Upregulation of Transient Receptor Potential Vanilloid Type-1 Channel Activity and Ca\(^{2+}\) Influx Dysfunction in Human Pterygial Cells

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Pterygium (conjunctivae) is a common ocular surface hyperproliferative tumorous disorder. Its risk factors include not only dust, wind, heat, dryness, and smoke, but also chronic UV-B light exposure, which is the main risk factor. In addition, viral infection and genetic factors contribute to initiating and promoting this disease.1,2 Specifically, pterygium incidence is greater in regions near the equator (pterygium belt),1,3–5 whereas in Europe it is substantially less.1,3–6 Surgical resection is the only option for reducing visual impairment caused by a pterygium-related disturbance. Because of its high recurrence rate (10%–70%), often repeated surgery may be needed, which heightens the likelihood of regrowth, inflammation, and scar formation.7 Advanced pterygium frequently leads to precorneal tear film dysfunction and dry eye disease (DES). In the long term, it often results in reduced vision due to astigmatism, tear film alterations, and movement restriction of the bulbus.6

Vascular endothelial growth factor (VEGF) and its cognate receptor are upregulated in pterygium tissue (PT).8–10 Retention of the pterygium cell phenotype appears to be location specific, as this tissue does not infiltrate into the cornea, suggesting that the corneal milieu is not supportive of this process.2

Transient receptor potential (TRP) channels constitute a superfamily of ligand-gated, nonselective cation channels subdivided into seven subfamilies according to differences in amino acid sequence homology (reviewed by Ramsey et al.11). The heat-sensitive TRP vanilloid receptor 1 (TRPV1, capsaicin [CAP] receptor) channel is the most investigated TRP channel and is the only one whose three-dimensional structure was determined.12,13 Besides being activated by heat (>43°C) and CAP exposure to acidity and hyperosmolarity can also induce this response.14–16 Furthermore, changes of TRPV1 expression (in human skin) can be detected after UV irradiation.17 Transient receptor potential V1 mediates heat shock–induced matrix metalloproteinase-1 (MMP-1) expression in human keratinocytes.18 However, diverse TRPV1 activation mechanisms induced through various (physical) stimuli have not yet

**Purpose.** The heat-sensitive transient receptor potential vanilloid type-1 (TRPV1) channel (i.e., capsaicin [CAP] receptor) is upregulated in numerous cancers. This study determined if this response occurs in fresh and cultured hyperplastic human pterygial epithelial tissues.

**Methods.** Reverse transcriptase PCR and quantitative real-time PCR, along with immunohistochemistry and Western blotting, characterized TRPV1 expression patterns in pterygial and healthy conjunctival tissue, primary and immortalized pterygial cells (hPtEC), and primary and immortalized conjunctival epithelial cells (HCjEC). Imaging of Ca\(^{2+}\) and planar whole-cell patch-clamping evaluated TRP channel activity. An MTS assay measured cell metabolic activity and a cell growth assay monitored proliferation.

**Results.** Capsaicin (20 μM) and elevating bath temperature above 45°C activated Ca\(^{2+}\) transients more in hPtEC than HCjEC. Capsaicin induced corresponding changes in inward currents that were inhibited by 20 μM capsazepine (CPZ). Vascular endothelial growth factor (VEGF) also increased Ca\(^{2+}\)-influx and induced corresponding inward currents more in hPtEC than in HCjEC, whereas CPZ (20 μM), BCTC (20 μM), or La\(^{3+}\) (500 μM) reduced these responses, respectively. Whereas epidermal growth factor (EGF) increased proliferation more in hPtEC than in HCjEC, VEGF had no effect on this response. Capsazepine suppressed hPtEC proliferation induced by EGF and VEGF whereas it was cytotoxic to HCjEC.

**Conclusions.** Mitogenic responses to EGF and VEGF are mediated through TRPV1 transactivation. Only in hPtEC do the increases in proliferation induced by EGF exceed those in HCjEC. Therefore, TRPV1 is a potential drug target whose clinical relevance in treating pterygium warrants further assessment.

Keywords: pterygium, human conjunctival epithelium, transient receptor potential vanilloid 1 channel, VEGF, EGF, intracellular Ca\(^{2+}\), planar patch-clamp technique.
been elucidated. High-resolution analysis of TRPV1 structure identified conformational changes in an outer pore domain and lower gate domain through ligand-binding. AllostERIC coupling between both domains is responsible for a broad physiological range of stimuli activating TRPV1 and other TRP channels.[19,20,22] Ogawa et al.[23] showed that the oxidative status of Cys residues in the TRPV1 sequence modulated by reactive oxygen species triggers TRPV1 activation through changes in the redox environment. Additionally, TRP channels are mechanically activated directly or by signaling cascades that alter lipid composition, change conformation of plasma membrane and open TRPs by mechanical force (reviewed in Liu and Montell[22]). In Dro sophila, photoreceptors and mammalian photosensitive retinal ganglion cell phototransduction is mediated by G protein-activated phospholipase C (PLC), which generates a force in the lipid-bilayer opening TRP-Canonical channels.[23,24]

Transient receptor potential V1 is expressed in human conjunctival epithelial cells (hCEC),[25] human corneal endothelial cells,[26] corneal fibroblasts,[27] and corneal epithelial cells (HCEC)[28,29] (reviewed in Ref.[30]). Cellular constituent release caused by a severe chemical injury also induces TRPV1 activation, followed by corneal opacification and chronic dysregulated inflammation during wound healing.[27,30,31] As increases in cell proliferation in cancer are associated with rises in functional TRPV1 expression and activity,[32] we hypothesized that this change is associated with conjunctival hyperplasia in pterygium.

We show here that TRPV1 channel expression is upregulated in human PT. This upregulation is associated with dysfunctional Ca2+ influx. Furthermore, the mitogenic responses to EGF and VEGF are dependent on their respective cognate receptors transactivating TRPV1, as suppression of TRPV1 activation by capsazepine (CPZ) inhibited the increases in proliferation induced by these cytokines in human pterygial cells (hPTECs).

**Materials and Methods**

**Materials**

Capsazepine (CPZ) and icilin were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). N-(4-tert-butyl-phenyl)-4-(3-chloropyridin-2-yl) tetrahydro-pyrazine-1(2H)-car boxamide (BCTC) and fura-2/AM were obtained from TOCRIS Bioscience (Bristol, UK). Vascular endothelial growth factor was purchased from ThermoFisher Scientific (Waltham, MA, USA). Medium and supplements for cell culture came from Life Technologies Invitrogen (Karlsruhe, Germany) or Biochrom AG (Berlin, Germany). All other reagents were purchased from Sigma (Deisenhofen, Germany).

**Tissue and Cell Preparation**

Human PT was obtained after surgical removal from 21 patients with primary pterygium conjunctivae (no recurrent lesions and no bilateral pterygia were included in the study) by the Department of Ophthalmology, Charité University Medicine Berlin, according to the tenets of the Declaration of Helsinki. All patients provided written consent, which was approved by the ethics committee of Charité, University Medicine Berlin, Germany. For RT-PCR and Western blot analysis, eight different PT samples were used and eight others for immunohistochemistry analysis. For functional studies, PT from five additional patients was mechanically reduced to small aggregates, incubated with 0.1 mg/mL collagenase type IV for 1 hour at 37°C, 5% CO2, 21% O2). After rinsing with 1× PBS, aggregates were incubated again with 0.01 mg/mL collagenase IV for another 24 hours. Cells were rinsed again in 1× PBS and isolated by centrifugation (5 minutes, 145 g). Afterward, cells were transferred and cultivated in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 containing 10% fetal calf serum, 1 µg/mL insulin, 5 µg/mL hydrocortisone, and 100 U/mL penicillin/streptomycin. After two to four passages, cells were used for functional studies. In two of these cell preparations, primary hPTEC underwent spontaneous immortalization after several cell passages. Primary conjunctival epithelial cells obtained from healthy individuals were grown under the same conditions only for two to four passages and then discarded to avoid possible passage-dependent electrophysiological changes. In addition, an immortalized hCEC line, was used and cultivated as previously reported.[25,29] Furthermore, an SV40-immortalized human cornea epithelial cell line (HCEC)[33] was cultured according to our previous study.[34]

**Reverse Transcriptase PCR**

Surgically removed PT was stored in RNAlater (Invitrogen, Karlsruhe, Germany) at 4°C and later lysed in 1 mL pegGOLD Trifast reagent (Peqlab, Erlangen, Germany) in an innuSPEED Lysis Tube placed in a SpeedMill plus homogenizer (both Analytik Jena AG, Jena, Germany). After centrifugation (10 minutes, 15,000 g), supernatants were used for RNA isolation. Pterygium tissue DNA contamination was eliminated by digestion with RNase-free DNase I (30 minutes, 37°C). DNase was heat inactivated for 10 minutes at 65°C. Reverse transcription of RNA samples to first-strand cDNA was performed by RevertAidTM H Minus M-Mul. V Reverse Transcriptase Kit (Fermentas, St. Leon-Rot, Germany) according to manufacturer’s protocol. Two micrograms total RNA and 10 pmol Oligo (dT) 18 primer (Fermentas) were used for each reaction. Each PCR reaction contained 2 µL cDNA in 2.5 µL 10X PCR buffer, 1 µL 50 mM MgCl2, 1 µL 10 mM dNTP mix (Fermentas), 0.5 µL 10 pmol forward primer, 0.5 µL 10 pmol reverse primer, 0.2 µL (5 U/µL) Taq DNA Polymerase (Invitrogen) diluted with RNase-free diethylpyrocarbonate-treated water. Polymerase chain reaction amplification underwent an initial cycle at 95°C for 5 minutes followed by 35 cycles at 95°C for 30 seconds, primer-specific annealing temperature for 30 seconds, 72°C for 30 seconds, and a final elongation at 72°C for 5 minutes as well as a temperature hold at 4°C. Gene-specific intron-spanning primer sequences, annealing temperatures, cycle numbers, and product sizes are shown in the Table. Primers were synthesized at Metabion International AG (Stein kirchen, Germany). Polymerase chain reaction products were resolved by electrophoresis in a 1.5% agarose gel and visualized via fluorescence. Base pair (bp) values were compared with Genbank data. Reverse transcriptase PCR product identity was confirmed by sequencing and sequence alignment (data not shown). Genomic contamination was prevented by omission of reverse transcriptase. Housekeeping β-actin gene expression assessed integrity and stability of the reverse transcribed cDNA.

**Real Time RT-PCR**

Each reaction was performed in a final volume of 20 µL containing 10 µL LightCycler 480 SYBR Green Mastermix (Roche, Penzberg, Germany), 0.5 µL gene-specific primer mix (10 pmol), 7.5 µL nuclease-free water, and 2 µL sample cDNA. Each plate was run at 95°C for 2 minutes, followed by 45 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 10 seconds, and gene-specific transcript amplification was confirmed by obtaining a melting curve profile (60–95°C). The cyclic threshold (Ct) parameter was defined by second
Table. Gene-Specific Intron-Spanning Primer Sequences, Annealing Temperatures, Cycle Numbers, and Product Sizes of Biomarkers and TRPs

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Immunohistochemistry

Surgically removed PT fixed in 4% formalin was embedded in paraffin, sectioned (7 μm) and deparaffinized. Immunohistochemistry was performed with anti-TRPV1 (ACC-050; Alomone Labs, Jerusalem, Israel), TRPM8 (HPA024117; Sigma Aldrich, Munich, Germany), cytokeratin (CK-13 (sc-57003; Santa Cruz Biotechnology, Heidelberg, Germany), CK-15 (CBL1587417; Millipore, Darmstadt, Germany), and CK-19 (sc-53003; Santa Cruz Biotechnology) antibodies, as previously described in detail.\(^{25,57}\) Transient receptor potential V1 and -M8 antibody specificity was confirmed based on elimination of antibody reactivity by antibody preadsorption with 1 μg of a respective blocking peptide per μg antibody. Furthermore, control sections were incubated with nonimmune IgG to determine possible nonspecific binding of IgG. The slides were examined with a Keyence BZ 9000 microscope (Keyence Germany, NeuIsenburg, Germany).

Western Blot Analysis

Cells were lysed with 1% Triton X-100 for 30 minutes on ice and centrifuged at 17,950 g for 5 minutes. Pterygium tissue was lysed with peqGOLD TriFast reagent (Peqlab). Total protein was isolated from phenol/chloroform phase as dissolved in 10 M urea/50 mM dithiothreitol and sonicated (HTU Soni130; G. HEINEMANN, Schwäbisch Gmünd, Germany) for 3 seconds. Supernatant protein concentration was determined with the Bradford assay. For Western blot analysis, 20 μg total protein was boiled for 5 minutes with a reducing buffer containing β-mercaptoethanol and loaded onto 12% SDS-PAGE, separated by electrophoresis and transferred to a nitrocellulose membrane by blotting. Blots were blocked in 5% nonfat milk/TBST (1 mL Tween 20/1 L Tris-buffered saline) for 1 hour and probed with the primary antibody (1:100 dilution) overnight at 4°C. Washing with TBST was followed by incubation with horseradish peroxidase–conjugated secondary antibody (dilution 1:5000, 2 hours at room temperature). Signal readout was determined by chemiluminescence with an enhanced chemiluminescence substrate (Millipore) in Biorad Universal All II (Munich, Germany). Furthermore, every blot was stripped with β-mercaptoethanol, 20% SDS, 1M Tris-HCl pH 6.8 containing buffer at 70°C for 30 minutes. Afterward, glyceraldehyde 3-phosphate dehydrogenase detection for normalization was performed. Blots were analyzed and quantified by Quantity One Software v4.6.9 (Biorad).

Proliferation Assay

Spontaneously immortalized hPtEC as well as HCJEC cells were seeded at 10⁵ cells/mL in 24-well cell culture plates. Cells were cultivated under aforementioned standard conditions in 10% fetal calf serum–containing DMEM/Ham’s F12 medium (Control). Cells were cultivated with 10 ng/mL epidermal growth factor (EGF), 10 ng/mL VEGF in the presence and absence of 10 μM CAP as well as 10 or 20 μM CPZ. Medium with/without additives was replaced every 48 hours. At the indicated time points, cells were trypsinized and counted under a phase-contrast microscope (Axiovert 40 CFL; Zeiss, Oberkochen, Germany) according to manufacturer’s protocol, respectively. Metabolic cell viability was measured after 24 hours by photometry in a plate reader (MWG, Ebersberg, Germany) at 490 nm. Human pterygium epithelial cells and HCJEC without additives were used as controls.

Metabolic Cell Viability Assay

Using the CellTiter 96 AQueous MTS Assay System (Promega, Mannheim, Germany) according to manufacturer’s protocol, cells were cultured in serum-free medium supplemented with either 10, 20, or 100 μM CAP as well as 10 or 20 μM CPZ, respectively. Metabolic cell viability was measured after 24 hours by photometry in a plate reader (MWG, Ebersberg, Germany) at 490 nm. Human pterygium epithelial cells and HCJEC without additives were used as controls.

Fluorescence Calcium Imaging

Cells were seeded on sterile glass cover slips and loaded with fura-2/AM (2 μM) for 20 to 50 minutes at 37°C. Loading was
stopped by replacing the dye containing culture medium with a Ringer-like (control) solution optimized for TRP detection\(^\text{38}\) whose composition was in mM: 150 NaCl, 6 CsCl, 1 MgCl\(_2\), 10 glucose, 10 HEPES, and 1.5 CaCl\(_2\) at pH 7.4. Washed cover slips were put into a bath chamber containing the aforementioned Ringer-like solution on the stage of a microscope (Olympus BX51WI; Olympus Europa Holding GmbH, Hamburg, Germany) in conjunction with a digital imaging system having UV excitation capability (TILL Photonics, Munich, Germany). Cells were alternately excited at 340 and 380 nm and fluorescence emission was detected from cell clusters every 500 ms at 510 nm. The ratio ($I_{340nm}/I_{380nm}$) is a relative index of intracellular Ca\(^{2+}\) ($[\text{Ca}^{2+}]_i$) levels.\(^\text{38}\) The 340- and 380-nm signals were always detectable and did not distort the ratio. The measuring field was adapted to the number of cells (TILL Photonics viewing system). Before the experiments, stable control baseline levels were obtained for 8 to 10 minutes. The control traces are designated with open circles in the figures. All experiments were performed at a constant room temperature (\(\sim 20^\circ - 23^\circ C\)) because experiments performed at higher temperatures led to increased Ca\(^{2+}\) levels due to increased open probability of thermosensitive-TRPs in ocular surface cells.\(^\text{25,29}\) Stock solutions of drugs were prepared in dimethyl sulfoxide (DMSO). Dimethyl sulfoxide concentrations below 0.1% had no detectable effect on intracellular Ca\(^{2+}\) regulation (data not shown).\(^\text{37,40}\)

Planar Patch-Clamping
The whole-cell mode ("Port-a-Patch"; Nanion, Munich, Germany) was used in combination with an EPC 10 patch-clamp amplifier (HEKA, Lamprecht, Germany) and PatchMaster software (Version 2.4; HEKA).\(^\text{25,40}\) In brief, the standard intracellular solution contained in mM: 50 CsCl, 10 NaCl, 60 CsF, 20 EGTA, and 10 HEPES-acid at pH of approximately 7.2 and approximately 288 mOsM, which was applied to the inner side of the microchip. The external solution contained in mM: 140 NaCl, 4 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 5 D-glucose monohydrate, and 10 HEPES (pH \(\sim 7.4\) and osmolality \(\sim 298 \text{ mOsm}\)) was present on the external side of the microchip (resistance of 2.5–3.0 MΩ). A single cell was held in place in the aperture by applying negative pressure (software-controlled pump). The mean membrane capacitance of HCjEC was 13 pF \(\pm\) 1 pF. Mean access resistance was 11 \(\pm\) 1 MΩ (both \(n = 26\)). For hPtEC, the corresponding values were 9 pF \(\pm\) 1 pF and 37 \(\pm\) 4 MΩ (both \(n = 26\)), respectively. Series resistances, and fast and slow capacitance transients were compensated by the patch-clamp amplifier. Current recordings were all leak-subtracted and cells with leak currents above 100 pA were excluded from analysis. The I-V response patterns were generated by imposing voltages in 10 mV steps from \(-60\) mV to \(+130\) mV.\(^\text{41}\) The holding potential was set to 0 mV to eliminate any possible contribution of voltage-dependent Ca\(^{2+}\) channels.

**Statistical Analysis**
Student’s \(t\)-test for paired data (\(P\) values: 2-tailed) was used if the data passed a normality test. If this failed, the nonparametric Wilcoxon matched pairs were used. For nonpaired data, Student’s \(t\)-test for unpaired data was used, after passing a normality test. Alternatively, the nonparametric Mann-Whitney \(U\) test was performed. Welch’s correction was applied if data variance of the two groups was not at the same level. Probabilities of \(P < 0.05\) (indicated by asterisks [*] and hashtags [#]) were considered to be significant. The number of repeats is shown in each case in brackets, near the traces or bars. All values are means \(\pm\) SEM. All plots were generated with SigmaPlot software version 12.5 (Systat Software, San Jose, CA, USA). Bar charts were plotted with GraphPad Prism (version 5) (La Jolla, CA, USA).

**RESULTS**
**Validation of hPtEC Phenotype**
Reverse transcriptase PCR documented biomarker gene expression of pterygium (MMP7 and Ki-67) and conjunctiva (MUC5AC, CK-13 and -15)\(^\text{15}\) in spontaneously immortalized hPtECs and in PT (Fig. 1A). Western blot analysis shows increased protein levels of CK-13, -15, and -19 in hPtEC compared with HCjECs. In hPtECs, there was a 2.9-fold increase in CK-13, 11.4-fold increase in CK-15 and 96.9-fold
increase in CK-19 protein expression compared with HCjEC (Figs. 1B, 1C). In formalin-fixed paraffin-embedded (FFPE) sections of PT obtained from eight different patients, CK-13, -15, and -19 expression were identified in hPtEC (Fig. 1D). There was pronounced intracytoplasmic CK-15 reactivity in intermediary and superficial pterygial epithelial cell layers, but no reactivity in the basal cell layer. Weak CK-15 expression was evident in all the pterygial epithelial layers along with CK-19 immunoreactivity in the intermediate cell layer. In the superficial epithelial cells, CK-19 expression was more intense. There were no significant differences in CK expression in these different pterygial samples irrespective of age or sex. Control sections with nonimmune IgGs were negative (not shown).

**Temperature-Sensitive TRP Channel Expression**

In hPtEC, PT, immortalized HCEC, HCjEC, and limbal epithelial cells, there was TRPV1, 2, 3, and 4 and TRPM8 gene expression (Fig. 2A). The TRP gene expression profiles were similar to one another and the β-actin control PCR (298 bp) products were invariant in all samples. Real-time RT-PCR analysis revealed significant TRPV1 gene expression upregulation (∆P = 0.042) in hPtEC compared with HCjEC. In the no template controls (NTC), contaminating genomic DNA was absent. Transient receptor potential V1 and weaker than those in HCEC and HCjEC (Fig. 2B). In addition, there was TRPV1 and TRPM8 localized expression in FFPE sections of pterygia (Fig. 2C). Transient receptor potential V1 protein expression levels were approximately 2-fold and 4.8-fold higher in hPtEC and PT, respectively, approximately 2-fold and 4.8-fold higher in hPtEC and PT, respectively, than in HCEC and HCjEC (Fig. 2B). In addition, there was TRPV1 and TRPM8 localized expression in FFPE sections of pterygia (Fig. 2C). Transient receptor potential V1 protein expression levels were approximately 2-fold and 4.8-fold higher in hPtEC and PT, respectively, than in HCEC and HCjEC (Fig. 2B).

**Functional Heat-Sensitive TRPV1 Expression**

In hPtEC expanded from five patients, the f_340nm/f_380nm ratio irreversibly increased after heating (>45°C) from 1.197 ± 0.004 to 1.556 ± 0.045 (at 320 seconds; n = 22; ***P < 0.005, paired tested; Fig. 3A, left), but this response was partially suppressed by the TRP channel blocker lanthanum-III-chloride (La^3+; 100 μM) (1.377 ± 0.058; n = 18; **P < 0.01; Fig. 3A, right). Similar results were obtained in HCjEC (Fig. 3B; 1.628 ± 0.049; n = 5; ***P < 0.01, paired tested) and HCEC (Fig. 3C; 1.440 ± 0.065; n = 18; **P < 0.01, paired tested), which could also be suppressed by La^3+ (Figs. 3B, 3C). However, there was a sustained increase in intracellular Ca^2+ after heat stimulation only in hPtEC. This was not obvious in HCjEC and HCEC and the Ca^2+ transients were not significantly different shortly after heat stimulation. Specifically, after heat stimulation for 4 minutes, the duration of the TRPV1 response was longer in hPtEC than in HCjEC and HCEC (Fig. 3D). Moreover, the transient decline was more gradual in hPtEC than HCjEC (Fig. 3A versus Fig. 3C). With hPtEC at 320 seconds, the f_340nm/f_380nm ratio rose to 1.556 ± 0.045; n = 22 and fell slightly to 1.478 ± 0.059; n = 22 at 480 seconds. On the other hand, with HCjEC, the f_340nm/f_380nm ratio rose less, from 1.200 ± 0.045; n = 11; ***P < 0.005, paired tested; Fig. 4A, 4B). Subsequent to reaching a peak, it partially declined to 1.315 ± 0.012 in HCjEC (n = 11; *P < 0.05, paired tested). Although the Ca^2+ transient peaks were not different from one another in HCjEC and hPtEC (Fig. 3), at 480 seconds intracellular Ca^2+ level remained at a higher level in hPtEC (480 seconds; Fig. 3D). Using instead primary freshly isolated hPtEC and comparing
its heat-induced \([\text{Ca}^{2+}]_i\) transient with that measured in healthy HCjEC, the difference was larger than obtained with immortalized hPtEC (Fig. 4). Taken together, the larger and less reversible heat-induced \(\text{Ca}^{2+}\) influx in primary passaged hPtEC indicates a longer and greater TRPV1 open probability than in the normal healthy HCjECs.

**FIGURE 3.** Increase in \(\text{Ca}^{2+}\) entry through heat-sensitive TRPs caused by heat exposure in hPtEC, HCjEC, and HCEC. The thermal changes were inflicted at the time points indicated by arrows. Data are mean ± SEM of 4 to 22 experiments. (A) Rise of bath solution temperature from room temperature (≈22°C) to over 43°C resulted in an irreversible \(\text{Ca}^{2+}\) elevation in hPtEC (n = 22; left). In the presence of La\(^{3+}\) (100 μM), the heat-induced \(\text{Ca}^{2+}\) increase was clearly suppressed (n = 18; right). (B–C) Same set of experiments as shown in (A), but with HCjEC (B) and HCEC (C), respectively. The heating effect was at lower levels between HCEC and hPtEC at 480 seconds. (D) Summary of the experiments with heat stimulation and La\(^{3+}\) in hPtEC, HCjEC, as well as HCEC. The asterisks (*) indicate significant differences between controls (\(\text{Ca}^{2+}\) base levels before heating) and heat-induced \(\text{Ca}^{2+}\) increase on \([\text{Ca}^{2+}]_i\); (n = 18–22) (paired Student’s t-test). The hash marks (#) indicate significant differences between \(\text{Ca}^{2+}\) influx with and without La\(^{3+}\) (unpaired Student’s t-test) and heat-induced \(\text{Ca}^{2+}\) influx between hPtEC and HCEC (n = 10–22).

**FIGURE 4.** Increased heat-induced \(\text{Ca}^{2+}\) influx in primary cultivated hPtEC. The thermal change was carried out at the time point indicated by an arrow. Data from five patients with pterygium are mean ± SEM of 22 experiments. Data from one healthy individual are mean ± SEM of 11 experiments. (A) Rise of bath solution temperature from room temperature (22°C) to over 43°C resulted in \(\text{Ca}^{2+}\) elevation in healthy primary cultivated HCECs from one healthy individual and hPtECs from patients with pterygium. Traces show intracellular \(\text{Ca}^{2+}\) measured of several neighboring healthy HCjECs (open circles) (n = 11) and primary cultivated hPtECs from patients with pterygium (filled circles) (n = 22) (control base line; f340nm/f380nm = 1.2). In hPtEC from patients with pterygium, the heat-induced \(\text{Ca}^{2+}\) influx was clearly increased (##P < 0.01; n = 11–22). (B) Summary of the experiments with healthy HCjEC from a healthy patient and hPtEC from five patients with pterygium. The asterisks (*) indicate significant differences between controls (\(\text{Ca}^{2+}\) base levels; n = 11–22) and heat-induced \(\text{Ca}^{2+}\) increase on \([\text{Ca}^{2+}]_i\); (n = 11–22) (paired Student’s t-test). The hash marks (#) indicate significant differences between healthy HCjEC and hPtEC from patients with pterygium (unpaired Student’s t-test).

**TRPV1–Induced Currents in Immortalized hPtEC**

To validate that the heat-induced \(\text{Ca}^{2+}\) transients are attributable to TRPV1 activation, they were compared with the underlying currents induced by 20 μM CAP. It increased outwardly rectifying currents (+130 mV) from 81 ± 13 pA/pF up to 199 ± 44 pA/pF (*P < 0.05; n = 16, paired tested) (Figs. 5C, 5D). This change was...
Thermo-Sensitive TRP Upregulation in Pterygia

Heightened Functional TRPV1 Behavior in hPtEC

To validate that the difference in inward currents is accountable for larger Ca^{2+} influx in immortalized hPtEC than HCjEC, we compared the magnitudes of the Ca^{2+} transients induced by CAP. Results shown in Figures 6D-F indicate that the CAP-induced Ca^{2+} transients were at higher levels in immortalized hPtEC than in HCjEC. Specifically, the f_{340nm/f380nm} ratio increased from 1.201 ± 0.002 to 1.231 ± 0.045 after CAP application (20 μM) in immortalized hPtEC (n = 31; ***P < 0.005, paired tested; Fig. 6D), whereas in HCjEC, this ratio increased less from 1.200 ± 0.0003 to 1.209 ± 0.003 (n = 12; **P < 0.01, unpaired tested; Figs. 6D, 6F). These increases were smaller than those induced by heating to 45°C, as heating is less specifically activating TRPV1 than CAP. A difference in selectivity also is apparent between exposure to CAP and a hypertonic stress because the latter condition induced larger Ca^{2+} transients in immortalized HCjEC (Figs. 6A-C). This difference among the effects of CAP, hypertonicity, and heating are not attributable to baseline variations, because in the absence of CAP, the baseline levels remained constant and were identical to one another in HCjEC and hPtEC (Figs. 6D-F; n = 13-19; open circles). Taken together, these results suggest that functional TRPV1 expression in hPtEC is at higher levels in immortalized hPtEC than in HCjEC.

Dependence of VEGF-Induced Ca^{2+} Influx on TRPV1 Transactivation in hPtEC

As shown in Figure 7B, VEGF increased the f_{340nm/f380nm} ratio from 1.202 ± 0.002 to 1.225 ± 0.006 (t = 480 seconds) in hPtEC (n = 6; ***P < 0.05, paired tested), whereas the VEGF effect was smaller in HCjEC (Fig. 7A). On the other hand, in hPtEC, 20 μM CPZ as well as a mixed TRPV1/TRPM8 antagonist, 20 μM BCTC,43 both caused the Ca^{2+} transients to even fall below their baseline (Figs. 7C, 7D). Corresponding effects of 10 ng/mL VEGF on inward currents indicate that they increased from −18 ± 2 pA/pF to −54 ± 11 pA/pF (n = 6–12; **P < 0.005; unpaired tested in hPtEC; Fig. 8H), whereas rises in outward currents were smaller (Figs. 8D, 8H). Notably, 20 μM CPZ inhibited the inward currents (−20 ± 4 pA/pF; n = 8; ***P < 0.01; unpaired tested), whereas 500 μM La^{3+} only partially decreased these currents (Figs. 8A-D). This agreement between the suppressive effects of CPZ on VEGF-induced rises in inward currents and Ca^{2+} transients supports the notion that the Ca^{2+} transients induced by VEGF are mediated through TRPV1 transactivation.

Human Pterygium Epithelial Cells and HCjEC Proliferation Behavior

As functional TRPV1 activity is larger in immortalized hPtEC than in HCjEC, we hypothesized that hyperplastic hPtEC activity is dependent on enhanced TRPV1 channel activity.
mediating larger Ca\textsuperscript{2+} influx. To test this hypothesis, time-dependent increases in cell proliferation were compared between spontaneously immortalized hPtEC and HCjEC. The results shown in Figure 9A indicate that the number of cultivated hPtECs was significantly greater at all time points examined (*P < 0.05, **P < 0.01, ***P < 0.001). During the long exponential growth phase extending from 24 to 168 hours, the population-doubling time of immortalized hPtEC was 39.09 hours compared with 54.27 hours for HCjEC, to 168 hours, the population-doubling time of immortalized hPtECs was significantly greater at all time points (**P < 0.01) and 100 μM (***P < 0.001). This partial inhibitory effect of CPZ suggests that other pathways besides TRPV1 contribute to mediating a rise in Ca\textsuperscript{2+} influx needed to induce an increase in proliferation. Capsaicin (10 μM) did not affect proliferation in HCjEC until 96 hours when it began to instead inhibit this response (Fig. 10A). Similarly, CPZ inhibited HCjEC cell proliferation and became cytotoxic (Figs. 10B, 10D; ###P < 0.001). After another 48 hours of exposure to CPZ, there were no remaining viable HCjECs. Taken together, these results suggest that larger functional TRPV1 activity in hPtEC than HCjEC is sufficient to induce hyperplastic pterygium epithelial cell behavior. Nevertheless, TRPV1 activation con-

**Capsazepine Suppresses EGF-Induced Cell Proliferation in hPtEC**

The mitogenic responses by hPtEC and HCjEC to 10 ng/mL EGF were compared to determine if they corresponded with differences in TRPV1 channel activity between these cell types. Epithelial growth factor–induced mitogenic responses were larger in hPtEC than in HCjEC. (Figs. 10A, 10C). After 96 hours, cocultivation of hPtEC with EGF/CPZ cell amount was higher compared with that with CPZ alone (##P < 0.01). The partial inhibitory effect of CPZ suggests that other pathways besides TRPV1 contribute to mediating a rise in Ca\textsuperscript{2+} influx needed to induce an increase in proliferation. Capsaicin (10 μM) did not affect proliferation in HCjEC until 96 hours when it began to instead inhibit this response (Fig. 10A). Similarly, CPZ inhibited HCjEC cell proliferation and became cytotoxic (Figs. 10B, 10D; ###P < 0.001). After another 48 hours of exposure to CPZ, there were no remaining viable HCjECs. Taken together, these results suggest that larger functional TRPV1 activity in hPtEC than HCjEC is sufficient to induce hyperplastic pterygium epithelial cell behavior. Nevertheless, TRPV1 activation con-
**Figure 7.** Vascular endothelial growth factor–induced increases of intracellular Ca$^{2+}$ in immortalized HCjEC and in hPtEC. Data are mean ± SEM of 4 to 11 experiments. (A) Application of 10 ng/mL VEGF resulted in Ca$^{2+}$ elevation in HCjEC. Traces show intracellular Ca$^{2+}$ measurements with VEGF (filled circles, $n = 11$) and without VEGF ($n = 4$) (control Ca$^{2+}$ baseline). (B) Similar experiments as shown in (A) but with hPtEC. Traces show intracellular [Ca$^{2+}$], measurements with VEGF (filled circles, $n = 6$) and without VEGF ($n = 4$) (control Ca$^{2+}$ baseline). (C) Similar experiments as shown in (B) but in the presence of 20 μM CPZ (left) or 20 μM BCTC (right). The VEGF-induced Ca$^{2+}$ entry was completely abolished in the presence of CPZ or BCTC. (D) Comparison of Ca$^{2+}$ entries in HCjEC and immortalized hPtEC. The Ca$^{2+}$ entry in hPtEC was at higher levels compared with those of HCjEC (480 seconds). The asterisks (*) indicate significant differences between controls (Ca$^{2+}$ baseline; $n = 12$) and VEGF-induced Ca$^{2+}$ increases on [Ca$^{2+}$] (n = 31) (paired Student’s t-test). The hash marks (#) between the filled bars indicate significant differences between HCjEC and immortalized hPtEC and VEGF effect with and without CPZ/BCTC (unpaired Student’s t-tests).

**Figure 8.** Vascular endothelial growth factor–induced increases of whole-cell currents in hPtEC. (A) Time course recording showing the current increases by 10 ng/mL VEGF and current density levels after adding La$^{3+}$ (500 μM). (B) Original traces of VEGF-induced current responses to voltage ramps. Current densities are shown before application (labeled as [A]), during application of VEGF (labeled as [B]) and after addition of La$^{3+}$ (labeled as [C]). Data were obtained from the data shown in (A) but in the presence of 20 μM CPZ or 20 μM BCTC. The VEGF-induced Ca$^{2+}$ entry was completely abolished in the presence of CPZ or BCTC. (C) Similar recordings as shown in (A), but with CPZ (20 μM) from approximately 250 seconds. (D) Similar traces as shown in (B) but with CPZ instead of La$^{3+}$. Notable, there was a clear decrease of VEGF-induced inward currents in the presence of CPZ (lower trace). (E) Similar diagrams as shown in (C) but with CPZ instead of La$^{3+}$. (F) Summary of the experiments with VEGF and CPZ. The asterisks (*) indicate statistically significant differences of VEGF-induced increases of inward currents with and without CPZ ($n = 6–8$; **$P < 0.01$ at the minimum; unpaired tested).
containing DMSO were used as controls and set to 100%. Values represent mean ± SEM of four independent experiments (n = 4). For statistical evaluation of cell counts, 2-way ANOVA and Bonferroni posttests were performed. (B) Metabolic cell activity is affected by TRPV1 agonist CAP in both cell lines, whereas TRPV1 antagonist CPZ affected only hPtEC. Human conjunctival epithelial cells and hPtECs were incubated with 5, 20, and 100 μM CAP, as well as 10 and 20 μM CPZ for 24 hours. Metabolic cell activity was colorimetric analyzed by MTS assay. Cells cultivated in standard medium containing DMSO were used as controls and set to 100%. Values represent mean ± SEM of eight independent experiments (n = 8). Statistical significance was calculated by 1-way ANOVA and Dunnet’s posttests and indicated by asterisks $CP < 0.05$; **$P < 0.01$; ***$P < 0.001$) and hashtags (#$P < 0.001$).

**DISCUSSION**

By comparing TRPV1 characteristics in clinical pterygial samples and a spontaneously immortalized pterygial epithelial cell line (hPtEC) with those in freshly isolated conjunctival tissue and an immortalized HCjEC counterpart, our results indicate that hyperplastic pterygial cell activity is associated with heightened TRPV1 channel expression levels and functional activity. This association suggests that larger TRPV1 activation may support an increased cell proliferation as a consequence of increased Ca$^{2+}$ influx. Such an effect may more fully activate the mitogen-activated protein kinase (MAPK) signaling pathways mediating growth factor receptor control of proliferation.28,44

Functional TRPV1 activity was characterized in these different tissue preparations by contrasting the effects of raising the bath temperature above 43°C (specific for TRPV114,15) with those induced by CAP on Ca$^{2+}$ transients and underlying ionic currents. The involvement of TRPV1 in inducing these changes was validated by showing that these responses were suppressed during exposure to either La$^{3+}$ or CPZ. These TRPV1-mediated Ca$^{2+}$ transients and current response patterns correspond to those described in many studies using different cell types, including tumor cells.26,27,38,40,45–48 Because TRPV1 expression and activity are elevated in cancerous tissue, it is conceivable that TRPV1 and possible endogenous modulators may be a potential drug target to suppress proliferation not only in malignant tumors but also in benign hyperplastic tumors, such as pterygium (reviewed by Santoni et al.49).

**TRPV1 Expression and Functional Relevance**

An association between increases in functional TRPV1 activity and proliferation in immortalized hPtEC relative to that in HCjEC is similar to what was described in retinoblastoma cells and in various other tumorous tissues.46 Increased functional TRPV1 expression in immortalized hPtEC (Figs. 4–6) suggests that reducing its activity may resolve tumor progression if increases in its activity precede or occur as a consequence of pterygial development. Dysregulated TRP channel activation contributes to also triggering, aberrant differentiation, and resistance to apoptotic cell death leading to uncontrolled tumor invasion (spread) (reviewed by Santoni et al.49).

Transient Receptor Potential V1 (also classically known as a pain receptor50–52) is a nociceptor that is a potential drug target for inhibiting cancer pain in bone metastases, pancreatic cancer, and most likely in other cancers (reviewed in Refs.34,53). Additionally, TRPV1 is also expressed at a multitude of nonneuronal sites, which is prompting many studies probing for their possible involvement in disease progression. Besides TRPV1, our RT-PCR results indicate that there is also TRPV2 and TRPM8 gene expression in PT. It is noteworthy that TRPV2 mRNA level in PT appears to be higher than in HCjEC (Fig. 2). Similar to TRPV1, TRPV2 is also elevated in cancerous cells compared with their healthy counterparts.54 Furthermore, TRPV2 expression may be relevant for supporting the pterygial phenotype, because activation of TRPV2 is associated with release of VEGF in RPE cells.45 Similarly, TRPM8 expression may contribute to the pterygial transition, as this channel shows a role in cancer diseases concerning by modulating proliferation and cell cycle progression.55 Notably, TRPM8 is functionally expressed at higher levels in hPtEC (data not shown) compared with human corneal epithelial cells and conjunctival epithelial cells,45,56 whereas TRPM8 mRNA expression was not significantly different (Fig. 2). These disconnects show that there is not always an association between changes in TRP mRNA expression and their functional expression.

**TRPV1 Characteristics**

Inducing TRPV1 activation by a thermal stress led to continuous larger sustained intracellular Ca$^{2+}$ increases after initiating heating in primary hPtEC (Fig. 3A) than in immortalized HCjEC or HCEC (Figs. 3B, 3C). This difference in the Ca$^{2+}$ response patterns suggests a possible Ca$^{2+}$ signal remodeling in tumorigenic from that in nontumorigenic cells.57 More specifically, the time-dependent Ca$^{2+}$ transient recovery subsequent to reducing the bath temperature back to 22°C was different between hPtEC and HCjEC. In primary hPtEC, there was no reversal, whereas in HCjEC, temperature restoration to 22°C caused a partial recovery toward its baseline level (Fig. 3B). This limited baseline restoration in
HCjEC is somewhat similar to the complete recovery that occurred in immortalized HCEC29 (Fig. 3C). Even though these are immortalized cell lines, their similar recovery patterns indicate that Ca\(^{2+}\) extrusion mechanism activation is sufficient to offset Ca\(^{2+}\) influx. In primary HCjEC, the recovery pattern was similar to that in immortalized HCjEC (Fig. 4A, open circles). Another suggestion of Ca\(^{2+}\) regulation remodeling in hPtEC is that the intracellular Ca\(^{2+}\) level was always higher in primary hPtEC than in normal tissues (Fig. 4A, filled circles). Such remodeling may involve TRPV1 upregulation along with overexpression. This suggestion of Ca\(^{2+}\) signal remodeling is based on similar differences in Ca\(^{2+}\) transients between other hyperproliferative cancerous and normal tissues. 57,58 For example, Stewart et al. 57 suggested, in their hypothetical remodeling of Ca\(^{2+}\) signaling, that lack of recovery is linked with a sustained increase in intracellular Ca\(^{2+}\) tumorigenic

**FIGURE 10.** Cell proliferation in hPtEC and HCjEC with and without TRPV1 modulators as well as pterygium-associated growth factors. Capsazepine (10 \(\mu\)M) suppressed EGF- but not VEGF-induced cell proliferation in hPtEC at long-term cultivation. Capsaicin (10 \(\mu\)M) did not affect cell proliferation of hPtEC. Both TRPV1 modulators had a cytotoxic effect in HCjEC. (A–D) Cell proliferation assays. Human pterygial cells (A, C), as well as HCjEC (B, D), were cocultivated with CAP (10 \(\mu\)M), CPZ (10 \(\mu\)M), EGF and VEGF (each 10 ng/mL), and CPZ together (EGF/CPZ and VEGF/CPZ) as well as without additives (control) up to 96 hours. Total numbers of cells were counted every 24 hours. (E–H) Detailed statistical analysis of cell proliferation in hPtEC (E, G) and HCjEC (F and H). All data are presented as mean ± SEM of four independent experiments (\(n = 4\)). For statistical evaluation of cell counts, 1-way ANOVA and Bonferroni posttests were performed. Statistical significance is indicated by asterisks (*\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\)) and hashtags (**\(P < 0.01\); ###\(P < 0.001\)). n.d., no detectable cells.
cells. Notably, this indication of Ca\(^{2+}\) remodeling may account for the lack of recovery in both immortalized pterygial cells and in uveal melanoma cells.\(^57\) The more prolonged Ca\(^{2+}\) transient in tumor cells suggests that TRPV1 open probability time is longer than in healthy controls. Another possibility for the lack of recovery is that calcium extrusion mechanism (i.e., Na\(^+/\)Ca\(^{2+}\) exchanger and/or plasma membrane calcium ATPase) may be reduced in pterygial cells.

The correspondence between the heat-induced Ca\(^{2+}\) transients and underlying ionic currents was validated by showing that CAP and CPZ had similar modulatory effects on TRPV1 activity. Another result reflective of TRPV1 involvement is that the reversal potential was always near 0 mV, which is consistent with behavior of a nonselective cation channel. On the other hand, any possible contributions by Cl\(^-\) channel currents are unlikely because isosmotic substitution of NaCl with Na-glucuronate Ringers did not change whole-cell currents.\(^20\) The CAP-induced inward current densities were larger in hPtEC than in HCjEC, whereas the outward current densities were not significantly different from one another (Fig. 5). This larger inward current indicates that Ca\(^{2+}\) influx is greater in hPtEC due to TRPV1 activation eliciting larger Ca\(^{2+}\) influx along a favorable bath to cell electrochemical Ca\(^{2+}\) gradient. However, the outward currents involve other ions than Ca\(^{2+}\), which do not permeate against an unfavorable electrochemical gradient from cell to bath. Such a difference between the effects of CAP on the inward and outward currents supports the notion that the larger inward currents in cultivated hPtEC are attributable to greater functional TRPV1 activity in hyperplastic immortalized pterygial cells than in HCjEC.

**Differential Responses by Cell Types to TRPV1 Channel Modulators**

Capsaicin induced larger Ca\(^{2+}\) transients and whole-cell currents in immortalized hPtEC than in HCjEC, which were blocked by CPZ and BCTC (Figs. 7A–D, 8E–H). As this difference may be associated with hyperplastic activity in immortalized PT, we determined if mitogenic responses to EGF were dependent on Ca\(^{2+}\) influxes induced by TRPV1 activation. As CPZ had corresponding inhibitory effects on increases in Ca\(^{2+}\) influx and immortalized hPtEC proliferation, EGF receptor activation transactivates TRPV1. Another indication that larger TRPV1 activity provides the needed Ca\(^{2+}\) influx to support immortalized hPtEC hyperplastic behavior is that transient recovery in HCEC and HCjEC recovery was evident, whereas in immortalized hPtEC it was delayed. The correspondence between enhanced cell proliferation in fresh clinical pterygial samples and hPtEC shows that results obtained with immortalized hPtEC are relevant for gaining insight into the pterygial samples and hPtEC shows that results obtained with immortalized hPtEC are relevant for gaining insight into the pterygium pathogenesis.\(^2\) Another indication of the suitability of hPtEC as a model is that CAP increased metabolic activity in immortalized hPtEC, extrusion by this MDR transporter of CAP may prevent it from accumulating to cytotoxic levels, whereas in HCjEC, such a protective effect may be diminished. On the other hand, CPZ increased metabolic activity in immortalized hPtEC, suggesting that suppressing TRPV1 activity improved cell viability. The lack of an inhibitory effect by CPZ on proliferation in HCjEC may indicate that functional TRPV1 activity is at a much lower level than in immortalized hPtEC (Fig. 9B). Heparin-bound EGF as well as EGF levels rise in pterygium during UV-B exposure\(^2\) and contribute to mediating increases in cell proliferation. Tran-...
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