Basal Calcium Entry in Retinal Pigment Epithelial Cells Is Mediated by TRPC Channels

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PURPOSE. Ca²⁺ is a major regulator of cell function. In the retinal pigment epithelium (RPE), intracellular free Ca²⁺ concentration ([Ca²⁺]) is essential for the maintenance of normal retinal function. Therefore, accurate control of [Ca²⁺] is vital in these cells. Because Ca²⁺ is permanently extruded from the cytosol, RPE cells need a basal Ca²⁺ entry pathway that counteracts this Ca²⁺ efflux. The purpose of this study was to identify the molecular basis of basal Ca²⁺ entry into the RPE.

METHODS. [Ca²⁺], was measured using Fura-2-loaded ARPE-19 cells. The expression pattern of TRPC channels was investigated by RT-PCR with RNA extracted from ARPE-19 cells and freshly isolated RPE cells from human donor eyes.

RESULTS. In most cells, basal [Ca²⁺] is highly controlled by cell membranes that are only slightly permeable to Ca²⁺ and by the activity of Ca²⁺ pumps and transporters. The authors show here that RPE cells have a basal Ca²⁺ conductance that is dose dependently blocked by La³⁺. Basal [Ca²⁺], was also strongly reduced by the TRP channel blockers Gd³⁺, Ni²⁺, 2-APB, and SKF96365 and was insensitive to blockers of other Ca²⁺ channels. In confirmation of this pharmacologic profile, RPE cells expressed TRPC1 and TRPC4 channels, as shown by RT-PCR experiments.

CONCLUSIONS. Ca²⁺ is needed for several permanently occurring regulatory processes in RPE cells. The Ca²⁺ influx pathway identified in this study is essential to define a resting basal [Ca²⁺]. This resting [Ca²⁺], may contribute, for example, to basal cytokine secretion essential for the maintenance of normal retinal function. (Invest Ophthalmol Vis Sci. 2007;48: 5767–5772) DOI:10.1167/iovs.07-0412

Many cellular processes are controlled by the intracellular Ca²⁺ concentration ([Ca²⁺]).² These include excitation, secretion, cell differentiation, gene expression, endocytosis, and apoptosis. Altered Ca²⁺ homeostasis may lead to physical impairment, as seen in genetic diseases associated with Ca²⁺ transporters and channels.³ Generally, two pathways for the elevation of [Ca²⁺], exist: Ca²⁺ may enter through plasma membrane Ca²⁺ channels or Ca²⁺ may be released from intracellular Ca²⁺ stores by the activation of ryanodine or inositol trisphosphate receptors. For each case, the cells use a variety of Ca²⁺-transporting proteins.² The Ca²⁺ extrusion is mediated by Ca²⁺ exchangers and pumps. In resting cells in which the [Ca²⁺], is approximately 100 nM, the Ca²⁺ influx, efflux, and intracellular Ca²⁺ buffering are in equilibrium.

The retinal pigment epithelium (RPE) is located between the neural retina and the choroidal vasculature.³⁴ With its tight junctions, it forms part of the blood-retina barrier and is important for the maintenance of retinal function. Its pigment absorbs excess light, it reisomerizes all-trans retinal to 11-cis retinal and delivers it to the photoreceptors, it controls the transport of metabolites, nutrients, ions, and water between the subretinal space and the choroidal vessels, and it phagocytes shed outer segments of photoreceptors. As a target and source of a variety of growth factors, the RPE maintains retinal integrity and is part of the immune privilege of the subretinal space.³⁴ Many of these tasks are influenced by changes in the [Ca²⁺].⁷ Therefore, accurate control of [Ca²⁺] in the RPE is vital.

In the RPE, it has been shown that Ca²⁺ influx through the voltage-operated L-type Ca²⁺ channel α1D plays a role in the control of growth factor secretion.³⁶ Additionally, a specific block of L-type channels led to a reduced light peak in electrotetrograms in mice and rats, indicating that these channels are involved in light-induced responses of the RPE.³⁷,³⁸ Furthermore, purinergic stimulation of RPE cells leads to an increase in [Ca²⁺], which seems to at least partially mediated by ionotropic purinergic receptors (P2X).¹¹ This ATP-induced [Ca²⁺], increase leads to increased transepithelial Cl⁻ and water transport.

Both L-type and purinergic receptor channels open only in response to specific stimulation, such as depolarization (α1D) or binding of ATP (P2X). Ca²⁺ is permanently extruded from RPE cells through constitutively active plasma-membrane Ca²⁺ ATPases and Na⁺/Ca²⁺ exchangers.¹²,¹³ Therefore, unstimulated RPE cells seem to have a Ca²⁺ leak pathway for Ca²⁺ influx that stabilizes the basal [Ca²⁺], of 100 nM. In another study we have already demonstrated that this Ca²⁺ influx is not driven by a reverse mode of the Na⁺/Ca²⁺ exchanger.¹⁴ Here we show in addition that it is not driven by the already identified voltage-operated Ca²⁺ channels or the ionotropic purinergic receptor-operated channels. Instead, we show by pharmacologic reduction of the basal [Ca²⁺], that this background Ca²⁺ influx is carried out by a member of the transient receptor potential (TRP) channels.

MATERIALS AND METHODS

Cell Culture

The human retinal pigment epithelial cell line ARPE-19 was cultured in Dulbecco modified eagle medium/F-12 nutrient mixture (D-MEM/F-12) containing 10% fetal bovine serum, insulin-transferrin-sodium (Roche, Basel, Switzerland), nonessential amino acids, and penicillin/streptomycin at 37°C in a humidified ambient atmosphere containing 5% CO₂. They were passaged twice per week. For Fura-2 measurements, they were seeded onto coverslips and cultured to confluence.

For primary cultures of human RPE cells, the anterior part of human donor eyes, including vitreous and retina, were removed. The RPE and choroid were carefully separated from the sclera, washed with PBS,
Table 1: Oligonucleotides Used to Amplify Transcripts of TRPC1–7

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
</table>
| TRPC1 | NM_003504 | Forward: 5'-TGGTACAACTGCTGGTG-3' 
Reverse: 5'-AACCTGGGGCAGGGTTACG-3' | 243 | 59 |
| TRPC3 | NM_003505 | Forward: 5'-GACTTGGAGAATGTGTG-3' 
Reverse: 5'-ATTGGCTGGGAATGTG-3' | 264 | 59 |
| TRPC4 | NM_016179 | Forward: 5'-TACCTCCCTAATGTCAT-3' 
Reverse: 5'-TTACAGGGTCCTCATAACG-3' | 183 | 54 |
| TRPC5 | NM_012471 | Forward: 5'-CAACTGGCTGGAATGTG-3' 
Reverse: 5'-AGTGCTTCGCAATCAGAGT-3' | 244 | 59 |
| TRPC6 | NM_004621 | Forward: 5'-GGCTCTTCATTACTGGTTG-3' 
Reverse: 5'-GTCGCTGGTTCTATTAGGAAG-3' | 171 | 54 |
| TRPC7 | NM_020389 | Forward: 5'-GCCACACCATACCAAGAAA-3' 
Reverse: 5'-CAACCTCAGGTTCTTGT-3' | 241 | 56 |

and incubated overnight with collagenase IA/IV (0.5 mg/mL each) in serum-free culture medium. The dissociated cells were collected by centrifugation (50g, 5 minutes) and cultured on coverslips in the same medium as the ARPE-19 cells. These still pigmented RPE cells were used for Ca2+ measurements after they reached confluence. Human material was used in accordance with the tenets of the Declaration of Helsinki.

Measurement of Intracellular Free Ca2+ Concentrations

ARPE-19 cells grown on coverslips to confluence were washed with Ringer solution (130 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 5 mM glucose, 10 mM HEPES, pH 7.3, with NaOH) and loaded with Fura-2 AM ester (Fluka, Buchs, Switzerland) for 40 minutes in the dark at room temperature in Ringer solution containing 10 μM Fura-2 AM. Cells were washed and incubated for at least 30 minutes with Ringer solution. The coverslips were placed into a bath chamber perfused constantly with Ringer solution and mounted onto an inverted microscope (Axiovert 35; Carl Zeiss, Oberkochen, Germany) equipped with a 40× objective (Fluar; Carl Zeiss). To exclude the possible stimulation of mechanosensitive channels by the perfusion system, we stopped the perfusion and found no change in [Ca2+]i. We performed ratiometric measurement Fura-2 fluorescence at 5-second intervals using a high-speed polychromator system (VisiChrome; Visitron Systems, Puchheim, Germany) altering the wavelength of excitation light between 480 and 510 nm. Data were collected with MetaFluor software (Universal Imaging) and analyzed (MetaAnalysis software; Universal Imaging). Intracellular free Ca2+ ([Ca2+]i) was calculated from the Fura-2 fluorescence ratio (F488/F510). Mean fluorescence intensities after excitation with 340 nm were between 153 and 197 arbitrary units, indicating equal loading of the cells throughout all experiments.

RNA Isolation and RT-PCR

Human RPE was obtained from organ donors within 24 hours of death. After the cornea was removed for transplantation, the eyes were subjected for preparation of for organ culture. The anterior parts of the eyes, including the vitreous and the retina, were removed. The posterior part was rinsed with ice-cold PBS (without Ca2+ and Mg2+) to wash away residual material from the neural retina. With the use of fine forceps, the RPE was gently brushed away. RPE cells were collected and lysed in lysis buffer (RNeasy Mini Kit; Qiagen, Valencia, CA). Total RNA from ARPE-19 cells was prepared from confluent cultures grown in a 25-cm2 culture flask. RNA was isolated (RNeasy Mini Kit; Qiagen) according to manufacturer’s instructions. RNA (1 μg) was reverse transcribed at 37°C for 1 hour in the following reaction mixture: 1 μg oligo dT primer (Invitrogen, Carlsbad, CA), 1 mM of each dNTP, 20 U RNaseguard (Amersham Biosciences, Freiburg, Germany), and 20 U M-MLV reverse transcriptase (Invitrogen). For control PCR reactions, human total brain RNA (Stratagene, La Jolla, CA) was reverse transcribed under the same conditions. PCR experiments were performed with 1 μl cDNA in 50-μl PCR reaction mixtures with Taq DNA polymerase (Stratagene) and 1.5 pmol of sense and antisense oligonucleotides specific to the various TRPC channel subunits (Table 1). The identity of the amplification product was confirmed by sequencing.

Data Analysis

Results were presented as mean ± SEM. Statistical significance was tested using one-way analysis of variance (P < 0.05; statistical significance; P < 0.01, strong statistical significance; P < 0.001, very strong statistical significance).

RESULTS

Blocking of the plasma membrane Ca2+ ATPase in RPE cells by the application of 2 mM orthovanadate led to a pronounced increase in [Ca2+]i. The [Ca2+]i increased to 216.78% ± 10.46% of the basal value (n = 4; Fig. 1). Given that plasma membrane Ca2+ ATPases are responsible for Ca2+ efflux to maintain the low [Ca2+]i, this increase must have been caused by a permanent Ca2+ influx through a sustained membrane conductance for Ca2+.

It has already been shown that RPE cells have some types of Ca2+-conducting ion channels, such as voltage-operated L-type Ca2+ channels and purinergic receptors. Therefore, we first tested the influence of blockers of these currents on the resting [Ca2+]i by applying 2 mM orthovanadate. The [Ca2+]i increased to 216.78% ± 10.46% of the basal value (n = 4; Fig. 1). Given that plasma membrane Ca2+ ATPases are responsible for Ca2+ efflux to maintain the low [Ca2+]i, this increase must have been caused by a permanent Ca2+ influx through a sustained membrane conductance for Ca2+.

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![Image](image-url)
TRPC1 and TRPC4 were expressed in the RPE. TRPC7 was also expressed in freshly isolated cells (Fig. 7).

DISCUSSION

In this study, we showed for the first time that RPE cells have large basal membrane permeability for Ca$^{2+}$, as indicated by an increase in [Ca$^{2+}$], after inhibition of the plasma membrane Ca$^{2+}$ ATPase. This basal Ca$^{2+}$ conductance was inhibited by blockers with the same pharmacologic profiles as TRPC channels. We were able to exclude the contribution of voltage-operated Ca$^{2+}$ channels and purinergic receptors to basal Ca$^{2+}$ entry. By RT-PCR we confirmed the expression of TRPC channels in the RPE.

The RPE is a target for and a source of various cytokines whose intracellular signaling cascades are coupled to [Ca$^{2+}$]. Furthermore, [Ca$^{2+}$] changes are involved in many other RPE functions, such as photoreceptor outer segment phagocytosis, transcellular fluid and ion transport, cell differentiation, and the control of gene expression. Nevertheless, until now, only voltage-operated L-type Ca$^{2+}$ channel α1D expression has been shown in RPE cells. These channels are involved in growth factor signaling in the RPE. In addition to these studies about voltage-operated channels, only one other study based on pharmacologic evidence of purinergic receptors suggests that ionotropic purinergic receptors (P2X) are involved in the regulation of fluid and ion transport across the RPE. In addition, it has been reported that NMDA receptors are expressed in the RPE, though it seems unlikely that these channels may contribute to resting [Ca$^{2+}$], because they need glutamate for stimulation.

Because lanthanides are known to inhibit Ca$^{2+}$ influx, we tested the effect of La$^{3+}$ and Gd$^{3+}$ on basal [Ca$^{2+}$], in RPE cells. Both ions reduced [Ca$^{2+}$] significantly; 100 μM La$^{3+}$ led to a reduction to 25.26% (n = 10), and 100 μM Gd$^{3+}$ to 14.57 ± 2.67% (n = 5) of the resting [Ca$^{2+}$] (Fig. 5). Although La$^{3+}$ is an unspecific blocker of different Ca$^{2+}$ channels, the sensitivity of the individual Ca$^{2+}$ channel types to La$^{3+}$ is very different. Therefore, we investigated the concentration dependence of the La$^{3+}$ effect on the basal [Ca$^{2+}$]. At the lowest concentration of 0.2 μM, La$^{3+}$ already reduced basal [Ca$^{2+}$] to 39.7% ± 11.72% (n = 7; Fig. 4). Gd$^{3+}$ is also known to inhibit Ca$^{2+}$ influx through different Ca$^{2+}$ channels. Application of 2 mM Ni$^{2+}$ reduced the [Ca$^{2+}$] to 33.08% ± 8.95% (n = 6).

Lanthanides and Ni$^{2+}$ are relatively unspecific inhibitors that affect various ion channels and transporters, but at low concentrations they are known to be efficient blockers of some members of the TRP channel family. In addition, we used blockers influencing a more narrow number of channels, including TRPC channels. The application of 2-APB and SKF 96365 to RPE cells resulted in a decrease of [Ca$^{2+}$], comparable to that seen with La$^{3+}$ or Gd$^{3+}$. 2-APB (75 μM, n = 8) and SKF 96365 (50 μM, n = 8) led to a decrease of [Ca$^{2+}$]$_{i}$ to 23.55% ± 5.83% and 63.78% ± 8.85%, respectively (Fig. 5). This reduction of basal [Ca$^{2+}$], was qualitatively reproduced with primary cultures of RPE cells from human donor eyes (Fig. 6). The basal [Ca$^{2+}$], was reduced in these cells by the application of 75 μM 2-APB and 50 μM SKF 96365 to 55.7% ± 9.38% and 67.36% ± 4.63%, respectively.

The pharmacologic profile of blockage of basal Ca$^{2+}$ entry observed here suggested that it was driven by members of the TRPC subfamily. RT-PCR with RNA from the RPE cell line ARPE-19 and from freshly isolated RPE cells showed that distinct from voltage-operated Ca$^{2+}$ channels and P2X channels expressed in these cells.

In this study, we showed for the first time that RPE cells have large basal membrane permeability for Ca$^{2+}$, as indicated by an increase in [Ca$^{2+}$], after inhibition of the plasma membrane Ca$^{2+}$ ATPase. This basal Ca$^{2+}$ conductance was inhibited by blockers with the same pharmacologic profiles as TRPC channels. We were able to exclude the contribution of voltage-operated Ca$^{2+}$ channels and purinergic receptors to basal Ca$^{2+}$ entry. By RT-PCR we confirmed the expression of TRPC channels in the RPE.

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Thus, considering the multitude of different \([Ca^{2+}]_i\)-regulated RPE cell functions, additional \(Ca^{2+}\) channels are likely to be expressed in the RPE. TRP channels are good candidates because they are coupled to a variety of intracellular signaling pathways. RPE cells use energy to continuously extrude \(Ca^{2+}\) effectively from the intracellular space through a plasma-membrane \(Ca^{2+}\) ATPase and a \(Na^+/Ca^{2+}\) exchanger. Because some of the \(Ca^{2+}\)-induced processes, such as basal VEGF secretion, must persist without stimulation, RPE cells need an equally effective \(Ca^{2+}\) entry pathway. To our knowledge, such a leakage \(Ca^{2+}\) pathway is only described for vascular smooth muscle cells, osteoblastlike cells, and chromaffin cells.20

Our findings that \(La^{3+}, Gd^{3+}, Ni^{2+}, 2-APB, and SKF 96365\) inhibited this nonstimulated \(Ca^{2+}\) entry indicated that TRP channels are the molecular correlate of this \(Ca^{2+}\) leak. Although none of these blockers is specific for a particular class of TRP channels, conclusions concerning the molecular identity of the channels can be drawn from the combination of their effects. TRPV channels are either insensitive to or activated by 2-APB.27 Accordingly, these channels can be excluded from their possible role in basal \(Ca^{2+}\) entry in RPE cells. TRPM channels can be excluded because of their insensitivity to \(Ni^{2+}\). Thus far, it has been found that TRPM channels are highly permeable to \(Ni^{2+}\).28,29 Therefore, we concentrated on the TRPC channels.

Our RT-PCR experiments revealed that TRPC1 and TRPC4 are expressed in ARPE-19 and cultured RPE cells from human donor eyes. In the latter, we also found TRPC7 to be expressed. \(La^{3+}\) and \(Gd^{3+}\) block a variety of TRP channels.30 They also inhibit a variety of other ion channels and transporters.31–35 All non-TRP channels, however, have a much lower \(La^{3+}\) sensitivity than the leak \(Ca^{2+}\) channel investigated in this study. Voltage-operated \(Ca^{2+}\) channels had an IC\(_{50}\) of 1.1 mM.34 Most TRP channels have much higher \(La^{3+}\) sensitivity than voltage-operated \(Ca^{2+}\) channels. Nevertheless, their sensitivity is much higher (IC\(_{50}\), 3–100 \(\mu M\)) than the IC\(_{50}\) for \(La^{3+}\) block of basal \(Ca^{2+}\) entry in RPE cells.36–39

Some study results conflict with ours. In these studies, TRPC4-mediated currents were potentiated rather than blocked, as in our study, by \(La^{3+}\) in micromolar concentrations.40–42 In other studies, TRPC4 channels were blocked by \(La^{3+}\).43–45 Comparative studies of wild-type and TRPC4 knockout mice revealed very high \(La^{3+}\) sensitivity of these native TRPC4 channels in the nanomolar range.43,46 This discrepancy might be explained by the fact that the potentiation of these currents could only be observed in the heterologous expression system, whereas studies indicating high \(La^{3+}\) sensitivity of

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932948/)

**Figure 4.** Concentration-response curve for the influence of \(La^{3+}\) on basal \([Ca^{2+}]_i\). (a) Stepwise reduction of \([Ca^{2+}]_i\) after application of increasing concentrations of \(La^{3+}\) calculated from Fura-2 measurements. (b) Influence of increasing concentrations \(La^{3+}\) on \([Ca^{2+}]_i\). Compared are the steady state values with the different \(La^{3+}\) concentrations. Mean ± SEM \([Ca^{2+}]_i\), normalized to the concentration before \(La^{3+}\) application \((n = 2–15)\). Note that the x-axis is interrupted between 15 and 45 \(\mu M\) \(La^{3+}\).

![Figure 5](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932948/)

**Figure 5.** Influence of 2-APB and SKF 96365 on basal \([Ca^{2+}]_i\), in RPE cells. (a) \([Ca^{2+}]_i\), changes after application of 2-APB (75 \(\mu M\)) or SKF 96365 (50 \(\mu M\)) calculated from Fura-2 measurements. (b) \([Ca^{2+}]_i\), before (black bars) and during (gray bars) application of the TRP channel blockers 2-APB \((n = 8)\) and SKF 96365 \((n = 8)\). Both molecules reduced \([Ca^{2+}]_i\), significantly. *\(P < 0.05\). **\(P < 0.001\).

![Figure 6](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932948/)

**Figure 6.** Influence of 2-APB and SKF96365 on basal \([Ca^{2+}]_i\), in primary cultures of RPE cells. (a) \([Ca^{2+}]_i\), changes after the addition of SKF96365 (50 \(\mu M\)) calculated from Fura-2 measurements. (b) Mean \([Ca^{2+}]_i\), before (black bars) and during (gray bars) application of SKF96365 \((n = 5)\) and 2-APB \((n = 5)\). Both molecules reduced \([Ca^{2+}]_i\), significantly in all five independent experiments. *\(P < 0.05\).
TRPC4 were conducted with endogenously expressed channels in native cells.

TRPC4 has been shown to coassemble with TRPC1 to form heteromultimeric channels.47 These heteromultimeric channels might be dominated by the high La3+ sensitivity of native TRPC4 channels.

In another single study on TRP channels in the RPE, it was found that only TRPC1 is expressed in ARPE-19 cells.48 TRPC4 is known to be differentially spliced.41,49,50 Because we amplified different parts of the gene, our results may be attributed to a splice variant that Bollimuntha et al.48 could not detect with their oligonucleotides. In addition, we confirmed the expression of TRPC1 and TRPC4 in native RPE cells. We also found TRPC7 to be expressed in native RPE cells. It has been thought that heteromultimers could only be formed among TRPC1, TRPC4, and TRPC5 and among TRPC3, TRPC6, and TRPC7.47 Nevertheless, some studies show that heteromultimers can also be formed between these groups.51,52 Hence, the additional expressed TRPC7 might be a part of a heteromultimeric channel carrying the basal influx into native RPE cells.

Ca2+ is one of the fundamental intracellular signaling molecules. TRPC channels mediating the leak Ca2+ entry to the RPE may be involved in basal cellular processes controlled by [Ca2+]i, such as basal secretion of cytokines. Additionally, TRPC channels are coupled to intracellular signaling pathways that are activated by G protein-coupled receptors. On stimulation, these channels may contribute not only to the adjustment of resting [Ca2+]i, but also to that of elevated [Ca2+]i.53 Because TRPC channels are nonselective cation channels, the TRPC channels identified in this study may also contribute to the resting membrane potential in RPE cells. Human RPE cells have a resting membrane potential of approximately -45 mV.54,55 Given that they have high K+ permeability,54 the observed resting potential requires an additional Na+-permeable current that shifts the resting potential to values positive to the equilibrium potential of K+. TRPC channels are known to set the membrane potential and the basal Ca2+ entry56 in other cell types. They may also do so in the RPE.

Acknowledgments

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References


Figure 7. Expression profile of TRPC channels in ARPE-19 cells compared with the expression pattern in freshly isolated RPE cells from human donor eyes.


