The role of calcium-independent phospholipase A$_2\gamma$ in modulation of aqueous humor drainage and Ca$^{2+}$ sensitization of trabecular meshwork contraction

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Pattabiraman PP, Lih FB, Tomer KB, Rao PV. The role of calcium-independent phospholipase A$_2\gamma$ in modulation of aqueous humor drainage and Ca$^{2+}$ sensitization of trabecular meshwork contraction. Am J Physiol Cell Physiol 302: C979–C991, 2012. First published January 11, 2012; doi:10.1152/ajpcell.00396.2011.—The contractile and relaxation characteristics of trabecular meshwork (TM) are presumed to influence aqueous humor (AH) drainage and intraocular pressure. The mechanisms underlying regulation of TM cell contractile properties, however, are not well understood. This study investigates the role of calcium-independent phospholipase A$_2$ (iPLA$_2$), which controls eicosanoid synthesis, in regulation of TM cell contraction and AH outflow using mechanism-based isoform specific inhibitors (R)-bromoenol lactone (R-BEL, iPLA$_2$ specific) and (S)-bromoenol lactone (S-BEL, iPLA$_2$$\beta$ specific). Immunohistochemical analysis revealed intense staining for both iPLA$_2$$\beta$ and $\gamma$ isoforms throughout the TM, juxtacanalicular tissue, and Schlemm’s canal of human eye. Inhibition of iPLA$_2$$\gamma$ by R-BEL or small interfering RNA-mediated silencing of iPLA$_2$$\gamma$ expression induced dramatic changes in TM cell morphology, and decreased actin stress fibers, focal adhesions, and myosin light-chain (MLC) phosphorylation. AH outflow facility increased progressively and significantly in enucleated porcine eyes perfused with R-BEL. This response was associated with a significant decrease in TM tissue MLC phosphorylation and alterations in the morphology of aqueous plexi in R-BEL-perfused eyes. In contrast, S-BEL did not affect either of these parameters. Additionally, R-BEL-induced cellular relaxation of the TM was associated with a significant decrease in the levels of active Rho GTPase, phospho-MLC phosphatase, phospho-CPI-17, and arachidonic acid. Taken together, these observations demonstrate that iPLA$_2$$\gamma$ plays a significant and isoform-specific role in regulation of AH outflow facility by altering the contractile characteristics of the TM. The effects of iPLA$_2$$\gamma$ on TM contractile status appear to involve arachidonic acid and Rho GTPase signaling pathways.

Rho GTPase; arachidonic acid; glaucoma

GLAUCOMA IS AN OPTIC NEUROPATHY that leads to irreversible blindness. Primary open-angle glaucoma (POAG) is the most common form of glaucoma and is associated with elevated intraocular pressure (IOP). Elevated IOP is a major risk factor for glaucoma (41, 54), and arises mainly due to an abnormally increased resistance to the drainage of aqueous humor (AH) through the conventional outflow pathway comprising of trabecular meshwork (TM) and the Schlemm’s canal (SC) (28, 54). Pharmacological interventions to treat glaucoma have been classically aimed at lowering elevated IOP by either decreasing secretion of AH by the ciliary body, or augmenting AH drainage by the uveoscleral and conventional pathways (29). Recently, significant advances have been made towards identifying the role of cellular processes involved in regulating AH outflow dynamics and the potential of specific signaling pathways to serve as therapeutic targets for enhancing AH outflow through the TM tissue (2, 9, 15, 29). Among the cellular processes that influence outflow facility via the conventional pathway, regulation of actomyosin organization and contractile characteristics of TM and SC by calcium-dependent (e.g., myosin light-chain kinase) and independent (e.g., Rho/Rho kinase and CPI–17) mechanisms has been confirmed to play a significant role (24, 43, 45, 52, 55). The TM tissue is thought to possess smooth muscle-like properties and agents that disrupt the normal actin cytoskeletal network and myosin II activity in TM and SC, thereby resulting in cellular relaxation, has been shown to increase outflow facility and a subsequent decrease in IOP (12, 43, 52). These findings indicate the existence of a possible association between contractile status of TM tissue and outflow facility (12, 42, 43, 52, 55, 57). Therefore, it is important to identify the different cellular mechanisms that regulate the contractile characteristics of TM cells and determine how these may impact AH drainage and IOP, to further the goal of developing novel glaucoma therapies.

Phospholipase A$_2$ (PLA$_2$) constitutes a growing family of enzymes that catalyzes the hydrolysis of fatty acids from the sn-2 position of phospholipids to generate arachidonic acid (AA) and subsequent eicosanoid derivatives (27, 48). These products can then serve as second messengers to regulate a variety of cellular processes including tissue contraction and cytoskeletal changes (16, 48, 50). PLA$_2$ enzymes are divided into Ca$^{2+}$-dependent and Ca$^{2+}$-independent classes (8, 38, 48). The former class includes several groups of secretory PLA$_2$ (sPLA$_2$) and cytosolic PLA$_2$ (cPLA$_2$) (38, 48), while the Ca$^{2+}$-independent forms (iPLA$_2$) are mainly intracellular and are classified under the group VI PLA$_2$ (38, 47, 48). The iPLA$_2$ class includes two major, well-characterized isoforms: iPLA$_2$$\beta$ (mass = 85 kDa), found mostly in the cytosol, and iPLA$_2$$\gamma$ (mass = 88.5 kDa), which is mostly membrane-associated (1, 4, 21, 22, 32, 51). The $\beta$ and $\gamma$ isoforms of iPLA$_2$ are homologous in the catalytic region but have different NH$_2$-terminal regions. iPLA$_2$ represents an intensely studied lipoly-
tic enzyme that plays a key role in diverse cellular responses including general lipid metabolism, membrane homeostasis, cell proliferation, signal transduction, and production of pro-inflammatory mediators such as prostaglandins and leukotrienes through the release of AA from membrane phospholipids (8, 10, 34, 38, 47, 48). iPLA2 and its lipid products have been shown to be required for agonist-induced Ca\(^{2+}\) sensitization of contraction in vascular smooth muscle cells (18, 31, 44, 56). Additionally, the lyso phosphatidic acid generated by PLA2-catalyzed hydrolysis of membrane phospholipids (3) alters the contractile properties of cells (26, 37), and influences aqueous humor outflow facility (36).

While the distribution profiles of different subtypes of PLA2 have been documented in the human eye AH outflow pathway including TM (46), the role of these enzymes in regulation of AH outflow has remained unexplored. This study aims to determine whether iPLA2 plays a role in regulation of AH drainage and to investigate the cellular mechanism(s) underlying any observed effects of iPLA2 on AH outflow dynamics. Bromoenol lactone (BEL) is a potent, irreversible, mechanism-based small-molecule chemical compound that inhibits iPLA2 activity selectively. Its enantiomers S-BEL and R-BEL inhibit the activities of iPLA2\(\beta\) and iPLA2\(\gamma\), respectively, with great selectivity and specificity in the low micromolar range (20, 21). These inhibitors have been exploited extensively to explore specific roles for iPLA2 in both in vivo and cell culture systems (8, 38, 48). Using these isoform-specific inhibitors along with additional molecular approaches, in this study we demonstrate that iPLA2 regulates, in an isoform-specific manner, the drainage of AH via the conventional outflow pathway. The iPLA2-mediated increase in AH outflow appears to involve changes in Ca\(^{2+}\) sensitization of TM contraction and alterations of cell morphology and actomyosin organization.

MATERIALS AND METHODS

Bromoenol lactone (BEL) [6E-(bromoethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one], S-bromoenol lactone (S-BEL) [6E-(bromoethylene)tetrahydro-3S-(1-naphthalenyl)-2H-pyran-2-one], R-bromoenol lactone (R-BEL) [6E-(bromoethylene)tetrahydro-3R-(1-naphthalenyl)-2H-pyran-2-one], AA, and arachidonic acid-d\(_8\) (AA-d\(_8\)) were purchased from Cayman Chemical (Ann Arbor, MI). Rabbit anti-iPLA2\(\gamma\) (HPA020083), phalloidin-tetramethylrhodamine B iso-thiocyanate (P1951), and rabbit anti-vinculin (no. V9139) antibody were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-phospho myosin light chain (MLC) (no. 3672), rabbit anti-total MLC (no. 3671), and rabbit anti-pMARCKS (no. 2741) were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-p-cPI-17 (no. 07–344) and rabbit anti-phospho-myosin phosphatase targeting subunit 1 (MYPT1; no. ABS45) were purchased from Millipore (Danvers, MA). Protease inhibitor cocktail tablets (complete, Mini, EDTA-free) and phosphatase inhibitor cocktail tablets (PhosSTOP) were from Roche (Basel, Switzerland). The rabbit anti-iPLA2\(\beta\) was a generous gift from Richard Gross from Washington University (St. Louis, MO). Alexa Fluor 594 goat anti-rabbit (A-11012) antibody was from Invitrogen (Carlsbad, CA).

Cell cultures. Human primary TM cells (HTM) were cultured from TM tissue isolated from freshly obtained corneal rings after they had been used for the corneal transplantation at the Duke Ophthalmology clinical service. Initially, the extracted TM tissue was chopped into small pieces in serum, which were then placed under a glass coverslip in six-well plastic culture plates and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% FBS and penicillin (100 U/500 ml)-streptomycin (100 μg/500 ml)-glutamine (4 mM). Similarly, porcine TM cells (PTM) were isolated and cultured by collagenase treatment of TM tissue obtained from freshly enucleated eyes from a local abattoir, as we described previously (36). All experiments were conducted using confluent cultures between four and six passages, and cells were cultured at 37°C under 5% CO\(_2\), in DMEM containing 10% FBS and penicillin-streptomycin-glutamine. All cell culture experiments were performed after serum starvation for at least 24 h unless mentioned otherwise.

RT-PCR. Total RNA was extracted from HTM and PTM cells using RNeasy Micro kit (Qiagen, Valencia, CA) as directed per the user manual. Five micrograms of total RNA (free of DNA) were used for first-strand cDNA synthesis using Superscript first-strand synthesis system (Life Technologies, Carlsbad, CA). The cDNA pool was subsequently screened for iPLA2\(\beta\) and iPLA2\(\gamma\) transcripts using the respective sense and antisense primer sets: 5’-CCA TAG TGC TGC TGA CCC AC-3’/5’-CTC CAT CAC GCA ACA GCA GG-3’ and 5’-AGG ACC CGG GCA TTA GTT CA-3’/5’-AAC ACC TTT CAC GCA GCA GA-3’, as we described earlier (40). Reactions lacking reverse transcrip-tase (−RT) controls were carried out simultaneously. The PCR products were separated on a 1% agarose gel, visualized, and imaged using ethidium bromide and Foto Dyne imaging system. PCR products were sequenced to confirm identity.

Immunohistochemistry. Tissue sections from formalin-fixed, paraform-embedded human donor eye whole globes were immunostained as previously described (30). Briefly, five-micron thick tissue sections were deparaffinized and rehydrated using three changes of xylene, followed by three changes of absolute ethyl alcohol and washing with three changes of water. To unmask the antigen epitopes, heat-induced antigen retrieval was performed using 0.1 M citrate buffer pH 6.0 (Vector Laboratories, Burlingame, CA) for 20 min at 100°C. The slides were then blocked for nonspecific interaction with Biocare Medical’s (Concord, CA) Sniper Background Reducer (BS966). Tissue sections were then incubated overnight at 4°C with primary antibodies (rabbit anti-iPLA2\(\beta\) or rabbit anti-iPLA2\(\gamma\), at dilutions of 1:30) in a humidified chamber. All primary antibody dilutions were made in 1% bovine albumin in Tris-buffered saline (TBS). After incubation, the slides were washed with TBS three times and incubated with Alexa Fluor 594 goat anti-rabbit secondary antibodies for 2 h at room temperature. The immunostaining was carried out in triplicate, and a negative control (in the presence of secondary antibody alone) was run simultaneously. After final staining, the tissue sections were coverslipped using Aqua mount (Lerner Laboratories, Pittsburgh, PA). The slides were then viewed and imaged using a Nikon C1 Digital Eclipse confocal system (Nikon Instruments, Melville, NY).

Immunofluorescence staining. The TM cells were grown on gelatin (2%)-coated glass coverslips until they attained confluence. After appropriate treatments, cells were washed in PBS twice and then fixed, permeabilized, and stained or immunostained for actin stress fibers and focal adhesions as we described earlier (40). The slides were viewed and imaged using a Nikon C1 Digital Eclipse confocal system (C1 Digital Eclipse).

Immunoblot analysis. Total protein cell lysates were prepared from serum-starved confluent cultures of HTM and PTM cells with or without drug treatments. Bio-Rad protein assay reagent (no. 500-0006) was used to estimate protein concentration. Samples containing equal amounts of protein were mixed with Laemmli buffer and separated by SDS-PAGE (8 and 15% acrylamide), followed by transfer of resolved proteins to nitrocellulose membranes. Membranes were blocked for 2 h at room temperature in TBS containing 0.1% Tween 20 and 5% (wt/vol) nonfat dry milk and subsequently probed with primary antibodies (anti-MYPT1, anti-cPI-17, or anti-MARCKS) in conjunction with horseradish peroxidase-conjugated secondary antibodies. Detection of immunoreactivity was performed by enhanced chemiluminescence (ECL). Densitometry of immunoblot films was performed using ImageJ software (National Institutes of Health, AJP-Cell Physiol • doi:10.1152/ajpcell.00396.2011 • www.ajpcell.org.
Bethesda, MD). Data were normalized to the specified loading controls.

**Myosin light-chain phosphorylation assay.** The effects of R-BEL and S-BEL on TM cell MLC phosphorylation were determined by urea-glycerol gel electrophoresis and immunoblot analysis using anti-phospho-MLC antibody, as we described earlier (36). Densitometry of immunoblot films was performed as described above, and data were normalized to total MLC.

**Aqueous humor outflow facility.** Porcine eyes (obtained fresh from a local abattoir) were perfused with either R-BEL (25 μM) or S-BEL (25 μM) by the standard constant pressure technique using a Grant stainless steel corneal fitting (42). Initial baseline outflow measurements were established at 14 mmHg and 37°C with perfusion medium containing Dulbecco’s phosphate-buffered saline (DPBS; pH 7.4) and 5.5 mM d-glucose. After this, the anterior chamber aqueous of one eye of each pair was exchanged either with R-BEL (25 μM) or S-BEL (25 μM) dissolved initially in DMSO and perfused with the same concentration of drugs for a period of 5 h, while the contralateral control eye was perfused with equal concentration of DMSO (0.1%) in perfusion medium. Outflow measurements were continuously recorded using a pressure transducer (DXT Plus DT-XX; BD Biosciences) connected to a PowerLab data acquisition system (ML870/P PowerLab 8/30, AD instruments, Colorado Springs, CO) and PowerLab software. The transducers were calibrated before each experiment to ensure accurate pressure measurement. Effects of each drug are expressed as the percentage change in outflow facility (compared with baseline values) over 5 h, in drug-perfused versus sham-treated paired controls. Values are expressed as means ± SE. Data were analyzed by a paired two-tailed Student’s t-test to determine significance.

**Histology.** Immediately at the end of a 5-h perfusion period, three eyes from the sham control and drug-treated fellow eyes were fixed for histologic examination by perfusing them overnight with 2.5% glutaraldehyde and 2% formaldehyde at 14 mmHg pressure, as we described earlier (42). Tissue quadrants obtained from drug-perfused and control eyes were fixed in 1.0% osmium tetroxide in 0.1 M sodium cacodylate buffer and then stained with 1% uranyl acetate. Finally, sections obtained by microtome (70 nm) were stained with KMOx and Sato’s stain and photographed using an electron microscope (Jem-1400 EX, JEOL, Tokyo, Japan) and a light microscope (Carl Zeiss Meditec, Thornwood, NY) as we described earlier (42).

**iPLA2 activity.** iPLA2 activity was measured in HTM cells using a PLA2 activity assay kit and following the manufacturer’s instructions (Cayman Chemical). Briefly, the HTM cell pellet was suspended in 500 μl of 50 mm HEPES, pH 7.4, containing 1 mm EDTA and sonicated (Micronson Ultrasonic Cell disruptor) on ice. Cell homogenates were cleared by centrifugation at 10,000 rpm for 15 min at 4°C. Cell lysates (100 μg of protein in a total volume of 45 μl) were added to microtiter plate wells containing 5 μl of assay buffer (80 mm HEPES, pH 7.4, 150 mm NaCl, 10 mm CaCl2, 4 mm Triton X-100, 30% glycerol, and 1 mg/ml bovine serum albumin), either with or without 10 μM BEL. The reaction was initiated by the addition of 200 μl of arachidonoyl thiophosphatidylcholine dissolved in 2× assay buffer and incubated at room temperature for 60 min. The reaction was then terminated by the addition of 10 μl of a solution containing 25 mm 5,5′-dithio-bis(2-nitrobenzoic acid) and 475 μm EGTA in 0.5 mM Tris-HCl (pH 8.0), and the absorbance was measured at 414 nm in a SpectraMax 190 microtiter plate reader (Molecular Devices, Sunnyvale, CA). To determine iPLA2 activity, the optical density obtained in the presence of BEL was subtracted from the total optical density, and the resulting values were converted into nanomoles of substrate hydrolyzed per minute per milliliter using the 5,5′-dithio-bis (2-nitrobenzoic acid) extinction coefficient value at 414 nm.

**Small interfering RNA transfection.** Small interfering RNA (siRNA) directed against the human iPLA2γ sequence (sc-89772) and a fluorescein isothiocyanate (FITC)-conjugated corresponding scrambled control siRNA (sc-36869) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HTM cells were transfected using a 120 picomolar concentration of iPLA2γ siRNA or control siRNA using an endothelial nucleofector Kit (Lonza, Basel, Switzerland) as per the manufacturer’s instructions. iPLA2γ siRNA was cotransfected with the green fluorescent protein (GFP) plasmid pmaxGFP provided in the kit. Following transfection, cells were plated either on glass coverslips or in plastic petri plates. Expression of GFP or appearance of FITC in the cells was followed to confirm transfection efficiency. Morphological changes were observed within 72 h after transfection after which the cells were fixed and immunostained or immunoblotted for proteins of interest.

**Rho GTPase activation assay.** Porcine TM cells were serum starved for 24 h and then treated with either S-BEL or R-BEL (10 μM for 1 h), and cell lysates were prepared using 50 mM Tris buffer, pH 7.5, containing 10 mM MgCl2, 0.5 M NaCl, 1% Triton X-100, and a cocktail of phosphatase and protease inhibitors. Cell lysates were then subjected to a Rho-GTP pull-down assay to quantify the levels of activated Rho GTPase (Rho-GTP) using rhoteikin-Rho binding domain (RBD) beads (Millipore) and immunoblot analysis, as we described earlier (36). Appropriate positive and negative controls for the assay were simultaneously carried out through the assay.

**Quantification of arachidonic acid by liquid chromatography-tandem mass spectrometry.** PTM cells (3 × 105) were serum starved for 24 h and then treated with either R-BEL (10 μM) or 10% serum for 30 min. DMSO (0.1%)-treated samples served as a control. Cellular lipids were extracted by the Folch method (13). Nonhydrolyzed lipid samples were chromatographed on an Agilent (Santa Clara, CA) 1200 capillary liquid chromatography instrument and subjected to tandem mass spectrometry using an API 3000 (AB Sciex, Foster City, CA) equipped with a Turbo Ionspray source and operated in negative ion selected reaction monitoring mode. Precursor to product ion transitions were 303.4 to 259.2 for AA, 311.4 to 267.2 for AA-d6, and 310.4 to 266.2 for arachidonic acid-d7 (AA-d7). AA was quantified against the summed signal of AA-d6 and AA-d7. Calibration samples contained 25 pg/μl AA-d6 and 25–2,500 pg/μl AA. AA levels are expressed as nanograms per total number of cells (3 × 105) used for each extraction.

**Statistical analyses.** All data represent the average results of at least three independent experiments. Quantitative data were analyzed by Student’s t-test, and minimum values of P < 0.05 were considered as statistically significant.

**RESULTS**

**Expression and distribution of iPLA2 isoforms in the AH outflow pathway.** Total RNA extracted from two different HTM cultures (derived from donor eyes aged 18 and 57 yr) was analyzed by RT-PCR to determine the expression profile of iPLA2 isoforms in TM cells. The analysis confirmed expression of both iPLA2β and -γ in HTM cells (Fig. 1A). Additionally, immunoblot analysis of the same HTM cell culture samples and of PTM cell-derived total protein extracts using isoform-specific antibodies (Fig. 1B) confirmed that both HTM and PTM cells express iPLA2β (~85 kDa) and γ isoforms (~88 kDa).

Furthermore, the anterior chamber angle of formalin-fixed paraffin-embedded human eyes exhibited cells staining immunopositive for both iPLA2β and γ isoforms. The TM stained intensely for both iPLA2β and iPLA2γ isoforms, with immunopositive signal distributing to the uveoscleral and corneo-scleral TM regions (Fig. 1C). Immunopositive staining was also evident in the endothelial cell lining of the SC and the juxtanaculcular tissue for both the isoforms (Fig. 1C).
Detection of iPLA₂ activity in HTM cells. To determine the percent contribution of iPLA₂ activity to total PLA₂ activity in HTM cells, equal amounts of total protein derived from HTM cells (obtained from three different donor eyes aged 18, 57, and 65 yr) were assayed for total cPLA₂ activity in the presence or absence of 10 \( \mu \)M BEL using arachidonoyl thiophosphatidylcholine as a substrate. The total cPLA₂- and iPLA₂-specific activities were found to be 5.6 ± 0.24 and 2.4 ± 0.2 nmol/min per 100 µg of HTM protein, respectively (Fig. 1D), indicating that iPLA₂ activity (both β and γ) accounts for nearly ~40% of the total cellular PLA₂ activity in HTM cells.

Effect of S-BEL and R-BEL on TM cell morphology and actin cytoskeletal organization. Treatment of serum-starved PTM cells (grown to confluence on gelatin-coated glass coverslips) with either S-BEL or R-BEL (5 and 10 µM) for 90 min resulted in concentration and time-dependent changes in cell morphology in the case of R-BEL alone, with changes being detectable within 30 min of treatment initiation. After 90 min of R-BEL (5 µM and 10 µM) treatment, PTM cells exhibited cell-cell separation and cell rounding (Fig. 2A), while S-BEL (10 µM) was without effect. R-BEL was noted to exert the same morphological effects in HTM cells as well (data not shown).
shown). R-BEL-induced morphological changes were not associated with cytotoxicity as determined by in vivo staining using propidium iodide and fluorescein diacetate (data not shown) (42).

To explore a possible association between the morphological changes induced by R-BEL in TM cells and actin cytoskeletal reorganization, R-BEL-treated (10 μM for 90 min) PTM cells were stained for F-actin using tetramethylrhodamine isothiocyanate (TRITC)-phalloidin. While staining for actin stress fibers was intense and spread throughout the cell body in control cells, R-BEL-treated cells exhibited a dramatic reduction in staining of actin stress fibers (Fig. 2B). Additionally, this decrease in actin stress fibers was associated with reduced vinculin-based focal adhesions in the R-BEL-treated cells relative to control PTM cells.
tile characteristics. No noticeable differences were evident in actin stress fibers or focal adhesions in S-BEL (10 μM for 90 min)-treated PTM cells, relative to control cells (Fig. 2, B and C, respectively).

**Effect of S-BEL and R-BEL on myosin light-chain phosphorylation in PTM cells.** Since cellular contraction and relaxation status in most cell types is determined predominantly by the phosphorylation status of MLC (50), we evaluated the effects of S-BEL and R-BEL on MLC phosphorylation in TM cells using anti-phospho-MLC antibody-based immunoblot analysis. PTM cells treated (for 90 min) with either 5 or 10 μM S-BEL or R-BEL revealed a dose-dependent and significant (P < 0.01, n = 4) decrease (>80%) in MLC phosphorylation in response to R-BEL (10 μM) (Fig. 3). The levels of total MLC in control and drug-treated PTM cells, on the other hand, were found to be comparable (Fig. 3).

**R-BEL affects the calcium sensitization of TM cell contractile characteristics.** To explore the possible influence of iPLA2γ on calcium-independent (also referred to as “calcium sensitization”) regulation of cellular contraction (50, 53), we measured the levels of active Rho GTPase (Rho-GTP) using a pull-down assay, and the phosphorylation status of MYPT1 and CPI-17, each of which is known to regulate MLC phosphorylation and contractile activity, using TM cells treated with R-BEL. Cell lysates derived from the confluent cultures of serum-starved (24 h) PTM cells treated for 1 h with 10 μM of R-BEL exhibited a significant (P < 0.01, n = 3) decrease in the levels of Rho-GTP, relative to lysates from control cells and cells treated with S-BEL (Fig. 4A). For these analyses, RhoA-GTP protein levels were normalized to the total RhoA. Moreover, cell lysates preincubated with either GDP (negative control) or GTPγS or 10% serum for 30 min (positive control) were subjected to the same protocol along with S-BEL- and R-BEL-treated samples for confirming the specificity of the Rho-GTP pull-down assay (Fig. 4A).

Under similar conditions, R-BEL-treated TM cells showed a significant (P < 0.01, n = 3) decrease in the levels of phospho-MYPT1 as well as CPI-17 as compared with the control cells or S-BEL-treated cells (Fig. 4B). R-BEL treatment also led to a significant reduction (P < 0.01, n = 3) in the levels of phospho-MARCKS, which is involved in actin cytoskeletal dynamics and membrane stability (19) and is a known substrate of both Rho kinase and protein kinase C (19, 39).

**Effects of silencing of iPLA2γ expression in HTM cells.** To obtain additional confirmation for the specific involvement of iPLA2γ in the TM cell contractile process and actin cytoskeletal organization as evidenced by the effects of R-BEL on these events (Figs. 2 and 3), we utilized siRNA technology to suppress expression of iPLA2γ. Transfection of HTM cells with iPLA2γ-specific siRNA (pool of 3 target-specific sequences) resulted in a reduction of iPLA2γ protein levels by 80% (P < 0.01, n = 4) within 72 h, based on immunoblot analysis and relative to cells transfected with a scrambled siRNA control (Fig. 5B). This knockdown of iPLA2γ expression in TM cells led to changes in cell morphology and decreased actin stress fibers (Fig. 5A) mimicking the effects of R-BEL (Fig. 2). Moreover, the iPLA2γ siRNA-treated TM cells showed a significant (P < 0.01, n = 4) decrease (by >80%) in the levels of phospho-MLC (Fig. 5B). Total MLC was immunoblotted in the same cell lysates to normalize for protein loading between the iPLA2γ siRNA-treated and control samples (Fig. 5B).

**Modulation of aqueous humor outflow facility in enucleated porcine eyes perfused with R-BEL.** To determine the role of iPLA2 in AH outflow, freshly enucleated porcine eyes were perfused with either 25 μM of R-BEL or S-BEL at a constant pressure of 14 mmHg. After establishment of a baseline outflow facility with DPBS buffer containing D-glucose at 37°C, basal rates of outflow facility in the control and R-BEL-perfused eyes were found to be 0.41 ± 0.028 and 0.63 ± 0.051 μl·min⁻¹·mmHg⁻¹, respectively (Fig. 6A). Following R-BEL perfusion, the outflow facility increased significantly by 57% (P < 0.05, n = 6) by the first hour. This effect was progressive, and resulted in an 80% (P < 0.01) increase by 3 h and plateauing by 4 h (Fig. 6A). The fellow paired control eyes showed an expected change in outflow facility (~ 20–30%, washout response) from the corresponding initial baseline values. As compared with R-BEL, the S-BEL (25 μM)-perfused eyes under similar conditions did not show any significant changes in outflow facility compared with the contralateral control eyes over 5 h of perfusion (Fig. 6B).

To determine the effects of R-BEL perfusion on TM tissue MLC phosphorylation status, the TM tissue homogenates (tissue from three eyes were pooled per sample) derived from the eyes perfused with R-BEL or from sham control eyes were analyzed by immunoblot analysis. The R-BEL-perfused TM tissue exhibited a significant (P < 0.01, n = 3) decrease in...
MLC phosphorylation status (by >80%) compared with the sham-treated controls (Fig. 6C). Contrarily, there was no significant change in phospho-MLC levels in the S-BEL-perfused TM tissue as compared with the respective control (Fig. 6D).

**R-BEL induced morphological changes in the AH outflow pathway.** After 5 h of perfusion with R-BEL, porcine eyes were fixed and AH outflow pathway tissues were examined for histological changes. Four specimens were prepared from different quadrants of two eyes in each group. Under light microscope examination, we did not see any gross deformation of TM or aqueous plexi (the Schlemm’s canal equivalent in porcine). Interestingly though, the giant vacuoles (invaginations of inner wall of Schlemm’s canal) in the endothelial cells of aqueous plexi were found to be more...
Fig. 5. Effects of iPLA₂γ knockdown by small interfering RNA (siRNA) on human TM cell morphology, actin stress fibers, and phosphorylation of MLC. To confirm the independent means of iPLA₂γ-specific involvement in TM cells, iPLA₂γ expression was downregulated in TM cells using an siRNA approach as described in MATERIALS AND METHODS. Transfected cells were examined for morphological changes and actin cytoskeletal organization, while cell lysates were probed for phospho-MLC status in conjunction with appropriate controls. A: after 72 h of treatment with iPLA₂γ siRNA, human primary TM cells exhibited easily detectable changes in cell morphology and decreased actin stress fibers [stained with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin] as compared with cells treated with a control scrambled siRNA. Scale bar, 50 μm. These morphological changes in TM cells were associated with decreased levels of iPLA₂γ by at least 70% as compared with the respective control, as shown in B. B: in TM cells in which iPLA₂γ expression was downregulated by siRNA, levels of phospho-MLC were significantly decreased as compared with cells transfected with the scrambled siRNA control. Histograms show the densitometry-based differences in protein levels of iPLA₂γ and phospho-MLC derived from 3 independent analyses. Total MLC and β-tubulin were immunoblotted and used for loading controls. *P < 0.05.

prominent with increased number (by ~ 3 to 5 per 100 μm of aqueous plexi) in the R-BEL-perfused group (Fig. 7B) compared with the controls (Fig. 7A). These changes were not associated with noticeable cell loss or accumulation of cell debris in the TM of eyes perfused with R-BEL, ruling out involvement of tissue destruction in the noted effects of drug treatment.

R-BEL decreased the levels of arachidonic acid in TM cells. One of the important products of iPLA₂-catalyzed breakdown of membrane phospholipids is AA, which can in turn act directly as a signaling messenger to modulate cellular activity and other cellular responses, or serve as precursor for various eicosanoids (5, 6, 35, 49). To determine the role of iPLA₂γ in regulating endogenous levels of AA in TM cells, R-BEL (10 μM for 30 min)-treated PTM cells were extracted for total free fatty acids, which were then analyzed by LC-MS/MS to quantify AA levels using a stable isotope dilution method. An external calibrator was prepared and AA was quantified based on the area ratio of AA:AA-d₈. Serum-starved (24 h) PTM cells treated with 10 μM R-BEL for 30 min showed a significant (P < 0.001, n = 3) decrease (by ~50%) in the levels of AA compared with untreated controls [expressed as nanograms of AA per total number of cells used (3 × 10⁶) for the extraction]. Serum stimulation (10% serum for 30 min) of serum-starved PTM cells, on the other hand, led to a significant (P < 0.001, n = 3) increase (~25%) in the levels of AA (Fig. 8A). Representative LC-MS/MS chromatograms for nondeuterated AA (top) and deuterated AA (bottom) are shown in Fig. 8B. Deuterated molecules elute slightly earlier than the nondeuterated form; hence there is a 0.1-min difference in the retention time between these two species.

**DISCUSSION**

In this study we tested the hypothesis that iPLA₂, which generates arachidonic acid (AA) and its eicosanoid derivatives through catalytic hydrolysis of membrane phospholipids (48), regulates TM cell contractile activity and actin cytoskeletal organization, and thereby AH drainage, through the conventional pathway. This study demonstrates that inhibition of iPLA₂γ in the AH outflow pathway increases AH outflow facility through the TM in association with decreased TM tissue MLC phosphorylation and altered morphology of the inner wall of aqueous plexi in enucleated porcine eyes. Additionally, in cultured TM cells, inhibition of iPLA₂γ activity induced changes in cell morphology, decreases in actin stress fibers, phosphorylation of MLC, CPI-17, and MYPT1 (myosin binding subunit of myosin phosphatase), and a reduction in levels of active Rho GTPase (Rho-GTP) and AA. These observations collectively reveal a critical role for iPLA₂γ in regulation of AH drainage and IOP, possibly via AA and Rho GTPase-mediated effects on contraction and actomyosin organization in cells of the AH outflow pathway. Interestingly, the effects of iPLA₂ appear to be rather specific to the iPLA₂γ isofrom. To our knowledge, this is the first study to report a specific role for iPLA₂γ in modulation of AH outflow through the trabecular pathway, and to reveal the significance of AA and likely its eicosanoid derivatives in the underlying process.

To investigate a possible role of iPLA₂ in AH outflow, we first evaluated the expression and distribution profile of individual iPLA₂ isoforms (β and γ isoforms), which are known to play distinct role(s) in different cellular processes, in cultured TM cells and in tissues of the AH outflow pathway. These
Fig. 6. Effects of R-BEL and S-BEL on aqueous outflow facility in enucleated porcine eyes. Freshly enucleated porcine eyes were perfused with either 25 μM R-BEL (A) or 25 μM S-BEL (B) at a constant pressure of 14 mmHg, after baseline outflow facility was established with perfusion media containing D-glucose at 37°C. Aqueous humor (AH) outflow facility was observed to increase significantly from baseline outflow facility after 1 h of perfusion (n = 6 eyes) with R-BEL (*P < 0.05). Outflow facility continued to increase significantly at each of the next two (hourly) time points in R-BEL-perfused eyes, reaching a maximum and significant increase of ~80% (**P < 0.01 relative to control eyes) at 3 h, after which a plateau was attained. Fellow-paired control eyes were perfused with media containing DMSO (0.1%) (n = 6) and showed a marginal and expected increase (by ~20%, washout) over the baseline facility. To determine whether the drug-induced increase in AH outflow facility was associated with changes in MLC phosphorylation, TM tissue was extracted from R-BEL and S-BEL-perfused eyes and from the respective control eyes and examined for changes in the level of phospho-MLC by immunoblot analysis (C and D). As shown in C, the R-BEL-perfused eyes showed a dramatic and significant decrease in the levels of phospho-MLC as compared with their respective control and S-BEL-treated eyes (D). Protein loading for the immunoblots was normalized to total MLC. Histograms are based on the three pooled specimens and values represent means ± SE. *P < 0.05.
initial studies confirmed expression of both the β and γ isoforms of iPLA2 in the human AH outflow pathway including TM and SC tissues, and in cultured PTM and HTM cells, and revealed that iPLA2 activity (BEL-sensitive) in TM cells appears to account for ~40% of total PLA2 activity. Further studies using iPLA2 inhibitors revealed that treatment of HTM and PTM cells with R-BEL, which is specific for the γ isoform of iPLA2, resulted in changes in cell morphology, while S-BEL, which inhibits the β isoform of iPLA2, had little to no effect on this parameter, relative to control cells at the concentrations used in this study. This early indication of the significance of iPLA2γ was reinforced when we examined the effects of R-BEL and S-BEL on actomyosin organization and focal adhesions in TM cells. Consistent with the changes in cell morphology noted in early experiments, treatment of TM cells with R-BEL also elicited marked reductions in actin stress fibers and focal adhesions, both of which are dependent on actin cytoskeletal dynamics and myosin II activity (53). R-BEL also caused significant decreases in phosphorylation of MLC, which is a regulatory subunit of myosin II, in TM cells. Importantly, each of R-BEL-induced pharmacological responses was mimicked in TM cells in which iPLA2γ expression was silenced using the iPLA2γ-specific siRNA. Since MLC phosphorylation is crucial for myosin II activity and increases and decreases in MLC phosphorylation-induced cellular contraction and relaxation, respectively (49), the R-BEL-induced changes in TM cells were concluded to occur as a result of changes in cellular contractile and relaxation properties, and to highlight a role for iPLA2γ in regulating these responses.

Elevation of contractile tension can be achieved without changes in the levels of intracellular calcium through inhibition of MLC phosphatase in various smooth muscle tissues (49, 53). This response is termed as “Ca2+ sensitization” and is regulated predominantly by Rho/Rho kinase and CPI-17 in smooth muscle tissues (49). Interestingly, iPLA2 and its AA products have been shown to regulate Rho GTPase and Rho kinase activities and calcium sensitization of contraction in vascular smooth muscle cells (49). Since iPLA2 activity is known to be calcium-independent (5), we speculated that R-BEL-induced...
changes in TM cell MLC phosphorylation might be independent of changes in the levels of intracellular calcium, and recruit elements of the Ca\(^{2+}\) sensitization pathway instead. To explore this possibility, we evaluated the effects of iPLA\(_2\) on Rho GTPase activation, phosphorylation-dependent inhibition of MLC phosphatase, and activation of CPI-17, which is an inhibitor of MLC phosphatase whose activity is dependent on protein kinase C and Rho kinase-mediated phosphorylation (23, 25, 50), in R-BEL-treated TM cells. Interestingly, inhibition of iPLA\(_2\gamma\) in TM cells not only decreased the levels of active Rho GTPase (Rho-GTP) but also decreased the levels of phospho-MLC phosphatase and phospho-CPI-17 in association with significant decreases in MLC phosphorylation. These changes in signaling proteins involved in regulation of MLC phosphorylation and myosin II activity in R-BEL-treated TM cells underscore the significance of iPLA\(_2\) activity in controlling the contractile properties and cell adhesive interactions in TM cells in a Rho GTPase/Rho kinase-dependent manner. Since CPI-17 is known to be phosphorylated by both PKC and Rho kinase, additional studies are required to decipher whether the decreased levels of phospho-CPI-17 in R-BEL-treated cells are attributable solely to compromised activity of the Rho/Rho kinase or the PKC pathway, or to inhibition of both pathways. However, further evidence for compromised Rho kinase and perhaps PKC activity as well, in iPLA\(_2\) inhibited TM cells, comes from the observation that these cells exhibit a decrease in levels of phospho-MARCKS, which is a known substrate of both kinases and is involved in actin cytoskeletal organization (19, 39).

This study also identifies a possible cause for the compromised Ca\(^{2+}\) sensitization of TM contractile activity observed upon inhibition of iPLA\(_2\) activity. One of the predominant products of iPLA\(_2\) catalytic activity is AA, which can in turn regulate Rho GTPase activity by activating G protein-coupled receptors, and by independently activating Rho kinase activity and MLC phosphatase (14, 16, 50). In R-BEL-treated TM cells, the levels of free AA were found to be decreased significantly in TM cells as determined by LC-MS/MS, revealing the importance of iPLA\(_2\) activity and its catalytic products such as AA in regulating Ca\(^{2+}\) sensitization of TM cell contraction and thereby cell morphology, perhaps by activating the Rho/Rho kinase signaling pathway. However, we cannot rule out involvement of iPLA\(_2\) and its products regulating TM cell contractile properties and actin cytoskeletal dynamics independent of their influence on the Rho/Rho kinase pathway.

Having found evidence that iPLA\(_2\) behaves as a critical regulator of actomyosin organization in TM cells, we then assessed whether iPLA\(_2\) is capable of modulating AH outflow facility. Perfusion of enucleated porcine eyes with R-BEL revealed a time-dependent and significant increase in AH outflow facility, while S-BEL was without effect. Importantly, the change in AH outflow facility induced by R-BEL was associated with a significant decrease in MLC phosphorylation in the drug-perfused TM tissue, indicating altered contractile properties of the TM tissue. Additionally, the morphology of inner wall of aqueous plexi, which depends on cytoskeletal integrity of its endothelial cell lining, exhibited notable changes such as an increase in the number of giant vacuoles in R-BEL-perfused eyes. Although the cellular and molecular basis underlying the morphological changes (e.g., giant vacuoles) is not completely clear, one of the commonly noted observations associated with altered AH outflow facility is a change in the giant vacuole size and their number in the inner wall of SC (7, 11, 17, 42). Therefore, it is conceivable that R-BEL-induced changes in the contractile properties of aqueous plexi and TM and their subsequent influence on cell stiffness and cell adhesion are partly responsible for the in-
creased number of giant vacuoles in the inner wall of aqueous plexi in porcine eyes, which are structurally similar to the human SC.

In conclusion, this study provides molecular evidence for the involvement of iPLA2γ in regulation of TM cell contractile and cell adhesive properties possibly via Rho GTPase/Rho kinase signaling and AA. Importantly, inhibition of iPLA2γ was found to increase AH outflow facility, indicating a critical role for this enzyme in regulation of aqueous humor outflow through the conventional pathway and homeostasis of IOP. Future studies are needed to determine the changes in AH outflow and IOP in the iPLA2γ knockout mouse model which has been developed (33) recently to extend the R-BEL-induced cell biological and ex vivo observations of this study to the in vivo significance of iPLA2γ in homeostasis of IOP.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

P.P.P. and F.B.L. performed the experiments; P.P.P., F.B.L., and P.V.R. analyzed the data; P.P.P., F.B.L., and P.V.R. interpreted the results of the experiments; P.P.P. and F.B.L. prepared the figures; P.P.P., F.B.L., K.B.T., and P.V.R. approved the final version of the manuscript; K.B.T. and P.V.R. conceived and designed the research. P.P.P. and F.B.L. drafted the manuscript; P.P.P., F.B.L., K.B.T., and P.V.R. edited and revised the manuscript; P.P.P., F.B.L., K.B.T., and P.V.R. approved the final version of the manuscript; K.B.T. and P.V.R. drafted the manuscript; P.P.P., F.B.L., K.B.T., and P.V.R. edited and revised the manuscript; P.P.P., F.B.L., K.B.T., and P.V.R. approved the final version of the manuscript; K.B.T. and P.V.R. conception and design of the research.

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