Involvement of TRPV1 and TRPV4 Channels in Retinal Angiogenesis


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PURPOSE. We investigate the contribution of TRPV1 and TRPV4 channels to retinal angiogenesis.

METHODS. Primary retinal microvascular endothelial cells (RMECs) were used for RT-PCR, Western blotting, immunolabeling, Ca²⁺ signaling, and whole-cell patch-clamp studies while localization of TRPV1 also was assessed in retinal endothelial cells using whole mount preparations. The effects of pharmacologic blockers of TRPV1 and TRPV4 on retinal angiogenic activity was evaluated in vitro using sprout formation, cell migration, proliferation, and tubulogenesis assays, and in vivo using the mouse model of oxygen-induced retinopathy (OIR). Heteromultimerization of TRPV1 and TRPV4 channels in RMECs was assessed using proximity ligation assays (PLA) and electrophysiologic recording.

RESULTS. TRPV1 mRNA and protein expression were identified in RMECs. TRPV1 labelling was found to be mainly localized to the cytoplasm with some areas of staining colocalizing with the plasma membrane. Staining patterns for TRPV1 were broadly similar in endothelial cells of intact vessels within retinal flat mounts. Functional expression of TRPV1 and TRPV4 in RMECs was confirmed by patch-clamp recording. Pharmacologic inhibition of TRPV1 or TRPV4 channels suppressed in vitro retinal angiogenesis through a mechanism involving the modulation of tubulogenesis. Blockade of these channels had no effect on VEGF-stimulated angiogenesis or Ca²⁺ signals in vitro. PLA and patch-clamp studies revealed that TRPV1 and TRPV4 form functional heteromeric channel complexes in RMECs. Inhibition of either channel reduced retinal neovascularization and promoted physiologic revascularization of the ischemic retina in the OIR mouse model.

CONCLUSIONS. TRPV1 and TRPV4 channels represent promising targets for therapeutic intervention in vasoproliferative diseases of the retina.

Keywords: TRPV1, TRPV4, angiogenesis

Angiogenesis has a fundamental role in many physiologic and pathologic processes. In the eye, abnormal angiogenesis contributes to visual impairment in several retinal diseases, including proliferative diabetic retinopathy, neovascular (NV)-AMD, and retinopathy of prematurity. Current therapies for retinal NV diseases include laser photocoagulation, vitrectomy surgery, and intravitreal injection of anti-VEGF agents. Laser and surgical treatments, however, can be associated with severe side effects, which in themselves can lead to vision loss. Anti-VEGF agents have been particularly successful for treating NV-AMD but these require regular retreatment and many patients become refractory over time. Evidence also suggests that prolonged use of anti-VEGF therapy may trigger retinal atrophy and the development of fibrotic changes in the retina. Evidently, new treatment options are needed to improve outcomes in patients with retinal angiographic disorders. From this perspective, a better understanding of the cellular mechanisms that regulate pathologic angiogenesis in the retina may reveal new therapeutic targets for disease prevention and treatment.

Angiogenesis is characterized by endothelial cell (EC) activation, vascular destabilization, EC migration and proliferation, and finally the formation of patent vascular tubes. EC Ca²⁺ signaling is a key intracellular signaling mechanism involved in the regulation of angiogenesis. Blocking Ca²⁺ signaling, using intracellular Ca²⁺ chelators or nonspecific Ca²⁺ channel antagonists, for example, results in inhibition of EC proliferation, motility, and tubulogenesis in vitro, and suppresses angiogenesis in vivo. Presently, little is known about the molecular identity of the Ca²⁺ influx pathways in retinal microvascular ECs that stimulate the angiogenic process. One study, however, has reported the involvement of the transient receptor potential (TRP) channel, TRPC4, in regulating retinal neovascularization in the murine model of oxygen-induced retinopathy (OIR).

Two Ca²⁺-permeable channels that are known to be highly expressed at the transcript and protein level in retinal microvascular ECs are TRPV1 and TRPV4. We and others have recorded TRPV4-mediated plasma membrane currents in these cells, although functional TRPV1 channel activity has yet to be explored. To date, relatively few studies have examined the contribution of EC TRPV1 and TRPV4 channels to the angiogenic process. Nonetheless, using in vitro tubulogenesis and in vivo Matrigel plug assays, exogenous and
endogenous activators of TRPV1 have been reported to elicit an angiogenic response.\textsuperscript{18–20} TRPV4 has been studied mainly from the perspective of tumor angiogenesis, with some conflicting results reported depending on the type of tumor EC examined. In breast tumor-derived ECs, activation of TRPV4 has been shown to exert a proangiogenic effect,\textsuperscript{21} while in ECs from prostate cancer, TRPV4 appears to act as a negative regulator of angiogenesis.\textsuperscript{22,23} A recent study has suggested that TRPV4 may be involved in modulating retinal angiogenic signaling in vitro,\textsuperscript{24} but a detailed analysis of this channel in the development of pathologic angiogenesis in the retina has yet to be performed.

We investigated the relevance of TRPV1 and TRPV4 channels to retinal angiogenesis in vitro and in vivo. Our results strongly suggested that inhibition of either channel could offer new therapeutic opportunities for treatment of retinal NV disorders. We also provided the first evidence to our knowledge to suggest that TRPV1 and TRPV4 are capable of forming functional heteromeric channels in the vascular endothelium.

**Materials and Methods**

**Ethical Approval**

Animal procedures were approved by Queen’s University of Belfast Animal Welfare and Ethical Review Body (AWERB) and authorized under the UK Animals (Scientific Procedures) Act 1986. Animal use conformed to the standards of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with European Directive 210/63/EU. C57BL/6J mice were purchased from Harlan Laboratories (Harlan, Bicester, UK) and bred in-house (Biological Services Unit, Queen’s University of Belfast). The mice were housed in standard micro-isolator cages with a 12-hour light/dark cycle and provided food and water ad libitum.

**Cell Culture**

Primary retinal microvascular endothelial cells (RMECs) were isolated and cultured from bovine retinas as described previously.\textsuperscript{12} The cells were used between the second and fifth passages. All experiments were repeated a minimum of three times using different RMEC isolations, with three technical replicates per batch of cells. The endothelial characteristics of RMECs were confirmed by immunolabeling with CD31 (PECAM-1), von Willebrand factor (vWF), β-catenin, ZO-1 (Tight Junction Protein-1), and vimentin (Supplementary Fig. S1). The cells were negative for markers of smooth muscle cells (α-SMA) and macrophages (CD14) (Supplementary Fig. S1).

**Immunolabeling Studies**

Immunolabeling was performed on bovine retinal whole-mounts and RMECs. For whole-mount preparations, fresh bovine eyes were obtained from a local abattoir and transported to the laboratory on ice. Retinas were dissected out, fixed in 4% paraformaldehyde (PFA) for 20 minutes, and then washed in PBS. They subsequently were placed overnight in permeabilization and blocking buffer (0.5% Triton X-100 and 1% donkey serum in PBS) and incubated for 24 hours with rabbit polyclonal antibody against TRPV1 (1:50; SC-28759; Santa Cruz Biotechnology, Dallas, TX, USA). Retinas were costained with a monoclonal mouse antibody targeting eNOS (1:200; 610297; BD Biosciences, Oxford, UK) to positively identify vascular endothelial cells. After washing, tissues were incubated overnight at 4°C with donkey anti-rabbit IgG labeled with Alexa-488 and donkey anti-mouse IgG labeled with Alexa-568 (both 1:200; Life Technologies, Paisley, UK). For RMECs, cells were grown on #0 glass coverslips and fixed in 4% PFA for 5 minutes at room temperature. Fixed cells were washed in PBS and the plasma membranes stained using isoclin B4 (1:50; 1 hour at room temperature; L2140; Sigma-Aldrich Corp., Dorset, UK) and Streptavidin 568 (1:500; overnight at 4°C; Life Technologies). Cells then were blocked and permeabilized for 1 hour (PBS, 1% donkey serum. 0.05% Triton X-100) followed by incubation with primary anti-TRPV1 antibody (1:200; overnight at 4°C; ab63083; Abcam, Cambridge, UK). Donkey anti-rabbit Alexa-488 (1:200; overnight at 4°C; Life Technologies) was used for TRPV1 channel detection. Retinal whole-mounts and RMECs were labeled with TOPRO3 nuclear stain (1:1000; Life Technologies; pseudo-colored blue in relevant images) before mounting in Vectashield (Vector Laboratories, Peterborough, UK). Images were captured using a Leica SP5 confocal laser scanning microscope (HCX PL APO x63 oil immersion lens; Leica Microsystems, Milton Keynes, UK). Images were acquired in sequential scanning mode to minimize bleed through. All secondary-only controls were negative for staining.

**RT-PCR Amplification**

RNA was extracted from RMECs using an RNeasy Micro kit (Qiagen, Crawley, UK). RT-PCR was performed according to previously established protocols within our laboratory\textsuperscript{16} using primers designed to amplify bovine TRPV1 (forward primer 5' GTAGACGCGACACCCCTAACT 3', reverse primer 5' GAAAGATGCGCTTGACA 3'; expected product size 104 base pairs [BP]; Eurogentec, Southampton, UK). A ‘no RT’ control was generated with the omission of reverse transcriptase.

**Western Blotting**

Western blot extracts were prepared by lysing RMECs in ice-cold RIPA buffer in the presence of protease inhibitors (Thermo Scientific Laboratories, Rockford, IL, USA). After vortexing and centrifugation to separate cell debris, protein yield was measured using the BCA protein assay kit (Thermo Scientific Laboratories). Then, 50 μg protein was loaded onto 8% polyacrylamide gels and following separation transferred to Immobilon-P PVDF membranes (Millipore Limited, Watford, UK). Binding of primary antibody against TRPV1 (1:200; ab63083; Abcam) was detected with a LI-COR Odyssey imaging system using IRDye800-conjugated secondary antibody (1:10000, LI-COR Biosciences, Cambridge, UK).

**Patch-Clamp Electrophysiology**

RMECs were seeded onto #0 coverslips and transferred to a glass-bottomed recording chamber perfused with modified Hanks’ solution (see Solutions and Drugs) at 37°C. Ionic currents were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices, San Jose, CA, USA). Data acquisition and analysis was performed using pClamp9 software (Molecular Devices). Pipettes with tip resistances of 0.5 to 5 MΩ were pulled from filamented borosilicate glass capillaries (Harvard Instruments, Kent, UK). Liquid junction potentials (11 mV), cell capacitance (+1.52 ± 10.0 pF) and series resistance (5.1 ± 0.4 MΩ) were routinely compensated. Whole-cell currents were elicited using voltage ramp protocols from −80 to +80 mV applied over 2.5 seconds from a holding potential of 0 mV. Voltages ramps were repeated every 10 seconds and current amplitudes at −80 and +80 mV measured offline to obtain time course plots. Current densities were calculated by dividing current amplitudes by the whole-cell...
capacitance. Drugs were applied using a gravity-driven perfusion system with an exchange time of approximately 1 second.25

**Sprouting Angiogenesis Assay**

The effects of TRPV1 and TRPV4 antagonists on in vitro angiogenesis were investigated using a sprouting angiogenesis assay. Briefly, 1 × 10^5 RMECs were resuspended in 25 μL Dulbecco’s modified Eagle medium (DMEM) containing 20% porcine serum (PS) and mixed in a 1:1 ratio with growth factor-reduced Matrigel (Corning, Boston, MA, USA). The cell-Matrigel mixture (50 μL) was spotted into the center of each well of a 12-well plate and incubated at 37°C for 45 minutes to allow the Matrigel to polymerize. The Matrigel spots were covered with DMEM containing 10% PS and incubated at 37°C for a further 48 hours. The culture medium then was aspirated and a second layer of Matrigel diluted 1:1 with DMEM (20% PS) was layered evenly over the primary spots. After polymerization, the duplex cultures were incubated for another 48 hours in DMEM containing 10% PS at 37°C. In each treatment, test substances were added to the secondary Matrigel layer and the surrounding culture media. At 48 hours after creating the duplex cultures, endothelial sprouts could be observed easily invading the secondary Matrigel layer. The number of sprouts around the circumference of each spot was quantified and data expressed as a percentage of nontreated control experiments.

**Cell Viability**

RMEC viability was assessed using the Promokine Live/Dead Cell Staining Kit II (Promokine, Heidelberg, Germany). Cells were grown to 80% confluency on 1% gelatin-coated 24-well plates and treated with test substances for 24 hours. Media then was replaced with serum-free DMEM supplemented with 2 μM Calcein-AM and 4 μM EthD-III and cells incubated for 45 minutes at 37°C. After washing, RMECs were imaged by confocal microscopy (Nikon TE-2000 C1 confocal system; Nikon Ltd, Kingston upon Thames, UK) and the numbers of live and dead cells quantified using ImageJ.26

**Intracellular Ca2+ Measurements**

Confocal Ca2+ imaging and FLIPR Calcium 6QF Assays (Molecular Devices) were used to study the involvement of TRPV1 and TRPV4 channels in VEGF-induced Ca2+ responses in RMECs. For confocal Ca2+ imaging, RMECs were seeded onto coverslips for 24 to 48 hours and incubated for 1 hour at 37°C with normal Hanks’ solution at 37°C. Cells then were labeled with Calcein Green AM (0.3 μM) and incubated for a further 20 minutes. Cells then were blocked for 30 minutes in Duolink blocking solution, and incubated for 1 hour at room temperature with mouse anti-TRPV1 (1:50; ab203103; Abcam) and rabbit anti-TRPV4 (1:50; sc-98592; Santa Cruz Biotechnol-

**Proliferation**

RMECs were seeded at a density of 1 × 10^4 cells/well on 1% gelatin-coated 96-well plates and cultured for 24 hours in DMEM containing 10% PS. The media then was refreshed with media containing test compounds and the cells incubated for a further 24 hours. Cell proliferation was determined using a bromodeoxyuridine (BrdU) incorporation assay (Roche, Mannheim, Germany) as described previously.12

**Cell Migration**

The scratch wound assay was used to analyze RMEC migration. Briefly, RMECs were grown to 80% confluency in DMEM containing 10% PS on 1% gelatin-coated 6-well plates. A uniform scratch wound was created across the center of each well using a sterile 200 μL pipette tip. The cells then were washed and media changed to one containing test substances. Scratch wounds were imaged at 0 and 16 hours after wounding using a Nikon Eclipse TS100 inverted microscope equipped with a Nikon Coolpix 5400 camera (Nikon Ltd). Percentage wound repair was determined using ImageJ.26 All scratch wound assays were undertaken in the presence of 5-fluorouracil (1 mM) to prevent cell proliferation.

**Tubulogenesis Assay**

An in vitro tubulogenesis assay was used to examine the ability of RMECs to cluster and interact with one another to form capillary-like tubes. RMECs (5 × 10^4 cells) were resuspended in culture media (DMEM + 20% PS) supplemented with test substances and 50% growth factor-reduced Matrigel (BD Biosciences). Then, 50 μL aliquots were spotted onto 24-well plates and let to polymerize for 45 minutes at 37°C. After polymerization, spots were submerged in DMEM containing 10% PS and corresponding test substances at 37°C for 48 hours. Cells then were labeled with Calcein Green AM (0.3 μg/mL for 45 minutes; Thermo Scientific Laboratories) and capillary tube-like structures imaged by laser confocal scanning microscopy (Nikon TE-2000 C1 confocal system). Tube length and area were analyzed in four randomly selected low power fields (magnification ×10) from each well using NIS Elements software (Nikon).

**Proximity Ligation Assay**

Protein-protein interactions between TRPV1 and TRPV4 were analyzed using the Duolink in situ proximity ligation assay (PLA; Olink Biosciences, Sigma-Aldrich Corp.) according to the manufacturer’s instructions. RMECs were grown on #0 coverslips, fixed with 4% paraformaldehyde for 20 minutes, and permeabilized in PBS containing 0.05% Triton X-100 for 1 hour. Cells then were blocked for 30 minutes in Duolink blocking solution, and incubated for 1 hour at room temperature with mouse anti-TRPV1 (1:50; ab203103; Abcam) and rabbit anti-TRPV4 (1:50; sc-98592; Santa Cruz Biotechnol-
ogy) primary antibodies in Duolink antibody diluent solution. Cells subsequently were labeled with Duolink PLA anti-rabbit PLUS and anti-mouse MINUS probes for 1 hour at 37°C. Following incubation in Duolink ligation-ligase solution at 37°C for 30 minutes, RMECs were incubated in a Duolink amplification-polymerase solution for 100 minutes at 37°C. TOPRO-3 (1:1000; Life Technologies) then was added to the coverslips for 15 minutes at room temperature. Images were acquired using a Leica SP5 confocal microscope equipped with a ×63/1.4NA oil objective lens and LAS- AF software (Leica Microsystems). Antibodies were chosen for the PLA assays on the basis that they had been validated previously in tissues from TRPV1 and TRPV4 knockout animals. Controls were performed by incubating with both primary antibodies separately.

Oxygen-Induced Retinopathy (OIR) Model

To investigate the role of TRPV1 and TRPV4 channels in mediating retinal neovascularization in vivo, we used the OIR model of ischemic retinopathy. Briefly, litters of C57BL/6 mouse pups with their nursing dams were exposed to 75% oxygen (Oxycycler A84X0; BioSpherix, Ltd, Parish, NY, USA) from postnatal day 7 (P7) to P12, before being returned to normal room air between P12 and P17. Since preretinal neovascularization in this model begins at approximately P15 and reaches a maximum at approximately P17, mice were intravitreally injected with TRPV1 or TRPV4 inhibitors at P15 and sacrificed at P17. Before intravitreal injections, mice were anesthetized by intraperitoneal (IP) administration of ketamine (60 mg/kg) and xylazine (6 mg/kg). The intravitreal injections were performed using a 33-gauge beveled needle that was attached to a Nanofil syringe (World Precision Instruments, Sarasota, FL, USA). Reported drug concentrations represent the estimated end vitreal concentrations, assuming a vitreal volume of approximately 5 l. In a P15 mouse eye. Mouse pups received a single injection into the left eye (12 animals per treatment group). The mice were euthanized using an overdose of sodium pentobarbital (200 mg/kg) administered by IP injection and their eyes collected for quantification of retinal NV, ischemic, and vascularized areas.

Enucleated eyes were fixed in 4% PFA for 2 hours. Retinas were dissected and stained en face with biotin-labeled isolectin-B4 (1:50; Sigma-Aldrich Corp.) and Alexa Fluor Streptavidin 488 (1:500; Life Technologies) to visualize the retinal vasculature. After whole-mounting, retinas were imaged using an upright fluorescent microscope (Nikon Eclipse E400). NV, avascular, and normal vascular areas were quantified as a percentage of the total retinal area using custom software developed in our laboratory (OIR Select for ImageJ). The normal vascular area comprises vessels from the superficial, intermediate, and deep capillary plexuses of the retina.

Solutions and Drugs

Normal Hanks’ solution contained 140 mM NaCl, 6 mM KCl, 1.3 mM MgCl₂, 2 mM CaCl₂, 5 mM D-glucose, 10 mM HEPES (pH 7.4 with NaOH). The composition of the modified Hanks’ solution used for electrophysiologic recording was (mM): NaCl, 140; CsCl, 6; D-glucose, 5; CaCl₂, 2; MgCl₂, 1.3; HEPES, 10; pH set to 7.4 with Tris. The pipette solution was (mM): Cs-aspartate, 100; CsCl, 20; BAPTA, 10; CaCl₂, 0.08; Na₂ATP, 4; MgCl₂, 1; HEPES, 10; adjusted to pH 7.2 with Tris. Reagents used in this study were purchased from the following sources: Resiniferatoxin (RTX), A784168, Capsazepine (CapZ), GSK1016790A (GSK101), HC067047 (HC06), and RN1734 (Tocris, Bristol, UK); recombinant human VEGF₁₆₅ (R&D Systems, Abingdon, UK); and Fluo-4AM (Thermo Fisher Scientific, Loughborough, UK). Drugs were dissolved in dimethyl sulfoxide (DMSO) as concentrated stock solutions and diluted at least 10,000-fold. This yielded a final DMSO concentration of 0.01% or less, a concentration that was found not to affect the results of any of the assays.

Statistics

Summary data are presented as mean ± SEM. Statistical comparisons were performed on raw data or following arcsin transformation (percentage values) using Prism V5.03 (GraphPad Software, San Diego, CA, USA). Data normality was tested using the D’Agostino-Pearson normality omnibus test. Parametric data were analyzed using Student’s t-tests or 1-way ANOVA with a Newman-Keuls post hoc test. Nonparametric data were compared by Mann-Whitney U tests or the Kruskall-Wallis test with Dunn’s post hoc comparison. Curve fitting and half maximal inhibitory concentration (IC₅₀) determinations were performed using GraphPad Prism V5.03. In all experiments, P < 0.05 was considered statistically significant.

RESULTS

Molecular and Functional Expression of TRPV1 and TRPV4 in Retinal ECs

To our knowledge, TRPV1 molecular and functional expression has not been investigated previously in detail in retinal ECs. Therefore, we began by examining the localization of TRPV1 channels in the bovine retinal vasculature and cultured RMECs. In bovine whole-mount preparations, TRPV1 colocalized with eNOS-positive ECs throughout the retinal vascular tree (Fig. 1A). Staining was broadly distributed across the cells and mainly punctate in nature. Strong immunolabeling for TRPV1 also was detected in RMECs (Fig. 1B). Most of the TRPV1 labeling was cytosolic, with some punctate staining colocalizing with the plasma membrane marker, isoelectin B4 (Fig. 1B). TRPV1 expression in RMECs also was substantiated by RTPCR (Fig. 1G) and Western blot analysis (Fig. 1Ci). In Western blot experiments, 95 and 113 kDa bands were detected corresponding to unglycosylated and glycosylated forms of TRPV1 (Fig. 1Ci). Lower molecular weight bands between 70 and 80 kDa also were noted, consistent with previous reports in other cell types. These lower molecular weight bands may represent TRPV1 splice variants, which have been widely reported in the literature. The functional expression of plasma membrane TRPV1 channels in RMECs was assessed by whole-cell patch clamp recording. Application of the TRPV1 activator, RTX (10 nM), triggered inward and outward currents at ~80 and +80 mV, respectively (Fig. 1Di,ii). These currents peaked and then plateaued within 1 minute and were reversible upon washout of the drug. Currents induced by RTX were prevented by treating the cells with the TRPV1 antagonists, CapZ (10 μM) or A784168 (100 nM; Fig. 1Dii-vi).

TRPV4 expression and functional activity has been characterized extensively in native and cultured retinal ECs. Therefore, we limited our experiments to consolidating previous findings showing that RMECs express functional TRPV4 channels. In whole-cell patch-clamp experiments, stimulation of RMECs with the TRPV4 agonist, GSK101 (300 nM), elicited membrane currents closely resembling those described previously in these cells (Supplementary Fig. S2A). GSK101-activated currents were blocked in the presence of the TRPV4 antagonists, HC06 (20 μM) or RN1734 (15 μM) (Supplementary Figs. S2B, S2C).
TRPV1 and TRPV4 Channels Regulate In Vitro Retinal Angiogenesis

An in vitro sprouting angiogenesis assay was initially used to test the significance of TRPV1 and TRPV4 channels to retinal angiogenesis. TRPV1 and TRPV4 antagonists produced a concentration-dependent block of vessel sprouting (Fig. 2). The TRPV1 inhibitors, CapZ and A784168, blocked sprout formation with IC_{50} values of 452 and 9.8 nM, respectively (Figs. 2Aii, 2Aiii). For the TRPV4 antagonists, the corresponding IC_{50}s were 3.6 µM for HC06 and 4.6 µM for RN1734 (Figs. 2Bii, 2Biii).

To ensure that the effects of the TRPV1 and TRPV4 inhibitors on in vitro angiogenesis could not be attributed to a loss of cell viability, we used a live/dead cell assay kit. Prolonged incubation (24 hours) of RMECs with CapZ, A784168, or RN1734 did not result in cell death or loss of cell viability.

**Figure 1.** Molecular and functional expression of TRPV1 in retinal microvascular endothelial cells. (Ai–Aiii) Confocal images of bovine retinal vessels within retinal flat mount preparations immunolabeled for TRPV1 (green), eNOS (red), and TO-PRO nuclear dye (blue). In addition to the retinal microvascular ECs, TRPV1 staining also was apparent in the vascular smooth muscle cells of the retinal arteries (regions adjacent to the eNOS staining in [Ai]) and some RGCs within the retinal neuropile (Aiii). Scale bars: 50 µm. (Bi) Confocal images of two RMECs immunostained for TRPV1 (green), isolectin B4 (IsoB4; red), and TO-PRO nuclear dye (blue). Scale bar: 10 µm. (Bii) Magnified inset of the dashed boxed area in (Bi) showing TRPV1 colocalization with the plasma membrane marker, isolectin B4 (yellow puncta). Scale bar: 2 µm. (C) RT-PCR analysis of TRPV1 mRNA expression in RMECs. No product was seen when the RT enzyme was omitted (RT−/−). (Cii) Glycosylated (113 kDa) and unglycosylated (95 kDa) TRPV1 was detected in RMECs by Western blot analysis. Several lower molecular weight bands also were present consistent with reports in other cell types. (Di) Time course record showing RMEC current activation at −80 and +80 mV upon application of TRPV1 agonist, RTX. In this and all subsequent patch-clamp figures, current plots have been normalized to cell capacitance and the dashed line indicates the zero current level. (Dii) Summary data showing RMEC current densities before and following application of RTX. *P < 0.05 for the indicated comparison. (Diii–Dvi) Time course records and summary data showing that the TRPV1 antagonists, CapZ (Diii, Div), and A784168 (Dv, Dvi) prevent activation of RTX-induced currents in RMECs. The data in (Div) and (Dvi) are presented as the change in current density (Δ [pA/pF]) relative to baseline (i.e., before drug addition).
A784168, HC06, and RN1734 had no effect on cell viability at concentrations that elicited a maximal block of sprouting angiogenesis (Supplementary Fig. S3).

In a separate set of experiments, the effects of the TRPV1 agonist, RTX, on sprouting angiogenesis was tested. Contrary to expectations, RTX caused a concentration-dependent inhibition of vessel sprouting with an IC50 value of 700 nM (Supplementary Fig. S4A). To explain these findings, we considered the possibility that RTX might be causing desensitization of RMEC TRPV1 channels, thereby decreasing the activity of these channels over time. To ascertain whether RTX does cause desensitization in RMECs, we undertook whole-cell patch-clamp experiments where RTX was applied twice to each cell, with a 1-minute interval between additions. Although the first treatment with RTX consistently elicited TRPV1 currents, no significant currents were seen during the second application of this drug (Supplementary Fig. S4B). These findings are consistent with the view that RTX can induce TRPV1 desensitization in RMECs.

Experiments subsequently were undertaken to investigate whether TRPV1 and TRPV4 channels contribute to VEGF-induced angiogenesis. Exposure of RMECs to VEGF nearly doubled the number and total length of vascular sprouts in the in vitro sprouting angiogenesis assay (Figs. 3A, 3Bi, 3Ci). Despite this, CapZ, A784168, HC06, and RN1734 had no effect on VEGF-stimulated sprouting in RMECs (Figs. 3A, 3Bi, 3Ci). These findings strongly suggest that VEGF-induced retinal angiogenesis in vitro does not require activation of TRPV1 or TRPV4 channels.

The above results imply that VEGF-induced signaling in RMECs does not rely upon TRPV1 and TRPV4 channel activation. In follow-up experiments, Ca2+ imaging studies were done to investigate this more directly. Confocal Ca2+ imaging revealed considerable heterogeneity in VEGF-induced Ca2+ responses among individual RMECs (Fig. 4Ai). Although VEGF-induced Ca2+ responses in most cells were initiated as spreading intracellular Ca2+ waves that propagated throughout the cell (Supplementary Videos S1–S3), the amplitude and temporal profile of the Ca2+ signals varied widely among different RMECs over time, even within the same field of view (Fig. 4Ai). Some cells displayed a single Ca2+ transient, while others exhibited more complex oscillatory Ca2+ signaling events. In addition, following VEGF exposure, Ca2+ returned to basal levels in some cells, while in others a sustained increase in Ca2+ was observed. Using confocal Ca2+ imaging, we observed no effects of CapZ or HC06 on VEGF-induced Ca2+ signals in RMECs (Figs. 4Ai, 4ii; Supplementary Videos S1–S3). Given the considerable variability in RMEC Ca2+ responses to VEGF at the single cell level combined with limitations on the number of cells that could be analyzed by confocal Ca2+ imaging, we undertook FLIPR-based high throughput Ca2+ imaging experiments to confirm our findings in RMEC cell populations. At the cell population level (average Ca2+ level across all cells in a well), VEGF induced a biphasic Ca2+ response in RMECs, comprising of an initial transient rise in
Ca\(^{2+}\), followed by a smaller sustained Ca\(^{2+}\) elevation that remained above basal Ca\(^{2+}\) levels for the duration of the experiment (~10 minutes; Fig. 4Bi). Again, CapZ and HC06 had no effect on VEGF-induced Ca\(^{2+}\) signaling in these experiments (Figs. 4Bi, 4Bii). Taken together, these Ca\(^{2+}\) data are consistent with the view that VEGF does not trigger TRPV1 and TRPV4 channel activation in RMECs.


**TRPV1 and TRPV4 Channels Act by Specifically Modulating Tubulogenesis**

Retinal angiogenesis is a complex multistep process that involves EC migration, proliferation and capillary tube formation. Therefore, experiments were undertaken to clarify which steps in the angiogenic process are regulated by TRPV1 and TRPV4 channels.

To establish whether TRPV1 and TRPV4 have a role in RMEC migration, we undertook scratch-wound assays. Blockade of TRPV1 (CapZ, 10 \(\mu\)M or A784168, 100 nM) or TRPV4 (HC06, 20 \(\mu\)M or RN1734, 15 \(\mu\)M) had no discernible effect on the degree of RMEC monolayer recovery (Fig. 5A).

The BrdU ELISA assay demonstrated that the level of cell proliferation did not differ significantly in RMECs treated with TRPV1 or TRPV4 inhibitors when compared to control cells (Fig. 5B).

The ability of RMECs to form capillary-like tubes was investigated in the absence and presence of TRPV1 or TRPV4 antagonists using a Matrigel-based in vitro tubulogenesis assay. Forty-eight hours after culturing RMECs within a Matrigel matrix, control cells exhibited a robust tubulogenic response (Fig. 5Ci). Tube areas and lengths were significantly attenuated in RMECs exposed to CapZ, A784168, HC06, or RN1734 (Figs. 5Ci–iii). Collectively, these results suggested that TRPV1 and TRPV4 channels regulate retinal angiogenesis in vitro by specifically modulating the tubulogenesis process.

**TRPV1 and TRPV4 Form Functional Heteromeric Channels in RMECs**

Previous work has demonstrated that TRPV1 and TRPV4 subunits can coassemble to form heteromeric channels in heterologous expression systems. The similarities in our data examining the effects of TRPV1 and TRPV4 antagonists on retinal angiogenesis in vitro, prompted us to explore the possibility that these channels may physically interact to form functional heteromeric channels in RMECs.

We conducted in situ PLA assays as a first step in addressing this issue. PLA assays allow the detection of proteins that are in very close proximity to each other (<40 nm) and has been used in a range of studies to detect heteromeric ion channel complexes. Use of anti-TRPV1 and anti-TRPV4 antibodies raised in different species resulted in a large number of PLA puncta in RMECs (Fig. 6Ai). PLA signals were detected in the cytoplasm as well as being distributed in a pattern consistent with a plasma membrane localization (Fig. 6Ai). No PLA signals were detected when RMECs were incubated with the TRPV1 or TRPV4 antibodies alone, or when the PLA probes were omitted from the protocol (Fig. 6Aii).

Our PLA assay results strongly suggested that TRPV1/TRPV4 heteromeric channels are present in RMECs. To test whether these heteromeric channels are functional, whole-cell patch recordings were performed. Specifically, the effects of TRPV4...
channel blockade on TRPV1-agonist evoked currents was examined, and vice versa. Inward and outward currents elicited by the TRPV1 agonist, RTX, were inhibited by approximately 80% following application of the TRPV4 antagonist, HC06 (20 μM; Figs. 6Bi, 6Bii). Similar results were obtained when the TRPV1 inhibitor, CapZ (10 μM), was applied following activation of TRPV4 channel subunits using GSK101 (Figs. 6Biii, 6Biv). Taken together, these data provided strong evidence for the presence of functional TRPV1/TRPV4 heteromeric channels on RMEC plasma membranes.

TRPV1 and TRPV4 Channels Regulate Retinal Angiogenesis In Vivo

Given the apparent role of RMEC TRPV1 and TRPV4 channels in regulating angiogenic responses in vitro, we subsequently used the OIR mouse model of ischemic retinopathy to test the involvement of these channels in mediating pathologic retinal angiogenesis in vivo. We injected CapZ, A784168, HC06, or RN1734 into the vitreous cavity of OIR mice at P15 and compared the degree of preretinal neovascularization at P17 with that of OIR control (non- and vehicle-injected) mice. All four drugs significantly inhibited pathologic retinal angiogenesis in this model (Figs. 7A, 7Bi). Interestingly, for each of these drugs, blockade of preretinal neovascularization was accompanied by an unexpected increase in normal intraretinal vascular areas (Figs. 7A, 7Bii). For CapZ and RN1734 a significant decrease in avascular areas (Figs. 7A, 7Biii) also was observed. Overall, these findings confirmed a role for TRPV1 and TRPV4 channels in the development of retinal neovascularization in vivo, but also indicated that blockade of these channels may help to stimulate physiologic revascularization of the ischemic retina.

**DISCUSSION**

In this study, we have demonstrated an important role for TRPV1 and TRPV4 channels in modulating retinal angiogenesis. TRPV1 and TRPV4 inhibitors suppressed in vitro sprouting angiogenesis by specifically disrupting EC tubulogenesis, with no effect on cell migration or proliferation. Our data also showed that TRPV1 and TRPV4 can coassemble in retinal microvascular ECs to form functional heteromeric channels. In the mouse OIR model of retinopathy of prematurity, blockade of TRPV1 or TRPV4 channels inhibited neovascularization, while simultaneously enhancing vascular recovery within the ischemic retina. As discussed below, these findings extended our understanding of the mechanisms controlling retinal angiogenesis and may provide new targets for therapeutic intervention in vasoproliferative diseases of the retina.

Although TRPV4 channel activity has been characterized previously in retinal microvascular ECs, to our knowledge our study is the first to demonstrate the functional expression of TRPV1 channels in these cells. In retinal whole mounts, TRPV1 exhibited a punctate localization pattern throughout the ECs of the retinal arteries, capillaries, and veins. Punctate TRPV1 expression also was observed at the plasma membrane and within the cytoplasm of cultured RMECs. Punctate TRPV1 staining is consistent with observations in other cell types, where these proteins have been reported to form channel clusters. In sensory nerves, clustering of TRPV1 channels is mediated by A-kinase anchoring proteins (AKAPs) which enables them to operate in a cooperative, or “coupled,”
Functional plasma membrane TRPV1 channel activity was confirmed in RMECs by electrophysiologic recording. Administration of the potent TRPV1 agonist, RTX, to RMECs induced inward and outward currents that could be completely abolished in the presence of the TRPV1 antagonists, CapZ or A784168. These currents activated slowly (taking ~1 minute to reach peak amplitude), reversed at $\theta_C = -9.6 \pm 3.2$ mV, and were outwardly rectifying. In general, these biophysical features are consistent with RTX-induced TRPV1 currents previously reported in heterologous expression systems. In RMECs, however, the outward rectification of the current appears much more pronounced. This discrepancy could, at least in part, be explained by the demonstrated existence of TRPV1/TRPV4 heteromultimers in these cells (Fig. 6Ai). Indeed, it is well established that when TRPV subunits coassemble into heteromeric channels, they can exhibit modified biophysical properties when compared to their respective homomeric counterparts.

Pharmacologic inhibition of TRPV1 or TRPV4 channels caused a concentration-dependent block of retinal angiogenic sprouting in vitro. In general, the IC$_{50}$ values that we obtained for the different antagonists used in this work corresponded well with published IC$_{50}$ values for the effects of these drugs on TRPV1 and TRPV4 channels in other cells and tissues. Notably, however, the IC$_{50}$ value that we obtained for the inhibition of angiogenic sprouting by HC06 (3.6 $\mu$M) is approximately 30- to 200-fold greater than that previously reported for the actions of this compound on heterologous expressed human, rat, and mouse TRPV4 channels. This discrepancy could be explained by species-specific differences in the sensitivity of bovine TRPV4 channels to HC06 (bovine RMECs were used in this study) when compared to their human and rodent orthologues. The responses of TRP channels to agonists and antagonists are known to vary among species and for some TRP channel ligands species-specific differences have been used to elucidate the structural basis of channel activation and inhibition mechanisms.

Our data have shown that TRPV1 and TRPV4 channels regulate retinal angiogenesis in vitro by specifically modulating tubulogenesis. Tubulogenesis is a complex process that involves multiple endothelial cell functions, including cytoskeletal reorganization, assembly of intracellular junctional complexes, and cell polarization. The involvement of TRPV1 and TRPV4 channels in retinal endothelial cell tubulogenesis may be linked to the requirement for intracellular Ca$^{2+}$ signaling during the formation of adherens and tight junction complexes. In particular, Ca$^{2+}$ signals initiated at points of cell-to-cell contact are known to have a central role in the recruitment of junctional proteins from intracellular sites to the plasma membrane. Previous studies have found TRPV1 and TRPV4 to be enriched at sites of cell-to-cell contact, suggesting that these channels may contribute to the Ca$^{2+}$ signaling events that trigger cell-to-cell adhesion. Consistent
with this idea, TRPV4-deficient mice have been reported to display impaired intracellular Ca$^{2+}$ signaling and a disruption in the development and maturation of cell-cell junctions in epithelia of the skin.65

Structurally, TRP channels are formed by four subunits that are organized around a central cation-selective pore.66 While early work suggested that these channels preferentially assemble into homomeric channels,67 over recent years it has become evident that heteromerization of TRP channel subunits of either the same subfamily or different subfamilies can occur.68 Heteromerization of TRP channel subunits is thought to serve as a mechanism to increase TRP channel diversity and, hence, the capacity of cells to respond to changes in their environment.68 Previous investigators have reported coassembly of TRPC1-C4,69 TRPC3-C4,70 and TRPV4-C1-P271 heteromeric channels in the vascular endothelium, but to our knowledge our study is the first to demonstrate the presence of functional TRPV1-V4 complexes in these cells. Our findings concurred with those in dorsal root ganglion (DRG) neurons suggesting that TRPV1 and TRPV4 can form heteromeric channel complexes in native tissues.44 Not all cells, however, that coexpress these proteins form functional heteromeric TRPV1-V4 channels. In a subset of RGCs, for example, TRPV1 and TRPV4 were found to colocalize, but failed to functionally interact.72 The reasons for cell-specific differences in the heteromerization of TRP channels are not fully understood at this time, but could relate to differences in TRP channel splicing patterns among different cell types.73

Intravitreal injection of TRPV1 or TRPV4 inhibitors suppressed ischemia-driven neovascularization in the OIR mouse model of retinopathy of prematurity. Thus, in addition to regulating retinal angiogenic signaling in vitro, these channels also appear to have an important role in mediating pathologic retinal angiogenesis in vivo. We were surprised to find, however, that blockade of these channels also enhanced physiologic revascularization of the ischemic retinal tissue. These results might be explained by the fact that TRPV1 and TRPV4 are not only expressed in vascular endothelial cells of the retina, but also other retinal cell types including retinal ganglion cells (RGCs).74,75 During retinal ischemia, RGCs have been shown to produce vasorepellent factors, such as members of the secreted class III semaphorins (semaphorin 3A and 3E), which can suppress new blood vessel growth into ischemic regions of the retina.76,77 Semaphorin expression has been shown to be regulated in a Ca$^{2+}$-dependent manner78 and activation of Ca$^{2+}$ signaling through TRPV1 and TRPV4 channels has been implicated in RGC responses to ischemia.75,79 These observations provide a possible mechanism through which TRPV1 and TRPV4 antagonists might enhance reparative angiogenesis in the ischemic retina (i.e., by modulating RGC Ca$^{2+}$, and thus, semaphorin levels), although other explanations cannot be excluded. Our data open new avenues for future studies aimed at better understanding the molecular mechanisms that regulate reparative angiogenesis in the ischemic retina.

In summary, this study demonstrated that TRPV1 and TRPV4 channels have a critical role in retinal angiogenesis. We showed that these channels are capable of forming heteromeric complexes in retinal microvascular ECs and that they contribute to retinal angiogenesis by specifically modulating...
the tubulogenesis process. Our findings suggested that targeting these channels could offer a new therapeutic strategy for inhibiting pathologic angiogenesis in the retina that may be either an alternative or a complement to existing anti-VEGF approaches. The observation that blockade of these channels also enhances physiologic revascularization of the ischemia retina may be viewed as being particularly advantageous with regards to exploiting these targets for therapeutic use.

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