$_2\alpha$) regulation and its association to platelet-derived growth factor (PDGF)-induced cell proliferation.

Methods. cPLA$_2\alpha$ was examined using human lens epithelial (HLE) B3 cells. Reactive oxygen species (ROS) generation induced by PDGF was analyzed by luminescence assay. Cell proliferation was measured by cell counting and by BrdU assay. Human *cPLA$_2\alpha$* gene was cloned via RT-PCR followed by site-directed mutagenesis to construct HLE B3 cells expressing either inactive cPLA$_2\alpha$ enzyme with S228A mutation (S228A), or cPLA$_2\alpha$ truncated at the calcium-binding C2 domain (C2D). Activity of cPLA$_2\alpha$ was measured by arachidonic acid (AA) release from cell membranes using [^{3}H]-arachidonic acid prelabeled cells. The effect of intracellular calcium level on cPLA$_2\alpha$ function was examined by treating cells with ionomycin (calcium influx), thapsgargin (endoplasmic reticulum [ER] calcium store release) or 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (BAPTA; calcium chelator). Activation of extracellular signal–regulated kinases (ERK), JNK, p38, or Akt was detected by Western blot analysis using specific antibodies.

Results. S228A mutant showed suppressed PDGF-induced reactive oxygen species generation, ERK and JNK activation (no effect on p38 or Akt), and cell proliferation in comparison with the vector alone (Vec) control. Calcium-binding C2 domain cells lost the ability of membrane translocation and activation of cPLA$_2\alpha$. PDGF cell signaling was calcium-dependent, and the calcium was supplied either from the external flux or endoplasmic reticulum store. However, enrichment of cellular calcium not only augmented PDGF function, but also demonstrated a cPLA$_2\alpha$-dependent calcium-signaling cascade that led to cell proliferation.

Conclusions. cPLA$_2\alpha$ is regulated by calcium mobilization and mitogen-activated protein kinases (MAPK) activation. Both PDGF mitogenic action and calcium signaling are cPLA$_2\alpha$-dependent. (*Invest Ophthalmol Vis Sci.* 2011;52:8231–8240) DOI:10.1167/iovs.11-7542

Cytosolic phospholipase A$_2\alpha$ (cPLA$_2\alpha$) belongs to the large phospholipase A$_2$ (PLA$_2$) family that is widely involved in various fundamental cellular physiological functions, including neurochemical processes, normal brain function, regulation of protein kinase C and modulation of ion channels.[1,2] cPLA$_2\alpha$ is widely distributed in mammalian tissues and can be activated by extracellular stimuli such as growth factors, cytokines, neurotransmitters, or endotoxins,[2] and selectively hydrolyzes glycerophospholipids containing arachidonic acid (AA) at the sn-2 position. The released AA can be further metabolized to leukotrienes, prostaglandins, or platelet-activating factor for various biological needs.[3–5] The subcellular localization and target of cPLA$_2\alpha$ may vary depending on the cell type; however, cPLA$_2\alpha$ requires cellular calcium binding at the C2 domain to be translocated to the membrane for its catalytic action. For instance, in endothelial cells, cPLA$_2\alpha$ is mobilized from the cytosol to the perinuclear region (endoplasmic reticulum, Golgi, and nuclear envelope) where the AA produced is metabolized to eicosanoids[6] while in neutrophils and granulocytes, cPLA$_2\alpha$ is recruited to plasma membrane in which the AAs released are needed for the generation of reactive oxygen species (ROS) from NADPH oxidase. Translocating cPLA$_2\alpha$ to the membrane is an important step as it allows cPLA$_2\alpha$ to be phosphorylated and activated by ERK so that it can interact with its phospholipid substrate on the membrane to release AA.[7] Serine residues in positions 228, 437, 454, 505, and 727 of cPLA$_2\alpha$ have been identified to be the sites of phosphorylation. In particular, the site at Ser 228 is known to control cPLA$_2\alpha$ activity.

Calcium ion (Ca^{2+}) is an important biological regulator, which is broadly involved in various cellular signaling pathways of physiologic or pathologic events.[8] The normally low intracellular level of calcium can be enriched by mobilization of the internal store in the endoplasmic reticulum (ER) or from the extracellular medium. Typically, stimulation of G-protein coupled receptor or tyrosine kinase receptors by outside stimuli can activate the isoforms of phospholipase C (PLC), which in turn hydrolyzes phosphatidyl inositol 4,5-tri-phosphate (PIP$_2$) to release the secondary messenger of inositol triphosphate (IP$_3$). The subsequent binding of IP$_3$ to the receptor on the ER membrane allows the calcium stored inside to be released into the cytoplasm. On the other hand Ca adenosine triphosphatase (ATPase) located on the ER membrane can pump calcium back into ER to replenish the store. Homeostasis of intracellular calcium is essential to the health of cells and tissues. For instance, it is known that calcium is needed in cell proliferation,[9] however, too great an influx of calcium or disruption of the ER store can both lead to cell death and diseases.[10–12] Recently, attention has been drawn to the relationship between Ca^{2+} and ROS with the demonstration that Ca^{2+} is required in many ROS-related cellular activities,[13,14] one of which is cell proliferation.

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Sundaresan et al.[15] first reported that platelet-derived growth factor (PDGF) mitogenic action is mediated by ROS generation. Bae et al.[16] later showed that epidermal growth factor (EGF) signaling for cell proliferation was also regulated by ROS production. Therefore, redox signaling has been suggested in many cell types to be the key regulator for growth factor- and/or cytokine-induced cellular functions, including cell proliferation, migration, and wound healing. Recently ROS signaling has also been recognized as the essential regulator for PDGF function in the lens[17] and EGF function in the cornea.[18] In both lens and corneal epithelial cells, $cPLA_2\alpha$ was shown to play a major role, as $cPLA_2\alpha$ inhibition led to ceased ROS generation from NADPH oxidase,[19] and subsequently halted cell proliferation, migration, and even wound healing in cells stimulated with PDGF or EGF.[18]

Even though $cPLA_2\alpha$ is critical for growth factor-induced ROS generation in the lens epithelial cells,[19] the detailed mechanism underlying $cPLA_2\alpha$ activation and function remains unclear in the ocular tissues. While the cellular level of calcium, especially that in the calcium store in ER, appears to be closely associated with EGF-stimulated growth and survival of lens epithelial cells,[20] little is known about any possible role played by calcium in the function of $cPLA_2\alpha$ in ocular tissues. In the present study, we have investigated the significance of calcium ion in PDGF-induced ROS signaling by manipulating intracellular calcium level with ionomycin to increase external calcium influx, thapsigargin to release ER calcium stores, or a chelating agent to deplete calcium. We have examined the regulation of $cPLA_2\alpha$ by using a stable dominant-negative $cPLA_2\alpha$ (S228A) HLE B3 cell line, and a transient cell line with truncated C2 calcium binding domain (C2D) to examine their effects on PDGF signaling. Our results indicate that an intact and active $cPLA_2\alpha$ as well as the presence of adequate intracellular calcium are essential for PDGF-elicited ROS production and cell proliferation. For simplicity, $cPLA_2\alpha$ IV will be referred to as $cPLA_2$ from this point on in this report.

Materials and Methods

Materials

Minimum Eagle essential medium (MEM) and fetal bovine serum (FBS) were supplied by Invitrogen Corporation (Carlsbad, CA). HEPES, trypsin, arachidonic acid, and lucigenin were obtained from Sigma-Aldrich (St. Louis, MO). [^{3}H]-AA was obtained from Amersham Biosciences Inc. (Piscataway, NJ). Ionomycin, a calcium ioophore, thapisgargen, an endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, and 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA-AM), a calcium-chelating agent, and arachidonyl trifluoromethyl ketone (AACOCF3), a specific inhibitor to $cPLA_2$, were purchased from Biomol International, LP (Plymouth Meeting, PA). The chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate) and the protease inhibitor (Halt Protease Inhibitor Cocktail) were from Pierce Biotechnology, Inc. (Rockford, IL). Phospho-p42/44 MAPK (P-ERK1/2), total ERK [ERK1/2], phospho-Akt (P-Akt), phospho-p38 (P-p38), phospho-JNK (P-JNK), total JNK, $cPLA_2$, and phospho-$cPLA_2$ (P-$cPLA_2$) antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies and glyceraldehyde-3-phosphodehydrogenase (G3PD) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Nitrocellulose membrane (Hybond ECL) was from Amersham Biosciences Inc. (Piscataway, NJ). All chemicals were of reagent grade.

Cell Culture

The human lens epithelium (HLE) B3 cell line was a gift from Usha Andley (Washington University, St. Louis, MO). The cells were cultured in MEM containing 20% FBS until they reached confluence. Before performing experiments, cells were cultured in MEM with 2% FBS overnight followed by serum-free MEM for another 24 hours. For experiments requiring Ca^{2+}-free conditions, MEM was first mixed with 2 mM EGTA for 30 minutes before use.

The Construction of Dominant-Negative $cPLA_2$ (C228S) HLE B3 Stable Cell Line

Total RNA was extracted from HLE B3 cells using an RNA extraction kit (Qiagen; Valencia, CA) and quantified by spectrophotometry. The RNA was used for cDNA synthesis by in vitro reverse transcription. PCR was performed by using specific primers: 5′-ACGCGTATGTCATTTATAGATCCTTACCAGCAC-3′ and 5′-GTCGACCTATGCTTTGGGTTTACTTAGAAACTC-3′.

The cloned *$cPLA_2\alpha$* was digested by restriction endonuclease and ligased with expression vector (pCR3.1; Invitrogen). The site-directed mutagenesis at 228th residue from serine to alanine (S228A) on *$cPLA_2\alpha$* was performed (QuickChange Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA) following manufacturer's instructions. The expression vector containing the mutated insert was introduced into HLE-B3 cell by electroporation, and the cells were cultured in medium with geneticin (G418, 1.0 mg/mL) for 4 weeks followed by single-colony selection. Selected $cPLA_2$-S228A cells were cultured in MEM containing 20% FBS until confluence and then stored in −80 °C for further use.

Generation of a Transient C2D HLEB3 Cell Line

To construct an expression vector containing $cPLA_2\alpha$ without its C2 domain (residues 127–749), PCR was performed by using primers flanking $cPLA_2$ DNA sequence (nucleotide 379-2250) with appropriate restriction enzyme cutting sites added: 5′-GGGGAAGCTTATGGTCACTGAAATGGTTCTAGAAATGTCT-3′ and 5′-GTCGACCTATGCTTTGGGTTTACTTAGAAACTC-3′. PCR product was cloned into expression vector (PCR3.1; Invitrogen) and the plasmid was named $cPLA_2$-C2D. HLE B3 cells were transiently transfected with $cPLA_2$-C2D expression vector (Lipofectamine 2000; Invitrogen)-engaged system and all procedures followed manufacturer's instructions.

Detection of AA Release

Either wild type HLE-B3 or S228A mutant cells (1.3×10^6) were incubated with 0.5 μCi/mL [^{3}H]-AA for 15 hours. After incubation, unincorporated [^{3}H]-AA in the medium was removed, and the cells were treated with 5 ng/mL PDGF. Aliquots of media (100 μL) were collected at 0 (control), 2.5, 5, 10, and 20 minutes and counted with a scintillation counter for quantifying the released [^{3}H]-AA.

Analysis of Cell Proliferation by Cell Counting and BrdU Analysis

The detection of cell proliferation was done by using either manual cell counting with a hemocytometer or by using a cell proliferation chemiluminescent assay kit (Roche Applied Science, Indianapolis, IN). The protocol for BrdU analysis was based on manufacturer's instructions with slight modifications. In short, the trypsinized cells were cultured on a 96-well plate (8×10^3 cells per well) in FBS-free medium for 24 hours. The MEM containing 5 ng/mL of PDGF was used to treat the cells for 30 minutes. After treatment, each well was washed thoroughly with MEM and loaded with MEM containing BrdU from the cell proliferation chemiluminescent assay kit (Roche Applied Science). The cells were incubated at 37 °C for 8 hours followed by the chemiluminescent immunoassay for quantifying cell proliferation by using a luminometer.

Detection of ROS

The level of extracellular ROS released from cells was assayed using lucigenin as a substrate based on the method of Zhang et al.[19] with slight modifications. Lucigenin interacts with free radicals such as superoxide anion and emits luminescence, which can be quantitatively detected and analyzed by a luminometer. The cells (1×10^6) were trypsinized from the culture plates, washed thoroughly with PBS, and kept in Krebs buffer (pH 7.4, 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$,

1.8 mM $CaCl_2$, 5 mM glucose, 35 mM phosphoric acid, 20 mM HEPES) until further use. To measure ROS level, the cells were put in luminescence buffer (Krebs buffer containing 0.5 mM lucigenin), and then nicotinamide adenine dinucleotide phosphate (NADPH; 0.1 mM) was added as the substrate. The emitted luminescence was detected with a luminometer (FluoStar Optima; BMG Labtech, Durham, NC). For experiments under calcium-free condition, a modified Krebs buffer was prepared by replacing the 1.8 mM $CaCl_2$ with 1.8 mM NaCl.

Manipulation of Intracellular Calcium Level with Ionomycin, Thapsigargin or BAPTA-AM

Stock solutions of ionomycin and thapsigargin were prepared according to manufacturer's instructions and stored in −20 °C. Proper dilution of the reagents was carried out before the experiment. An adequate amount of HLE B3 cells were cultured on a 10-cm dish, 6-cm dish, or 96-well plate depending on experiment needs. HLE B3 cells were cultured overnight in MEM containing 2% FBS. For ROS detection or measurement of intracellular calcium, the culture medium was changed to MEM containing ionomycin (0.01 μM) or thapsigargin (0.01 μM), or BAPTA-AM (5 μM) for 30 minutes followed with or without PDGF stimulation. For BrdU-based cell proliferation assay, the culture medium was changed to MEM containing ionomycin (0.01 μM) or thapsigargin (1 nM-10 μM) or BAPTA-AM (5 μM) and cultured for 6 hours.

Detection of Intracellular Calcium Level in HLE B3 Cells

The intracellular calcium levels were assayed (Fluo-4 Direct Calcium Assay Kit; Invitrogen). All procedures in this experiment were done following the manufacturer's instructions. The detection of fluorescence, which represents the intracellular calcium level, was performed with a fluorometer (FluoStar Optima; BMG Labtech) with excitation at 494 nm and emission at 516 nm.

Isolation of Cellular Membrane Fraction

The membrane fraction was isolated with the method used by Seth et al.[21] with modifications. Cells (6×10^6) were washed three times with ice-cold PBS before being lysed by sonication in lysis buffer. The lysate

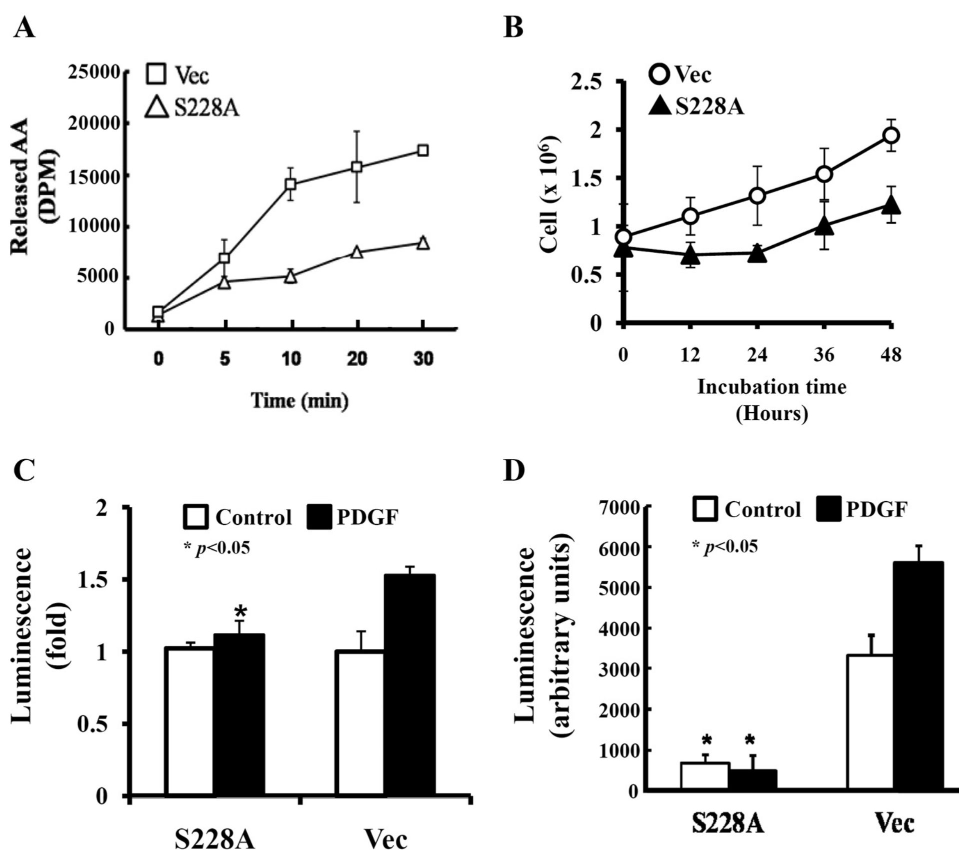

FIGURE 1. The effect of expressing dominant negative form of cPLA$_2\alpha$ (S228A) on AA release and ROS generation stimulated by PDGF in HLE cells. (**A**) Arachidonic acid release: cells were treated with PDGF (5 ng/mL) and the medium was collected at different times as described. The released [^{3}H]-AA in cell culture medium was determined by scintillation counting. (**B**) Cell proliferation: Vec and S228A HLE B3 cells (8×10^5 per dish) were cultured in MEM containing 20% FBS for 48 hours. The cell number was counted every 12 hours using a cell counter (Invitrogen). (**C**) DNA synthesis: proliferation of HLE B3 cells expressing normal or S228A mutated cPLA$_2\alpha$ induced by PDGF (5 ng/mL, 6-hour incubation) was detected by determination of neosynthesized DNA with a BrdU-based cell proliferation chemiluminescent assay kit (Roche Applied Science). The final result is the average of three independent experiments and is shown as mean ± SD. * $P < 0.05$ versus Vec cells with the same treatment condition. (**D**) ROS production: cells transfected with empty vector alone (Vec) and cells expressing cPLA$_2\alpha$-S228A (S228A) were treated with PDGF (5 ng/mL) and the levels of extracellular ROS within 5 minutes after PDGF stimulation were detected using a lucigenin-based luminescence assay. The results are presented based on five independent experiments and expressed as mean ± SD. * $P < 0.05$ compared with Vec cells of the same experimental conditions.

was centrifuged at 1000*g* to remove debris and nuclei and then centrifuged at 100,000*g* to isolate the membrane and cytosolic fractions. The supernatant (cytosolic fraction) was saved for future use and the pellet was mixed in lysis buffer containing 1% Triton X-100 followed by ultracentrifugation at 100,000*g*. The supernatant (membrane fraction) was then collected for further experiments.

Western Blot Analysis

Cells (1.3×10^6) were washed thoroughly with ice-cold PBS and lysed in 500 μL lysis buffer containing 1% Triton X-100. The proteins in the lysate (25 μg/well) were separated by 10% SDS-PAGE, and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences Inc.). After the transferring process, the membranes were blocked with blocking solution containing 5% nonfat milk or 5% BSA and then probed with anti- P-ERK1/2, ERK1/2, P-JNK, JNK, P-p38, P-Akt, $cPLA_2$, P-PLA2, or G3PD antibodies, respectively, based on the procedures described by Chen et al.[17] The immunoblots were labeled with horseradish peroxidase (HRP)-conjugated secondary antibodies and bands were detected with an imaging system (VersDoc; Bio-Rad, Hercules, CA) with enhancement by a chemiluminescence reagent.

Determination of Protein Concentration and Statistical Analysis

All protein concentrations were measured by the BCA protein assay according to Smith et al.[22] For all tests, the data were analyzed using Student's *t*-test. A P value < 0.05 was considered significant.

Results

The S228A Mutation in $cPLA_2$ Changes Its Enzyme Activity and the Level of Cell Proliferation in HLE B3 Cells

AA-release is widely used for measuring the enzyme activity of $cPLA_2$. To test if a point mutation at the active site of $cPLA_2$ would obliterate its activity, we produced a dominant negative form of $cPLA_2$ (S228A) mutant and used it for this purpose. After PDGF treatment, we found that the HLE B3 cells expressing the S228A mutant released much less free AA into the medium than the HLE B3 cells transfected with vector alone (Vec) (Fig. 1A), confirming that the S228A mutation reduces the activity of $cPLA_2$. We further examined the effect of the S228A mutation on cell proliferation by manual cell counting and by BrdU-based luminescence assay. After 12-hour incubation in MEM containing 20% FBS, a difference in cell numbers was already apparent between the S228A and Vec cells. At the end of 48 hours, the Vec cells had doubled, whereas the S228A cells had only increased approximately 20% over the control (Fig. 1B). By using BrdU-based luminescence assay, we were able to quantify the neogenesis of cellular DNA for cell proliferation. As shown in Figure 1C, within 6 hours of PDGF treatment, the Vec cells show a 50% increase in DNA synthesis over the unstimulated control, while the mutant cells display a negligible increase. To understand the effect of mutated $cPLA_2$ in ROS generation in HLE B3 cells, we used a luminescence assay to measure PDGF-induced ROS generation from both Vec and S228A cells. As shown in Figure 1D, in the absence of PDGF, the S228A cells generate only about 25% of ROS in comparison with the Vec cells. Treatment with PDGF induced approximately 40% ROS increase in Vec cells but had essentially no effect on S228A cells.

The Effect of the S228A Mutation and Calcium Level on Membrane Translocation of $cPLA_2$

The activation of $cPLA_2$ is accompanied by membrane-translocation phenomenon and is required for the PDGF-induced signaling pathway, which leads to ROS generation and cell proliferation.[19,23,24] To examine how dominant negative $cPLA_2$ could affect its translocation after PDGF stimulation, and whether the translocation was calcium-dependent, we used Western blot analysis to detect $cPLA_2$ in the membrane fraction isolated from Vec or S228A cells treated with and without PDGF for 10 minutes. As shown in Figure 2, the translocation of $cPLA_2$ to the membrane fraction was observed in both Vec and S228A cells after PDGF stimulation (see the normalized bar graph). However, when the cells were pretreated with BAPTA-AM (5 μM)), a membrane-permeable Ca^{2+} chelator to sequester calcium, translocation of $cPLA_2$ was extensively inhibited in both S228A and Vec cells (Fig. 2). It is worth noting that the $cPLA_2$ band was stronger in the S228A cells than in the Vec cells (first and second rows) because the former overexpressed the mutant form of $cPLA_2$. Gp91 (Gp91phox, the major membrane NOX2) and glyceraldehyde-3-phosphate dehydrogenase (G3PD) were used as markers for membrane protein and cytosolic protein, respectively, to ensure equal protein loading.

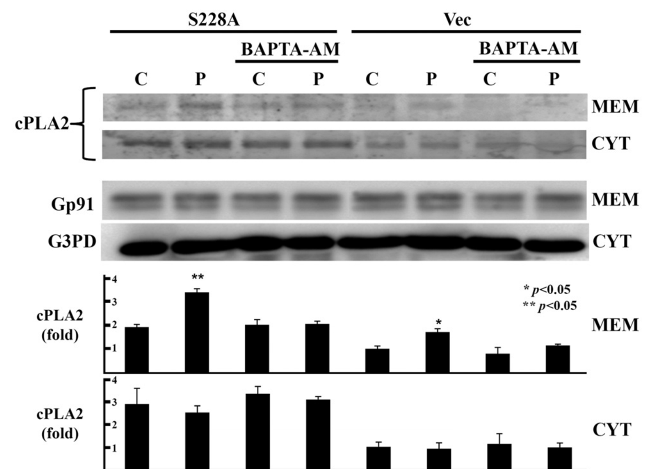

FIGURE 2. The effect of calcium on PDGF-induced membrane translocation of $cPLA_2$ in HLE cells. HLE B3 cells expressing $cPLA_2$-S228 (S228A) or cells transfected with vector alone (Vec) were serum-starved and treated with PDGF (5 ng/mL) for 10 minutes. Another set of cells was pretreated with a calcium chelator (BAPTA-AM) before PDGF stimulation. Cells were lysed and the membrane fractions were isolated by ultracentrifugation. Western blot analysis was performed to detect $cPLA_2\alpha$ in both membrane (MEM) and cytosolic fraction (CYT). Gp91 (Gp91phox, the major membrane subunit of NADPH oxidase 2 [NOX2]) and G3PD (glyceraldehyde-3-phosphate dehydrogenase) were probed as loading controls for membrane and cytosolic fraction, respectively. The quantitative results are based on the Western blot analysis of $cPLA_2$ normalized by reference to respective marker proteins, and the magnitude (fold) of each is compared with Vec untreated cells (normalized to 1.0). The data in the graphs are averages of three independent experiments, and are presented as mean ± SD. * $P < 0.05$ versus Vec cells without treatment. ** $P < 0.05$ versus S228A cells without treatment. C, control; P, PDGF treatment.

The Effect of $cPLA_2$-S228A Mutation on PDGF-Induced Activation of Signaling Factors

It is well known in the upstream process of PDGF mitogenic action that PDGF receptor binding can lead to its autophosphorylation with subsequent stimulation of the MAPK pathway. The resultant activated ERK1/2, or P-ERK1/2, has been suggested to be one of the kinases that can activate $cPLA_2$ translocated on the membrane.[23,25] To further examine the effect of dominant negative $cPLA_2$ on the activation of mitogenic signaling factors, we performed Western blot analysis to determine the phosphorylation or activation of MAPK family

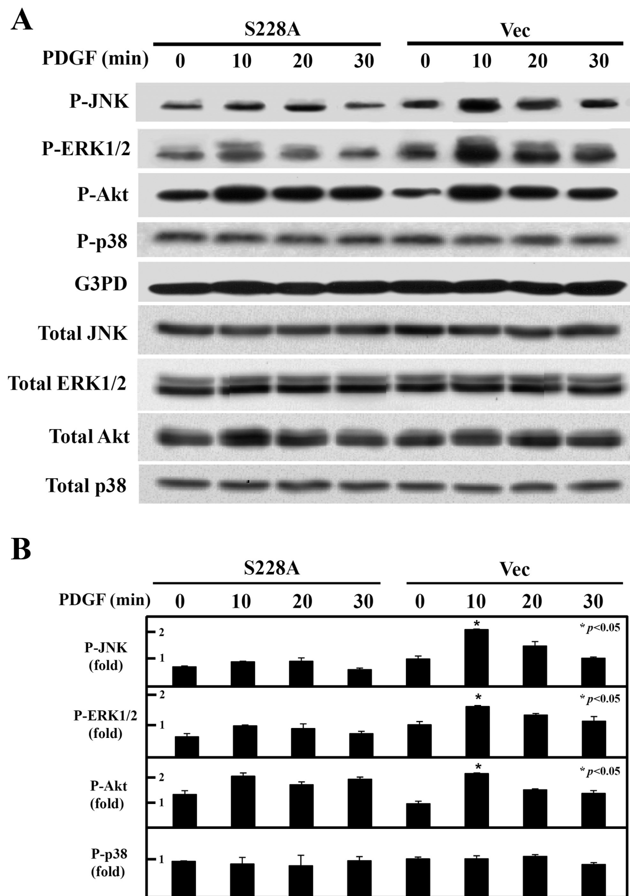

FIGURE 3. Comparison of the PDGF-induced activation of signaling factors in HLE cells expressed intact and S228A $cPLA_2$. (**A**) HLE B3 cells expressing normal or S228A mutated $cPLA_2\alpha$ were serum-starved and treated with PDGF (5 ng/mL). Cell lysates were collected every 10 minutes, and Western blot analysis was performed to detect the phosphorylation of ERK, JNK, Akt, and p38, as well as the total protein levels of ERK, JNK, Akt, and p38. G3PD was probed as loading control. (**B**) The results of Western blot analysis in Figure 3A were analyzed quantitatively and the bar graph showing P-JNK, P-ERK1/2, P-Akt, and P-p38 was each normalized with total JNK, ERK1/2, Akt, and p38, respectively. The magnitude (fold) of each is compared with Vec untreated cells (normalized to 1.0). The Western blot analysis represents a typical pattern from three separate studies while the data in the bar graphs are the averages of three independent experiments, and are presented as mean ± SD. * $P < 0.05$ versus Vec cells without treatment.

proteins, including ERK1/2, JNK, and p38, and the survival factor Akt. G3PD was also probed as a control for sample loading. As shown in Figure 3A, P-ERK1/2 in Vec cells is transiently induced by PDGF with the maxima activation at 10 minutes. However, mutation of $cPLA_2$ weakened the transient P-ERK1/2 and P-JNK signals throughout the entire 30 minutes of the experiment, and lowered over 50% of the relative magnitude of PDGF-induced activation (see the bar graph on the normalized P-ERK1/2 and P-JNK in Fig. 3B). Mutation of $cPLA_2$ showed no effect on total ERK, total JNK or P-Akt (Fig. 3A). As expected, PDGF did not stimulate p38 and the mutation of $cPLA_2$ did not affect the P-p38 signal, which was included as a negative control for the PDGF mitogenic study.

Effect of Intracellular Calcium Levels on ROS Generation and Cell Proliferation

In many cell types, including the lens epithelial cells, the downstream PDGF mitogenic action depends on NADPH oxidase to produce ROS to mediate cell proliferation.[24] NADPH oxidase activation requires the assembly of cytosolic subunits to be translocated onto the membrane in the proximity of the gp91 and p22 catalytical units.[24] Calcium-dependent PKC activation plays a major role in this critical event for ROS generation.[19] To study the possible collaboration between calcium and $cPLA_2$ in PDGF-induced redox signaling, we compared ROS production in both Vec and S228A cells with and without PDGF stimulation under the following conditions: no pretreatment; pretreated with ionomycin (Ca^{2+} agonist); or pretreated with BAPTA-AM (Ca^{2+} chelator). As shown in Figure 4A, the mutant cells have a very low basal ROS level compared with the Vec cells, and no significant PDGF-induced ROS increase is observed in S228A mutant cells while the Vec cells responded to PDGF stimulation by generating 74% more ROS over the basal level. Ionomycin-induced calcium influx into the Vec cells not only elevated the basal ROS in the unstimulated cells but also augmented PDGF to produce nearly 83% more ROS over the nonstimulated control. By contrast, the mutant cells did not respond to PDGF stimulation although the basal ROS was elevated by the presence of ionomycin. However, when the calcium in the medium was chelated by BAPTA-AM, no

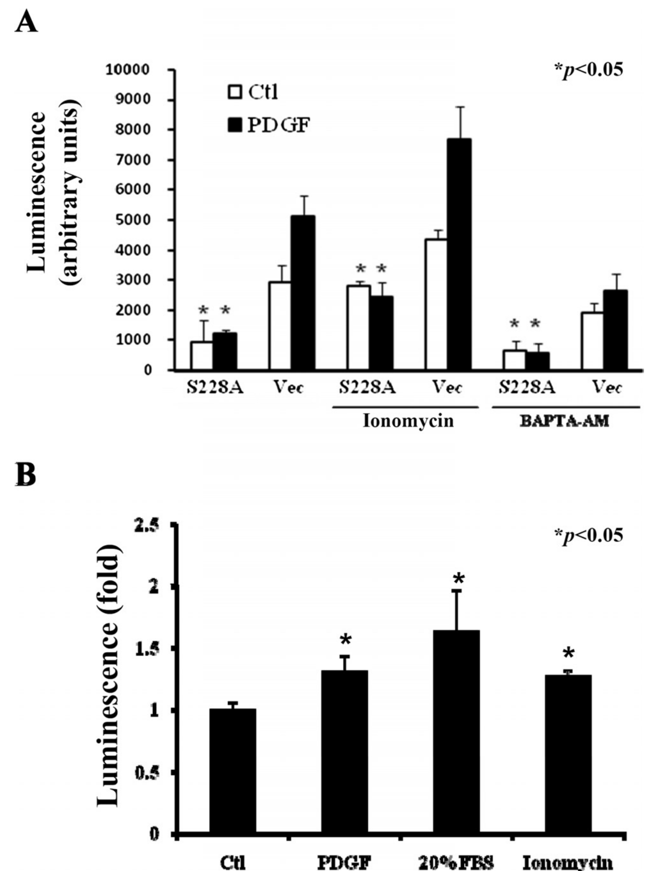

FIGURE 4. The effect of calcium on PDGF-induced ROS generation through $cPLA_2$ in HLE cells. (**A**) HLE B3 cells expressing $cPLA_2\alpha$-S228A with or without the pretreatment of ionomycin or BAPTA-AM for 30 minutes were stimulated with PDGF (5 ng/mL) and the extracellular ROS levels were detected within 5 minutes after PDGF stimulation by lucigenin-based luminescence assay. The results are the average of five independent experiments and are expressed as mean ± SD. * $P < 0.05$ versus Vec cells group in the same PDGF treatment condition. (**B**) HLE B3 cells were treated with or without MEM containing PDGF (5 ng/mL), 20% FBS or ionomycin (0.01 μM) for 6 hours, and the cell proliferation was assayed by BrdU-based neosynthesized DNA quantification. The luminescence represents the level of neosynthesized DNA as detected by a luminometer. The results are the average of three independent experiments and are presented as mean ± SD.

significant ROS generation was detected in Vec cells with PDGF stimulation, while the mutant cells remained nonresponsive to PDGF (Fig. 4A).

Because the above results suggested that ionomycin could independently stimulate ROS production besides augmenting PDGF, we explored next if ionomycin was able to stimulate cell growth and its association with $cPLA_2$. Figure 4B shows the BrdU analysis of cells cultured under various conditions, in which both PDGF (5 ng/mL) and FBS (20%) provided positive cell growth (positive controls) in comparison with that of the untreated control. Interestingly, ionomycin at $0.01\mu M$ was as effective a cell growth stimulator as that of PDGF. These results suggest that ionomycin-enriched intracellular calcium may have initiated calcium signaling that is capable of mobilizing and activating $cPLA_2$ for the downstream process needed for cell proliferation.

The Role of Endoplasmic Reticulum (ER)-Stored Calcium in ROS Generation

To investigate the source of calcium used for NADPH oxidase activation during PDGF mitogenic action, we compared PDGF-stimulated ROS generation in HLE B3 cells under the conditions of no treatment (control); pretreatment with ionomycin, pretreatment with an ER calcium store-releasing agent (thapsigargin), or pretreatment with a calcium binding agent (BAPTA-AM). A parallel study was conducted with cells cultured in regular medium (Fig. 5A) and cells cultured in calcium-free medium (Fig. 5B). As shown in Figure 5A, both ionomycin and thapsigargin pretreated cells are able to double the basal ROS level over the control untreated cells, and amplified the cell's response to PDGF stimulation. BAPTA-AM pretreatment totally eliminated the basal ROS elevation observed in ionophore and thapsigargin treatment, and the cellular response to PDGF stimulation. The same pattern for ROS generation was also observed in cells cultured in calcium-free medium (Fig. 5B). While thapsigargin is known to release calcium stored in the ER specifically, ionomycin is also capable of releasing calcium stored in the ER as well as infusing calcium extracellularly. Our results confirmed this feature of ionomycin in the HLE B3 cells (Fig. 5B). The same PDGF-stimulated ROS production, with and without calcium in the medium, indicates that although calcium may come from the extracellular source (Fig. 5A), the calcium from the ER store is sufficient to carry out the PDGF signaling function (Fig. 5B).

To further clarify the role of $cPLA_2$ in calcium-mediated ROS generation, we measured PDGF-induced ROS generation with a lucigenin-based luminescence assay in Vec and S228A cells cultured in calcium-free medium and pretreated 30 minutes in the following conditions: no treatment (control), treated with PDGF alone, treated with thapsigargin (10 μM), or treated with PDGF plus thapsigargin. As shown in Figure 5C, the S228A mutant does not respond to any of the stimuli and its ROS level is even less than the untreated control Vec cells. Interestingly thapsigargin itself stimulated ROS generation as much as that of PDGF, and displayed synergistic effect with PDGF. These data suggest that calcium released from the ER store must have mediated the PDGF-induced ROS generation, and also independently initiated signaling events, but both requires an intact cellular $cPLA_2$.

Next we used the BrdU assay to examine if thapsigargin could stimulate cell proliferation. HLE B3 cells were treated with various affectors for 6 hours, including thapsigargin (1 nM), AACOCF3 (10 μM, $cPLA_2$ specific inhibitor), and thapsigargin plus AACOCF3. We found that thapsigargin stimulated

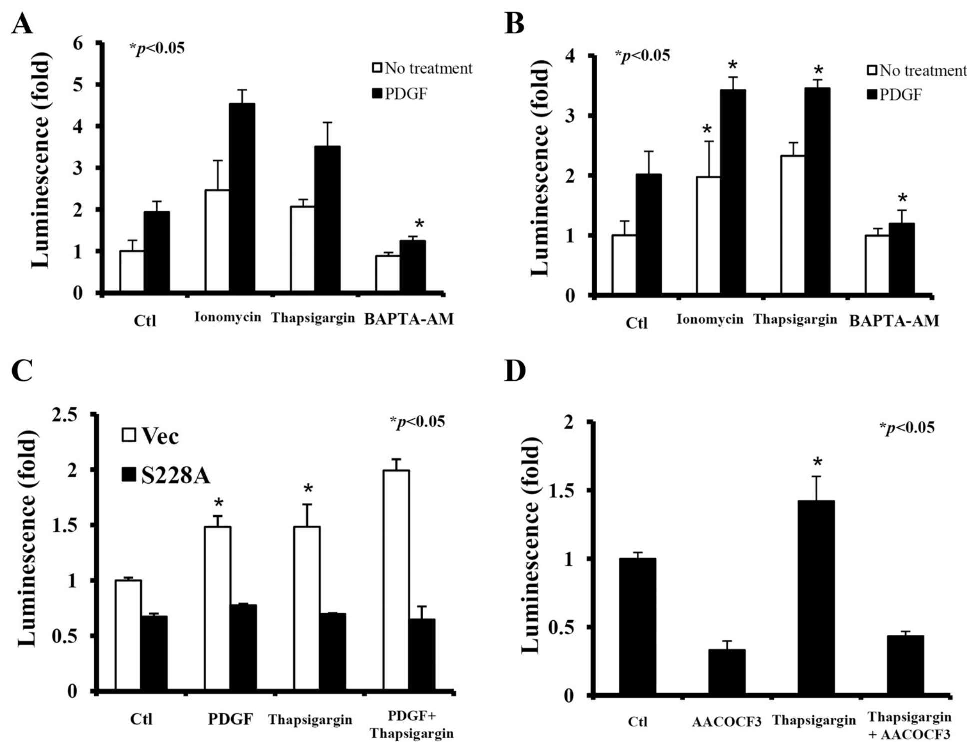

FIGURE 5. The effect of ER-stored calcium on ROS generation through $cPLA_2$ in HLE cells. (A) HLE B3 cells were pretreated with ionomycin (10 minutes), thapsigargin (30 minutes), or calcium-chelator BAPTA-AM (30 minutes), before PDGF stimulation. The extracellular ROS levels at 10 minutes after PDGF treatment were determined using the luciginin-based luminescence assay. The results are averages of three independent experiments and are expressed as mean ± SD. * $P < 0.05$ versus the control (Ctl) group. (B) HLE B3 cells cultured in EGTA-pretreated MEM (Ca^{2+}-free MEM) for 1 hour were pretreated with ionomycin (10 minutes), thapsigargin (30 minutes), or BAPTA-AM (30 minutes), followed by PDGF stimulation. The extracellular ROS levels at 10 minutes after PDGF stimulation were assayed as before. The results are averages of three independent experiments and are expressed as mean ± SD. * $P < 0.05$ versus the Ctl cells group. (C) HLE B3 cells transfected with $cPLA_2\alpha$-S228A or vector alone were pretreated with or without thapsigargin for 30 minutes before stimulation with or without PDGF (5 ng/mL). The extracellular ROS levels were determined by the lucigenin-based luminescence assay at 10 minutes after PDGF stimulation. The results are the averages of three independent experiments and are expressed as mean ± SD. * $P < 0.05$ versus the same cell line in the Ctl group. (D) HLE B3 cells were treated with arachidonyl trifluoromethyl ketone (AACOCF3) at 10 μM, thapsigargin (1 nM), or thapsigargin (1 nM) plus AACOCF3 (10 μM) for 6 hours followed by BrdU-based cell proliferation analysis. The results are the averages of three independent experiments and are shown as mean ± SD. * $P < 0.05$ compared with the Ctl group.

cell growth approximately 40% over the control, but this increase was totally abolished and suppressed below the control level when the cells were cotreated with a $cPLA_2$ inhibitor, similar to the level of suppression when cells were treated with the inhibitor alone (Fig. 5D). This indicates that the mitogenic effect triggered by the calcium released from the ER depends on the activity of $cPLA_2$. However, the positive effect of thapsigargin was only demonstrated when a low dosage of 1 nM was used. Concentrations of thapsigargin at the range of 0.01 to 10 μM all showed negative effects (data not shown).

The Intracellular Calcium Pool and the Effect on ERK Activation

To investigate the possible mechanism of calcium-regulated cell proliferation, we used ionomycin and thapsigargin to alter the intracellular calcium level in HLE B3 cells and to detect the activation of ERK1/2 signaling. Figure 6A shows that 20% FBS increases intracellular calcium level approximately 30%, and both ionomycin (0.01 μM) and thapsigargin (10 μM) can elevate intracellular calcium 20% over that of the untreated HLE B3 cells. Because ERK1/2 is one of the important MAPK signaling factors that govern mitogenic effect, we investigated whether pretreating cells with either thapsigargin or ionomycin (30 minutes) could activate ERK1/2. We found that thapsigargin (10 μM) and ionomycin (0.01 μM) each transiently activated ERK1/2, and the P-ERK1/2 signal peaked at 10 minutes and returned to near basal level at the end of 30 minutes (Fig. 6B).

Calcium-Dependent Membrane Translocation Affecting the Activity of $cPLA_2$

To understand the relationship between the activation of $cPLA_2\alpha$ and its membrane translocation property, we transfected HLE B3 cells with expression vector to overexpress the C2 domain-deleted $cPLA_2$ ($cPLA_2$-C2D) of 70 kDa, and monitored its membrane translocation by Western blot analysis. Figure 7A shows that after 10 minutes of PDGF (5 ng/mL) stimulation, $cPLA_2$-C2D does not translocate to the membrane, whereas the full-length $cPLA_2$ (85 kDa) does. To investigate whether the C2D-truncated $cPLA_2$ can be activated by PDGF treatment, we performed Western blot analysis to detect phospho-$cPLA_2$ (phosphorylated at Ser505), which is known to be the location governing enzyme activity and a phosphorylation target of ERK1/2. Figure 7B shows that none of the $cPLA_2$-C2D is phosphorylated at Ser505 (indicated by an arrow head in the upper panel). However, a small amount of P-$cPLA_2$ from the endogenous full-length $cPLA_2$ in the C2D cells could be detected, same as that of the Vec cells (see the arrow at the upper panel, and the bar graph below). These results indicate that the activation of $cPLA_2$ requires its C2 domain, and the event of translocation precedes activation.

Discussion

In a previous study, we have found that when $cPLA_2$ is inhibited by a chemical inhibitor (AACOCF3), exogenous AA can intercept and directly process the downstream signals to stimulate ROS generation in human lens epithelial cells, and we have thus proposed that $cPLA_2$-released AA can provide positive feedback regulation of PDGF and amplify the mitogenic signaling.[19] Our current results suggest that the reason $cPLA_2$ mutated at S228A could not respond to PDGF-induced ROS production or cell proliferation is likely due to its inability to release sufficient AA from membrane phospholipids to aid the activation of NADPH oxidase for ROS production and subsequent cell proliferation (Fig. 1). The S228A mutant with inac-

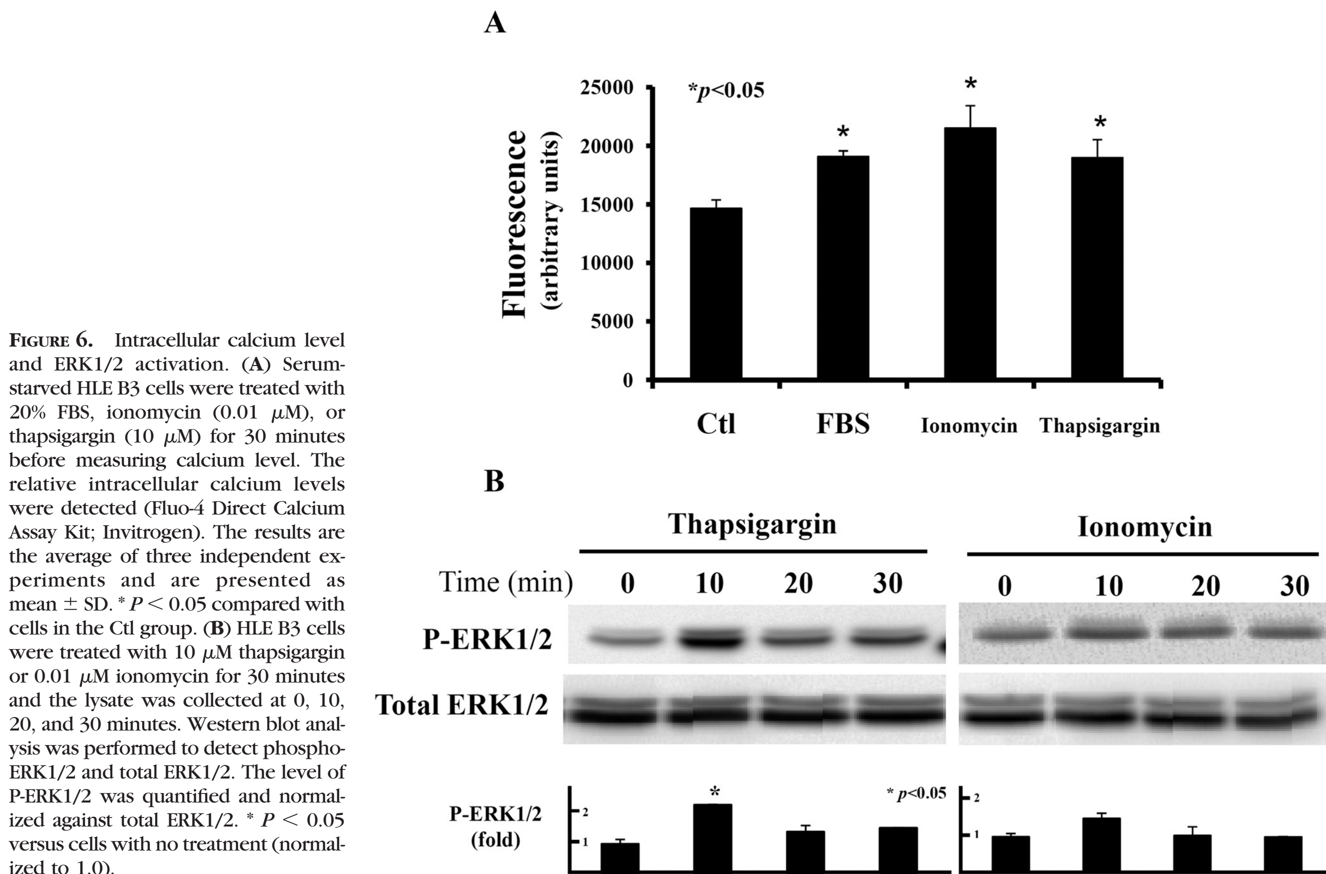

Figure 6. Intracellular calcium level and ERK1/2 activation. (**A**) Serum-starved HLE B3 cells were treated with 20% FBS, ionomycin (0.01 μM), or thapsigargin (10 μM) for 30 minutes before measuring calcium level. The relative intracellular calcium levels were detected (Fluo-4 Direct Calcium Assay Kit; Invitrogen). The results are the average of three independent experiments and are presented as mean $\pm$ SD. $^*P < 0.05$ compared with cells in the Ctl group. (**B**) HLE B3 cells were treated with 10 μM thapsigargin or 0.01 μM ionomycin for 30 minutes and the lysate was collected at 0, 10, 20, and 30 minutes. Western blot analysis was performed to detect phospho-ERK1/2 and total ERK1/2. The level of P-ERK1/2 was quantified and normalized against total ERK1/2. $^*P < 0.05$ versus cells with no treatment (normalized to 1.0).

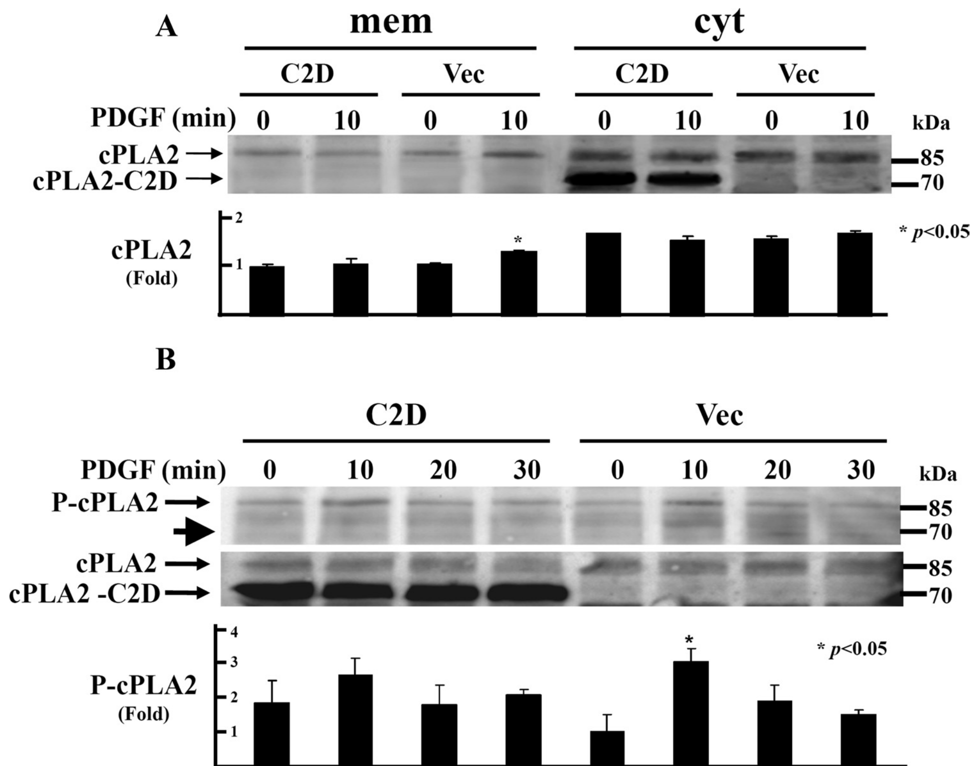

FIGURE 7. The relationship between the C2 domain and the activation of $cPLA_2\alpha$. (A) HLE B3 cells expressing $cPLA_2\alpha$-C2D (C2D) were stimulated with 5 ng/mL PDGF for 10 minutes. The cells were then lysed and the membrane (mem) and cytosolic (cyt) fractions were separated by ultracentrifugation before performing Western blot analysis to detect endogenous $cPLA_2\alpha$ and $cPLA_2\alpha$-C2D. HLE B3 cells transfected with vector alone were used as control (Vec). Each lane contains 40 μg of protein. The level of $cPLA_2$ was quantified based on three independent experiments and expressed as mean ± SD. * $P < 0.05$ versus membrane fraction of Vec cells without treatment. (B) HLE B3 cells transfected with vector alone (Vec) or $cPLA_2\alpha$-C2D (C2D) were treated with 5 ng/mL PDGF for 30 minutes and the lysates were collected at 0, 10, 20, and 30 minutes of treatment. Western blot analysis was performed to detect $cPLA_2\alpha$ and phospho- $cPLA_2\alpha$. Each lane contains 25 μg of protein and the result represents a typical outcome of three independent repeats. The level of P-$cPLA_2$ was quantified based on three independent experiments and expressed as mean ± SD. * $P < 0.05$ versus Vec cells without treatment (normalized to 1.0).

tive $cPLA_2$ made in this study agrees with the reports in the literature,[2] and also confirm that the active site of $cPLA_2$ in lens epithelial cells is similar to that of other cell types. The mutant cell line grew more slowly than the Vec cells but the cells were still viable with no obvious sign of cell death. It has been reported that genetic deletion of $cPLA_2$ in mice did not lead to a significant change in life span or any obvious phenotypic defects,[4,26] indicating that defected $cPLA_2$ in cells or tissues may be overridden by other compensatory pathways.

Membrane translocation is an important event in the activation process of $cPLA_2$. The translocation is not only required for interaction between $cPLA_2$ and its substrate (phospholipids) but also allows $cPLA_2$ to move closer to other downstream enzymes in AA metabolism, such as lipoxygenase (LOX) and cyclooxygenase (COX),[6] and other target proteins such as NADPH oxidase for ROS generation.[27,28] It has been reported that translocation of PLA2 family enzymes can be calcium-dependent ($cPLA_2$) and calcium-independent ($iPLA_2$). However, $cPLA_2\alpha$ IV isozyme examined in the present study is known to be calcium-dependent in other cell types.[29] Our data also clearly demonstrated that calcium is required for $cPLA_2$ membrane translocation in the lens epithelial cells (Fig. 2). In addition, $cPLA_2$ carrying S228A mutation could still be translocated onto the membrane as long as calcium was available in the microenvironment. It has been demonstrated that $cPLA_2$ has a calcium-dependent phopholipid binding domain (C2D) located in the N-terminal region.[30] The mutation of S228A apparently does not disturb this membrane-binding region.

Our data also clearly demonstrated that the presence of calcium binding C2 domain on $cPLA_2$ is essential for this translocation as $cPLA_2$ without the C2 domain (the C2D mutant) lost the ability to move onto the membrane with or without intracellular calcium (Fig. 7A). Furthermore, the activation or phosphorylation of $cPLA_2$ requires it to be in place on the membrane where a MAP kinase, such as ERK1/2, can phosphorylate at Ser505 and activate the enzyme. Therefore, the $cPLA_2$ mutant truncated at the C2 domain was also unable to be activated. The mass difference between the intact $cPLA_2$ (85 kDa) and the shortened $cPLA_2$-C2D (70 kDa), allowed the two proteins to be separated quite clearly on the gel. Therefore, the Western blot analysis (Fig. 7B) was able to distinguish a transiently intensified P-$cPLA_2$ at 10 minutes post-PDGF treatment, which was the nascent and intact $cPLA_2$ expressed in the transient mutant cells, while the major bulk of $cPLA_2$-C2D expressed as the 70 kDa protein remained unphosphorylated on PDGF stimulation.

Many different signaling factors are involved in cell proliferation. Previously, we have found in lens epithelial cells[17,19] that PDGF or AA were able to induce transient activation of cell signaling factors, including mitogen-activated protein kinase/ERK kinase (MEK), ERK1/2, and JNK.[19,24] We have also observed that inhibition of MEK activation by U0126, not only

prevented the downstream ERK1/2 from activation, but also eliminated the cell's response to PDGF stimulation for ROS production or cell proliferation.[19] In the present study, we observed that the PDGF-induced transient activation of ERK1/2 and JNK was present, likely due to the basal $cPLA_2$ that is expressed in the mutant cells. However, the signals during the entire 30 minutes of PDGF stimulation were much lower (approximately 50%) than that of the Vec cells (Fig. 3). Importantly, the signal loss in P-ERK1/2 and P-JNK (Fig. 3A) corroborated with the lowered ROS level (Fig. 4A) and the suppressed growth rate (Fig. 1B) observed in the same mutant. This suppressed activation of JNK and ERK1/2 in S228A mutant implies that the role of AA as a positive feedback regulator for PDGF signaling is compromised due to the deficient AA release from a defective $cPLA_2$. p38, which is known as the cellular stress signal, showed no difference between the Vec and S228A cells (Fig. 3), indicating that both cell populations were not stressed during the experimental conditions.

Interestingly, the activation of Akt induced by PDGF was not affected by the S228A mutation in $cPLA_2$. It has been suggested that Akt controls cell survival and proliferation signals, and that Akt can be highly activated by the treatment of PDGF.[31–33] No change in PDGF-induced Akt phosphorylation between Vec and S228A cells may indicate that Akt is not a downstream factor in $cPLA_2$-related mitogenic cell signaling pathways. This phenomenon also suggests that $cPLA_2$ may govern multiple PDGF-induced ROS sources leading to the induction of different downstream factors such as JNK, ERK1/2, and Akt. Further studies on this direction of research are required.

The association of calcium signaling with cell proliferation has been studied extensively in many cell types,[8] including lens epithelial cells,[34,20] however, the requirement of calcium ion for growth factor-induced ROS generation has never been explored in ocular tissues or cells. Our present study clearly showed (Figs. 4A and 5A and B) that calcium ion was required for PDGF-induced ROS generation in HLE B3 cells, and that a calcium ionophore showed synergism with PDGF (Fig. 4A). This finding is in agreement with the observation in lymphocytes, in which another calcium ionophore A23187 synergized with PMA, a PKC activator, promotes cell growth.[35] Interestingly, ionomycin alone raised the basal level of ROS in both Vec and S228A mutant cells but the effect was eliminated in the presence of a calcium chelator (Fig. 4A), indicating that the elevated intracellular calcium could stimulate the native $cPLA_2$ expressed in the quiescent mutant and the Vec cells. Thus, it is not surprising that ionomycin stimulated cell proliferation as efficiently as PDGF shown in Figure 4B.

Besides the influx from external sources, intracellular calcium can also be enriched from the internal stores, induced by the growth factor receptor-IP3 signaling processes. Wang et al.[20] showed that thapsigargin-induced calcium release from ER stores could transiently elevate internal calcium level, which either impeded serum or EGF-stimulated cell growth or elicited cell death, depending on the concentration of thapsigargin used (0.1–1 μM). In our present study, we have found that cells pretreated by thapsigargin at a low level (1 nM) could stimulate cell growth (Fig. 5D), which was suppressed to below the level of control cells when a specific inhibitor (AACOCF3) was present. Additionally, in a 30-minute pretreatment study, higher level of 10 μM thapsigargin directly stimulated ROS production as effectively as that of PDGF in Vec cells but not in S228A mutant (Fig. 5C). Apparently such a physiological function of thapsigargin is only effective when the enzyme $cPLA_2$ is intact. In addition, thapsigargin, similar to ionomycin, could stimulate a transient ERK1/2 activation (Fig. 6B), indicating a crosstalk between calcium signaling and MAPK pathway, similar to other cell types.[8]

Wang et al.[20] also observed activation of ERK1/2 in thapsigargin-treated cells, but their other results contradicted with ours. This may possibly be due to the differences in experimental conditions as we only used thapsigargin to pretreat the cells for 30 minutes before embarking on various studies while Wang et al.[20] placed thapsigargin in the cell culture medium during the entire incubation period of 24 hours. We also observed an adverse effect of thapsigargin at dosage > 1 nM when conducting the 6-hour BrdU cell proliferation studies (data not shown). This is reasonable because many agents do display an opposite effect, depending on the dosage and duration of exposure as we have experienced with N-acetylcysteine during treatment of corneal wound healing.[18]

In conclusion, our findings indicate that $cPLA_2$ in the lens epithelial cells is regulated by the calcium binding-assisted membrane translocation for its orientation and activation on the membrane. The intact enzyme is necessary for the downstream NADPH oxidase activation and ROS production in the PDGF-stimulated cell growth of HLE B3 cells. Both ionomycin and thapsigargin raised intracellular calcium level as much as those of fetal bovine serum (Fig. 6A), and our data indicate that this influx of calcium appears to participate in $cPLA_2$ regulation. Our present study not only clarified the regulatory mechanism for $cPLA_2$ and its association with PDGF mitogenic action, it also revealed for the first time that a $cPLA_2$-dependent calcium signaling for cell proliferation is present in the HLE B3 cells, and likely in other cell types.

Acknowledgments

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