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Phospholipase D1 modulates protein kinase C-epsilon in retinal pigment epithelium cells during inflammatory response

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ABSTRACT

Inflammation is a key factor in the pathogenesis of several retinal diseases. In view of the essential role of the retinal pigment epithelium in visual function, elucidating the molecular mechanisms elicited by inflammation in this tissue could provide new insights for the treatment of retinal diseases. The aim of the present work was to study protein kinase C signaling and its modulation by phospholipases D in ARPE-19 cells exposed to lipopolysaccharide. This bacterial endotoxin induced protein kinase C-α/βII phosphorylation and protein kinase-ε translocation to the plasma membrane in ARPE-19 cells. Pre-incubation with selective phospholipase D inhibitors demonstrated that protein kinase C-α phosphorylation depends on phospholipase D1 and 2 while protein kinase C-ε activation depends only on phospholipase D1. The inhibition of α and β protein kinase C isoforms with Go 6976 did not modify the reduced mitochondrial function induced by lipopolysaccharide. On the contrary, the inhibition of protein kinase C-α, β and ε with Ro 31-8220 potentiated the decrease in mitochondrial function. Moreover, inhibition of protein kinase C-ε reduced Bcl-2 expression and Akt activation and increased Caspase-3 cleavage in cells treated or not with lipopolysaccharide. Our results demonstrate that through protein kinase C-ε regulation, phospholipase D1 protects retinal pigment epithelium cells from lipopolysaccharide-induced damage.

1. Introduction

The retinal pigment epithelium (RPE) is a monolayer of pigmented cells located between the choroid and the neural retina which because of their unique position are able to sense circulating toxins and inflammatory cytokines. Furthermore, these cells constitute the outer blood-retinal barrier, secrete several growth factors and cytokines and transport nutrients and water to the retina (Simo et al., 2010; Strauss, 2005). RPE cells are also essential for the integrity and function of the retina since they protect against photo-oxidation and mediate the re-isomerization of all-*trans*-retinal and the renewal of photoreceptor outer segments by phagocytosis (Simo et al., 2010; Strauss, 2005). In view of the essential role of the RPE in photoreceptor viability and visual function, elucidating the effects of the inflammatory process in these cells could lead to the discovery of new therapeutic targets for the treatment of retinal degenerative and inflammatory diseases.

Inflammation is a common factor in the pathogenesis of several retinal diseases that eventually end in vision loss and blindness, such as age-related macular degeneration, diabetic retinopathy, retinitis pigmentosa and uveitis (Perez and Caspi, 2015; Perez et al., 2013; Viringipurampeer et al., 2013; Bazan et al., 2011; Leung et al., 2009). It has been demonstrated that RPE cells can respond to the bacterial endotoxin lipopolysaccharide (LPS) since they express the primary LPS receptor, CD14, and its membrane-linked co-receptor, toll-like receptor 4 (TLR4) (Mateos et al., 2014; Elner et al., 2005). LPS can reach the RPE in patients with bacterial endophthalmitis (posterior segment eye infection). This unusual pathology can appear as a consequence of intravitreal injections, trauma, eye

Abbreviations: COX-2, cyclooxygenase 2; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; HRP, horseradish peroxidase; LPS, lipopolysaccharide; LPPs, lipid phosphate phosphatases; MEF, membrane-enriched fraction; PA, phosphatidic acid; PC, phosphatidylcholine; PGs, prostaglandins; PKC, protein kinase C; PLD, phospholipase D; PVDF, polyvinylidene fluoride; RasGRP, Ras guanine-releasing protein; RPE, retinal pigment epithelium; TCL, total cell lysates; WB, western blot.

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surgery, or sepsis (endogenous endophthalmitis) (Vaziri et al., 2015; Haddock et al., 2014; Holland et al., 2014; Pollreisz et al., 2012), has generally poor prognosis and can lead to vision loss (Haddock et al., 2014; Holland et al., 2014).

We have recently reported that the human RPE cell line ARPE-19 expresses both classical phospholipase D isoforms (PLD1 and PLD2) and that LPS stimulates PLD activity (Mateos et al., 2014). Classical PLDs hydrolyze phosphatidylcholine (PC) to generate the lipid second messenger, phosphatidic acid (PA), and choline. PA can be further dephosphorylated by lipid phosphate phosphatases (LPPs) in order to generate diacylglycerol (DAG), another lipid messenger (Peng and Frohman, 2012). Thus, the PLD/LPPs pathway can modulate the activity of DAG-responding proteins, such as classical and novel protein kinases C (PKCs), chimaerins, DAG kinases, protein kinases D, and Ras guanine-releasing protein (RasGRP), all of which present at least one DAG-binding C1 domain (Newton, 2010; Caloca et al., 2008; Carrasco and Merida, 2007; Yang and Kazanietz, 2007; Wang, 2006). PKC phosphorylation is necessary to render PKC in a catalytically competent conformation and also to protect it from degradation. These kinases are acutely activated by their translocation to membranes (mediated by second-messenger binding to their regulatory domains), the release of the autoinhibitory pseudosubstrate domain from the active site and the shift into an active and open conformation (Antal and Newton, 2014; Wu-Zhang and Newton, 2013). Conventional PKCs (cPKCs), such as PKCα, βI, βII and γ, are allosterically activated by binding to Ca^{2+} and DAG while novel PKC isoforms (nPKCs), such as δ, ε, η and θ, are activated only by DAG. Atypical isoforms (aPKCs), namely ζ and ι, are activated neither by Ca^{2+} nor DAG, but are instead regulated by phosphatidylserine and protein–protein interactions (Antal and Newton, 2014; Wu-Zhang and Newton, 2013).

The participation of PLDs in several cell biological events, such as *exo-* and endocytosis, vesicle trafficking, proliferation, cell migration, autophagy, and apoptosis, has been widely studied (Frohman, 2015; Nelson and Frohman, 2015; Klein et al., 1995). Furthermore, with the development of pharmacological PLD inhibitors, the PLD pathway has been recently postulated as a possible therapeutic target for the treatment of hypertension, cancer, and autoimmune, thrombotic and neurodegenerative diseases (Frohman, 2015). In this connection, our previous study constituted the first evidence of classical PLDs participation in the LPS-induced inflammatory process in RPE cells through extracellular signal-regulated kinase (ERK1/2) activation and cyclooxygenase-2 (COX-2) expression (Mateos et al., 2014). Our results demonstrated that after LPS treatment the PLD2-dependent activation of ERK1/2 and the subsequent induction in the expression of COX-2 and production of prostaglandin E2 (PGE_2) mediate RPE cell damage. In addition, PLD1 appears to play a dual role in LPS-exposed RPE cells, by promoting cell damage through COX-2 induction and by modulating cellular protective mechanisms (Mateos et al., 2014). Our aim in the present paper was to study the role of PLDs in PKC signaling in RPE cells exposed to LPS and thus arrive at a better understanding of the role of DAG-mediated signaling pathways in RPE cells exposed to inflammatory conditions.

2. Materials and methods

2.1. Reagents

Triton X-100 (octyl phenoxy polyethoxyethanol), dimethyl sulfoxide (DMSO), U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), lipopolysaccharide from *Klebsiella pneumoniae* (LPS, L4268) and MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were from Sigma-Aldrich (St. Louis, MO, USA). VU0359595 (or EVJ) and VU0285655-1 (or APV) were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) was from Life Technologies Corporation (Grand Island, NY,USA). Ro 31-8220 (Bisindolylmaleimide IX) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Go 6976 (5,6,7,13-tetrahydro-13-methyl-5-oxo-12*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-12-propanenitrile) was from Abcam (Cambridge, MA, USA). All other chemicals were of the highest purity available.

2.2. Antibodies

Rabbit polyclonal antibodies anti-PKCα (#2056), anti-phospho PKCα/βII($Thr^{638/641}$) (#9375), anti-cleaved Caspase-3 (#9661) and mouse monoclonal anti-phospho Akt (Ser^{473}) (#4051) and anti-Akt (#9220) and were from Cell Signaling (Beverly, MA, USA). Mouse monoclonal antibodies anti-PKCβ (#610127) and anti-PKCε (#610085) were from BD Biosciences (San José, CA, USA). Mouse monoclonal anti-α Tubulin (DM1-A) (CP06) was from EMD/Biosciences-Calbiochem (San Diego, CA, USA). Rabbit polyclonal anti-Bcl-2 antibody (sc-492) and polyclonal horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (sc-2004) and polyclonal HRP-conjugated goat anti-mouse IgG (sc-2005) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Alexa Fluor®546 goat anti-rabbit (A11035) and Alexa Fluor®546 goat anti-mouse (A11003) were from Life Technologies Corporation (Grand Island, NY, USA).

2.3. Retinal-pigmented epithelium cell cultures and treatments

Human retinal-pigmented epithelium cells (ARPE-19) from the American Type Culture Collection (ATCC, Manassas, VA) were generously donated by Dr. E. Politi and Dr. N. Rotstein (INIBIBB, Bahía Blanca, Argentina). The human retinal pigment epithelium cell line D407 was a generous gift from Dr. E. Rodriguez-Bouland (Weill Medical College of Cornell University, New York, NY, USA). Cells (in passage 5–12) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Natocor, Argentina) and antibiotic-antimycotic (Anti-Anti 100X, Gibco by Life Technologies) at 37 °C under 5% CO_2. Except for the microscopy experiments, cells were grown to 100% confluence on plastic 35 mm diameter culture dishes. Confluent cell cultures were serum-starved for 2 h prior to stimulation and treated for 5 min or 2, 24 or 48 h with different doses (5 and 10 μg/ml) of LPS in serum-free DMEM or the same volume of sterile ultra pure water (control condition). LPS stock (4 mg/ml) was prepared in sterile ultra-pure water. ARPE-19 cells were pre-incubated with EVJ (0.15 μM) to inhibit PLD1 activity or with APV (0.5 μM) to inhibit PLD2 for 1 h at 37 °C prior to cell stimulation with LPS. DMSO (vehicle of the inhibitors) was added to all conditions to achieve a final concentration of 0.0025%. ARPE-19 and D407 cells were pre-incubated with 2 μM Go 6976 (a PKCα and β inhibitor), or 10 μM Ro 31-8220 (a PKCα, β, γ, and ε inhibitor) for 1 h at 37 °C prior to LPS exposure for 48 h. DMSO (vehicle of the inhibitors) was added to all conditions to achieve a final concentration of 0.005%.

2.4. MTT reduction assay

After 48 h LPS treatment, mitochondrial function was assessed by MTT reduction assay. MTT, a water-soluble tetrazolium salt, is reduced by mitochondrial dehydrogenases of metabolically viable cells to a colored, water-insoluble formazan salt. MTT (5 mg/ml, prepared in phosphate buffer saline) was added to the cell culture medium at a final concentration of 0.5 mg/ml. The culture dishes were incubated for 1 h at 37 °C in a 5% CO_2 atmosphere, cells were washed twice with phosphate buffer saline (PBS) and lysed

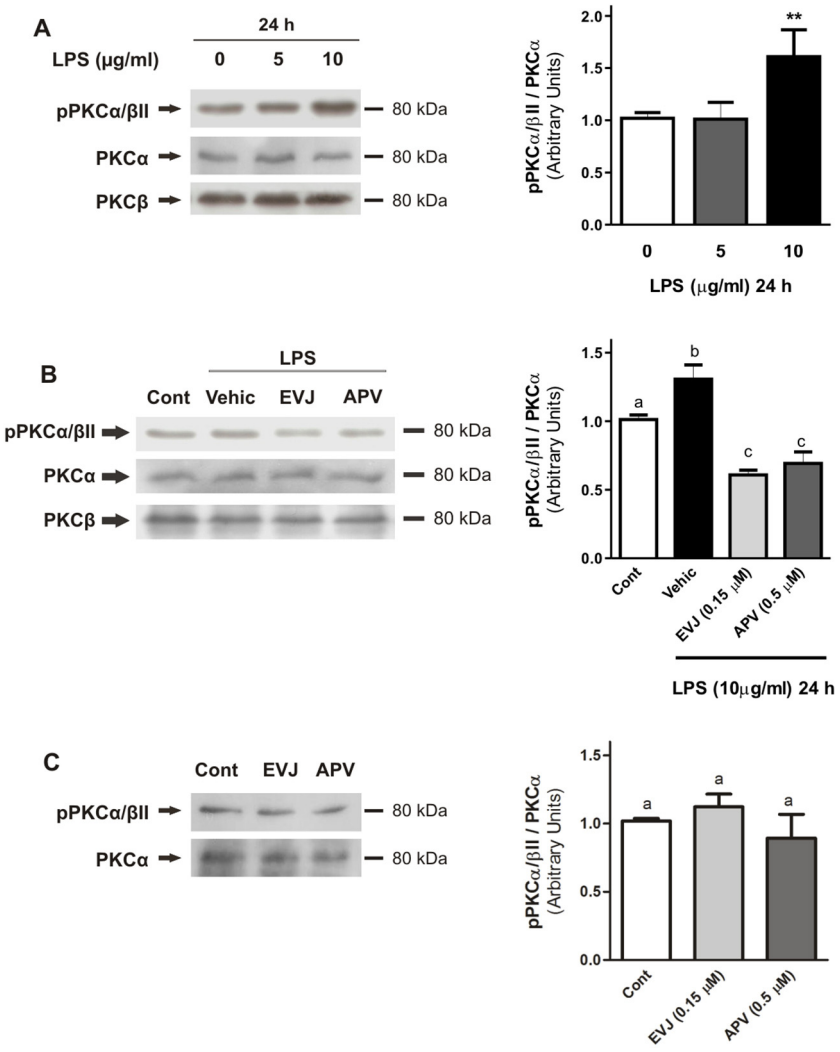

Fig. 1. (A) PKCα/βII phosphorylation in ARPE-19 cells exposed to LPS for 24 hARPE-19 cells were treated with LPS (5 or 10 μg/ml) or ultra-pure water (control condition) for 24 h. PKCα/βII phosphorylation was evaluated by WB as detailed in Materials and Methods using anti-phosphoPKCα/βII (pPKCα/βII) antibody. Numbers to the right indicate molecular weights and the bar graph shows the densitometry values of pPKCα/βII/PKCα expressed in arbitrary units as a ratio of the control. Asterisks indicate significant differences with respect to the control condition (** $p < 0.01$). (B) Role of classical PLDs in LPS-induced PKCα/βII phosphorylation. ARPE-19 cells were pre-incubated with 0.0025% DMSO (Control and Vehicle conditions), 0.15 μM EVJ or 0.5 μM APV for 1 h before LPS treatment. Cells were treated with 10 μg/ml LPS or ultra-pure water (control condition) for 24 h and PKCα/βII phosphorylation was evaluated by WB. Numbers to the right indicate molecular weights and the bar graph shows the densitometry values of pPKCα/βII/PKCα expressed in arbitrary units as a ratio of the control. Conditions designated with different letters (a–c) present significant differences ($p < 0.05$). (C) Effect of PLD inhibitors on PKCα/βII phosphorylation under control conditions. ARPE-19 cells were incubated with 0.0025% DMSO (Control condition), 0.15 μM EVJ or 0.5 μM APV for 24 h and PKCα/βII phosphorylation was evaluated by WB using anti-phosphoPKCα/βII (pPKCα/βII) antibody. Numbers to the right indicate molecular weights and the bar graph shows the densitometry values of pPKCα/βII/PKCα expressed in arbitrary units as a ratio of the control.

with 1 ml of a buffer containing 10% Triton X-100 and 0.1N HCl in isopropanol. The extent of MTT reduction was measured spectrophotometrically at 570 nm using a V-630 spectrophotometer (JASCO, Analytical Instruments). Results are expressed as arbitrary units with respect to the control condition.

2.5. Western blot (WB)

After experimental treatment the medium was removed from confluent 35 mm dishes, cells were washed three times with PBS and scraped off with 80 μl ice-cold RIPA lyses buffer [10 mM Tris–HCl (pH 7.4), 15 mM NaCl, 1% Triton X-100, 5 mM NaF, 1 mM Na_2VO_4 and the complete protease inhibitor cocktail]. Protein content of total cell lysates was determined by the Bradford method (Bradford, 1976) (Bio-Rad Life Science group, #500-0006) and samples were denatured with Laemmli sample buffer at 100 °C for 5 min (Laemmli, 1970). 30 μg protein were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 10% BSA in TTBS buffer [20 mM Tris–HCl (pH 7.4), 100 mM NaCl and 0.1% (w/v) Tween 20] at room temperature for 2 h and subsequently incubated with primary antibodies overnight at 4 °C. After three washes with TTBS, membranes were exposed to the appropriate HRP-conjugated secondary antibody for 2 h at room temperature. Membranes were washed again three times with TTBS and immunoreactive bands were detected by enhanced chemiluminescence (Pierce®ECL Western Blotting Substrate, #32209, Thermo scientific) using UltraCruz# Autoradiography Film, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Densitometry values of the immunoreactive bands were determined using ImageJ 1.38 software. The molecular weight of bands was determined using the spectra multicolor broad range protein ladder (26634, Thermo Scientific).

2.6. Immunocytochemistry and wide-field fluorescence microscopy

For immunocytochemistry assays 60,000 cells were seeded onto 12 mm coverslips on plastic 35 mm diameter culture dishes. After LPS treatment (10 μg/ml for 24 h) cells were washed twice with ice-cold PBS, fixed with 2% paraformaldehyde for 40 min and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Cells were then blocked with 2% BSA in PBS for 15 min and exposed to primary antibodies (1:100 in blocking solution) and Alexa Fluor®546-conjugated secondary antibodies (1:500 in blocking solution) for 1 h. Finally, nuclei were stained with DAPI for 10 min at room temperature. The whole immunocytochemical method was performed at room temperature and three washes with ice-cold PBS were performed between each step of the procedure and after DAPI staining.

Coverslips were mounted for examination with a Nikon Eclipse E-600 microscope. Imaging was done with an SBIG model ST-7 digital charge-coupled device camera (765 × 510 pixels, 9.0 × 9.0 μm pixel size; Santa Barbara, CA, USA). The ST-7 CCD camera was driven by CCDOPS software package (SBIG Astronomical Instruments, version 5.02). For all experiments a 40× (1.0 N.A.) oil-immersion objective was used. Appropriate dichroic and emission filters were used to avoid crossover of fluorescence emission. 16-bit TIFF images were exported. Fluorescence intensity values were determined using ImageJ 1.38 software.

2.7. Subcellular fractionation

Confluent 60 mm culture dishes were harvested into ice-cold lysis buffer (20 mM Tris–HCl (pH 7.5), 5 mM EGTA, 1 mM NaF, 100 μM Na 2VO4 and the complete protease inhibitor cocktail), sonicated and fractionated by ultracentrifugation, as described by Caloca and collaborators (Caloca et al., 2008). The cytosolic fraction was obtained by collection of the supernatant after centrifugation of the total cell lysate (TCL) (1 h at 100,000*g* at 4 °C). The pellet was treated for 1 h with 1% Triton X-100 in lysis buffer and subsequently centrifuged (1 h at 100,000*g* at 4 °C). The resulting supernatant represents the Triton X-100 soluble membrane-enriched fraction (MEF), and the remaining pellet is the Triton X-100-insoluble fraction (cytoskeleton-enriched). Protein concentration was determined by the Bradford method and equal amounts of protein of TCL and MEF fractions were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted for PKCε detection as described in 2.5.

2.8. Statistical analysis

Statistical analysis of the raw data was performed using ANOVA followed by Bonferroniís test to compare means. *p*-values lower than 0.05 were considered statistically significant. Results are expressed as a ratio of the control. Data represent the mean value ± SD of at least three independent experiments. The WBs shown are representative images of samples from three independent experiments.

3. Results

3.1. LPS-induced PKCα/βII phosphorylation in RPE cells is dependent on classical PLDs

To study the effect of LPS on PKC signaling in RPE cells, ARPE-19 cells were treated with 0 (control condition), 5 and 10 μg/ml LPS for 24 h and PKCα/βII phosphorylation (pPKCα/βII) was evaluated by WB as described in Materials and Methods. As shown in Fig. 1A, 10 μg/ml LPS treatment for 24 h increased PKCα/βII phosphorylation by 40% with respect to the control condition. To study the role of classical PLDs on PKCα/βII phosphorylation the RPE cells were pre-incubated with selective PLD1 and PLD2 inhibitors prior to LPS treatment. The pre-incubation of the cells with the PLD1 inhibitor (0,15 μM EVJ) or the PLD2 inhibitor (0,5 μM APV) completely abolished the increase in the pPKCα/βII/PKCα ratio observed after 24 h exposure to LPS (Fig. 1B). Under control conditions, the incubation with PLD inhibitors did not modify PKCα/βII phosphorylation, demonstrating that neither EVJ nor APV have a direct effect on basal PKCα/βII phosphorylation (Fig. 1C). Shorter periods of treatment with LPS (5 and 120 min) did not modify the PKCα/βII phosphorylation state (Data not shown). These results demonstrate that sustained LPS treatment induces PKCα/βII phosphorylation in RPE cells and that this priming process depends on both classical PLDs. Since PKC phosphorylation is necessary to render PKC in a catalytically competent conformation and also to protect PKC from degradation, our results demonstrate that PLD inhibition prevents PKCα/βII activation.

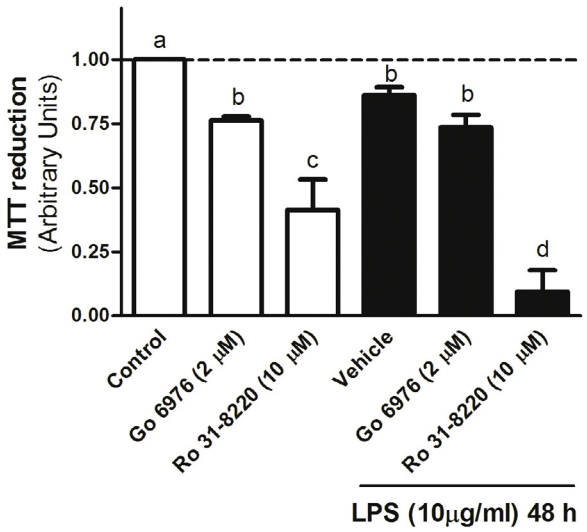

Fig. 2. Effect of PKC inhibition on RPE mitochondrial function. ARPE-19 cells were pre-incubated for 1 h with 0.005% DMSO (Control and Vehicle conditions), 2 μM Go 6976 or 10 μM Ro 31-8220 and then treated with 10 μg/ml LPS (black bars) or ultra-pure water (control condition, white bars) for 48 h. Mitochondrial function was evaluated using the MTT reduction assay as detailed in Materials and Methods. Data represent the mean value ± SD of three independent experiments. Results are expressed in arbitrary units as a ratio of the control. Conditions designated with different letters (a–d) present significant differences ($p < 0.05$).

3.2. The reduced RPE mitochondrial function induced by LPS is differentially affected by PKCs inhibitors

RPE cells are extremely resistant and able to deal with threats, such as UV light, toxins and oxidative stress. Previously, we demonstrated that LPS (10 μg/ml) treatment for 48 h reduced ARPE-19 cells mitochondrial function by 15% with respect to the control condition (Mateos et al., 2014). In the present work we studied the role of PKCs in the loss of mitochondrial viability observed in RPE cells exposed to LPS. To this end ARPE-19 cells were pre-incubated with 2 μM Go 6976 (a PKCα and β inhibitor), or 10 μM Ro 31-8220 (a PKCα, β, γ, and ε inhibitor) prior to LPS exposure for 48 h and mitochondrial function was measured using the MTT reduction assay. As shown in Fig. 2, under control conditions the inhibition of PKCs for 48 h with Go 6976 and Ro 31-8220 reduced MTT reduction by 24% and 60%, respectively. Interestingly, no differences in MTT reduction were observed between cells pre-incubated with Go 6976 and treated or not with LPS (Fig. 2). This result suggests that even though PKCα/βII is phosphorylated by LPS in ARPE-19 cells,

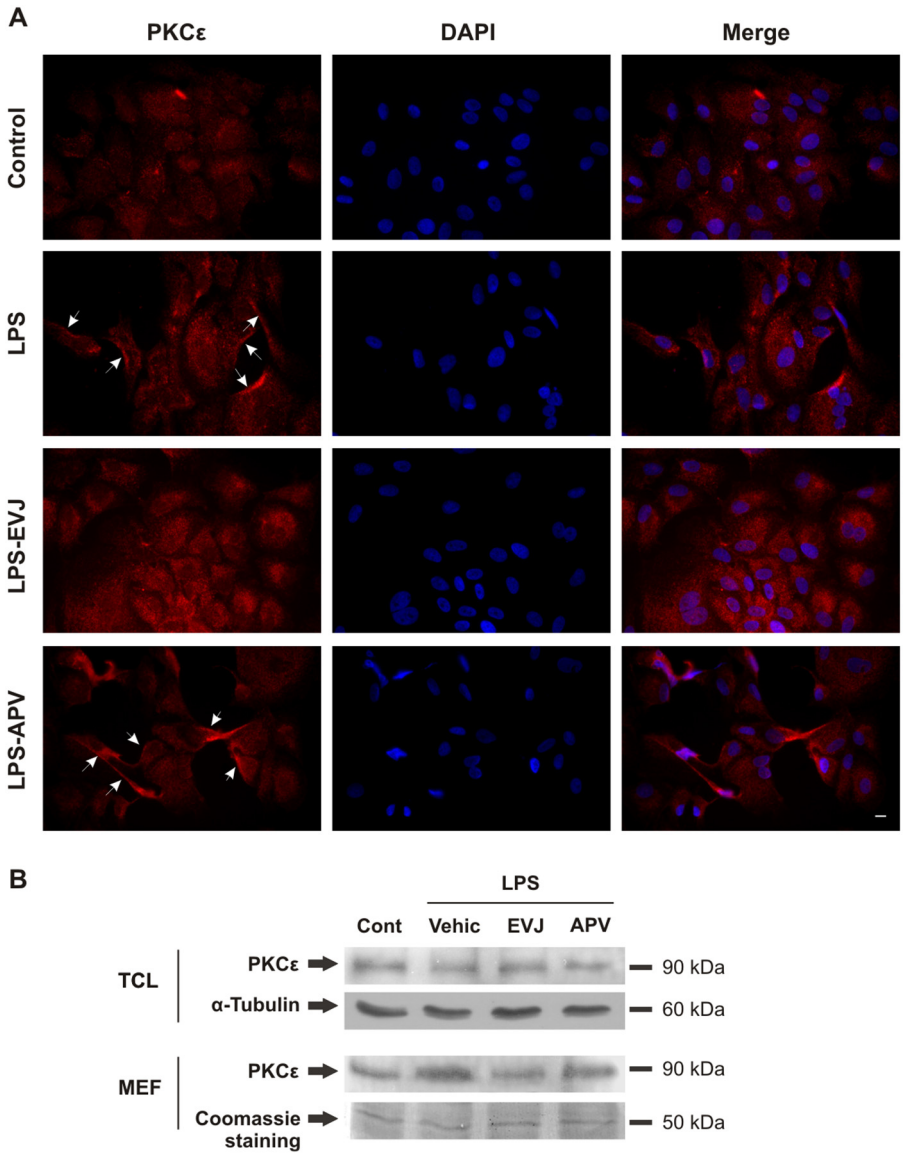

Fig. 3. LPS effect on PKCε subcellular localization in RPE cells. (A) ARPE-19 cells grown onto 12 mm coverslips were pre-incubated with 0.0025% DMSO (control and LPS conditions), 0.15 μM EVJ (LPS-EVJ condition) or 0.5 μM APV (LPS-APV condition) for 1 h before being exposed to LPS (10 μg/ml) or to the ultra-pure water (control condition) for 24 h. After the experimental treatment immunocytochemistry assays were performed as described in Materials and Methods. Representative microphotographs from three independent experiments show PKCε distribution (first column), DAPI staining (second column) and the merge image (third column). Scale bar = 10 μm. (B) Confluent ARPE-19 cell cultures were pre-incubated with 0.0025% DMSO (control and LPS conditions), 0.15 μM EVJ (LPS-EVJ condition) or 0.5 μM APV (LPS-APV condition) for 1 h before being exposed to LPS (10 μg/ml) or to the ultra-pure water (control condition) for 24 h. Subcellular fractionation was performed as described in Materials and Methods and equal amounts of protein of total cell lysates (TCL) and membrane-enriched fraction (MEF) fractions were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted for PKCε and α Tubulin detection. Numbers to the right indicate molecular weights.

these conventional isoforms are not involved in the loss of mitochondrial function induced by LPS. On the contrary, in cells treated with Ro 31-8220, LPS was able to drastically reduce MTT reduction (90%) with respect to the control condition (Fig. 2). Since it was previously reported that human RPE do not express PKCγ (Yu et al., 2007; Wood and Osborne, 1997) these results suggest that PKCε (which is inhibited by Ro 31-8220 but not by Go 6976) could be activated by LPS to mediate cell survival.

The MTT reduction assay was also performed in the human RPE cell line D407 and the results obtained were similar to those obtained in ARPE-19 cells. Pre-incubation with Go 6976 reduced MTT reduction by 22% in D407 cell cultures. Moreover, D407 cells appeared to be more sensitive to LPS and to PKC inhibition. In D407 cells 48 h LPS treatment reduced mitochondrial functionality by 33% with respect to the control condition and pre-incubation with 10 μM Ro 31-8220 drastically reduced MTT reduction (by 85%), both in cells treated or not with LPS (Data not shown).

3.3. *LPS-induced PKCε translocation to the plasma membrane is dependent on PLD1 but independent of PLD2*

In view of the results obtained with the PKC inhibitor Ro 31-8220, we performed immunocytochemistry assays in order to study the effect of LPS on PKCε subcellular localization. PKCs are acutely activated by their translocation to membranes mediated by second-messenger binding to their regulatory domains (Antal and Newton, 2014; Wu-Zhang and Newton, 2013). Fig. 3A shows that LPS induced a change in PKCε subcellular localization, with PKCε staining at the plasma membrane zone in ARPE-19 cells treated with LPS for 24 h (Fig. 3A, indicated with white arrows in the sec-

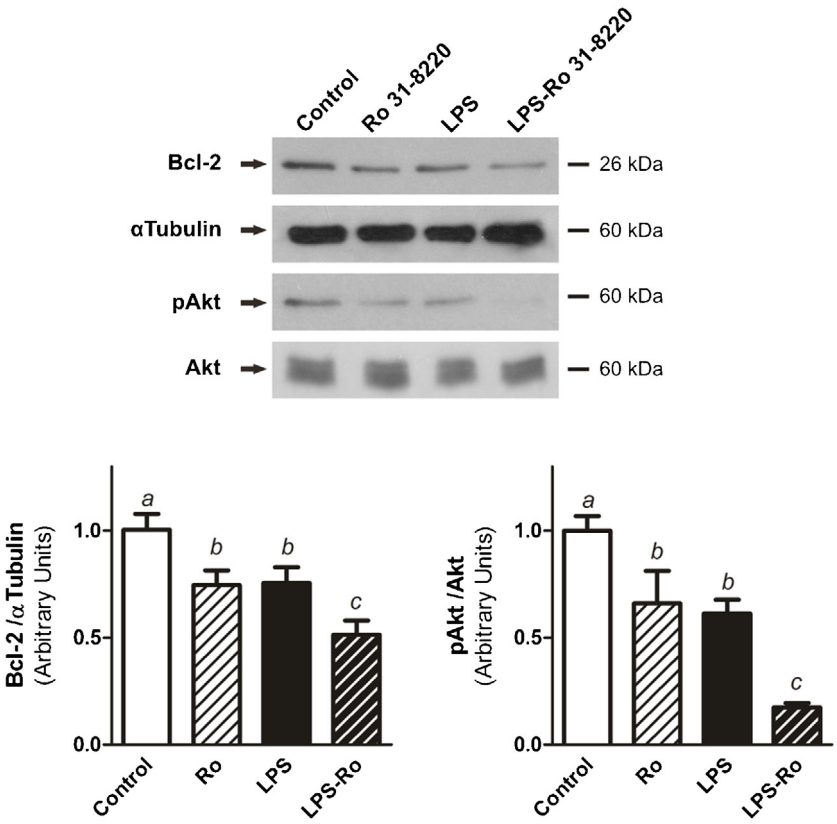

Fig. 4. Effect of Ro 31-8220 and LPS on Bcl-2 expression and Akt activation. ARPE-19 cells were pre-incubated for 1 h with 0.005% DMSO (Control and LPS conditions) or 10 μM Ro 31-8220 and then treated with LPS (10 μg/ml) or to the ultra-pure water (control conditions) for 48 h. Bcl-2 expression and Akt phosphorylation (pAkt) was evaluated by WB as detailed in Materials and Methods. Bar graphs show the densitometry values of Bcl-2/α Tubulin and pAkt/Akt expressed in arbitrary units as a ratio of the control. Conditions designated with different letters (a–c) present significant differences ($p < 0.05$).

ond row of images). This translocation was completely abolished by pre-incubation with the PLD1 inhibitor EVJ but unaffected by the PLD2 inhibitor APV (Fig. 3A, third and fourth row of images, respectively). To further confirm the PKCε translocation subcellular fractionation studies of 100% confluent cultures were performed as described in Material and Methods. WBs shown in Fig. 3B demonstrate that PKCε effectively translocates to the membrane-enriched fraction (MEF) upon LPS stimulation and that this translocation is inhibited by the PLD1 inhibitor (EVJ) but not by the PLD2 inhibitor (APV). Together, these results demonstrate that in RPE cells, LPS induces PKCε activation in a PLD1-dependent manner.

3.4. PKCε inhibition reduces Bcl-2 expression and Akt activation and increases Caspase-3 cleavage

To further study the effect of PKCε inhibition on cell survival WB assays were performed in order to study the Akt phosphorylation state (a cannonical survival pathway) and the expression of the anti-apoptotic protein Bcl-2. Immunocytochemistry assays were also performed to evaluate Caspase-3 cleavage. Fig. 4 shows that pre-incubation with Ro 31-8220 and LPS treatment for 48 h reduced Bcl-2 expression (by 25%) and Akt phosphorylation (by 35%). In cells pre-incubated with Ro 31-8220 and treated with LPS, the expression of Bcl-2 was reduced by 50% and Akt phosphorylation by 83%. In addition, the pre-incubation with Ro 31-8220 and LPS treatment for 48 h increased Caspase-3 cleavage (Fig. 5). Caspase-3 cleavage and activation was even more pronounced in cells pre-incubated with Ro 31-8220 and treated with LPS (Fig. 5). Changes in Bcl-2 expression, Akt phosphorylation and Caspase-3 activation are in agreement with the reduced mitochondrial function under experimental conditions favoring PKCε inhibition (Fig. 2).

Pre-incubation with the conventional PKC inhibitor Go 6976 reduced Akt phosphorylation but, in agreement with the MTT reduction, no changes were observed in Akt activation between cells pre-incubated with Go 6976 and treated or not with LPS. In addition, pre-incubation with Go 6976 did not affect Bcl-2 expression, in cells whether treated or not with LPS (Data not shown). These results demonstrate that conventional PKCs are not involved in the reduced Bcl-2 expression and Akt activation induced by LPS.

4. Discussion

In a previous paper we reported for the first time that classical PLDs participate in the inflammatory signaling induced by LPS in ARPE-19 cells and we demonstrated that upon LPS stimulation PLD1 translocates from the perinuclear region to the plasma membrane and that this translocation is dependent on PLD1 activation (Mateos et al., 2014). However, the differential effects of selective PLD inhibitors (EVJ and APV) on ERK1/2 activation and mitochondrial function demonstrate that both PLD isoforms are activated by LPS (Mateos et al., 2014). Interestingly, while LPS-induced cell damage was prevented by PLD2, ERK1/2 and COX-2 inhibitors, the inhibition of PLD1 was not able to restore MTT reduction to control levels, even though it did reduce COX-2 expression (Mateos et al., 2014). These results led us to postulate that PLD1 could play a dual role in RPE cells exposed to inflammatory injury, on the one hand by promoting cell damage through COX-2 induction and on the other by mediating cellular protective mechanisms (Mateos et al., 2014). To further investigate DAG-mediated cellular signaling in RPE cells exposed to LPS, in the present work we studied PKC signaling in this inflammatory paradigm.

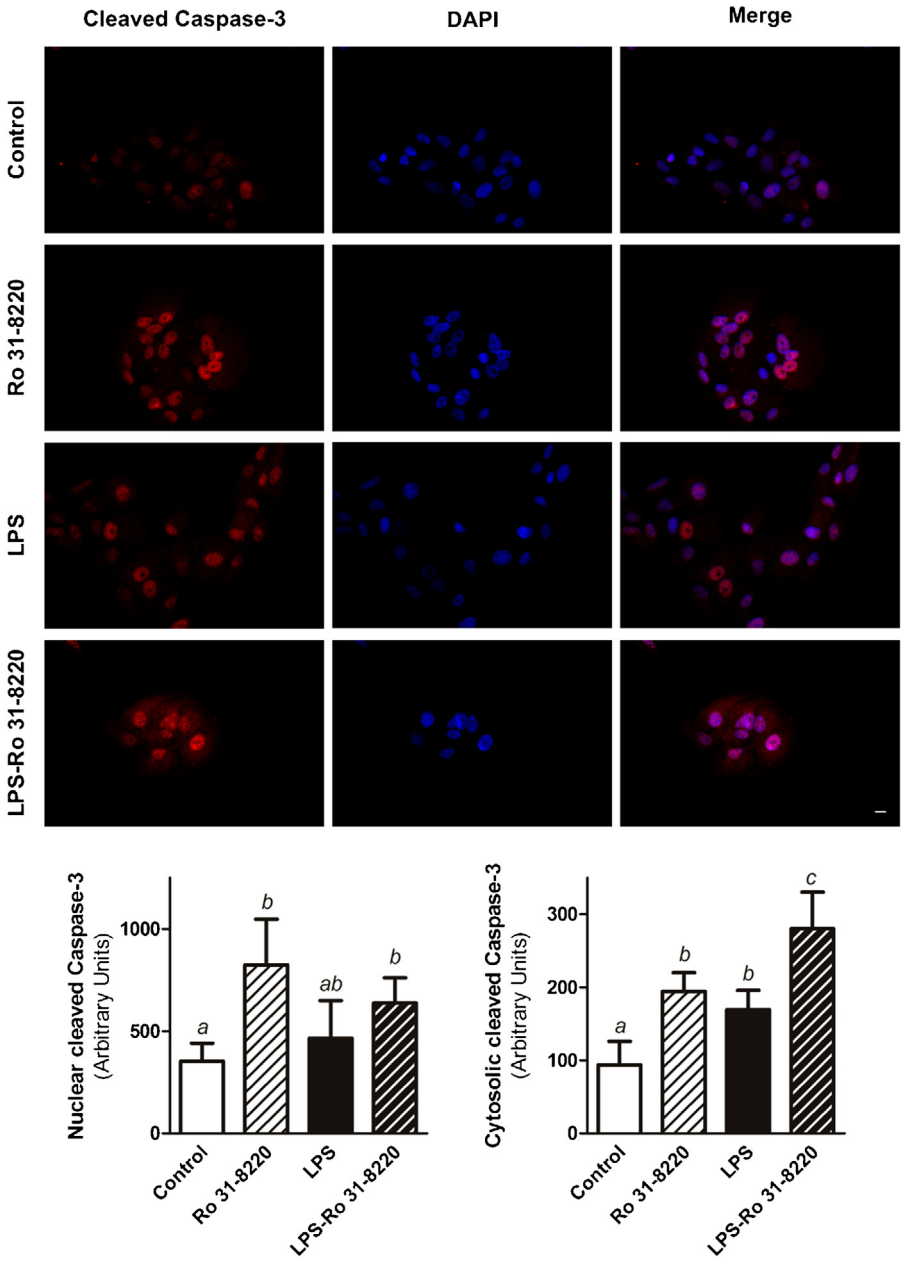

Fig. 5. Effect of Ro 31-8220 and LPS on Caspase-3 cleavage. ARPE-19 cells grown onto 12 mm coverslips were pre-incubated with 0.005% DMSO (Control and LPS conditions) or 10 μM Ro 31-8220 and then treated with LPS (10 μg/ml) or to the ultra-pure water (control conditions) for 48 h. After the experimental treatment immunocytochemistry assays were performed as described in Materials and Methods. Representative microphotographs from three independent experiments show cleaved Caspase-3 staining (first column), DAPI staining (second column) and the merge image (third column). Scale bar = 10 μm. Bar graphs show cytosolic or nuclear cleaved Caspase-3 fluorescence intensity expressed as arbitrary units. Conditions designated with different letters (a–c) present significant differences ($p < 0.05$).

PKCs transduce a wide range of DAG-mediated extracellular signals, thereby regulating cell survival, growth, proliferation, migration, apoptosis, and inflammatory reaction, among other cellular events (Bradley et al., 2015; Antal et al., 2015; Brognard et al., 2009; Bertolaso et al., 1998; Zauli et al., 1996; Watanabe et al., 1992; Aihara et al., 1991). Elucidating the molecular mechanisms of PKC signaling is therefore crucial to understanding disease mechanisms in a wide range of fields (Antal and Newton, 2014; Wu-Zhang and Newton, 2013). PKC activity has previously been reported to mediate migration and phagocytosis in RPE cells (Finnemann and Rodriguez-Boulan, 1999; Murphy et al., 1995; Sheu et al., 1994) and the expression of PKCα, βII, δ, ε, θ, ξ, ι and μ,but not PKCγ and PKCη, was reported in cultured human RPE cells isolated from donor eyes (Yu et al., 2007; Wood and Osborne, 1997). In LPS-challenged RPE cells, interleukin 8 (IL-8) production induced by LPS treatment was drastically reduced by the PKC inhibitor Ro 31-8220 (Elner et al., 2003). However, results shown in the present work constitute the first evidence that LPS-induced PKC signaling in RPE ARPE-19 cells is differentially modulated by classical PLDs.

The data presented here demonstrate that LPS treatment of ARPE-19 cells for 24 h increased PKCα/βII phosphorylation (Fig. 1A) and that this phosphorylation was able to be prevented by pre-incubation with the PLD1 (EVJ) and the PLD2 (APV) inhibitors (Fig. 1B). These results are in agreement with the increased PLD activity previously reported in RPE cells exposed to LPS for 24 h (Mateos et al., 2014). Moreover, neither PKCα phosphorylation (Data not shown) nor PLD activation was observed after short term LPS treatment (2 h) (Mateos et al., 2014). Altogether, these

results demonstrate that sustained LPS exposure induces PKCα/βII phosphorylation in ARPE-19 cells in a PLD1 and PLD2-dependent manner.

As mentioned above, we previously reported that 48 h LPS treatment reduced ARPE-19 mitochondrial function (Mateos et al., 2014). To study the role of PKC signaling in the reduced RPE mitochondrial function observed after LPS treatment, two PKC inhibitors were used, Go 6976 (a PKCα and β inhibitor) and Ro 31-8220 (a PKCα, β, γ, and ε inhibitor). Even under control conditions, incubation for 48 h with both PKC inhibitors markedly reduced MTT reduction (Fig. 2). Since no differences in MTT reduction were observed between cells pre-incubated with Go 6976 and those treated or not with LPS (Fig. 2), it can be concluded that, although phosphorylated, PKCα is not involved in the reduced mitochondrial function induced by LPS and further experiments are required to elucidate the role of PKCα in RPE cells exposed to inflammatory conditions.

Since it was previously reported that human RPE do not express PKCγ (Yu et al., 2007; Wood and Osborne, 1997) the drastic reduction in mitochondrial function observed in cells pre-incubated with Ro 31-8220 and treated with LPS suggests that PKCε (which is inhibited by Ro 31-8220 but not by Go 6976) could be activated by LPS to mediate cell survival (Fig. 2). This result together with the LPS-induced PKCε translocation to the plasma membrane (Fig. 3) suggests that this novel PKC is activated by LPS to exert protective mechanisms in RPE cells. In addition, LPS-induced PKCε translocation was prevented by the PLD1 inhibitor but unaffected by the inhibition of PLD2 (Fig. 3). Our results suggest that after sustained exposure to LPS (24 h), PLD1 and PKCε are subsequently activated to mediate protective cellular mechanisms against LPS-induced damage. These results are consistent with the fact that PLD1 inhibition is not able to restore the reduced RPE cell viability induced by LPS and support the hypothesis that PLD1 plays a dual role in RPE cells exposed to inflammatory conditions (Mateos et al., 2014). Furthermore, we demonstrated that PKCε protects against LPS-induced cell damage in RPE cells by promoting a decrease in Caspase-3 cleavage and also by regulating Bcl-2 expression and Akt activation (Figs. 4 and 5). PLD-mediated PKCε activation was also reported in primary cortical cell cultures, in which PKCε activation was reduced by the PLD1 and PLD2 inhibitor FIPI (Bjornstrom et al., 2014).

It was previously reported that the presence of non-selective PKC inhibitors for more than 24 h triggers apoptosis in human RPE cells (Wood and Osborne, 1997). In agreement with our results, several studies have shown that PKCε plays a protective role during cell death in cardiomyocytes, leukemia cells and monocytes (Malavez et al., 2015; Loi et al., 2015; Waza et al., 2014). Not only does PKCε promote cell growth but it can also function as an anti-apoptotic protein inhibiting both the mitochondrial and the receptor-mediated apoptotic pathways (Basu and Sivaprasad, 2007). In line with this, PKCε inhibits pro-apoptotic signaling by increasing the levels of anti-apoptotic Bcl-2 protein in different cell types (Sivaprasad et al., 2007; Gubina et al., 1998) and by inhibiting Bax activation (Lu et al., 2007). Moreover, it was demonstrated that PKCε is able to phosphorylate and activate Akt in breast cancer cells (Lu et al., 2006).

In summary, this paper demonstrates for the first time that PKC signaling elicited by LPS in RPE cells is modulated by the PLD pathway. While PKCα phosphorylation depends on both classical PLDs, PKCε is modulated only by PLD1. Moreover, the PLD1-PKCε pathway appears to mediate cell survival by preventing the increased Caspase-3 cleavage and the reduced Bcl-2 expression and Akt activation induced by LPS. These results, together with our previous findings, point to the potential use of PLD2 as a therapeutic target for the treatment of ocular inflammatory diseases. Conversely, the inhibition of PLD1 prevents cellular protective mechanisms, such as PKCε activation (Fig. 6). Our results also underline the importance of developing selective PLD inhibitors enabling these phospholipases to be used as therapeutic targets.

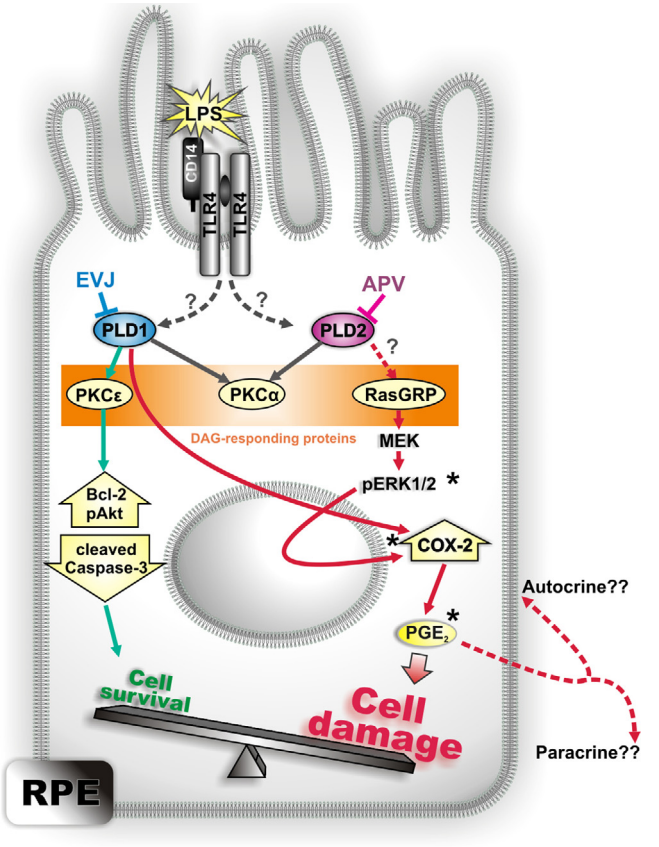

Fig. 6. Schematic view of signaling events elicited by LPS in RPE cells. Dashed arrows and question marks indicate possible, unstudied mechanisms. Asterisks indicate signaling pathways described in a previous publication from our lab (Mateos et al., 2014).

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Author contributions

PET performed the experiments. MVM and GAS designed the experiments and supervised the study. MVM wrote the manuscript. NMG and GAS provided equipment and reagents and revised the manuscript.

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