Emergent Temporal Signaling in Human Trabecular Meshwork Cells: Role of TRPV4-TRPM4 Interactions

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Trabecular meshwork (TM) cells are phagocytic cells that employ mechanotransduction to actively regulate intraocular pressure. Similar to macrophages, they express scavenger receptors and participate in antigen presentation within the immunosuppressive milieu of the anterior eye. Changes in pressure deform and compress the TM, altering their control of aqueous humor outflow but it is not known whether transducer activation shapes temporal signaling. The present study combines electrophysiology, histochemistry and functional imaging with gene silencing and heterologous expression to gain insight into Ca2+ signaling downstream from TRPV4 (Transient Receptor Potential Vanilloid 4), a stretch-activated polymodal cation channel. Human TM cells respond to the TRPV4 agonist GSK1016790A with fluctuations in intracellular Ca2+ concentration ([Ca2+]i) and an increase in [Na+]i. [Ca2+]i oscillations coincided with monovalent cation current that was suppressed by BAPTA, Ruthenium Red and the TRPM4 (Transient Receptor Potential Melastatin 4) channel inhibitor 9-phenanthrol. TM cells expressed TRPM4 mRNA, protein at the expected 130-150 kDa and showed punctate TRPM4 immunoreactivity at the membrane surface. Genetic silencing of TRPM4 antagonized TRPV4-evoked oscillatory signaling whereas TRPV4 and TRPM4 co-expression in HEK-293 cells reconstituted the oscillations. Membrane potential recordings suggested that TRPM4-dependent oscillations require release of Ca2+ from internal stores. 9-phenanthrol did not affect the outflow facility in mouse eyes and eyes from animals lacking TRPM4 had normal intraocular pressure. Collectively, our results show that TRPV4 activity initiates dynamic calcium signaling in TM cells by stimulating TRPM4 channels and intracellular Ca2+ release. It is possible that TRPV4-TRPM4 interactions downstream from the tensile and compressive impact of intraocular pressure contribute to homeostatic regulation and pathological remodeling within the conventional outflow pathway.

Keywords: Trabecular meshwork, TRPV4, TRPM4, calcium oscillations, glaucoma, conventional outflow facility, GSK1016790, immune
INTRODUCTION

Biomechanical factors such as intraocular pressure (IOP) are an important determinant of the ocular environment, with roles in growth, cellular signaling that subserve ocular health and pathology (1). IOP is homeostatically regulated by the trabecular meshwork (TM), a mechanosensitive phagocytic tissue composed of smooth muscle-, macrophage- and fibroblast-like cells that function as an adaptive valve for outflow of aqueous humor from the anterior eye (1, 2). In healthy cells, acute IOP elevations engender an increase in outflow resistance that is followed by an adaptive response that guides gradual recovery of tissue permeability to fluid flow (3) whereas chronic mechanical stress triggers transdifferentiation of TM cells into stiff and contractile myofibroblasts that subserve a lasting increase in outflow resistance (4–6). TM cells express major histocompatibility complex proteins and Toll-like receptor 4 receptors (TLR4) and help maintain the immune privilege in the anterior eye via phagocytosis and TGFβ2 signaling (7). TLR4 expression is increased in glaucoma, TGFβ-TLR4 crosstalk is involved in production of ECM and regulation of IOP, whereas a mutation in TLR4 inhibited TGFβ2-induced ocular hypertension in mice (7). These observations suggest that the innate immune system might be involved in pressure-dependent differentiation of TM cells into profibrogenic myofibroblasts (8) yet the molecular mechanisms that underlie pressure-dependent TLR4 activation, TGFβ signaling and ECM remodeling remain unknown. Recent studies showed that TM cells sense acute and chronic mechanical stressors via arrays of mechanosensitive proteins that include integrins, the cytoskeleton, and stretch-activated ion channels (SACs) (9–11) such as TRPV4. This nonselective cation channel responds to TM substrate deformation with Ca2+ influx that regulates cytoskeletal and lipid dynamics, Rho signaling and cell-ECM interactions to facilitate myofibroblast differentiation (12–14). Excessive TRPV4 activity was suggested to drive the pressure-induced increase in TM stiffness and contractility (5, 14, 15) but it is not known how the channel responds to sustained activation.

The human TRPV4 gene contains 16 exons that encode a protein consisting of a proline-rich (PRD) domain, phosphoinositide binding site, ankyrin repeats and a helix-turn-helix (HTH) linker domain within the N-terminus, six transmembrane domains with the pore between S5-S6, and modulatory sites (TRP domain, MAP7-binding domain, CaM/IP3R-binding domain, PDZ domain) in the C-terminus that endow the channel with sensitivity to temperature, metabolites of arachidonic acid, nociceptive and mechanical stimuli (16, 17). Gain-and loss-of-function mutations in the TRPV4 gene cause debilitating skeletal abnormalities, sensorimotor neuropathies and vision loss (18), indicating that the channel is required for homeostatic transduction of sensory information. TRPV4 overactivation and exposure to TGFB induce transdifferentiation of epithelial, endothelial, and smooth muscle cells into hypersecretory and contractile myofibroblasts (19, 20). Interactions with a wide array of proteins (aquaporins, BK channels, kinases, actin) and processes (Ca2+ release from intracellular stores, receptor-operated and store-operated Ca2+ influx) (13, 21–24) indicate that TRPV4 exerts its functions within a multiplicity of biological contexts.

The aim of this study was to simulate sustained application of mechanical stress in the absence of confounding effects of the in situ milieu and attendant activation of auxiliary stretch-activated channels (SACs) such as TREK-1 and Piezo1 (10, 11). We report that continual chemical stimulation of TRPV4 channels [P_{Ca/PNa} = 0.12 (26)] that has been linked to hypertension and Ca2+ oscillations and Ca2+ release from intracellular compartments in lymphocytes and macrophages (27–30). TRPV4 and TRPM4 were also implicated in the maturation of phagocytic function, with disruption of either TRPV4 or TRPM4 signaling in monocytes and macrophages resulting in profound dysregulation of Ca2+ homeostasis, LPS-stimulated phagocytosis and survival (31, 32). These findings identify a mechanism that couples TM mechanosensing to endogenous oscillatory activity, with potential functions in innate immune regulation and outflow resistance in the anterior eye.

MATERIALS AND METHODS

Primary Cell Isolation and Culture

Primary trabecular meshwork cells (pTM) were isolated from juxtanaculicular and corneoscleral regions of the human donors’ eyes (56 years-old male, 62 years-old female) with no recorded history of eye disease as described (10, 11, 13). The tissues were obtained through the Lions Eye Bank at the Moran Eye Center at the University of Utah and were used in concordance with the tenets of the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. A subset of biochemical experiments was conducted in parallel with immortalized cells (hTM), isolated from the juxtanaculicular region of the human eye (ScienCell Research Laboratories, Carlsbad, CA, USA). The cell phenotype was periodically profiled for expression of MYOC, TIMP3, AQP1, MGP, ACTA2 (α-smooth muscle actin, αSMA) genes and DEX-induced upregulation of myocilin protein (11, 14).

Passage 2 – 6 cells were seeded onto Collagen I-coated coverslips and grown in Trabecular Meshwork Cell Medium (ScienCell, Catalog#6591; Carlsbad, CA, USA) supplemented with 2% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, at 37°C and pH 7.4. Cells were transfected with scrambled shRNA (Sc-shRNA) or Trpm4 shRNA using Lipofectamin™ 3000 (5 μg per 25 cm² tissue culture flask). shRNA -transfected cells were identified by green (GFP) or red (mCherry) fluorescence. Knockdown efficiency of TRPM4 shRNA was validated in HEK 293T cells overexpressing GFP-hTRPM4, scrambled shRNA or one of three TRPM4 shRNA constructs. shRNA1 and shRNA2, with target sequences GGACATTGCCAGAGTGAACT (1218–1238) and GGAAAGACCTGCGGTCAAGT (1835–1855), showed >65% knockdown efficiency (Supplementary Figure 1). Experiments were conducted 3 - 4 days post- transfection.

Reagents

GSK1016790A (GSK101) is a highly specific agonist (16, 17, 23) and HC067047 (HC-06) antagonist, of TRPV4 channels. Both were
purchased from Sigma (St. Louis, MO) or Cayman Chemical (Ann Arbor, MI). Salts were purchased from Sigma (St. Louis, MO) or VWR (Radnor, PA), CBA (4-chloro-2-[(2-chlorophenoxoy) acetyl aminobenzoic acid from Tocris Bioscience (Minneapolis, MN), and BAPTA (1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate acid) from Alfa Aesar (Tewksbury, MA). The TRPM4 antagonist 9-phenanthrol (9-PA) was from Tocris Bioscience (Bristol, UK). GSK101 (1 mM), and HC-06 (20 mM) DMSO stocks were diluted in extracellular saline (98.5 mM NaCl, 5 mM KCl, 3 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, 10 mM D-glucose, 93 mM mannitol), with final DMSO concentration not exceeding 0.1%.

**Immunocytochemistry**

**Cells**

Cells were plated on collagen Type 1-coated glass cover slip 1 day prior to fixation with the 4% PFA for 10 minutes at RT. The samples were blocked with phosphate-buffered saline containing 0.3% Triton X-100 and 5% FBS for 30 minutes at a room temperature (RT). The primary polyclonal rabbit antibody was diluted 1:1000 in PBS (2% BSA and 0.2% Triton X-100) and incubated overnight at 4°C (32). After rinsing, cells were incubated for 1 hour at RT with goat anti-mouse and goat anti-rabbit IgG (H + L) secondary antibodies conjugated to fluorophores (Alexa Fluor 488 nm, 568 nm and/or 594 nm; 1:500; Life Technologies, Carlsbad, CA, USA). Unbound antibody was rinsed, and conjugated fluorophores protected with Fluoromount-G (Southern Biotech, Birmingham, AL, USA) prior to mounting coverslips. Images were acquired on a confocal microscope (FV1200; Olympus, Center Valley, PA) at 1024 x 1024 pixels with a 20x water superobjective (1.00 N.A.; field size: 158.565 x 158.565 µm; 0.155 µm/pixel; sampling speed: 10.0 us/pixel; 12 bits/pixel). ≥50 cells per experiment were acquired for at least 4 independent experiments (n ≥ 200 in total; N ≥ 4).

**Tissue Immunohistochemistry**

Anterior chambers were fixed in 4% paraformaldehyde for 1 hour, cryoprotected in 15 and 30% sucrose gradients, embedded in Tissue-Tek® O.C.T. (Sakura, 4583), and cryosectioned at 12 µm, as described (13, 30). Sections were probed with a polyclonal rabbit TRPM4 antibody (1:100 (32); and aquaporin-1 mouse monoclonal antibody (1:1000; Santa Cruz Biotechnology Sc-25287). Secondary antibodies were anti-rabbit IgG DyLight 488 (Invitrogen, 35552) and anti-mouse IgG DyLight 594 (Invitrogen, 35511). Sections were coverslipped with DAPI-Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA), and imaged with a confocal microscope. Images were acquired using identical (HV, gain, offset) parameters.

**Semi quantitative Real Time-PCR**

Total RNA was extracted using Arcturus PicoPure RNA Isolation Kit (ThermoFisher, Cat. No.: KIT0204) (33, 34). 100 ng of total RNA was used for reverse transcription. First-strand cDNA synthesis and PCR amplification of cDNA were performed using qScript™ XLT cDNA Supermix (Quanta Biosciences, Cat. No. 95161). The samples were run on 2% agarose gels using ethidium bromide staining along with the 100-bp DNA ladder (ThermoFisher Scientific, Waltham, MA, Cat. No.: S0323). The primers used in the study are listed in Table 1.

**Western Blot**

TM cells were detached from culture flasks by trypsinization and centrifuged at 2000 rpm for 3 minutes. The cell pellet was washed with PBS and lysed in a RIPA Buffer System (Santa Cruz Biotechnology, Dallas, TX). Cell lysates were separated by 10% SDS-PAGE followed by electrophoretic transfer to polyvinylidene difluoride membranes (Bio-Rad, Hercules CA). Membranes were blocked with 5% skim milk in PBS containing 0.1% Tween 20 and incubated at 4°C overnight with the TRPM4 antibody, following by 3 washes with PBS containing 0.1% Tween-20, blotted with HRP-conjugated GAPDH antibody for 30 min at room temperature and washed with PBS containing 0.1% Tween 20. The signals were visualized with an enhanced chemiluminescence system (FluorChem Q, Cell Biosciences, Santa Clara, CA).

**Ion Imaging**

Pharmacological experiments were conducted on a microscope stage in a fast-flow chamber (RC26GLP, Warner Instruments, Hamden CT) connected to a gravity-fed perfusion system. The flow rate of the solution was regulated via individual pinch valves (VC-6; Warner Instruments; Holliston, MA). Primary cells were loaded with Fura-2 AM (3 μM; Invitrogen/ThermoFisher Scientific) for 45 min at RT. The cells were perfused with extracellular solution containing (mM): 135 NaCl, 2.5 KCl, 1.5 MgCl2, 1.8 CaCl2, 10 HEPES, 5.6 D-glucose (pH = 7.4; osmolarity = 300 - 303 mOsm). Epifluorescence imaging was performed on Nikon microscopes using 40x (1.3 N.A., oil or 0.80 N.A., water) objectives, 340 nm and 380 nm excitation filters (Semrock, Lake Forest, IL, USA) and a Xenon arc lamp (DG4, Sutter Instruments). Fluorescence emission at 510 nm, in response to alternating 340/340 excitation, was captured with cooled

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**TABLE 1 | Primer sequences used for PCR analysis.**

| Name  | Forward primer | Reverse primer | Product size (bp) | NCBI reference number |
|-------|----------------|----------------|-------------------|-----------------------|
| TRPM4 | GATGCAACCCACCGAGAA | AGAGCCCGGGAATTGCTG | 91 | NM_017636.4 |
| TRPV1 | GGCAGCAGTTCGACCAATC | TGGCCAGTQAGACGTACCA | 169 | NM_080704.3 |
| TRPV4 | TCCATGTCTGTCAGCCAC | AGGQCTGTAGCCTGAGATA | 217 | NM_021625.4 |
| MYOC  | CACCTGCGAGATCGACACA | TCCAGTGGCCTAGGATAT | 118 | NM_000261.1 |
| AQP1  | TGGAGCAGCTTGGCGTTCGTT | CGTGTGCTCAGCTGCAA | 174 | NM_198098.3 |
| GAPDH | CTGCTGTTCGAGAAGCCC | GACTGCGACTTGACGCTTC | 89 | NM_000246.5 |
EMCCD or CMOS cameras (Photometric, Tucson, AZ). Data acquisition was controlled by NIS Elements 3.22 software (Nikon). Typically, ~5-10 cells per slide were averaged across ~3-6 slides per experiments, with at least 3 independent experiments (n ≥ 50; N ≥ 3). The experiments were performed at room temperature (20 - 22°C).

Ca²⁺ imaging. Cells were loaded with 3 µM Fura-2 AM (Invitrogen/ThermoFisher) (Kd at RT = 225 nM) for 45 min. ΔR/R (peak F₃₄₀/F₅₀₀ ratio – baseline/baseline) was used to quantify the amplitude of Ca²⁺ signals (e.g. (13, 35). Only transient Ca²⁺ events with amplitudes exceeding three standard deviations of baseline fluctuations were included in analysis.

Intracellular Na⁺ imaging. Cells were loaded with 3 µM NaTRIUM Green™-2 AM (TEFLabs Austin, TX) for 50 – 60 min in a cell incubator at 37°C. Fluorescence was acquired at 484 nm excitation and 520 nm emission (Semrock, Rochester, NY). Na⁺ signals were normalized to the average baseline (F/F₀) obtained at the beginning of the experiments.

**Electrophysiology**

Whole cell and single-channel techniques were used to record membrane currents (voltage clamp) or voltage (current clamp) (11, 35, 36). Borosilicate patch pipettes (WPI, Sarasota, FL) were pulled to resistances of 5 - 8 MΩ (P-2000; Sutter Instruments, Novato CA). The standard pipette solution contained (in mM): 125 NaCl, 10 KCl, 10 HEPES, 1 MgCl₂, 2 ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N'-tetraacetic acid (EGTA), 0.3 Na-GTP (pH 7.3). The pipette solution for recording Ca²⁺-activated current contained (in mM): 125 Na-glucuronate, 10 HEPES, 1 MgCl₂, 0.01 CaCl₂, 0.3 Na-GTP (pH 7.3). Nominally Ca²⁺-free pipette solution (standard) contained (in mM): 125 Na-glucuronate, 10 HEPES, 1 MgCl₂, 5 BAPTA, 0.3 Na-GTP (pH 7.3). The standard extracellular solution contained (in mM): 135 NaCl, 2.5 KCl, 1.5 MgCl₂, 1.8 CaCl₂, 5.6 D-glucose, 10 HEPES (pH 7.4). The extracellular solution for recording Ca²⁺-activated current contained (in mM): 135 Na-glucuronate, 10 HEPES, 1 MgCl₂, 1.8 CaCl₂, 0.3 Na-GTP (pH 7.3). The bathing solution for excised (inside-out) patch clamp experiments contained (in mM): 140 NaCl, 2.5 KCl, 1.5 MgCl₂, 1.8 Ca²⁺ or 10 EGTA, 5.6 D-glucose, 10 HEPES (pH 7.4, adjusted with NaOH). The pipette solution used in excised patch recordings contained 140 NaCl, 2.5 KCl, 1.5 MgCl₂, 1.8 CaCl₂, 5.6 D-glucose, 10 HEPES (pH 7.4, adjusted with NaOH). Experiments were performed at room temperature of 21-22°C.

Patch clamp data in whole-cell and single-channel configurations was acquired with a Multiclamp 700B amplifier and a Digidata 1550 interface, and controlled by Clampex 10.7 (Molecular Devices, Union City, CA). Cells were held at -100 mV or +100 mV, with currents sampled at 5 kHz, filtered at 2 kHz with an 8-pole Bessel filter and analyzed with Clampfit 10.7 (Molecular Devices) and Origin 8 Pro (Origin Lab, Northampton, MA). Whole-cell currents were elicited by voltage ramps ascending from -100 mV to 100 mV (0.2 V/sec) from the holding potential of 0 mV. The time course of Ca²⁺-activated currents was obtained by subtracting voltage ramp-evoked currents traces recorded 40 s after obtaining the whole-cell configuration from preceding and following traces.

1-V curves of Ca²⁺-activated currents were assessed by subtracting currents recorded 40 s after obtaining the whole-cell configuration from steady state currents recorded 4 min after obtaining the whole-cell configuration.

Patch clamp combined with fluorescent imaging. Cells were loaded with a calcium indicator Fluo-4 (Invitrogen/ThermoFisher) through a patch pipette. Fluorescence signals in voltage-clamped cells were detected using the excitation filter 484 nm and the emission filter 520 nm (Semrock, Lake Forest, IL). VOCC-mediated current was recorded in the extracellular solution containing as following (mM): 115 NaCl, 2.5 KCl, 20 BaCl₂, 1.5 MgCl₂, 10 HEPES, 5.6 D-glucose.

**Outflow Facility Measurement: iPerfusion**

Procedures on living mice were carried out under the authority of a UK Home Office project license and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The outflow facility in enucleated eyes from C57BL/6J mice (N = 5 mice; 11-week-old males; Charles River UK Ltd., Margate, UK) was measured using iPerfusion, a custom-made microfluidic setup (37, 38). Following cervical dislocation, eyes were enucleated and affixed to a support platform using tissue glue. Eyes were submerged in a PBS bath kept at 35°C throughout the perfusion. Using a micro-manipulator under a dissection microscope, the anterior chamber was cannulatated within 10 minutes of death using a glass micropipette pulled to have a 100 µm diameter beveled tip. The perfusion fluid was Dulbecco’s PBS containing divalent cations and 5.5 mM glucose (DBG) that was sterile filtered (0.25 µm) prior to use.

One eye was perfused with 9-PA (25 µM) whilst the contralateral eye was perfused with vehicle (perfusion fluid containing the same concentration of DMSO). IOP was set to 9 mmHg for 1 hour to pressurize and acclimatize the eye to the perfusion environment and to allow sufficient time for 9-PA to reach the outflow tissues. Flow into the eye was then measured over 8 increasing pressure steps from 6.5 to 17 mmHg. Steady state for each step was evaluated when the ratio of the flow rate to pressure changed by less than 0.1 nl/min/mmHg per minute over a 5-minute window (37). The stable pressure, P, and flow rate, Q, were calculated over the last 4 minutes of each step, and a power-law relationship of the form

\[
Q = C_r \left( \frac{P}{P_c} \right)^\beta P \quad \text{Eq. 1}
\]

was fit to the Q – P data. The reference outflow facility, \(C_r\), represents the value of outflow facility at a reference pressure \(P_c\) of 8 mmHg, and \(\beta\) characterizes the non-linearity of the Q-P relationship (37). The relative difference in \(C_r\) between treated and untreated contralateral eyes was then calculated as the ratio of \(C_r\) in the treated eye relative to that in the contralateral control eye minus unity. We evaluated whether the relative difference in facility was statistically different from zero using a weighted t-test on the log-transformed data, as previously described (37). Facility values and relative changes in facility are reported in terms of the geometric mean and the 95% confidence interval on the mean.
IOP Measurement
Male and female C57BL/6J mice (JAX; Bar Harbor, ME) and homozygous pan-TRPM4 KO mice (39) were maintained in a pathogen-free facility with a 12-hour light/dark cycle and ad libitum access to food and water. The experimental protocol was approved by the animal ethics committee at the University of Utah and adhered to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. No sex differences were noted in the data, which were pooled. A TonoLab rebound tonometer was used to measure IOP in mice between noon and 2 PM, as described (13). In animals habituated to handling for several days preceding the IOP measurement. An IOP reading represents an average of 10 to 20 tonometer readings.

Statistical Analysis
Student’s paired t-test or two-sample t-test (OriginPro 9.0; OriginLab, Northampton, MA) were applied to estimate statistical significance of results. P < 0.05 was considered statistically significant. Results are presented as the means ± S.E.M. A preprint of this article was submitted at https://biorxiv.org/cgi/content/short/2021.12.15.472700v1.

RESULTS

Activation of TRPV4 Induces Fluctuations of [Ca\(^{2+}\)]\(_i\)
Unstimulated TM cells from healthy donors have low basal [Ca\(^{2+}\)]\(_i\) and rarely show Ca\(^{2+}\) fluctuations (13) but previous studies of mechanically evoked Ca\(^{2+}\) signaling in TM cells have been limited to short stimulation paradigms (5-10 min) (11, 13) and little is known about how the TM regulates [Ca\(^{2+}\)]\(_i\) in the presence of sustained TRPV4 activity. To investigate the mechanisms that underlie Ca\(^{2+}\) signaling downstream from the channel, Fura-2-loaded human TM cells were stimulated for 30 - 45 min with bath-applied agonist GSK101 (25 nM). Agonist-evoked [Ca\(^{2+}\)]\(_i\) increases peaked within 2-3 min, followed by a gradual decline to a steady plateau at ~27% of the peak (Figures 1, 2). A subset (~50%) of cells showed an increase in the frequency of Ca\(^{2+}\) fluctuations during the plateau phase whereas treated with the TRPV4 antagonist HCO67047 (HC-06; 5 μM) (Figures 1B, C), broad-spectrum inhibitors of TRP channels such as Ruthenium Red, or Ca\(^{2+}\) -free saline (Figure 2), did not exhibit this time-dependent behavior.

Oscillatory Ca\(^{2+}\) signals can reflect activation of voltage-operated mechanisms within the membrane and/or release from internal compartments. Voltage-dependent mechanisms were tested by stimulating the cells in the current-clamp mode. The agonist evoked a ~15 mV shift in the membrane potential (Figures 3A, C) that was maintained over the course of the experiment (e.g., 40 min after agonist application; red trace in Figure 3B) and was not associated with changes in temporal behavior.

L-type voltage-operated Ca\(^{2+}\) channels have been implicated in calcium oscillations and pressure-dependent contractility in smooth muscle cells (40). To test their involvement, we combined imaging in Fluo-4-loaded cells with whole-cell recording. As shown in Figure 3D, stepping the holding potential from the holding potential of -70 mV to 0 mV had no detectable effect on the Ca\(^{2+}\) signal. Similarly, extracellular BaCl\(_2\) (20 mM) which permeates voltage-operated Ca\(^{2+}\) channels without inactivating the channel pore or stimulating Ca\(^{2+}\) release from ER stores (41–43) did not induce depolarization-evoked inward currents or Ca\(^{2+}\) spiking (Figure 3E) whereas retinal ganglion cells responded with an inward current (Figures 3Eii, F) that was blocked by the L-type channel blocker nimodipine (1 μM) (Figure 3F). These data suggest that TRPV4-dependent Ca\(^{2+}\) oscillations in human TM cells do not involve L-type voltage-operated Ca\(^{2+}\) channels activity.

TRPV4-Induced Ca\(^{2+}\) Fluctuations Require TRPM4
To further investigate the mechanism that underlies TRPV4-induced calcium oscillations, cells were loaded with the Na\(^+\)-sensitive indicator NaTRIUM Green-2 and exposed to GSK101. The agonist stimulated an increase in [Na\(^+\)]\(_i\) in 54% (39/73) cells (5.96 ± 0.81% above baseline; p < 0.0001; n = 39). The increase in Na\(^+\) concentration was not associated with an oscillatory component, indicating that TRPV4-induced Na\(^+\) influx is distinct from mechanisms that subserve Ca\(^{2+}\) oscillations. We tested the potential involvement TRPM4, a Ca\(^{2+}\)-activated channel permeable to monovalent ions that has been implicated in [Ca\(^{2+}\)]\(_i\) oscillations. In T cells, cardiomyocytes, and smooth muscle cells (27, 28, 40) but with no known functions in the eye by exposing the cells to GSK101 in the presence of 9-phenanthrol (9-PA, 40 μM), an intracellularly acting benzoquinolizinium inhibitor of the channel (44, 45). 9-PA reversibly attenuated the increase in [Na\(^+\)]\(_i\), evoked by GSK101 (P < 0.001) (Figures 4A, B), and obliterated Ca\(^{2+}\) fluctuations during the plateau response phase (Figures 4C, D) (P < 0.0001).

Histology: TM Cells Express TRPM4
Transcriptional profiling of immortalized (hTM) and primary (pTM) cells showed robust expression of TRPM4 and TRPV4 transcripts (Figure 5A) together with TM markers myocilin and aquaporin 1, at bp sizes that matched expected sizes for their respective DNAs. The overall levels of TRPM4 transcripts in hTM cells were comparable to TRPV4 mRNA, and ~35% of TRPV4 mRNA in primary cells (Figure 5B). In both cell lines, a validated antibody (32) labeled a Western blot band at ~130 kDa (Figure 5C), corresponding to the known M.W. of the TRPM4b protein variant (46). Confocal examination of TRPV4-immunoreactive cells and intact tissue further showed labeling of the plasma membrane and cytosolic puncta (Figure 5Dii) and cytosolic puncta. In the preparation from the mouse anterior eye, TRPM4-ir was distributed across the juxtacanalicular and corneoscleral TM regions (green), where it colocalized with the TM marker Collagen IV. Additional, albeit more modest, TRPM4-ir signals were also detected in the ciliary body and the retina.

Electrophysiology: TM Cells Functionally Express TRPM4
We next assessed the properties of the TRPM4-mediated current in voltage-clamped cells by probing for a Ca\(^{2+}\)-sensitive monovalent
FIGURE 1 | GSK101 triggers intracellular Ca\textsuperscript{2+} oscillations in hTM cells. (A) Representative traces of [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in GSK101 treated TM cells. (B) GSK101-induced oscillations are absent in cells pretreated with HC-06. (C) Quantification of results shown in (A, B), shown as average ± S.E.M. ****P < 0.0001, ***P < 0.001 (paired-sample t-test), ###P < 0.001 (two-sample t-test), n = 49 cells and n = 38 cells for control/GSK101 and control/HC06+GSK101 groups, respectively.

FIGURE 2 | GSK101-induced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations requires activity of Ca\textsuperscript{2+}-permeable and Ca\textsuperscript{2+}-dependent TRP channels. Shown are representative traces. (A, C) GSK101-induced [Ca\textsuperscript{2+}]\textsubscript{i} fluctuations are abolished by the nonselective TRP blocker Ruthenium Red (RuR; 10 µM) and in the absence of free extracellular Ca\textsuperscript{2+}. (B, D) RuR and Ca\textsuperscript{2+}-free solution abolish GSK101 induced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. Mean ± S.E.M. ***P < 0.001, ****P < 0.0001, paired t-test, n = 51 and n = 43 cells for (B, D), respectively.
current (47, 48). Cells were dialyzed with pipette solutions containing 10 µM or zero Ca^{2+} and Ca^{2+}-activated K^+ and Cl^- conductances were minimized by replacing extra/intracellular K^+ ions (with Na^+) and Cl^- ions with gluconate (47). ~50% of cells dialyzed with the Ca^{2+}-containing solution showed time-dependent increases in inward and outward whole-cell currents. The current-voltage relationship of the Ca^{2+}-dependent current showed modest outward rectification and peaked within ~4 min after obtaining the whole-cell configuration (Figure 6A), had a reversal potential of 1.6 ± 2.9 mV and amplitudes of -122.7 ± 61.5 pA and 211.2 ± 93.7 pA at holding potentials of -100 mV and 100 mV, respectively (Figure 6B). The current was inhibited by 9-PA (20 µM; n=7/7) and was not observed in cells dialyzed with Ca^{2+}-free solutions or exposed to bath-applied Ruthenium Red (10 µM) (n = 5/5) (Figure 6A).

Single channel properties of the Ca^{2+}-activated current were additionally investigated in inside-out membrane patches using symmetrical Na^+ gluconate-based pipette and extracellular solutions. Patch excision in 1.8 mM Ca^{2+}-containing saline triggered a rapidly inactivating channel with a linear current-voltage relationship (n = 15/85 patches; Figures 6C, F). The slope conductance of the unitary current was 18.9 ± 0.6 pS and the amplitude at 100 mV was 2.05 ± 0.04 pA (Figures 6D–F). The channel was facilitated at positive membrane potentials, inhibited by 9-PA (Figures 6C, D), and typically inactivated within ~5 min after patch excision. Its activity was abolished in Ca^{2+}-free saline (Figure 6C). Both whole-cell and single channel properties of the Ca^{2+}-induced current in TM cells are consistent with TRPM4 activity.

TRPM4 Is Required for TRPV4-Dependent Ca^{2+} Oscillations
To more specifically test the role of TRPM4 in [Ca^{2+}]_i fluctuations, we transfected the cells with TRPM4-specific short-hairpin (shTRPM4-GFP) or “scrambled” (Sc-GFP) control shRNAs (Figure 7A). Of the 3 TRPM4-shRNA constructs, we utilized Sh#1 which downregulated TRPM4 mRNA by 80% (Supplementary Figure 1). Compared to Sc-shRNA-expressing controls, TRPM4 shRNA-transfected cells showed ~80% reduction in the frequency of TRPV4-induced Ca^{2+} fluctuations (Figures 7B–D).
We also tested the TRPM4-dependence of TRPV4-induced calcium signals by transfecting HEK293 cells with TRPV4 and/or TRPM4 DNA. TRPV4 only-transfected cells responded to GSK101 with a peak 

\[\text{Ca}^{2+}\] i increase that inactivated to a plateau without showing time-dependent \(\text{Ca}^{2+}\) oscillations whereas cells co-transfected with TRPV4 + TRPM4 DNA displayed robust oscillations in the presence of GSK101 (Supplementary Figure 2).

**TRPM4 Does Not Regulate Trabecular Outflow or Steady-State Intraocular Pressure**

Chronic TRPV4 activation was suggested to suppress aqueous humor drainage in hypertensive mouse eyes (13). Given that 9-PA inhibits TRPV4-dependent \(\text{Ca}^{2+}\) oscillations in vitro (Figures 8A, B), we tested whether it influences the trabecular component of pressure-induced fluid outflow (“outflow facility”). The effect of 9-PA on outflow facility was examined in enucleated mouse eyes using the iPerfusion system (37, 38). In response to 25 \(\mu\text{M}\) 9-PA, outflow facility decreased by -5% [-23%, 18%] (geometric mean [95% CI]; Figure 8A) relative to contralateral eyes that were perfused with vehicle, but this difference was not statistically significant \((p=0.38, n=5 \text{ pairs})\). \(C_{\text{f}}\) for 9-PA treated eyes was 5.4 [4.2, 6.9] nl/min/mmHg and 5.5 [4.6, 6.7] nl/min/mmHg for vehicle-treated eyes (Figure 8A).

Finally, we investigated whether genetic deletion of TRPM4 channels affects intraocular pressure homeostasis in the mouse eye. TRPM4\(^{-/-}\) eyes showed intraocular pressure levels (11.86 ± 0.14 mm Hg that were comparable to controls (12.10 ± 0.16 mm Hg; \(p > 0.05\); \(n = 14\) eyes and \(n = 36\) eyes for WT and TRPM4\(^{-/-}\), respectively), indicating that TRPM4 may not be required for steady-state intraocular pressure regulation (Figure 8C). Overall, these results suggest that TRPM4 signaling is not required for step-induced fluid drainage across the or for steady-state intraocular pressure regulation.

**DISCUSSION**

The goal of this study was to define the properties of calcium homeostasis during sustained stimulation of TRPV4, a transducer of mechanical stimuli that has been implicated in the regulation of conventional outflow and intraocular pressure (13). We show that long-term TRPV4 stimulation evokes \(\text{Ca}^{2+}\) oscillations that are independent of the membrane potential and require expression of TRPM4. Both TRPV4 and TRPM4 were strongly expressed in primary and immortalized TM cells, with functional coupling between the two cation channels revealed by the rise of the \(\text{Ca}^{2+}\)-activated monovalent current and loss of oscillatory response.
following TRPM4 knockdown. Our findings identify novel interactions between TRPV4, TRPM4 and intracellular calcium stores and build a new working model that may help increase our understanding of homeostatic and time-dependent signaling within the primary outflow pathway.

Changes in $[\text{Ca}^{2+}]_{i}$ represent one of the earliest responses of nonexcitable cells to mechanical stress. Resting human TM cells do not exhibit spontaneous calcium activity whereas sustained stimulation of TRPV4 channels produced three types of time-dependent calcium behavior. (i) The initial increase in $[\text{Ca}^{2+}]_{i}$ evoked by GSK101 peaked within 2-4 min and showed slow onset kinetics that was likely shaped by the messenger pathway involving obligatory activation of phospholipase A2 and production of epoxyeicosatrienoic messengers that bind the pocket formed by S2-S3/S4-S5 linker residues (13, 17). (ii) The subsequent relaxation to the steady-state plateau phase reflects $\text{Ca}^{2+}$-dependent channel inactivation, internalization, interactions with modulatory sites within N- and C-termini (16) or negative regulation of $\text{Ca}^{2+}$ influx by TRPM4 (44). (iii) The relaxation phase was associated with $\text{Ca}^{2+}$ fluctuations in a frequency range similar to signals reported in glaucomatous TM (45) and pressure-, stretch- and GSK101-stimulated fibroblasts and chondrocytes (49, 50).

Several pieces of evidence suggest that the oscillatory response requires TRPM4 signaling. (i) TRPV4-induced fluctuations were suppressed by 9-PA, which also induced a reversible decrease in $[\text{Na}^{+}]_{i}$; (ii) TRPM4 knockdown in TM cells preserved the GSK101

**FIGURE 5** | TRPM4 is expressed in TM cells. (A) Representative PCR gels show expression of TRPM4 and TRPV4 transcripts together with markers aquaporin 1 (AQP1), TRPV1, and myocilin (MYOC) in immortalized (hTM) and primary (pTM) cells. (B) Semi-quantitative qRT-PCR. Relative mRNA abundance of TRPM4 vs. TRPV4; expression is normalized to hTM TRPM4; mean ± S.E.M, N = 3. (C) Western blot; hTM and pTM cells show a TRPM4 band at the expected M.W. [(D), upper panel] Cultured cells immunolabeled for TRPM4, with a magnified image of the region selected by the square (inset) showing punctate signals within the plasma membrane. Scale bar = 50 µm. (E) Anterior eye section from a wild type C57 mice, labeled with TRPM4. The TM region shows pronounced TRPM4-ir (arrows) that colocalizes with the marker Collagen IV (Col IV, blue). Moderate signal is seen in the ciliary body (arrows) and ciliary vasculature (asterisk).
response but eliminated the oscillatory calcium component, and (iii) Heterologous expression of TRPV4 and TRPM4 channels reconstituted fluctuations in HEK293 cells. TRPM4 expression in TM cells was confirmed by transcriptional profiling and immunohistochemistry, with TRPM4 mRNA levels reaching ~30-70% of TRPV4 mRNA, and immunoblots showing a band at the predicted M.W. As in other cell types (51, 52), the TRPM4 antibody labeled plasma membrane and intracellular compartments. The former corresponds to full-length TRPM4b variant which forms the Ca\(^{2+}\)-activated Ca\(^{2+}\)-impermeant cation channel and appears to congregate into punctate clusters within the cell membrane (Figure S5ii) whereas intracellular puncta may correspond to truncated N-terminal variants with unknown functions that localize to the ER (32, 53, 54). The aggregation of TRPM4-ir

![Functional expression of a calcium-activated non-selective cation channels.](image)

**FIGURE 6** | Functional expression of a calcium-activated non-selective cation channels. (A) Time course of the whole-cell current recorded at the holding potentials 100 and -100 mV. The pipette solution contained 10 µM Ca\(^{2+}\) in the standard saline (black, n = 6), with addition of Ruthenium Red (RuR; 10 µM; olive, n = 11), 9-PA (20 µM; magenta, n = 15) in extracellular solution, or Ca\(^{2+}\)-free pipette solution (red, n = 15). (B) Averaged current-voltage relationship of the Ca\(^{2+}\)-activated current in A subtracted by the Ca\(^{2+}\)-free component (n = 6). (C) Representative trace demonstrating the activity of a Ca\(^{2+}\)-activated channel in an inside-out patch preparation at +100 mV. (D) Representative traces of single channel activity. (E) Histogram of unitary current amplitude, fit with Gaussian function (red trace; R-Square = 96.6%). The data were binned at 0.02 pA. (F) Current-voltage relationship of single channel current, n = 6 patches. (A, B, F) show mean ± S.E.M.
signals resembles mechanosensory clusters enriched in TRPV4, paxillin and pFAK described in TM cells and embryonic fibroblasts (14, 55). Consistent with biochemical analyses, TM cells showed current-voltage relationship and pharmacological profiles characteristic of nonselective calcium-activated monovalent current mediated by TRPM4 (i.e., inhibition by Ruthenium Red, 9-PA and shRNA). Its quasi-linearity points at the membrane presence of PIP2 (56) whereas inhibition by BAPTA indicates that the channel is activated within membrane microdomains within which TRPV4 and TRPM4 functionally interact. Ca2+ fluctuations and calcium-activated monovalent current were observed in ~50% of recorded cells, possibly because the cells were derived from multiple TM populations or existed in different stages of the cell cycle. An immortalized atrial cardiomyocyte cell line similarly showed TRPM4-dependent Ca2+ oscillations in ~30% cells (28).

The advantage of the synthetic agonist GSK101 was that it allowed us to track signaling mechanisms associated with TRPV4 in the absence of concurrent activation of Piezo1 and TREK-1 stretch-activated channels (e.g (10, 11). In contrast to previous studies in myocytes, smooth muscle cells, neurons and immune cells (27–29, 57) which linked TRPM4-dependent [Ca2+]i oscillations to obligatory voltage-gated Ca2+ influx, TM cells did not show functional voltage-operated calcium channels expression under our experimental conditions. Thus, depolarization had no discernible effect on [Ca2+]i, and the transmembrane current, and Ba2+ concentrations that reliably trigger voltage-operated calcium channels-mediated spiking and suppress store release in retinal neurons (15, 41) had no discernable effects on TM signaling. The absence of coupling between the membrane potential, [Na+], and Ca2+ oscillations indicates that Ca2+ fluctuations reflect intracellular and/or voltage-independent mechanisms. Previous investigation of TRPM4-TRPC3 interactions in HEK293 cells showed that cell depolarization caused by Na+ influx transiently suppresses TRP-mediated Ca2+ influx by reducing the inward electrochemical driving force for Ca2+ (48). TRPM4 signaling and oscillations in cardiomyocytes were attributed to redistribution of mitochondrial Ca2+ stores into the cytosol (28) but it remains to be determined whether Ca2+ transients that underlie the oscillatory response involve \( \psi_{na} \) and mitochondrial transporters (Na+/Ca2+ -Li+ exchangers, Ca2+/H+ exchange, and/or MCU uniporters). A simple model delineating the potential players associated with TRPV4-dependent calcium oscillations is shown in Figure 9.

Another potential source could include reciprocal interactions between Ca2+ influx and the IP3R store (55, 55) mediated through contacts between the C-terminus CaM-binding site of TRPV4 and

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**FIGURE 7** | TRPM4 knockdown disrupts GSK101-triggered [Ca2+]i fluctuations. (A) TM cells treated with Sc-GFP, and TRPM4 shRNA-GFP constructs. (B, D) Sc-shRNA-transfected cells exhibit fluctuating [Ca2+]i signals, which show (C, D) reduced frequency in TRPM4-shRNA-treated cells. Data shown as mean ± S.E.M. ***P < 0.001, NS P > 0.05, paired t-test, n = 116 cells and n = 27 cells for Sc- and TRPM4 shRNAs, respectively.
the IP3R receptor (58) and depolarization-dependent reduction of the driving force for Ca$^{2+}$ influx through CRAC/Orai channels (27).

TRPM4 was suggested to be directly activated by membrane stretch (59) but this conclusion has been controversial (58, 60, 61). A compelling aspect of our findings is that its role in mechanosignaling might instead reflect activation downstream from stretch-activated channels. Chemical activation of TRPV4 reproduced time-dependent behavior of pressure-induced Ca$^{2+}$

**FIGURE 8** TRPM4 does not regulate trabecular outflow and steady-state intraocular pressure. (A) Representative flow-pressure (Q – P) plot for contralateral eyes perfused with 25 µM 9-PA versus vehicle. Curves show the optimal fit from Equation 1, with 95% confidence bounds and error bars show 95% confidence intervals. (B) Relative difference in outflow facility between contralateral eyes perfused with 25 µM 9-PA versus vehicle. The relative difference in facility is defined as the ratio of Cr in the experimental eye perfused with 9-PA relative to vehicle-perfused contralateral eye minus unity, expressed as a percentage. Each data point represents the relative difference in facility for an individual mouse. Error bars are 95% confidence intervals. Shaded regions represent the best estimate of the sample distributions, with the central white line representing the geometric mean. Dark central bands represent the 95% CI on the mean, and the outer white lines represent the limits encompassing 95% of the population. (C) Averaged results for intraocular pressure in wild-type (WT, n = 14 eyes) and TRPM4 KO (n = 36 eyes) mice. N.S. = P > 0.05.

**FIGURE 9** Model of TRPM4-dependent calcium oscillations downstream from TRPV4. Mechanical stretch triggers cation influx through TRPV4 to elevate [Ca$^{2+}$] and activate TRPM4, a Ca$^{2+}$-dependent Na$^+$ channel to depolarize the TM cells and increase [Na$^+$]. TRPM4 activation is required for voltage-independent oscillatory release of Ca$^{2+}$ from a yet-unknown intracellular compartment.
events in human TM cells (62) and oscillatory phenotypes in TRPV4-expressing macrophages/monocytes, fibroblasts, keratinocytes, endothelial cells, chondrocytes, and glia (50, 55) that have been linked to cellular microcontractions, ECM remodeling and transfer of mechanical force across load-bearing focal adhesions (49, 50, 63). In macrophages, TRPM4 regulates cellular calcium oscillations and contractility via FAK and Rac GTPases (27, 53, 55), processes that may be downstream from TRPV4 (14; Figure 4). Together, these data implicate TRPV4-TRPM4 interactions in endogenous, cell-generated control of stretch-sensitive tyrosine phosphorylations, RhoA signaling and contractility. Another intriguing possibility is that TRPV4-dependent Ca\(^{2+}\) oscillations operate at the threshold of contractile, RhoA-dependent (14) vs. relaxing, nitric oxide-dependent (64) states associated with myogenic-like contractility (62, 65, 66). TRPM4 shares the regulatory sulphonylurea receptor (SUR1/2) with inward rectifying K\(^+\) (Kir6.1 and Kir6.2) subunits that form K\(_{ATP} \) channels sensitive to [Ca\(^{2+}\)]\(_i\) and [Na\(^+\)]\(_i\). Analogy with other load-bearing and immune-like cells, suggests that a persistent pacemaking flux, release and Ca\(^{2+}\)-activated channels have been linked to both TRPV4 and TRPM4 activation (19, 31, 39). Owing to their role in translayer Ca\(^{2+}\), Na\(^+\) and water transport dynamics, myofibroblast transformation and immune regulation, TRPV4-TRPM4 interactions could therefore be targeted by translational interventions aimed at fine-tuning the homeostatic functions of the conventional outflow pathway and regulation of intraocular pressure.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee - University of Utah.

AUTHOR CONTRIBUTIONS

OY, TP, FV, JB, Jv, SR, ML, CR and JB carried out the experiments. OY, JB, SR and Jv performed the statistical analyses. EH and MF provided reagents/animal models. OY and DK drafted the manuscript. OY, TP, FV, JB, Jv, SR, ML, CR and JB conceived and coordinated the study. The authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.805076/full#supplementary-material

**Supplementary Figure 1** | Validation of TRPM4 shRNA constructs. (A) Typical WB results representing knockdown effects of the TRPM4 shRNA constructs in the HEK-293 overexpression system. (B) Quantification of results shown in (A). The results are representative of three independent experiments.

**Supplementary Figure 2** | HEK293 cells. TRPV4 and TRPM4 coexpression is sufficient to sustain calcium oscillations in the presence of GSK101. Representative traces of ratiometric signals in (A) TRPV4 + TRPM4 DNA-transfected cells and (B) Cells transfected with TRPV4 alone. (C) Averaged frequency of [Ca\(^{2+}\)]\(_i\) transients in TRPV4+TRPM4 expressing cells before and after GSK101 exposure.
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