Temperature-Sensitive Transient Receptor Potential Channels in Corneal Tissue Layers and Cells

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Abstract
We here provide a brief summary of the characteristics of transient receptor potential channels (TRPs) identified in corneal tissue layers and cells. In general, TRPs are nonselective cation channels which are Ca 2+ permeable. Most TRPs serve as thermosensitive molecular sensors (thermo-TRPs). Based on their functional importance, the possibilities are described for drug-targeting TRP activity in a clinical setting. TRPs are expressed in various tissues of the eye including both human corneal epithelial and endothelial layers as well as stromal fibroblasts and stromal nerve fibers. TRP vanilloid type 1 (TRPV1) heat receptor, also known as capsaicin receptor, along with TRP melastatin type 8 (TRPM8) cold receptor, which is also known as menthol receptor, are prototypes of the thermo-TRP family. The TRPV1 functional channel is the most investigated TRP channel in these tissues, owing to its contribution to maintaining tissue homeostasis as well as eliciting wound healing responses to injury. Other thermo-TRP family members identified in these tissues are TRPV2, 3 and 4. Finally, there is the TRP ankyrin type 1 (TRPA1) cold receptor. All of these thermo-TRPs can be activated within specific temperature ranges and transduce such inputs into chemical and electrical signals. Although several recent studies have begun to unravel complex roles for thermo-TRPs such as TRPV1 in corneal layers and resident cells, additional studies are needed to further elucidate their roles in health and disease.

Introduction
Temperature-sensitive transient receptor potential channels (thermo-TRPs) are a superfamily which is subdivided into 7 groups according to differences in amino acid sequence homology. Specifically, TRPs are subdivided into canonical receptors (TRPC1–6), vanilloid receptors (TRPV1–7), melastatin receptors (TRPM1–8), ankyrin receptor (TRPA1), polycystins (TRPP1–5) and mucolipins (TRPML1–3). Another TRP subfamily is assigned to mechanosensory channels (TRPN), which are not expressed in mammals. TRPV1–4, TRPM8 and TRPA1 belong to the thermo-TRP family. Modulation of thermo-TRP activity by environmental thermal challenges is essential for tissues
to offset declines in tissue function that result from thermal exposure [1]. Such changes in different TRP subtypes are elicited for the most part through transient increases in intracellular Ca\(^{2+}\) influx as a consequence of channel activation. These rises in turn activate downstream-linked Ca\(^{2+}\)-sensitive signaling pathways. The magnitude and duration of the Ca\(^{2+}\) influx encodes downstream signaling responses that induce either a homeostatic or lethal response. Therefore, identifying which TRP subtypes respond to physiological and pathogenic stresses imposed on corneal tissue can aid in identifying novel drug targets.

This insight can then be utilized to target specific subtypes with drugs or genetic techniques to reverse losses in corneal function which are caused by maladaptive TRP function. At this point, it is increasingly apparent that thermo-TRPs play an important role in eliciting such control. Okada, Reinach and colleagues [2, 3] have recently shown in different studies that inhibition of both TRPV1 and TRPA1 activation after an alkali burn markedly reduces corneal fibrosis inflammation and opacification. On the other hand, TRPV1 activation hastens corneal epithelial healing following epithelial debridement, suggesting that increasing TRPV1 activity in a clinical setting could decrease the likelihood of corneal infection. Although there are some relevant indications of putative roles of thermo-TRPs in corneal cells, there is scant information about the cellular mechanisms modulating TRP channel activation and deactivation in corneal tissues and cells. This realization has prompted efforts to fill this void, which is needed to develop drugs or gene expression protocols that can selectively modulate TRP behavior without having side effects.

This review describes the functional importance of thermo-TRPs in corneal tissues and cells in maintaining corneal homeostasis. Table 1 provides a summary listing corneal TRP isotype characteristics, functional roles and cell type localization. We also present evidence that TRP malfunction can contribute to the underlying pathophysiology of inappropriate wound healing responses to corneal injury. Finally, the possible translational relevance of the insight gained from this characterization is discussed.

### Corneal Epithelium

In the last decade, different types of TRP isotype functional expression were identified on corneal epithelial nerves and also on corneal epithelial cell membranes.
TRPC4 is a thermally insensitive member of the TRP canonical subfamily which can be activated by intracellular Ca$^{2+}$ store depletion induced by epidermal growth factor (EGF) [5]. TRPC4 activation occurs as a consequence of Ca$^{2+}$ store emptying, which leads to increases in store operated Ca$^{2+}$ channel (SOCC) activity. It is uncertain whether this TRP subtype is the sole component of SOCCs or whether it is a contributor to its function. This uncertainty exists because TRPs are composed of tetrameric subunits, which can form both homomeric and heteromeric configurations. A homomeric TRP is made up of 4 subunits from one subfamily member, whereas a heteromeric TRP counterpart can have subunits derived from different members of a subfamily. These alternatives extend the complexity of delineating the functional roles of TRPs, since the regulation of heteromeric channels can differ from homomeric channels. In any case, in this study, it was shown that SOCC activation leads to capacitative calcium entry, which refills intracellular stores of their depleted Ca$^{2+}$ content [6]. TRPC4 involvement in capacitative calcium entry is apparent, since in TRPC4 siRNA gene-silenced cells, EGF-induced SOCC activation and proliferation were suppressed [5]. TRPC4 expression was also identified in bovine corneal endothelial cells and is also involved in regulating SOCC activity [7].

The vanilloid TRP protein isoform TRPV1 is functionally expressed in both human corneal epithelial cells and corneal endothelial cells [8, 9]. It is activated in nonneuronal cells by numerous stimuli including heat, voltage, vanilloids, cannabinoids, lipids and protons/cations [10, 11]. In this context, complex regulatory mechanisms are involved in encoding specific responses through Ca$^{2+}$ signaling [12]. Since TRPV1 activity is to some extent voltage dependent [13], membrane depolarization by TRPV1 channels suppressed Ca$^{2+}$ entry through calcium release-activated calcium channel protein 1 (ORAI1), a pore-forming transmembrane protein whose activation elicits SOCC activation in nonexcitable cells (fig. 2). Specifically, ORAI1, which is encoded by the ORAI1 gene, elicits the calcium release-activated Ca$^{2+}$ current when activated by Ca$^{2+}$ store depletion [14]. The process of ORAI1 channel activation is thought to depend on relocation to the plasma membrane of intracellular store membrane-associated stromal interaction molecule 1 (STIM1). This protein senses the Ca$^{2+}$ filling state of intracellular stores and moves to open SOCCs by interacting with ORAI1 upon intracellular Ca$^{2+}$ store depletion. The contribution by other SOCCs such as TRPC1 still remains to be elucidated. However, the role so far of TRPV1 in connection with ORAI1 has not been investigated in corneal tissues and cells.

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**Fig. 1.** Histological structure of the human cornea. Vertical section of the human cornea. The corneal endothelium, consisting of only one cell layer, is located at the posterior cornea, facing the anterior chamber of the eye. The corneal epithelium is located at the outside of the cornea, facing the air. In between, the corneal stroma is located. HE. ×100.

**Fig. 2.** Schematic representation of TRPV1 (capsaicin receptor). TRPV1 modulates driving forces for Ca$^{2+}$ entry. It is an ion channel and a polymodal receptor for various inflammatory stimuli, hypertonicity, protons, heat and lipid metabolites. TRPV1 possesses endogenous activators such as endocannabinoids (anandamide, NADA). TRPV1 can also be activated by heat (>43°C) and is permeable for monovalent and divalent cations like other unspecific cation channels. Notably, membrane depolarization (V_m↑) by TRPV1 channels results in a reduced Ca$^{2+}$ entry (Ca$^{2+}$↓) via ORAI. This Ca$^{2+}$ process accordingly modulates TRPV1 function to modify the intracellular Ca$^{2+}$ content.
Another characteristic of TRPs is that they are able to modulate driving forces for Ca\(^{2+}\) entry. On the one hand, membrane depolarization by TRPs results in a reduced Ca\(^{2+}\) entry through ORAI, as mentioned before. On the other hand, hyperpolarization of the membrane by big potassium or maxi-K channels or small conductance calcium-activated potassium channels results in an increased Ca\(^{2+}\) influx [12]. This Ca\(^{2+}\) change subsequently modulates TRP and big potassium or maxi-K channel as well as small conductance calcium-activated potassium channel function to fine-tune the intracellular Ca\(^{2+}\) concentration. Irrespective of K\(^{+}\) channel involvement, TRPV1 activation via capsaicin initially leads to increases in intracellular Ca\(^{2+}\) levels. Their rises can induce inflammatory cytokine release through mitogen-activated protein kinase (MAPK) signaling in corneal epithelial cells [8]. TRPV1 receptors play a role in mediating corneal epithelial and stromal cell inflammatory mediator infiltration and subsequent hyperalgesia based on results obtained with a mouse corneal wound healing model [3]. In this study, it was shown that in homozygous TRPV1 knockout mice, inflammation and fibrosis were clearly suppressed relative to what occurred in their wild-type counterpart [3]. A similar favorable outcome was obtained in wild-type mice systemically treated with a TRPV1 antagonist subsequent to alkali having burnt their corneas. Therefore, TRPV1 is a potential drug target for improving the outcome of inflammatory/fibrogenic wound healing [3]. Regarding TRPV1-mediated Ca\(^{2+}\) signaling, Yang et al. [15] recently showed that activation of a G protein-coupled receptor such as the cannabinoid receptor type 1 (CB1) suppressed TRPV1-induced inflammatory responses to corneal epithelial debridement. A suggested Ca\(^{2+}\) signal transduction pathway activated by the CB1 agonist WIN, TRPV1 can selectively be activated by heat (>43°C) or capsaicin and blocked by capsazepine. The CB1 receptor coupled to G\(_{i/o}\) proteins can be activated by WIN. If TRPV1 is activated by capsaicin, WIN blocks TRPV1, which leads to a reduction of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)). Probably, WIN may also directly activate TRPV1 by a CB1-independent mechanism.

**Fig. 3.** Suggested Ca\(^{2+}\) signal transduction pathways activated by the CB1 agonist WIN. TRPV1 can selectively be activated by heat (>43°C) or capsaicin and blocked by capsazepine. The CB1 receptor coupled to G\(_{i/o}\) proteins can be activated by WIN. If TRPV1 is activated by capsaicin, WIN blocks TRPV1, which leads to a reduction of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)). Probably, WIN may also directly activate TRPV1 by a CB1-independent mechanism.
Corneal Endothelium

The corneal endothelium is a single layer of postmitotic cells arranged in a hexagonal array at the posterior side of the cornea (fig. 1). By eliciting osmotically coupled fluid flow from the stroma into the anterior chamber, this tissue maintains corneal deturgescence and transparency [21, 22]. For these functions to be maintained, a critical endothelial cell density along with close cell-to-cell apposition is needed for its barrier property to prevent corneal...
swelling. Besides voltage-operated Ca\(^{2+}\) channels, the non-temperature-sensitive TRPM2 channel is putatively expressed in this tissue [23, 24]. Given that TRPM2 is activated by oxidative stress (H\(_2\)O\(_2\)), this suggests that corneal endothelial cells can withstand some levels of oxidative stress [25]. In a study, it was shown that a block of voltage-operated Ca\(^{2+}\) channels (L-type) could only partially suppress the H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx [24]. Furthermore, the cooling agent icilin induced Ca\(^{2+}\) increases, suggesting functional thermosensitive TRPM8 expression [26]. This suggests that corneal endothelial cell function can adapt to thermal variations and probably also to oxidative stress through activation of these TRP subtypes. TRPV1–3 functional expression in this cell layer also contributes to thermal sensing [9]. Modulation of their activity by temperature changes underlies essential homeostatic mechanisms, contributing to the support of corneal endothelial function under different ambient conditions. Interestingly, similar observations were made in the immortalized clonal cell populations HCEC-B4G12 and HCEC-H9C1 also under serum-free culture conditions [27]. The osmosensor TRPV4 was also identified in human corneal endothelial cells, because exposure to a hypotonic environment increased the intracellular Ca\(^{2+}\) concentration and TRP-like whole-cell currents in these cells [28]. Therefore, the commonality in thermosensitive TRP functional expression in the corneal epithelial and endothelial layers suggests that these tissues are well poised to compensate for losses in function that are caused by exposure to similar thermal stresses. Nevertheless, it is still unclear whether there are differences in their functional expression patterns, since only some of the numerous thermosensitive TRP subtypes have been evaluated.

**Corneal Stroma**

The stroma is located between the two outer limiting corneal layers and accounts for up to about 90% of the corneal full thickness (fig. 1). It is formed by an extracellular matrix of regularly arranged collagen fibers fixed in a geometric array and interspersed with keratocytes. TRPV1 expression was identified in primary cultured human fibroblasts [29]. Yang et al. [29] suggested that transforming growth factor (TGF)-\(\beta_1\)-induced fibrosis and inflammation are dependent on TRPV1 transactivation on fibroblasts in an injury severe enough for epithelial-derived TGF-\(\beta_1\) to gain stromal access. This dependence is evident, since in TRPV1 knockout mice, the corneal wound healing response to an alkali burn has a far more favorable outcome [3]. These undesirable effects in wild-type mice may result in part from an increased cytokine secretion caused by TGF-\(\beta_1\) stimulation of its cognate receptor, which mediates phospho-p38 MAPK signaling required for persistent Smad2/3 phosphorylation [29]. The fibrotic response that occurs subsequently to Smad2/3 activation is due to myofibroblast transdifferentiation, which leads to scarring and corneal opacification [30]. This response is dependent on activation of a positive feedback signaling transduction loop wherein p38 MAPK induces TRPV1 activation, leading to prolongation of both phospho-p38 MAPK and Smad2/3 activation through concerted TRPV1 and TGF-\(\beta\) receptor interaction.

Taken together, stromal TRPV1 activation by injury on stromal resident fibroblasts initially mediates innate immune responses, which provide an adaptive advantage against pathogenic infiltration. However, if this response is not self-limiting and instead becomes dysregulated, the resulting proinflammatory cytokine storm can lead to blindness. As the scarring response is dependent on TRPV1-induced prolongation and enhancement of TGF-\(\beta\) receptor signaling, drug inhibition of TRPV1 activation in a clinical setting may reduce fibrosis occurring during wound healing. Very recent studies suggest that kerocytes also express TRPM8, since its functional activity was recently identified in an established human corneal stromal cell line [31; Tuercker, Mergler et al., unpubl. data]. On the other hand, it is evident that TRPV1 activation in vivo has a double-edged sword aspect, since its activation following epithelial debridement instead increases epithelial cell proliferation and migration and hastens wound closure [2]. In an earlier study, it was shown that such promotion of wound healing occurs through the transactivation of EGF receptor [2, 15]. EGF receptor stimulation contributes to increases in cell proliferation and migration, which are essential responses for wound closure.

**Corneal Nerve Fibers**

The corneal stroma contains nerve fibers that form a dense midstromal and subepithelial plexus with fibers extending into the basal corneal epithelial layer [32]. Various studies demonstrate also an expression of thermo-TRPs in noncorneal primary sensory neurons [33]. TRPV1 and TRPM8 are more extensively described in corneal nerve fibers of mice, guinea pigs and humans [34–37]. Specifically, in mouse corneal nerve fibers, the TRPM8 agonist menthol induced TRPM8 activation. This response is also elicited at the same temperature character-
istic of TRPM8 activation and induces increased basal lacrimation, while TRPM8 gene silencing failed to induce this response. A low concentration of menthol increased lacrimation via TRPM8 channels without evoking nociceptive behavior [37]. Physical cooling of the cornea in humans similarly increased lacrimation, whereas warming had an opposite effect [36]. Therefore, it is suggested that TRPM8 expression in corneal nerves provides a cold receptor function on the ocular surface and may also serve as a ‘humidity detector’. These studies broaden our understanding of mechanisms that mediate ocular surface homeostasis. They suggest that tear hyposcretion in dry eye syndrome (DES) may be a consequence of impaired TRPM8 function on corneal nerves, since TRPM8 activity contributes to the regulation of basal tear flow in mice.

In conclusion, thermo-TRPs not only play a role in the reflexogenic protection of the ocular surface and anterior eye but also most likely mediate adaptive responses that are essential for maintaining corneal transparency. In this regard, Parra et al. [36] pointed out that prompt responses which are needed to maintain ocular surface hydration during exposure to a desiccating stress may depend on TRPM8 functional expression. Such maintenance may not be solely dependent on TRPM8 expression in corneal nerves but may also depend on TRPV isotype expression in corneal epithelial, stromal and endothelial cells. A possible functional interaction between expression of thermo-TRPs on nerve fibers and neighboring corneal tissues and cells has not been investigated so far.

TRP Drug Targeting in a Clinical Setting

The realization that a variety of different thermosensitive TRP isotypes and a thermoinsensitive counterpart are expressed on the corneal nerves and the cells of the epithelial, endothelial and stromal layers has prompted interest in developing drugs that selectively control their functional expression. This interest stems from studies demonstrating that these TRPs are activated by sensory stimuli and induce responses to environmental stresses that are of adaptive value by reducing declines in corneal function. TRP activation by such challenges can offset declines in epithelial barrier function caused by an anisosmotic stress, hasten corneal reepithelialization and reduce pathogenic infiltration, provided the inflammatory response is self-limiting rather than dysregulated. In addition, thermosensitive TRP activation may be essential to offset declines in tear fluid flow needed for sustaining anterior surface ocular health.

Out of the 27 candidate TRP mammalian genes, only 2 different TRPs so far are of potential value for drug development: TRPV1 and TRPM8. TRPV1 has attracted interest because its activation by injury on afferent corneal sensory nerves in some cases elicits debilitating pain. Another reason for targeting TRPV1 is that after a severe penetrating injury, corneal wound healing is inappropriate due to fibrosis and chronic inflammation. In the first case, suppressing TRPV1 activation through agonist downregulation can initially make the pain more severe due to TRPV1 stimulation, which wanes with time. Therefore, efforts are underway to develop novel selective antagonists that block TRPV1-induced nociception. Many candidates have to be screened because it has to be shown that any decline in TRPV1 functional expression does include neither a systemic nor an off-target effect. It is now also apparent that restoring corneal function with a TRPV1 antagonist should be restricted to usage after a severe injury rather than epitelial debridement. This distinction has to be made, since only after an injury that breaches the basement membrane is it possible for TGF-β to gain access to the stroma and induce fibrosis and chronic inflammation by transactivating TRPV1. Otherwise, after subsequent mild injury, it is not beneficial to inhibit TRPV1 activation, since increases in Ca²⁺ influx elicited by this channel are required to stimulate EGF signaling pathways leading to increases in cell proliferation and migration, both of which hasten wound closure and restoration of barrier function. Another reason to pursue the development of selective TRPV1 antagonists is that inhibition of TRPV1 may reduce inflammation that is symptomatic in some DES patients whose tears are hypertonic. These individuals may also benefit in the future from the development of TRPM8 agonists whose activation by temperature lowering can be promoted to enhance basal tear flow and contribute to the maintenance of ocular surface hydration.

Clinical Aspects: Thermography

Thermography is an investigative technique which allows a rapid color-coded display of the temperature across a wide surface by means of infrared detection using thermography devices such as the novel thermography device TG 1000 (Tomey Corp., Nagoya, Japan) or a thermal camera (noncontact thermographic camera, FLIR P 620) [38, 39]. Thermal pictures of ocular inflammation or noninflammatory ocular pathology can be taken to compare them with pictures of healthy ocular physiology [40]. Accordingly, inflammatory ocular conditions can be easily...
distinguished from noninflammatory conditions. Thermography can also be utilized in the evaluation of tear film in DES. Notably, the relation between ocular surface temperature (OST) and physical parameters of the anterior eye is important. OST can be specifically assessed by dynamic thermal imaging. Studies using thermal imaging revealed that OST is principally related to the tear film and less related to other corneal characteristics (e.g. corneal thickness, corneal curvature and anterior chamber depth) [41]. So far, however, nothing is known about TRPs in context with thermography. On the other hand, inflammatory processes are associated with an increased temperature of the affected tissue and increased levels of thermo-TRP expression. At this point, older studies demonstrated that administration of cryotherapy caused a decrease in the infiltration of inflammatory cells in alkali-burned corneas (the activity of collagenase was inhibited) [42]. Based on the recent and growing knowledge about the role of TRPs in connection with clinical aspects of eye surface biology (e.g. DES and keratoaplasty), especially the TRPV1 subtype could provide an excellent drug target to reduce inflammation and the discomfort experienced by individuals afflicted with DES. Inflammatory inhibition could be achieved by suppressing its channel activity or expression. Based on future characterizations of other TRP functions, novel ligands could be developed to modify TRP function. This could contribute to an efficient targeted therapy program to achieve a better clinical outcome.

Conclusions

Although there is an increasing awareness of the functional importance of thermo-TRP expression in the maintenance of corneal homeostasis during exposure to environmental stresses, it is still not clear whether channelopathies underlie the pathogenesis of some corneal diseases. Such clarification is needed to determine whether they are potential drug targets to reduce the declines in corneal deturgescence associated with disease. Another important area warranting additional investigation is the functional interplay between TRPM8 expressed on nerve fibers and adjacent corneal stromal and epithelial cells. Nevertheless, the insight gained from characterizing TRP functional expression may have an immediate benefit in identifying novel drug targets for improved treatment of DES and reducing sight-compromising fibrosis after a severe injury that breaches the epithelial basement membrane.

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