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Dependence of Regulatory Volume Decrease on Transient Receptor Potential Vanilloid 4 (TRPV4) Expression in Human Corneal Epithelial Cells

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Abstract

TRPV4 is a non-selective cation channel with moderate calcium permeability, which is activated by exposure to hypotonicity. Such a stress induces regulatory volume decrease (RVD) behavior in human corneal epithelial cells (HCEC). We hypothesize that TRPV4 channel mediates RVD in HCEC. Immunohistochemistry revealed centrally and superficially concentrated TRPV4 localization in the corneal tissue.

Immunocytochemical and Fluorescence Activated Cell Sorter (FACS) analyses identified TRPV4 membrane surface and cytosolic expression. RT-PCR and Western blot analyses identified TRPV4 gene and protein expression in HCEC, respectively. In addition, 4α-PDD or a 50% hypotonic medium induced up to three-fold transient intracellular Ca²⁺ ([Ca²⁺]_i) increases. Following TRPV4 siRNA HCEC transfection, its protein expression level declined by 64%, which abrogated these [Ca²⁺]_i transients. Similarly, exposure to either ruthenium red or Ca²⁺-free Ringer's solution also eliminated this response. In these transfected cells, RVD declined by 51% whereas in the non-transfected counterpart, ruthenium red and Ca²⁺-free solution inhibited RVD by 54% and 64%, respectively. In contrast, capsazepine, a TRPV1 antagonist, failed to suppress [Ca²⁺]_i transients and RVD. TRPV4 activation contributes to RVD since declines in TRPV4 expression and activity are associated with suppression of this response. In conclusion, there is TRPV4 functional expression in HCEC.

Keywords

Transient receptor potential vanilloid 4; Calcium transients; Regulatory volume decrease; Corneal epithelium; Small interfering RNA

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1. Introduction

Corneal epithelial integrity is required for maintaining tissue transparency and deturgescence. This outermost tear-side facing layer provides a barrier function to protect the cornea from noxious insults. This protective role is dependent on adequate tight junctional resistance [1]. If such resistance is disrupted by corneal injury or infection, corneal swelling and opacification may develop compromising visual performance. These changes result from decreases in transepithelial net osmolyte-coupled fluid flux from the stroma to tears, which is inadequate to overcome the innate tendency for the stroma to imbibe fluid and thicken [2]. On the other hand, epithelial barrier function is maintained despite continuous shedding of terminally differentiated superficial layers provided there is continuous cytokine-modulated corneal epithelial renewal [3–5]. Therefore, corneal transparency and normal vision are dependent on the ability of the corneal epithelium to undergo continuous renewal and preserve its tight junctional integrity and resistance [6].

Fluctuations in tear film osmolarity can occur in daily living and challenge corneal epithelial barrier function by inducing acute epithelial cell volume changes. In some types of dry eye disease, losses in tear film integrity and changes in its composition can chronically stress this tissue layer [7,8]. Nevertheless, under physiological conditions, corneal epithelial cells (CEC) withstand such stresses by mounting regulatory volume (RV) responses [9]. During exposure to a hypertonic challenge, these cells initially shrink, which is rapidly compensated for by activation of a RV increase (RVI) response [10]. RVI restores cells to their isotonic cell volume by stimulating the net uptake of osmolytes and subsequent osmotically coupled water influx. Such restoration is in part dependent on activation of Na-K-2Cl¹ co-transporter (NKCC1)-induced osmolyte influx coupled to Na-K pump stimulation [11]. On the other hand, during exposure to a hypotonic challenge, CEC initially swell, and activate a RV decrease (RVD) response to restore their isotonic volume [12]. In human CEC (HCEC), RVD is dependent on increases in K⁺ and Cl⁻ efflux through volume-sensitive ion channels [9,12] and K-Cl co-transporter 1 (KCC1) [13]. Each of these regulatory volume responses is essential for maintaining corneal transparency, since they restore CEC isotonic volume and prevent compromise of their barrier function [14].

During exposure to an anisotonic stress, mitogen-activated protein kinase (MAPK) superfamily activation in HCEC induces RV needed for restoration of isotonic cell volume. Exposure to hypertonicity induces protein-protein interaction between p38 and NKCC1. Their association in HCEC is requisite for NKCC1 activation and RVI induction [15]. On the other hand, activation of MAPKs ERK and JNK is required for RVD whereas p38 stimulation occurs subsequent to RVD in rabbit CEC (RCEC) [12].

Ca²⁺ signaling is critical to induce RVD during exposure to a hypotonic challenge. In RCEC, this response is dependent on ryanodine-sensitive channel stimulation resulting in Ca²⁺ release from intracellular Ca²⁺ stores (ICS). ICS depletion is followed by transient increases in plasma membrane Ca²⁺ influx [16]. This response suggests that in RCEC plasma membrane Ca²⁺ influx through store operated channels is an important contributor to inducing RVD. In many other tissues, these Ca²⁺ transients, in turn, stimulate MAPK and volume-sensitive K⁺ (i.e. Maxi-K⁺, intermediate K⁺) channels [17,18]. However, the identity of plasma membrane associated Ca²⁺ permeable pathways has not been described in HCEC.

The transient receptor potential (TRP) superfamily forms tetrameric cation permeable channels of varying selectivity. Many members of this superfamily are polymodally activated by a diverse assortment of environmental challenges, including osmotic, mechanical, and thermal stresses and cues [19,20]. For a variety of tissues, there is emerging evidence that regulatory

volume responses are dependent on increases in Ca^{2+} influx through pathways comprising several different members of the TRP superfamily [21–24].

The TRP Vanilloid subfamily in mammals has 6 members (TRPV 1–6) and they were identified in both excitable and non-excitable cells of multiple tissues [25]. These members were first characterized in invertebrates by a mutagenesis approach: in *Drosophila melanogaster*, they are encoded by the *nan* [26] and *iav* [27] genes, and in *Caenorhabditis elegans* by the *ocr 1–4* [28] and *osm-9* genes [29]. The TRPV1–4 members are moderately Ca^{2+} selective ($P_{\text{Ca}}/P_{\text{Na}} = 5–10$) whereas the TRPV5–6 members are highly Ca^{2+} selective ($P_{\text{Ca}}/P_{\text{Na}} > 100$) [30]. In HCEC, TRPC4 and TRPV1 functional expression was identified [31]. EGF-induced HCEC proliferation is dependent on activation of store operated channels containing TRPC4 whereas TRPV1 stimulation mediates inflammatory cytokine release via MAPK pathways [31,32].

TRPV4 activation in various studies occurs in either a direct or indirect manner, although definitive evidence of direct activation is still missing [33]. Moreover, different stimuli use distinct pathways to indirectly activate TRPV4 [34]. Cell swelling can activate TRPV4 following cPLA₂ stimulation to mobilize plasma membrane polyunsaturated fatty acid release, resulting in the formation of arachidonic acid (AA). Subsequent to AA conversion to epoxyeicosatrienoic acids (i.e., 5', 6'-EET and 8', 9'-EET) by cytochrome P450 epoxygenase, these metabolites activate TRPV4 although it is unclear whether these endogenous ligands can directly bind to the channel [35,36]. Contrarily, heat and phorbol esters induced TRPV4 activation is associated with a tyrosine residue (Tyr-556) in the N-terminal portion of TM3 [37].

The activated TRPV4 channel exhibits outwardly rectifying current with a single-channel conductance of 60–310 pS [30,38,39]. This wide range may be attributed to differences in measurement conditions in different studies and/or TRPV4 heterologous assembling as well as other factors. Sensitivity and temporality of TRPV4-induced currents to hypotonicity are similar to those of $[\text{Ca}^{2+}]_i$ transients and to those of cation channels activated by cell swelling in TRPV4-transfected HEK293 cells [40]. A similar role for endogenous TRPV4 has been described in rat and mouse renal ascending thin limb cells where substantial transcellular osmotic gradients occur [41].

Growing evidence indicates that the TRPV4 channel mediates cell volume homeostasis based on the fact that RVD induction requires extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$). TRPV4-transfected-Chinese hamster ovarian (CHO) cells exhibited RVD during a hypotonic challenge whereas in the non-transfected counterpart there was only persistent swelling [21]. Moreover, TRPV4 expression in epithelial cells appears to be a determinant of membrane fluid permeability since its activation enhances both transcellular and paracellular cytoplasmic membrane permeability. Increases in paracellular fluid permeability are attributable to TRPV4 activation induced tight junction (TJ) changes, including declines in claudin protein expression, and leakage-linked changes in TJ morphology in mouse mammary gland cells [42]. At the transcellular level, rises in transcellular electrolyte permeability are linked with changes in TRPV4 interaction with aquaporin 5 (AQP5), which is also required for RVD [22,43]. There is a disparity between how these two proteins interplay in response to hypotonic challenge. In human and mouse salivary gland cells, hypotonicity increased membrane abundance and colocalization of TRPV4 and AQP5 in the apical region of salivary gland cells whereas the absence of either impaired Ca^{2+} transients and RVD. Additionally, N-terminal deletion of AQP5 suppressed its membrane translocation, TRPV4 activation and RVD, suggesting these two proteins in concert mediate RVD, where hypotonicity induced TRPV4 stimulation is dependent on AQP5 activity. On the other hand, in mouse lung epithelial cells, hypotonic challenges reduced AQP5 surface abundance. This reduction may be dependent on TRPV4 activation since by inhibition of TRPV4 stimulation or in non-transfected HEK293 cells, such decreases were eliminated. These

results indicate that TRPV4 mediates hypotonicity-induced losses of surface-delimited AQP5 content. Apparently, in AQP5⁺/TRPV4⁺ epithelia their responses to hypotonic stimulation are cell type specific due to differences in interplay between TRPV4 and AQP5.

The importance of TRPV4 expression for osmotic stress detection has been documented in vivo in both invertebrates and vertebrates. Mechanical- and hypertonicity-induced avoidance, but not chemical induced avoidance, was (partially) restored by transgenic expression of mammalian TRPV4 in head-sensory neurons of the *osm-9* mutated *C. elegans* [19]. Similarly, TRPV4^{-/-} mice show reduced fluid intake and impaired responses to (hyper-) and hypo-osmotic stimuli, as well as thermal, mechanical sensitivity [44,45].

In the present study, we describe TRPV4 expression in the intact human corneal epithelium as well as in primary HCEC (pHCEC) and in SV40-immortalized HCEC. TRPV4 is not only localized in the cell periphery, but it is also somewhat evident in the perinuclear domain. Such expression is functional since the either TRPV4 agonist, 4α-PDD, or exposure to a hypotonic medium mediated Ca²⁺ transients. Furthermore, TRPV4 expression is requisite for RVD since TRPV4 knockdown by effective siRNAs, but not of mismatched siRNAs, markedly suppressed Ca²⁺ transients and RVD responses. Therefore, TRPV4 activation is essential for mediating a RVD response.

2. Methods

2.1 Cell culture

SV40-adenovirus-immortalized HCEC, a generous gift from Dr. Araki-Sasaki, (Kagoshima Miyata Eye Clinic, Kagoshima, Japan), were cultured as previously described [46]. pHCEC were purchased from Cascade Biologics, Inc. (Portland, OR, USA). These cells were grown and maintained in EpiLife medium (Cascade Biologics), supplemented with human corneal growth supplement (HCGS) containing bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, and mouse epithelial growth factor (EGF) in the absence of antibiotics and antimycotics, according to the manufacturer's instruction. Second- or third-passage cells were used for experimentation.

2.2 Immunohistochemistry

Human corneal tissues unsuitable for transplant were provided by Upstate New York Transplant Services Inc. (Buffalo, NY, USA). Human corneas were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h, embedded in paraffin, and processed for histology. Paraffin sections, 5 μm thick, were deparaffinized, rehydrated, and subjected to immunohistochemistry. Following a brief wash with phosphate-buffered saline (PBS), samples were blocked with 5% non-fat milk in PBS for 30 min at room temperature. An epitope-specific rabbit anti-TRPV4 (0.6 mg/ml) (Alomone Labs, Jerusalem, Israel) antibody was diluted in 5% non-fat milk/PBS and incubated with the samples overnight at 4° C. After incubation, three 10-min washes with PBS removed unbound antibodies. Samples were then incubated for 3 h at room temperature with the secondary antibody, goat anti-rabbit IgG-Alexa 488 (10 μg/ml, Molecular Probes, Eugene, OR, USA), diluted in 5% non-fat milk/PBS and exposed to 4',6-diamidino-2-phenylindole (DAPI) (300 nM, Sigma, St. Louis, MO, USA) for 5 min at room temperature. After three 10-min washes with PBS, sections were mounted directly onto glass slides using Mowiol mounting medium (Calbiochem, San Diego, CA, USA) and coverslip-sealed.

Confocal microscopy was performed on an LSM 510 upright confocal microscope (Zeiss, Oberkochen, Germany). The fluorochromes were excited with argon laser light of the

appropriate wavelength. Images were captured with Zeiss LSM 510 software and exported to Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, CA, USA).

2.3 Immunocytochemistry

Experiments were performed as previously described [47]. Briefly, HCEC were grown on a two-well Lab-Tek chamber slide system (NUNC, Rochester, NY, USA), fixed on ice in 4% paraformaldehyde/PBS for 30 min, and permeabilized using 0.1% Triton-X100 solution. After blocking unspecific binding sites with normal goat serum, cell monolayers were exposed to rabbit anti-TRPV4 antibody at a dilution of 1:200 (Alomone) in HEPES-buffered Ringer's solution containing 1% bovine serum albumin (BSA) overnight at 4°C. HCEC were then visualized by incubation with Texas Red-conjugated goat anti-rabbit IgG at a dilution of 1:400 (Santa Cruz Biotech, CA, USA) for 1 h at room temperature avoiding light. SYTO-16, 3 μ M, for 5 min (Molecular Probes) was used for nuclei counterstaining. Control experiments were performed using 10% goat serum in place of the primary antibody. Imaging was performed using a Nikon Eclipse TE2000-U fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) linked to a Pixelfly digital CCD camera; a 60x oil objective (NA = 1.40) was used. Images were processed using Image-Pro Express software (Media Cybernetics Inc., Silver Spring, MD, USA) and Photoshop 5.5.

2.4 Fluorescence-activated cell sorting (FACS) analysis

Cell surface expression of TRPV4 was determined by flow cytometry (Epics XL, Beckman-Coulter, Fullerton, CA, USA) with a laser power of 5.76 mW. The instrument was calibrated before each measurement using standardized fluorescent particles (Immunocheck, AMAC Inc., Westbrook, ME, USA). Fluorescence cell signals were measured simultaneously with three photomultiplier tubes and optical filters. Measurements are expressed as the mean log fluorescence intensity of the cell population within the gate. HCEC were first permeabilized/fixed in lysis solution (Optilyse; Beckman-Coulter, Fullerton, CA, USA) at room temperature for 5–10 min, then incubated with the anti-TRPV4 antibody (1:200, Alomone) on ice for 60 min, washed three times, incubated with a phycoerythrin (PE)-conjugated secondary antibody for 30 min on ice avoiding light, and finally analyzed by flow cytometry. Cells were initially identified by the characteristic forward- and side-scatter parameters of unstained cells, and then confirmed by staining with PE-conjugated primary anti-human HLA-DR (Beckman-Coulter). Data are expressed as mean relative fluorescence units (RFU) and percentage of positive cell-staining. Isotype primary conjugated antibodies served as a negative control. Samples were prepared and analyzed in duplicate, and a minimum of 5,000 cells was counted in each sample.

2.5 Reverse transcriptase polymerase chain reaction (RT-PCR)

cDNAs were synthesized directly from lysates of HCEC and pHCEC using Superscript™ III CellsDirect cDNA Synthesis System (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Polymerase chain reaction (PCR) was performed on a PCR cycler (Mastercycler Gradient, Eppendorf Scientific, NY, USA) using Platinum Taq DNA polymerase (Invitrogen). The TRPV4 (accession no. NM_021625) primers were 5'-AACTGAACAAGAACTCGAACCCG-3' (forward, nt 2581) and 5'-TGCGGACGCGGACTCGACGTA-3' (reverse, nt 3107), giving a 480 bp product [48]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to synthesize a housekeeping gene (1.15 kb) for normalizing TRPV4 product. Forty cycles of PCR amplification followed, as described previously [48] but with a slight modification: 94°C for 2 min, denaturation at 95°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min. PCR products were separated by electrophoresis in a 1.2% ethidium bromide-containing agarose gel (Invitrogen), sequence analysis verified their identity. To verify that there was no

genomic DNA contamination, parallel reactions were run on each sample in the absence of reverse transcriptase.

2.6 Western blot analysis

Western blot was carried out as described previously, with several modifications [11]. Briefly, HCEC cultured on 33 mm culture dishes were lysed using lysis buffer containing (mM): 20 Tris, 150 NaCl, 1 EDTA, 1 EGTA, 1% Triton X-100, 2.5 sodium pyrophosphate, 1 β -glycerolphosphate, and 1 Na_3VO_4 , pH 7.5, with a protease inhibitor mixture (1 mM PMSF, 1 mM benzamidine, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin) for at least 10 min. Cells were scraped with a rubber policeman followed by sonication (4 s by 4 cycles at 50 mV) and centrifugation (13,000 rpm for 15 min at 5°C). Supernates were harvested and stored at -80°C until analysis. The protein concentration of each lysate was determined by bichinchoninic acid assay (micro BCA protein assay kit, Pierce Biotechnology, Rockford, IL, USA). After boiling samples for 5 min, equal amounts of protein were fractionated onto 10% SDS-polyacrylamide gels, followed by electrophoresis and blotting onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with blocking buffer–5% fat-free milk in 0.1% Tris-buffered solution/Tween-20 (TBS-T)–for 1 h at room temperature, then probed overnight at 5 °C with rabbit anti-TRPV4 (Alomone) antibody (1:1500). Anti-TRPV4 antibody was prepared by pre-incubation with PBS containing 1% BSA for 1 h, followed by dilution with blocking buffer (1:1500). Pre-adsorption of anti-TRPV4 antibody with its blocking peptide was performed to validate antibody specificity. After three washes with blocking buffer, membranes were incubated with goat anti-rabbit IgG for 1 h at room temperature, then consecutively washed for 10 min each with TBS-T, TBS, and distilled water. Immunobound antibodies were visualized using the chemiluminescence (ECL)-plus detection system (Amersham Biosciences, Piscataway, NJ, USA). Membranes were reprobed with anti- β -actin antibody after being stripped with restoration buffer (Pierce, Rockford, IL, USA). Images were analyzed by densitometry using Photoshop 5.5.

Results were normalized to controls and are expressed as mean $\pm$ SEM in triplicate, unless otherwise indicated.

2.7 siRNA transfection

siRNA transfection was performed as described [32]. Briefly, cells were grown in six-well plates to 50–60% confluence and 1.5 ml of 10% fetal bovine serum (FBS)-containing medium was added to each well following washes with PBS. Then 75 nM of TRPV4 siRNAs (Table 1) (Ambion, Austin, TX, USA) was mixed with 3.6 μl of lipofectamine RNAiMAX (Invitrogen) in 500 μl of OptiMEM I-reduced serum medium (Invitrogen). Mixtures were incubated for 20 min at room temperature and then added to each of the culture wells. At 24 h following transfection, culture medium was refreshed with 10% FBS-containing DMEM/F12 medium. All experimental measurements were performed 48 h following transfection. RT-PCR and Western blot analyses were performed to evaluate the extent of knockdown of TRPV4 gene and protein expression. Mismatched siRNAs (siControl, Dharmacon Research Inc, Lafayette, CO, USA) were used as a control for monitoring non-sequence-specific effects.

2.8 Intracellular calcium fluorescence imaging

Relative changes in $[\text{Ca}^{2+}]_i$ were measured with ISEE 5.5.9 analytical imaging software, a single-cell fluorescence imaging system (Inovision Corp., Raleigh, NC, USA) as described [32,47]. Briefly, HCEC grown on circular 22-mm coverslips were loaded with 3 μM fura2-AM (Invitrogen-Molecular Probes) at 37°C for 50 min with or without test compounds. Cells were then washed with NaCl Ringer's solution (145 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM KH_2PO_4 , 1 mM MgCl_2 , 10 mM glucose, and 10 mM HEPES, pH 7.4, 300 mOsm/L). Ca^{2+} -free solution was supplemented with 2 mM EGTA. Coverslips were placed on the stage

of a Nikon Diaphot 200 inverted microscope (Nikon, Tokyo, Japan), where cells were alternately illuminated every 5 s at 340 and 380 nm; signal emission was monitored at 510 nm using a Roper Scientific CCD camera (Photometrics, Tucson, AZ, USA). Microscopic fields containing 5–10 cells were examined; at least three coverslips were used for each condition. Results were plotted as mean of F340/F380 nm $\pm$ SEM from at least three independent experiments.

2.9 Relative cell volume measurement and RVD analysis

Time-dependent changes in relative cell volume were measured using a fluorescence microplate analyzer [9]. Briefly, HCEC were plated into 24-well culture plates (Fisher Scientific, Suwanee, GA, USA) and grown to 80–90% confluence prior to use. HCEC were washed twice with pre-warmed PBS and loaded with 10 μ M calcein-AM (Invitrogen-Molecular Probes) for 1 h. Cells were then washed with PBS and incubated for 30 min with 200 μ l of isotonic Ringer's solution per well with or without test compounds. Relative cell volume measurements were performed using a Fusion™ Universal Microplate Analyzer (Perkin-Elmer, Boston, MA, USA) at 37°C. Calcein fluorescence excitation was measured at 485 nm and emission at 530 nm. Each well was read every 30 s for up to 40 min. A 5-min isotonic baseline was recorded prior to a 150 mOsm hypotonic challenge. This was done by adding 200 μ l pre-warmed (37°C) distilled water (0 ± 5 mOsm) to isotonic Ringer's solution. Data were analyzed using independent Student's two-tailed t-test ($p < 0.05$). Results are reported as mean RVD value $\pm$ SEM for at least four independent experiments unless otherwise indicated.

3. Results

3.1 TRPV4 expression in human corneal epithelium

To examine whether there is TRPV4 gene and protein expression in HCEC, we performed RT-PCR, immunohistochemistry, FACS, and Western blot analyses, respectively. Figure 1A shows a micrograph of the intact human cornea. TRPV4 staining is evident throughout the intact corneal epithelial layer (Fig. 1A, right panel). Staining is most intense in the central superficial layer, and diminishes towards the basal and limbal regions. There is minimal TRPV4 staining in the basal limbal zone (Fig. 1A, middle panel) and absent in the stroma. Either omitting the primary antibody (Fig. 1A, right panel) or pre-adsorbing it with its blocking peptide (data not shown) eliminated staining. This negative result documented the appropriate specificity of the anti-TRPV4 antibody.

TRPV4 localization was further evaluated by performing immunocytochemistry (Fig. 1B). Punctate staining is apparent along the cell margins and, at a much lower density, in the perinuclear region (Fig. 1B, upper panel). This primary antibody has adequate selectivity since its replacement with normal goat serum diminished the staining (Fig. 1B, lower panel). FACS analysis (Fig. 2) reveals that TRPV4 expression occurs along the cell surface periphery since exposure to this anti-TRPV4 antibody induced a rightward fluorescence band shift and band broadening relative to that of the unbound control peak [49,50]. This result substantiates that some TRPV4 expression is plasma membrane-associated.

We validated the relevance of using SV40-transformed HCEC to characterize the functional significance of TRPV4 by evaluating its expression in its primary counterpart. Using the same primer pair to assess TRPV4 gene expression in HCEC, the PCR product obtained with pHCEC cDNA is identical to that from HCEC (i.e., 480 bp) (Fig. 3A). Sequence analysis further substantiated the identity of the products. Consistently, Western blot analysis (Fig. 3B) revealed TRPV4 protein bands of identical apparent molecular weights (MW = 90 kDa) in the two different samples. When equal amounts of protein in the HCEC and pHCEC lysates were

loaded on the gel, the band optical densities were similar to one another, suggesting similar TRPV4 protein expression levels. This similarity validates the relevance of results obtained with HCEC.

3.2 TRPV4 expression knockdown

The functional role of TRPV4 was determined through evaluating the effects of TRPV4 knockdown on Ca^{2+} signaling and RVD. HCEC were transfected with three different siRNA sequences, each of which is selective for suppression of TRPV4 gene expression. Under the following conditions, transfection has been shown to result in the maximal suppression of TRPV4 gene and protein expression without cytotoxic effects: 75 nM siRNAs, 0.24% oligofectamine, and 24-h of transfection followed by an additional 24-h incubation period (c.f. Table 1). As indicated in Fig. 4A, TRPV4 gene expression was suppressed by siRNA2 and siRNA3. Oligofectamine RNAiMAX as a vehicle *per se* or mismatched siControl transfection had no effect on TRPV4 gene expression. Genomic contamination was not a contributing factor to TRPV4 gene expression since RT omission eliminated PCR product formation. The invariant levels of GAPDH gene expression (Fig. 4A, lower panel) indicate equivalent loading conditions.

Western blots (Fig. 4B) indicate that siRNA2 and siRNA3 reduced TRPV4 protein expression by 52 and 64%, respectively, compared with their non-transfected counterparts. These declines mirror reductions in TRPV4 gene expression following transfection with the abovementioned siRNAs. Furthermore, there was no difference in TRPV4 expression between non-transfected control cells, siControl- and siRNA1-transfected cells. Identical loading conditions were confirmed by uniform β -actin protein expression (Fig. 4B, lower panel). These results indicate that two of the three TRPV4 siRNA sequences were sufficiently selective to suppress TRPV4 expression. Hence, we used these two sequences to knock down TRPV4 protein expression, permitting us to determine its functional importance.

3.3 TRPV4 channel mediation of hypotonicity- and 4 α -PDD-induced Ca^{2+} influx

TRPV4 functionality was also determined by measuring the Ca^{2+} transients induced during exposure to either 4 α -PDD (3 μM) or 50% hypotonic challenge. Figure 5A shows that exposure to 4 α -PDD in the Ca^{2+} -containing Ringer's solution transiently increased the F340/F380 ratio by as much as three-fold ($\Delta\text{ratio} = 0.7 \pm 0.1$, $n = 8$) for 15 min. In TRPV4-knockdown cells incubated with the same solution, 4 α -PDD-induced Ca^{2+} transients were suppressed by 89% relative to those in nontransfected control ($p < 0.001$). Similarly, exposure to either ruthenium red (10 μM)—a pan Ca^{2+} permeable pathway inhibitor (Fig. 5B) or Ca^{2+} -free isotonic solution (Fig. 5C) obviated such a Ca^{2+} response (94.1% and 98.9%, respectively). Conversely, Ca^{2+} responses to 4 α -PDD were unaffected by the TRPV1 channel blocker, capsazepine (CPZ) (10 μM) (Fig. 5D).

In non-transfected control cells, exposure to a 150-mOsm hypotonic solution induced 2.5-fold Ca^{2+} transients (Fig. 5E, 0.6 ± 0.2 , $n = 7$). This transient response lasted about 15 min, which was similar to that induced by 4 α -PDD (c.f. Figs. 5A and 5E). On the other hand, in cells transfected with siRNA3, the transient rise was only 10% of the peak in the non-transfected counterpart. These results indicate that there is functional TRPV4 activity.

3.4 TRPV4 channel activation mediates RVD

RVD is dependent on increases in plasma membrane Ca^{2+} influx in various tissues [16,51, 52]. We first determined whether such influx is also required for RVD in HCEC. As shown in Fig. 6A, exposure to a Ca^{2+} -free solution blocked this response by 62% ($n = 4$, $p < 0.001$). Consistently, exposure to ruthenium red also inhibited RVD. This blocker at 1 μM suppressed RVD by 37% ($n = 4$, $p < 0.001$). By increasing its concentration to 10 μM , RVD inhibition

reached 54%. Exposure to either Ca^{2+} -free medium or ruthenium red affected RVD as early as 5 min following the challenge. We then examined if TRPV4 mediates RVD since this Ca^{2+} -permeable channel can be activated by hypotonicity [21,23]. To make this assessment, we compared RVD responses in non-transfected control cells with those in their TRPV4 knockdown counterparts. As indicated in Fig. 6B, RVD recovery declined by 38% and 51% in siRNA2- and siRNA3-transfected cells ($n = 4$, $p < 0.001$), respectively, relative to those of the non-transfected control. RVD suppression was also evident at 5 min following initiation of the hypotonic stress. However, RVD responses in cells transfected with either siControl or siRNA1 were similar to those in control cells. These data suggest that TRPV4 activation is required for inducing RVD. On the contrary, the TRPV1 antagonist CPZ (10 μM) failed to affect RVD behavior, suggesting that TRPV1 is not involved in inducing this process (Fig. 6A) [40].

4. Discussion

TRPV4 protein expression was detected in HCEC and its primary counterpart as well as in the intact human cornea. Its expression is functional because: 1) 4 α -PDD resulted in Ca^{2+} transients; 2) either ruthenium red, removal of $[\text{Ca}^{2+}]_o$, or TRPV4 siRNA knockdown obviated these transients; 3) RVD behavior was selectively inhibited following effective siRNA TRPV4 knockdown whereas this response was not affected by mismatched or nonfunctional siRNA; 4) pre-exposure to CPZ failed to suppress 4 α -PDD-induced Ca^{2+} transients and RVD. These pieces of evidence for functional TRPV4 expression suggest that its presence is essential for maintaining corneal epithelial barrier integrity during exposure to hypotonic challenges.

The rationale for evaluating TRPV4 involvement in mediating RVD arose from previous studies in RCEC, which showed that this response is dependent on increases in plasma membrane Ca^{2+} influx resulting from emptying intracellular store (ICS) Ca^{2+} content through ryanodine-sensitive channels [16]. In HCEC, TRPV4 channels can be stimulated by 4 α -PDD and hypotonicity irrespective of the ICS Ca^{2+} filling state, suggesting a store-operated channel independent Ca^{2+} influx pathway.

Although some studies suggest TRPV4 functions as an osmosensor, there is no evidence of direct activation of TRPV4 by hypotonicity. One possibility is that it may serve as a component of osmo-sensing pathway since several upstream events leading to TRPV4 stimulation have been found, such as cPLA₂ induced AA and EETs formation [34], AQP5 induction [22], and Src family tyrosine kinase-dependent tyrosine phosphorylation of TRPV4 at residue Tyr-253 [53]. Further study is needed to clarify which of these mechanisms account for TRPV4 stimulation in HCEC.

TRPV4 gene expression was validated by showing that the primer pair, which was used in a study employing synoviocytes, yielded the same-sized (i.e. 480 bp) cDNA product (Fig. 3A) [48]. Sequence analysis revealed that the base composition was identical to that predicted. The apparent MW of the TRPV4 protein is 90 kD (Fig. 3B), a figure similar to that reported in rat cortical astrocytes [54]. Furthermore, the antibody selectivity used to examine such expression was confirmed based on demonstrating that pre-adsorption of the antibody with the TRPV4 epitope peptide eliminated the staining seen in Western blot or immunohistochemistry.

TRPV4 expression in the intact human corneal epithelium is polarized in that its expression is most prominent in the central superficial layers whereas lessening in the epithelial peripheral and basal regions. This pattern of expression is similar to that described for the epithelial cell lining brain ventricles [45]. Moreover, as in HCEC, in the vascular endothelium isolated from wildtype mice which expressed endogenous TRPV4, TRPV4 activation was elicited by either a hypotonic medium or 4 α -PDD. However, in TRPV4^{-/-} mice this response could not be

induced [35]. To further resolve TRPV4 cellular localization we used immunocytochemistry in parallel with FACS. Punctate TRPV4 staining is evident along the cell periphery and to a less extent in the cytosolic and nuclear regions. This uneven distribution pattern is similar to that seen in rat cortical astrocytes expressing endogenous TRPV4 [54], suggesting TRPV4 clusters in the plasma membrane to form a tetrameric transmembrane channel although higher resolution techniques that can pinpoint the actual formation of TRPV4 are needed to confirm this assumption. Cytosol-localized TRPV4 may serve as a reservoir for plasma membrane insertion since some other TRP members are immediately translocated to the plasma membrane in response to various stimuli [55]. Therefore, TRPV4 cytosolic localization could be poised to respond to variations in tear film osmolarity.

Clarifying the role of TRPV4 channels has been somewhat hindered by the lack of a selective TRPV4 antagonist. Despite this shortcoming, TRPV4 function was assessed by determining if Ca^{2+} influx could be altered 1) during exposure to either ruthenium red or to a Ca^{2+} -free solution, 2) in a TRPV4^{-/-} animal [44]. Recently, siRNA technology has been used to selectively knock down TRPV4 gene and protein expression. In our case, two of the three candidate siRNAs (i.e., siRNA2 and 3) significantly reduced TRPV4 gene and protein levels (Fig. 4A). The magnitude of the TRPV4 protein expression knockdown 24 h following siRNA transfection is similar to that of endogenous TRPV4 suppression in M-1 cells following 48 h transfection [33]. Thus, our transfection protocol is appropriate to assess TRPV4 activity. In HCEC, TRPV4 was evaluated based on the declines in 4 α -PDD- and hypotonic-induced Ca^{2+} transients and hypotonic-induced RVD following TRPV4 knockdown. In non-transfected cells, both stimuli induced biphasic Ca^{2+} transients with an initial up to 3-fold [Ca^{2+}]_i rise followed by a partial recovery to the baseline values after 15 min (Fig. 5A and E). Such [Ca^{2+}]_i changes were also observed in human airway smooth muscle cells following 4 α -PDD stimulation [56]. In siRNA3-treated cells, these Ca^{2+} transients were almost abolished. On the other hand, even though there is also functional TRPV1 expression in HCEC, it is not involved in mediating these responses since pre-incubation with capsazepine, did not affect the Ca^{2+} transients or RVD.

TRPV4 mediated Ca^{2+} influx makes an important contribution to eliciting RVD. This association is consistent with our finding that the magnitudes of TRPV4 knockdown obtained with the three different TRPV4 siRNA candidates agree with the magnitudes of decline and retardation of RVD responses, namely; siRNA3 > siRNA2 > siRNA1. However, even the most effective siRNA sequence (siRNA3) did not completely abolish RVD. The residual RVD may be attributable to remaining TRPV4 expression or other swelling activated Ca^{2+} influx pathways. The latter is tenable because RVD inhibition following siRNA transfection was smaller than those induced by ruthenium red and calcium removal (i.e., calcium removal > ruthenium red > siRNA3). Possible alternative Ca^{2+} pathways were described in murine aortic myocytes, human kidney and liver cells. They include TRPV2, TRPM3 and TRPC1 [55,57, 58]. Further experiments are needed to clarify this question. Nevertheless, TRPV4 activation by a hypotonic stimulus appears to account for more than 50% of the overall RVD response.

In conclusion, there is TRPV4 protein expression in HCEC. Such expression is functional because 4 α -PDD and the hypotonic challenge induced similar Ca^{2+} transients. TRPV4 functionality is further validated by the fact that following siRNA TRPV4 knockdown, RVD responses were suppressed and retarded. The presence of TRPV4 in human corneal epithelium suggests that its activation during exposure to tear film hypotonicity could be essential for preserving the tissue integrity.

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Figure 1.

TRPV4 localization in intact human corneal epithelium and HCEC. **A:** Immunohistochemistry indicates that there is TRPV4 expression throughout the intact human corneal epithelium, predominantly in the central superficial layer (red). This expression wanes towards the basal and limbal regions. Cell nuclei were counterstained with DAPI (blue). Merged images show both TRPV4 expression and nuclei (upper panel). Omission of anti-TRPV4 antibody eliminated its staining (Right panel), which is the same result obtained with the primary antibody pretreated with its blocking peptide. Calibration bar: 10 μ m. **B:** Immunocytochemistry shows that punctate staining of TRPV4 is localized within the plasma membrane and perinuclear area. This staining was abolished by replacing anti-TRPV4 antibody

with normal goat serum (lower panel) or pre-exposure of anti-TRPV4 antibody to its blocking peptide (data not shown). Calibration bar: 15 μ m.

Figure 2. Validation of TRPV4 plasma membrane localization. FACS was used to validate TRPV4 localization. Left traces represent isotype control, which is similar to background autofluorescence. Right traces (arrow) represent PE-conjugated anti-TRPV4 antibody labeling. These traces show a rightward shift and peak broadening relative to those on the left indicative of TRPV4 surface expression.

Figure 3.

TRPV4 gene and protein expression in pHCEC and HCEC. **A:** RT-PCR reveals TRPV4 expression in pHCEC and HCEC since a predicted 480 bp cDNA product was obtained. Levels of housekeeping GAPDH gene expression were compared to validate results. (lower panel).

B: Western blot analysis shows that these two types of HCEC express similar amounts of a 90 kDa protein. Pre-adsorption of anti-TRPV4 to its epitope peptide was performed to validate antibody selectivity (middle panel). Stripped membranes were exposed to anti- β -actin antibody to validate equal loading conditions (lower panel).

Figure 4.

siRNA transfection knockdown of TRPV4 gene and protein expression. **A:** RT-PCR analysis indicates TRPV4 gene expression in HCEC 48 h following exposure to lipofectamine RNAiMAX (Con), siControl siRNA (siCon), and the three sequences of TRPV4-specific siRNAs (siRNA1, siRNA2, siRNA3). Negative control (Neg.) was performed by omission of the RT step. Levels of housekeeping GAPDH gene expression were compared to validate results. (lower panel). **B:** Western blot analysis validates TRPV4 protein knockdown. Results are presented in triplicates as means $\pm$ SEM and summarized in lower panel. * $p = 0.05$ compared with Con.. A.U., arbitrary unit.

Figure 5.

TRPV4 mediation of hypotonic- and 4 α -PDD-induced $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was monitored by a single-cell fluorescence imaging system following exposure of fura2-loaded cells to either 3 μ M 4 α -PDD or 50% hypotonic medium. Traces shown are presented as means $\pm$ SEM of 5–10 cells in at least three independent experiments for each condition. **A:** TRPV4 siRNA knockdown suppressed 4 α -PDD-induced Ca^{2+} transients. **B:** Pre-exposure of HCEC to ruthenium red (RR) (10 μ M) inhibited 4 α -PDD-induced Ca^{2+} transients. **C:** Removal of extracellular Ca^{2+} eliminated 4 α -PDD-induced Ca^{2+} transients. **D:** The presence of 10 μ M capsazepine (CPZ) did not affect 4 α -PDD-induced $[Ca^{2+}]_i$. **E:** TRPV4 knockdown in HCEC suppressed hypotonicity-induced Ca^{2+} transients.

B

Figure 6.

TRPV4 mediation of 50% hypotonic-induced RVD. **A:** 50% hypotonicity-induced RVD was evaluated for 40 min in calcein-loaded HCEC using a fluorescence microplate analyzer. The 30-min pre-exposure to Ca^{2+} -free medium containing 2 mM EGTA, or RR (1, 10 μM) inhibited RVD responses by 62, 37 and 54%, respectively. Such inhibition occurred as early as 5 min after imposing the hypotonic challenge. The presence of CPZ (10 μM) did not affect this response. **B:** TRPV4 knockdown inhibited RVD. Transfection of TRPV4-specific siRNA2 or 3 suppressed the hypotonicity-induced RVD by 38 and 51%, respectively. The suppression started 5 min following initiation of the hypotonic challenge. Incubation with mismatched siControl (siCon) or TRPV4-siRNA1 did not affect RVD behavior. Traces are presented as

means $\pm$ SEM in at least four independent experiments. Apparent cellular volumes at 40 min are summarized in the lower panel. ** $p = 0.001$ relative to control (Con) in A or to siCon in B. Arrow indicates application of the hypotonic challenge.

Table 1

Characteristics of siRNAs			
siRNA	Target sequence (5' → 3')	Location in gene sequence	Percent G/C (%)
V4-siRNA1	GGAGUUUCGAGAGCCAUCU	638	48
V4-siRNA2	GAAAGCACCCAUGGACUCA	387	48
V4-siRNA3	GUCUUCAACCGGCCUAUCC	529	52
siControl	UAGCGACUAAACACAUCAA	N/A	42