Published in final edited form as:

J Membr Biol. 2013 February ; 246(2): 167–175. doi:10.1007/s00232-012-9517-4.

Ionic currents of human trabecular meshwork cells from control and glaucoma subjects

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Abstract

Increasing evidence suggests that trabecular meshwork (TM) cells participate in the regulation of intraocular pressure by controlling the rate of filtration of the aqueous humor. Ionic conductances that regulate cell volume and shape have been suggested to play an important role in TM cell volume regulation. Here we compared ionic currents from TM cells derived from a normal subject (CTM) and from an individual affected by glaucoma (GTM). We found that while the ionic currents types are similar, the current amplitudes and percentage of cells endowed with specific current at baseline are different in the two cell lines. Thus, we found that the majority of CTM cells are endowed with a swelling-activated Cl⁻ current at baseline, whereas in the majority of GTM cells this current is not active at baseline and becomes activated only after perfusion with a hypotonic solution. An inward rectifier K⁺ current is also more prevalent in CTM than in GTM cells. Our work suggests that dysregulation of one or more of these ionic currents may be at the basis of TM cells participation in the development of glaucoma.

Keywords

Trabecular Meshwork; Ion Channels; Glaucoma

One of the hallmarks of glaucoma is elevated intraocular pressure (IOP). Elevated intraocular pressure is thought to result in optical nerve damage and eventually blindness. The IOP is balanced by the rate of production and drainage of the aqueous humor (Morrison & Acott, 2003). The aqueous humor is filtered through the trabecular meshwork (TM), a tissue located in the anterior chamber angle and drained via Schlemm's canal (Morrison & Acott, 2003; Tripathi, 1972). The rate of filtration through the TM is influenced by the tone of the ciliary muscle, which sends tendons into the TM tissue (Kaufman, 2008; Tamm, 2009). However, increasing evidence suggest that the TM tissue itself can modulate the filtration rate by changing the shape and volume of the cells that compose it (Goel et al., 2012). The volume and shape of cells is regulated by various systems including the cytoskeleton (Kaufman, 2008) and membrane ionic conductances (Ellis, 2011; Stumpff & Wiederholt, 2000). For example, studies in different types of tissues, including TM cells, established that chloride currents that are activated by cell swelling participate in cell volume regulation (Bond et al., 1998; Soto et al., 2004; Volk et al., 2008). Other types of channels sensitive to membrane stretch or sheer force can influence cell shape, thus affecting the outflow rate (Goel et al., 2012; Stumpff & Wiederholt, 2000). Finally, the ion

channel makeup of TM cells has the potential to impact the function of the TM tissue and consequently the outflow rate of the aqueous humor. Therefore, in order to fully understand the participation of the TM tissue to the filtration rate of the aqueous humor, a good understanding of the ionic conductances present in these cells is imperative. Moreover, a comparison between the ionic conductances present in TM cells from normal versus glaucoma subjects can give insight into the mechanisms that are dysregulated in glaucoma.

Previous studies by other laboratories identified and characterized ionic conductances of TM cells (Comes et al., 2006; Dismuke & Ellis, 2009; Gasull et al., 2003; Llobet et al., 2001; Nilius et al., 1998; Soto et al., 2004; Stamer et al., 1995). A swelling activated Cl^- current, an inward rectifier K^+ current and an outward rectifier Ca^{2+} -sensitive K^+ current, among others, were found to be present in TM cells (Dismuke & Ellis, 2009; Gasull et al., 2003; Llobet et al., 2001; Mitchell et al., 2002; Soto et al., 2004). Though, these studies were focused only on TM cells isolated from normal subjects or from animal models. The goal of the present work was to compare the ionic currents present in human TM cells from normal and glaucoma subjects. We show here that, similarly to what previously reported, TM cells are endowed with at least three major distinct types of currents: a swelling-activated Cl^- current, an outwardly rectifying K^+ current and an inwardly rectifying K^+ current. However, the ratio of cells in which each current is prevalent varies considerably in normal versus glaucoma derived TM cells. Our work shows that intrinsic electrophysiological properties are distinct between normal and glaucoma TM cells and it suggests that these differences could be considered as possible contributors to the development of the disease state.

Methods

Ethics statement

This study was performed adhering to the guidelines of the Institute Review board of the University of Miami. Human samples were handled in accordance with the principles expressed in the Declaration of Helsinki. Cadaver human eyes were obtained from National Disease Research Interchange, Philadelphia with the approval (exempt under category 4 of NIH guidelines) of the Institute Review board of the University of Miami. The Human TM cell culture protocol was approved by the Institute Review board of the University of Miami.

Cell lines development and culture

Two TM cell lines were developed from a normal (CTM35) and a glaucoma (GTM35) donor. Both donors were Caucasian males age 35 at the time of death and enucleation. The eyes were collected within 3 hours of death and stored immediately in pre-cooled Optisol GS and subsequently maintained at 4°C. These eyes were dissected within 24 hours under a dissecting microscope into four quadrant of the anterior chamber. The trabecular meshwork with minimum contamination of sclera and cornea was prepared from each quadrant. The excised tissue was immersed in 70% ethanol and all subsequent procedures were carried out in a sterile hood with sterile instruments. The tissues from each quadrant were placed in 10 cm plates containing enough sterile phosphate buffered saline to cover approximately about ~1 mm height. With a fine scalpel, the cells from the middle of these dissected tissues were scrapped and scrapped cells were removed into areas away from the tissue mass and further gently meshed with a forcep. These cells were allowed to grow in DMEM medium with 10% FBS for three days. Both cells were subjected to stable transfection with a Myc T58A mutant construct (Sears et al., 2000; Yeh et al., 2004). The cells were selected in presence of Neomycin for stable transfection. These cell lines show continuous growth beyond 30 passages, whereas the primary TM cells fail to grow in culture beyond 20–22 passages. However, cells used for current investigation were stored from same initial stock and are approximately from 6th to 10th passage. Both CTM35 and GTM35 cell lines were

maintained in DMEM with 10% FBS and 1% antibiotic/antimycotic. For electrophysiology, cells were plated at roughly 10% confluence in dishes containing 12mm round glass coverslips and allowed to adhere overnight before electrophysiological recordings.

Electrophysiology

Ionic currents were recorded at room temperature using the whole cell configuration of the patch clamp technique. An Axopatch 200 B (Molecular Devices, CA), Digidata 1322A and the pCLAMP suite of programs were used for data acquisition and analysis. Patch pipettes were pulled from boro-silicate capillaries (World Precision Instruments, FI) and fire-polished to have a resistance of 2–4 MΩ. Pipettes were filled with a solution containing (in mM): K-aspartate 150, MgCl₂ 3, CaCl₂ 1, EGTA 5 and Hepes 10, pH 7.3 (265 mosm). The isotonic extracellular solution contained (in mM): NaCl 145, KCl 5, MgCl₂ 3, CaCl₂ 1 and Hepes 10, pH 7.4 (295 mosm); the hypotonic extracellular solution contained (in mM): NaCl 80, KCl 5, MgCl₂ 3, CaCl₂ 1 and Hepes 10, pH 7.4 (184 mosm); the solution at high K⁺ concentration contained (in mM): KCl 150, MgCl₂ 3, CaCl₂ 1 and Hepes 10, pH 7.4 (289 mosm). The extracellular solutions in which the concentration of Cl[−] was reduced contained (in mM): NaCl 40, Na-aspartate 40, KCl 5, MgCl₂ 3, CaCl₂ 1 and Hepes 10, pH 7.4 (180 mosm) and Na-aspartate 80, KCl 5, MgCl₂ 3, CaCl₂ 1 and Hepes 10, pH 7.4 (176 mosm). The osmolality of the solutions was measured using an osmometer (Advanced Micro-osmometer Model 3MO plus, Advanced Instruments, MA). Values of cell capacitance were derived from the amplifier reading after correction of the pipette capacitance. The average cell capacitance was 67.7 ± 9.7 pF (n=19) and 51.2 ± 5.5 pF (n=47) for CTM and GTM cells respectively. Means were not statistically different. 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) were acquired from Tocris (UK) and Invitrogen (NY) respectively. Stock solutions were prepared in DMSO and H₂O at a concentration of 0.1 M and 0.5 M respectively.

Results

Trabecular meshwork cells from a normal subject express a swelling-activated Cl[−] current

To determine the types of ionic currents present in normal trabecular meshwork cells (CTM), we applied the patch clamp technique in the whole-cell mode. We used extracellular and pipette solutions that mimic the ionic composition of the extracellular and intracellular spaces respectively. In 11 of the 12 CTM cells we patched, we detected a rapidly activating outwardly rectifying current (Fig. 1A). This current was reminiscent of the swelling-activated Cl[−] current previously described in TM cells (Comes et al., 2006; Soto et al., 2004). Thus, to establish if the current we detected in CTM cells was sensitive to cell swelling, we perfused the cells with a hypotonic solution containing 80 mM instead of 145 mM NaCl. We found that the outward rectifying current increased in amplitude in hypotonic solution (Fig. 1 B and D). The reversal potential of the current also shifted towards less negative potentials (Fig. 1 C). These results suggested that the swelling activated current might be a chloride current.

To test this hypothesis, we used the Cl[−] channel blocker 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Mitchell et al., 2002). We found that application of 100 μM NPPB substantially blocked the swelling-activated current (Fig. 2 A–D). We conclude that the majority of CTM cells express a swelling-activated Cl[−] current.

An outward K⁺ current in the majority of glaucoma trabecular meshwork cells

Surprisingly, when we studied ionic currents in glaucoma trabecular meshwork (GTM) cells, we found that the majority of these cells expressed an outwardly rectifying current with

features distinct from the current we detected in CTM cells (Fig. 3 A and B). First, this current did not activate instantaneously but following a time course that became more rapid at more depolarized voltages. Second, it reversed at more negative potentials (Fig. 3 B and C). These features suggested that this current is likely carried by outwardly rectifying K^+ channels. To confirm this, we perfused cells with a high K^+ solution. We found that under these conditions the reversal potential of the current shifted towards more positive potentials (from -75 to -7.5 mV) and that a substantial inward component at negative potentials appeared (Fig. 3 D–F). These results confirmed that indeed this current is carried by K^+ ions.

In $\sim 1/3$ of the GTM cells though, we did detect the swelling-activated Cl^- current in isotonic solution (Fig. 4 A and B). When we compared the amplitude of the swelling-activated Cl^- current in CTM and GTM cells in isotonic solution, we found that it was similar (Fig. 4 C). Moreover, in GTM cells in which the current was not active in isotonic solution, this could be activated by perfusion with the hypotonic solution to amplitudes similar to the ones seen in CTM cells (Fig. 4 D). Moreover, the swelling-activated Cl^- current of GTM cells was blocked by NPPB to an extent similar to the swelling-activated current of CTM cells (Fig. 4 E). In addition, we found that $500 \mu M$ 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) blocked the swelling-activated current of GTM cells by a similar degree, further supporting that this current is a Cl^- current. Using GTM cells, we also tested the effect of changing the concentration of extracellular chloride on the reversal potential of the current, while maintaining all the other permeant ions at a constant concentration. We found that lowering the extracellular Cl^- concentration shifted the reversal potential towards less negative values (Fig. 4 F and table 2), as expected for a current carried by Cl^- ions. To conclude, we found that the prevalent current present in glaucoma trabecular meshwork cells at baseline is an outwardly rectifying K^+ current and that in only $\sim 1/3$ of these cells the swelling activated Cl^- current is active in isotonic solution. However, the swelling activated Cl^- current could be activated in GTM cells by perfusion with the hypotonic solution. In contrast, the swelling-activated Cl^- current is prevalent in isotonic solution in normal trabecular meshwork cells (table 1). Our data suggest that the swelling activated Cl^- current is present in both GTM and CTM cells, but that it is active at baseline in higher percentage of CTM than GTM cells.

Since we detected the outwardly rectifying K^+ current in only one CTM cell, we wondered whether the swelling activated Cl^- current present at baseline in most CTM cells, masked the outward K^+ current that can be readily recorded in GTM cells. To test this possibility, we analyzed the NPPB-resistant currents in both CTM and GTM cells. We found that these currents were slowly activating, outwardly rectifying and reversed at ~ -50 mV (Fig. 5). These results suggest that these currents are likely K^+ currents. We conclude that in CTM cells the outward K^+ current is likely present and just masked by the swelling-activated Cl^- current.

An inwardly rectifying K^+ current in normal and glaucoma trabecular meshwork cells

We found that 8 out of 12 (66%) CTM and 8 out of 21 (38%) GTM cells expressed an inward rectifier current, whose amplitude increased and whose reversal potential shifted from negative to 0 mV when extracellular $[K^+]$ was increased from 5 to 150 mM (Fig. 6 A–C). These features indicate that this current is an inwardly rectifying K^+ current (Llobet et al., 2001). Note that in the few cells in which the inward rectifying K^+ current was the major current present, the current displayed strong inward rectification (Fig. 6 A and B). However, the strong inward rectification was in most cells less apparent due to the presence of other overlapping currents, including the swelling-activated Cl^- current (Fig. 6 C and D). When we compared the amplitude of the inwardly rectifying K^+ current in CTM and GTM cells, we found that it was larger in CTM cells (Fig. 6 D). To conclude, while an inward rectifying

K⁺ current was found in both CTM and GTM cells, this current was larger and present in a higher percentage of CTM cells.

Discussion

In this work we compared ionic currents from two cell lines derived from a normal subject (CTM) and from an individual affected by glaucoma (GTM) respectively. We found that three major ionic currents are expressed in both cell lines, but that current amplitudes and the fraction of cells that express them at baseline are different. More specifically, we found that while in the majority of CTM cells a swelling-activated Cl⁻ current is prevalent at baseline in isotonic solution, in the majority of GTM this current is activated only upon perfusion with hypotonic solution. Moreover, an inward rectifying K⁺ current is of larger amplitude in CTM versus GTM cells.

A swelling-activated Cl⁻ current has been previously described in TM cell primary cultures from humans and animal models (Mitchell et al., 2002; Soto et al., 2004). Based on pharmacological and selectivity properties, it was proposed that this current is likely ICl swell, a chloride current found in many cell types and involved in regulatory volume decrease (RVD). RVD is a phenomenon by which cells that undergo hypotonic shock resulting in swelling of the cell are able to return to their baseline volume. Return to baseline volume requires efflux of Cl⁻ and K⁺, which is followed by water outflow (Cala, 1980). ICl swell is thought to be the major Cl⁻ current that mediates Cl⁻ efflux that might influence RVD (Bond et al., 1998). Following the discovery of ICl swell in TM cells, it was suggested that this current might play a role in RVD in these cells following osmotic shock with hypotonic solution (Soto et al., 2004). This hypothesis was tested using tamoxifen, a blocker of the swelling-activated Cl⁻ current. It was indeed found that in TM cells treated with tamoxifen, RVD was reduced and slowed significantly (Soto et al., 2004). Because RVD of TM cells was suggested to be involved in aqueous humor outflow, Soto and colleagues also tested the outflow facility of the aqueous humor following osmotic shock in the absence and presence of tamoxifen. They found that blocking of ICl swell, significantly reduced outflow. Reduction of outflow rate of the aqueous humor is associated with increase of intraocular pressure, which is a hallmark of glaucoma. These results suggest that one of the features of TM cells of glaucoma patients might be reduced amplitude of the ICl swell current. In our experiments, we found that ICl swell is present in a lower percentage of glaucoma TM cells as compared to normal cells, when the cells are perfused with isotonic solution (at baseline). However, when we challenged the cells with the hypotonic solution, ICl swell was activated in GTM cells and its amplitude reached the amplitude found in CTM cells. These results suggest that GTM cells might have dysregulated ICl swell channels rather than a lower number of them. One possibility is that ICl swell channels in GTM cells are less sensitive to membrane stretch. Alternatively, an intracellular molecule that regulates ICl swell amplitude might be at lower or higher concentrations in GTM versus CTM cells. One candidate such molecule is cAMP, which is known to inhibit ICl swell (Voets et al., 1996). cAMP is known to also inhibit inward rectifying K⁺ currents in TM cells (Llobet et al., 2001). Coincidentally, we found not only that a lower percentage of GTM cells expressed the inward rectifying K⁺ current, but also that its amplitude was smaller as compared to CTM cells. Further experiments are needed to establish if cAMP levels are higher in GTM versus CTM cells and/or whether ICl swell becomes activated in GTM cells later during the patch clamp recording, when intracellular components have been diluted through the pipette. However, regardless of the basis of the difference in ICl swell and inward rectifying K⁺ current between GTM and CTM cells, our data suggest that such differences need to be taken into account when considering the possible mechanisms underlying the development of glaucoma.

We found that both CTM and GTM cells express an outwardly rectifying K^+ current. Previous work by Soto and colleagues showed that TM cells are endowed with an outwardly rectifying Ca^{2+} activated K^+ current (BK) that is activated by osmotic shock with hypotonic solution. Soto and colleagues showed that inhibition of the BK current reduced RVD and aqueous humor outflow (Soto et al., 2004). The BK current might indeed be the major pathway for K^+ efflux needed for RVD.

In summary, our finding show that the basic electrophysiological properties of glaucoma TM cells differ from the ones of normal cells. Ionic channels expressed are the same, but current amplitudes and activities of channels at baseline are different, suggesting that the number of channels and/or channel regulation of glaucoma TM cells are different than those of normal cells. Our work suggests consideration of such differences in basic electrophysiological properties when weighting factors that might influence the development of glaucoma.

Acknowledgments

This work was supported by American Cancer Society RGS-09-043-01-DDC and NIH RO1NS070969 grants to LB and by EY016112 and A RPB career award grants to SKB.

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Figure 1. CTM cells express a swelling-activated current

A. Example of ionic currents recorded in a CTM cell perfused with an isotonic physiological solution. Currents were elicited by voltage steps from -80 to +80 mV (20 mV increments) from a holding potential of -50 mV. **B.** Same as in A while perfusing the cell with a hypotonic solution. In both panels the dashed line represents the zero current level. **C.** Current/voltage relationships of currents shown in A and B. Arrows point to the reversal potentials of the currents. In isotonic ($[Cl^-]_o = 158$ mM, open squares) the reversal potential was -50 mV, perfusion with the hypotonic solution ($[Cl^-]_o = 93$ mM, closed squares) shifted the reversal potential to -34 mV. **D.** Time-course of current activation at +80 mV. Shaded area represents perfusion with the hypotonic solution.

Figure 2. The swelling-activated current of CTM cells is inhibited by NPPB

A. Example of currents recorded in a CTM cell perfused with the hypotonic solution. Currents were elicited by voltage steps from -120 to +80 mV in 20 mV increments. The holding potential was -50 mV. **B.** The same cell shown in A was perfused with the hypotonic solution plus 100 μ M NPPB, a chloride channel blocker. **C.** NPPB-sensitive current obtained by subtracting current shown in B from current shown in A. In panels A-C the dashed line represents the zero current level. **D.** Average current-voltage relationships normalized by the cell capacitance in hypotonic solution (filled circles, n=5) and hypotonic solution plus 100 μ M NPPB (open circles, n=5). In the inset, current-voltage relationship of the NPPB-sensitive current.

Figure 3. Outward K⁺ currents in GTM cells

A. Example of outward K⁺ currents in a GTM cell perfused with the isotonic solution. Voltage steps were from -120 to +80 in 20 mV increments. The holding potential was -50 mV. Dashed line is the zero current level. **B.** Average current-voltage relationship for currents similar to the ones shown in panel A normalized by the cell capacitance in GTM cells. N is 14. **C.** Average reversal potential of currents recorded in GTM (n=21) and CTM (n=12) cells in isotonic solution. ** indicates p < 0.01 by t Test. **D.** Another example of outwardly rectifying currents in GTM cells, recorded in a physiological isotonic solution. The same voltage protocol used in A was used here. **E.** The same cell shown in D was perfused with the high K⁺ solution. **F.** Average current-voltage relationships for the outwardly rectifying K⁺ current recorded in physiological solution (filled triangles) and in high K⁺ (open triangle). Note the shift in the reversal potential (from -75 to -7.5 mV) consistent with this current being carried by K⁺ ions (n=4).

Figure 4. The swelling-activated chloride current in GTM cells

A. Example of ionic current recorded in a GTM cell perfused with the isotonic solution. **B.** The same cell was perfused with the hypotonic solution. The voltage protocol used in Figure 1 A and B was used here. The dashed line is the zero current level. **C.** Average current-voltage relationships normalized by the cell capacitance of the swelling-activated chloride current in CTM (filled squares, $n=11$) and GTM (filled triangles, $n=6$) cells, recorded in isotonic solution. **D.** Same as in C for cells in which the current was activated by the hypotonic solution. CTM (open squares, $n=5$) and GTM (open triangles, $n=9$) cells. **E.** Effect of NPPB and DIDS on the swelling activated current in GTM (black bars, $n=4$ and 3)

and CTM (dashed bar, n=5) cells. **F.** Current-voltage relationships for the swelling activated Cl^- current recorded while perfusing a GTM cell with 93, 53 and 13 mM Cl^- in the extracellular solution. The arrows indicate the reversal potential of the current.

Figure 5. The outward K^+ current in CTM cells

A. Example of outwardly rectifying NPPB-resistant currents in a CTM cell. The cell was perfused with hypotonic solution + 100 μ M NPPB. Voltage steps were from -120 to +80 in 20 mV increments. The holding potential was -50 mV. The dashed line is the zero current level. **B.** Average current-voltage relationship obtained from currents similar to the one shown in A, $n=3$. **C** and **D** same as A and B for GTM cells, $n=5$.

Figure 6. CTM and GTM cells express an inward rectifier K⁺ current

A. Example of inward K⁺ currents elicited by in a CTM cell perfused with a physiological solution containing 5 mM K⁺. The membrane was stepped from -120 to +80 mV from a holding potential of -50 mV. **B.** The same cell shown in A was perfused with a solution containing 150 mM K⁺. The dashed line is the zero current level. **C.** Average current-voltage relationships of CTM cells inward K⁺ current in 5 mM (open squares, n=7) and 150 mM (filled squares, n=7) K⁺. Note the shift in the reversal potential from ~-50 mV to 0 mV when extracellular K⁺ is increased to 150 mM. **D.** Average current-voltage relationships normalized by the cell capacitance of the inward K⁺ current in CTM (filled squares, n=8) and GTM (filled triangles, n=8) cells perfused with 150 mM K⁺.

Table 1

Percentage of CTM and GTM cells that are endowed with the hypotonic activated Cl^- current at baseline, while being perfused with the isotonic solution.

	cells with hypotonic-activated Cl^- current
CTM	11/12 (91%)
GTM	7/20 (35%)

Table 2

Average shift in reversal potential of the hypotonic-activated Cl^- current when the extracellular concentration of Cl^- was changed from 158 to 93 mM ($\Delta V1$), from 93 to 53 mM ($\Delta V2$) and from 53 to 13 mM ($\Delta V3$).

$\Delta V1$	$\Delta V2$	$\Delta V3$
22.6 ± 6.7 (n=6)	4.7 ± 1.2 (n=6)	11.9 ± 1.7 (n=6)

Values were obtained from GTM cells. Data are expressed as mean $\pm$ SE.