Modulation of Aqueous Humor Outflow by Ionic Mechanisms Involved in Trabecular Meshwork Cell Volume Regulation

David Soto,1,2 Núria Comes,1,2 Elisa Ferrer,1 Miguel Morales,1 Artur Escalada,5 Jordi Palés,1 Carles Solsona,5 Arcadi Gual,1 and Xavier Gasull1

PURPOSE. Trabecular meshwork (TM) cell shape, volume, contractility and their interactions with extracellular matrix determine outflow facility. Because cell volume seems essential to TM function, this study was conducted to investigate further the ionic channels and receptors involved in regulatory volume decrease and their roles in modulating outflow facility.

METHODS. Primary cultures of bovine TM cells were used. K+ and Cl− currents were studied with whole-cell patch clamping. Swelling was induced by hypotonic shock. [Ca2+]i was measured in TM cells loaded with fura-2. Bovine anterior segments were perfused at constant pressure to measure outflow facility.

RESULTS. Hypotonic media activated both the high-conductance Ca2+-activated K+ channel (BKCa) and swelling-activated Cl− channel (Clswell) currents and induced release of adenosine 5′-triphosphate (ATP) from TM cells. ATP activated P2Y2 receptors with the following profile: ATP = uridine 5′-triphosphate (UTP) > adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S) > adenosine 5′-diphosphate (ADP) = uridine 5′-diphosphate (UDP), and increased BKCa current. Hypotonic medium initially decreased outflow facility in perfused anterior segments, which recovered with time to baseline levels. Addition of tamoxifen or iberiotoxin (Cl− swell and BKCa blockers, respectively) lengthened the recovery phase, which implies that these channels participate in cell volume regulation. In contrast, an activator of BKCa (NSI619) produced the opposite effect.

CONCLUSIONS. Cell swelling activates a regulatory volume decrease mechanism that implies activation of K+ and Cl− currents and participation of P2Y2 receptors. Because previous studies have shown that intracellular volume of TM cells is an important determinant of outflow facility, it seems feasible that cell volume regulation would be part of the homeostatic mechanisms of the TM, to regulate the outflow pathway. (Invest Ophthalmol Vis Sci. 2004;45:3650–3661) DOI:10.1167/iovs.04-0060

In humans and primates, most of the aqueous humor (AH) produced by the ciliary body flows through the trabecular meshwork (TM) and the Schlemm’s canal endothelium to reach the aqueous veins, exiting the eye. The correct performance of this route, as well as the uveoscleral one, is determinant to maintain intraocular pressure (IOP) in the physiological range. On the contrary, when the outflow is somehow impaired, the increase in AH volume raises IOP, which is a major risk factor for the development of glaucoma. TM physiology is still largely unknown, but it has been proposed that TM cells modulate the pathway permeability by showing volume and contractile responses.1-3 Several mechanisms have been proposed to play a role in TM function, including contraction/relaxation of both TM and ciliary muscle; stretch; composition and remodeling of the extracellular matrix; pore formation in the Schlemm’s canal endothelium; and changes in gene expression,1,2,14 cell shape;1,5,15,16 or cell volume.5,17 Endothelial cells of Schlemm’s canal may also participate in the regulation of outflow facility, showing some of these mechanisms, especially in monkey and human eyes, where Schlemm’s canal has a higher degree of organization.18 TM cell volume appears to be an important determinant of tissue function, since swelling or shrinking the cells modifies AH outflow facility.1-3 Mitchell et al.18 recently showed that TM cells possess a regulatory volume decrease (RVD) mechanism. Thus, after cell swelling, the RVD is mediated, at least in part, by activation of swelling-activated Cl− channels (Clswell) and K+−Cl− symports. In this sense, the Na+/H+ antiport has also been suggested to participate in volume regulation, and therefore inhibitors of this antiport may increase outflow facility.19 Another player is the Na+−K+−Cl− cotransport that would modulate outflow facility by regulating cell volume in response to different conditions.18,22,23 But bumeonanide (an Na+−K+−Cl− cotransport blocker) unexpectedly did not have any effect on outflow facility in monkeys.20 Moreover, bumeonanide did not reduce IOP in monkey or mouse.20,21 Despite all the players identified and possibly involved in cell volume regulation, a direct relationship between cell volume regulation and outflow facility has not yet been demonstrated.

In other cell types,22-24 osmotic cell swelling is thought to activate Clswell. Simultaneously, cell swelling induces the release of adenosine 5′-triphosphate (ATP) that, in turn, acts as an autocrine/paracrine signal to activate purinergic P2Y receptors. Stimulation of purinergic receptors is thought to facilitate activation of K+ channels. Finally, Cl− and K+ efflux from the cell followed by water outflow permits the cell to recover its volume.25 It remains to be elucidated whether these mechanisms of cell volume regulation are essential to TM function. In this sense, several mechanisms such as the Na+−K+−Cl− cotransport or the high-conductance Ca2+-activated K+ channel (BKCa)26 are tightly modulated by several drugs, and alterations in their regulation may affect TM function and therefore...
outflow facility. Our purpose was to identify and characterize the receptors and channels involved in the RVD of TM cells and to determine their functional role in outflow facility modulation as part of an homeostatic system that may regulate TM resistance. We used hypotonic shocks to trigger RVD and to study the receptors and channels involved in this process. Our findings show that changes in TM cell volume modify outflow facility and that regulatory mechanisms to restore cell volume actively participate to maintain AH outflow.

Methods

Bovine TM Cell Culture

Bovine TM (BTM) cells were cultured by using a modification of the technique described by Stamer et al.7,8 As described previously,9 BTM strips were digested with 2 mg/ml collagenase (Sigma-Aldrich, Madrid, Spain) and 0.5 mg/ml bovine serum albumin (BSA; Sigma-Aldrich) at 37 °C for 2 hours. After mechanical digestion, the supernatant was collected, centrifuged, and resuspended. The resuspended solution was seeded in culture flasks containing Dulbecco’s modified Eagle’s medium (DMEM; BioWhitaker, Barcelona, Spain) plus 10% fetal bovine serum, 100 mg/ml l-glutamine (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (BioWhitaker). Cell growth was observed 2 to 4 days after seeding and cells reached confluence 12 to 15 days later. Cell passages were performed using trypsin-EDTA (Bio-Whitaker). Cells from passages 1 to 3 were used for electrophysiology, [Ca 2+]i, and ATP measurements.

Cytosolic Free-Ca2+ Measurement

Measurement of [Ca 2+]i was performed as described in detail previously.3,28 Briefly, BTM cells were plated on 25-mm diameter glass coverslips (VWR Scientific Inc., Philadelphia, PA) and then loaded with 5 μM fura-2/AM (Calbiochem, San Diego, CA) for 60 minutes at 37 °C in incubation buffer (121 mM NaCl, 4.7 mM KCl, 5 mM NaHCO3, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 10 mM glucose, 10 mM HEPES, and 0.01% BSA [pH 7.4], adjusted with NaOH, 287 ± 2 mOsm/kg; mean ± SD). Coverslips with fura-2-loaded cells were transferred into an open flow chamber (1 ml incubation buffer) mounted on the heated stage of an inverted epifluorescence microscope (Diaphot-300; Nikon, Tokyo, Japan). Fluorescence images were obtained by a charge-coupled device (CCD) camera (CH250; Photometrics, Tucson, AZ) and were digitized, stored, and analyzed on computer (Macintosh #60AV; Apple, Inc., Cupertino, CA). After a stabilization period of 10 minutes, image pairs were obtained alternately every 4 seconds, and, for a total of 8 minutes, at excitation wavelengths of 340 and 380 nm (10-nm bandwidth filters), to excite the Ca 2+ bound and Ca 2+ free forms of this ratiometric dye, respectively. The emission wavelength was 510 nm (120-nm bandwidth filter). [Ca 2+]i levels were calculated in single cells by determining the 340- to 380-nm fluorescence ratios at each time point.29 In both control and experimental groups, Ca 2+ was recorded for 1 minute before drug application and thereafter, for 7 minutes. Cells were considered as responders when [Ca 2+]i increased more than 100% above the resting value.

Patch-Clamp Procedures

TM cells were plated onto small glass coverslips and studied 24 to 48 hours thereafter. Coverslips were transferred to a special chamber (0.2 mL) in the stage of an inverted microscope (IX70; Olympus, Tokyo, Japan) to perform the recordings. External solutions were superfused at a rate of 3 to 4 μL/min by gravity. Before the recording session was started, culture medium was replaced with a bath recording solution. Recordings were performed in 2-hour sessions at 21 °C to 23 °C. Patch pipettes were pulled in an electrode puller (P97; Sutter Instruments, Novato, CA) and had a filled tip resistance of between 3 to 6 MΩ. Pipette capacitance to ground was neutralized after the seal was formed. An Ag/AgCl wire bath electrode was used except when recording chloride currents where the Ag/AgCl electrode was mounted in a 3 M KCl agar bridge. As previously described,6 we took care to avoid stretch activation of BKCa when performing the patches. Before the pipette had entered the bath and until cell contact, positive pressure was applied.

Whole-cell currents were recorded using a patch-clamp amplifier (List EPC7, Heka, Lambrecht/Pfalz, Germany) as described previously.5 Data acquisition and command potentials were controlled by computer (WinWCP 3.2 software; John Dempster, University of Strathclyde, UK), using an interface (CED1401; Cambridge Electronic Design Ltd., Cambridge, UK). Whole-cell currents were recorded at 10 kHz.

Chloride Currents

CIC functions currents were recorded using the whole-cell configuration of the patch-clamp technique. After achieving the whole-cell configuration, cells were allowed to stabilize and dialyze for 3 to 4 minutes before recording started. Whole-cell currents were recorded at 10 kHz without any leak current subtraction protocol. Cells were clamped at 0 mV, and voltage pulses from −80 to +120 mV were applied in 20-mV steps to evoke CIC currents. The intracellular pipette solution contained (in mM): 150 N-methyl-D-glucamine (NMDG)Cl, 1.2 MgCl2, 1.0 EGTA, 2 ATP, 0.5 guanosine 5’-triphosphate (GTP), 10 HEPEs (pH 7.35) adjusted with Tris (305 ± 4 mMol/kg; mean ± SD). The standard intracellular bath solution contained (in mM): 150 NMDGCl, 0.5 MgCl2, 1.3 CaCl2, and 10 HEPEs (pH 7.35) adjusted with Tris (219 ± 5 mMol/kg). The solution osmolarities were measured with an osmometer (Vapro, Wescor, UT). To study channel relative permeabilities, we recorded voltage ramps from −100 to +100 mV for Cl−, HCO3−, Br−, F−, and gluconate. In these experiments, the bath hypotonic solution used was (in mM): 90 NaX (X = anion), 10 NMDGCl, 0.5 MgCl2, 1.3 CaCl2, and 10 HEPEs (pH 7.35) adjusted with 0.5 M Tris. Data were corrected with the calculated theoretical junction potential values for each solution.

Potassium Currents

Potassium currents were recorded in whole-cell configuration, either with the ruptured-patch or the perforated-patch (with Nystatin; Sigma-Aldrich) technique. Leak current and residual capacitative current were subtracted using a P/4 protocol where N was −4. Cells were clamped at −60 mV, and depolarizing pulses were applied in 10 or 20-mV steps to evoke outward K+ currents. In whole-cell recordings, the solution in the pipette was (in mM): 140 KCl, 2 MgCl2, 0.1 EGTA, and 10 HEPEs (pH 7.2), with 0.5 M KOH. The bath physiological solution was (in mM): 140 NaCl, 4.3 KCl, 1.3 CaCl2, 1.0 MgCl2, and 10 HEPEs (pH 7.4), with NaOH (301 ± 5 mMol/kg). Hypotonic solution in which NaCl and KCl concentrations were reduced was (in mM): 100 NaCl, 2 KCl, 1.3 CaCl2, 1 MgCl2, and 10 HEPEs (pH 7.4), with NaOH (213 ± 4 mMol/kg; mean ± SD). Outward potassium currents were blocked with a specific BKCa blocker, iberiotoxin (IBTX, 50 mM) obtained from Sigma-Aldrich (St. Louis, MO). In the whole-cell perforated-patch technique, pipettes were backfilled with intracellular solution containing (in mM): 100 K-acetate, 40 KCl, 1.3 MgCl2, 1.36 EGTA, and 10 HEPEs (pH 7.2) with KOH in which Nystatin (150 μg/mL) was dissolved. The solution was made daily from a stock solution (3 mg/60 mL dimethyl sulfoxide [DMSO]) and sonicated to the final concentration. Slowly developing capacitative transients indicated establishment of the whole-cell perforated-patch configuration.

We continuously monitored series resistance to avoid sudden drops in this parameter indicating that ruptured-patch configuration was achieved. Only experiments with constant series resistance during the whole recording were considered for analysis.

Measurement of ATP Release

ATP release detection was performed in cultured TM cells 3 to 4 days after cell passage. Experiments were performed at 22 °C in cells grown...
in 35-mm Petri dishes. Culture medium was replaced by physiological solution (mM): 140 NaCl, 4.5 KCl, 1.3 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose (pH 7.4), with NaOH (291 ± 2.9 mOsM/kg) and allowed to rest for 30 minutes to minimize a possible ATP release due to medium exchange. Twenty percent and 40% hypotonic medium was obtained by adding distilled water to the physiological solution and resulting osmolalities were 242 ± 2 and 206 ± 2 mOsM/kg (mean ± SD), respectively. A vial of firefly lantern luciferase extract from Photinus pyralis (Sigma-Aldrich) was diluted in 1 mL of isotonic physiological solution or distilled water containing 5 mg/mL β-luciferin (Sigma-Aldrich). The resultant suspension was centrifuged for 30 seconds in a benchtop centrifuge. The supernatant was desalted in a 10-mL column (10 DG; Bio-Rad, Hercules, CA) equilibrated with recording solution. A photomultiplier tube (R464; Hamamatsu, Hamamatsu City, Japan) located over the Petri dish was used to detect photons. The resultant electric signal was filtered at 1 kHz, digitized, and recorded on computer (X-Chart software; Heka). Calibration curves were calculated by adding known doses of ATP (1, 3, 4, and 6 picomoles). Regardless of the different ionic strength of the isotonic and hypotonic solutions used, no significant differences were found between the calibration curves in the 1- to 6-picomole range. Therefore, data were not corrected for each solution and all calculations were done with the calibration values in isotonic conditions. Also, to test a possible lysis due to excessive cell swelling when hypotonic solutions were added, we measured lactate dehydrogenase (LDH) activity in the bath solution. No significant amounts of LDH activity were encountered before and after addition of isotonic or hypotonic solutions.

**Perfusion of Anterior Segments**

Eyes of 3- to 6-month-old calves were obtained at the local abattoir 0.5 to 2 hours after death and kept in phosphate-buffered saline (PBS) at 4°C for not more than 1.5 hours. Isolation of bovine anterior segments was performed as described previously. The perfusion technique has been described elsewhere. Briefly, bovine anterior segments were placed in a specially designed perfusion chamber. The anterior segments, located in their respective chambers together with force transducers (Leticia, Barcelona, Spain) and the tubing system, were placed in an incubator (Selecta, Barcelona, Spain) at 36°C and 5% CO₂. Perfusion was performed with DMEM. The pressure of the artificial anterior chamber was monitored and recorded throughout the experiments with a pressure transducer (91624; Mallinckrodt, Northampton, UK) and was maintained with a suspended reservoir at 10 mm Hg in bovine eyes. Outflow facility (C) was averaged during periods of 15 minutes (mean of 450 values). Baseline facility (C₀) was calculated during the first 90-minute period of stable C. When a different experimental condition or drug was added to the perfusion medium, the tubes and the anterior chamber were flushed and replaced with the new medium. This change was made by rapidly replacing the contents of the artificial anterior chamber by opening the exit needle until 200% of the volume had been exchanged. This exchange was always made at a pressure below 10 mm Hg. Recording of C started after stabilization of the flow.

The perfusion procedure was performed using a protocol with two periods: perfusion with control isotonic DMEM (301 ± 1 mOsM/kg) for 90 minutes, to establish the C₀ and perfusion with hypotonic (244 ± 2 mOsM/kg) DMEM for 360 minutes to determine changes in outflow facility after five experimental conditions: hypotonic medium, hypotonic + tamoxifen, hypotonic + IBTX, hypotonic + tamoxifen + IBTX, and hypotonic + BKca channel opener (NS1619; 1,3-dihydrop-1(2-hydroxy-5-(trithuromethyl)-phenyl)-5(trithuromethyl)-2H-benzimidazol-2-1)).

**Drugs**

ATP, uridine 5′-triphosphate (UTP), adenosine 5′-O-(3-thiotriphosphate) (ATPγS), adenosine 5′-diphosphate (ADP), uridine 5′-diphosphate (UDP), suramin sodium salt, 2MeS-ATP (2-methylthio adenosine 5′-triphosphate tetrasodium salt), thapsigargin, EGTA, U73122 (1-[6-[(17β)-3-methoxyestra-1,3,5(10)-tri-en-17-yl]amino][hexyl]-1H-pyrrrole-2,5-dione), ryanodine, cyclopiazonic acid (CPA), 4,4′-disothiocyana-toxystilbene-2,2′-disulfonyl acid disodium salt hydrate (DIDS), NMDG, tamoxifen ((Z)-1-p(dimethylamino)oxyphenyl)-1,2-diphenyl-1-buten, IBTX, firefly lantern extract (luciferyl-luciferase), the BKca channel opener NS1619, nystatin, and AR67156, were obtained from Sigma-Aldrich. PiPADS (pyrdoxal-phosphate-6-zophenyl2,4′-disulfonyl acid) was purchased from Calbiochem.

**Data Analysis**

Results are given as the mean ± SEM. Data were analyzed with the paired or unpaired Student’s t test, the Fisher exact test, and the χ² test. Two-way ANOVA with the Bonferroni posttest was used to evaluate differences between control and drug effects in the recorded currents. P < 0.05 was considered statistically significant.

**RESULTS**

**Modulation of K⁺ and Cl⁻ Currents in the TM by Cell Swelling**

It has been reported that hypertonicity triggers an RVD in TM cells and that it also decreases outflow facility in perfused anterior segments. In the present study, we further examined the effects of hypertonic stimuli on whole-cell currents mediated by the BKca and the possible activation of Clswell in TM cells. As shown in Figure 1A, whole-cell currents were first recorded in isotonic physiological solution (control) and afterward bath solution was replaced by hypotonic medium. Cells were clamped at −60 mV, and depolarizing pulses to +80 mV were applied in 10-mV steps to evoke outward BKca currents. In hypotonic medium, the total outward K⁺ current increased significantly (ANOVA; P < 0.001) in 15 of 15 whole-cell experiments (87% of the cells). Bonferroni posttests showed significant differences between the hypotonic and isotonic groups, at −10 mV (P < 0.05) and from 0 to +80 mV (P < 0.001). Two of 15 cells did not show significant changes. Figure 1A shows a typical recording and the current-voltage (I/V) relationship in isotonic and hypertonic conditions. In agreement with previous data, hypertonic solution did not modify [Ca²⁺]; thus, it is not likely that the BKca current increase was due to an increase in [Ca²⁺].

In a different set of experiments, cells were voltage clamped at 0 mV, and whole-cell currents were elicited by using specific solutions to record Cl⁻ currents (see the Methods section). Under isotonic conditions, TM cells possessed a small current of −3.0 ± 1.3 pA/pF (mean ± SEM) at an applied potential of −80 mV and 2.8 ± 1.2 pA/pF at +80 mV (Fig. 1B). When isotonic medium was replaced by a hypotonic one, Clswell currents were activated within 4 minutes and reached a maximum between 10 and 12 minutes (−8.2 ± 2.1 pA/pF at −80 mV and 14.4 ± 4.5 pA/pF at +80 mV; mean ± SEM). Accounting all the cells recorded in the whole study, Clswell current activation was seen in 140 (85%) of 164 cells. In the experimental group shown in Figure 1B, cells in isotonic medium (n = 5) were first exposed to hypotonic medium and, on maximum Clswell current activation, hypotonic medium was replaced, returning to isotonic medium. Statistical comparison of the I/V curves in isotonic and hypotonic conditions showed significant differences for the whole voltage range (−80 to +120 mV; ANOVA, P < 0.001). Bonferroni posttests showed significant differences between the isotonic and hypotonic groups, at +80 mV (P < 0.01) and +120 mV (P < 0.001; Fig. 1B). When returning to isotonic medium, Clswell currents decreased, and significant differences between isotonic and hypotonic conditions were also recorded at +80 mV (P < 0.01) and +120 mV (P < 0.001). All recordings were obtained with ATP and GTP in the intracellular solution. In a group of recordings (n = 5) where these compounds were omitted, no Clswell
Figure 1. Hypotonic medium increased K+ and Cl− currents. (A) Left: representative experiment showing whole-cell BKCa currents in a TM cell in isotonic and hypotonic media. Vh = −60 mV. Right: current–voltage curve showing outward BKCa currents of TM cells in the whole-cell configuration (n = 15). Currents are expressed as current densities (pA/μF). Data are for isotonic (301 mOsm/kg) and hypotonic (213 mOsm/kg) media. BKCa current increased significantly (ANOVA, P < 0.001). **P < 0.01, ***P < 0.001 hypotonic versus isotonic medium (Bonferroni posttest). Outward currents were blocked by IBTX (data not shown). (B) Left: representative experiment showing whole-cell Clswell in a TM cell in isotonic, then hypotonic, and returning to isotonic media. Vh = 0 mV. Right: Current–voltage curve showing outward Clswell currents of TM cells in the whole-cell configuration (n = 5). Data are shown for isotonic medium baseline, hypotonic medium, and isotonic medium return to baseline. Clswell current increased significantly (ANOVA, P < 0.001, hypotonic versus isotonic medium (Bonferroni posttest). + +P < 0.01, + + +P < 0.001 return to isotonic baseline versus hypotonic.

Current activation was seen in hypotonic medium (data not shown). This result is in agreement with previous data in other cell types showing the ATP dependency of Clswell current activation.31

As shown in the recordings displayed in Fig. 1B, Clswell currents are typically characterized by an outward rectification and a time-dependent decay at depolarizing voltages. To characterize Clswell currents further, we replaced Cl− for different anions to establish the channel anion permeability sequence. Figures 2A–D show mean voltage ramps for each anion after Cl− replacement in the hypotonic solution (SEM was omitted for clarity). Calculation of reversal potential (Vrev) and relative anion permeabilities (P/PCl, Fig. 2, table) showed an anion permeability sequence I− > Br− > Cl− > F− > gluconate− which is in agreement with previous data for this channel.32–34

Pharmacological characterization of Clswell currents is shown in Figure 3. After activation of Clswell currents in hypotonic solution, Cl− channel blockers were added to the solution, and their effects at depolarized and hyperpolarized potentials were evaluated. Tamoxifen (100 μM; n = 5), a known selective Clswell blocker, decreased outward and inward Clswell currents in a voltage-independent manner along the entire IV relationship (ANOVA P < 0.001, hypotonic versus hypotonic + tamoxifen). As shown in Figure 3A, tamoxifen blocked Clswell currents both at hyperpolarized (55% at −80 mV; t-test, P < 0.05 vs. hypotonic) and depolarized potentials (60% at +80 mV; t-test, P < 0.05). Next, we tested the effect of DIDS, a nonselective Cl− channel blocker. By contrast, addition of DIDS (100 μM; n = 6 cells) to the hypotonic medium significantly blocked only the outward currents (ANOVA, I-V relationship hypotonic + DIDS versus hypotonic, P < 0.001). DIDS decreased outward current by 40% (at +80 mV; t-test, P < 0.05), but did not affect inward current (6% at −80 mV; t-test, NS). Blocking effects of both tamoxifen and

<table>
<thead>
<tr>
<th>Anion (x)</th>
<th>Vrev (mV)</th>
<th>Px / PCl</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>I−</td>
<td>1.3 ± 1.2</td>
<td>1.36</td>
<td>6</td>
</tr>
<tr>
<td>Br−</td>
<td>2.6 ± 1.7</td>
<td>1.31</td>
<td>7</td>
</tr>
<tr>
<td>Cl−</td>
<td>6.3 ± 1.0</td>
<td>1.0</td>
<td>9</td>
</tr>
<tr>
<td>F−</td>
<td>17.8 ± 1.0</td>
<td>0.71</td>
<td>5</td>
</tr>
<tr>
<td>Gluconate−</td>
<td>34.7 ± 2.1</td>
<td>0.37</td>
<td>6</td>
</tr>
</tbody>
</table>

Vrev values were obtained from I-V plots as indicated in Methods. Relative anion permeabilities (Px / PCl) were calculated using the Goldman-Hodgkin-Katz equation.

Figure 2. Effect of Cl− ion replacement on reversal potentials of Clswell. Left: reversal potential (mean ± SEM), relative permeability, and number of experiments for each anion. Right: mean recordings in the whole-cell configuration of the patch-clamp technique for (solid lines) Cl− and (dotted lines) I− (A), Br− (B), F− (C), and gluconate− (D). SEM was omitted for clarity. Voltage ramps where recorded after activation of Clswell current in hypotonic medium. Cl− was replaced by each anion.
DIDS are characteristic of Cl<sub>swell</sub> currents, as described previously. These results are in agreement with electrophysiological characterization and further corroborate that the currents recorded are mediated by Cl<sub>swell</sub> channels. To characterize the currents further, we tested the effect of extracellularly applied ATP (1 mM), which has also been reported to block Cl<sub>swell</sub> currents. Extracellular ATP blocked Cl<sub>swell</sub> currents in a voltage-dependent manner along the entire I-V curve (ANOVA, \( P < 0.001; n = 6 \); Fig. 3C). Extracellular ATP blocked Cl<sub>swell</sub> currents both at hyperpolarized (28% at \(-80 \) mV; \( t \)-test, \( P < 0.05 \) vs. hypotonic) and depolarized potentials (52% at \(+80 \) mV; \( t \)-test, \( P < 0.01 \)).

**Cell Swelling’s Effects on ATP Release in TM Cells**

It has been reported that ATP can be released to function as an autocrine/paracrine signal in several cell types after osmotic challenge or in response to drug stimulation. In TM cells grown in 35-mm culture dishes, DMEM was replaced by an isotonic physiological solution (see the Methods section), and cells were allowed to rest for 30 minutes to minimize a possible ATP release due to medium exchange. Stimulation of TM cells with isotonic medium did not elicit a significant release of ATP into the extracellular solution (0.004 \pm 0.0006 picomoles or 0.001 \pm 0.002 nM; mean \pm SEM; \( n = 3 \); Figs. 4A, 4B). When medium hypotonicity was reduced by 20%, 2.76 \pm 0.18 picomoles of ATP was released, showing the same kinetics (1.56 \pm 0.08 nM; \( t \)-test \( P < 0.001 \) vs. isotonic; \( n = 5 \)). After reaching a peak, ATP release decreased exponentially to recover baseline levels within 400 to 500 seconds after stimulation. When medium hypotonicity was reduced by 40%, 4.35 \pm 0.23 picomoles of ATP was released, showing the same kinetics (1.56 \pm 0.08 nM; \( t \)-test \( P < 0.001 \) vs. isotonic; \( n = 4 \)).

**FIGURE 3.** Cl<sub>swell</sub> pharmacology. \( V_h = 0 \) mV in all three experiments. (A) Left: representative experiment showing whole-cell Cl<sub>swell</sub> in a TM cell in isotonic, hypotonic, and hypotonic + tamoxifen (100 \( \mu \)M) media. Tamoxifen decreased outward and inward currents. Right: mean \pm SEM outward (at \(+80 \) mV) and inward (at \(-80 \) mV) current densities (pA/pF) recorded in five TM cells. (B) Left: representative experiment showing whole-cell Cl<sub>swell</sub> in a TM cell in isotonic, hypotonic, and hypotonic + DIDS (100 \( \mu \)M) media. DIDS decreased outward but not inward currents. Right: Current densities are as in (A) and were recorded in six TM cells. (C) Left: representative experiment showing whole-cell Cl<sub>swell</sub> currents in a TM cell in isotonic, hypotonic, and hypotonic + extracellular ATP (1 mM) media. ATP decreased outward and inward currents. Right: Current densities are as in (A) and were recorded in six TM cells. Note that some Cl<sub>swell</sub> currents inactivated at strong depolarized potentials (hypotonic in A), but the common pattern was an increasingly rapid inactivation as the depolarization increased (hypotonic in B and C). Significant differences were recorded between isotonic and hypotonic medium (*\( P < 0.05 \); \( t \)-test) and between hypotonic medium and hypotonic + blocker (\(* * P < 0.05 \) and \(* * * P < 0.001 \); \( t \)-test).
Effect of ATP on Purinergic P2Y<sub>2</sub> Receptors in TM Cells

The fact that TM cells release ATP (and maybe other nucleotides) in response to certain stimuli suggests that these cells may express purinergic receptors to permit a physiological function of this nucleotide in the outflow pathway. Therefore, we tested this hypothesis by stimulating TM cells with different nucleotides while recording intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>). Resting [Ca<sup>2+</sup>]<sub>i</sub> in TM cells was found to be between 58 and 89 nM. In the control group, only 3% of the cells increased [Ca<sup>2+</sup>]<sub>i</sub> after drug vehicle application (Table 1). In contrast, ATP induced significant increases in [Ca<sup>2+</sup>]<sub>i</sub> in 99% of TM cells between 10<sup>−6</sup> and 10<sup>−4</sup> M (Table 1; Fig. 5). At lower concentrations, the percentage of response varied between 2% and 70% of the cells (10<sup>−10</sup>-10<sup>−7</sup> M; Table 1), therefore ATP stimulation followed a sigmoidal dose-response curve with an EC<sub>50</sub> of 2.64 × 10<sup>−8</sup> M (Fig. 5H). Figure 5A shows a typical profile of [Ca<sup>2+</sup>]<sub>i</sub> mobilization induced by ATP. [Ca<sup>2+</sup>]<sub>i</sub> increased rapidly to reach a peak and then decayed slowly to resting levels. [Ca<sup>2+</sup>]<sub>i</sub> peaks followed a dose-re-

<table>
<thead>
<tr>
<th>Drug</th>
<th>Basal [Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt; (nM)</th>
<th>Peak [Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Vehicle)</td>
<td>64 ± 5</td>
<td>169 ± 19</td>
</tr>
<tr>
<td>ATP dose-response relationship</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10&lt;sup&gt;−10&lt;/sup&gt; M</td>
<td>72±7§</td>
<td>215 ± 51</td>
</tr>
<tr>
<td>10&lt;sup&gt;−9&lt;/sup&gt; M</td>
<td>112±11§</td>
<td>128 ± 12</td>
</tr>
<tr>
<td>10&lt;sup&gt;−8&lt;/sup&gt; M</td>
<td>109±110§</td>
<td>234 ± 71</td>
</tr>
<tr>
<td>10&lt;sup&gt;−7&lt;/sup&gt; M</td>
<td>97±99§</td>
<td>311 ± 11§</td>
</tr>
<tr>
<td>10&lt;sup&gt;−6&lt;/sup&gt; M</td>
<td>311±315§</td>
<td>515 ± 121§</td>
</tr>
<tr>
<td>10&lt;sup&gt;−5&lt;/sup&gt; M</td>
<td>128±129§</td>
<td>567 ± 161§</td>
</tr>
<tr>
<td>10&lt;sup&gt;−4&lt;/sup&gt; M</td>
<td>82±83§</td>
<td>587 ± 177§</td>
</tr>
<tr>
<td>Purinergic agonists and antagonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTP 1 μM</td>
<td>516±522§</td>
<td>414 ± 8§</td>
</tr>
<tr>
<td>ADP 1 μM</td>
<td>145±427‡‡</td>
<td>359 ± 11§</td>
</tr>
<tr>
<td>UDP 1 μM</td>
<td>108±324‡‡</td>
<td>314 ± 14§</td>
</tr>
<tr>
<td>ATP-γS 1 μM</td>
<td>414±438‡</td>
<td>371 ± 7§</td>
</tr>
<tr>
<td>2-MeS-ATP 1 μM</td>
<td>29±106‡</td>
<td>422 ± 40§</td>
</tr>
<tr>
<td>PPADS 10 μM + ATP 1 μM</td>
<td>520±352§</td>
<td>387 ± 10§</td>
</tr>
<tr>
<td>Suramin 100 μM + ATP 1 μM</td>
<td>311±453‡‡</td>
<td>386 ± 10§</td>
</tr>
<tr>
<td>Intracellular mechanisms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP 1 μM (0 Ca&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td>456±464§</td>
<td>464 ± 8§</td>
</tr>
<tr>
<td>ATP-γS 1 μM (0 Ca&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td>115±116§</td>
<td>376 ± 18§</td>
</tr>
<tr>
<td>Thapsigargin 1 μM + ATP 1 μM</td>
<td>6±235‡‡</td>
<td>351 ± 47§</td>
</tr>
<tr>
<td>U73122 1 μM + ATP 1 μM</td>
<td>185±419‡‡</td>
<td>324 ± 10§</td>
</tr>
<tr>
<td>CPA 1 μM + ATP 1 μM</td>
<td>80±292‡‡</td>
<td>338 ± 10§</td>
</tr>
<tr>
<td>Ryanodine 1 μM + ATP 1 μM</td>
<td>171±176§</td>
<td>371 ± 14§</td>
</tr>
</tbody>
</table>

[Ca<sup>2+</sup>]<sub>i</sub> values are mean ± SEM. [Ca<sup>2+</sup>]<sub>i</sub> peaks were considered significant when they were at least two times basal levels and means were calculated only in the responder cells. Comparisons between the proportion of responder cells in the control group and the ATP-treated groups (*) were made using the Fisher exact test. Comparisons between the average [Ca<sup>2+</sup>]<sub>i</sub> peak elicited by BTM cells in the control group and in ATP-treated groups (†) were made using Student’s unpaired t-test. ‡<sup>P</sup> < 0.001 by Fisher exact test. †<sup>P</sup> < 0.05, ††<sup>P</sup> < 0.01 and †††<sup>P</sup> < 0.001 by Student’s unpaired t-test. Comparisons between the proportion of responder cells in the ATP 1 μM-treated group and the other groups (‡) were made using the Fisher exact test. Comparisons between the average [Ca<sup>2+</sup>]<sub>i</sub> peaks elicited by BTMs cells in the ATP 1 μM-treated group and the other groups (§) were made using Student’s unpaired t-test. ‡‡<sup>P</sup> < 0.01, ‡‡‡<sup>P</sup> < 0.001 by the Fisher exact test. §<sup>P</sup> < 0.05, §§<sup>P</sup> < 0.001 by Student’s unpaired t-test.
response curve with a maximum increase of 587 ± 17 nM at 10⁻⁴ M. To characterize further the receptors involved in the ATP response, we stimulated TM cells with different purinergic agonists and determined the response profile. UTP (1 μM) like ATP at the same concentration, induced [Ca²⁺]ᵢ mobilizations in 99% of the cells (Table 1, Figs. 5B, 5G). Nevertheless, the diphosphate nucleotides (ADP and UDP; 1 μM) increased [Ca²⁺]ᵢ in only 34% and 33% of the cells, respectively (Fig. 5C, 5D, 5G; Table 1; *P < 0.01 vs. ATP). Also, [Ca²⁺]ᵢ peaks were smaller than the ones elicited by ATP (1 μM). ATP-γ-S (1 μM), a nonhydrolyzable analogue, showed the same results as ATP. 2-MeS-ATP (1 μM), an agonist for P2Y₁ and P2X receptors, evoked [Ca²⁺]ᵢ mobilizations in 27% of TM cells (**P < 0.001 vs. ATP; Fig. 5G, Table 1). Finally, we tested ATP application in the presence of well-known purinergic receptor antagonists (Table 1; Figs. 5E–G). In the presence of 100 μM suramin, the percentage of response to ATP application decreased to 69% (P < 0.05 vs. ATP alone) while PPADS did not modify the percentage of responding cells to ATP when present in the bath solution. These results show a response profile to the different nucleotides as ATP = UTP > ATP-γ-S > ADP = UDP which suggests the presence of a P2Y₂ purinergic receptor. Moreover, as described previously, P2Y₂ receptors are partially inhibited by suramin but not by PPADS, which argues in favor of the presence of those receptors. Also, the effect of 2-MeS-ATP suggests the presence of P2Y₁ receptors.

We further examined the intracellular mechanisms involved in the ATP-induced [Ca²⁺]ᵢ increase in TM cells. In nominally Ca²⁺-free solution (+1 mM EGTA), both ATP and ATP-γ-S (1 μM), stimulated the same percentage of cells as ATP in the normal buffer solution (Table 1). No differences were seen on intracellular Ca²⁺ peak amplitude compared with ATP in the presence of calcium. Nevertheless, recovery phase to resting [Ca²⁺]ᵢ was shorter than with ATP in normal buffer solution (Fig. 6A). An ATP-induced [Ca²⁺]ᵢ increase was prevented by preincubation of cells with thapsigargin (***P < 0.001; Table 1, Figs. 6A, 6B). A significant decrease in the percentage of cells that had increased [Ca²⁺]ᵢ, after ATP exposure was seen in the presence of U73122, an inhibitor of phospholipase C.³⁸ (P < 0.001) and CPA, a Ca²⁺-ATPase inhibitor,³⁹ (P < 0.001), but not after preincubation with ryanodine.

As previously stated, hypotonic stimuli did not modify [Ca²⁺]ᵢ, which is inconsistent with release of ATP. To explore this possibility, we measured [Ca²⁺]ᵢ, after a hypotonic stimuli.
similar to the ones obtained with ATP (Fig. 6C). Ca\(^{2+}\) peaks elicited by hypotonic stimuli in the presence of ARL67156 were blocked by suramin (100 \(\mu M\)).

**ATP Increases BK\(_{ca}\) Current**

One of the possible ATP functions after activation of purinergic receptors, in particular, would be to increase K\(^+\) efflux from the cell thus contributing to cell volume recovery. We performed experiments to test this hypothesis using the whole-cell perforated patch technique, which prevents dialysis of intracellular content. After achieving this configuration, we clamped TM cells at -60 mV and BK\(_{ca}\) currents were recorded in resting conditions with a physiological solution in the bath (\(n = 6\); Fig. 7A). Superfusion of ATP at 10 \(\mu M\) significantly increased BK\(_{ca}\) currents along the 1:1 relationship (ANOVA \(P < 0.001\) vs. baseline physiological solution) in seven (78%) of nine tested. Bonferroni posttests showed significant differences at +60 (\(P < 0.05\)), +80 (\(P < 0.01\)), and +100 (\(P < 0.001\)) mV versus the baseline recording. After drug washout, BK\(_{ca}\) currents returned to baseline values, as can be seen in the recording and I-V plot displayed in Figure 7A. Next, we performed experiments in the presence of suramin (100 \(\mu M\); \(n = 5\); Fig. 7B) to test the receptor-mediated effect of ATP. After recording BK\(_{ca}\) currents in physiological solution (baseline) and in the presence of suramin (no effect was seen in the presence of suramin alone), we applied ATP at the same concentration as that used in the previous experiment. In the presence of the blocker, ATP induced a slight nonsignificant increase in BK\(_{ca}\) currents (Fig. 7B). These results confirm that the effects on BK\(_{ca}\) currents are mediated through activation of specific purinergic receptors present in TM cells.

**Participation of RVD Mechanisms in Outflow Facility Modulation**

It has been reported that hypotonic medium decreases outflow facility.\(^{2,3}\) In the current study, we perfused eye anterior segments (\(n = 6\)) for 90 minutes, to establish baseline outflow facility and therefore isotonic perfusion medium (301 \(\pm\) 1 mOsm/kg) was replaced with a hypotonic one (244 \(\pm\) 2 mOsm/kg) and maintained during 360 minutes (Fig. 8A). This moderate hypotonic shock decreased outflow facility significantly at 15 minutes (20\%, \(P < 0.001\); Fig. 8), and then it recovered gradually to baseline levels (3\% over baseline outflow facility, \(t\)-test, NS). The results obtained permitted us to hypothesize that outflow facility is closely linked to TM cell volume and that RVD mechanisms may contribute to recovery of outflow facility after hypotonic shock or other insults that may affect cell volume. We tested this hypothesis by adding drugs known to block or activate selectively the ionic channels that participate in RVD mechanisms. Anterior segments (\(n = 6\)) perfused in the presence of 100 \(\mu M\) tamoxifen showed a more pronounced decrease in outflow facility (25\%, \(P < 0.001\)) after 15 minutes in hypotonic medium compared with the perfusions with hypotonic medium alone (Figs. 8A, 8B). More interesting, they did not recover baseline outflow facility even after 360 minutes (12\% below baseline values; \(P < 0.001\) vs. hypotonic medium). Similar results were obtained when hypotonic medium was supplemented with IBTX (10 nM). When both drugs (IBTX and tamoxifen) were added together (\(n = 6\)), not only was the initial decrease in outflow facility more pronounced than in the previous groups (34\%, \(P < 0.001\)), but the recovery phase was the slowest. After 360 minutes, outflow facility was still 17\% below baseline (\(P < 0.001\)). On the contrary, perfusion with hypotonic medium plus a BK\(_{ca}\) channel opener (NS1619; 30 \(\mu M\), \(n = 6\)) showed opposite results. The initial decrease in outflow facility was smaller than in hypotonic medium alone (14\%, \(P < 0.001\)) and recovery to
baseline was faster than in the absence of the drug. In fact, outflow facility achieved baseline within ~45 minutes after hypotonic shock (Figs. 8A, 8B).

**DISCUSSION**

In this study, cellular mechanisms involved in TM cell volume regulation were essential in determining AH outflow facility. We put together data from the cellular level with measurements of AH evacuation showing that modulation of certain channels or receptors may have a great influence on outflow facility.

Different factors have been involved in modulating outflow resistance through the TM (see the introduction). Several studies\(^2\,5\,17\,19\) have focused on the changes in TM cell volume, since previous data from our laboratory\(^3\) and others\(^2\,5\,19\) have shown that osmolality changes in the perfusion medium evoke swelling or shrinkage of TM cells that modify outflow facility. Also, it has been reported that TM cells trigger an RVD mechanism in response to osmotic insults that may be involved in outflow facility modulation.\(^19\) In fact, several different membrane proteins that are thought to participate in cell volume regulation have been identified in TM cells, such as the BK calcium channel,\(^6\,26\) a Cl\(^-\)swell channel,\(^19\) an Na\(^+-\)K\(^+\)-2Cl\(^-\) symporter,\(^19\) an Na\(^+/\)H\(^+\) antiporter,\(^41\) a K\(^+\)-Cl\(^-\) symporter,\(^19\) and a Cl\(^-\)/HCO\(_3\)\(^-\) exchanger.\(^19\) Moreover, other mechanisms such as ATP release from nerve terminals or due to cell swelling may also be involved in cell volume regulation.\(^23\,25\)

AH appears to be slightly more hypertonic than plasma,\(^4\,2\,45\) and it is likely that changes in AH tonicity influence TM cell volume, although they have not been described to date. More important may be drug-induced changes in TM cell volume (e.g., norepinephrine and adenosine agonists).\(^17\,40\,44\) O’Donnell et al.\(^17\) showed that bumetanide, ethacrynic acid, 8Br-cAMP, and 8Br-cGMP among others, decreases volume in cultured TM cells through inhibition of the Na\(^+\)-K\(^+\)-2Cl\(^-\) symporter. In contrast, phorbol 12-myristate 13-acetate (PMA) increases cell volume. Bumetanide also increases outflow facility in human and calf eyes.\(^7\) Moreover, it has been reported recently that adenosine receptor agonists cause cell shrinkage in TM cells, probably because of Ca\(^+\) influx and Cl\(^-\) efflux.\(^19\) In fact, the adenosine agonist cyclohexyladenosine (CHA) has been reported to increase outflow facility in rabbit eyes,\(^45\) but it is unknown whether this effect is due to changes in cell volume in the TM. All these results seem to agree that TM cell shrinkage increases outflow facility, whereas cell swelling decreases it, as previously reported by changing perfusate osmolality.\(^7\,3\)

Our results show cell swelling activates two ionic conductances: a BK\(_{Ca}\) and a Cl\(_{swell}\). As described in several cell types, K\(^+\) and Cl\(^-\) effluces are accompanied by water to restore normal cell volume (RVD).\(^23\,25\) In particular, BK\(_{Ca}\) currents had an approximately threefold increase over the resting values in

![Figure 7](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932925/)
isotonic medium. Cell swelling activated $I_{\text{Cl,sellw}}$ as soon as 4 minutes after bath exchange to hypotonic medium and reached a maximum between 10 and 12 minutes. $I_{\text{Cl,sellw}}$ currents decreased when returning to isotonic medium (Fig. 1B) showing that their activation is a reversible process. Also, the effects of well-known blockers (tamoxifen and DIDS) of $I_{\text{Cl,sellw}}$ currents and their ionic selectivity are in agreement with previous reports in TM cells and other cell types. Special mention should be made to the effects of extracellular ATP on $I_{\text{Cl,sellw}}$ currents. After activation by hypotonicity, addition of ATP to the recording bath partially blocked $I_{\text{Cl,sellw}}$ currents (Fig. 3C). This effect has also been previously described, but its physiological relevance is unknown. We have also found, as previously reported (Cui M, et al. IOVS 2001;42:ARVO Abstract 745), that hypotonic stimuli trigger the release of ATP from TM cells. ATP, through activation of purinergic receptors, increased BK$_{Ca}$ currents (Fig. 7), perhaps contributing to the RVD. Nevertheless, the increase of ATP concentration in the extracellular environment may be part of a physiological mechanism to prevent excessive activation of $I_{\text{Cl,sellw}}$, since the blocking effect is only at high concentrations (in the millimolar range) but not at lower concentrations (micromolar) where the effect is to stimulate BK$_{Ca}$ currents. It should be mentioned that in the absence of hypotonic stimulus, ATP (in micromolar concentrations) activates $I_{\text{Cl,sellw}}$ currents in TM cells (data not shown) as seen in other studies. We should note that ATP can be released due to mechanical stimulation in TM cells (Cui M, et al. IOVS 2001;42:ARVO Abstract 745) or can reach the TM after being released in the AH by different ocular tissues and nerves (Fleischhauer SMJ, et al. IOVS 2001;42:ARVO Abstract 3146). Also, we cannot exclude that ATP can be liberated on cell stimulation with several agonists, as seen in other cell types. Therefore, it can be hypothesized that ATP acts in an autocrine/paracrine manner in the TM, regulating outflow facility by volume changes in TM cells. Moreover, Fleischhauer et al. have recently shown that released ATP may be a source of adenosine and that adenosine agonists reduce TM cell volume. Results obtained in the present study coincide with a profile for a P2Y$_{2}$ receptor. The presence of this receptor has been suggested (Cui M, et al. IOVS 2001;42:ARVO Abstract 745), and its activation leads to stimulation of phospholipase C, release of calcium from intracellular stores (IP$_{3}$-sensitive), and sustained influx of extracellular Ca$^{2+}$ (Fig. 6). The characteristics of Ca$^{2+}$ mobilization triggered by ATP and UTP are similar to the ones induced by other drugs such as bradykinin, carbamylcholine, or adenosine agonists.

As previously mentioned, ATP stimulation of TM cells increased BK$_{Ca}$ currents. This effect was partially blocked in the presence of suramin, implying a receptor-mediated mechanism. It is possible that BK$_{Ca}$-s were stimulated directly due to the increase in [Ca$^{2+}$]$_{i}$, but it is also possible that purinergic receptor stimulation triggers an intracellular cascade of second messengers to activate BK$_{Ca}$-s, probably through PKC. These two possibilities, which remain to be tested, are not exclusive, and both may be involved in the same final effect on BK$_{Ca}$-s. Of note, ATP released by hypotonic stimuli did not increase [Ca$^{2+}$]$_{i}$ in TM cells, probably due to a rapid degradation of this nucleotide by nucleotidases. Experiments performed with an inhibitor of nucleotidases in the hypotonic solution showed Ca$^{2+}$ peaks with characteristics similar to the ones elicited by ATP. This fact suggests that in cultured cells ATP is rapidly cleaved but in vivo locally released ATP may be sufficient to stimulate purinergic receptors and activate BK$_{Ca}$ currents. Further investigation should be conducted to describe the transduction pathway. In this study, BK$_{Ca}$-s were involved in ATP signaling and volume regulation, but they have also been shown to mediate the effects of purinergic agonists, nitric oxide, tyrosine kinase, and flufenamic acid and to detect membrane stretching. Therefore, BK$_{Ca}$-s play a central role in mechanical stimulation in TM cells, probably regulating the contractile state and the aqueous outflow facility. Taken together, these effects would finally regulate the AH outflow through the TM.

Hypotonic solutions decrease outflow facility and after an initial decline (Fig. 8), this parameter recovers to achieve baseline levels within ~2 hours, probably due to an RVD mechanism in TM cells, as shown by Mitchell et al. Outflow facility did not recover in the presence of tamoxifen, a $I_{\text{Cl,sellw}}$ blocker, and/or IBTX, a BK$_{Ca}$ blocker. The initial decline in

**FIGURE 8.** Influence of RVD mechanisms on modulation of outflow facility. (A) Outflow facility ratio (normalized with baseline outflow facility) is plotted against time. Anterior segments were perfused with control medium (DMEM) for 90 minutes (baseline), and then perfusion medium was replaced (arrow) by hypotonic DMEM with or without drugs, and the solution was maintained until the end of the experiment. Data are shown for hypotonic (n = 6), hypotonic+tamoxifen (100 µM, n = 6), hypotonic+tamoxifen+IBTX (10 nM, n = 6), hypotonic+BK$_{Ca}$ opener (NS1619, 30 µM, n = 6), and hypotonic+IBTX (10 nM, n = 6). The initial decrease in outflow facility was reversed in the presence of K$^{+}$ and Cl$^{-}$ channel blockers. Also, recovery phase to baseline was impaired in the presence of the blockers. On the contrary, the BK$_{Ca}$ channel opener decreased the initial decline in outflow facility in hypotonic medium and shortened the recovery phase. (B) Mean ± SEM outflow facility ratios measured after 15 minutes in the hypotonic medium (or hypotonic medium+drugs) and after 360 minutes of perfusion in hypotonic medium. Time after 15 minutes is representative of the initial decrease in outflow facility due to hypotonic medium. Measures after 360 minutes in hypotonic medium are representative of the recovery phase. Significant differences (t-tests) are shown between each group and the control group (baseline in isotonic medium): *P $<$ 0.05, **P $<$ 0.01, ***P $<$ 0.001.
outflow facility after 15 minutes in hypotonic medium is even greater in the presence of Cl− and K+ blockers (Figs. 8A, 8B) suggesting that RVD quickly activates to recover cell volume, which shows a direct correlation with outflow facility values. The opposite occurs when using N1619, a BKCa activator, the initial decrease in outflow facility is smaller and the time to recover baseline values is shorter. Therefore, activators of BKCa may exert a protective effect against osmotic insults or drugs that modify cellular volume.40 It may also be possible that activators of BKCa like nitric oxide or cGMP may be endogenously released to modulate outflow facility, although further investigations are necessary to test this hypothesis. Finally, specific activators of Clswell (unknown to date with the exception of ATP at low concentrations) would also be interesting molecules to modulate outflow facility. Because TM cell function can be modulated by changes in cell volume, selective drugs targeting cell volume regulatory mechanisms would increase outflow facility without damaging either the cells or the extracellular matrix. The present results and others17,19,20,40,44,53 support this idea.

In summary, our findings show that cellular mechanisms that regulate cell volume in TM cells play an important role in outflow facility regulation. In particular, K+ and Cl− channel activation is directly involved in the recovery of baseline outflow facility after hypotonic shock. Therefore, cell volume regulation appears to be part of the homeostatic mechanisms involved in the regulation of the outflow pathway, actively contributing to TM function.

Acknowledgments
The authors thank Pablo Perez de la Ossa for advice and assistance in the LDH determinations.

References
33. Schmid A, Blum R, Krause E. Characterization of cell volume-sensitive chloride currents in freshly prepared and cultured pan-
40. Sabirov RZ, Dutta AK, Okada Y. Volume-dependent ATP-conduc
tive large-conductance anion channel as a pathway for swelling-induced ATP release. J Gen Physiol. 2001;118:251–266.
46. Sabirov RZ, Dutta AK, Okada Y. Volume-dependent ATP-conduc
tive large-conductance anion channel as a pathway for swelling-induced ATP release. J Gen Physiol. 2001;118:251–266.
51. Tanihara H, Ohuchi T, Yoshimura N, Negishi M, Ito S. Heteroge