Trabecular Meshwork TREK-1 Channels Function as Polymodal Integrators of Pressure and pH

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Purpose. The concentration of protons in the aqueous humor (AH) of the vertebrate eye is maintained close to blood pH; however, pathologic conditions and surgery may shift it by orders of magnitude. We investigated whether and how changes in extra- and intracellular pH affect the physiology and function of trabecular meshwork (TM) cells that regulate AH outflow.

Methods. Electrophysiology, in conjunction with pharmacology, gene knockdown, and optical recording, was used to track the pH dependence of transmembrane currents and mechanotransduction in primary and immortalized human TM cells.

Results. Extracellular acidification depolarized the resting membrane potential by inhibiting an outward K^+-mediated current, whereas alkalinization hyperpolarized the cells and augmented the outward conductance. Intracellular acidification with sodium bicarbonate hyperpolarized TM cells, whereas removal of intracellular protons with ammonium chloride depolarized the membrane potential. The effects of extra- and intracellular acid and alkaline loading were abolished by quinine, a pan-selective inhibitor of two-pore domain potassium ($K2_P$) channels, and suppressed by shRNA-mediated downregulation of the mechanosensitive $K2_P$ channel TREK-1. Extracellular acidosis suppressed, whereas alkalosis facilitated, the amplitude of the pressure-evoked TREK-1–mediated outward current.

Conclusions. These results demonstrate that TM mechanotransduction mediated by TREK-1 channels is profoundly sensitive to extra- and intracellular pH shifts. Intracellular acidification might modulate aqueous outflow and IOP by stimulating TREK-1 channels.

Keywords: trabecular meshwork, mechanotransduction, K2p channels, TREK-1

The pH of the aqueous humor (pH_{AH}) reflects the activity of regulatory mechanisms and buffers in the anterior eye.[1–3] Intracellular protons are generated by respiration, anaerobic glycolysis, and calcium clearance, sequestered by buffering mechanisms, and extruded via Na^+/H^+ exchange, Na^+-driven Cl^-/HCO_3^- transport, lactate export, glutamine import, and other processes.[4–6] In addition to biological mechanisms, clinical interventions such as cataract surgery, antiglaucoma drugs, and contact lens insertion may lower pH_{AH} from its average level of ~7.4 to below 6.0.[7–9] Although the (patho)-physiologic significance of such pH_{AH} shifts has not been studied at the cellular/molecular levels, impetus for such investigations comes from early clinical investigations that showed that metabolic acidosis, diabetic acidosis, respiratory alkalosis, and exercise markedly lower IOP,[10–13] and from studies showing that pH affects the delivery and biodegradation of IOP lowering drugs.[14] pH shifts may modulate HCO_3^- and AH production/transport in the ciliary body[3] and permeability of the blood–ciliary barrier,[15] however, recent studies, which localized TREK-1, a mechanosensitive ion channel with exquisite sensitivity for pH[16] in mouse and human trabecular meshwork cells,[17,18] raise the question whether the pH dependence of IOP observed in clinical studies[10–13] might include the effects of pH on TM mechanotransduction itself.

The 426-residue TREK-1 (TWIK-related potassium; OMIM 603219) channel subunit encoded by the human *KCNK2* gene contains two pore-forming P domains and four transmembrane segments. Its expression covers the brain, heart, kidneys, ovaries, and eye, as well as smooth muscle cells and mechanosensitive neurons that innervate the colon and bladder.[19–22] TREK-1 gating is inhibited by extracellular protons but somewhat uniquely among ion channels, the channel activity can be potentiated by intracellular protons.[23] Studies in recombinant systems identified its cytosolic C-terminal domain as the integrator of the modulatory effects of heat, mechanical force, and pH on TREK-1 currents[24–26]: substitution of the proton-binding E306 residue locks the channel in an open configuration,[27] whereas external proton sensors include H126 in the first extracellular loop and W275 in the fourth transmembrane domain.[16,28,29] Despite the importance of proton binding for the gating of recombinant TREK-1 channels, the physiologic significance of this process and its relevance for multimodal integration remain unknown.

We recently identified TREK-1 as a principal regulator of the membrane potential, calcium homeostasis, and pressure sensitivity in trabecular meshwork (TM) cells[18]: mechanosensitive, smooth muscle-like cells in the iridocorneal angle that control the aqueous fluid outflow in the mammalian eye[30] and play a central role in the etiology of glaucoma.[31] The ability of

TM cells to sense mechanical stress[32] and match mechanotransduction to fluctuations in the local physicochemical environment allows them to maintain IOP within an acceptable physiologic range[33]; however, TM function is adversely impacted by glaucoma and may involve aberrant TREK-1 signaling.[17,34] Given the significance of protonation for TREK-1 activity,[27,28,35] we wondered how acidic and alkaline pH shifts encountered under physiologic and pathologic conditions might influence signals across the TM membrane and whether they affect TREK-1–dependent mechanosensitivity. We report that the background membrane conductance and sensitivity to pressure in these cells can be modulated by external and internal protons and that pH shifts act almost exclusively via TREK-1 channels. These findings implicate activity-dependent, metabolic, and pathologic pH shifts in the regulation of TREK-1–dependent pressure sensing, multimodal transduction, and control of the conventional outflow pathway in the primate eye.

Methods

Cell Culture and Transfection

Human trabecular meshwork (hTM cells), isolated from the juxtacanalicular and corneoscleral regions of the human eye (ScienCell Research Laboratories, Carlsbad, CA, USA), were grown in Trabecular Meshwork Cell Medium (ScienCell, Catalog#6591) at 37°C and 5% CO_2. Confluent cells showed the flattened phenotype that is typical of cultured hTMs and expressed TM marker genes, including *Aqp1*, *Timp3*, *Myoc*, *Cryab1*, and *Acta2* (Supplementary Fig. S1). The phenotype of the hTM cell line was further confirmed by testing for steroid-induced upregulation of the myocilin gene. As shown in Supplementary Figure S2, 120-hour incubation with dexamethasone (DEX; 500 nM) potentiated *Myoc* expression. These data are consistent with our previous characterizations of the same cell line.[18,36] A subset of experiments was conducted with primary TM (pTM) cells isolated from corneal rims from three donors (aged between 35 and 60 years) and dissected out of the eye of two additional donors with no history of eye disease. The tissues were acquired and used in concordance with the tenets of the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

Cells were transiently transfected with TREK-1 shRNA (Catalog No: TL312003; OriGene Technologies, Inc., Rockville, MD, USA) or scrambled shRNA-mCherry using Lipofectamine 3000 reagent by manufacturer's instructions. The efficiency of knockdown estimated by RT-PCR analysis was 30%; however, construct-expressing cells were identified for patch-clamp experiments by green fluorescent protein (GFP) reporter fluorescence. Transfected cells were studied at the third or fourth day after transfection.

Electrophysiology

Whole-cell patch-clamp data were collected as described,[18,37] using pClamp 10.7 acquisition software, a Multiclamp 700B amplifier, and DigiData 1550 (Molecular Devices, San Jose, CA, USA). Recordings were sampled at 5 kHz and filtered at 2 kHz. Pipettes, fabricated from borosilicate glass capillaries (WPI; outer diameter 1.5 mm, inner diameter 0.84 mm) had a resistance of 6 to 9 MOhm when filled with the K-gluconate–based pipette solution. Whole-cell current was elicited by –100- to 100-mV ramps from the holding potential of –40 mV. RAMP pulses were of 1-second duration and applied at 0.1 Hz. Membrane potential was recorded in the current-clamp mode in which no holding current was applied. The standard extracellular recording solution contained the following (mM): 140 NaCl, 2.5 KCl, 1.5 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 5.5 D-glucose. The pipette solution contained the following (mM): 120 K-gluconate, 10 KCl, 10 HEPES, 1 $MgCl_2$, 4 Mg-ATP, 0.6 Na-GTP, and 0.5 EGTA; pH was adjusted to 7.3 with KOH. All experiments were performed at room temperature (22–23°C). In experiments using NH_3Cl, 10 mM NaCl was replaced with an equimolar amount of NH_3Cl, and in experiments using $NaHCO_3$, 90 mM NaCl was replaced with an equimolar amount of $NaHCO_3$. The pipette solution in this experiment contained the following (mM): 135 K-gluconate, 10 KCl, 2 HEPES, 1 $MgCl_2$, 4 Mg-ATP, 0.6 Na-GTP, and 0.5 EGTA; pH was adjusted to 7.3 with KOH.

High-Speed Pressure Clamp

Membrane stretch was induced with the HSPC-1 device from ALA Scientific Instruments (Farmingdale, NY, USA). Positive pressure steps (15 mm Hg, 3-second duration) were delivered through the recording electrode. Acquisition of the pressure-induced current was controlled via pClamp 10.7 (Molecular Devices). Before pressure applications, 100 μM 4,4′-Diisothiocyano-2,2′-stillbenedisulfonic acid (DIDS) was added to the extracellular solution to inhibit volume-regulated anion conductance.

Fluorescent Imaging

pH_i levels were tracked with BCECF AM (5 μM; 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester), as described.[36,38,39] Emitted epifluorescence was visualized on an upright E600 FN microscope (Nikon Instruments, Tokyo, Japan) with 40× (0.8 N.A.) water objectives following sequential excitation at 480 nm. The acquisition was controlled, backgrounds were subtracted, and the ratios were computed by Nikon Elements software. The saline perfusate contained the following (mM): 140 NaCl, 2.5 KCl, 1.5 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 5.5 D-glucose, with 10 mM NaCl in some experiments replaced with equimolar NH_4Cl. Intracellular acidification was achieved by application of external solution contained 90 mM $NaHCO_3$. BCECF fluorescence was normalized to averaged baseline values measured 60 to 0 seconds before application of $NaHCO_3$ or NH_4Cl. Results are plotted as average values (three to four slides, each containing 5–10 cells) from at least two separate experiments.

PCR

Total RNA was isolated from hTM cells using RNAeasy mini kit (Qiagen, Hilden, Germany). The complement DNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). RT-PCR was used to detect the mRNA expression of TM markers with the primers listed in Supplementary Figure S1. The PCR amplification was done using T100 Thermal Cycler (Biorad, Hercules, CA, USA) with the program (95°C for 3 minutes; 95°C for 20 seconds, 60°C for 30 seconds, 72°C for 30 seconds, 45 cycles; 75°C for 5 minutes, 4°C for ∞. For quantitative PCR, hTM cells were treated with or without 500 nM dexamethasone for 5 days and then harvested for RNA isolation. The expression of the myocilin mRNA was normalized to an internal control (β-tubulin). The relative mRNA value was calculated from Ct value by using the $2^{-\Delta\Delta CT}$ method for relative gene expression analysis.

Reagents

The reagents used for extracellular and intracellular (pipette) solutions were obtained from Sigma-Aldrich (St. Louis, MO,

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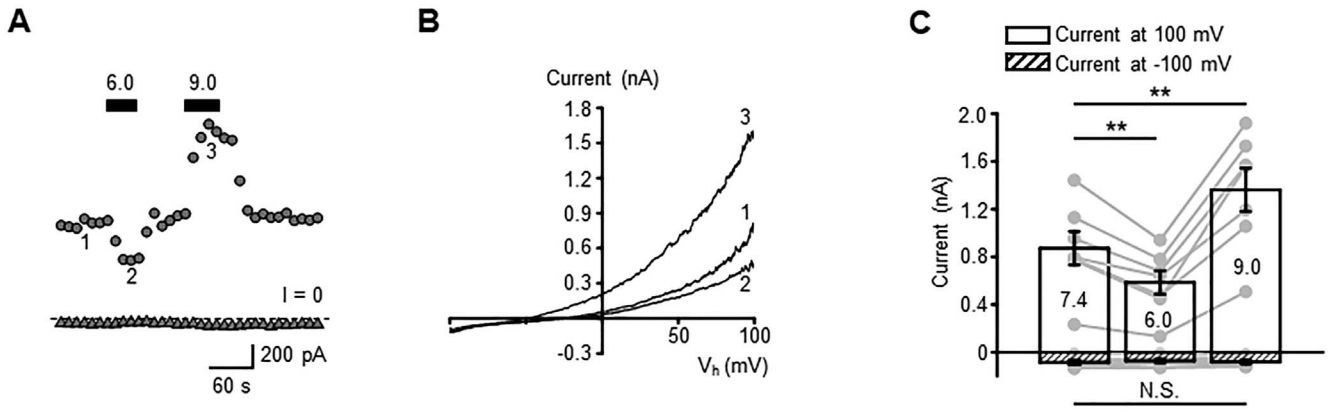

Figure 1. External pH modulates the transmembrane current and the plasma membrane potential of hTM cells. (**A**) A representative trace of the whole-cell current illustrating inhibition and potentiation of current by extracellular acidification and alkalinization. The cells were held at +100 (*circles*) and −100 mV (*triangles*); *solid bars* indicate the application time of the external solution with specified pH. (**B**) Current–voltage relationship of current recorded at time points shown in **A**. (**C**) Summary of experiments shown in **A** and **B**, with the average ± SEM of current amplitudes recorded at +100 (*open bars*) and −100 mV (*patterned bars*). $^{**}P < 0.01$; Pair-sample *t*-test, $n = 8$ cells.

USA) or Calbiochem (San Diego, CA, USA). BCECF AM was from Molecular Probes (Eugene, OR, USA).

Data Analysis

Clampfit 10.7 and OriginPro8 were used for patch-clamp data analysis. Imaging data were analyzed using Nikon NIS-Elements software. Unless specified, the two-sample *t*-test was used to compare two mean values. The difference between the two mean values was considered significant if $P < 0.05$.

Results

pH_o Modulates TM Pressure Sensing

We first determined how external pH affects the resting current in whole-cell recordings from unstimulated hTM cells that were voltage clamped at ±100 mV. Acidification decreased the current amplitude from 872.2 ± 140.6 (control) to 585.2 ± 99.0 pA, whereas alkalinization increased it to 1361.8 ± 181.2 pA (Figs. 1A–1C; $P < 0.01$). The amplitude of the inward current was not significantly modulated by acidic and alkaline pH_o shifts (Fig. 1C). Hence, changes in the extracellular proton concentration mainly affect the outward current that subserves the resting TM conductance.

We next studied whether pH_o affects mechanosensitive TM currents by stretching the plasma membrane with steps of positive pressure delivered with high-speed pressure clamp.[40] The *I-V* relationship of the pressure-induced whole-cell current showed weak outward rectification, presumably due to the presence of extracellular magnesium.[41] When the membrane potential was held at +100 mV, 15 mm Hg steps elicited a robust outward current that peaked at 3898.8 ± 1104.8 pA ($n = 8$ cells; $P < 0.01$). At −100 mV, the pressure-activated inward current reached a maximum of −368.3 ± 67.5 pA (Figs. 2A–2C). Following the outward and inward peaks, the current relaxed to a steady-state plateau at 1186.7 ± 108.8 pA ($n = 8$ cells; $P < 0.05$) and −314 ± 68.3 pA ($n = 8$ cells; $P < 0.01$), respectively (Figs. 2A–2C). Primary TM cells isolated from human donor eyes responded similarly to extracellular pH shifts (Fig. 2C), with inward and outward peaks at −965.18 ± 530 and 3752.3 ± 1260 pA and steady-state plateaus at −182.6 ± 43.3 and 1110.7 ± 225.1 pA ($n = 7$ cells, $N = 2$ donors), respectively. Acidic conditions (superfusion with pH 6.0 saline) suppressed the outward plateau current from 1254.9 ± 118.9 (control) to 666.4 ± 99.5 pA, a 47% decrease ($n = 8$ cells; $P < 0.01$), whereas alkaline (pH 9.0) conditions increased the current to 1996 ± 238 pA, which is a 59% increase ($n = 8$ cells; $P < 0.01$; Figs. 2D–2F).

TM $K2_P$ Channels Function as pH_o Sensors

The principal fraction of resting and pressure-evoked outward currents in human TM cells is mediated by $K2_P$ channels.[18] Consistent with this, the pan-$K2_P$ inhibitor quinine (100 μM) reduced the peak outward current to 950.6 ± 153 pA ($n = 8$ cells; $P < 0.05$) without affecting inward current significantly (−507.4 ± 127.9 pA; $n = 8$ cells; $P > 0.05$), whereas the plateau responses were reduced to 393 ± 142.9 ($n = 8$ cells; $P < 0.001$) and −154 ± 99.4 pA ($n = 8$ cells; $P > 0.05$), respectively. Under these conditions, TM cells lost their responsiveness to acid and alkaline shifts (Fig. 3A). Hence, the pH sensitivity of the resting current is mediated through steady-state activation of TREK-1. Figure 3B shows that the effect of pH on the membrane current (and thus TREK-1 activation) is augmented in cells with active mechanotransduction (i.e experiencing pressure/stretch). This finding predicts that cells experiencing elevated IOP levels will be subject to stronger TREK-1 modulation by protons.

We investigated how the pH_o dependence of transmembrane current translates to changes in the cells' resting potential (V_{rest}) by exposing them to extracellular acid and alkaline shifts under current-clamp conditions. Acidification from pH 7.4 to pH 6.0 depolarized the cells from −36.6 ± 2.8 (control) to −24.1 ± 4.0 mV ($n = 8$ cells; $P < 0.01$; Figs. 4A, 4B), whereas alkalinization to pH 9.0 evinced a significant ($P < 0.001$) hyperpolarization (to −56.9 ± 2.9 mV; $n = 8$ cells). V_{rest} in pTM cells was similarly sensitive to pH_o (Fig. 4B). Quinine abolished the pH_o dependence of the TM membrane potential, depolarizing V_{rest} to −7.0 ± 2.0 mV ($n = 8$ cells, $P < 0.001$) and occluding the effects of acidification and alkalinization ($n = 8$ cells, $P > 0.05$; Figs. 4C, 4D).

To establish whether the pH_o dependence of the resting conductance reflects modulation of TREK-1 channels, cells were transfected with scrambled (control) (Sc-shRNA) and TREK-1-specific short hairpin RNAs (TREK1-shRNA). Transfection efficiency of the construct was evaluated with RT-PCR, and transfected cells were identified by a reporter (GFP) fluorescence (Figs. 5A, 5B). At pH 7.4, Sc-shRNA–treated TM cells had a V_{rest} of −30.9 ± 3.4 mV and showed pH_o dependence that mirrored wild-type cells. Thus, acidification

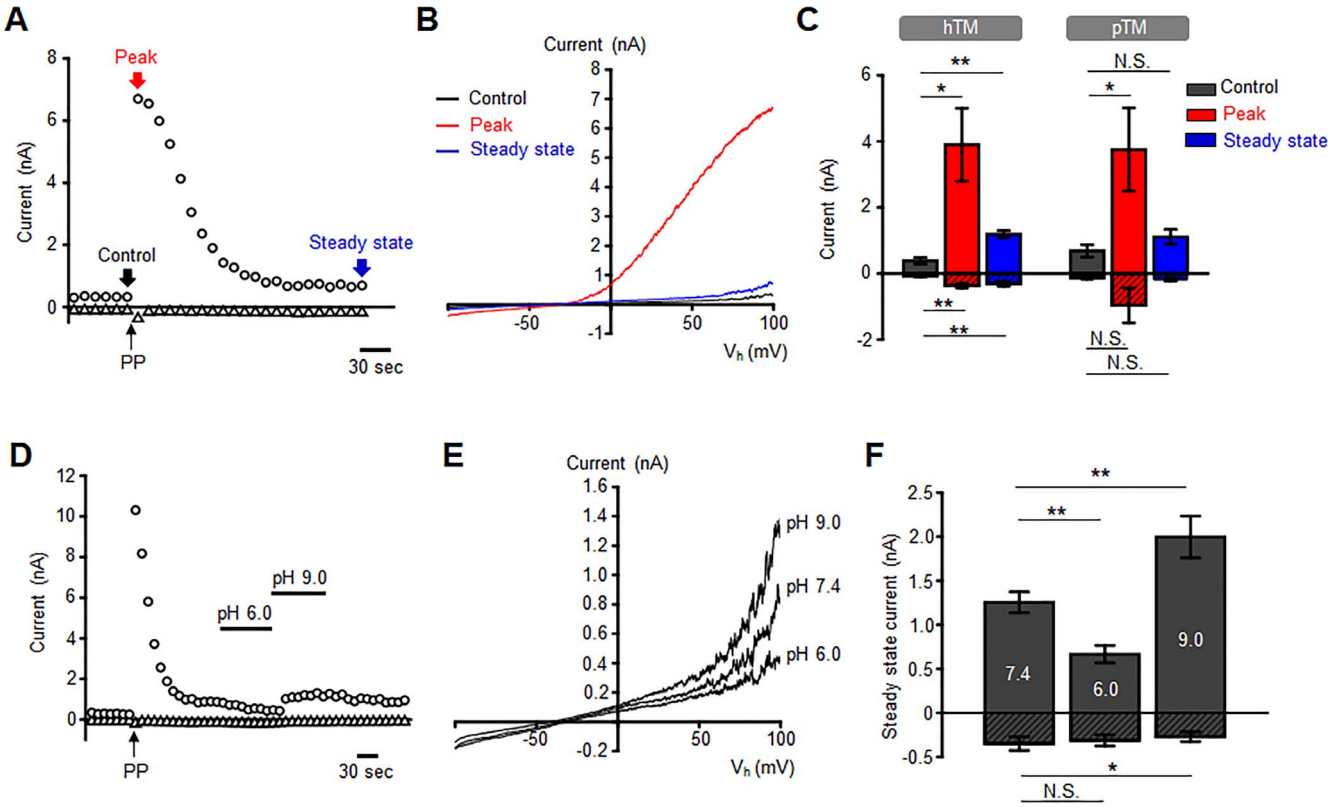

Figure 2. External pH modulates mechanogated current in hTM cells. (**A**) A representative time course of pressure-induced current in hTM cells induced by a pressure pulse (PPs) (*arrow*) in the presence of 100 μM DIDS; the holding potentials were −100 (*triangles*) and +100 mV (*circles*). (**B**) Current–voltage relationship of control, peak, and steady-state current recorded in time-points shown in **A**. (**C**) Summary of peak and steady-state components of the pressure-induced current (−100 mV, *patterned bars* and +100 mV, *open bars*) for hTM ($n = 8$ cells) and pTM cells ($n = 8$ cells, $N = 2$ donors). Shown are the mean ± SEM. $^{\text{Not Significant (N.S.)}}P > 0.05$; $^{*}P < 0.05$. (**D**) The steady-state component of pressure-induced current is sensitive to pH_o. (**E**) The current–voltage relationships from the experiment is shown in **D**. (**F**) Summary of the experiments in **D** and **E**. *Patterned* and *open bars* represent the currents recorded at −100 and +100 mV, respectively, and plotted as means ± SEM. Pair-sample *t*-test. $n = 8$ cells. $^{\text{N.S.}}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$.

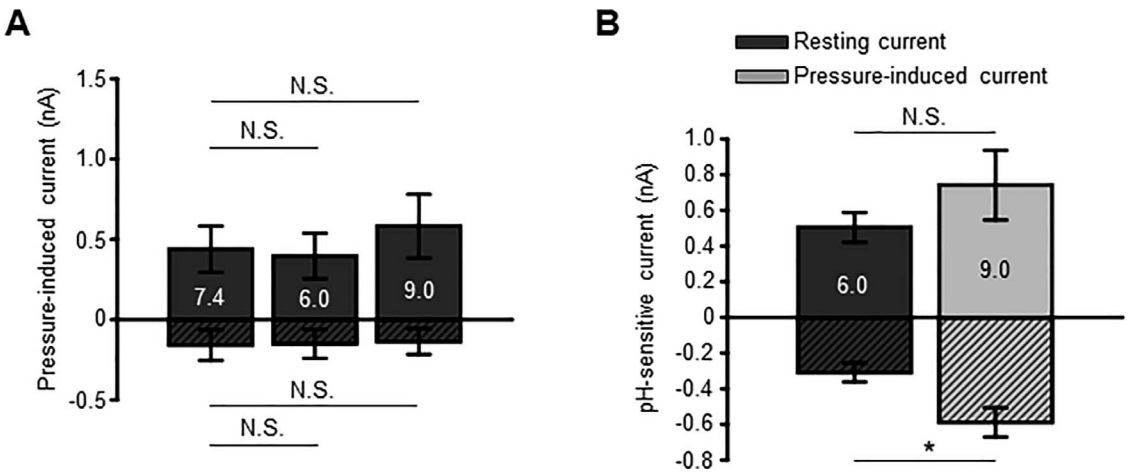

Figure 3. The sensitivity of hTM cell transmembrane current to pH_o is mediated by $K2_P$ channels. (**A**) Quinine abolishes modulation of the transmembrane current by pH_o. *Patterned* and *open bars* represent the currents recorded at −100 and 100 mV, respectively. Mean ± SEM. $^{\text{N.S.}}P > 0.05$. Pair-sample *t*-test. $n = 9$ cells. $P > 0.05$. (**B**) Quinine affects the acid sensitivity of the steady-state component of pressure-induced current. The pH-sensitive current was calculated by subtracting the current at pH 7.4 from the minimum and maximum currents recorded at pH 6.0 and 9.0, respectively. *Patterned* and *open bars* represent the currents inhibited by pH 6.0 and potentiated by pH 9.0, respectively. $^{*}P < 0.05$; $^{\text{N.S.}}P > 0.05$; Two-sample *t*-test, $n = 7$ cells and $n = 9$ cells for background (resting) and pressure-induced currents, respectively. Quinine (100 μM) was applied before pressure stimulation.

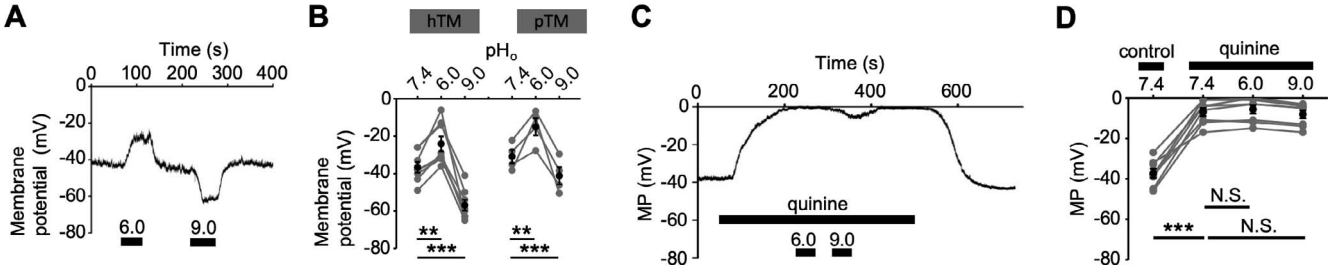

Figure 4. Inhibition of K2p channels abolishes pH_o-dependent modulation of the membrane potential. (**A**) Representative trace of the membrane potential: pHo shifts profoundly modulate V_{rest}. (**B**) Summary of results illustrated in **A**. Means (*black symbols*) ± SEM and individual values (*gray symbols*). $^{**}P < 0.01$; $^{***}P < 0.001$. Pair-sample *t*-test, $n = 8$ cells. pTM stands for primary TM cells; hTM for immortalized TM cells. (**C**) Quinine depolarizes hTM cells and suppresses their sensitivity to pH_o. (**D**) Summary of the data presented in **C**, with the mean ± SEM of the average membrane potential values (*black symbols*) and individual values (*gray symbols*). $^{N.S.}P > 0.05$; $^{***}P < 0.001$. Pair-sample *t*-test, $n = 8$ cells.

induced a ~48% depolarization (to −16.7 ± 4.0 mV; $n = 5$ cells; $P < 0.001$), whereas alkalinization hyperpolarized Sc-shRNA–treated cells to −48.9 ± 6.8 mV ($n = 5$ cells; $P < 0.001$) (Figs. 5C, 5D). Consistent with TREK-1 channel's role in maintaining the TM background conductance,[18] its knockdown depolarized the cells to −14.2 ± 3.2 mV and largely obliterated pH_o-dependent modulation of V_{rest}. TREK-1 shRNA-treated cells responded to external acidification with a small depolarization (~2 mV; $n = 5$ cells), whereas alkalinization evinced a hyperpolarization (~2 mV), neither of which were statistically significant ($P > 0.05$; Figs. 5E, 5F). These results identify TREK-1 channels as the primary effectors of V_{rest} modulation by external pH.

TREK-1 Functions as a Transducer of Trabecular pH_i

Uniquely among pH-sensitive $K2_P$ channels, the TREK family can be modulated by both external and internal pH.[23,27] Because human TM cells do not express TREK-2,[18] pH_i dependence of TREK gating is expected to involve the TREK-1 isoform. Two complementary approaches, consisting of intracellular acidification through exposure to external HCO_3^-, and alkalinization delivered via the "ammonium prepulse," were used to modulate TREK-1 via pH_i. Intracellular acidification, imposed by application of 90 mM $NaHCO_3^-$, was tracked by the proton indicator dye BCECF AM. Figure 6A shows representative traces of simultaneous recordings from "non-patched" cells (gray traces) and a current-clamped cell (red trace), all of which showed an increase in the proton loading caused by the diffusion of dissolved CO_2. Intracellular bicarbonate loading and acidosis resulted in sustained and reversible changes in the TM membrane potential. Consistent with TREK-1 activation, HCO_3^- loading hyperpolarized the membrane by −16.9 ± 1.2 mV ($n = 5$ cells; $P < 0.001$; Figs. 6B, 6C).

The ammonium prepulse is based on the dissolution of ammonium chloride NH_4Cl in the external saline; as the ammonia gas (NH_3) crosses into the cell interior, it removes protons by ionizing into NH_4^+, effecting an intracellular alkalinization that is visualized by BCECF.[38] This is shown in Figure 6D: NH_4Cl (10 mM) alkalinized three intact cells (gray traces), whereas NH_4Cl removal resulted in transient acidification. A simultaneously recorded cell was current-clamped (red trace). In this cell, alkalinization was associated with a significant ($P < 0.001$) and reversible depolarization from the resting potential of −30.8 ± 3.0 to −19.7 ± 1.9 mV ($n = 7$ cells; Figs. 6E, 6F). Quinine did not affect NH_4Cl-induced changes in pH_i (Fig. 6G), whereas NH_4Cl had no additive depolarizing effects in quinine-treated cells ($n = 5$ cells; $P > 0.05$; Figs. 6H, 6I). These data support the conclusion that the pH_i dependence of V_{rest} is mediated largely via the internal proton binding site(s) of TREK-1. Occasionally, cytosolic alkalinization under control conditions evoked a small hyperpolarization (arrow in Fig. 6E) that appeared to be missing in quinine-treated cells but was not examined further. Altogether, these results suggest that modulation of intracellular pH (by metabolism, cell activity, external modulators) may regulate the TM plasma membrane via the proton dependence of TREK-1.

Discussion

This study demonstrates that pH changes control trabecular mechanotransduction by modulating TM-intrinsic mechanosensitive K^+ channels. Extracellular acidification depolarized the resting potential and suppressed pressure sensitivity by inhibiting an outwardly rectifying current, whereas intracellular acidification promoted TREK-1 activation. These effects were abolished through pharmacology and by shRNA-mediated inhibition of TREK-1 channels. Our findings, therefore, suggest that acid/alkaline loading of the aqueous humor and TM cytosol could profoundly influence TREK-1–dependent physiologic states such as IOP.[17]

TM function in the healthy eye requires the ability to sense pressure stimuli and adjust fluid outflow to match an increase in pressure.[32,42] In a predominantly glycolytic tissue such as TM,[43] pH_i and pH_o changes that might modulate the pressure dependence and physiology of TM-resident ion channels could arise from multiple sources that include metabolic activity, proton clearance, buffering, and lactate release. The relative involvement of carbonic anhydrases and concentration of the AH HCO_3^- seem to vary across species, with pH_{AH} ranging between slightly alkaline (~7.6) in rabbits to acidic (~7.18) in pigs and neutral in nonhuman primates (~7.44) and humans (~7.38).[8,9,14,44] Although its functions in bicarbonate production/transport point at the ciliary body as a critical regulator of pH_{AH},[45,46] our results are, to the best of our knowledge, the first to identify TM mechanotransduction as a potential target of pH shifts in the anterior eye.

Our voltage- and current-clamp data show that altering the pH_i or pH_o to match the conditions that might be expected during drug/laser exposure can substantially affect the resting potential and pressure sensitivity of TM cells. pH_o lowering to 6.0 depolarized the cells by ~10 mV, with comparable hyperpolarizations seen in response to extracellular alkalinization. Intracellular acid loads (mediated by HCO_3^-) and alkalinization (mediated via ammonium prepulse), respectively, hyperpolarized and depolarized, V_{rest}. We identified the principal target of pH_o/pH_i as the mechanosensitive TREK-1 channel. The sensitivity to arachidonic acid, spadin, insensitiv-

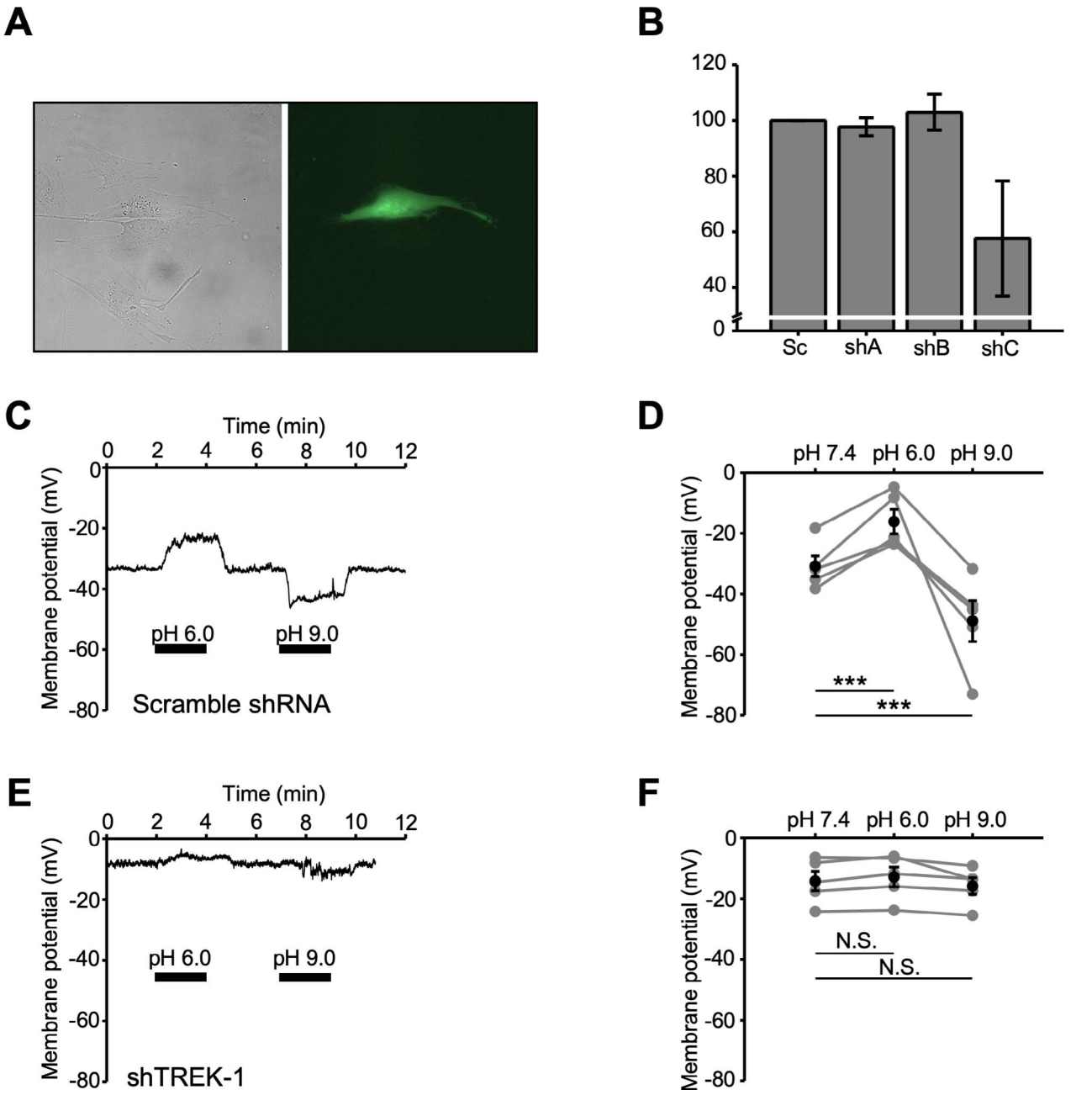

Figure 5. TREK-1 knockdown abolishes the effects of external pH on the plasma membrane potential. (**A**) Representative bright-field (*left*) and fluorescent (*right*) images of TM cells. The fluorescent cell expresses TREK-1 shRNA constract. (**B**) Bar graphs summarizing knockdown eficiency of shRNA constructs. (**C**, **E**) pH_o-dependent modulation of V_{rest} is abolished in TREK-1 shRNA (shTREK-1) but not scrambled shRNA (Sc-shRNA)–overexpressing hTM cells. Shown are representative traces. (**D**, **F**) Summary of data shown in **C** and **E**. Mean ± SEM (*black symbols*) and individual membrane potential values (*gray symbols*). $P > 0.05$; $^{***}P < 0.001$. Pair-sample *t*-test, $n = 5$ cells for shTREK-1 and Sc-shRNA cohorts.

ity to "classical" K^+ channel blockers and absence of TREK-2 and TRAAK expression[18] established that V_{rest} and pressure-activated K^+-mediated current in TM cells are subserved by TREK channels rather than four- and six-pore K^+ channels (e.g., K_v, K_{ir}, K_{ATP} and K_{Ca} channels). Consistent with this, pan-$K2_P$ blockers and TREK-1–specific shRNA suppressed the depolarization and inhibition of the outward current induced by external protons together with blocking the hyperpolarization and current facilitation mediated by external alkaline shifts.

Further identifying TREK-1 as the central target of proton modulation was the abrogation of acid/alkaline loading effects on the membrane potential and current by TREK-1 inhibition/deletion. The pH dependence of the TREK-1 current in TM cells largely mirrors the properties of recombinant channels and smooth muscle cells,[20,47] with the relatively weak outward rectification seen at positive potentials presumably reflecting intracellular Mg^{2+} modulation at physiologic (asymmetrical) K^+ concentrations.[41,48] Although TASK-1 is expressed in hTM cells and sensitive to pH,[49] its minimal contribution to V_{rest} and absence of mechanosensitivity[18] argue against its involvement in the pH dependence of TM mechanotransduction. Mild internal acidosis resulting from metabolic activity might, therefore, be expected to facilitate constitutive TREK-1 opening by shifting the pressure–activation curve toward

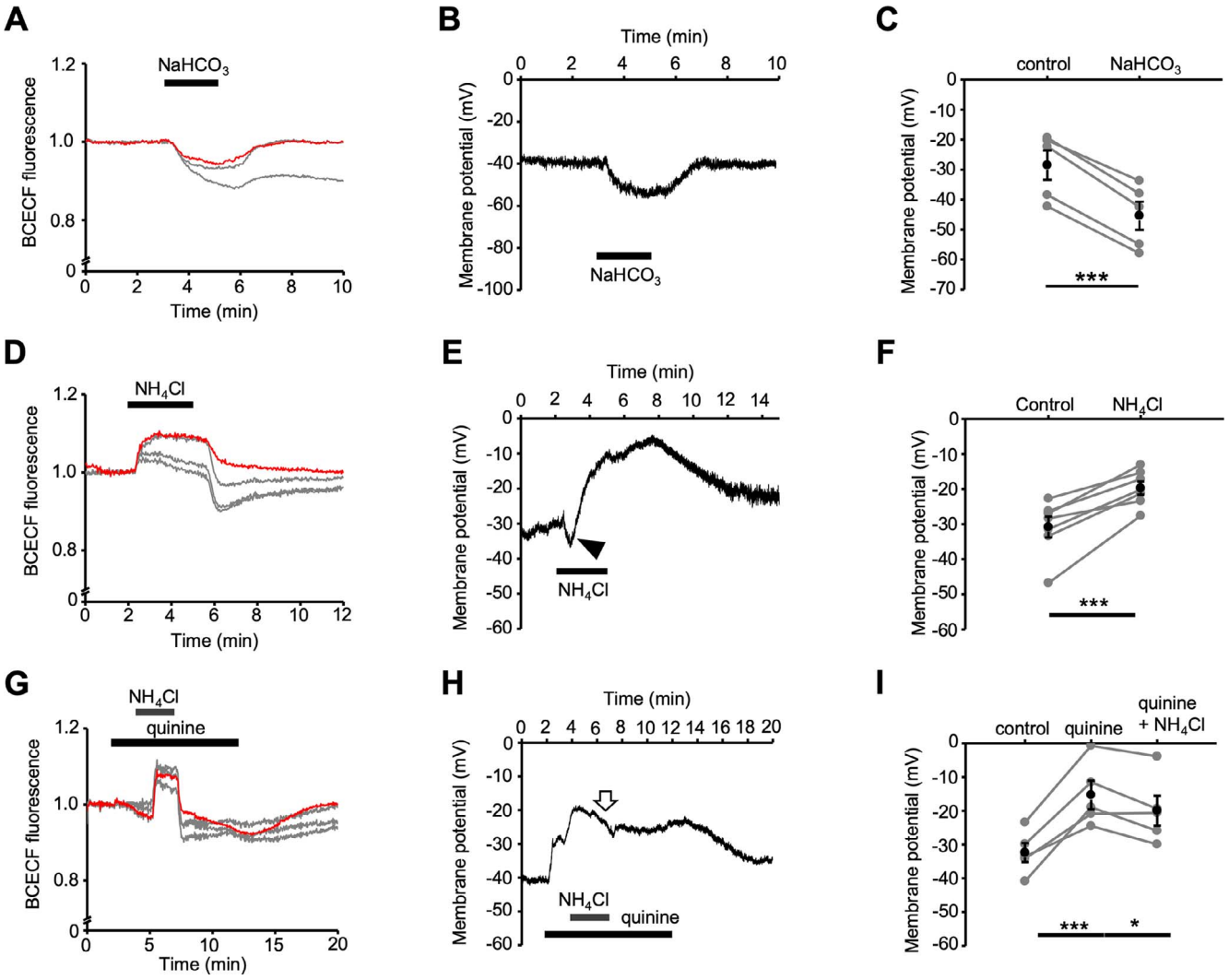

Figure 6. The hTM membrane potential is pH_i dependent. (**A**) Bicarbonate loading. $NaHCO_3$ acidifies pH_i recorded from intact (*gray traces*) and patched (*red trace*) cells loaded with BCECF-AM. (**B**) The patched cell (*red trace* in **A**) hyperpolarizes during the acid load. (**C**) Summary of the data in **B**, with the mean ± SEM (*black symbols*) and individual V_{rest} values (*gray symbols*). $^{***}P < 0.001$. Pair-sample *t*-test, $n = 7$ cells. (**D**) Effects of NH_4Cl on pH_i in intact (*gray traces*) and patched (*red trace*) cells. (**E**) Current-clamp. Intracellular alkalinization induces a transient hyperpolarization (*arrowhead*) followed by sustained depolarization. (**F**) Summary for **D** and **E**, with the mean ± SEM (*black symbols*) and individual values (*gray symbols*). $^{***}P < 0.001$. Pair-sample *t*-test, $n = 7$ cells. (**G**) The NH_4Cl prepulse alkalinizes, then acidifies the pH_i in intact (*gray traces*) and patched (*red trace*) hTM cells. Quinine does not affect ΔpHi. (**H**) Representative current-clamp recording. Quinine inhibits NH_4Cl-induced depolarization. (**I**) Summary of data illustrated in **G** and **H**; mean ± SEM (*black symbols*) and individual values (*gray symbols*). $^{*}P < 0.05$, $^{***}P < 0.001$. Pair-sample *t*-test, $n = 5$ cells.

positive pressures and converting the channel from the voltage-dependent into a "leak" mode.[23,24] The opposite would be the case during respiratory acidosis when IOP elevation[13] would result from suppression of TREK-1.

Pharmacologic or surgical interventions could affect TM-dependent regulation of fluid outflow by modulating the TREK-1 selectivity filters at conserved sites (e.g., W275 within TM4) that govern multimodal transduction of heat, pressure, and protons,[28] PIP_2-interacting domains that are responsible for acidic activation (e.g., E306 within the C terminus)[16,27] and residues that mediate external pH sensing (e.g., H126).[29] For example, antiglaucoma medications were reported to lower pH_{AH} to ~5.3 to 5.8,[8] levels at which we observe substantial modulation of TREK-1. Although it remains to be seen whether protons regulate (conventional and uveoscleral) outflow, TM mechanotransduction is modulated by acidifications resulting from inhibition of carbonic anhydrases (Trusopt), stimulation of $PGF_{2\alpha}$ receptors (Xalatan), and a block of β-adrenergic receptors (Timoptol).[8] Another clinical intervention that may transiently affect pH_{AH} and TM signaling is the use of femtosecond laser during cataract surgery, reported to lower pH to <6.4.[9]

pH-dependent generation and sensing of forces by TM cells almost certainly involve additional extra-/intracellular inputs that modulate TREK-1 channels (Fig. 7) together with other pH-sensitive ion channels, cytoskeletal elements, polyunsaturated fatty acids, and phagocytosis/secretion processes. TRPV4, a nonselective TM-resident cation channel activated by stretch and pressure[36] that may counter TREK-1 activation by depolarizing the TM membrane,[18] appears to be activated by acidosis at ~pH_o 6.0 with peak activation at ~pH 4.0.[50] Intracellular acid loading could modulate contractility by displacing Ca^{2+} ions from internal buffers,[38] depressing the effectiveness of actin–myosin interactions,[51] and modulate the actomyosin contractility.[52] Signaling pathways downstream from stretch sensors might themselves contribute to cytosol

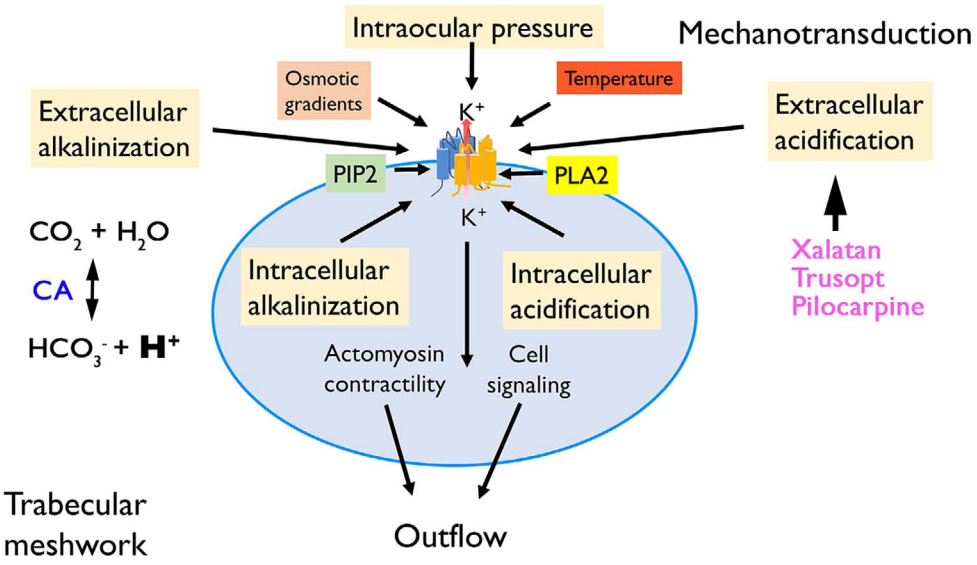

Figure 7. Hypothetical model of pH dependence of trabecular mechanotransduction. The mechanosensitivity of TM cells is modulated by the effects of intra- and extracellular pH shifts on TREK-1 gating and pressure transduction. The process is influenced by the polymodality of TREK-1 activation, which is sensitive to temperature, membrane content of phosphatidylinositol bisphosphate (PIP2), phospholipase A2 (PLA2) activation, cell swelling, fluid shear, and potentially antiglaucoma drugs. A key player in local pH regulation is the carbonic anhydrase (CA), which regulates the local availability of bicarbonate ions and protons. Through their effects on the pressure dependence of TREK-1, the mechanisms that produce, buffer, and remove protons modulate the drainage of the aqueous fluid from the anterior eye.

acidification by producing lactate.[53] By analogy with tissues from TREK-1–null mice that are more sensitive to a variety of external stressors,[54] we propose that TREK-1 activation (by intracellular acidosis and extracellular alkalosis) is protective because it counters the overactivation of mechanosensitive TRP channel isoforms.[36] Our finding that TREK-1 channels integrate the TM response to pH_o, pH_i, and pressure represents only a fraction of its overall polymodal potential given that TREK-1 in vivo is likely to combine mechano- and pH-transduction with temperature sensing (TREK-1 might sense the temperature gradient across the anterior chamber that controls the velocity of the AH[55–57]) and phospholipid modulation[21] (Fig. 7). It also must be noted that many studies of trabecular outflow have been conducted in enucleated eyes, which are likely to show profound lowering of pH_{AH}[14]; it is thus possible that trabecular mechanosensitivity and potentially outflow facility might be affected by postmortem changes in proton/bicarbonate activities. Taken together, the results presented in this study suggest that IOP sensing can only be understood by taking into account the dynamic properties of the intra- and extracellular milieu that include the production and removal of protons. Given the widespread expression of TREK-1[21,58] and ubiquitous nature of proton homeostasis, the significance of pH-dependent mechanotransduction transcends the eye. Relevant examples can be found in cancer and cardiovascular biology, where pathology remodels tissue mechanics and architecture. High lactic acid production and carbonic anhydrase activity in the hypertrophic heart and tumors may promote reactive oxygen generation, atrial fibrillation, cancer progression, and metastasis[59,60] due to interstitial acidification and pH dependence of mechanotransduction.

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