Identification of Intracellular Phospholipases A2 in the Human Eye: Involvement in Phagocytosis of Photoreceptor Outer Segments

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PURPOSE. To identify intracellular phospholipases A2 (PLA2) in the human retina and to explore the role of these enzymes in human retinal pigment epithelium (RPE) phagocytosis of photoreceptor outer segments (POS).

METHODS. PCR amplification and Western blot analysis were used to identify mRNA and protein expression of intracellular PLA2 subtypes in the retinal pigment epithelial cell line ARPE-19. Immunohistochemical staining of normal human eye sections was performed to reveal the cellular location of the enzymes. A model of RPE phagocytosis of POS was used to explore the role of intracellular PLA2 in phagocytosis. An activity assay was used to evaluate PLA2 activity, and inhibitors of specific PLA2 were applied to evaluate the role of PLA2 in RPE phagocytosis.

RESULTS. Genes encoding calcium-independent (i)PLA2, group VIA; calcium-dependent cytosolic (c)PLA2, groups IVA, IVB, and IVC; and iPLA2, group VIIB, were identified in the human RPE cell line ARPE-19. Furthermore, protein of iPLA2-VIA, cPLA2-IVA, and iPLA2-VIB were identified in ARPE-19 cells and in various parts of the normal human eye. iPLA2-VIA protein levels were upregulated during phagocytosis, and iPLA2-VIA activity was found to be specifically increased 12 hours after ARPE-19 cells were fed with POS. Finally, RPE phagocytosis was inhibited by the iPLA2-VIA inhibitor bromoenol lactone.

CONCLUSIONS. Various intracellular PLA2 subtypes are present in the human retina. iPLA2-VIA may play an important role in the regulation of RPE phagocytosis of POS and may also be involved in the regulation of photoreceptor cell renewal.

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human RPE cell line ARPE-19 compared with the low-molecular-weight sPLA₂. The present study therefore evaluated the known high-molecular-weight PLA₂ in human RPE cells and explored their possible role in ARPE-19 phagocytosis of POS.

**METHODS**

**ARPE-19 Cultures**

Human ARPE-19 cells (purchased from ATTC-LGC Promochem AB, Boras, Sweden) were maintained at 37°C in a humidified chamber of 5% CO₂ in Dulbecco’s modified Eagle’s medium–F12 (DMEM) containing 5 mM glucose and supplemented with 15% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen-Gibco, Carlsbad, CA). The cells were grown in six-well plates, and the culture medium was replaced with fresh medium twice a week. The cells were harvested at different time points and kept at −80°C until use.

**PCR Amplification**

Total RNA was extracted from 2-week-old ARPE-19 cultures. cDNA was then generated (ThermoScript cDNA kit; Invitrogen-Gibco). One microgram of total RNA was used for each reaction with polyT primers and amplified (AmpliTaQGold; Applied Biosystems, Foster City, CA). To exclude genomic contamination, controls were made by excluding the polyT primers during reverse transcription. Furthermore, primer sets were all designed to span an intron. Volumes of cDNA used were from 100 ng RNA for each of the PLA₂. Amplification was performed in an automated thermal cycler (model 2400; Perkin Elmer, Norwalk, Connecticut, USA) followed by visualization using BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium) substrate reagent and finally visualized by the use of AEC (3-amino-9-ethylcarbazole) substrate reagent. The amplified products were ligated into PCR-II vectors (Invitrogen-Gibco) and sequenced, thereby confirming the specificity of the PCR reactions.

**Western Blot Analysis**

ARPE-19 cells were kept in growth medium and incubated with POS or PBS. The cells were collected after 12 and 24 hours and homogenized in 500 μL lysis buffer. Samples were centrifuged at 4000 g for 25 minutes at 4°C, and samples containing 25 μg protein were loaded. Blots were preincubated overnight in TBS (0.2 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk and incubated with one of the following primary antibodies in TBS-1% (150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk and iPLA₂-VIA (CAY-160507, 1:200; Cayman Chemical, Ann Arbor, MI); cPLA₂-IVA (SC-454, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA); or iPLA₂-VIB (1:100), prepared as described by Poulsen et al. (manuscript submitted). Extracellular signal-regulated kinase (ERK) expression did not change during phagocytosis and blots incubated with an antibody recognizing ERK were used as loading controls (see Fig. 4B). Blots were washed in TBS and incubated with goat anti-rabbit IgG alkaline phosphatase secondary antibody (TriChem ApS; Interkemi, Frederikssund, Denmark) followed by visualization using BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium) substrate reagent and finally visualized by the use of AEC (3-amino-9-ethylcarbazole) substrate reagent. The amplified products were ligated into PCR-II vectors (Invitrogen-Gibco) and sequenced, thereby confirming the specificity of the PCR reactions.

**Immunoprecipitation**

Protein was isolated from 3-week-old ARPE-19 cells as just described. Precleared supematant was incubated with a goat anti-iPLA₂-VIA antibody (SC-14463; 1:400; Santa Cruz Biotechnology, Inc.) overnight at 4°C. To monitor nonspecific binding, we omitted the antibody from a similar sample. After incubation, 50 μL of protein G-Sepharose beads (GE Healthcare, Hillerod, Denmark) was added, and samples were incubated at 4°C for a further 4 hours. The resin was collected by centrifugation and washed five times in buffer. Bound protein was eluted by boiling the resin in 40 μL of 2× loading buffer and resolved by SDS gel electrophoresis. Blots were stained with a rabbit anti-iPLA₂-VIA (CAY-160507, 1:200; Cayman Chemical).

**Immunohistochemistry**

Human eyes were obtained from the Eye Pathology Institute, University of Copenhagen, in accordance with the guidelines of the Declaration of Helsinki for research involving human tissue. Sections were deparaffinized, boiled for 20 minutes in a microwave in TE buffer (pH = 9.5) for antigen retrieval and incubated with 3% H₂O₂ for 8 minutes at room temperature, to inhibit endogenous peroxidase activity. Sections were exposed to antibodies that specifically recognize iPLA₂-VIA (1:200), iPLA₂-VIB (1:200), or cPLA₂-IVA (1:200); washed twice with PBS; and exposed to biotinylated anti-rabbit IgG followed by incubation with a dilution of streptavidin peroxidase complex reagent and finally visualized by the use of AEC (3-amino-9-ethylcarbazole) chromogen (Dako Inc., Copenhagen, Denmark). Relevant positive and negative controls were used. Immunostained sections were examined by light microscopy.

**Confocal Microscopy**

ARPE-19 cells were detached with trypsin, transferred to glass coverslips in 24-well plates, and incubated in normal glucose medium. The cells were grown to confluence and stained with an iPLA₂-VIA-specific antibody (SC-160507; 1:200) overnight at 4°C. They were washed three times with PBS and fixed in formaldehyde 3.7% for 15 minutes at
room temperature, followed by membrane permeabilization by 0.2% Triton X-100 in TBS for 10 minutes. They were washed three times in blocking buffer (TBS + 5% BSA), and Alexa 488-phalloidin (1:20; Invitrogen-Gibco) was added to for 2 hours at room temperature in the dark. Finally, the cells were washed three times in blocking buffer. Coverslips were moved to slides and sealed with fluorescence-preserving mounting medium (Vector Laboratories, Burlingame, CA). A confocal laser scanning image system (LSM 510; Carl Zeiss Meditec GmbH, Düsseldorf, Germany) was used to detect immunofluorescence.

**Isolation of POS**

Normal bovine eyes were obtained from an abattoir, and preparation was initiated within 6 hours after enucleation. POS isolation was performed as described by Hall.\(^1\) The retina was removed and transferred into PBS. The retinal tissue was trypsinized on ice and fragmented by a magnetic stirrer for 30 minutes. Large tissue fragments were eliminated by precipitation for 20 minutes at 4°C, and supernatants were centrifuged at 800g for 10 minutes. The samples were rinsed twice with PBS, and aliquots containing 40 × 10^6 POS were stored at −70°C until use.

**Labeling of POS and Quantification of POS Phagocytosis by ARPE-19 Cells**

POS were labeled with the biotin-streptavidin method described by Schraermeyer and Stieve.\(^13\) POS suspended in PBS were centrifuged at 1000g for 10 minutes. The pellet, containing the outer segments, was then biotinylated with 1:5 (wt/vol) 0.8 mg/ml NHS-LC biotin (Sigma-Aldrich, St. Louis, MO) dissolved in PBS for 1 hour in the dark and on ice. POS was washed twice in PBS and in a second step, the biotin residues on the outer segments were labeled 1:5 (wt/vol) with 0.05 mg/ml streptavidin-AlexaFluor568 red (Jackson Immunoresearch, West Grove, PA) for 1 hour in the dark and on ice and washed twice in PBS to remove unbound conjugates. Finally, POS was resuspended 1:2 (wt/vol) in PBS. RPE cells were grown in 96-well plates. Media were removed, and 200 μL of Locke's solution (154 mM NaCl, 5.6 mM KCl, 0.6% glucose, 2.2 mM CaCl₂, and 5 mM HEPES buffer adjusted to pH 7.4) was added to all wells. Five microliters POS were gently added on top of confluent monolayer cultures of ARPE-19 cells grown in 96-well plates. Five microliter's of Locke's was added to the control wells. Incubations were continued in darkness for various time intervals at 37°C in an atmosphere of 5% CO₂ and 95% air. Phagocytosed fluorescent POS were detected with a gel and blot imager (Typhoon 9410; GE Healthcare) equipped with a 532-nm excitation filter. Background fluorescence of the system, as assayed without cells, was very low and was automatically subtracted. Bromoenol lactone (BEL; 0.1, 1, 5, 10, or 20 μM) arachidonoyl trifluoromethyl ketone (AACOCF₃; 10 μM) or propranolol (150 μM) was added to cells simultaneously with the POS. To evaluate the phagocytosis of POS, we used Alexa-red-labeled POS to ARPE-19 cells grown on glass coverslips, as described earlier. After 12 hours, the cells were washed three times with PBS and fixed. Alexa 488-phalloidin (1:20; Invitrogen) was added to the cells for 2 hours at room temperature without light. Finally, the cells were washed and visualized with a confocal laser scanning image system.

**Cytotoxicity Assay**

After an overnight incubation with different concentrations of BEL (n = 6 for each condition) cell viability was determined by a colorimetric method, which is based on the conversion of yellow MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) to an insoluble purple formazan dye by mitochondrial dehydrogenase of living cells. Briefly, the assay was performed by removing the cell culture medium and replacing it with 100 μL fresh culture medium containing 5.0 mg/ml MTT. After 4 hours of incubation at 37°C, the cells were solubilized overnight with 100 μL of a solution containing 50% dimethylformamide and 20% SDS (pH 4.7), and absorbance was measured at 560 nm. The background readings (blank wells with medium, MTT, and solubilization buffer) were subtracted from the average absorbance readings of the BEL-treated wells, to obtain an adjusted absorbance reading that represented cell viability. This reading was divided by the adjusted absorbance reading of untreated cells in control wells, to obtain the percentage of cell survival.

**Measurement of PLA₂ Activity**

ARPE-19 cells, with or without preincubation with POS, were collected and homogenized in 150 μL lysis buffer (50 mM HEPES, 1 mM EDTA, 1 mM Na-orthovanadate, and protease inhibitor cocktail [1:100; Sigma-Aldrich]). Samples were centrifuged at 2000g for 30 minutes at 4°C. The supernatants were collected and subsequently spun through 30-KD cutoff filters (12 minutes, 14,000g; Microcon YM-30; Millipore, Hundested, Denmark). Arachidonoyl thioester bond at the sn-2 position by PLA₂ releases free thiol, which is detected by Ellman’s reagent. PLA₂ activity was determined in the supernatant with a cPLA₂ assay kit (Cayman Chemical), in the presence and absence of a specific inhibitor of iPLA₂ BEL, which was incubated for 15 minutes at 25°C at a concentration of 10 μM before the assay. Activity was calculated by measuring the absorbance at 405 nm, using the 5,5′-dithiobis(2-nitrobenzoic acid; DTNB) extinction coefficient of 10.66/mM, and reported as nanomoles per minute per gram cytosolic protein.

**Statistics**

Quantitative results are expressed as the mean ± SD. Statistical significance between experimental groups was assessed by Student’s t-test.

**RESULTS**

**Expression of mRNAs and Proteins of Intracellular PLA₂ in ARPE-19 Cells**

Primers recognizing genes encoding iPLA₂-VIA, cPLA₂-IVA, and iPLA₂-VIB revealed significantly higher expression of iPLA₂-VIA than of cPLA₂-IVA and iPLA₂-VIB. cPLA₂-IVA (P < 0.001, n = 4) and iPLA₂-VIB (P < 0.001, n = 4) mRNA expression was 12% and 67% of iPLA₂-VIA mRNA expression (n = 4), respectively (Fig. 1A). RT-PCR primers designed to detect splice variants of iPLA₂-VIA documented the expression of the known alternative genes encoding the iPLA₂-VIA region, with and without exon 9, ankyrin 1, and ankyrin 2.\(^20\) We furthermore identified a new splice variant containing intron 4 (Fig. 1B).

**Western blot analysis, using antibodies against human iPLA₂-VIA, human cPLA₂-IVA, and human iPLA₂-VIB, revealed protein expression of all the intracellular PLA₂s in ARPE-19 cells. POS did not express any of the known PLA₂s (Fig. 2A). iPLA₂-VIA identified a ~85-kDa band and a ~70-kDa band, previously described in the literature.\(^21\) The cPLA₂-IVA antibody recognized a ~100-kDa band and finally iPLA₂-VIB recognized a ~88, ~77, ~63, and ~48-kDa band. Immunohistochemical staining of ARPE-19 cells confirmed the protein expression of all the intracellular PLA₂s (Fig. 2B). The authenticity of the ~70-kDa protein to which the CAY-160507 (Cayman Chemical) antibody reacts, was evaluated by immunoprecipitation. An iPLA₂-VIA-directed antibody (SC-14463; Santa Cruz Biotechnology, Inc.) was used to immunoprecipitate iPLA₂-VIA from ARPE-19 cells. In this precipitate, both the ~85- and the ~70-kDa proteins were recognized by Western blot analysis using the CAY-160507 antibody, strongly indicating that this protein is an iPLA₂-VIA protein product (data not shown).
Cellular Location of Intracellular PLA₂ in the Normal Human Eye

Six normal eyes were used for evaluation of the expression pattern of intracellular PLA₂. Antibodies recognizing iPLA₂-VIA, cPLA₂-IVA, and iPLA₂-VIB were used as described in the Methods section. Table 2 presents the reactivity of the various ocular structures.

In the cornea, moderate detectable expression of iPLA₂-VIA was found in the epithelial cells, whereas strong iPLA₂-VIA expression was observed in the endothelium (Fig. 3A). Weak to moderate iPLA₂-VIA expression was found in the dilator and sphincter muscles. Pigmented and nonpigmented ciliary epithelial cells as well as the ciliary muscle revealed moderate iPLA₂-VIA expression. Strong expression of iPLA₂-VIA was observed in various layers of the retina (Fig. 3C). The most abundant expression was detected in the RPE cells, the ganglion cells, and the nerve fiber layer. Moderate iPLA₂-VIA expression was found in the photoreceptor inner segment and the inner and outer plexiform layers, whereas no expression appeared in the nuclear layers and the POS (Figs. 2B, 3C). Moderate iPLA₂-VIA expression appeared in both the anterior (Fig. 3D) and equatorial lens epithelium.

cPLA₂-IVA expression also showed widespread expression in the human eye. In the cornea moderate detectable expression was found in the epithelial cells and in the endothelium (Fig. 3E). Pigmented epithelial cells of the ciliary body (Fig. 3F) as well as the ciliary muscle revealed moderate cPLA₂-IVA expression, whereas strong staining appeared in the nonpigmented epithelial cells (Fig. 3F). Moderate expression was detected in the photoreceptor inner segment of the retina (Fig. 3H). Weak expression of cPLA₂-IVA was found in the anterior (Fig. 3I) and equatorial lens epithelium.

In contrast to the expression of iPLA₂-VIA and cPLA₂-IVA, iPLA₂-VIB appeared both in the cytosol and the nuclei of various cells in the human eye. Strong iPLA₂-VIB was found in the corneal epithelium and moderate expression was detected in the corneal endothelium (Fig. 3I). Pigmented and nonpigmented epithelial cells of the ciliary body (Fig. 3J) and ciliary muscle revealed moderate iPLA₂-VIB expression. In the retina, moderate iPLA₂-VIB expression was found in the photoreceptor inner segment, the inner nuclei layer, in the ganglion cells, in the nerve fiber layer and in the RPE (Fig. 3K). Strong iPLA₂-VIB expression appeared in both the anterior (Fig. 3L) and equatorial lens epithelium.

![FIGURE 1](A) PCR amplification and semiquantitative analysis of genes encoding iPLA₂-VIA, cPLA₂-IVA, and iPLA₂-VIB. (B) PCR amplification of genes encoding iPLA₂-VIA splice variants and cPLA₂-IV subtypes.

![FIGURE 2](A) Western blot analysis using iPLA₂-VIA-, cPLA₂-IVA-, and iPLA₂-VIB-specific antibodies on protein isolated from ARPE-19 cells and bovine POS. (B) Immunohistochemical staining of normal human retinas with antibodies specifically recognizing iPLA₂-VIA, cPLA₂-IVA, and iPLA₂-VIB. Note strong reactivity in the RPE with iPLA₂-VIA and only weak reactivity with the other two antibodies. RPE, retinal pigment epithelium; OS, photoreceptor outer segments; POS, photoreceptor outer segments; IS, photoreceptor inner segments; ONL, outer nuclear layer. (C) Immunohistochemical staining of ARPE-19 cells. Red: iPLA₂-VIA reactivity; green: cytoskeleton, stained by Alexa 488-phalloidin. Note the iPLA₂-VIA reactivity in the cytosol of the ARPE-19 cells. Bar, 5 µm.
and equatorial lens epithelium. In control eyes stained with rabbit serum, no reactivity appeared (Fig. 3M). In control eyes stained with preimmune serum from the rabbits used to produce the iPLA2-VIB antibody, weak reactivity appeared in the photoreceptor inner segment, in the inner and outer plexiform layers, and in the nerve fiber layer (Fig. 3N).

Cellular Location of iPLA2-VIA in ARPE-19 Cells
ARPE-19 cells stained with an iPLA2-VIA-specific antibody confirmed the location of iPLA2-VIA in the cytosol of the RPE cells (Fig. 2C).

Validation of POS and Latex Bead Uptake by ARPE-19 Cells
ARPE-19 cells were incubated with Alexa-red–labeled POS or beads for 12 and 24 hours. The identity of the fluorescence-labeled cells was confirmed by confocal microscopy revealing perinuclear localization of POS or beads in RPE cells. No staining was evident when nonlabeled POS were added to the cells or when no POS or beads were added to the media (data not shown).

Expression of PLA2 after POS Phagocytosis
ARPE-19 cells were fed with POS, as described in the Methods section. After 12 and 24 hours of incubation, the cells were harvested and the protein isolated for Western blot analysis. The 70-kDa iPLA2-VIA was upregulated by 1.6-fold after 12 hours (P < 0.001, n = 4) and 1.7-fold after 24 hours (P < 0.001, n = 4; Fig. 4B), whereas the 85 kDa iPLA2-VIA was downregulated by 0.2-fold after 12 and 24 hours (P < 0.05, n = 4). cPLA2-IVA and iPLA2-VIB revealed no significant change in expression after POS phagocytosis (n = 4; Fig. 4B).

No change in iPLA2-VIA, cPLA2-IVA or iPLA2-VIB expression was found after phagocytosis of the beads (data not shown).

### Table 2. Staining Intensity of iPLA2-VIA, cPLA2-IVA, and iPLA2-VIB Protein Expression in the Human Eye

<table>
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* The staining intensity was scored as negative (−), weak (+), moderate (++), strong (+++), or very strong (++++) The intensity shown is an average of results in six normal eyes.

ARPE-19 Phagocytosis of POS
Alexa red–labeled POS (n = 3) or latex beads (n = 3) were added to ARPE-19 cultures as described in the Methods section. The cells were washed after 1, 2, 4, 6, 8, 10, 12, 16, and 24 hours. Maximum absorbance was found 12 hours after addition of both POS (Fig. 4A) and beads (data not shown).
Induction of iPLA₂-VIA Activity by ARPE-19 Phagocytosis of POS

ARPE-19 cultures were fed with POS, and protein was isolated 4, 8, and 12 hours after POS addition. PLA₂ activity was measured in conditions with and without POS and was found to be 0.24 micromoles/per milligram protein in untreated ARPE-19 cells (n = 14). In ARPE-19 cells fed with POS, PLA₂ activity was measured to be 0.24 micromoles/per milligram protein (P < 0.05, n = 4) after 4 hours, 0.34 micromoles/per milligram protein (P < 0.05, n = 4) after 8 hours, and 0.35 micromoles/per milligram protein (P < 0.001, n = 18) after 12 hours (Fig. 5A). The iPLA₂-VIA-specific inhibitor BEL (10 μM) was added to the proteins to estimate PLA₂ activity and was found to inhibit all activity induced by phagocytosis of POS (Fig. 5A). Addition of beads to the ARPE-19 cells did not induce iPLA₂-VIA activity, which was measured at 0.23 micromoles/per milligram protein after 12 hours (n = 8; Fig. 5A). Inhibition with the specific cPLA₂-IVA inhibitor AACOCF₃ did not reveal a significant role of cPLA₂-IVA in the increased PLA₂ activity induced by phagocytosis (not shown).

Inhibition of ARPE-19 Phagocytosis by BEL

The specific iPLA₂-VIA inhibitor BEL (10 μM) significantly reduced ARPE-19 phagocytosis of POS (24%; P < 0.01, n = 16; Fig. 4).
**FIGURE 5.** (A) PLA₂ activity in ARPE-19 cells fed with POS or beads. Bars represent total PLA₂ activity (±SD). Black portions indicate iPLA₂-VIA-specific activity. (B) Inhibition of ARPE-19 phagocytosis of POS by the specific iPLA₂-VIA inhibitor BEL (10 μM) or the cPLA₂-IVA inhibitor AACOCF3 (10 μM). Bars represent the mean percentage of inhibition (±SD). (C) Irreversible inhibition of ARPE-19 phagocytosis of POS 24, 48, and 72 hours after addition of BEL. POS was repeatedly added every 12 hours. Bars represent the mean BEL inhibition (±SD). (D) Cytotoxicity of various concentrations of BEL using an MTT assay. Bars represent the mean percentage of cytotoxicity after addition of the different BEL concentrations (±SD). (E) Inhibition of ARPE-19 phagocytosis of POS by different BEL concentrations. Bars represent mean percentage of inhibition (±SD). Significance levels: *P < 0.05, **P < 0.001.

Fig. 5B). The inhibition was sustained after readdition of POS after 24, 48, and 72 hours (21%; P < 0.05, n = 4), 26% (P < 0.05, n = 4), and 32% (P < 0.05, n = 4), respectively (Fig. 5C). BEL toxicity was evaluated by MTT as described in the Methods section. Significant toxicity was observed when cells were exposed to BEL concentrations above 20 μM (Fig. 5D). BEL concentrations between 5 and 20 μM had an equal inhibitory effect on ARPE-19 phagocytosis of POS (Fig. 5E). The cPLA₂-IVA inhibitor AACOCF3 (n = 15; Fig. 5B) and propranolol (n = 15; data not shown) did not significantly reduce phagocytosis.

**DISCUSSION**

RPE phagocytosis of POS is essential for retinal function, and it has been suggested that alterations in POS degradation may lead to age-related macular degeneration (AMD), the most common cause of irreversible blindness in the Western world.²¹,²² Besides phagocytizing POS, RPE cells have various additional physiological functions such as supporting the neuroretina, creating part of the blood–brain barrier, and transporting metabolites and water.²³ Studies of other phagocytizing cells may be helpful in understanding RPE phagocytosis of POS. PLA₂ activity has been demonstrated in macrophages, and it has been shown that PLA₂ activity increases during phagocytosis. Among other specific PLA₂ subtypes, iPLA₂-VIA has been shown to be essential in macrophage phagocytosis.⁷,⁹,²⁴,²⁵ Lyso-phosphatidylcholine, a metabolite of iPLA₂-VIA activity, seems to generate a signal for phagocytosis, and macrophage phagocytosis can be inhibited by the iPLA₂-specific inhibitor BEL.³ Evidence on cPLA₂-IVA involvement in macrophage phagocytosis has also been suggested,¹³,²⁶ whereas no studies have explored a role of iPLA₂-VIB in phagocytosis.

In the retina, the literature has described PLA₂ activity in the RPE cells as well as in the neuroretina. However, no specific PLA₂ has been identified in these tissues, and the current knowledge is based on indirect evidence of substrate-specific PLA₂ activity.¹⁶,¹⁷,²⁷ Based on the previous findings, the purpose of this study was to identify intracellular PLA₂ in the human eye, with emphasis on the outer retinal layers, and to evaluate the role of intracellular PLA₂ in RPE phagocytosis of POS. Intracellular PLA₂ expression was found in various parts of the eye, as outlined in Table 2. Abundant expression of genes encoding iPLA₂-VIA were present in the human ARPE-19 cell line, whereas RPE expression of cPLA₂-IVA and iPLA₂-VIB was less abundant (Fig. 1A). The various known splice variants of iPLA₂-VIA were identified, including a new splice variant without exon 4, which creates a frameshift and thereby a new possible regulator of iPLA₂-VIA activity.¹⁶ Based on the previous findings, the purpose of this study was to identify intracellular PLA₂ in the human eye, with emphasis on the outer retinal layers, and to evaluate the role of intracellular PLA₂ in RPE phagocytosis of POS. Intracellular PLA₂ expression was found in various parts of the eye, as outlined in Table 2. Abundant expression of genes encoding iPLA₂-VIA were present in the human ARPE-19 cell line, whereas RPE expression of cPLA₂-IVA and iPLA₂-VIB was less abundant (Fig. 1A). The various known splice variants of iPLA₂-VIA were identified, including a new splice variant without exon 4, which creates a frameshift and thereby a new possible regulator of iPLA₂-VIA activity.¹⁶ Because all splice variants of iPLA₂-VIA seem to be present in the ARPE-19 cells, there may be tightly regulated functions of iPLA₂-VIA, not only in the phagocytosis of POS but also in other functions of these multifunctional cells. However, the physiological relevance of the splice variants remains to be shown.

On the protein level, expression of iPLA₂-VIA, cPLA₂-IVA, and iPLA₂-VIB were identified, both in the normal human eye sections and the ARPE-19 cells (Fig. 2). Western blot analysis of
iPLA$_2$-VIA revealed ~85- and ~70-kDa bands. The ~85-kDa band has been shown to be a catalytically active iPLA$_2$-VIA, and evidence from pancreatic islets and insulinoma cells shows the significance of the ~70 kDa iPLA$_2$-VIA. In the present study the specificity of the ~70 kDa band was furthermore strengthened by immunoprecipitation.

The ~70 kDa band of iPLA$_2$-VIA was upregulated 12 and 24 hours after the addition of POS to ARPE-19 cells indicating a role in RPE phagocytosis. It has been suggested that the high-molecular-weight iPLA$_2$ is proteolytically cleaved to create the low-molecular-weight iPLA$_2$. Because the present Western blot analysis reveals decreased expression of the high-molecular-weight iPLA$_2$-VIA in phagocytizing ARPE-19 cells, the induction of the ~70-kDa band may be due to cleavage of the ~85-kDa band. Furthermore, the ~85-kDa band has been shown to include a membrane-binding site and it is therefore tempting to suggest that cleavage possibly leads to the smaller ~70-kDa band, which may relocate and thereby obtain ability to act in the phagocytosis process. Future studies are necessary, however, to clarify these issues.

Previous studies have shown a role of COX-2 in rat RPE phagocytosis and it is possible that cPLA$_2$-IVA may play a role in this induction. However, no significant upregulation of cPLA$_2$-IVA was found (Fig. 4B) and the present study could not confirm an involvement of this subtype in RPE phagocytosis of POS. Finally, no significant regulation of iPLA$_2$-VIB was observed (Fig. 4B). Involvement of sPLA$_2$ in macrophage phagocytosis has previously been shown and this subgroup of PLA$_2$ may also be involved in RPE phagocytosis of POS. However, the present study did not evaluate possible roles of sPLA$_2$, and future studies should investigate this question. In comparison with published data on PLA$_2$ activity in different cell types, basal PLA$_2$ activity in ARPE-19 cells appears to be relatively high. In the present study, we showed increased PLA$_2$ activity in phagocytosing RPE cells 8 (not shown) and 12 hours (Fig. 5A) after ARPE-19 cells were fed with POS, whereas no upregulation was seen 4 hours after addition of POS. The delay on increased PLA$_2$ activity suggests that PLA$_2$ activity may be a response to POS engulfment and thereby a mechanism to degrade the membranes and thereby regulate phagocytosis. Of interest, the increased activity could be blocked by BEL, which strongly supports the role of iPLA$_2$-VIA in RPE phagocytosis of POS (Figs. 5B, 5C). When inhibiting phagocytosis with the iPLA$_2$-VIA specific inhibitor BEL, we significantly reduced phagocytosis, whereas no effect was found with the cPLA$_2$-IVA-specific inhibitor AACOCF$_3$. Because PLA$_2$-VIA activity has been implicated in the involvement of normal phospholipid remodeling and several reports have suggested that iPLA$_2$-VIA is one of the main modulators of PC catabolism, our present data support this hypothesis.

In conclusion, in the present study, we explored intracellular PLA$_2$ in the human retina. We confirmed that RPE elicits PLA$_2$ activity and identified iPLA$_2$-VIA, cPLA$_2$-IVA, and iPLA$_2$-VIB on both the mRNA and the protein levels. We report a significant role of iPLA$_2$-VIA in RPE phagocytosis of POS, whereas cPLA$_2$-IVA and iPLA$_2$-VIB do not seem to be essential in this process. Because RPE phagocytosis is critical for vision and is damaged during pathologic processes such as AMD, it is tempting to suggest that iPLA$_2$-VIA could be a target for future pharmacological intervention.

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References


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