TREK-1 channels regulate pressure sensitivity and calcium signaling in trabecular meshwork cells

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Mechanotransduction by the trabecular meshwork (TM) is an essential component of intraocular pressure regulation in the vertebrate eye. This process is compromised in glaucoma but is poorly understood. In this study, we identify transient receptor potential vanilloid isoform 4 (TRPV4) and TWIK-related potassium channel-1 (TREK-1) as key molecular determinants of TM membrane potential, pressure sensitivity, calcium homeostasis, and transcellular permeability. We show that resting membrane potential in human TM cells is unaffected by “classical” inhibitors of voltage-activated, calcium-activated, and inwardly rectifying potassium channels but is depolarized by blockers of tandem-pore K+ channels. Using gene profiling, we reveal the presence of TREK-1, TASK-1, TWIK-2, and THIK transcripts in TM cells. Pressure stimuli, arachidonic acid, and TREK-1 activators hyperpolarize these cells, effects that are antagonized by quinine, amlodipine, spadin, and short-hairpin RNA–mediated knockdown of TREK-1 but not TASK-1. Activation and inhibition of TREK-1 modulates [Ca2+]TM and lowers the impedance of cell monolayers. Together, these results suggest that tensile homeostasis in the TM may be regulated by balanced, pressure-dependent activation of TRPV4 and TREK-1 mechanotransducers.

Introduction

Intraocular pressure (IOP) is the most significant risk factor for glaucoma (Kass et al., 2002; Leske et al., 2003), with current treatment largely limited to IOP-lowering agents that target the secretion of aqueous humor from the ciliary body or its drainage through the pressure-insensitive “uveoscleral” pathway. However, by far the largest outflow component (~90%) in the primate eye is mediated by the trabecular meshwork (TM), which filters and funnels aqueous humor into the canal of Schlemm (Lütjen-Drecoll and Rohen, 1989). Unlike the ciliary body and muscle, this conventional TM pathway is mechanosensitive and protects the eye from hypertension by autoregulating fluid outflow under different pressure regimens (Brubaker, 1975; Lei et al., 2011). The trabecular outflow mechanism is often compromised in glaucoma, as TM cells adopt the properties of contractile myofibroblasts that chronically augment the tissue resistance to fluid outflow (Flügel et al., 1991; Last et al., 2011). Given that TM has remained intractable to antiglaucoma medications, understanding how its cells sense and transduce pressure is a matter of considerable academic and clinical interest.

We recently identified the nonselective cation channel TRPV4 as a cell volume sensor (Toft-Bertelsen et al., 2017) and likely TM transducer of pressure, swelling, and strain (Ryskamp et al., 2016). In vitro and in vivo experiments revealed that TRPV4 plays a central role in Ca2+-dependent cytoskeletal up-regulation, TM resistance to fluid outflow, and regulation of IOP. TRPV4 antagonists lowered IOP in chronically hypertensive eyes but had no effect on healthy eyes (Jo et al., 2016; Ryskamp et al., 2016), suggesting that steady-state tensile homeostasis and mechanoadaptation in TM cells rely on additional mechanosensing mechanisms. In this study, we investigated the background pressure dependence in human TM cells using electrophysiology, pharmacology, RNA silencing, and impedance measurements. We demonstrate that TREK-1, a polymodal mechanosensitive tandem-pore potassium (K2P) channel (Meadows et al., 2000), represents a crucial molecular link between the membrane potential of primary and immortalized TM cells and their sensitivity to pressure. TREK-1 is activated by multiple types of mechanical force (strain, swelling, shear flow, and stretch) and functions as a regulator of mechanical thresholds, nociception, and stretch-in-
duced contractility in neurons, bladder, colon, and uterine cells (Patel et al., 1999; Talley et al., 2001; Gruss et al., 2004; Heurteaux et al., 2004; Alloui et al., 2006; Nayak et al., 2009; Baker et al., 2010; Monaghan et al., 2011; Noël et al., 2011). Its sensitivity to temperature, pH, long-chain polyunsaturated fatty acids (such as arachidonic acid [AA]), and widely used anesthetics, antidepressants, and neuroleptics (Enyedi and Czirják, 2010; Brohawn et al., 2014; Feliciangeli et al., 2015) allows these channels to integrate the cells’ electrical properties with mechanotransduction to tune a wide spectrum of ambient signals. TRPV4 and TREK-1 channels were reported to regulate the TM cytoskeleton and were implicated in glaucoma (Ryskamp et al., 2016; Carreon et al., 2017), yet it is unclear whether they can be activated by physiologically relevant pressures and how they collaborate in pressure transduction. Here, we characterize the response of TREK-1 to pressure steps and AA and delineate its function as a gatekeeper for Ca2+ homeostasis and ECM adhesion. We show that the TM pressure response involves opposing activation of TRPV4 and TREK-1, which cooperate in control of pressure-dependent signaling, calcium homeostasis, and cell–ECM interactions. These findings place TREK-1 in the center of mammalian IOP regulation, and therefore vision, and suggest a novel mechanism for pressure dysregulation in open-angle glaucoma.

Materials and methods

Cell culture and transfection

Immortalized cells, isolated from the juxtacanalicular region of the human eye (hTM cells), were purchased from ScienCell Research Laboratories. Key physiological features (e.g., TREK-1 dependence of the membrane potential) were also tested in primary TM cells (pTM cells) isolated from corneal rims from three donors (aged between 35 and 60 yr) with no history of eye disease. The rings 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. The identity of TM cells was validated as described (Ryskamp et al., 2016; Stamer and Clark, 2017), with hTM/pTM exhibiting similar genetic/biochemical/molecular signatures (expression of TM-specific markers, smooth muscle actin, and DEX-induced up-regulation of myocilin), responsiveness to ambient stimuli, and expression of K0.5 and TRP channel transcripts. Cell cultures were maintained in the Trabecular Meshwork Cell Culture medium (ScienCell, 6591) at 37°C and 5% CO2 and studied at passages 1–10 and 1–3, respectively. For some experiments, cells were transiently transfected with GFP-tagged myosin, DEX-induced up-regulation of myocilin, and DEX-induced up-regulation of myocilin, and expression of K0.5 and TRP channel transcripts. Cells were cultured in the Trabecular Meshwork Cell Culture medium (ScienCell, 6591) at 37°C and 5% CO2 and studied at passages 1–10 and 1–3, respectively. For some experiments, cells were transiently transfected with GFP-tagged short hairpin RNA (shRNA) constructs targeting human TREK-1, TASK-1, or scrambled shRNA (shTREK-1, shTASK-1, and scRNA, respectively), using Lipofectamine 3000 according to the manufacturer’s (Thermo Fisher Scientific) instructions. The transfected cells showed no obvious changes in morphology, confluence, and viability and were used for experiments at the second to third day after transfection.

Reverse transcription (RT)-PCR assay

RNA was isolated as described (Ryskamp et al., 2016). Total RNA was extracted with TRI-Reagent (Sigma) with 2 μg used for complementary DNA (XLT cDNA super mix kit; Quanta). Quantitative RT-PCR was performed with Apex-Cyber Green on CFX96 Touch Real-Time PCR (Bio-Rad). PCR conditions were as follows: 95°C for 15 min; 95°C for 15 s, 60°C for 1 min, 40 cycles. The primer sequences and data are shown in Table 1 as mean value of 2−ΔΔCt ± SEM.

Western blots

hTM/pTM cells were lysed in a buffer containing the standard protein inhibitor cocktail (Santa Cruz Biotechnology). Total protein was heat inactivated for 3 min at 95°C in Laemli buffer (Bio-Rad). 30 μg total protein per lane was loaded in 8% mini polyacrylamide gels. Electrophoresis was performed at 90 V for 1 h in the Mini-PROTEAN Tetra apparatus (Bio-Rad; running buffer 25 mM Tris, 195 mM glycine, and 0.1% SDS). Proteins were transferred to a polyvinylidene fluoride membrane (0.2 μM, Bio-Rad) in transfer buffer (25 mM Tris, 195 mM glycine, and 20% methanol) in the Mini-PROTEAN Tetra apparatus at 4°C overnight. The membrane was blocked with 5% skim milk for 30 min, incubated overnight with anti–TREK-1 antibody (1:1,000, Santa Cruz Biotechnology) and anti-TASK-1 antibody (1:1,000, Alomone Labs), and washed with the TBS-T solution (TRIS-base [19 mM], NaCl [150 mM] and Tween-20 [0.1%] diluted in ddH2O, pH 7.5) for 3 × 5 min each. Membranes were incubated with anti-rabbit HRP (1:2,000, Abcam) or GAPDH (1:5,000, Abcam) antibodies for 2 h at room temperature. Protein bands were detected on x-ray film by using enhanced chemiluminescence (Pierce) and developer (AFIP Imaging Corp.).

Immunohistochemistry

Cells were plated on type I collagen–coated glass coverslips for 24 h and treated with DMSO (control group) or TREK-1 agonists for 1 h at 37°C. After fixation in 4% PFA, the cells were washed in PBS, permeabilized with 0.1% Triton X-100, and exposed to the blocking solution (1% BSA, 0.3% Triton X-100/PBS, and 0.1% NaNO3).

Electrophysiology

Whole-cell recordings were conducted with borosilicate patch pipettes (tip resistance 6–7 MΩ). To avoid the potentially confounding contributions from intercellular junctions, the recordings were generally conducted in preconfluent cells with spindle-like morphology that is typical of metabolically active cells from the juxtacanalicular TM (Stamer and Clark, 2017). Whole-cell currents and the membrane potential were measured as described (Jo et al., 2016; Molnár et al., 2016) using a Multi-clamp 700B amplifier, Digidata 1550 board, and Clampex 10.7 acquisition software (Molecular Devices). Cells were typically held at −30 mV, with current–voltage (I–V) relationships determined from voltage ramps imposed from −100 to 100 mV. Currents were sampled at 10 kHz, filtered at 5 kHz with an eight-pole Bessel filter, leak-subtracted after data acquisition, and analyzed with Clampfit 10.7 (Molecular Devices) and OriginPro 8 (Origin Lab). shRNA-transfected cells were recognized by GFP expression.

The external solution contained (in mM) 150 NaCl, 2.5 KCl, 1.5 MgCl2, 1.5 CaCl2, 2.5 HEPES, 12 MgSO4, 25 NaHCO3, and 10 d-glucose (pH 7.4 was adjusted with NaOH). In some experiments, 5 mM NaCl was substituted with equimolar TEA-Cl. HEPE replaced...
NaH₂PO₄ and NaHCO₃ in experiments that used pH changes to test the sensitivity of the resting membrane potential (Vrest). The extracellular saline used to record inward rectifying K⁺ currents contained (in mM) 128 KCl, 1.5 MgCl₂, 1.5 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 d-glucose. The intracellular (pipette) solution contained (in mM) 135 potassium d-gluconate, 10 KCl, 1 MgCl₂, 10 HEPES, 0.5 EGTA, 0.01 CaCl₂, 0.3 Na-GTP (pH 7.3, adjusted with KOH), and 1 Mg-ATP. All recordings were conducted at room temperature (22–24°C).

**High-speed pressure clamp**

Pressure pulses were generated with the high-speed system from ALA Scientific Instruments. Pressure steps were delivered through the patch pipette and controlled through pClamp 10.7. 100 µM 4,4'-Diisothiocyanatostibene-2,2'disulfonic acid disodium salt (DIDS) was added to the extracellular solution to inhibit volume-regulated anion conductances.

Calcium imaging was conducted as described (Ryskamp et al., 2016; Jo et al., 2017; Phuong et al., 2017). Briefly, cells were loaded for 30–40 min with the calcium indicator Fura-2 ace-toxymethyl ester (5 µM), placed in recording chambers on an upright epifluorescence microscope (Nikon E600 FN; 20×/0.5 or 40×/0.8 Nikon Fluor objectives), and perfused with isotonic saline containing (in mM) 126 NaCl, 2.5 KCl, 1.5 MgCl₂, 2 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 d-glucose. 340/380-nm excitation was delivered from a 150-W Xenon arc source (DG-4; Sutter Instruments). Fluorescence emission was high-pass filtered at 510 nm and captured with cooled EMCCD cameras (Photometrics). Data acquisition, ratio calculation, and background subtraction were accomplished using NIS Elements (Nikon). Results represent averages across cells (three to six slides, each containing 10–30 cells) from at least three separate experiments.

**Measurement of hTM monolayer resistance**

Real-time impedance of hTM monolayers was measured using the electric cell-substrate impedance sensing (ECIS) Zθ system (Applied BioPhysics), as described (Wang et al., 2016). In brief, cells were seeded on 8W10E+ arrays coated with rat collagen before seeding. Once a stable monolayer was established, the cells were exposed to test agents (TREK-1 activators and inhibitors) or control media, and the resistance was measured in each well for 1 h at 10-s intervals in response to a 4-kHz AC frequency (40 electrodes per well). Resistance for each well was normalized to the baseline resistance before the addition of agonist/antagonist to account for baseline differences in the electrodes. Treatment wells were normalized to the control resistance at each time point to determine change in resistance relative to control.

**Table 1. Primer list**

| Name   | Primer  | Sequences (5′-3′) | Amplicon (bp) | NCBI reference  |
|--------|---------|-------------------|---------------|-----------------|
| TREP1  | Forward | AGGGATTTCATCTTGCGGC | 100           | NM_001017424.2  |
|        | Reverse | CAAGAACGTGGGCTCCTGTTG |               |                 |
| TREP2  | Forward | CTTTCTCCCTGCGCAAAGGA | 249           | NM_138317.2     |
|        | Reverse | GTGCCATCCATCCTGCGCT |               |                 |
| TREP1  | Forward | CGACGTGAGGTTCAAGCTA | 311           | NM_002246.2     |
|        | Reverse | CACCCTTTGGCTCTTGTCTC |             |                 |
| TREP2  | Forward | TCTTACAAAGAGGTGTTGTA | 202           | NM_003740.3     |
|        | Reverse | TTTACACCCGCCACAAAGT |               |                 |
| TREP3  | Forward | GTTTCAGCCGCTCTGCTTTC | 122           | XM_017013530.1  |
|        | Reverse | AGCGTGCCATAACATGGA |               |                 |
| TREP1  | Forward | CTCTCCACCCATAGGTTTGG | 86            | NM_022054.3     |
|        | Reverse | AACACCCCAAAGGCCGTA |               |                 |
| TREP2  | Forward | GTGCTGCAACACTGCTTCG | 109           | NM_022055.1     |
|        | Reverse | TGAAAGACAGGATGGTCAG |               |                 |
| TREP1  | Forward | GGTCTGATGCTCCGATGC | 191           | XM_011544184.2  |
|        | Reverse | ATATTGGAGGCCCCAGCTT |             |                 |
| TREP2  | Forward | GAGGCTGACCCTCCTTACTT | 125           | XM_011527526.1  |
|        | Reverse | GAGGCTGCTGCACCTGCGG |               |                 |
| TREP1  | Forward | AGATAGAGCTGCTCCGTTCG | 193           | NM_033310.2     |
|        | Reverse | GAGGCTGCTGCACCTGGCG |               |                 |
| TREP1  | Forward | CTCTTGGTCAAGCTGACGC | 89            | NM_002046.5     |
|        | Reverse | GACTCCAGCTACCTTCTTC |               |                 |
we sought to determine the identity of ion channel subserv -
in the resting membrane conductance. Under current clamp,
rectifying conductance (Fig. 1, F and G). Pressure steps induced
pressure-dependent current was dominated by an outwardly
reduced by the selective antagonist HC067047 (2 µM; Fig. 1 E).
posed mechanosensing function of TRPV4 channels (Ryskamp
et al., 2016), the amplitude of the pressure-evoked current was
posed by TRPV4 inhibitors (Fig. 1, A–D). Consistent with the recently pro-
relationship showed low conductance at negative membrane
mV) is situated at the pressure-insensitive fulcrum for mecha-
J and J) and evinced ∼5% suppression of the outward current
(Fig. 2, K–M). pTM Vrest and outward currents were similarly
sensitive to quinine (P < 0.01 and P < 0.001 for Vrest and whole-
cell currents, respectively; Fig. 2, J and M). These data indicate
that the hyperpolarizing drive that maintains Vrest in human
TM cells under physiological conditions is largely controlled
by K2P channels.

The TM membrane potential is sensitive to a wide-spectrum
blocker of K2P channels
The K+ dependence of Vrest and insensitivity to classic K+ chan-
nel antagonists (Fig. 2 H) pointed at potential roles for the K2P
channel family, which has been associated with leak conduc-
tances in a variety of tissues, including smooth muscle cells,
which share molecular markers with the TM (Enyedi and
Crižjak, 2010; Stamer and Clark, 2017). The antimalarial alka-
loid quinine (100 µM), which is often used as a pan-K2P
channel blocker (Lesage and Lazdunski, 2000), depolarized hTM
cells from −37.7 ± 4.4 to −8.7 ± 2.6 mV (n = 7; P < 0.005; Fig. 2,
1 and J) and evinced ∼5% suppression of the outward current
(Fig. 2, K–M). pTM Vrest and outward currents were similarly
sensitive to quinine (P < 0.01 and P < 0.001 for Vrest and whole-
cell currents, respectively; Fig. 2, J and M). These data indicate
that the hyperpolarizing drive that maintains Vrest in human
TM cells under physiological conditions is largely controlled
by K2P channels.

Molecular expression of KCNK channels in TM cells
The human K2P channel family comprises at least 15 members that
have been placed into six subfamilies based on sequence identity
and functional characteristics. KCNK7 cannot be functionally
expressed, TRESK is restricted to the spinal cord and brain, and
TALK is confined to islets of Langerhans (Enyedi and
Crižjak, 2010; Feliciangeli et al., 2015). Analysis of the remaining genes
showed broadly consistent expression patterns across hTM/pTM
cells, together with the expression of mRNA that encodes TRPV4,
The key TM mechanotransducer (Fig. 3 A). The most abundant K<sub>Cp</sub> transcripts belonged to TASK-1 (TWIK-related acid-sensitive K<sup>+</sup> channel; KCNK3), TREK-1 (TWIK-related K<sup>+</sup> channel, KCNK2), TWIK-2 (two-P domain in a weakly inward rectifying K<sup>+</sup> channel; KCNK6) and THIK-2 (tandem pore domain halothane-inhibited K<sup>+</sup> channel; KCNK12) subunits. Western blots confirmed TREK-1 protein expression (Fig. 3 C), with the expected bands (de la Peña et al., 2012) at 47 and 58 kD. Moreover, immunostaining showed that TREK-1 and TASK-1 localize to the TM plasma membrane (Fig. 3 D).

The TM membrane potential is dominated by arachidonate-sensitive channels

K<sub>Cp</sub> isoforms were functionally characterized using pharmacological tools. In particular, the long-chain polyunsaturated fatty acid AA is an activator of TREK-1, which also inhibits TASK-1 and TWIK-1 (Honoré et al., 2002; Heurteaux et al., 2004). Because AA also activates TRPV4 (Ryskamp et al., 2015) and modulates chloride channels (Meyes, 2008), our experiments were generally conducted in the presence of HC-067047 (5 µM), the pan-TRP blocker Ruthenium Red (RuR; 10 µM), and/or DIDS.
to exclude the confounding contributions from cation/anion channels. Under these conditions, AA (100 µM) evoked sustained hyperpolarizations (arrow in Fig. 4, A and B) that reduced \( V_{\text{rest}} \) from \(-24.2 \pm 2.6 \, \text{mV} \) in unstimulated cells to \(-57.0 \pm 4.6 \, \text{mV} \) (\( n = 10 \) cells; \( P < 0.01 \), paired \( t \) test). AA-evoked hyperpolarizations were associated with an increase in the outwardly rectifying conductance from \( 342.4 \pm 49.0 \, \text{pA} \) in unstimulated cells to \( 2,078 \pm 213 \, \text{pA} \) in the presence of the polyunsaturated fatty acid (Fig. 4, C and D). This effect was antagonized by spadin, a selective inhibitor of TREK-1 (Mazella et al., 2010), which reversibly depolarized the cells to \(-46.1 \pm 4.8 \, \text{mV} \) (\( n = 9 \); Fig. 4, A and B) and partially but significantly (\( P < 0.001 \)) suppressed the amplitude of AA-evoked outward currents (to \( 1,518 \pm 188 \, \text{pA} \); Fig. 4, C and D). The transient depolarization induced by AA in the presence of TRPV4 blockade (arrow in Fig. 4 A) was not investigated further but could involve incomplete TRPV4 inhibition by micromolar HC-067047 and/or activation of arachidonate-regulated \( Ca^{2+} \) channels (e.g., Meves, 2008; Thompson and Shuttleworth, 2013).

We next explored whether the cohort of TREK-1 channels that maintain \( V_{\text{rest}} \) represents the maximal fraction of activatable channels by exposing the cells to small-molecule TREK-1 activators ML-335 and ML-402 (Lolicato et al., 2017). Both agonists shifted the membrane potential in the hyperpolarizing direction (\( \Delta V_{\text{m}} \) = \(-15.5 \pm 4.1 \) and \(-24.7 \pm 6.5 \, \text{mV} \) by ML-335 and ML-402, respectively) while augmenting the whole-cell conductance, with \( \Delta I \) = \( 707 \pm 98 \) and \( 1,458 \pm 159 \, \text{pA} \) for ML-335 and ML-402, respectively (Fig. 5, A–E). Hence, TM cells maintain a substantial fraction of "reserve" TREK-1 channels that are available for activation.
TREK isoforms have been differentiated from other K2P channels with the dihydropyridine analogue amlodipine (Liu et al., 2007). The blocker (10 μM) depolarized hTM cells from −33.4 ± 3.9 to −4.1 ± 1.7 mV and attenuated the amplitude of the outward current (Fig. 5, F–J). Similar inhibitory effects on the membrane potential and outward conductance were observed in pTM cells (Fig. 5, G and I), confirming that V_{rest} is largely subserved by tonic activation of TREK-1.

The prominent mRNA and protein expression of TREK-1 and TASK-1 in pTM/hTM cells (Fig. 3) prompted us to clarify their relative contributions to V_{rest}, with isoform-specific knockdown. Cells were transfected with lentiviral vectors carrying shRNA or scrambled (Sc) RNAs that were labeled with a fluorescent marker (GFP). Transcript analysis showed 0.57 ± 0.2 knockdown of TREK-1 (P < 0.2) and 0.43 ± 0.02 TASK-1 mRNAs (P < 0.0018) relative to Sc controls, with no apparent effects on cell morphology or survival. Cells transfected with Sc shRNA expressed the outwardly rectifying current (Fig. 6, B and C) typical of untransfected hTM/pTM (Fig. 1 A). Transfection with Sc shRNA had no effect on V_{rest} (−29.1 ± 2.9 mV), whereas TREK-1 knockdown depolarized the cells to −16.9 ± 2.1 mV (Fig. 6 A). TASK-1 knockdown had no significant effect on V_{rest} (Fig. 6 D). The transmembrane current in TREK-1 shRNA–transfected cells at V_h = 100 mV was reduced from 480 ± 60 pA in cells overexpressing Sc shRNA to 187 ± 23 pA in TREK-1 shRNA–expressing cells (Fig. 6, A and B). Down-regulation of TASK-1 had a slight but significant depolarizing effect on V_{rest} in pTM cells (P < 0.05; Fig. 6 J). Similar results were obtained from pTM cells (Fig. 6, G–I). These data confirm
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the dominant contribution of TREK-1 to the background “leak” conductance in TM cells and suggest a potential auxiliary role for TASK-1 in primary cells.

TREK-1 channels are required for TM pressure sensitivity

We investigated the role of K2P channels in mediating the TM responsiveness to physiological steps of pressure in the presence of HC-067047. Positive pressure steps (15 mm Hg, 3 s) hyperpolarized the Vrest by 3.52 ± 1.49 mV (n = 8 cells, P < 0.05; Fig. 7, A and B), evoking robust outward currents that reached the peak of 2,645 ± 624 pA within 10 s (n = 5; Fig. 7 C). Consistent with K2P involvement, quinine reduced the pressure-evoked outward current by ∼86% to 369 ± 78 pA (n = 5 and n = 6 cells for control and quinine groups, respectively; P < 0.01; Fig. 7, C and D). Given that TREK-1 is the sole TM-expressed member of the mechano-sensitive K2P group activated by poking, stretching, or pressuring the plasma membrane (Dyachenko et al., 2006; Brohawn et al., 2014), we assessed the pressure response after its knockdown. The pressure-induced current in control Sc-shRNA–transfected cells (3,412 ± 552 pA) was attenuated by ∼80% to 686 ± 63 pA after the transfection with TREK-1 shRNA (n = 11 for Sc shRNA and n = 11 for TREK-1 shRNA groups; respectively, P < 0.01; Fig. 7, E and F). Collectively, these results identify TREK-1 as a regulator of TM mechanosensitivity.

Inhibition of K2P channels results in an elevation of intracellular Ca²⁺

Given the dominant role of the second messenger calcium in TM transmembrane ion flux, volume regulation, contractility, and outflow resistance (Wiederholt et al., 2000; Gasull et al., 2003; Dismuke and Ellis, 2009; Ryskamp et al., 2016), we wondered whether TREK-1 activation coincides with altered Ca²⁺ homeostasis. To test this, cells were loaded with the calcium indicator Fura-2 AM, and the 340/380-nm fluorescence ratio was monitored in the presence of TREK-1 activators and blockers.

Unexpectedly, exposure to the TREK-1 activator ML-402 (40 µM) evoked rapid [Ca²⁺], elevations in ∼85% of hTM cells (Fig. 8, A and B). This effect was abolished by RuR (10 µM), suggesting that TREK-1-dependent hyperpolarizations stimulate Ca²⁺ entry via tonically activated TRP-like channels (Fig. 8 C). We tested this possibility in voltage-clamped cells by switching the membrane potential from −30 to 0 mV and −70 mV, respectively.

Figure 4.  TM membrane potential is potently modulated by arachidonate-activated currents. (A) Current clamp, hTM cells superfused with the TRPV4 blocker HC-067047 (2 µM). AA transiently depolarizes the cell, an effect followed by sustained hyperpolarization that is partially and reversibly antagonized by spadin (1 µM). (B) Quantification of experiments shown in A at 3 min after AA application. The black symbols show the mean ± SEM. Pair-sample t test, n = 9 cells. (C) The I-V relationship of the AA-evoked current shows an increase in the outward conductance that is sensitive to spadin. (D) Data summary for the experiments shown in C. The black symbols show the mean ± SEM values of currents recorded at +100 mV. Pair-sample t test, n = 9 cells. ***, P < 0.001 in C and D.
Consistent with the hypothesis that the cells experience constitutive TRP-mediated Ca\(^{2+}\) influx driven by the electrochemical gradient, we found that depolarizing steps decreased \([\text{Ca}^{2+}]_i\), whereas hyperpolarizations, conversely, increased \([\text{Ca}^{2+}]_i\). Reductions (−Δ1.8 mM) and increases (+Δ3 mM) in \([\text{Ca}^{2+}]_o\) induced corresponding decreases and increases in \([\text{Ca}^{2+}]_i\) (Fig. 8 D). Another surprising observation was that TREK-1 blockers quinine, amlodipine, and spadin also evoke robust increases in \([\text{Ca}^{2+}]_i\) in resting TM cells (Fig. 8, G–H). The source of Ca\(^{2+}\) signals induced by TREK-1 blockers remains to be determined but may include depolarization-activated Ca\(^{2+}\) channels and/or release from internal stores (e.g., Wiederholt et al., 2000).

TREK-1 regulates impedance of TM monolayers

To determine whether channel activators and inhibitors have an impact on the transcellular current flow in TM monolayers, we used ECIS. We have recently used this technique in endothelial monolayers to characterize the permeability of adherens junctions (Phuong et al., 2017), but current flow across nonendothelial monolayers that lack intercellular junctions was proposed to largely represent contractility-dependent adhesivity to the substrate (Qiu et al., 2008; Ramachandran et al., 2011). As illustrated in Fig. 9, 30-min exposure to TREK-1 agonists ML402 and ML67-33 that had been pipetted into the well dose-dependently lowered monolayer impedance. At 100 μM, ML402 and ML67-33 reduced normalized impedance by 9.0 ± 1.7% and 19.0 ± 2.6% (n = 4), respectively (Fig. 9, A–D). The effect of ML402 was transient, whereas ML67-33 demonstrated more sustained reduction of impedance without apparent recovery in the presence of the drug. Similar to its effect on Ca\(^{2+}\) signals, K\(_{\text{ATP}}\) inhibition likewise decreased the impedance of TM monolayers, with 0.6 ± 0.2% (n = 7) and 10.0 ± 1.4% decreases in peak impedance (n = 8) observed at 10 and 100 μM quinine (Fig. 9, E and F). In contrast to the effect of ML67-33, the effect of quinine was time dependent, as translayer impedance largely recovered in the presence of the drug. The observed TREK-1–dependent reductions in cell–substrate impedance are consistent with increased paracellular spacing between adjacent TM cells, altered cell contractility, and/or changes in cell–matrix adhesion and support a role for TREK-1 in TM–ECM interactions (Goel et al., 2012).

Discussion

We report that polymodal TREK-1 channels are required to maintain the membrane potential, Ca\(^{2+}\) homeostasis, and pressure sensitivity in human TM cells. Our findings include (a) the close relationship between the TM membrane potential and pressure sensitivity; (b) TREK-1 activation underlies the steady-state resting potential, with a reserve pool of nonactivated channels mediating the hyperpolarizing response to pressure and PUFAs; (c) novel links between TREK-1 activation and Ca\(^{2+}\) homeostasis; and (d) TREK-1 regulates cell–ECM adhesivity. Together, these results...
place TREK-1 in the center of mechanosensing and IOP regulation in the primate eye.

The current–voltage relationship and outward rectification of the macroscopic leak current in in physiological K⁺ gradients, together with the abrogation of the membrane potential gradient in symmetric transmembrane K⁺ gradients, indicate that TM $V_{\text{rest}}$ is controlled by the passive behavior of K⁺ leak channels predicted by the Goldman–Hodgkin–Katz equation. Among the variety of K⁺ channels expressed in bovine, mouse, and/or human TM cells are voltage-activated, inwardly rectifying, ATP-sensi-

Figure 6. **TREK-1 but not TASK-1 knockdown suppresses the transmembrane conductance and depolarizes $V_{\text{rest}}$ in hTM cells.** (A) Current clamp, hTM cells. Averaged $V_{\text{rest}}$ values in cells transfected with scrambled (Sc) shRNA ($n = 12$ cells) or TREK-1 shRNA (shTREK-1; $n = 10$ cells), two-sample t test. (B) Voltage clamp. Averaged I–V relationship for cells transfected with Sc ($n = 12$ cells; open circles) or shTREK-1 ($n = 10$ cells; closed circles). (C) Quantification of the TREK-1 dependence of the steady-state current. The current amplitude was significantly reduced by TREK-1 knockdown ($n = 12$ cells and $n = 10$ cells for Sc- and shTREK-1 cohorts, respectively). (D) Current clamp. Averaged $V_{\text{rest}}$ values in cells transfected with scrambled (Sc) shRNA (Sc; $n = 10$) or TASK-1 shRNA (shTASK-1; $n = 10$), shown as mean ± SEM. $P > 0.05$, two-sample t test. (E) Voltage clamp. Averaged I–V relationship for cells transfected with Sc ($n = 10$) or shTASK-1 ($n = 10$). (F) Quantification of TASK-1 dependence of the steady-state current in hTM cells. The whole-cell current amplitude was unaffected by TASK-1 knockdown. $P > 0.05$, two-sample t test. (G) Current clamp, pTM. Averaged $V_{\text{rest}}$ for Sc-shRNA ($n = 11$) and TREK-1 shRNA (shTREK-1; $n = 11$) transfected pTM cells, with mean ± SEM. $*, P < 0.05$, two-sample t test. (H) Voltage clamp. Averaged I–V curve for Sc ($n = 11$) and shTREK-1 ($n = 11$) transfected cells. (I) Quantification of TREK-1 dependence of the steady-state current in pTM cells ($n = 11$ and 11 cells for Sc and shTREK-1 cohorts, respectively). $*, P < 0.05$, two-sample t test. (J) Current clamp, pTM. Averaged I–V curve for Sc ($n = 20$ cells) and TASK-1 shRNA (shTASK-1; $n = 22$ cells) transfected cells. Two-sample t test. (K) Voltage clamp. Averaged I–V curve for Sc ($n = 20$ cells) and shTASK-1 ($n = 22$ cells) transfected pTM cells. (L) Quantification of TASK-1 dependence of the steady-state current in pTM cells. $n = 20$ cells and $n = 22$ cells for scrambled control and shTASK-1 cohorts, respectively. Two-sample t test. Shown in A–L are the mean ± SEM. N.S., $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$. 

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tive, Ca$^{2+}$-activated large-conductance (BK) and tandem pore K$^+$ channels (Lloret et al., 2001; Gasull et al., 2003; Grant et al., 2013), and volume-activated K$^+$ channels (Mitchell et al., 2002). Our observation that resting K$^+$ fluxes in hTM/pTM cells are insensitive to classic blockers of Kv, Kir, and KCa channels supports the conclusion that the hyperpolarizing component of the $V_{\text{rest}}$ is subserved almost exclusively by K$_{2P}$ channels. We identified TREK-1 as the key regulator of the background conductance based on the following evidence: (a) hyperpolarizations induced by AA, pressure, and small-molecule activators; (b) obliteration of the resting potential by amlodipine, spadin, and TREK-1 shRNA; (c) outward rectification of the TM current typical of TREK-1 activation in the presence of divalent cations (Maingret et al., 2002); and (d) antibody staining in cultured cells (Fig. 3) and intact tissue (Carreon et al., 2017). We were unable to discern obvious roles for TWIK/THIK-2 subunits, which may have been nonfunctional under our experimental conditions (Bichet et al., 2015), whereas TASK-1 may contribute a small residual outward component in pTM cells.

The extent to which TREK-1 dominates the TM membrane potential seems to be without precedent in eukaryotic cells. Typically, the channel is expressed in cells from organs that experience mechanical deformation (e.g., lung, uterus, stomach, intestine, colon, and bladder cells), but its contribution to $V_{\text{rest}}$ tends to be auxiliary (Fink et al., 1996; Reyes et al., 1998; Ferroni et al., 2003; Heurteaux et al., 2004; Honoré, 2007; Lembrechts et al., 2011; Cadaveira-Mosquera et al., 2012; Lei et al., 2014). Submaximal TREK-1 activation in resting TM cells may be caused by the low probability of opening at near-zero membrane tension (Fink et al., 1996), whereas TREK-1–mediated hyperpolarizations (~15–25 mV) induced by pressure, AA, and selective agonists indicate a substantial pool of activatable channels. Activation of this reserve pool might endow the TM with autoregulatory response to mechanical stressors, as documented in heterologously expressing cells (Brohawn et al., 2014). Chloride channels almost certainly contribute to the maintenance of the hyperpolarized state, because blocking Cl$^-$-permeable channels (e.g., volume-regulated anion channels, ClC2 and ClC3; Mitchell et al., 2002).
et al., 2002; Comes et al., 2005) evinced small (∼4–6 mV) depolarizations (O. Yarishkin and D. Križaj, unpublished observations). Studies in bovine TM cells similarly suggested that Cl⁻ fluxes supply ∼10% of the standing outward current (Wiederholt et al., 2000). Another interesting question pertains to the nature of the depolarizing component that balances the resting K⁺ and Cl⁻ fluxes. The identity of this component remains to be determined, with candidates including voltage-operated, store-operated Orai and/or TRP channels (Wiederholt et al., 2000; Abad et al., 2008; Tran et al., 2014).

In addition to maintaining Vrest, the TREK-1 response to pressure appears to modulate calcium influx and cell–ECM interactions. ECIS measurements revealed that channel agonists and antagonists induced significant decreases in monolayer impedance, with a time course that roughly matched that of the [Ca²⁺]i elevations evoked by TREK-1 activation and inhibition. The precise mechanism remains to be determined but may have involved Ca²⁺-dependent changes in TM contractility, integrin binding (Wiederholt et al., 2000; Campbell and Humphries, 2011), and/or integrin–ECM contacts (Ramachandran et al., 2011), given that Ca²⁺ regulates the interactions between TREK-1 and α-smooth muscle cell actin (Patel et al., 1998), MAPK activation (Bittner et al., 2013), strain-dependent formation of actin stress fibers, and secretion of fibronectin (Ryskamp et al., 2016). Low levels of VE cadherin and lack of adherens and occludens contacts (Bhatt et al., 1995; Pattabiraman et al., 2014) might have contributed to the low steady-state impedance compared with endothelial monolayers (Phuong et al., 2017) that is further decreased through TREK-1 modulation. It remains to be seen whether the increased impedance observed in glaucomatous cells (Torrejon et al., 2016) is mirrored by altered TREK-1 activation in hypertensive eyes.

Among the advantages of the high-speed pressure clamp technique over traditional cell poking assays is the ability to precisely control the timing and amount of force applied to the channel. Using this method, we found hyperpolarizations evoked by physiological pressure steps to be driven mainly by TREK-1. Because the unpressurized gigaseal patch itself imposes nonzero lateral tension on the channel, we did not estimate the threshold for activation by pressure. It is notable that forces that activate TREK-1 (Brohawn et al., 2014) also span the IOPs measured in the healthy eye, ranging from ∼3–7-mm diurnal fluctuations to transient ∼200–300-mm Hg increases evoked by blinking, saccadic eye movements, eye scratching, and sneezing (Downs, 2015). The pressure steps used in our study (15 mm Hg) increased the open probability of human TREK-1 by ∼19-fold (Lei et al., 2014), but it remains to be determined how its open probability is influenced by membrane tensions in hypertensive eyes in which TM cells are stiffer (Last et al., 2011; Pattabiraman et al., 2014) and whether gain/loss-of-function TREK-1 mutations contribute to hypertensive injury in the eye (e.g., Goel et al., 2012; Decher et al., 2017).

One of the conclusions of the present study is that TREK-1 in healthy TM cells functions as a voltage tuner that minimizes pressure responsiveness, Ca²⁺ dysregulation, and contractility through its control of Vrest (approximately −30 mV in human and bovine TM cells; Lepple-Wienhues et al., 1994; Llobet et al., 2001). Tensile steady-state may be so important for TM cells that deviations in depolarizing or hyperpolarizing directions induce a similar compensatory (calcium) response; however, we demonstrate that these compensatory responses involve...
different intracellular signaling pathways. RuR sensitivity of the hyperpolarizing response suggests that TREK-1 activators facilitate TRP channel-dependent Ca\textsuperscript{2+} influx, possibly via TRPV and/or TRPC channels (Abad et al., 2008; Ryskamp et al., 2016), whereas TREK-1 inhibition presumably depolarizes the cells and induces voltage-dependent Ca\textsuperscript{2+} influx (e.g., Lepple-Wienhues et al., 1991). This ingenious molecular arrangement allows TREK-1 to transduce decreases and increases in pres-
sure into calcium-dependent compensation to modulate TM tensile homeostasis.

We hypothesize that IOP-dependent tensional integration of the TM membrane involves concurrent modulation of at least two intrinsic mechanotransducers, TREK-1 and TRPV4. Compressive, tensile, and/or shear load prepressing the TM membrane by influencing its curvature and fluidity (Anishkin et al., 2014) and thereby facilitating force detection at the TREK-1-lipid interface. In contrast to TREK-1, TRPV4 channels do not regulate the standing conductance, calcium homeostasis, or pressure sensing, as indicated by the lack of effect of HC067047 on the V_{est} [Ca^{2+}], and resting IOP (Ryskamp et al., 2016). After the decrease in outflow facility, changes in aqueous shear flow, and/or changes in membrane stiffness that elevate IOP, both TRPV4 (Lakk et al., 2017) and TREK-1 (Brohawn et al., 2014) are activated and cooperate to set a new homeostatic tensile set point. We propose that such set points are likely to be modulated by phospholipid hydrolysis via phospholipase A2 and production of AA (C20:4 ω6), which regulates both TRPV4 and TREK-1. It is noteworthy that exposure to AA recapitulates many aspects of TRPV4- and TREK-1-induced cytoskeletal remodeling, including increases in [Ca^{2+}], up-regulation of actin stress fibers, and fibronectin release (Ryskamp et al., 2016; White et al., 2016; Carreon et al., 2017), whereas inhibition of AA production weakens stress fibers and increases aqueous fluid outflow (Pattabiraman et al., 2014). Hence, IOP elevations might regulate the channels directly influencing its curvature and fluidity (Anishkin et al., 2014) and an Unrestricted Grant from Research to Prevent Blindness to the Department of Ophthalmology at the University of Utah.

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