Regulatory Roles of Anoctamin-6 in Human Trabecular Meshwork Cells

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Purpose. Trabecular meshwork (TM) cell volume is a determinant of aqueous humor outflow resistance, and thereby IOP. Regulation of TM cell volume depends on chloride ion (Cl^-) release through swelling-activated channels ($I_{Cl,Swell}$), whose pore is formed by LRRC8 proteins. Chloride ion release through swelling-activated channels has been reported to be regulated by calcium-activated anoctamins, but this finding is controversial. Particularly uncertain has been the effect of anoctamin Ano6, reported as a Ca^{2+}-activated Cl^- (CaCC) or cation channel in other cells. The current study tested whether anoctamin activity modifies volume regulation of primary TM cell cultures and cell lines.

Methods. Gene expression was studied with quantitative PCR, supplemented by reverse-transcriptase PCR and Western immunoblots. Currents were measured by ruptured whole-cell patch clamping and volume by electronic cell sizing.

Results. Primary TM cell cultures and the TM5 and GTM3 cell lines expressed Ano6 3 to 4 orders of magnitude higher than the other anoctamin CaCCs (Ano1 and Ano2). Ionomycin increased cell Ca^{2+} and activated macroscopic currents conforming to CaCCs in other cells, but displayed significantly more positive mean reversal potentials (+5 to +12 mV) than those displayed by $I_{Cl,Swell}$ (−14 to −21 mV) in the same cells. Nonselective CaCC inhibitors (tannic acid>$CaCC_{inh}$–A01) and transient Ano6 knockdown strongly inhibited ionomycin-activated currents, $I_{Cl,Swell}$ and the regulatory volume response to hyposmotic swelling.

Conclusions. Ionomycin activates CaCCs associated with net cation movement in TM cells. These currents, $I_{Cl,Swell}$, and cell volume are regulated by Ano6. The findings suggest a novel clinically-relevant approach for altering cell volume, and thereby outflow resistance, by targeting Ano6.

Keywords: TMEM16F, $I_{Cl,Swell}$, regulatory volume decrease, calcium-activated Cl^- current, intraocular pressure, outflow facility

Glaucoma usually is associated with increased resistance (decreased outflow facility) of aqueous humor flow through the conventional trabecular outflow pathway.[1,2] For outflow to match inflow in glaucoma, IOP must rise to overcome the increased outflow resistance. The elevated IOP leads to death of retinal ganglion cells and optic atrophy. If optic atrophy is caused by elevated outflow resistance, the most direct intervention would be to lower trabecular outflow resistance.

Among other modulators of outflow,[3,4] cell volume within the trabecular meshwork (TM) pathway is linked to outflow resistance. Volume changes of TM, juxtacanalicular, and Schlemm canal cells produce transient changes in outflow resistance of human, nonhuman primate, and calf eyes.[5–7] These changes occur within approximately 15 minutes,[5,7] probably by distorting optimal fluid funneling in the juxtacanalicular regions of the outflow tract.[3,8] Volume changes also can exert slower, more sustained effects on outflow resistance over hours through a cascade initiated by altering TM-cell release of adenosine triphosphate (ATP).[9] Swelling of TM cells triggers release of ATP,[9] which in turn is converted to adenosine by ectoenzymes.[10,11] Adenosine then stimulates A_1 adenosine receptors to secrete metalloproteinases MMP-2[12] and MMP-9.[10] These metalloproteinases reduce outflow resistance.[13,14] The mechanism appears likely to be a rearrangement of the funneling of fluid destined for outflow by rearranging the morphology of the juxtacanalicular tissue and inner endothelial wall.[8] To the extent that Ca^{2+}-activated Cl^- channels (CaCCs) regulate TM cell volume,[15] these channels would be highly relevant for addressing glaucoma.

Cell volume regulation strongly depends on the swelling- and stretch-activated Cl^- channel ($I_{Cl,Swell}$, also known as VRAC, VSOR, VSOAC) in most cells[16] and specifically in human TM cells.[17] Following anisosmotic swelling, release of Cl^- through $I_{Cl,Swell}$ and K^+ through parallel K^+ channels drives water release, as well, restoring cell volume to isosmotic levels (regulatory volume decrease [RVD]). Chloride ion release through swelling-activated channels also releases organic osmolytes, such as taurine.[18] Trabecular meshwok cells display an RVD, both as isolated cells[17] and in intact human outflow

Table. Custom-Made Anoctamin Primers for RT-PCR

Target	Forward Primer	Reverse Primer	Product, bp	Annealing Temp
hAno1	CCTCACGGGCTTTGAAGAG	CTCCAAGACTCTGGCTTCGT	75	55°C
hAno2	TGGATGTGCAACAATTGAAGA	GCATTCTGCTGGTCACACAT	59	55°C
hAno3	TCAGAGCAGAAGGCTTGATG	AAACATGATATCGGGGCTTG	61	54°C
hAno4	TGACTGGGATTTGATAGACTGG	GCTTCAAACTGGGGTCGTAT	60	54°C
hAno5	TGGAAACATTAAAGAAGCCATTTA	GAGTTTGTCCGAGCTTTTCG	72	54°C
hAno6	AGGAATGTTTTGCTACAAATGGA	GTCCAAGGTTTTCCAACACG	72	55°C
hAno7S	GGCTCTTACGGGAGCACAG	CAAACGAGGACGAAGTCGAT	106	55°C
hAno7L	GCTCTGTGGTGATCGTGGT	GGCACGGTACAGGATGATAGA	71	55°C
hAno8	GGAGGACCAGCCAATCATC	TGCTCGTGGACAGGGAAC	72	55°C
hAno9	CAAACCCCAGCTGGAACTC	GGATCCGGAGGCTCTCTT	60	54°C
hAno10	CAGGGTCTTCAAACGTCCAT	TCATCGTTTCAAAAGCCAACT	73	55°C

tissue.[19] Studies using genome-wide RNAi screens have demonstrated that heteromers A, C, D, and E of the Leucine-Rich Repeat-Containing Protein 8 (LRRC8A,C,D,E) form an essential component of $I_{Cl,Swell}$.[18,20] Published evidence also suggests that a 10-member vertebrate family of calcium-activated anoctamins, Ano1-10 (*TMEM16* genes *A–K*) has an important role in cell volume regulation in other cells.[15] Agreement as to the role of anoctamins in cell volume regulation is incomplete, particularly since family members display several functions,[21] reportedly acting as CaCCs,[22–32] nonselective cation channels,[33] and scramblases or scramblase components, dissipating the phospholipid asymmetry across the plasma membrane.[34,35] Ano1[22–24,36,37] and Ano2[24,36,37] were the first and most clearly documented anoctamins to be CaCCs. More recently, Ano6 also has been demonstrated to function as an ion channel or channel component.[28,29,33,38] In contrast, Schreiber et al.[38] have reported that Ano9 and Ano10 expressed in Fisher Rat Thyroid (FRT) cells inhibit Ca^{2+}-activated Cl^- currents. However, the relative Na^+ to Cl^- permeability (P_{Na}/P_{Cl}) of Ano6 has been uncertain. In HEK293T cells overexpressing human Ano6, Shimizu et al.[29] found that Ano6 functioned exclusively as an anion channel, whereas Grubb et al.[28] reported that $P_{Na}/P_{Cl} = 0.3$. In contrast, Yang et al.[33] found that endogenous Ano6 in axolotl oocyte membranes and WT mouse megakaryocytes, as well as overexpressed mouse Ano6 in *Xenopus* oocyte membranes, acted as a nonselective cation channel ($P_{Na}/P_{Cl} \sim 7$).

In part, conflicting reports likely reflect known interactions of anoctamins with other anoctamins and other channels (Discussion) which may modify anoctamin function differentially in other cells, tissues, and organs. In addition, much information concerning anoctamins derives from overexpression in cell lines or oocytes. Thus, it currently is impossible to predict from published studies of other cells the potential role of anoctamin channels in TM cells.

Given the strong link between TM-cell volume regulation and outflow resistance, the potential role of anoctamins in outflow regulation, and the conflicting results obtained with other cells, we have tested whether anoctamins modulate the Ca^{2+}-activated currents, $I_{Cl,Swell}$, and RVDs of human TM cells in primary culture and transformed cell lines of normal and glaucomatous origin. The results suggested that Ano6 modulates TM-cell volume regulation, an observation of potential relevance in targeting outflow resistance.

Materials and Methods

Cellular Models

Transformed normal human TM cells (TM5) and glaucomatous TM cells (GTM3; both gracious gifts from Alcon Research, Inc., Fort Worth, TX, USA)[39] were maintained in Dulbecco's modified Eagle's medium (DMEM) high-glucose media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μg/ml of gentamicin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.[11] The glaucomatous GTM3 cells were studied in view of the relevance of CaCC cells to glaucoma (Introduction). Culture media were replaced every 3 days and cells subcultured 1:5 when reaching 90% confluence. Transformed normal human TM cells were studied in passages 20 to 38 and GTM3 cells in passages 23 to 102. Primary human TM cells (HTM)[38] were kept in DMEM low-glucose media with the same supplements; cells studied were from passages 4 to 7.[40] All reagents for cell culture were purchased from Gibco, Invitrogen (Carlsbad, CA, USA).

Reverse Transcription-PCR (RT-PCR)

Total RNA was isolated from cells with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and was treated with RNase-free DNase I to avoid possible contamination with genomic DNA. Reverse-transcription into cDNA then was performed with Taqman Reverse Transcription Reagents (Applied Biosystems [ABI], Foster City, CA, USA) following the manufacturer's instructions.[9] Polymerase chain reaction was performed with the AccuPrime Taq DNA polymerase High Fidelity Kit (Invitrogen) under the recommended conditions. Primers used for gene-specific amplification are shown in the Table. Polymerase chain reaction products were separated on 1% agarose gels containing 0.05% ethidium bromide. Bands were visualized under ultraviolet light, sized, and photographed by the Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA).

Real-Time Quantitative PCR (qPCR)

Cell cDNA templates were obtained as noted in the previous paragraph. The TaqMan gene expression assay was conducted at least in triplicate for each cDNA sample. TaqMan qPCR assays were done in 96-well plates with TaqMan 2X PCR Master Mix (P05837; ABI) using 7300 Real-Time PCR System (ABI) and default thermocycler program. Inventoried FAM-labeled MGB TaqMan probes for Ano1, Ano2, and Ano6 used in the assays were Hs00216121_m1, Hs00220570_m1, and Hs03805835_m1, respectively. The expression levels of indicated genes were calculated by the $2^{-\Delta\Delta Ct}$ method, with human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs99999905_m1, ABI) as the endogenous control.

Transient siRNA Knockdown of Ano6

Trabecular meshwork cells (0.2 million) were plated in the growth media specified above with serum, but without

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antibiotics, into 6-well tissue culture plates. After reaching 60% to 80% confluence, cells were transfected with siRNA directed against human Ano6 (20-60 pmol, sc-96071; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) using lipofectamine 2000 (Invitrogen). Control cells were transfected with scrambled RNA. Experimental and control cells were studied after reaching 90% confluence 48 hours later. Efficacy of transient knockdown was determined by qPCR.

Western Immunoblotting

Cells were lysed with RIPA buffer (Pierce, Rockford, IL, USA) supplemented with protease inhibitor cocktail (Complete Mini Tablets; Roche Diagnostics, Indianapolis, IN, USA).[11] The samples were sonicated for 30 seconds with a 50% pulse and cleared by centrifugation (10,000*g*) at 4°–8°C for 30 minutes. Supernatant protein concentrations were measured with bicinchoninic acid (BCA) protein assay reagent (Pierce). Homogenate containing 20 μg protein/lane was separated by SDS–PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Nonspecific binding was blocked with Protein-Free Blocking Buffers (Pierce) for 1 hour. Blots then were incubated overnight at 4°–8°C with primary antibodies, followed by 1-hour incubations with secondary antibodies conjugated to horseradish peroxidase (HRP), and finally developed by chemiluminescence detection (Super-Signal WestPico Substrate; Pierce). The primary antibodies used were to: Ano1 (rabbit polyclonal antibody ab72984, 1/500; Abcam, Cambridge, MA, USA); Ano6 (rabbit polyclonal antibody SC-136930, 1/100; Santa Cruz Biotechnology, Inc.); and GAPDH (mouse monoclonal antibody ab8245, 1/10,000; Abcam). Secondary sheep anti-mouse and goat anti-rabbit IgG-HRP antibodies (1/8000) were purchased from Amersham (GE Healthcare LifeScience, Buckinghamshire, UK).

Whole-Cell Patch Clamp

As previously described,[41,42] TM cells were trypsinized, resuspended, and allowed to settle on glass coverslips. Ruptured-patch, whole-cell currents in TM cells were measured with a MultiClamp 700B amplifier (Axon Instruments, Foster City, CA, USA) coupled to an external Bessel filter (model 900; Frequency Devices, Haverhill, MA, USA).

Micropipettes were prepared from Corning No. 7052 glass (World Precision Instruments [WPI], Sarasota, FL, USA) with a Flaming/Brown micropipette puller (P-97; Sutter Instruments Co., Novato, CA, USA), coated with Sylgard (WPI), and fire-polished with a microforge (MF-830; Narishige, Tokyo, Japan). Micropipette resistances were 2 to 4 MΩ, attaining several gigohms after seal formation. Potentials were measured in a perfusion chamber connected to a Ag/AgCl pellet in 3M KCl solution through a 3M KCl agar bridge. Step changes in potential from a holding potential (Vh) of 0 mV to values from +100 to −100 mV were applied in 20 mV decrements for 300 ms at 2-second intervals.

Data were recorded with a digital interface (Digidata 1322A; Molecular Devices, Union City, CA, USA) at 2 to 5 kHz and filtered at 500 Hz coupled with pClamp 9.2 software (Axon Instruments, Union City, CA, USA). Analysis was performed with the Clampfit 9.2 software (Axon Instruments). Unless otherwise stated, stimulations of current were measured at the maximum responses and inhibitions at the lowest values following drug application.

The micropipette filling solution contained (in mM): 24.2 NaCl, 110 aspartic acid, 120 *N*-methyl-D-glucamine base, 0.38 $CaCl_2$, 0.8 NaHEPES, 11.2 HEPES, 1.0 EGTA, 1.0 MgATP, and 0.01 GTP (~280 mosmol/kg H_2O, adjusted to pH 7.2). The isotonic bath contained (in mM): 110 NaCl, 6 HEPES, 6 NaHEPES, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 5 glucose, and 67 mannitol (~310 mosmol/kg H_2O, adjusted to pH 7.4). Swelling-activated Cl^- currents ($I_{Cl,Swell}$) were generated by removing the mannitol from the bath solution, reducing the osmolality to approximately 240 mosmol/kg H_2O at pH 7.4.

Cell Volume by Electronic Cell Sizing

Cell volume was monitored by electronic cell sizing using a Coulter counter (ZBI-Channelyzer II; Beckman Coulter, Inc., Brea, CA, USA) with a 100 μm aperture.[43] Transformed normal human TM cells were trypsinized and resuspended in isotonic solutions with or without drugs for 1 hour. The isotonic bath comprised (in mM): 110 NaCl, 15 HEPES, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 4.7 KCl, 1.2 KH_2PO_4, 30 $NaHCO_3$, and 10 glucose (~300 mOsm/kg H_2O, adjusted to pH 7.4). Thereafter, osmolality was reduced nearly 50% by lowering the NaCl concentration to 30.5 mM. The ensuing RVD was monitored by measuring cell volume at the time points indicated.

Intracellular Ca^{2+} Concentration by Fura-2

For measurements of free intracellular Ca^{2+} activity, cells grown on coverslips for 1 day were loaded in the dark with 5 μM fura 2-AM and 0.01% Pluronic F-127 (Molecular Probes, Eugene, OR, USA) for 60 minutes at room temperature and perfused with fura-free solution for 30 minutes before data acquisition was begun.[17] Coverslips were mounted on a Nikon Diaphot microscope and visualized with a ×40 oil-immersion fluorescence objective. The emitted fluorescence (520 nm) from approximately 75 cells at approximately 90% confluence was sampled at 1 Hz with the photomultiplier following excitation at 340 and 380 nm, and the ratio was determined with a Delta-Ram system and Felix software (Photon Technology International, Edison, NJ, USA). The ratio (R) of light excited at 340 nm to that at 380 nm was taken to be a direct index of free intracellular Ca^{2+} activity. That ratio was converted into free intracellular Ca^{2+} concentration with the method of Grynkiewicz et al.[44] An in situ Kd value for fura 2 of 350 nM was used.[45] The minimal ratio value (Rmin) was obtained by bathing cells in a Ca^{2+}-free isotonic solution of pH 8.0 containing 10 mM EGTA and 10 μM ionomycin. The maximal ratio value (Rmax) was obtained by bathing the cells in isotonic solution with 100 μM Ca^{2+} and 10 μM ionomycin. Calibration was performed separately for each experiment. Baseline levels from TM cells in the absence of fura 2 were subtracted from control records to correct for autofluorescence.

Statistics

Unless otherwise stated, Student's *t*-test was applied to compare two sets of data, and 1-way ANOVA was used for comparing three or more sets of data.[11] using the Holm-Sidak Multiple Comparison Procedure. Statistical analyses were conducted with SigmaStat (Aspire Software International, Ashburn, VA, USA). Results are presented as means ± SE, with *N* indicating the number of entries in the data set. A probability (*P*) less than 0.05 was considered statistically significant.

Results

Anoctamin Expression

Of the three anoctamins (Ano1, Ano2, and Ano6) most convincingly associated with plasma membrane ion currents,[24,46] Ano6 was most strongly detected by RT-PCR of mRNA from human TM5 cells. In the representative RT-PCR

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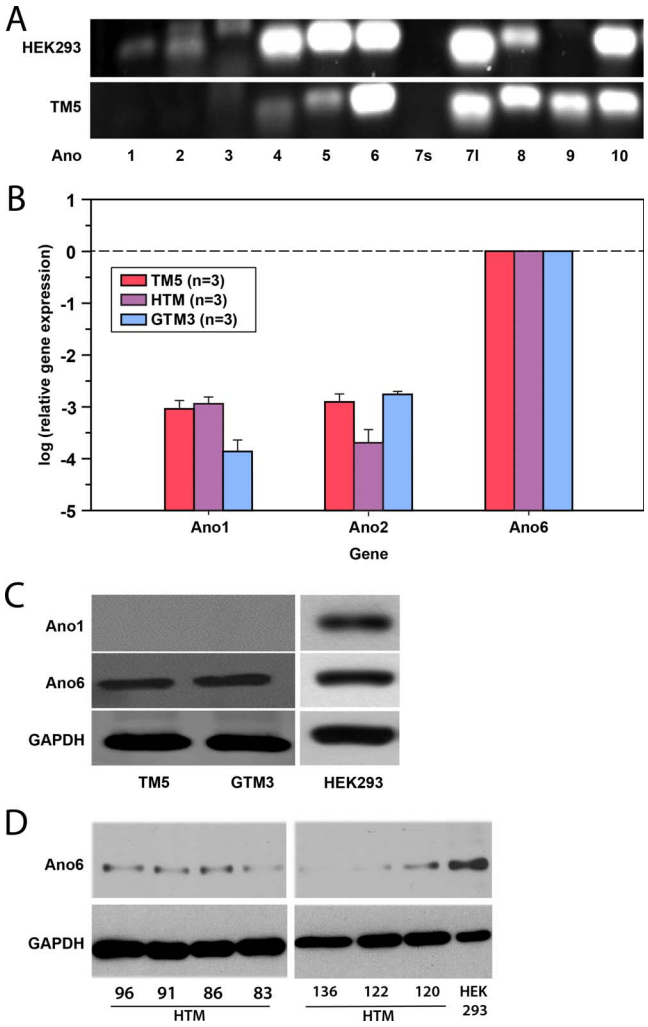

Figure 1. Expression of anoctamins in human TM cells at mRNA and protein levels. (**A**) Presents the gene expression of all human anoctamin isoforms Ano1-Ano10 (TMEM16A-K) from RT-PCR analyses ($N = 3$) of TM5 and HEK293 cells. The expected product sizes are entered in the Table. Ano7s is the short, and Ano7l the long, splice variant of Ano7 (TMEM16G). (**B**) *Bar graphs* display qPCR results indicating that TM5, HTM and GTM3 cells all express Ano1 and Ano2 three to four orders of magnitude lower than Ano6. Protein expression of Ano1 and Ano6 isoforms in TM5, GTM3, and HEK293 cells were assayed by Western blots (**C**), while Ano 6 protein expression in 7 different strains of human trabecular meshwork cells is shown in (**D**). Ano 1 protein was not detected in these human TM cell strains (not shown). The sizes of Ano1, Ano6, and GAPDH were 114kDa, 106kDa, and 36kDa, respectively.

analysis of RNA from TM5 cells of Figure 1A ($N = 3$), a strong band for Ano6 was observed but Ano1 and Ano2 were not detected. Bands for all three genes, *Ano1*, *Ano2*, and *Ano6*, were displayed for HEK293 cells, providing a positive control (Fig. 1A).

The relative gene expression of Ano1, Ano2, and Ano6 in the TM cell lines and in primary cultures of HTM cells was quantitatively measured by qPCR. Each measurement was conducted in triplicate. The means ± SE for three independent experiments are presented on the logarithmic ordinate scale of Figure 1B. The expression of Ano6 was 3 to 4 orders of magnitude greater than that of either Ano1 or Ano2 in each of the three cell preparations. For example, the expressions of Ano1 and Ano2 relative to Ano6 in HTM primary cultures were $(1.2 \pm 0.4) \times 10^{-3}$ and $(2 \pm 1) \times 10^{-4}$, respectively. In the absence of any dramatic difference in the relative expression of Ano1 or Ano2 to Ano6 in GTM cells from a glaucomatous patient, we did not pursue further studies with GTM cells.

Western immunoblot (Fig. 1C) verified in two independent experiments that protein product of Ano6, but not of Ano1, was expressed by the TM5 and GTM3 cell lines, whereas both proteins were readily detectable with HEK293 positive controls. We next screened seven different primary HTM cell strains and observed Ano6 protein expression in all studied (Fig. 1D).

Anoctamins Regulate Ca^{2+}-Triggered Plasma-Membrane Currents

In preliminary experiments ($N = 4$), increasing the Ca^{2+} concentration of the micropipette-filling solution to 15 μM triggered currents, but only after a delay of approximately 10 minutes. In contrast, perfusion with 5 μM ionomycin in the external bath raised intracellular Ca^{2+} concentration to approximately 650 nM (Fig. 2A) and consistently elicited CaCC-like currents (Fig. 2B), usually within 20 seconds. Because of the consistency and rapidity of the response, ionomycin was thereafter applied to elicit Ca^{2+}-activated currents. Ca^{2+}–activated Cl^- currents are characteristically outwardly-rectifying, displaying slow activation at highly positive intracellular potentials and deactivation at hyperpolarizing potentials.[47] Similar Ca^{2+}-activated currents were observed in all TM cell preparations, comprising the TM5 and GTM3 cell lines and the HTM primary cultures.

The reversal potentials (E_{rev}) of the currents activated in HTM and TM5 cells were positive. Measured 5 minutes after initiating ionomycin perfusion, the mean values of E_{rev} ± SE (in mV) were 12 ± 1 ($N = 15$, $P < 0.001$) and 5 ± 1 ($N = 8$, $P = 0.002$) for HTM and TM5 cells, respectively. Reversal potentials were 5 ± 3 ($N = 7$) in GTM3 cells, not significantly different from zero ($P = 0.141$). Probabilities were assessed by *t*-test after verifying normality by the Shapiro-Wilk test. The mean E_{rev} was significantly more positive for HTM cells than for TM5 ($P = 0.015$) and GTM3 cells ($P = 0.017$) by 1-way ANOVA, using the Holm-Sidak Multiple Comparison Procedure. Positive reversal potentials of ionomycin-activated currents are consistent with a dominant inflow of cations. For a positive control, E_{rev} of the swelling-activated Cl^- currents ($I_{Cl,Swell}$, Fig. 2C) at the same 5-minute endpoint was characteristically negative in TM cells, as previously reported.[17] In the present work, the E_{rev} values of $I_{Cl,Swell}$ were: −21 ± 3 ($N = 13$, $P < 0.001$), −14 ± 4 ($N = 13$, $P = 0.003$), and −19 ± 3 ($N = 4$, $P = 0.006$) for the HTM, TM5, and GTM3 cells, respectively. As for the ionomycin-activated currents, E_{rev} was measured approximately 5 minutes after hypotonic stimulation. The difference in E_{rev} between the ionomycin- and hypotonicity-stimulated currents was significant for each of the cell types, analyzed either by unpaired *t*-test when normality and equal variance were satisfied (GTM3 cells, $P < 0.001$) or otherwise by the Mann-Whitney Rank Sum Test (HTM cells, $P < 0.001$ and TM5 cells, $P < 0.001$). Reversal potentials for $I_{Cl,Swell}$ were not significantly different from one cell type to another ($P = 0.269$, 1-way ANOVA).

The effects of two nonspecific CaCC inhibitors on the Ca^{2+}-activated currents were measured after a baseline period of 5 minutes followed by ionomycin activation for 5 minutes before applying inhibitor. The percentage inhibitions after 5 minutes of exposure to inhibitor are presented in Figure 3A ($N = 4$–6). Both inhibitors reduced the Ca^{2+}-activated currents, tannic acid > $CaCC_{inh}$-A01[48,49] in TM5 and HTM cells. Tannic acid inhibits CaCC currents with an IC_{50} of 5.9 μM in FRT-TMEM16A cells and 3.1 μM in T84 cells (Fig. 3).[49] The CaCCinh-A01 inhibits CaCC currents with an IC_{50} of < 10 μM in T84 cells (Fig. 7).[48] Tannic acid also inhibited the Ca^{2+}-activated currents of GTM3

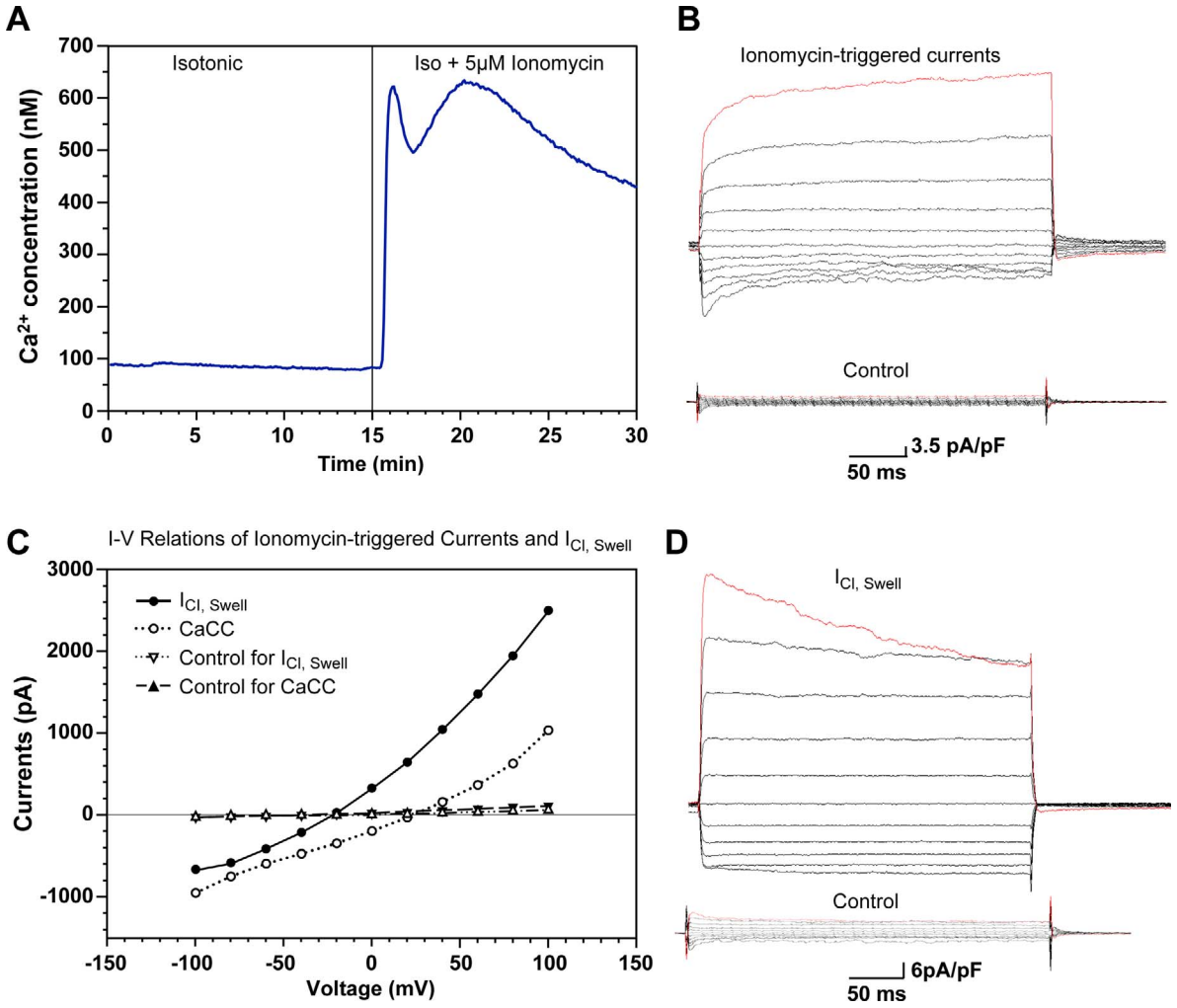

Figure 2. Effects of ionomycin and hypotonicity on TM-cell currents. (**A**) Displays the response of the free intracellular Ca^{2+} to perfusion with 5 μM ionomycin. (**B**) Presents representative traces of baseline whole-cell perforated patch currents and currents measured 3 minutes after perfusing the TM5 cell with ionomycin. The currents are the responses to clamping the membrane potential at test pulses from +100 to −100 mV at decrements of 20 mV from a holding potential of 0 mV. The peak current reached 1647 pA at +100 mV, the nadir was −963 pA at −100 mV, and the membrane capacitance was 28 pF. The reversal potential was +25 mV, far from the Cl^- Nernst potential of −39 mV and consistent with activation of net cation inflow. (**D**) Illustrates representative baseline currents and swelling-activated ($I_{Cl,Swell}$) currents 10 minutes after initiating hypotonic perfusion in another TM5 cell. The peak current reached 2491 pA at +100 mV, the nadir was −721 pA at −100 mV, and the membrane capacitance was 51 pF. The reversal potential was −21 mV, consonant with activation of net anion inflow. (**C**) Presents the current-voltage relationships for the experimental and baseline traces of (**B**) and (**D**), measured 14 msec after the application of the test pulses.

cells (Fig. 3A; $N = 5$). The effects of the CaCC inhibitors suggested that an anoctamin was subserving the Ca^{2+}-activated currents. The very high relative expression of Ano6 (Fig. 1B) pointed to that anoctamin as a likely conduit. This possibility was addressed by transient Ano6 knockdown (Fig. 3B). Knockdown efficiency was approximately 69 ± 15% in this study. The knockdown reduced the Ca^{2+}-activated currents in TM5 ($N = 8$, $P = 0.002$, unpaired *t*-test) and HTM cells ($N = 4$, $P = 0.02$, unpaired *t*-test) by 52 ± 7% and 49 ± 12%, respectively, in comparison with the scrambled control cells.

Role of Anoctamins in Regulating Plasma-Membrane Currents Triggered by Hypotonicity

In view of reports suggesting a role of anoctamins in cell volume regulation of other cells (Introduction), the CaCC inhibitors also were applied to hypotonically-activated TM cells. After a baseline period of 5 minutes followed by hypotonic activation for 10 minutes, inhibitions were assessed after exposure to: 100 μM tannic acid for 2 minutes or 50 μM $CaCC_{inh}$-A01 for 10 minutes. The percentage inhibitions are presented in Figure 4A.

Reducing osmolality by approximately 23% triggered typical $I_{Cl,Swell}$ currents[16,17,50] displaying modest outward rectification and time-dependent inactivation at highly depolarizing voltages (Figs. 2C, 2D). As in the case of the ionomycin-activated currents (Fig. 3A), tannic acid strongly reduced $I_{Cl,Swell}$ in TM5, HTM, and GTM3 cells (Fig. 4A) in comparison with the scrambled controls. The nonselective inhibitor $CaCC_{inh}$-A01 also reduced $I_{Cl,Swell}$ in TM5 and HTM cells.

The effect of transient Ano6 knockdown on $I_{Cl,Swell}$ also was examined in TM5 ($N = 7$) and HTM ($N = 5$) cells. As illustrated by Figure 4B, transient knockdown inhibited $I_{Cl,Swell}$ of TM5 cells by 73 ± 11% in comparison with the scrambled control cells ($N = 5$, $P = 0.028$, unpaired *t*-test). Transient Ano6 knockdown of HTM cells also was associated with a trend to inhibition by 54 ± 19% in comparison with scrambled controls ($N = 4$). This trend did not reach significance ($P = 0.054$). We have followed common convention in arbitrarily defining significance as a probability (P) of the null hypothesis of

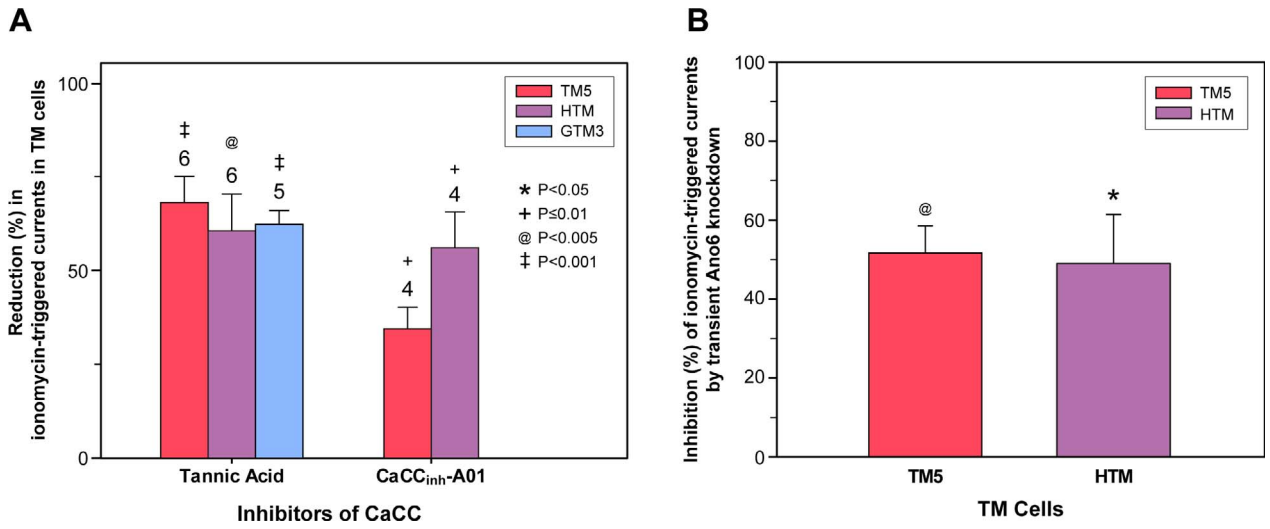

Figure 3. Effects of anoctamin inhibitors and transient Ano6 knockdown on ionomycin-triggered TM-cell currents. (**A**) Presents the percentage inhibition observed after 5 minutes of exposure of the ionomycin-activated TM cells to each inhibitor, 100 μM tannic acid, or 50 μM $CaCC_{inh}$-A01, using each cell as its own series control. The numbers of cells studied are indicated over the corresponding *bars*. The data conformed to a normal distribution by the Shapiro-Wilk test. The probability of the null hypothesis was calculated by Student's *t*-test: $^{*}P < 0.05$; $^{+}P < 0.01$; $@P < 0.005$, and $‡P < 0.001$. The symbols have the same significance in Figures 4 and 5, and are so defined in Figures 4A and 5A, as well. (**B**) Presents the percentage inhibition produced by transient Ano6 knockdown of three TM5 or three HTM cells in comparison with corresponding numbers of scrambled control cells. The probabilities of the null hypothesis were calculated from unpaired *t*-tests after verifying normality by the Shapiro-Wilk test and equal variance.

<0.05. However, this is arbitrary practice. Taken together with the effects of the CaCC inhibitors noted above in this section, the data suggested that Ano6 does enhance $I_{Cl,Swell}$ in TM cells.

Anoctamins Modulate Regulatory Volume Decrease of TM Cells

The foregoing results suggested that anoctamins regulate $I_{Cl,Swell}$ (Discussion). However, the primary issue of physiologic importance was whether anoctamins modulate cell volume regulation. This issue was addressed more directly by monitoring the regulatory volume response to anisosmotic swelling.

As illustrated by the control trajectories displayed in all four panels of Figure 5, reducing the osmolality of the perfusion solution by approximately 50% produced cell swelling, peaking 4 minutes later. Thereafter, progressive release of solute, and secondarily water, over the subsequent 26 minutes restored cell volume to within 1% to 6% of the initial isotonic volume. This RVD of TM cells primarily arises from KCl release through paired K^+ and Cl^- channels.[17]

Tannic acid, the more efficacious of the nonselective anoctamin blockers in inhibiting Ca^{2+}-activated currents and $I_{Cl,Swell}$, significantly slowed the RVD of the HTM and TM5 cells (Figs. 5A, 5B; $P < 0.05$, 1-way ANOVA at 30 minutes using the Holm-Sidak Multiple Comparison Procedure). The less efficacious nonselective anoctamin blocker $CaCC_{inh}$-A01 displayed a trend to slow the RVD, which did not reach significance.

Transient Ano6 knockdown also slowed the RVD of TM5 and HTM cells. In comparison with the scrambled controls (*N*

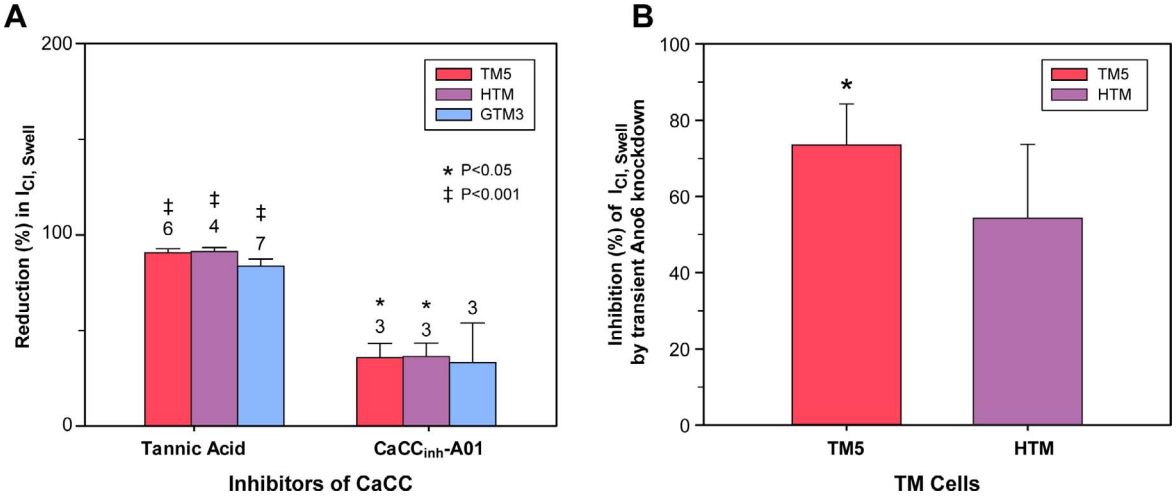

Figure 4. Effects of anoctamin inhibitors and transient Ano6 knockdown on hypotonicity-triggered TM-cell currents. After hypotonically triggering $I_{Cl,Swell}$, perfusion with 100 μM tannic acid or 50 μM $CaCC_{inh}$-A01 produced the inhibitions indicated by the bar graphs of (**A**). In general, the data were normally distributed by the Shapiro-Wilk test, permitting calculations of the probability of the null hypothesis with the *t*-test. These probabilities are indicated with the same symbols used in Figure 3. Transient Ano6 knockdown significantly inhibited $I_{Cl,Swell}$ of TM5 cells in comparison to the scrambled control cells ($N = 5$–7, $P = 0.028$) and displayed a trend to reduce those currents in HTM cells ($N = 4$–5, $P = 0.054$; [**B**]).

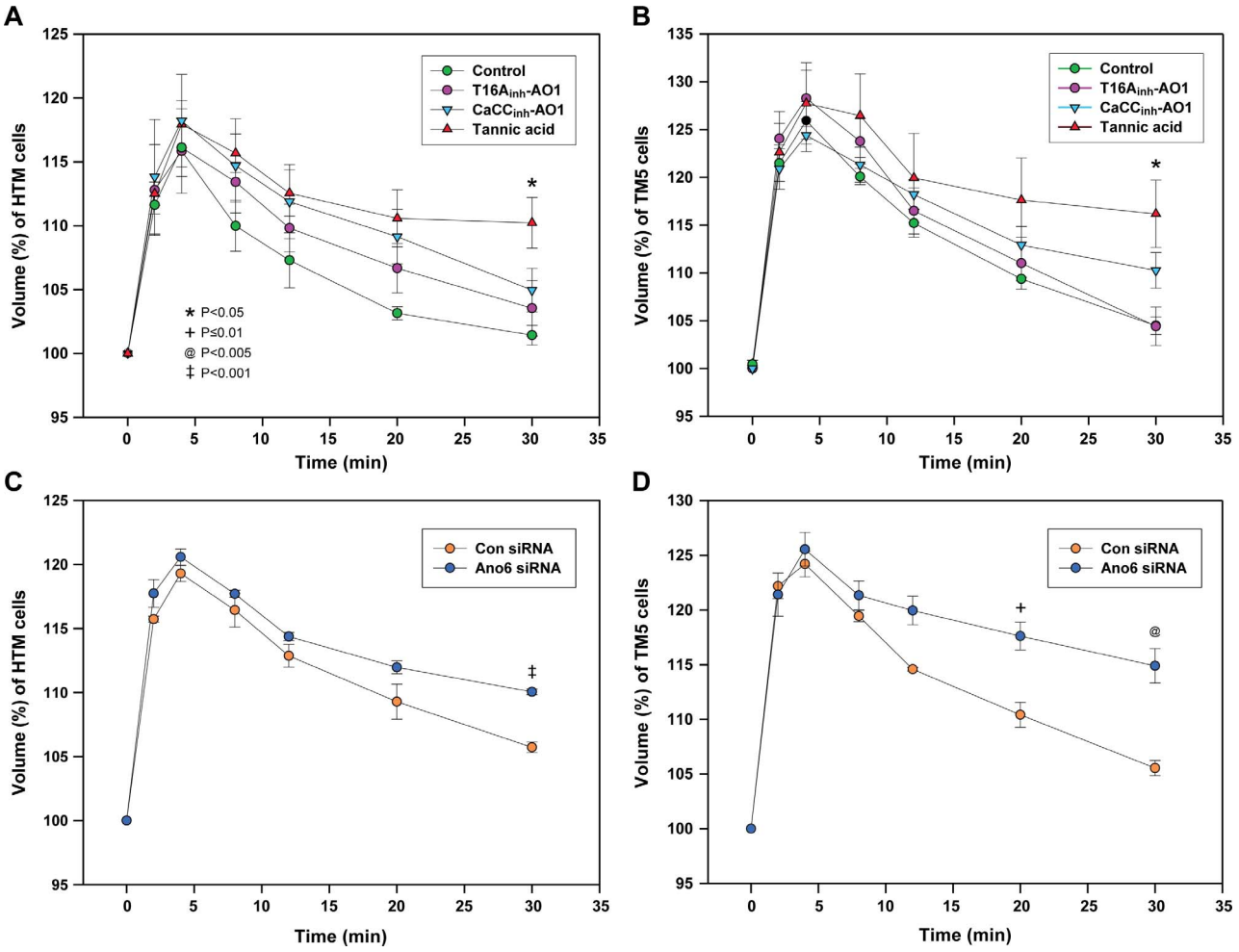

Figure 5. Effects of anoctamin inhibitors and transient Ano6 knockdown on RVD of TM cells. Halving the osmolality triggered swelling. Cell volume peaked 4 minutes later, followed by an RVD reflecting release of intracellular solute and water. Statistics were conducted either by 1-way ANOVA (**A**, **B**) or unpaired *t*-test (**C**, **D**) at each time point after first confirming that normality and equal variance were satisfied. The *symbols* indicate the same probabilities of the null hypothesis defined in Figure 3A, and are so defined in (**A**). Four experiments were averaged to generate the experimental trajectory of (**D**), and three experiments were averaged in generating the others. The nonselective CaCC inhibitor tannic acid slowed the control RVD of HTM cells (**A**) and TM5 cells (**B**). In both cases, TM cells remained significantly more swollen than the controls 30 minutes after applying hypotonicity. Similarly, transient Ano6 knockdown left the HTM cells (**C**) and TM5 cells (**D**) more swollen than the scrambled controls 30 minutes after establishing hypotonicity. The TM5 cells also remained more swollen at the 20-minute endpoint.

= 3), HTM cells with transient Ano6 knockdown ($N = 3$) remained significantly larger 30 minutes after hypotonic exposure ($P < 0.001$, unpaired *t*-test, Fig. 5C). Following transient Ano6 knockdown, TM5 cells ($N = 4$) remained larger than control cells ($N = 3$) both 20 minutes ($P = 0.010$) and 30 minutes ($P < 0.001$) after applying hypotonicity (unpaired *t*-test, Fig. 5D).

The foregoing data obtained with CaCC inhibitors and transient Ano6 knockdown suggested that Ano6 enhances the RVD of human TM cells.

Discussion

Relative Gene and Functional Expression of Ano1, Ano2, and Ano6

Of the 10-member vertebrate family of anoctamins, the three anoctamins clearly documented to subserve CaCCs in other cells are Ano1, Ano2, and Ano6 (Introduction). The current qPCR analyses indicated that gene expression of Ano6 in human TM cells is 3 to 4 orders of magnitude greater than those of Ano1 and Ano2 (Fig. 1C). The RT-PCR analyses (Fig. 1A) and Western blots (Fig. 1B) are consistent with that observation. Nonselective inhibition of the anoctamins strongly inhibited all three functional assays. These observations suggested that an anoctamin, presumably Ano6, regulates TM-cell Ca^{2+}-activated currents, $I_{Cl,Swell}$ and the RVD, an important physiologic regulator of TM cell volume and conventional outflow homeostasis.

Ano6 Regulation of Ca^{2+}-Activated Currents

In the presence of extracellular Ca^{2+}, the calcium ionophore ionomycin increased intracellular Ca^{2+} activity (Fig. 2A) and triggered membrane currents (Fig. 2B). The currents were modulated by Ano6, since they were reduced by nonselective CaCC inhibitors (Fig. 3A) and inhibited by partial transient Ano6 knockdown (Fig. 3B).

The macroscopic kinetics of the Ca^{2+}-activated currents were identical with those of Ca^{2+}-activated Cl^- currents.[47] However, the E_{rev} of the Ca^{2+}-activated currents ranged from +5 to +12 mV for the three TM cell types studied. In contrast,

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E_{rev} of the anion-selective channels $I_{Cl,Swell}$ was clearly negative under the same experimental conditions, ranging from −14 to −21 mV for the same TM cell types. With the current solutions, the theoretical Nernst reversal potentials for Cl^- and Na^+ were identical in magnitude but opposite in sign. Thus, the Ca^{2+}-activated currents of the TM cells reflect faster conduction of cation than anion, in contrast with human Ano6 currents homologously overexpressed in HEK293T cells[29] and mouse Ano6 heterologously overexpressed in HEK293 cells.[28] The current data suggested that P_{Na}/P_{Cl} was higher for Ca^{2+}-activated currents than $I_{Cl,Swell}$ by 6.3-, 2.7-, and 3.8-fold for the HTM, TM5, and GTM3 cells, respectively. The present observations are most consonant with the endogenous Ano6 currents in axolotl oocyte membranes, reported to display a P_{Na}/P_{Cl} approximately 7 (Yang et al.[39]).

Ano6 Regulation of Swelling-Activated Cl^- Currents ($I_{Cl,Swell}$)

As widely observed with other cells, hypotonic swelling triggers $I_{Cl,Swell}$ in TM cells (Fig. 2C) associated with the negative E_{rev} noted above (Section 4.2). Nonselective CaCC inhibitors inhibited $I_{Cl,Swell}$ in these cells (Fig. 4A) and transient Ano6 knockdown also strongly inhibited $I_{Cl,Swell}$ in TM5 TM cells (Fig. 4B). The RVD, which depends upon $I_{Cl,Swell}$, also was inhibited by the nonselective CaCC inhibitor tannic acid and by transient Ano6 knockdown in TM5 and HTM cells (Fig. 5).

Almaça et al.[7] first reported that selective transient knockdown of anoctamins 1, 6, 8, and 9 nonadditively reduced the whole-cell conductance of hypotonically-activated HEK293 cells. In contrast, Shimizu et al.[29] found that selective transient knockdown of either Ano6 or Ano10 had no effect on the current-voltage relationship of $I_{Cl,Swell}$ in HEK293T and HeLa cells. Juul et al.[46] have emphasized that the effects displayed by manipulating Ano6 strongly depend on the presence of extracellular Ca^{2+}. The latter investigators found that the RVD of Ehrlich ascites tumor cells was unaffected by stable Ano6 knockdown in the total absence of external Ca^{2+}, but was strongly inhibited in the presence of 0.5 mM Ca^{2+}. However, differences in bath Ca^{2+} are unlikely to account for the divergent results reported by Shimizu et al.[29] External Ca^{2+} was present in the solutions of Almaça et al.[7] (1.3 mM calcium gluconate) and Shimizu et al.[29] (2 mM $CaSO_4$ in baseline solutions). In the present work, 1.8 mM $CaCl_2$ was included in the external bath.

The mechanism by which Ano6 modulates $I_{Cl,Swell}$ cannot be identified from our data. The pore of $I_{Cl,Swell}$ is formed by LRRC8 heteromers.[51] Isomers A, C, D, and E of the LRRC8 family are incorporated in the channel.[16,17] The specific isomeric configuration determines $I_{Cl,Swell}$ inactivation kinetics,[16] and the isomeric combination varies with cell type.[16] Whether or not Ano6 interacts directly with LRRC8A,C,D,E in other cells is unknown. However, anoctamins do interact with other anoctamins as hetero-oligomers[27,38,52] and with other channels, such as CFTR,[26] TRPC2,[53] and TRPV4,[54] which may modulate anoctamin function variably in different cells and tissues.

Future Implications

The current results may well have clinical implications. The autocrine release of ATP that initiates purinergic regulation of outflow can be triggered equally well by hyposmotic swelling or cell stretch.[10] The duration of the stimulus determines the amount of ATP released, and, thus, the amount of MMP-2 and MMP-9 released downstream. The physiologic event that terminates ATP release is the RVD, which permits the TM cells to restore their baseline volumes, thereby removing the signal for ATP release. Early restoration of osmolality by adding mannitol terminates the period of ATP release. Pretreatment with dexamethasone also accelerates the RVD, reducing the period of ATP and MMP release.[10] In contrast, slowing the RVD with cytochalasin prolongs the period of cell stress and thereby increases ATP and downstream MMP secretion.[10] Our data indicated that Ano6 accelerates the RVD, reducing the period for ATP and MMP release. These results raise the possibility of pharmacologically targeting Ano6 or its link to LRRC8, the critical component of $I_{Cl,Swell}$. Inhibiting the action of Ano6 is expected to maintain the TM cell volume high, thereby enhancing the rate of ATP release and further reducing outflow resistance. It should be noted that a larger percentage inhibition of ionomycin-triggered currents and $I_{Cl,Swell}$ was noted with nonspecific inhibitors than by Ano6 siRNA knockdown. This suggests that anoctamins in addition to Ano6 may by having a role in TM cell volume regulation. The possibility of targeting Ano6 to modify TM cell volume, suggested by our studies of cultured cells, is supported by recent exon-level expression profiling of Ano6 in 10 ocular tissues.[55] The data can be accessed at https://genome.uiowa.edu/otdb/search?type=symbol&term=ANO6&set=extended, in the public domain. Not only is Ano6 expressed far more than any other trabecular meshwork anoctamin, but Ano6 is more highly expressed in trabecular meshwork than in the other 9 ocular tissues tested. Development of this novel approach for lowering IOP will require future identification of the LRRC8 isomers expressed by normal and glaucomatous TM cells, and the mechanism(s) of interaction between LRRC8 and Ano6 in human TM cells.

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