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Differential expression and localization of transient receptor potential vanilloid 1 in rabbit and human eyes

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Summary. Introduction: The superfamily of transient receptor potential (TRP) cation channels is involved in nociception. Members of this family, such as the vanilloid receptor type 1 (TRPV1) channel, are activated by a wide range of stimuli including heat (>43°C), low pH (<6.5), hypoxia, and hypertonicity. Here we report TRPV1 expression in rabbit and human eyes.

Material and methods: We analyzed the expression of TRPV1 mRNA by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and protein by immunohistochemistry in eyes of New Zealand White rabbits and humans.

Results: In rabbit and human eyes, TRPV1 protein was present in all layers of the corneal epithelium, but only in the basal layer of the conjunctiva. It was also in the ciliary and lens epithelia of both species as well as in the secretory cells of the rabbit lacrimal gland. The retinal pigment epithelium was positive for this protein in both species. TRPV1 was also present in rabbit Müller cells, where it had a similar pattern of expression to vimentin intermediate filaments. Analysis by qRT-PCR showed that TRPV1 mRNA was found in all of the structures where the protein was present. The highest level was in the lens and the lowest in the retina.

Conclusion: TRPV1 is expressed in cells that are particularly active in Ca^{2+} exchange as well as in cells with significant water transport activity. Because TRPV1 is a Ca^{2+} channel, it probably functions in the regulation of both water and Ca^{2+} movements in ocular tissues.

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Introduction

Transient receptor potential (TRP) is a superfamily of cation channels permeable to calcium. These channels are composed of six transmembrane spanning subunits in the form of tetramer. The TRP superfamily is divided into seven subfamilies (Venkatachalam and Montell, 2007): TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPN (NOMP, No-mechano-potential), TRPP (polycystin), and TRPV (vanilloid). The TRPV subfamily is composed of seven members (TRPV1-TRPV7) that contain three conserved ankyrin domains. TRPV1 was the first cloned by Caterina et al., (1997).

TRPV1 is a non-selective cation channel, activated by a wide range of stimuli, such as heat (>43°C), low pH (<6.5), a variety of inflammatory lipid metabolites, phosphorylation by protein kinase C, capsaicin, resiniferatoxin, endocannabinoids (anandamine) (Szallasi and Blumberg, 2007), hypoxia (Ristoiu et al., 2011) and hypertonicity (Liu et al., 2007). Initially, TRPV1 was thought to be restricted to primary afferent nociceptors of the trigeminal ganglion (Bae et al., 2004); however subsequent studies have shown a wide distribution of this receptor throughout the body. In addition to the presence of TRPV1 in peripheral sensory neurons (Caterina et al., 1997), it is also present in keratinocytes (Denda et al., 2001), hepatocytes (Rychkov and Barritt, 2011), visceral adipose tissue (Zhang et al., 2007b), central nervous system (Sasamura

and Kuraishi, 1999), heart (Buckley and Stokes, 2011), and arterial mesenteric beds (Poblete et al., 2005).

In the visual system, TRPV1 has been detected in the cornea and retina. In humans, rats and mice it is present in the corneal epithelial cells (Mergler et al., 2010; Nakagawa et al., 2009; Zhang et al., 2007a). Different roles have been assigned to TRPV1 in these cells, including inflammation and fibrogenesis (Okada et al., 2011), nociception (Nakagawa et al., 2009), and response to stress (Pan et al., 2011). In the corneal endothelial cell layer, TRPV1 has been detected only in humans (Mergler and Pleyer, 2007) where it appears to contribute to maintaining the transparency of the cornea in response to thermal stress (Mergler et al., 2010). In addition, abundant TRPV1 is present in nerve terminals that innervate the cornea and in the ophthalmic branch of the trigeminal nerve (Murata and Masuko, 2006).

In the retina, TRPV1 is expressed by different cells and appears to have different roles depending on the stage of development. In the adult retina, TRPV1 is expressed by retinal microglia (Kim et al., 2006) and by retinal ganglion cells in rodents, where it plays a role in microglial activation and ganglion cell death induced by high intraocular pressure (Sappington et al., 2009). During development, TRPV1 is found at very early stages in neurons and microglia of the retina, where it is implicated in apoptosis, neuronal and synaptic maturation and differentiation (Leonelli et al., 2009, 2011).

Because of the potential importance in ocular physiology and inflammation, we analyzed the distribution of TRPV1 and the relative expression levels of TRPV1 mRNA in the eyes of rabbits, a commonly used animal model for ocular research, and of humans. We found that TRPV1 is expressed and localized in cell types that are active in regulating the movement of fluids and in which Ca^{2+} plays an important role.

Materials and methods

Animals

Thirteen New Zealand White adult rabbits, weighing approximately 2 kg, were used in this study. The animals were kept on 12:12 h light/dark schedule with lights on at 8 am. Care was provided according to the guidelines of the Association in Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

The rabbits were euthanized with an intracardiac injection of sodium pentobarbital (Dolethal, Vetoquinol, Lure, France) under general anesthesia of ketamine hydrochloride 37.5 mg/kg (Ketolar, Parke-Davis S.A., Barcelona, Spain)/xylazine hydrochloride 5 mg/kg (Rompun, Bayer AG, Leverkusen, Germany). The eyes were enucleated and placed in 10% formaldehyde or dissected into cornea, lens, ciliary body, and retina. Portions of these same tissues and the lacrimal gland were prepared for quantitative reverse transcription polymerase chain reaction (qRT-PCR described below).

Human eyes

In accordance with all applicable statutes, the use of human tissues was approved by the ethics committees of Rio Ortega Hospital, Institute Oftalmo-Biología Aplicada (IOBA), and Fernandez Vega Ophthalmic Institute. Corneas and conjunctivas were obtained from pathology departments at the three institutions. Ciliary body, lens, and retina were provided by the Eye Bank of Oviedo after removal of the corneo-scleral ring for transplantation. The clinical histories of the eyes included melanoma and trauma; however the portions used in these studies were unaffected by disease or trauma. Each of the pathology departments prepared 7-μm paraffin sections of fixed tissues for immunohistochemistry.

RNA isolation and qRT-PCR

Rabbit ocular structures: Cornea, ciliary body, retina, whole lens, and lacrimal gland were immersed in RNAlater (Qiagen, Duesseldorf, Germany) and frozen. RNA was extracted from the homogenized ocular structures using TriZol (Life Technologies, Carlsbad, CA, USA) and cleaned by passing the samples through an RNAeasy column (Qiagen). The quantity and quality of the RNA were analysed using an Agilent Bioanalyser 2100 (Agilent, Santa Clara, CA, USA). Total RNA (4 μg) was retrotranscribed (High-Capacity cDNA Archive kit, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Real time PCR was performed using the StepOnePlus detection system (Applied Biosystems). The following gene-specific probes and primers available as Taqman assays were used: Oc03397394_m1 (TRPV1) and 824823 B3 (hypoxanthine phosphoribosyltransferase 1, HPRT1, Applied Biosystems). Each sample (500 ng) was amplified in a TaqMan 2X Universal Master Mix under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All real time qRT-PCR amplifications were performed in triplicate. Quantification data of TRPV1 gene expression were normalized to the expression of HPRT1 used as an endogenous control.

Immunohistochemistry

For immunohistochemistry, the following antibodies were used: rabbit anti-human TRPV1 polyclonal antibody (1:500, catalogue number NB100-98899 Novus Biologicals, Cambrige, UK), sheep anti-rabbit TRPV1 polyclonal antibody (1:1000, catalogue number NB 100-98890, Novus Biologicals). Mouse anti-rabbit vimentin monoclonal antibody (1:20, catalogue number NB 100-65191 Novus Biologicals). To reduce non-specific binding of antibodies, rabbit and human eye sections

were incubated in normal donkey serum (catalogue number D-9663 Sigma Aldrich, St. Louis, MO, USA) or normal goat serum (catalogue number G-9023 Sigma Aldrich) respectively for 1 h at room temperature. The sections were then incubated in anti-human TRPV1 or anti-rabbit TRPV1 for 24 h at 4°C. Following several washes with phosphate buffered saline, samples were incubated in goat anti-rabbit Alexa Fluor 488 (1:100, Molecular Probes, Invitrogen, Barcelona, Spain). Sections were counterstained with To-PRO-3 (Molecular Probes) or 4',6-diamidino-2-phenylindole (DAPI, catalogue number D9541, Sigma Aldrich). Negative controls consisted of the omission of primary antibodies. Other controls included the application of blocking peptides (NB100-988990P and NB100-98890P, Novus Biologicals) to rabbit anti-human TRPV1 polyclonal antibody and to sheep anti-rabbit TRPV1 polyclonal antibody, respectively, to determine the specificity of the primary antibodies.

Imaging of the stained sections was performed with a Leica confocal microscope (Leica TCS SP, Wetzlar, Germany.) and Leica Application Suite Advanced software. All samples were examined with 40x and 63x lenses.

Results

Rabbit

TRPV1 gene expression

TRPV1 mRNA levels were normalized against the levels present in rabbit cornea. The lens had the highest level of TRPV1 mRNA, with almost 56 times that present in the cornea. Expression in the ciliary body was almost 13 times that in the cornea. In contrast, expression in the lacrimal gland was half that of the cornea, and the retina was approximately 7 times less (Fig. 1).

Immunohistochemical detection of TRPV1 protein

Cornea and conjunctiva: TRPV1 was detected in the epithelium (Fig. 2A) and endothelium of the cornea (Fig. 2B). All of the layers of the corneal epithelium were labeled. TRPV1 labeling was reduced in the outer epithelial layers towards the limbus. In the conjunctival epithelium, only the basal layer was TRPV1 positive (Fig. 2C). Significant TRPV1 staining was absent in epithelial, endothelial and limbal-conjunctival immunohistochemical controls (Fig. 2D).

Uvea: In the vascular layer of the rabbit eye, the unpigmented and pigmented epithelia of the ciliary processes (Fig. 3A) and pars plana (Fig. 3B) were TRPV1 positive. Similarly, the bulk of the vascular endothelia in the choroid layer showed strong TRPV1 labeling (Fig. 3C). Significant TRPV1 staining was absent in ciliary processes, pars plana, and vascular immunohistochemical controls (Fig. 3D-F).

Lens: The epithelium of the lens was densely and uniformly immunostained for TRPV1 in the central, intermediate, and equatorial zones (Fig. 4). The lens fibers were unstained.

Retina: TRPV1 was present in several layers of the rabbit retina (Fig. 5). The distribution was uniform throughout the retina, with no changes between the peripheral and central regions. The retinal pigment epithelium (RPE) had the most intense staining (Fig. 5 A-C).

Very thin lines of positive staining arranged as columns were present in the outer nuclear layer (ONL), as well as punctate staining at the edges (Fig. 5A,D). In the inner nuclear layer (INL), TRVP1 was localized surrounding the neurons. The most prominent immunostaining was present at the end of the nerve fiber layer, at the conical expansions (white arrowheads). Double staining with anti-vimentin antibody, a marker for Müller cells, showed that TRPV1 was co-localized with the intermediate filaments throughout the retina (Fig. 5F arrowheads). Both the morphology and the pattern of expression of vimentin indicated that these TRPV1-positive cells were Müller cells (Fig. 5B,F).

Lacrimal gland: The cytoplasm of the epithelial cells in secretory clusters of the lacrimal gland was TRPV1 positive (Fig. 6.)

Human

Immunohistochemical detection of TRPV1 protein

Cornea: TRPV1 was present throughout the human corneal epithelium (Fig. 7A). In contrast, in the conjunctival epithelium TRPV1 was present only in the

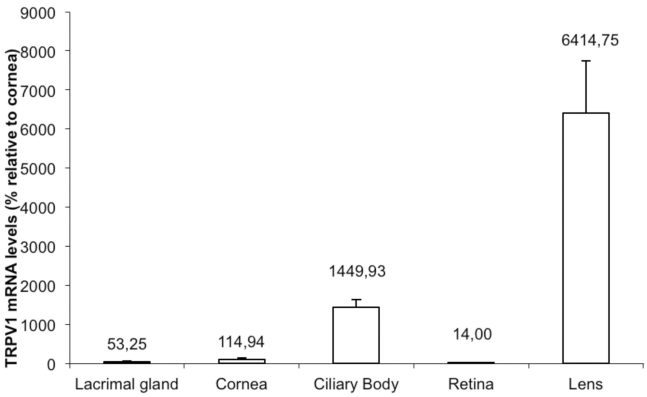

Fig. 1. Quantitative determination of TRPV1 mRNA expression levels in rabbit eye using qRT-PCR. Expression levels were normalized to that in the cornea.

basal layer and wing cells (Fig. 7B). The endothelium was also TRPV1 positive (Fig. 7C).

Uvea: In the human uvea, TRPV1 immunoreactivity was localized to both the pigmented and the unpigmented epithelia of the ciliary processes (Fig. 8A) and the pars plana (Fig. 8B). The endothelial cells of the choroidal vessels in the uvea were also strongly stained (Fig. 8C).

Lens: The epithelium of the human lens was strongly stained from the anterior pole to the equator (Fig. 8D). The fibres and posterior pole of the lens were not stained.

Retina: In the human retina, TRPV1 was restricted to the pigment epithelium (Fig. 8E). Staining of Müller cells was not evident.

Discussion

TRPV1 is a non-selective cation channel that is implicated in numerous physiological and pathological processes, such as neuronal transmission (Shen et al., 2009), nociceptive and thermosensitive sensory signalling (Di Marzo et al., 2002), inflammatory cytokine release (Zhang et al., 2007a), apoptosis (Leonelli et al., 2011), cell migration (Waning et al., 2007), and sensing the osmotic environment (Bourque et al., 2007). The role of TRPV1 in these physiological functions has been thoroughly studied in the nervous system where both expression and function appear to be species specific (Zhang et al., 2007a).

TRPV1 expression and localization within the eye has previously been described in mice and rats (Nakagawa et al., 2009). The aim of the present report was to assess the expression and cellular localization of this receptor/channel in rabbits, a very commonly used research model in ophthalmology. Additionally, we have assessed the immunoreactive localization of TRPV1 in human eyes and compared the two species.

Previous studies have shown the localization of TRPV1 protein in the epithelium of the mouse and

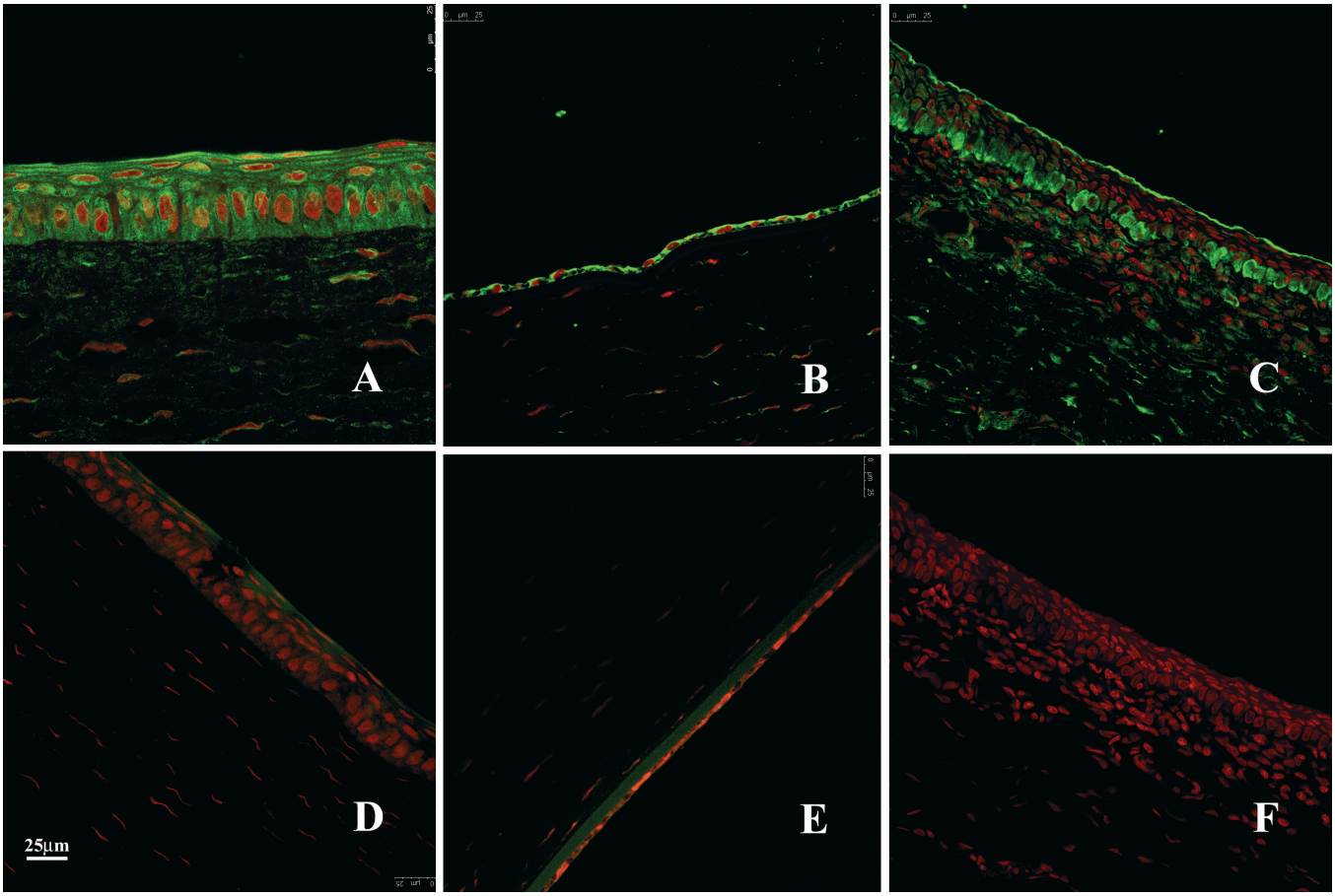

Fig. 2. TRPV1 in rabbit corneas. **A.** TRPV1 (green) was present in all layers of the epithelium. **B.** TRPV1 immunoreactivity was detected in the endothelium. **C.** In the limbal and conjunctival epithelia, TRPV1 localization was restricted to the basal cell layers. **D-F.** Negative controls for the corneal epithelium, endothelium, and limbal and conjunctival epithelium respectively. Red, nuclei counterstained with To-PRO-3.

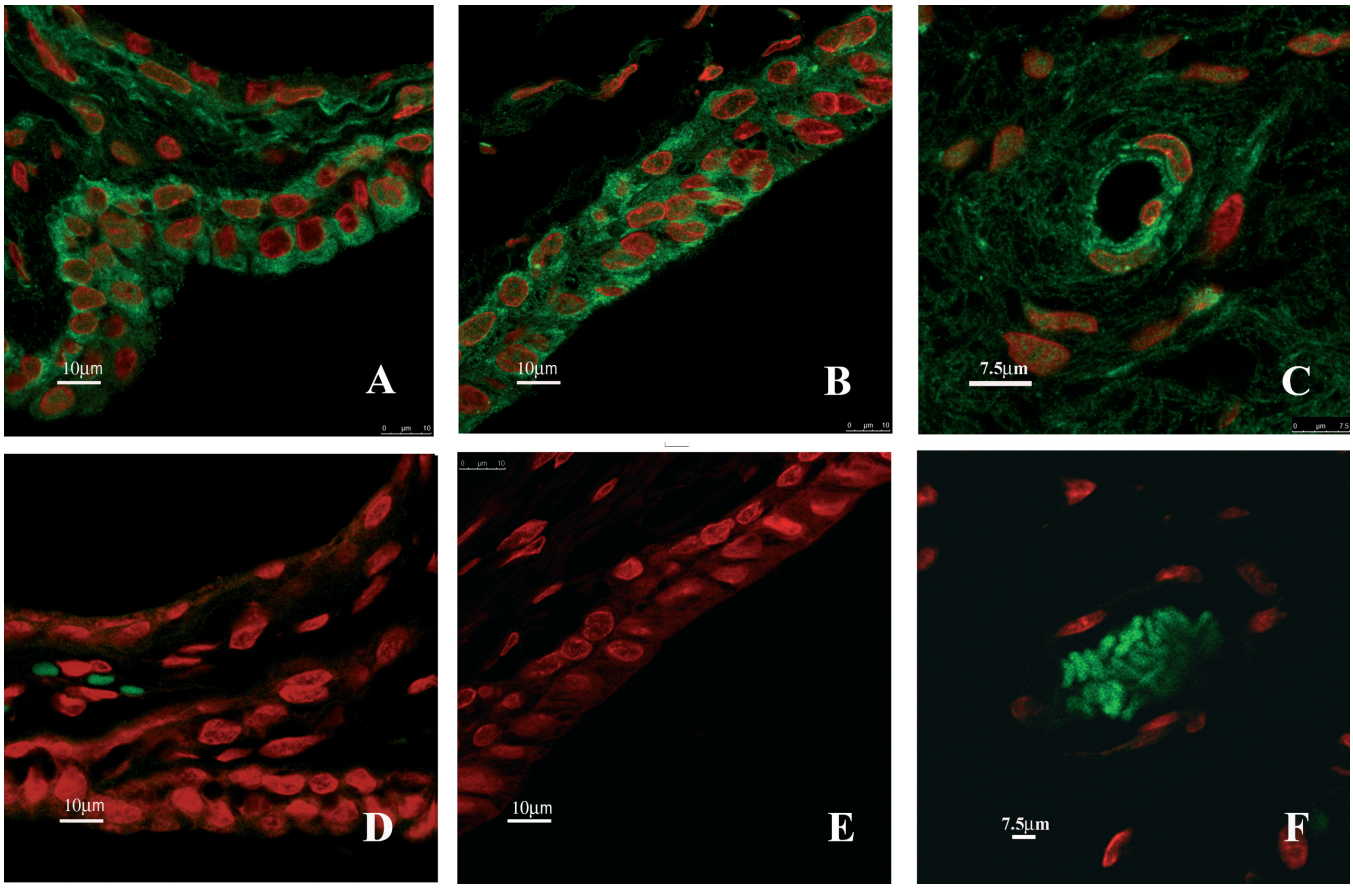

Fig. 3. Uvea of the rabbit eye. **A-B.** The pigmented and unpigmented epithelia of the ciliary body processes (**A**, pars plicata) and pars plana **(B)** had significant TRPV1 immunostaining. **C.** TRPV1 immunoreactivity was also present in endothelium of the uveal blood vessels. **D-E.** Negative controls for the pars plicata, pars plana, and vascular layer respectively. Red, nuclei counterstained with To-PRO-3.

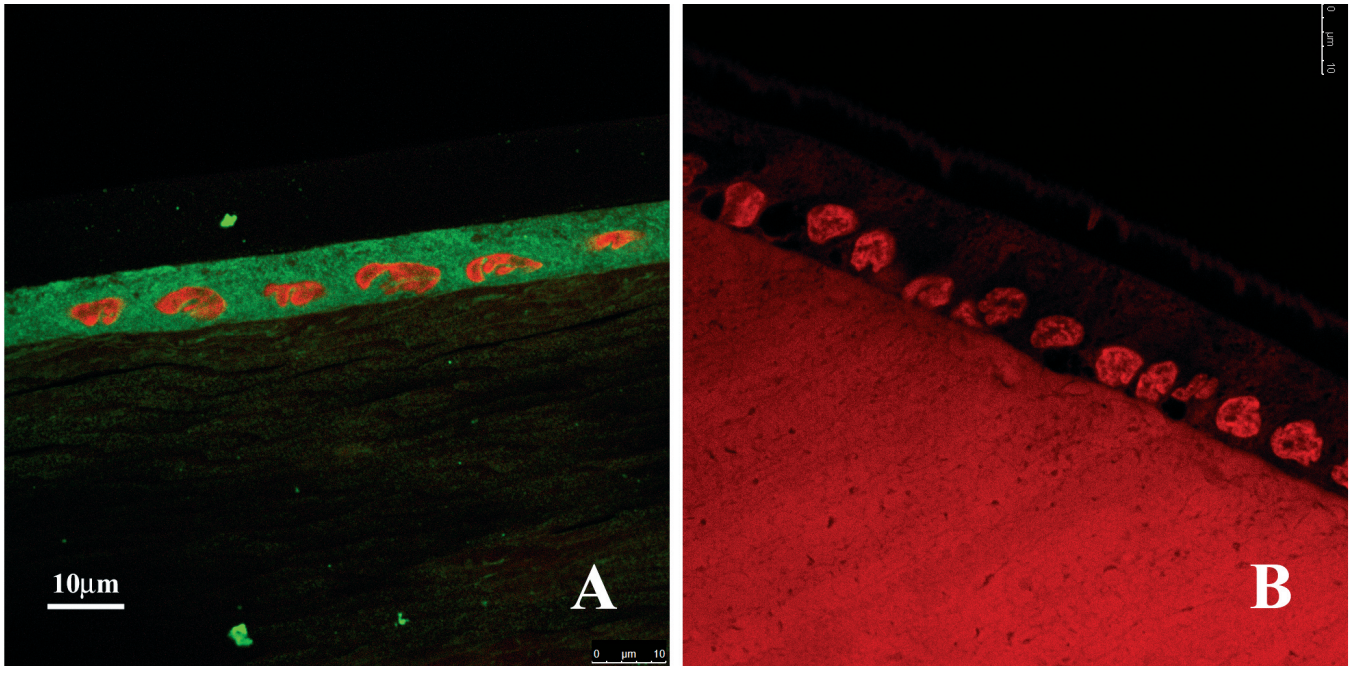

Fig. 4. Transverse sections of rabbit lens. **A.** TRPV1 was localized in the anterior epithelium. **B.** Negative control. Red, nuclei counterstained with To-PRO-3.

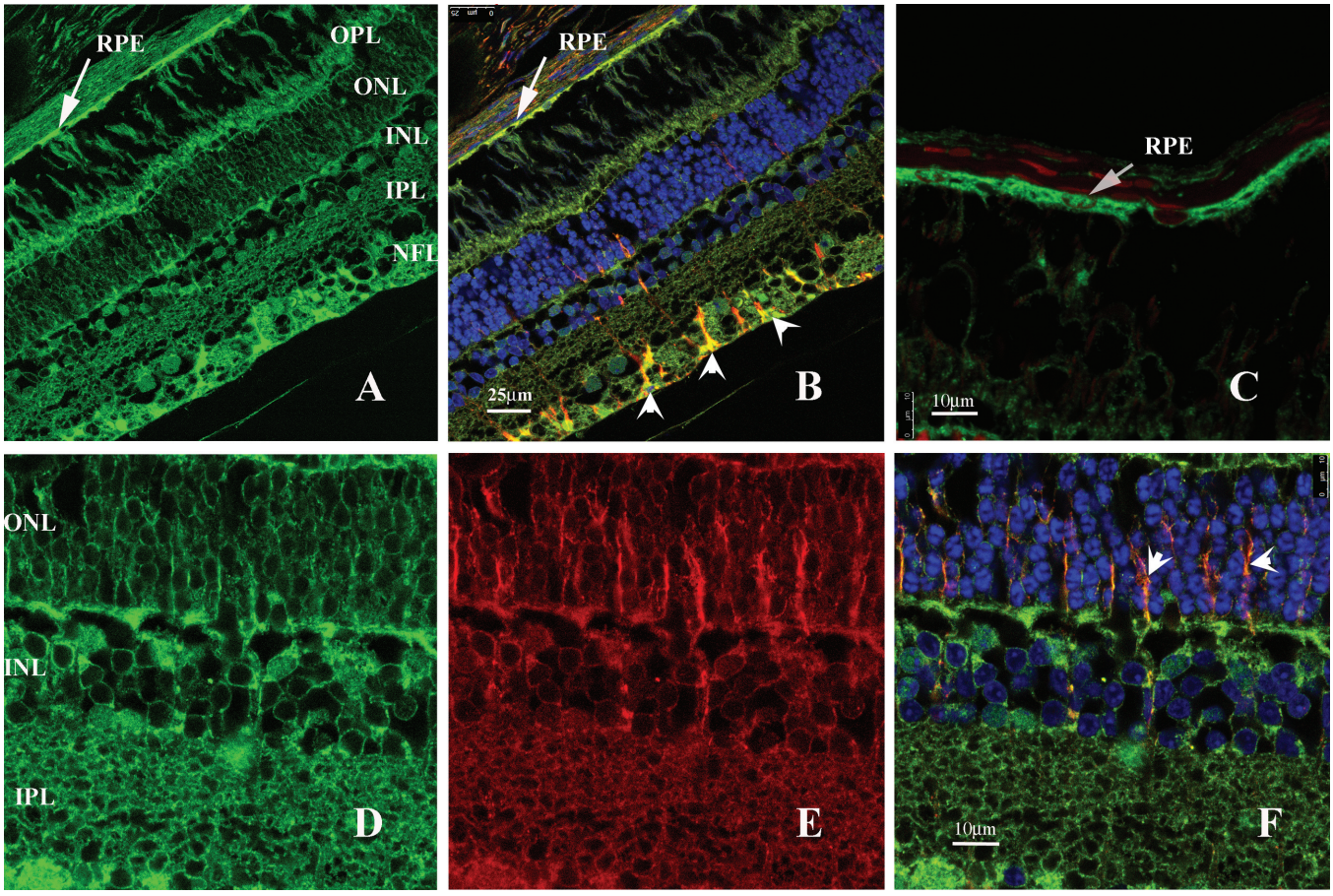

Fig. 5. Rabbit retina. **A.** TRPV1 protein was distributed throughout the rabbit retina. **B.** Double labeling with anti-TRPV1 antibody (green) and anti-vimentin (red). Arrowheads indicate conical formations. Nuclei were counterstained with DAPI (blue). Arrows indicate the RPE. **C.** Retinal pigment epithelium (RPE) at high magnification. Nuclei were counterstained with To-PRO-3 (red). **D.** In the outer nuclear layer (ONL) and inner nuclear layer (INL), TRPV1 was localized around the neurons. In the inner plexiform layer (IPL), it was present in a network of fibers. **E.** Anti-vimentin immunoreactivity. **F.** TRPV1 and vimentin co-localization (arrowheads).

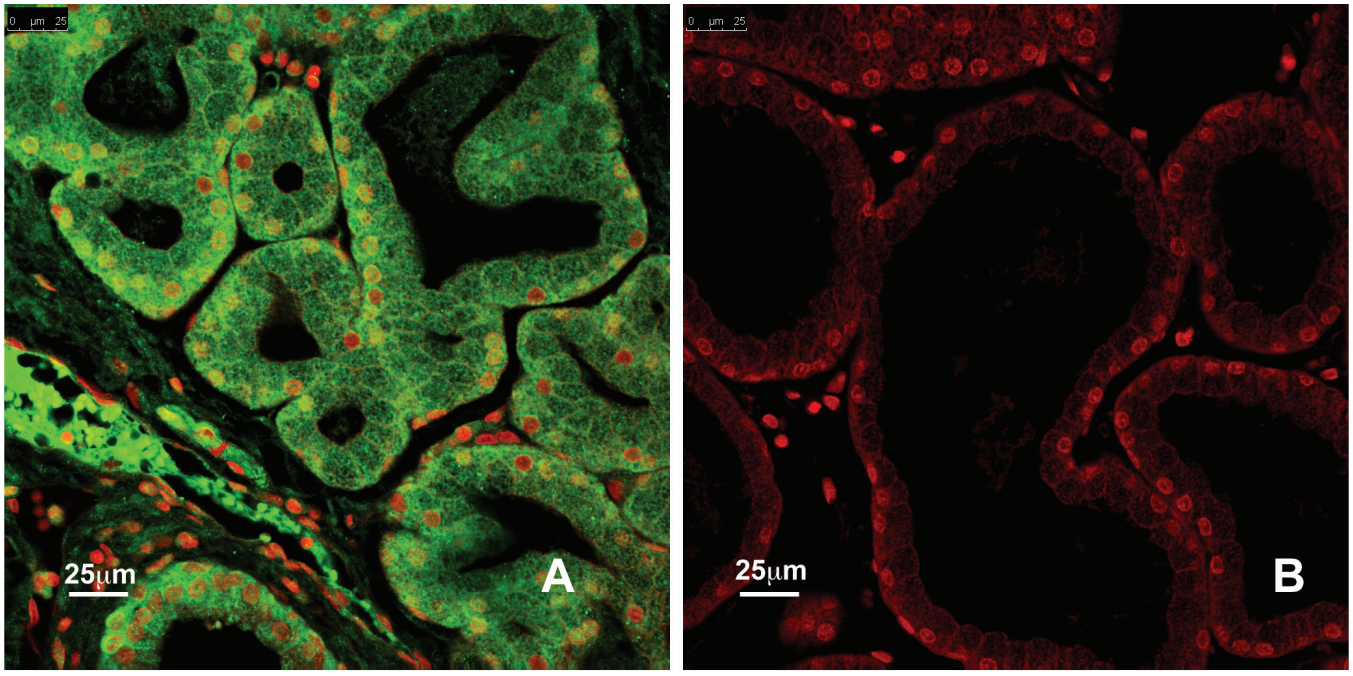

Fig. 6. Rabbit lacrimal gland. **A.** Distribution of TRPV1 receptor in the secretory cells of acini **B.** Negative control.

human corneas. In mice, TRPV1 is restricted to the basal layer and the wing cells of the corneal epithelium (Okada et al., 2011), whereas in humans it is present in the most apical cells of the epithelium (Zhang et al., 2007a). Our results showed relatively abundant TRPV1 mRNA expression in cornea and an abundant presence

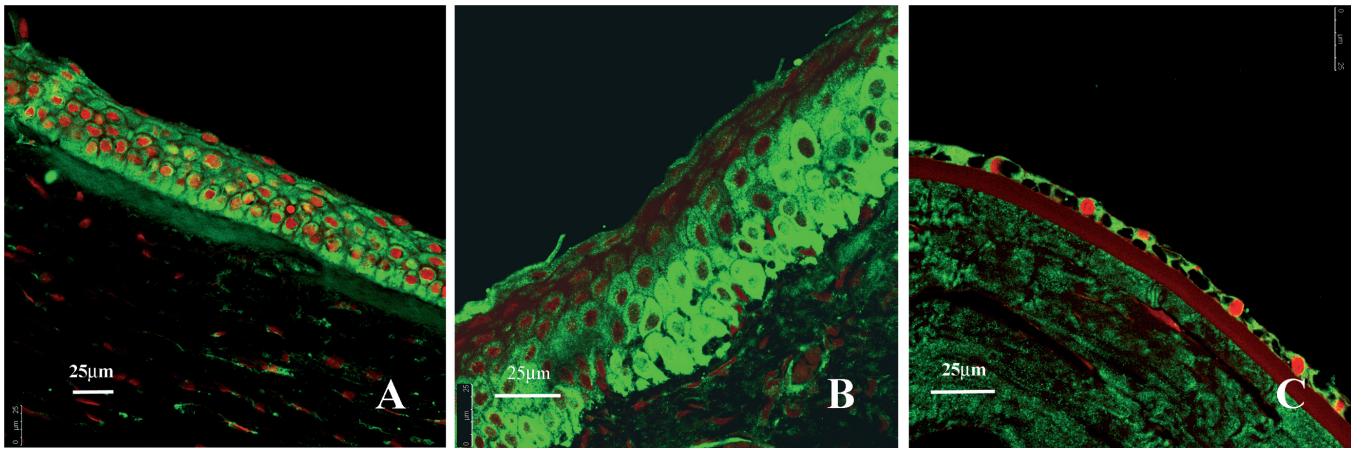

Fig. 7. Human cornea. **A.** TRPV1 was present in all layers of the epithelium. **B.** In the limbus, immunoreactivity was restricted to the basal and wing cells. **C.** Cells of the endothelium were also positively stained for TRPV1.

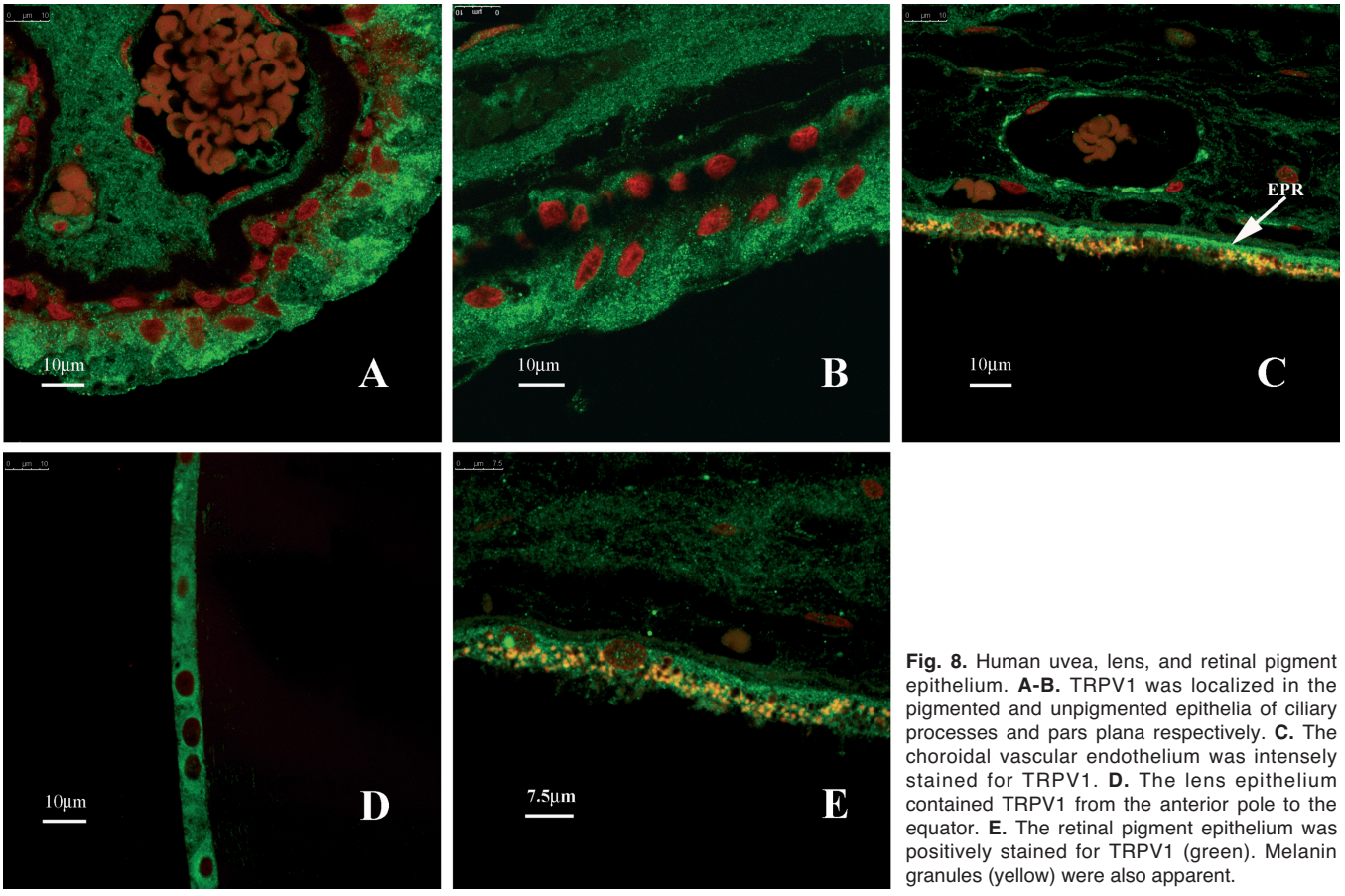

Fig. 8. Human uvea, lens, and retinal pigment epithelium. **A-B.** TRPV1 was localized in the pigmented and unpigmented epithelia of ciliary processes and pars plana respectively. **C.** The choroidal vascular endothelium was intensely stained for TRPV1. **D.** The lens epithelium contained TRPV1 from the anterior pole to the equator. **E.** The retinal pigment epithelium was positively stained for TRPV1 (green). Melanin granules (yellow) were also apparent.

of the protein in every layer of the epithelium in the central zone of rabbit and human corneas. In the limbal region of the cornea and in the conjunctiva it was restricted to the basal layer and wing cells. This localization was very different from that found by Mergler et al. (2012) in human conjunctiva, where it was present only in the superficial layers. These authors proposed that TRPV1 in this location acts as a regulator of tear volume, since they express hypertonic sensitivity.

We also found TRPV1 receptors/channels in both rabbit and human corneal endothelium. These results are in agreement with Mergler et al., (2010) who proposed that endothelial TRPV1 is responsive to temperature changes and this contributes to the regulation of Ca^{2+} homeostasis of the endothelium under different ambient conditions.

Perhaps the most remarkable finding in our study was the expression of TRPV1 mRNA and the localization of the protein in the rabbit and human ciliary body, especially in the epithelia of the pars plicata and pars plana. Although TRPV1 has not been described in the ciliary body, its presence there might be expected based on the importance of Ca^{2+} in the active ion channels of the ciliary epithelial cells (Fleischhauer et al., 2001).

TRPV1 protein was also present in the endothelium of the choroidal blood vessels of both rabbit and human eyes. Similar findings were described in the retinal vessels (Leonelli et al., 2009) and in the arterial mesenteric bed (Poblete et al., 2005) of the rat. Both sets of vessels can undergo vasodilatation due to calcium influx.

While several membrane calcium channels have been identified in the lens epithelium (Rhodes and Sanderson, 2009), this is the first study to report the presence of TRPV1 in the lens anterior epithelium of both rabbits and humans. Of all the rabbit ocular tissues examined, TRPV1 mRNA expression was highest in the lens. The protein was also strongly expressed in both the rabbit and human lens epithelia. Cataract formation is enhanced by high Ca^{2+} levels (Borchman et al., 1989). Thus, the presence of TRPV1 and other calcium channels in the lens epithelium suggests that they may be involved in regulating the passage of this ion from aqueous humor to the lens fibres.

Immunoreactivity for TRPV1 in rabbit and human RPE was very intense. This receptor is present in the RPE of developing rats (Leonelli et al., 2009) as well as in primary cultures of human RPE and in ARPE-19, a cell line derived from human RPE (Cordeiro et al., 2010). The role of TRPV1 in these tissues is unknown.

The neural retina of the rabbit expressed only low levels of TRPV1 mRNA. The protein was not detected in either the rabbit or human retinas. A previous study showed that TRPV1 protein was present in a large population of neurons in the INL and ganglion cells of the developing rat retina (Leonelli et al., 2009), whereas in the adult rat it was restricted to the ganglion cells, the synaptic vesicles in the plexiform layers, and the endothelial cells of blood vessels (Leonelli et al., 2011). Moreover, in zebrafish and goldfish retinas, immunoreactivity was present in the photoreceptor synaptic ribbons and in amacrine cells (Zimov and Yazulla, 2004, 2007).

TRPV1 is present in astrocytes of different brain areas in adult rats (Toth et al., 2005) and in retinas (Leonelli et al., 2009). While there are no reports that Müller cells in normal rat retinas contain TRPV1, it was present after acute axotomy (Leonelli et al., 2010). We found TRPV1 immunoreactivity in the Müller cells of rabbit eyes. Newman (2005) reported spontaneous and light evoked Ca^{2+} changes in Müller cells, and Agulhon (2007) found Ca^{2+} activity selectively in Müller cells during dark periods. Thus, the TRVP1 that we observed could be partially responsible for the changes in intracellular Ca^{2+} levels.

To our knowledge, this is the first report that describes the presence of TRPV1 mRNA and protein in the lacrimal gland. The levels of rabbit lacrimal gland mRNA were approximately 50% of the expression in cornea. The protein was localized in the cytoplasm of the epithelial cells of the acini where its function has not been described. The acinar cells secrete water, electrolytes and proteins. This secretion is regulated by acetylcoline and norepinephrine. Both neurotransmitters act through several signalling pathways that use Ca^{2+}-dependent enzymes. However, despite the importance of Ca^{2+}, there are no studies about Ca^{2+} channels in the lacrimal gland (Dartt et al., 1988; Hodges et al., 2006)

In summary, we showed that TRPV1 is present in multiple structures of rabbit and human eyes. All of the TRPV1-containing cells are involved in the maintenance of water homeostasis of different media: the pigmented and unpigmented epithelia of the ciliary body regulate aqueous humour, the pigmented and unpigmented epithelia of the pars plana regulate the vitreous humour hydration, the anterior epithelium of the lens regulates lens hydration, the RPE and Müller cells regulate the interphotoreceptor matrix, and the lacrimal gland regulates tear production. All of these are maintained by controlling water flow by means of active transport of ions. In each of these tissues, TRPV1 may also play a role in regulation of Ca^{2+} as an intracellular second messenger. Further physiological studies are necessary to elucidate each of these potential functions.

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