Cytoskeletal Dependence of Adenosine Triphosphate Release by Human Trabecular Meshwork Cells

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PURPOSE. To test whether adenosine triphosphate (ATP) release links cytoskeletal remodeling with release of matrix metalloproteinases (MMPs), regulators of outflow facility and intraocular pressure.

METHODS. ATP release was measured by luciferin-luciferase. Ecto-ATPases from transformed human trabecular meshwork (TM) cells (TM5) and explant-derived TM cells were identified by RT-PCR. Actin was visualized by phalloidin staining. Cell viability was assayed by lactate dehydrogenase and thiazolyl blue tetrazolium bromide methods and propidium iodide exclusion, gene expression by real-time PCR, and MMP release by zymography. Cell volume was monitored by electronic cell sorting.

RESULTS. Hypotonicity (50%) and mechanical stretch increased ATP release with similar pharmacologic profiles. TM cells expressed ecto-ATPases E-NPP1-3, E-NTPD2, E-NTPD8, and CD73. Prolonged dexamethasone (DEX) exposure (≥ 2 weeks), but not brief exposure (3 days), increased cross-linked actin networks and reduced swelling-triggered ATP release. Cytochalasin D (CCD) exerted opposite effects. Neither DEX nor CCD altered the cell viability, gene expression, or pharmacologic profile of ATP-release pathways. DEX accelerated, and CCD slowed, the regulatory volume decrease after hypotonic exposure. Activating A₁ adenosine receptors (A₁ARs) increased total MMP-2 and MMP-9 release. DEX reduced total A₁AR-triggered MMP release, and CCD increased the active form of MMP-2 release. The A₁AR agonist CHA and the A₁AR antagonist DPCPX partially reversed the effects of DEX and CCD, respectively.

CONCLUSIONS. Cytoskeletal restructuring modulated swelling-activated ATP release, in part by changing the duration of cell swelling after hypotonic challenge. Modifying ATP release is expected to modulate MMP secretion by altering ecto-enzymatic delivery of adenosine to A₁ARs, linking cytoskeletal remodeling and MMP-mediated modulation of outflow facility. (*Invest Ophthalmol Vis Sci.* 2011;52:7996–8005) DOI: 10.1167/iovs.11-8170

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Glaucoma is frequently associated with elevated intraocular pressure (IOP) that leads to the loss of retinal ganglion cells and atrophy of the optic nerve. The only intervention documented to slow the onset and progression of irreversible blindness is to lower IOP, even in patients with normotensive glaucoma.^{1–4} IOP can be reduced by decreasing inflow rate, increasing outflow rate through the uveoscleral exit pathway, or enhancing outflow facility (reducing resistance to outflow) of aqueous humor through the pressure-sensitive trabecular outflow route. Of particular promise in reducing outflow resistance are cytoskeleton-disrupting drugs that can lower IOP in humans⁵ and monkeys.⁶

Depolymerization of the actin cytoskeleton, directly with agents such as cytochalasin D (CCD) or indirectly with agents such as 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), reduces IOP^{5,6} and outflow resistance.⁶ The converse is also true. Enhancing cytoskeletal polymerization with dexamethasone (DEX)⁷ increases IOP in 30% to 40% of healthy humans.⁸ The responsive eyes display structural changes in the trabecular meshwork similar to those observed in primary open angle glaucoma.⁹

How cytoskeletal disruption reduces outflow resistance is uncertain. However, altered activity of matrix metalloproteinases (MMPs) not only accompanies cytoskeletal remodeling but likely mediates the effects of such remodeling on outflow resistance.¹⁰ Disrupting the cytoskeleton with CCD or latrunculin A activates MMP-2 in human trabecular meshwork (TM) cells,¹⁰ whereas enhancing polymerization with dexamethasone reduces the expression¹¹ and secretion¹² of MMPs in TM cells. The link between cytoskeletal remodeling and modulation of MMP activity and secretion is unknown.

The role of MMPs in modulating outflow resistance is even more strongly established in mediating the purinergic regulation of outflow. In the absence of cytoskeleton-remodeling agents, TM cells release MMP-2 after agonist activation of A₁ adenosine receptors (A₁ARs).¹³ The activation of A₁ARs also reduces outflow resistance in nonhuman primates¹⁴ and perfused bovine anterior segments¹⁵ and lowers IOP in several species.^{14,16–18} The outflow effect can be blocked by inhibiting MMP activity.¹⁵ The cascade of events linking A₁AR activation to MMP-2 release by human TM cells has been recognized.¹⁹ The event triggering this sequence is the delivery of adenosine to the A₁ receptors, through ecto-enzymatic metabolism of adenosine triphosphate (ATP) released by the TM cells. The mechanisms underlying swelling-activated ATP release by human TM cells have recently been identified.²⁰

We wondered whether cell ATP release, triggering purinergic regulation of MMP-mediated outflow resistance, might also be modulated by cytoskeletal modeling, thereby potentially influencing outflow. The present study tested whether assembly and disassembly of the actin cytoskeleton alters ATP release by human TM cells.

MATERIALS AND METHODS

Cellular Models

Transformed normal human trabecular meshwork (HTM) cells (TM5; Alcon Research Inc., Fort Worth, TX) and primary HTM cells were cultured as previously reported^{20–22} and studied from passages 20 to 40 and 4 to 7, respectively. Cytoskeleton-remodeled TM5 cells were obtained by the addition of DEX (1 μ M) or CCD (25 μ M) to the culture media for the periods specified.

Solutions and Pharmacologic Reagents

As previously described,^{20,23} the isotonic solution (295–305 mOsm/kg) with 0.1 mM external free Ca^{2+} was composed of 110 mM NaCl, 4.7 mM KCl, 5.1 mM CaCl_2 , 1.2 mM MgCl_2 , 30 mM NaHCO_3 , 1.2 mM KH_2PO_4 , 15 mM HEPES, 5 mM EGTA, and 10 mM glucose. Selectively omitting NaCl reduced osmolality to approximately 100 mOsm/kg (67% hypotonicity). Intermediate osmolalities were generated by appropriate mixing of the isotonic and hypotonic solutions. In some experiments, isotonicity was restored by combining 1 part of mannitol stock solution (1.5 M) with 10 parts of the hypotonic solution (50%). The final osmolalities were verified, and pH values were adjusted to 7.4 before each experiment. Biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO), except for probenecid (Alfa Aesar, War Hill, MA). Chemicals and media for cell culture were obtained from Gibco (Invitrogen, Carlsbad, CA). Ethanol (<0.1%) was used to solubilize hydrophobic drugs, exposing controls to the same concentration of vehicle. Unless otherwise noted, all experiments were conducted at room temperature.

Confocal Microscopy of F-Actins by Phalloidin Staining

Control and DEX-treated (1 μ M for either 3 days or ≥ 2 weeks) TM5 cells were trypsinized and seeded on noncoated coverslips for 12 hours to allow firm attachment to the underlying surface. CCD-treated samples were obtained by applying CCD (25 μ M) to coverslips with adherent cells for 1 hour before fixation. The cells were then fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 100 μ M digitonin for 10 minutes, followed by blocking with 1% bovine serum albumin-PBS for 1 hour. Samples were probed with Alexa Fluor-488 phalloidin (1:2000; Invitrogen) for 2 hours and rinsed with PBS three times. DAPI (0.15 μ g/mL) was added to counterstain the nuclei fluorescently. Coverslips mounted with reagent (Prolong Gold Antifade; Invitrogen) were observed under a confocal laser scanning microscope (FluoView 1000; Olympus America, Center Valley, PA). Single layers of 0.5- μ m thickness were photographed.

ATP Measurement

ATP release was measured by bioluminescent luciferin-luciferase reaction with light emission recorded using a microplate luminometer (Synergy 2; BioTek, Winooski, VT), as previously documented.^{20,23} Cells of the DEX group were treated continuously with 1 μ M DEX for either 3 days or over 14 days, as indicated, whereas those of the CCD group were treated with 25 μ M CCD for 1 hour. In some experiments, measurements were paused at designated time points for less than 1 minute before recordings resumed to restore isosmolality by the addition of hypertonic mannitol. Separate standard curves were used in experiments involving changes in ionic strength. No test substance interfered with the ATP assay at the specified concentration used in this study. Inhibition of the hypotonicity-induced enhancement of ATP release was calculated from the following equation, as previously described^{20,23}:

$$\text{Inhibition (\%)} = 100\% \cdot [(C_{\text{max}} - C_{\text{exp}})/C_{\text{max}} - C_{\text{con}}] \quad (1)$$

C_{max} was the maximal ATP concentration after hypotonic treatment without inhibitor, C_{con} was the control ATP concentration in the

isotonic bath at the same time point, and C_{exp} was the maximal ATP concentration after hypotonic treatment in the presence of inhibitor.

Stretch Chamber Experiment

Mechanical perturbation was performed by stretching TM5 cells in the chambers in accordance with the method of Tschumperlin et al.,²⁴ with some modification. Briefly, silicone membranes (Specialty Manufacturing Inc., Saginaw, MI) were autoclaved and coated with fibronection (50 μ g/mL; Millipore, Billerica, MA). A sterile hollow glass cylinder (1-cm diameter; Fisher Scientific, Pittsburgh, PA) was placed on the center of the membrane surface to keep cells growing within this designated area. TM5 cells were trypsinized and plated into the cylinder at a density of 1.27 million/cm² membrane (i.e., 0.1 million per cylinder-delimited area) and were cultured for 2 days until cells securely adhered to the membrane. Before study, the cylinder was removed, and the culture medium was replaced with 200 μ L isotonic solution with or without drugs for a 1-hour preincubation. Thereafter, 50 μ L solution was collected as the control (Stretch[−]). By screwing down the chamber for 7 full turns, the membrane was extended to 137%, and another 50 μ L solution was collected after stretch was maintained for 10 minutes (Stretch⁺). Sample ATP concentrations were determined using the method described in ATP Measurement. Cytoskeleton-remodeled cells were generated by treatment with DEX (1 μ M, ≥ 14 days) or CCD (25 μ M, 1 hour) by the time of stretching.

Cell Viability Assays

To quantify cell viability after treatment, the lactose dehydrogenase (LDH) assay and the thiazolyl blue tetrazolium bromide (MTT) assay were adapted for microplate-based experiments.

Activity of released LDH was measured with a cytotoxicity detection kit (Roche Diagnostic, Indianapolis, IN) in accordance with the manufacturer's instructions,²³ and the cell metabolic states were estimated by the MTT assay as previously described.²⁰ For stretch experiments, cells grown on the membrane after stretching were stained directly with propidium iodide (2 μ g/mL) for 10 minutes, and photographed under a fluorescence microscope (Eclipse Ti; Nikon Instrument Inc., Melville, NY).

Real-Time Quantitative PCR

Total RNA isolation, reverse transcription, and the following TaqMan gene expression assays were performed, as previously described.^{20,23} FAM-labeled MGB TaqMan probes used in the assays are listed in Supplementary Table S1A (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8170/-DCSupplemental>). The expression levels of indicated genes were all normalized to that of PX1 in specified control cells after $2^{-\Delta\Delta Ct}$ calculation, with human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control. For samples with cytoskeletal remodeling, TM5 cells were initially treated with DEX (1 μ M, ≥ 14 days) or CCD (25 μ M, 1 hour) before RNA extraction.

Cell Volume Measurement

Cell volume was monitored by electronic cell sorting, using a Coulter counter (ZBI-Channelyzer II; Beckman Coulter Inc., Brea, CA) with a 100- μ m aperture as previously reported.²⁵ TM5 cells were trypsinized and resuspended in isotonic solutions with or without drugs for 1 hour. Thereafter, 50% hypotonicity was applied, and cell volume changes were recorded at indicated time points. Samples with remodeled cytoskeletons were obtained by pretreating cells with either DEX (1 μ M, ≥ 14 days or ≤ 3 days) or CCD (25 μ M, 1 hour).

Reverse Transcription–Polymerase Chain Reaction

Cell cDNA templates were obtained as described. PCR was performed with a kit (AccuPrime Taq DNA polymerase High Fidelity; Invitrogen) according to the manufacturer's recommendations. Primers used for gene-specific amplification are shown in Supplementary Table S1B

(<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8170/-/DCSupplemental>). PCR products were separated on 1% agarose gels containing 0.05% ethidium bromide. Bands were visualized under ultraviolet light, sized, and photographed (Molecular Imager Gel Doc XR+ System; Bio-Rad, Hercules, CA). The successfully amplified products were recovered by gel extraction and further verified by sequencing in the DNA Sequencing Facility of the University of Pennsylvania.

Gelatin Zymography of Matrix Metalloproteinases

The secretion of MMP-2 and MMP-9 into external media was analyzed by gelatin zymography, as previously described,^{10,26} with minor modifications. In brief, cells were plated onto 48-well plates at a density of 0.2 million per well and were allowed to grow to confluence, followed by starvation for 24 hours in serum-free media (catalog no. 21063; Gibco). Thereafter, 140 μ L fresh DMEM with or without drugs was added to each well to condition cells for 20 hours at 37°C. The conditioned media were completely collected and cleared by centrifugation (10,000g) for 20 minutes. The supernatants were then mixed with protein sample buffer (Zymogram Sample Buffer; Bio-Rad) as suggested by the instructions, and 30 μ L per sample was loaded onto each lane of the precast polyacrylamide gel (10% Precast Zymogram Gels; Bio-Rad) for SDS-PAGE separation. After electrophoresis, gels were washed sequentially (Zymogram Renaturing Buffer for 3 hours, Zymography Developing Buffer for 24 hours at 37°C, and Coomassie Brilliant Blue R-250 Staining Solution for 8 hours; Bio-Rad) according to the manufacturer's recommendations. Gels were destained in the destaining solution (Coomassie Brilliant Blue R-250; Bio-Rad) until clear bands were visible against the blue background. The gels were subsequently scanned (Scanjet 3570c, Hewlett-Packard, Palo Alto, CA), and band density was analyzed by ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Statistical Analysis

Student's *t*-test, paired or unpaired as appropriate, was applied in comparing two sets of data, and one- or two-way ANOVA was applied to compare three or more sets of data. Statistical analyses were per-

formed with a statistical analysis program (SigmaStat; Aspire Software International, Ashburn, VA). Unless otherwise stated, the results are presented as mean $\pm$ SE, with *n* and *N* indicating the number of wells studied and the number of independent experiments performed, respectively. *P* < 0.05 was considered statistically significant.

RESULTS

Effects of Cytoskeletal Remodeling on Swelling-Activated ATP Release

Continuous exposure to 1 μ M DEX for 14 days or more produced substantial cytoskeletal changes in the phalloidin-stained actin cytoskeleton of TM5 cells. Polymerization of F-actin filaments was enhanced, and cross-linked actin networks (CLANs) were extensively formed (Figs. 1B, 1E). CLANs were rarely seen in control TM5 cells (Figs. 1A, 1D). Consistent with earlier reports,^{7,8,27} short-term addition (3 days) of DEX (1 μ M) did not lead to such structural changes (data not shown). In contrast, CCD (25 μ M) rapidly disrupted the actin cytoskeleton within 1 hour, so that F-actin filaments remained visible in only isolated, sparse areas (Figs. 1C, 1F), as previously reported by Sanka et al.¹⁰

TM5 cells release ATP after hypotonic swelling.^{20,28} We tested whether cytoskeletal remodeling modifies that release (Fig. 2). The ATP concentration was 9.7 ± 0.3 nM (*n* = 404 wells) after incubation of TM5 cells in control isotonic solution. This low baseline level was stable during the 2 hours of measurement. Hypotonicity (50%) triggered ATP release, raising the bath concentration by 3.2-fold to 41 ± 1 nM (*n* = 569; *P* < 0.001). Neither DEX (1 μ M for ≥ 14 days, *n* = 164) nor CCD (25 μ M for 1 hour, *n* = 234) treatment significantly affected baseline (*P* = 0.882 by one-way ANOVA). However, stimulating actin polymerization with 1 μ M DEX for ≥ 14 days substantially decreased swelling-elicited ATP release by $36\% \pm 3\%$ (*n* = 246; *P* < 0.001 vs. normal hypotonic control); depolymerizing actin filaments with CCD increased release by $45.9\% \pm 4.8\%$ (*n* = 303; *P* < 0.001). Incubating cells with 1 μ M DEX for

FIGURE 1. Cytoskeletal remodeling produced by prolonged DEX and brief CCD exposure. TM5 cells were treated with cytoskeleton-remodeling agents, including DEX (1 μ M, ≥ 14 days) and CCD (25 μ M, 1 hour). F-actin filaments were visualized by phalloidin staining (green), and nuclei were counterstained with DAPI (blue). Compared with nontreated controls (NT; A, D), DEX treatment strengthened actin filaments (B) and generated CLANs (E, red arrow). In contrast, CCD rapidly disrupted cytoskeleton, making the actin filaments visible only in isolated, sparse areas (C, F). Scale bar, 20 μ m.

FIGURE 2. Effects of DEX and CCD on swelling-induced ATP release. TM5 cells were exposed to cytoskeleton-remodeling agent DEX (1 μ M, ≥ 14 days or 3 days) or CCD (25 μ M, 1 hour). Prolonged DEX treatment enhanced actin polymerization and decreased swelling-triggered ATP release, whereas CCD produced opposite results. Short-term DEX treatment was ineffective. Isotonic baselines were unaffected by the drugs, compared with nontreated (NT) controls. * $P < 0.05$, one-way ANOVA. Numbers of wells analyzed are entered under the x-axis.

3 days did not alter isotonic ($n = 32$; $P = 0.194$) or hypotonic ($n = 48$; $P = 0.162$) bath ATP levels compared with nontreated controls. The observed effects of DEX and CCD suggested that the actin cytoskeleton is important in some way in regulating swelling-activated ATP release.

Cytoskeletal Remodeling Did Not Change the ATP-Releasing Pathways

We wondered whether DEX- and CCD-induced changes of ATP release after cell swelling might be attributed to alterations in these conduits. Hypotonicity-evoked ATP efflux is released through three major conduits in TM5 cells: pannexin-1 (PX1) and connexin (Cx) hemichannels and the purinergic P2X₇ receptor (P2RX₇).²⁰ Consistent with our previous data,²⁰ relatively selective blockers (Discussion) of PX1 (probenecid [PRO] 0.1 mM) and Cx hemichannels (heptanol [HEP] 1 mM), and P2RX₇ (KN62, 1 μ M) inhibited swelling-induced ATP release by $35\% \pm 1\%$ ($n = 75$), $49\% \pm 3\%$ ($n = 72$), and $37\% \pm 2\%$ ($n = 71$), respectively. The nonselective chloride-channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) decreased the release by $32\% \pm 2\%$ ($n = 64$) at the low concentration of 30 μ M. The inhibitor of vesicular ATP release, bafilomycin A1 (BAF, 2 μ M), had no effect ($1\% \pm 5\%$, $n = 48$; $P > 0.05$). Application of KN62 together with either PRO or HEP enhanced the inhibition to $53\% \pm 2\%$ ($n = 64$) and to $70\% \pm 1\%$ ($n = 80$), respectively. These less than additive enhancements are considered in the Discussion. Nearly complete inhibition ($91\% \pm 1\%$, $n = 48$) of the hypotonically stimulated ATP release from TM5 cells was achieved by simultaneous blocking of PX1, Cx hemichannels, and P2RX₇ using a combination of 30 μ M CBX, 1 mM HEP, and 1 μ M KN-62.

The ATP-releasing pharmacologic profile of the cytoskeleton-remodeled TM5 cells was similar to that of control cells (Fig. 3). We also tested whether gene expression of the three major conduits had been altered by DEX or CCD. The results obtained with real-time PCR indicated that the mRNA levels of PX1, Cx, and P2RX₇ remained unchanged after cytoskeletal remodeling (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8170/-/DCSupplemental>). These data indicated that cytoskeletal remodeling did not alter the number or properties of the ATP-releasing conduits in TM cells.

Because the different amounts of ATP released by cytoskeleton-remodeled cells might have reflected cell death caused by drug toxicity, LDH and MTT assays were performed. There was no indication from the LDH assay that remodeling agents decreased cell viability or from the MTT assay that the drugs altered the metabolic state in the concentrations specified

(Supplementary Fig. S2A, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8170/-/DCSupplemental>). The nearly complete abolition of hypotonicity-triggered ATP release from all cells by combined blockade of PX1, Cx hemichannels, and P2RX₇, together with the findings from the LDH and MTT assays, provided no indication of measurable loss of cell viability under our experimental conditions.

Role of Regulatory Volume Decrease in Swelling-Activated ATP Release

Cytoskeletal restructuring did not alter the exit pathways of hypotonicity-triggered ATP release; therefore, we considered the possibility that DEX and CCD might have altered either the magnitude or the duration of swelling produced by the hypotonic challenge. Figure 4 presents the time courses of cell volumes after application of 50% hypotonicity. The control and two experimental groups swelled rapidly, peaked at approxi-

FIGURE 3. Unchanged efficacy of blockers of swelling-evoked ATP release in TM5 cells after cytoskeletal restructuring. Inhibitors of previously identified conduits for hypotonicity-evoked ATP release, including probenecid (PRO, 0.1 mM, PX1 blocker), heptanol (HEP, 1 mM, Cx blocker), NPPB (30 μ M, nonselective chloride channel blocker), and KN62 (1 μ M, P2RX₇ blocker) displayed the same efficacy in nontreated (NT), DEX-treated (1 μ M, ≥ 14 days), and CCD-treated (1 μ M, 1 hour) cells. The combination of carbenoxolone (CBX, 30 μ M), HEP (1 mM), and KN62 (1 μ M) nearly abolished this release, whereas bafilomycin A1 (BAF, 2 μ M, vesicular ATP release blocker) was ineffective. $P > 0.05$ vs. NT controls by one-way ANOVA. The numbers of wells analyzed in the study are entered under the x-axis.

FIGURE 4. Effects of prolonged DEX treatment and transient CCD exposure on the RVD of TM5 cells. TM5 cells were treated with DEX (1 μ M, ≥ 14 days) and CCD (1 μ M, 1 hour) before challenge by hypotonicity (50%). The trajectories are three-parameter exponential fits. Results were significantly different among the DEX-treated ($n = 4$), CCD-treated ($n = 4$), and nontreated (NT) control ($n = 3$) cells ($P < 0.05$ by two-way ANOVA). The DEX group had a much shorter period for 50% recovery after hypotonic exposure ($T_{1/2}$) compared with the NT control group, whereas the CCD group had the longest.

mately 4 minutes, and thereafter exhibited a regulatory volume decrease (RVD). The time to and the magnitude of maximal swelling were identical among the three groups. Nevertheless, the RVD recovery phases of the control, DEX-treated, and CCD-treated cells were significantly different ($P < 0.05$, two-way ANOVA with Dunnett's multiple comparisons test). The times ($T_{1/2}$) for 50% return to isotonic volume were 21.2 ± 1.9 minutes (control), 16.3 ± 0.2 minutes (DEX), and 25.1 ± 1.7 minutes (CCD). Treatment with DEX for < 3 days did not alter the $T_{1/2}$ ($n = 8$; $P > 0.08$ vs. control group).

RVD strongly influences the rate of hypotonicity-triggered ATP release in certain cells.²⁹ We accordingly tested whether shortening the period of hypotonic swelling could reduce ATP release from TM5 cells. We have previously reported that ATP release from TM5 cells triggered by 50% hypotonicity peaks at 26.6 ± 0.3 minutes, with a time to half-peak response of 8.5 ± 0.1 minutes.²⁰ We have now found that restoring isotonicity by adding hypertonic mannitol at 0 minute, 5 minutes, and 20 minutes after hypotonic shock decreased ATP release by $89\% \pm 2\%$ ($n = 32$; $P < 0.001$ vs. the hypotonic control), $66\% \pm 3\%$ ($n = 32$; $P < 0.001$), and $-2\% \pm 4\%$ ($n = 32$; $P > 0.05$), respectively. These data strongly suggested that cytoskeletal remodeling modulated ATP release from TM5 cells by modifying the swelling produced by hypotonic challenge rather than targeting downstream conduits.

TRPV4 channels, members of the transient receptor potential channels, are stimulated by hypotonicity, leading to Ca^{2+} influx and activation of downstream events,³⁰ including the release of large molecules such as diadenosine tetraphosphate (Pintor JJ, et al. *IOVS*. 2011;52:ARVO E-Abstract 2052). We tested the potential mediation of TRPV4 in hypotonicity-triggered ATP release by applying the nonselective inhibitor of TRPV channels ruthenium red (RUR). RUR (20 μ M) had no effect on ATP from TM5 cells, either under isotonic conditions ($n = 16$; $P > 0.9$) or after hypotonic challenge ($n = 32$; $P = 0.997$). Therefore, TRPV4 does not play a role in hypotonicity-initiated ATP release from TM5 cells.

Conduits for Stretch-Activated ATP Release

The foregoing data were obtained by stimulating ATP release with cell swelling. In addition, pannexins³¹ and connexins³²

are known to be mechanosensitive. Given that the ATP-releasing pathways recruited by cells are likely stimulus dependent,²⁰ we identified the release mechanisms elicited by mechanical stretch.

Mechanically stretching the TM5 cells by 30% increased ATP release by nearly twofold ($194\% \pm 22\%$, $n = 82$; $P < 0.001$). The responses to separate application of inhibitors of PX1 (PRO), Cx (HEP), P2RX₇ (KN62), vesicular release (BAF), and the combined inhibition of PX1, Cx, and P2RX₇ (CBX + HEP + KN62) were identical with those to hypotonic swelling (Fig. 5). No detectable cell damage was found after stretching (Supplementary Fig. S2B, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8170/-DCSupplemental>). The pharmacologic results indicated that swelling- and stretch-elicited ATP efflux from TM5 cells were mediated mainly through PX1 and Cx hemichannels, together with P2X₇ ionoreceptors. The effects of cytoskeletal remodeling on stretch-activated ATP release were also similar to those on swelling-activated release. Increasing actin polymerization by prolonged DEX application (1 μ M, ≥ 14 days) reduced stretch-triggered release by $81\% \pm 1\%$ ($n = 33$; $P < 0.001$ vs. normal controls). Depolymerizing actin with CCD (25 μ M, 1 hour) increased ATP efflux by $32\% \pm 3\%$ ($n = 43$; $P < 0.001$). The results indicate that mechanical stretch and hypotonic swelling activate the same ATP-releasing pathways.

Effects of Cytoskeletal Remodeling on A₁ Adenosine Receptor-Mediated Secretion of MMP-2 and MMP-9

Agonist activation of A₁ adenosine receptors (A₁ARs) increases secretion of MMP-2 in cultured TM cells,¹³ resulting in the reduction of outflow resistance.^{14,15} In principle, ecto-enzymatic conversion of released ATP can lead to A₁AR activation. However, the expression profile of ecto-ATPases in TM cells is

FIGURE 5. Correlation of drug inhibitions on hypotonicity- and stretch-elicited ATP release. TM5 cells were mechanically stretched in the chambers, and the following drugs were used to block the release: PRO (0.1 mM, $n = 21$), HEP (1 mM, $n = 9$), KN62 (1 μ M, $n = 18$), BAF (2 μ M, $n = 11$), and a combination of 30 μ M CBX, 1 mM HEP, and 1 μ M KN62 ($n = 21$). The trajectory is a linear least-squares fit, with a slope of 1.04 ± 0.02 ($P < 0.001$) and a correlation coefficient of 0.999, indicating that the same conduits are recruited in both swelling- and stretch-elicited ATP release.

FIGURE 6. Multiple membrane-type nucleotide ectoenzymes expressed by trabecular meshwork cells. RT-PCR with subsequent DNA sequencing verified that both TM5 and HTM cells express CD73, E-NPP1, E-NPP2, E-NPP3, E-NTPD2, and E-NTPD8, and several transcript variants were identified as well. No product was detectable on omitting reverse-transcriptase [RTase(-)], confirming that the cDNA derived from total RNA was free of genomic DNA contamination.

unknown. By using RT-PCR followed by DNA sequencing, we found the expression of multiple ecto-ATPases in explant-derived TM cells and transformed TM5 cells (Fig. 6). The identification of ecto-enzymes CD73, E-NPP1-3, E-NTPD2, and E-NTPD8 provides the first molecular evidence that conversion of ATP to adenosine is physiologically feasible in TM cells.

We next confirmed that A₁AR activation stimulates MMP-2 secretion. TM5 cells were incubated either with the selective A₁AR agonist CHA (100 nM) or with the nonselective AR agonist NECA (1 μ M) for 20 hours, with or without the highly selective A₁AR antagonist DPCPX (20 nM). The results of gelatin zymography (Fig. 7A) indicated that adding CHA and NECA increased the secretion of MMP-2 by $38\% \pm 5\%$ ($n = 23$; $P < 0.001$ vs. the nontreated control) and $70\% \pm 5\%$ ($n = 12$; $P < 0.001$), respectively. The CHA- and NECA-stimulated enhancements were significantly reduced to $16\% \pm 5\%$ ($n = 23$) and $25\% \pm 5\%$ ($n = 12$), respectively, by the A₁AR antagonist DPCPX. DPCPX had no effect on baseline MMP secretion ($n = 18$; $P > 0.05$ vs. the nontreated control). For the first time, we also found that MMP-9, which was also abundant in external media, was upregulated by stimulating A₁ARs (Fig. 7A). The antagonist DPCPX strongly decreased both CHA- and NECA-stimulated MMP-2 and MMP-9 secretion, indicating that A₁ARs are the dominant AR mediators of this purinergic stimulation.

Cytoskeleton-remodeling agents strongly affected both baseline and A₁AR-stimulated secretion of MMP-2 and MMP-9 (Figs. 7B, 7C). Prolonged DEX treatment drastically inhibited MMP-2 and MMP-9 secretion by $70\% \pm 3\%$ ($n = 32$) and $92\% \pm 1\%$ ($n = 32$), respectively, in comparison with controls. In contrast, MMP-9 secretion was significantly increased by $48\% \pm 3\%$ ($n = 26$; $P < 0.05$) by CCD treatment. CCD application did not alter total MMP-2 secretion, in agreement with Sanka et al.,¹⁰ but did produce the appearance of the active form of MMP-2 (Fig. 7C). It is possible that the appearance of the active form of MMP-2 might have raised the total digesting ability of MMP-2, enzymatically reducing the proactive form secreted.

We also examined the interaction of cytoskeleton restructuring and A₁AR-active agents. Applying CHA (100 nM) to DEX-treated cells partially reversed DEX inhibition, stimulating the secretion of MMP-2 and MMP-9 from $8\% \pm 1\%$ and $30\% \pm 3\%$ in controls to $21\% \pm 2\%$ ($n = 22$) and $75\% \pm 3\%$ ($n = 22$), respectively (Fig. 7B). Similarly, adding DPCPX (20 nM) partially reversed the stimulation by CCD, reducing MMP-9 secretion from $148\% \pm 3\%$ to $119\% \pm 2\%$ ($n = 32$). DPCPX (20 nM) reduced the total and active forms of MMP-2 secretion after CCD treatment by $20\% \pm 4\%$ ($n = 36$) and $23\% \pm 5\%$ ($n = 36$; $P < 0.05$ vs. CCD group), respectively. The persistence of the active form of MMP-2 after the inhibition of A₁ARs suggested that CCD treatment stimulated MMP-2 secretion or activation through additional A₁AR-independent signaling pathways.

DISCUSSION

ATP release is the enabling step in purinergic regulation of outflow resistance and IOP. Consistent with our previous studies of TM5 and explant-derived human TM cells,²⁰ separate inhibition of PX1 hemichannels, Cx hemichannels, and P2X₇ receptors reduced swelling-activated ATP release from TM5 cells to comparable extents (Fig. 8). Simultaneous inhibition of all three pathways reduced that release by $>90\%$. Stretch triggered ATP release through the same pharmacologically defined pathways. Stretch may be an important physiological stimulus for TM cell ATP release. TM cells are likely to be displaced and stretched during oscillations of 2.4 ± 0.7 mm Hg produced by the cardiac cycle in the IOP of healthy humans.³³ Elevated ATP levels in the aqueous humor have been associated with primary angle closure glaucoma,³⁴ as well as chronic angle closure glaucoma,³⁵ possibly because of increased stretch of the anterior segment resulting from the very high intraocular pressures recorded.

As previously reported,²⁰ comparable inhibition of ATP release was observed by selectively blocking PX1 and Cx hemichannels of TM5 and explant-derived TM cells. We also reported that concurrent inhibition of PX1 and Cx hemichannels produced an additive reduction in ATP release from TM5 cells.²⁰ Estimating the contribution of P2X₇ receptors is more uncertain. In the present study, a separate block of the P2X₇ ionoreceptor with KN62 reduced swelling-activated ATP release by $37\% \pm 2\%$. However, KN62 produced a less than additive inhibition when administered simultaneously with an inhibitor of either PX1 or Cx hemichannels. In the latter conditions, KN62 appeared to be inhibiting ATP release by 16% to 19%. Evidently, P2RX₇ contributes more greatly to ATP release when the parallel ATP release pathways are operative, consistent with the expectation that the P2X₇ ATP receptor is activated in the presence of higher baseline ATP levels. In contrast to its importance in ATP release from TM cells, P2RX₇ appears to play no role in the release of ATP by the ciliary epithelial cells responsible for the formation of aqueous humor.²³

Pharmacologic estimations of the relative contributions of the parallel pathways to total ATP release are necessarily approximate, given possible cross-target inhibitions. However, our previous results obtained with 21 inhibitors and PX1 knockdown suggest that these estimations are likely correct for human TM cells.²⁰ An alternative strategy based on mimetic peptides has proved unsuccessful in generating selective inhibitors.³⁶ In contrast, probenecid is relatively selective in blocking PX1 and not Cx hemichannels,³⁷ and CBX also preferentially blocks PX1 hemichannels in low concentrations³⁸ with a reported IC₅₀ value 5- to 20-fold lower than that required to block Cx hemichannels. We found that 0.1 mM PRO and 3 μ M

FIGURE 7. MMP-2 and MMP-9 secretion modulated by adenosine A_1 receptors and cytoskeletal remodeling. (A) Stimulating A_1 ARs with either specific agonist CHA (100 nM) or wide-spectrum AR agonist NECA (1 μM) increased MMP-2 and MMP-9 secretion. Both stimulations were blocked by concomitant application of the selective A_1 AR antagonist DP-CPX (20 nM). Numbers of independent experiments are indicated along the abscissa. (B) Increasing actin polymerization with DEX (1 μM, ≥14 days) reduced MMP-2 and MMP-9 secretion considerably, and the reductions were significantly reversed by activating A_1 ARs with CHA (100 nM). Applying CCD (25 μM, 1 hour) raised MMP-9 secretion without affecting total MMP-2. DPCPX (20 nM) lowered both MMP-9 and total MMP-2 secretion of CCD-treated cells. Numbers of independent experiments are indicated along the abscissa. (C). The zymographic gel image shows the changes in MMP secretion produced by CCD (1 μM), an increase in the proform of MMP-9, and the appearance of the active form of MMP-2.

CBX produced identical inhibition ($\sim 36\%$) of ATP release by TM5 cells, consistent with an inhibition of $31\% \pm 2\%$ produced by partial knockdown of PX1.²⁰ Heptanol is a relatively selective blocker of Cx hemichannels at 1 mM,³⁹ but it also blocks PX1 hemichannels at 3 mM.⁴⁰ External Ca^{2+} selectively blocks Cx hemichannels.⁴¹ Once again, the two relatively selective blockers of Cx hemichannels produced similar inhibitions of ATP release, 1 mM heptanol inhibiting by $44\% \pm 3\%$ and 2.5 mM Ca^{2+} inhibiting by $36\% \pm 7\%$.²⁰ Blocking PX1 and Cx hemichannels concurrently produced an additive inhibition.²⁰ We have assayed the contribution of P2X₇ receptors with the blocker KN-62,⁴² which also inhibits calcium/calmodulin-dependent protein kinase II (Ca/CaMKII). However, other P2RX₇ inhibitors, brilliant blue G (BBG)⁴² and A438079,⁴³ produced the same inhibition of ATP release by TM5 cells, and the selective inhibitor of Ca/CaMKII lavendustin C⁴⁴ was ineffective.²⁰ Simultaneously blocking PX1, Cx, and P2RX₇ pathways inhibited ATP release from TM5 cells by $>90\%$.

We have used BAF to assess the vesicular release of ATP. This inhibitor, like monensin, interferes with vesicular uptake of ATP within the cell and is frequently used to reduce vesicular ATP transfer to the extracellular fluid.^{45–48} Neither

BAF nor monensin significantly affected ATP release from TM5 cells.²⁰ BAF was also tested on explant-derived TM cells and was found ineffective. Interestingly, Luna et al.⁴⁹ reported that monensin nearly completely inhibited ATP release from porcine TM cells stimulated with cyclic mechanical stretch. The basis for the difference is unclear, possibly reflecting species origin (porcine vs. human cells), stimulus used for ATP release, or other unidentified methodological factors.

Cytoskeletal remodeling modified ATP release. Disrupting the actin cytoskeleton with CCD increased swelling-activated ATP release by $46\% \pm 5\%$. The cytochalasins act by capping the barbed end of actin polymers, preventing elongation.^{6,50} Stimulating actin polymerization with glucocorticoid had the opposite effect. DEX inhibited swelling-activated ATP release by $36\% \pm 3\%$ after incubation for ≥ 2 weeks but had no effect on that release after incubation for 3 days. In agreement with previous reports,^{7,8} prolonged DEX administration increased actin polymerization and the number of CLANS, which were rarely seen in untreated cells. CLANS usually appear after 3 to 4 days of exposure to DEX,⁷ possibly by increasing the expression and activation

FIGURE 8. Cytoskeleton-dependent purinergic regulation of aqueous humor outflow. Swelling-activated ATP release is recognized to proceed through PX1 and Cx hemichannels and P2X₇ purinergic receptors.²⁰ The present study tests showed mechanical stretch triggers release through the same conduits, whereas remodeling actin cytoskeleton does not modulate this process. The present study also demonstrated ecto-enzymes CD73, E-NPP1-3, E-NTPD2, and E-NTPD8, which convert ATP to adenosine, are expressed in TM cells, consequently activating A₁AR to stimulate the secretion of MMP-9 in addition to MMP-2.

of $\beta 3$ integrins.⁵¹ The temporal association of CLAN formation with the modulation of ATP release raises the possibility that CLANs or a co-temporal structural change may play a role in modifying ATP release.

We wondered whether cytoskeletal remodeling might modulate swelling-activated ATP release by altering the gene expression or membrane trafficking of the ATP release pathways. However, neither gene expression nor the pharmacologic profile of the release mechanisms was changed. Alternatively, cytoskeletal restructuring could act by altering the signaling cascade linking swelling to ATP release. One possible mechanism would be to modify the cell volume response to hypotonic challenge. In most cells, swelling is not sustained during continued hypotonic exposure; rather, it displays a regulatory volume decrease (RVD) mediated by the release of solute and secondarily by water.^{52,53} Thus, the duration of hypotonic swelling is limited by the RVD so that slowing the RVD might be expected to increase swelling-activated ATP release. In support of this expectation, most ATP was found to be released during maximal swelling, before the initiation of RVD by A549 and 16HBE140 cells.²⁹ Similarly, we have now found that early restoration of isotonicity by the addition of mannitol within 5 minutes after hypotonic challenge reduced ATP release. We observed that CCD prolonged the period of hypotonic swelling by slowing the RVD, whereas prolonged exposure to DEX had the opposite effect. Brief incubation with DEX did not alter the RVD; the time required for 50% recovery from peak swelling ($T_{1/2}$) was unchanged. In contrast, prolonged DEX administration significantly reduced $T_{1/2}$ by approximately 5 minutes.

The hypothesis under study has been that changes in outflow resistance triggered by cytoskeletal remodeling might be mediated, at least partly, by modifying ATP release and thereby altering the delivery of adenosine to A₁ adenosine receptors. The tacit assumption is that TM cells express ecto-enzymes capable of converting ATP to adenosine, for which no evidence has yet been available. Our RT-PCR analysis, followed by DNA sequencing, has now demonstrated gene expression of multiple ecto-enzymes in both explant-derived human TM cells and the TM5 cell line, which can metabolize ATP to adenosine.⁵⁴ E-NPP1-3 are members of the ecto-nucleotide pyrophosphatase/phospho-

diesterase family of enzymes that convert ATP to adenosine monophosphate (AMP). E-NTPD2 and E-NTPD8 are members of the ecto-nucleoside triphosphate diphosphohydrolases that convert both ATP to ADP and ADP to AMP. Ecto-5'-nucleotidase (CD73) can convert AMP to adenosine. The observed expression of these six ecto-enzymes supports the plausibility that modifications of ATP release can alter the delivery of adenosine to the TM cells.

Activation of A₁ARs with the selective agonist CHA increased MMP-2 secretion, confirming the earlier observation by Shearer and Crosson.¹³ Furthermore, the selective A₁AR antagonist DPCPX abolished the increase in MMP-2 secretion initiated by the nonselective agonist A₁AR agonist NECA. In addition, we have observed for the first time that CHA and NECA enhance MMP-9 secretion, an enhancement that is almost entirely blocked by DPCPX. A₁ adenosine receptors appear to play a role in the altered MMP secretion produced by cytoskeletal remodeling. The A₁AR antagonist DPCPX sharply reduced the CCD-initiated stimulation of MMP-9 secretion but had no effect on baseline secretion of either MMP-9 or MMP-2. DPCPX also reduced secretion of the proactive form and the active form of MMP-2, but to a lesser extent, suggesting a role for additional, unidentified regulators of MMP-2 release.

We conclude that stretch and swelling of TM5 cells trigger ATP release through pharmacologically identical pathways, PX1 and Cx hemichannels, and P2X₇ receptors (Fig. 8). The role of P2RX₇ is enhanced by concurrent activity of the hemichannels. Both explant-derived TM cells and TM5 cells express ecto-ATPases capable of converting released ATP to adenosine. Adenosine activation of A₁ adenosine receptors stimulates TM-cell secretion of MMP-9, as well as MMP-2, gelatinase. Thus, the modulation of ATP release triggered by cytoskeletal restructuring can play a role in altering MMP release by the TM cells, leading to modified outflow resistance and intraocular pressure. The quantitative significance of this role is under investigation.

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