TRP Channels Localize to Subdomains of the Apical Plasma Membrane in Human Fetal Retinal Pigment Epithelium

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Submitted: September 24, 2014
Accepted: February 17, 2015

Citation: Zhao PY, Gan G, Peng S, et al. TRP channels localize to subdomains of the apical plasma membrane in human fetal retinal pigment epithelium. *Invest Ophthalmol Vis Sci*. 2015;56:1916–1923. DOI:10.1167/iov.14-15738

PURPOSE. Calcium regulates many functions of the RPE. Its concentration in the subretinal space and RPE cytoplasm is closely regulated. Transient receptor potential (TRP) channels are a superfamily of ion channels that are moderately calcium-selective. This study investigates the subcellular localization and potential functions of TRP channels in a first-passage culture model of human fetal RPE (hfRPE).

METHODS. The RPE isolated from 15- to 16-week gestation fetuses were maintained in serum-free media. Cultures were treated with barium chloride (BaCl₂) in the absence and presence of TRP channel inhibitors and monitored by the transepithelial electrical resistance (TER). The expression of TRP channels was determined using quantitative RT-PCR, immunoblotting, and immunofluorescence confocal microscopy.

RESULTS. Barium chloride substantially decreased TER and disrupted cell-cell contacts when added to the apical surface of RPE, but not when added to the basolateral surface. The effect could be partially blocked by the general TRP inhibitor, lanthanum chloride (LaCl₃, ~75%), or an inhibitor of calpain (~25%). Family member-specific inhibitors, ML204 (TRPC4) and HC-067047 (TRPV4), had no effect on basal channel activity. Expression of TRPC4, TRPM1, TRPM3, TRPM7, and TRPV4 was detected by RT-PCR and immunoblotting. The TRPM3 localized to the base of the primary cilium, and TRPC4 and TRPM3 localized to apical tight junctions. The TRPV4 localized to apical microvilli in a small subset of cells.

CONCLUSIONS. The TRP channels localized to subdomains of the apical membrane, and BaCl₂ was only able to dissociate tight junctions when presented to the apical membrane. The data suggest a potential role for TRP channels as sensors of [Ca²⁺] in the subretinal space.

Keywords: retinal pigment epithelium, TRP channels, tight junction, subretinal space

The role of calcium in the subretinal space remains under investigation. The subretinal space lies between the photoreceptors and the RPE. The RPE is a monolayer of pigmented cuboidal cells in the eye that supports visual function and forms the outer blood-retinal barrier. The RPE regulates the outer blood-retinal barrier, phagocytoses the outer segment discs shed by photoreceptors, secretes growth factors, regenerates 11-*cis*-retinal, and absorbs stray light. Many functions of the RPE are segregated to apical (facing the subretinal space) and basolateral (facing the choroid) domains of the plasma membrane.¹ The polarized distribution of proteins between these domains is maintained by the fence function of tight junctions, which encircle each cell in the plane of the monolayer. The barrier function of tight junctions results from binding neighboring cells with a partially occluding seal. This seal selectively retards diffusion between the subretinal space and the fenestrated choriocapillaris.² The combination of polarity and barrier function allow RPE to regulate transcellular transport, thereby regulating the ionic milieu of the subretinal space. This combination allows RPE to exchange retinal nutrients and metabolic waste with the choriocapillaris.³ In addition, the signaling function of the tight

junctions transmits extracellular signals that regulate various cellular functions.^{4,5}

Polarity and barrier function allow the RPE to maintain the neural retina in a relatively dehydrated state. As the RPE pumps chloride from the subretinal space into the choroid, cations and water passively follow. Further, RPE buffers the ion composition of the subretinal space to compensate for shifts caused by changes in photoreceptor activity throughout the day-night cycle. Light induces a rapid decrease of Ca²⁺ in the subretinal space ([Ca²⁺]_{SRS}) followed by a slow increase in volume and efflux of Ca²⁺ from photoreceptors into the subretinal space.^{6,7} The mechanisms by which RPE senses and regulates volume_{SRS} and [Ca²⁺]_{SRS} are unknown.

Intracellular free calcium ([Ca²⁺]_i) acts as a secondary messenger and/or directly modifies protein structure to regulate many cell functions. In the RPE, Ca²⁺_i is tightly regulated and affects transport of ions and water, secretion of growth factors, and phagocytosis of shed photoreceptor outer segments.^{3,8-10} For example, extracellular ligands can increase [Ca²⁺]_i to stimulate fluid transport.¹¹ The RPE continuously extrudes Ca²⁺ from the cytosol, using pumps and exchangers to move Ca²⁺ into the extracellular space, endoplasmic reticulum, and melanosomes. Continuous efflux is opposed by influx

through ion channels. The net effect is that $[Ca^{2+}]_i$ is maintained at approximately 100 nM, four orders of magnitude below the extracellular concentration. There are local variations in $[Ca^{2+}]_i$, as exemplified by the primary cilium of RPE.¹² The resting $[Ca^{2+}]_{\text{cilium}}$ is approximately 7 times higher than resting $[Ca^{2+}]_{\text{cytoplasm}}$, and regulation of hedgehog signaling was effected by manipulating $[Ca^{2+}]_{\text{cilium}}$ without changing $[Ca^{2+}]_{\text{cytoplasm}}$.

In RPE, Ca^{2+} influx from the extracellular space is mediated by three families of ion channels at the plasma membrane: voltage-dependent L-type channels, ligand-gated channels, and transient receptor potential (TRP) channels.³ Closed at physiologic membrane potentials, voltage-dependent L-type channels open when phosphorylated by tyrosine kinase.¹³ Ligand-gated channels are activated by extracellular purinergic compounds.¹¹ In contrast, TRP channels mediate the constitutive Ca^{2+} influx that defines the resting $[Ca^{2+}]_i$ for ARPE19 cells.¹⁴ The mammalian TRP channel superfamily is divided primarily into TRPC, TRPM, and TRPV subfamilies, though each individual subfamily member has diverse roles and mechanisms of activation. The specific TRP channels expressed in human RPE tissue are not well characterized.

To study TRP channels, we used a well-established culture model of human fetal RPE (hFRPE) isolated from 15- to 16-week gestation fetal eyes and maintained in defined serum-free media.¹⁵ Cultures of hFRPE are highly polarized, as reflected by a transepithelial electrical resistance (TER) $> 300 \Omega \times \text{cm}^2$ and by functional and electrophysiological properties attributed to native adult human RPE.^{16,17} Using Ba^{2+} as a Ca^{2+} surrogate, we show there are apical pathways for entry of divalent cations. These pathways are active in the absence of external stimulation and are blocked by the nonselective TRP channel inhibitor La^{3+} . We characterize the TRP channel expression profile of hFRPE, and show the localization of TRPC4, TRPM3, and TRPV4 to subdomains of the apical plasma membrane.

MATERIALS AND METHODS

Cell Culture

Primary cultures of hFRPE were obtained from Sheldon Miller (National Eye Institute). Research adhered to the tenets of the Declaration of Helsinki. Cultures were trypsinized and reseeded on 0.4- μm pore Transwell or Snapwell culture inserts (Corning, Inc., Corning, NY, USA) coated with 5% human extracellular matrix (BD Biosciences, Franklin Lakes, NJ, USA). After reseeding, cultures were maintained for 4 weeks in media containing 5% serum as previously described.¹⁷ After cultures became confluent and the TER stabilized, they were adapted to serum-free media (SFM-1) containing 70% Dulbecco's modified Eagle's medium (DMEM), 30% F-12, 2% B27, and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA).¹⁸ Cultures were maintained in SFM for at least 1 month before experiments. The TER was monitored using an EVOM resistance meter with Endohm electrodes (World Precision Instruments, Sarasota, FL, USA). All cultures used in these experiments had a TER of at least $300 \Omega \times \text{cm}^2$.

Barium and Calcium Studies

Experiments were performed by exchanging half of the SFM-1 in Transwell or Snapwell chambers with $\times 2$ concentrations of barium chloride ($BaCl_2$) or calcium chloride ($CaCl_2$) and inhibitors or ionophores that were diluted in SFM-1. Except caloxin 1b1, inhibitors were first dissolved in dimethyl sulfoxide (DMSO; AmericanBio, Natick, MA, USA) to create a stock solution and then diluted to the indicated concentrations

on the day of the experiment (DMSO $< 1\%$ of total media). Caloxin 1b1 (Anaspec, Inc., Fremont, CA, USA) was dissolved in SFM-1. Final concentrations were: 3.0 mM $BaCl_2$ (Thermo Fisher Scientific, Rockford, IL, USA), 6.3 mM $CaCl_2$ (Sigma-Aldrich Corp., St. Louis, MO, USA), 2.0 mM $LaCl_3$ (Sigma-Aldrich Corp.), 5.0 μM valinomycin (R&D Systems, Minneapolis, MN, USA), 10 μM nifedipine (Alfa Aesar, Ward Hill, MA, USA), 20 μM ML204 (Sigma-Aldrich Corp.), 1.0 μM HC-067047 (Sigma-Aldrich Corp.), 100 μM ALLM (Santa Cruz Biotechnology, Dallas, TX, USA), and 400 μM caloxin 1b1. Solutions were added to apical and basolateral media chambers unless otherwise specified. After media exchange, tissue culture plates were agitated gently to mix the media and incubated at 37°C . The TER was measured before media exchange, and at 2 and 4 hours of incubation.

Quantitative Real-Time Reverse-Transcription PCR (qRT²-PCR)

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The cDNA was reverse transcribed from 2.0 μg total RNA using the QuantiTect Reverse Transcription Kit (Qiagen). Reactions were performed using iTaq SYBR Green (Bio-Rad, Hercules, CA, USA) and the primers listed in Supplementary Table S1. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method, normalized to GAPDH and CLDN19.¹⁹ All reactions were performed in triplicate from a minimum of two cell samples from different primary cultures.

Immunoblotting

Samples were washed with ice cold PBS and lysed by sonication into a 25 mM Tris (pH 8.0) solution containing protease inhibitor and 1% SDS. Then, 20 μg protein extracts were diluted in Laemmli sample buffer, separated on a 7.5% Tris-HCl polyacrylamide gel (Bio-Rad), and transferred to polyvinylidene fluoride (PVDF) membranes (PerkinElmer, Inc., Santa Clara, CA, USA). After 1 hour incubation in Membrane Blocking Solution (Life Technologies, Frederick, MD, USA), membranes were incubated with primary antibodies (Supplementary Table S2) overnight at 4°C . Membranes were washed three times with PBS containing 0.2% Tween and incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibodies (1:3000, Thermo Fisher Scientific) for 1 hour. After three additional washes, proteins were visualized with SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific). Images were captured using the ChemiDoc XRS System (Bio-Rad). The M_r values were calculated relative to PrecisionPlus Dual Color Standards (Bio-Rad). Immunoblots were performed on at least two distinct cell samples from different primary culture flasks.

Immunocytochemistry

Subcellular distribution of TRP channels and ZO-1 was determined by indirect immunofluorescence. Samples were washed with PBS and fixed in 100% ethanol. Blocking was performed for 1 hour using 5% donkey serum and 1% BSA in PBS. After incubation with primary antibody overnight at 4°C , samples were washed three times in PBS and incubated with secondary antibody at room temperature for 1 hour. Primary and secondary antibodies are listed in Supplementary Table S2. Nuclear staining was done with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Corp.). After three additional PBS washes, samples were mounted to slides with CytoSeal 60 (Thermo Fisher Scientific) and cured overnight. Fluorescent channels were captured in grayscale using an LSM 410 Yokogawa

FIGURE 1. Effect of BaCl₂ on hRPE in culture media. The TER is reported relative to the TER at the start of the experiment (TER ≥ 300 Ω × cm²). Except where noted, the results are based on three independent experiments (mean ± SE). (A) The results for SFM-1 and SFM-1 plus caloxin 1b1 (Ca²⁺-ATPase inhibitor) were indistinguishable. The effect of adding CaCl₂ was transient with a small, but significant decrease ($P < 0.05$) at 2 hours. The decrease was prolonged in the presence of the inhibitor ($P < 0.05$ relative to Ca²⁺ alone). Barium chloride caused significant reduction of TER after 2 and 4 hours in SFM at 37°C. Similar results were obtained when the TER of hRPE was elevated by maintaining hRPE in a serum-containing culture medium (not shown).¹⁸ (B) Light microscopy revealed normal polygonal RPE morphology after 4 hours in 5 mM CaCl₂ (left) and loss of the apical junctional complex after 4 hours in BaCl₂ (right). (C) The TER decreased when BaCl₂ was added to the apical media chamber, but not when added to the basolateral chamber (mean ± SE). Valinomycin and nifedipine were ineffective at preventing TER reduction (mean ± range, $n = 2$). (D) Lanthanum chloride in the apical chamber partially blocked the Ba²⁺-mediated effect, but LaCl₃ in the basolateral chamber was ineffective. Inhibitors of TRPC4 (ML204) and TRPV4 (HC-067047), alone and in combination (ML/HC), failed to block the effect of Ba²⁺ (mean ± range, $n = 2$).

spinning-disc confocal microscope (Yokogawa Electric, Tokyo, Japan) and then processed and colorized with the AxioVision or Zen software suites (Carl Zeiss, Thornwood, NY, USA).

RESULTS

Barium Disrupts RPE Monolayers When Added to the Apical Surface

When extracellular [Ca²⁺] was increased from 1.3 to 6.3 mM by the additions of CaCl₂, the TER transiently decreased approximately 20% (Fig. 1A). Isoforms 1, 2, and 4 of the plasma membrane calcium-ATPase are found in human RPE.²⁰ The concentration of inhibitor, caloxin 1b1, used in this experiment was >3 to 10 times the pK_i of these isoforms, which would achieve a partial inhibition.²¹ With increased [Ca²⁺]_{extracellular} plus inhibitor the TER decreased approximately 40% and did not recover during the experiment. Inhibition of the plasma membrane calcium-ATPase by itself had no effect. To explore this effect further, we used Ba²⁺ as a surrogate for Ca²⁺, because it is a poor substrate for the plasma membrane calcium-ATPase and is not transported by the smooth endoplasmic reticulum calcium-ATPase.

Barium can block K⁺ channels and permeate through TRP channels.^{18,22,23} When 3 mM BaCl₂ was added to the culture medium, the TER decreased substantially within 2 hours at 37°C (Fig. 1A). There was no discernable change in morphology and the TER recovered several days after the Ba²⁺ was washed away. By 4 hours, cells in BaCl₂ disassembled the apical junctional complex (adherens and tight junctions), lost polygonal morphology, and began to detach from the filter

(Fig. 1B). To determine whether the Ba²⁺-mediated effect was polarized, BaCl₂ was added to either the apical or basolateral medium chamber. Reduction in TER was reproduced only when Ba²⁺ was added to the apical chamber (Fig. 1C). The K⁺ ionophore valinomycin was used to determine whether the effect on TER could be relieved by providing an alternative route for K⁺ to exit the cell. The blocker nifedipine was used to determine whether Ba²⁺ was exerting its effect through voltage-gated L-type channels. Neither valinomycin nor nifedipine could relieve the Ba²⁺-mediated decrease of TER (Fig. 1C). Based on these results we explored the hypothesis that Ba²⁺ may exert its effect after entering cells via TRP channels located on the apical membrane.

Lanthanum is a general TRP channel blocker,¹⁴ but it also would block Ba²⁺ entry through connexin hemichannels in the apical membrane. Apical channels transiently appear early in the development of chick RPE.^{24,25} However, connexin 43, the predominant connexin of human RPE,²⁰ was only detected in the apical junctional complex by immunofluorescence (Supplementary Fig. S1). At 2 mM, lanthanum chloride (LaCl₃) reduced the ability of Ba²⁺ to lower the TER by 75% when both were added to the apical medium chamber (Fig. 1D). In contrast, LaCl₃ was ineffective when only added to the basolateral chamber. The reduction in TER could not be attributed to a specific TRP channel through the use of selective inhibitors (Fig. 1D). We examined ML204, an inhibitor of activated TRPC4,²⁶ and HC-067047, an inhibitor of TRPV4,²⁷ and a combination of the two. The inhibitors were tested at concentrations 20-fold higher than their IC₅₀, but they did not block the effect of Ba²⁺ on TER.

FIGURE 2. Expression of TRP channels in hRPE cultures. (A) Quantitative RT-PCR showing gene expression relative to tight junction gene CLDN19. Horizontal line delineates 100-fold less expression than CLDN19. For the indicated genes, the PCR product was of predicted size (Supplementary Fig. S3). Transcripts that were undetected after 40 cycles of PCR are not included: TRPC5, TRPM6, TRPV5, TRPV6, and TRPA1. Error bars: SE, $n \geq 3$. (B) For genes that expressed high levels of mRNA, immunoblots revealed proteins of the appropriate M_r (see text). The extra protein band for TRPM1 also was observed in immunoblots of a cell line rich in TRPM1 (Supplementary Fig. S4). Preincubation of the anti-TRPV4 antibody with the peptide antigen prevented the labelling of TRPV4 (~90 kDa) and several minor protein bands.

mRNA and Protein Expression of TRP Channels

A comprehensive view of TRP channel gene expression was obtained by consulting an RNA-sequencing database generated from previous hRPE cultures (Supplementary Fig. S2).²⁰ High levels of gene expression were observed for TRPC1, TRPC4, TRPM1, TRPM3, TRPM7, and TRPV4. Using qRT²-PCR, we verified high expression levels of these six channels, as well as reduced (100-fold less than RPE marker claudin-19), or negligible, expression of the remaining TRP channels (Fig. 2A). These data were consistent across multiple cultures that were isolated from distinct donors. Primers were validated by confirming amplicon size on a DNA agarose gel (Supplementary Fig. S3).

Immunoblots were used to characterize the expression of TRP channel proteins with high mRNA expression (Fig. 2B). The TRPC1 protein was not detected. The TRPC4 was detected near 90 kDa, in agreement with the predicted molecular weight. Three TRPM channels were visualized at molecular weights different from their predicted protein sizes, which is

common for membrane proteins. The TRPM1 was visualized as three bands, one near 250 kDa and two in close proximity near 125 kDa. Corresponding bands were observed in a positive control, lysates from the TRPM1-expressing cell line 501mel (Supplementary Fig. S4). The TRPM3 was detected near 250 kDa, consistent with past studies.²⁸ The TRPM7 also was detected near 250 kDa, consistent with sizes detected in the manufacturer's positive controls. Anti-TRPV4 antibody stained an appropriately sized protein near 90 kDa. Several other weakly stained proteins also were observed. Labeling of these bands was blocked by preincubating the antibody with the peptide antigen.

TRP Channels Localized to Subdomains of the Apical Surface

Immunocytochemistry and confocal microscopy were used to determine the subcellular localization of TRP proteins. The TRPC4 colocalized with the tight junction protein ZO-1 (Fig. 3A). Because Ba²⁺ caused tight junctions to dissociate, we tested the hypothesis that a localized increase of Ba²⁺ near the tight junctions might activate calpain-mediated dissociation of the tight junctions.^{29,30} The calpain inhibitor, ALLM, slightly blocked the effect of Ba²⁺ on TER, but was less effective than La³⁺ (Fig. 4). There was no evidence that Ba²⁺ effected an enrichment of calpain near the tight junctions (data not shown).

The TRPM3 was concentrated in two locations along with a weaker immunofluorescence signal that corresponded to microvilli (Fig. 3B). An intense signal for TRPM3 colocalized with the tight junction marker ZO-1 at the apical end of the lateral membranes. The TRPM3 also was concentrated at a punctate locus near the center of the cell and apical to the nucleus. This location would correspond to the basal body, which lies at the base of the primary cilium. Antibodies to γ -tubulin were used to identify the basal body (Fig. 5).³¹ At the resolution of immunofluorescence, a γ -tubulin signal would overlap with a membrane protein at the base of the cilium. Double immunolabeling with anti-TRPM3 demonstrated colocalization of the two antigens. The length of the primary cilium was revealed using antiacetylated tubulin, and indicated that TRPM3 was confined to the base of the cilium.

In roughly 5% of cells, TRPV4 was observed in the punctate apical pattern typified by microvilli (Fig. 3C). The majority of cells showed weak staining. The immunofluorescence localization studies for TRPM1 and TRPM7 were inconclusive due to a weak, diffuse signal.

DISCUSSION

Environmental signals regulate the functions of epithelia. The diurnal fluctuation of [Ca²⁺]_{src} was identified 20 years ago,^{6,7} but it remains unknown whether this fluctuation has any functional consequences. The localization of TRP channels to microdomains of the apical membrane reopens this question. The TRP channel, PKD1L1-PKD2L1, has emerged as a potential [Ca²⁺]_{src} sensor that localizes along the length of primary cilium of RPE cultures that were derived from a 1-year-old child.^{12,32,33} The PKD1L1-PKD2L1 maintained resting [Ca²⁺]_{cilia} at least 0.4 μ m higher than resting [Ca²⁺]_i. Modulating [Ca²⁺]_{cilia} affected the subcellular localization of hedgehog pathway proteins. Although we failed to identify this channel in fetal-derived RPE by RNA-sequencing or qRT²-PCR, we found that TRPM3 localized to the base of the cilium. A second microdomain for TRP channels was the tight junction. As reported in other cells, TRPM3 and TRPC4 were concentrated here.^{34,35} In this perijunctional region, a higher [Ca²⁺]

FIGURE 3. Subcellular localization of TRP channels by confocal immunofluorescence microscopy. In merged images, the DAPI counterstain (blue) revealed the position of nuclei. The *inset* shows the *xz* plane. (A) The TRPC4 co-localized with ZO-1 in apical tight junctions. (B) The TRPM3 also localized to tight junctions and to a punctate structure near the center of the cell, in the plane of the apical junctional complex. (C) An immunosignal for TRPV4, appeared in only approximately 5% of cells. In positive cells, TRPV4 was observed in a punctate pattern at the apical surface that is typical of microvilli. Scale bar: 20 μ m.

might increase junction permeability by activating calpain, calmodulin, or protein kinase C. The TRPV4 localized to apical microvilli in a small subset of cells. We confirmed the presence of various RPE TRP channels,^{14,36,37} but also determined, their subcellular distribution. Although the presence of spurious bands on the immunoblots might be explained by degradation products or multimer formation in SDS, their presence means the data should be viewed with caution. The data suggest that RPE might express three types of calcium sensor in the apical membrane that mediate signaling via localized changes in $[Ca^{2+}]$.

Plasma membrane TRP channels provide a constitutive leak for divalent cations to enter the cell.^{14,38,39} To counter the constant influx of Ca^{2+} , Ca^{2+} -ATPases in the plasma membrane

and organelles keep $[Ca^{2+}]_{cytoplasm}$ low. This mechanism is less effective for Ba^{2+} . The sarco/endoplasmic reticulum Ca^{2+} -ATPase does not pump Ba^{2+} , and the plasma membrane Ca^{2+} -ATPase pumps Ba^{2+} at a slower rate than Ca^{2+} .^{40,41} A rise in $[Ba^{2+}]$ near the tight junction might activate calpain, but not calmodulin or protein kinase C.^{29,30,42}

Barium reversibly decreased the TER within 4 hours, but only when it was added to the apical medium chamber. The effect could be blocked by La^{3+} , a nonspecific inhibitor of TRP and other channels. Even with Ba^{2+} added to both media chambers, La^{3+} in the apical chamber was sufficient to block the effect of Ba^{2+} . The effect of Ba^{2+} could result from its ability to block K^{+} channels, principally Kir7.1, which localizes to the apical plasma membrane. This is unlikely, because valinomy-

FIGURE 4. Calpain inhibition reduces the effect of BaCl₂ on TER. Data were collected after a 2-hour incubation. The TER is reported relative to the TER at the start of the experiment (TER $\geq 300 \Omega \times \text{cm}^2$). The ALLM had a small, but significant, effect on the action of BaCl₂ ($P < 0.001$). Error bars: SE ($n = 3$).

cin, a K⁺-specific ionophore, failed to protect RPE from Ba²⁺. Alternatively, Ba²⁺ might enter cells through connexin hemichannels in the apical membrane as found in chick RPE during early development,^{24,25} but we found no evidence of connexin in the apical membrane.

Experiments with the calpain inhibitor, ALLM, supported a partial role for Ba²⁺-activated calpain in the decrease of TER. Evidence that Ca²⁺ could regulate RPE tight junctions comes from the observation that raising the [Ca²⁺]_{extracellular} to 6.3 mM concentration while inhibiting the plasma membrane Ca²⁺-ATPase inhibitor decreased the TER 40%. The TRP-specific inhibitors did not allow us to tie these effects to a particular channel. However, these inhibitors may not have targeted the correct isoform and some affect activity stimulated by G protein coupled receptors rather than basal activity.²⁶ There are additional potential functions for a tight junction associated ion sensor, including signaling networks that control cell size, shape, polarity, proliferation, and apoptosis.^{43,44}

A mechanism for the effect of Ba²⁺ has to explain the requirement that cells be maintained in a complete medium. Previous work from our lab showed that 3 mM BaCl₂ had the opposite effect of increasing the TER when the BaCl₂ was dissolved in a modified Ringer's solution that lacked bicarbonate.¹⁸ In this medium, the plasma membrane electrical resistance increases as the principal membrane transport mechanisms are inhibited. With tight junctions remaining intact, the TER approximated the resistance of the tight junctions.^{18,45} Together, these data indicate mechanisms that disassemble tight and adherens junctions requires a complete medium. When BaCl₂ was withdrawn from the medium after two hours the cells recovered within two days, which is consistent with the time course for junction disassembly/reassembly experiments performed in other epithelia.⁴⁶

The findings on expression of TRPV channels in RPE have been inconsistent. We found TRPV4 in the microvilli of a small subset of cells. Regional heterogeneity of RPE has been observed for other proteins in vivo and in culture.^{18,47} Cordeiro et al.³⁶ found expression of TRPV1-V4 in ARPE-19 and primary donor RPE cultured in media containing 10% fetal bovine serum. In contrast, Kennedy et al.³⁷ found expression of TRPV5 and TRPV6 in primary cultures of RPE cultured in 10% fetal calf serum plus 5% newborn calf serum. Our study used hRPE cultured in a serum-free media that promotes differentiation.¹⁷ The contradictory literature may reflect the

FIGURE 5. Localization of TRPM3 at the base of the primary cilium. The basal body, which lies at the base of the cilium was identified with antibodies to γ -tubulin. The cilium was identified with antibodies to acetylated tubulin (Ac-Tubulin). The confocal image was taken above the plane of the tight junctions and shows the cilium of two adjacent cells. In one cell, the cilium was perpendicular to the plane of the image (*arrowhead*) and the signals for each protein overlaps. In the other cell, the cilium lies in the plane of the image (*arrow*). Only the γ -tubulin and TRPM3 signals overlap to give an orange signal. Scale bar: 5 μm .

heterogeneity of different cell cultures, as well as differing patterns of protein expression in response to different media formulations.

Although TRPM1 was detected on immunoblots, expression could not be localized to a specific subcellular compartment. Lack of expression at the plasma membrane is consistent with prior studies.⁴⁸ The TRPM7 also was undetected by immunofluorescence microscopy despite its detection by immunoblotting. In vascular endothelial cells, TRPM7 channels localize to the plasma membrane in the presence of laminar fluid flow.⁴⁹ In the absence of a stimulus, TRPM7 might be diffusely localized in an internal pool.

The hRPE used in this study are highly differentiated and used within one passage since their isolation from 15- to 16-week gestation fetuses.^{15,50} Therefore, the selective pressure to adapt phenotype to culture conditions has been minimized, but this also means that our cultures represent a snapshot of development. In rodents, RPE's primary cilium is transiently present during development and is involved in the differentiation of photoreceptors.^{12,51} In humans, photoreceptors differentiate during the fifth month of gestation.⁵² This study identified all the TRP channels that are expressed by RPE at an earlier stage of human fetal development. It is limited by the availability and quality of antibodies and specific inhibitors. Further, TER is but one function of tight junctions that might be affected by a Ca²⁺ sensor. Given these limitations, our data indicated that TRP channels are polarized to the apical plasma membrane where they may monitor [Ca²⁺]_{SRS}.

Acknowledgments

The authors thank Arvydas Maminishkis, PhD, and Sheldon Miller, PhD, for the generous gift of hfRPE, Antonella Bacchicocchi for the gift of protein extract from 501mel cells, and John Geibel, MD, PhD, for many helpful discussions regarding ion channels.

Supported by grants from the Connecticut Regenerative Medicine Research Fund, 10-SCB-02 (LJR); Alonzo Family Fund (LJR); NIH CTSA-TL1 TR000141 (PYZ); International Retinal Research Foundation, Callahan Award (GG); National Natural Science Foundation of China, 30772381 (SP); Newman's Own Foundation (RAA); and Leir Foundation (RAA).

Disclosure: **P.Y. Zhao**, None; **G. Gan**, None; **S. Peng**, None; **S.-B. Wang**, None; **B. Chen**, None; **R.A. Adelman**, None; **L.J. Rizzolo**, None

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