Dihydropyridine-Sensitive Calcium Currents in Freshly Isolated Human and Monkey Retinal Pigment Epithelial Cells

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Purpose. The authors previously reported that rat retinal pigment epithelial (RPE) cells exhibit an ionic current through voltage-operated calcium channels that is dihydropyridine sensitive. They attempted to record the same current from freshly isolated fetal or adult primate RPE cells, as well as from cultured cells.

Methods. The whole-cell version of the patch-clamp technique was applied to RPE cells freshly isolated by enzymatic dissociation from fetal human and adult human and monkey eyes, as well as cultured fetal and adult human RPE cells. The cells were loaded with cesium to minimize potassium-related current and were bathed in an extracellular solution containing 40 mM barium to intensify the calcium currents.

Results. Freshly isolated cells, both fetal and adult, showed sustained and inward-going barium current through voltage-operated calcium channels with membrane depolarizations from a high holding voltage of −60 mV. This current was affected by dihydropyridine compounds. Cultured human RPE cells showed no sign of a calcium current of this type.

Conclusions. Freshly isolated fetal and adult human RPE cells, as well as adult monkey, exhibit calcium current through voltage-operated, dihydropyridine-sensitive channels, similar to the neuronal L-type, just as in rat RPE cells. Invest Ophthalmol Vis Sci. 1995;36:373-380.

Voltage-operated calcium channels (VOCCs) have been mainly observed in excitable cells. Neural cells possess several subtypes of VOCCs that may play a number of different roles.1-5 In muscle and in secretory cells, VOCCs have been reported as well, and they are thought to be involved, respectively, in the contraction mechanism and in hormone secretion.4-5 There are observations of VOCCs from so called non-excitable cells, such as glial cells and epithelial cells.6-7 Although the role of VOCCs is still not clear, they could act as part of an intracellular second messenger pathway.

Retinal pigment epithelial (RPE) cells form a monolayer of cuboidal cells that separate sensory retina from the choroidal vessels and function to phagocytize photoreceptor outer segments, transport water and metabolites, and maintain a suitable extracellular environment for the sensory retina, especially the photoreceptors. Calcium plays important roles in light adaptation in vertebrate photoreceptors.6-10 The calcium concentration around the photoreceptor outer segment is probably largely controlled by photoreceptor calcium permeability, by RPE cells through an hydration mechanism.11,12 and by unidentified mechanisms of calcium transport. Little is known, however, about the membrane properties of RPE cells with respect to calcium ions.

Recently, patch-clamp techniques have been used to investigate the ion channels of RPE cells. We previously reported that rat RPE cells, both fresh and cultured, possess VOCCs that are similar to the neuronal L-type.13 We were interested to discover if such calcium currents occur in primate RPE cells because ionic currents of RPE cells exhibit species differences (potassium currents14,15; chloride currents16-18).

In the present study, we asked if human and monkey RPE cells possess VOCCs. Human fetal and adult,
FIGURE 1. Inward current was observed from freshly isolated fetal human retinal pigment epithelial cells with membrane depolarization in 40 mM barium solution, and it reversibly disappeared in 4 mM cobalt solution. (A) Step pulses were applied from a holding voltage of −60 mV to between −50 and 50 mV in 20-mV steps. Sustained inward current was observed with depolarization, reached its maximum at 10 mV, and then became smaller. (B) This inward current was reversibly inhibited in 4 mM cobalt solution, which did not contain barium. (C) The average I–V relationship was drawn from the negative peak of the current in either 40 mM barium (control) or 4 mM cobalt solution (n = 3). I–V = current–voltage.

as well as monkey adult, fresh RPE cells showed VOCCs similar to those of rat cells. In contrast, cultured human fetal RPE cells did not have this type of channel.

MATERIALS AND METHODS

Cell Dissociation

Postmortem human eyes were obtained from organ donors after the cornea and sensory retina were removed. Information about sex or age was not always available and is not given in the text. Adult rhesus monkey eyes were obtained after the animal was killed for neuroscience experiments that had not affected the eyes. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In both cases, sensory retina had been removed, and the RPE–choroid–scleral segment was available. Then, RPE–choroids were peeled away from the sclera and incubated in Hank’s balanced salt solution (HBSS) containing collagenase (150 U/ml, type V, Sigma) for 30 minutes −2 hours at 36°C, pH 7. Retinal pigment epithelial monolayers were mechanically separated from choroid and triturated with a Pasteur pipette after bathing for 2 minutes in calcium- and magnesium-free HBSS containing trypsin (1 mg/ml, Difco, Detroit, MI). Growth medium was added to stop the enzyme, and cells were centrifuged and washed with growth medium. They were stored in Dulbecco’s minimum essential medium (DMEM) and suspended at 5°C before each experiment.

Fetal human eyes (14 to 22 weeks gestation) were obtained from the University of California Tissue Bank. They were bathed in HBSS, and the scleras and most of the choroid were mechanically peeled away. The eyes were incubated in HBSS containing collagenase (150 U/ml, type V, Sigma) for 30 minutes −2 hours at 36°C, pH 7. After residual choroid was removed from the bulb surface, the RPE was mechanically separated from sensory retina and cut into pieces, and the previously described method was followed.

Cell Culture and Dissociation of Cultured Cells

The isolated RPE cells were suspended in the growth medium, plated on 35-mm OD plastic culture dishes...
coated with extracellular matrix (derived from bovine endothelial cells), and incubated for 2 ~ 4 weeks at 38°C. The growth medium contained DMEM supplemented with 15% fetal bovine serum, 1 ng/ml basic fibroblast growth factor, and antibiotics. The cells lost their characteristic morphology and pigmentation, proliferated, and became hexagonally shaped after confluence. After several weeks, pigmentation reappeared. For passaging, cells were dissociated before confluence.

In some experiments, a serum-free medium was used instead. Confluent cells in the growth medium were switched to a serum-free medium of DMEM supplemented with 1 μg/ml insulin, 1 μg/ml transferrin, 5 ng/ml selenium, and 1 μg/ml bovine pituitary extract (Clonetics, La Jolla, CA) and incubated in 10% CO2 and 90% air for more than 20 days and then seeded to new extracellular matrix-coated plates and cultured in the serum-free medium.

To dissociate cultured cells, the dishes were washed with calcium- and magnesium-free HBSS. Then cells were incubated in calcium- and magnesium-free saline containing 0.05% trypsin and 0.02% versene at 20°C for 2 minutes. During the incubation, cells were examined with the microscope. When we observed cells that could be isolated, growth medium was added to stop the enzyme. Cells were centrifuged, washed, and stored in DMEM at 5°C.

Recording Conditions

The recording chamber was mounted on the stage of an inverted microscope (Olympus, Tokyo, Japan) with phase-contrast optics. A glass coverslip formed the bottom of the chamber, and the cells were allowed to settle for more than 10 minutes before starting the perfusion. Solutions (room temperature, 20 ~ 24°C) were gravity fed into the chamber at a rate of 2 ml/minute through a Y-tube and removed by suction.

Membrane currents were recorded by a patch-clamp system in the whole cell configuration. Patch pipettes were pulled from 1.5-mm OD Pyrex tubes with filament (Glass Company of America, Millville, NJ) by a vertical puller (Narishige, Tokyo, Japan), and the outer wall, except for the very tip, was coated with Apiezon wax (Apiezon Products, London, UK). The pipette solution contained (in mM) 160 CsCl, 5 BAPTA, 20 HEPES, 2 Mg-ATP, 1 c-AMP, 0.5 GTP, and 0.2 leupeptin to intensify calcium current and to minimize its run down, and also to suppress outward potassium current. Pipettes had resistance of 5 ~ 10 MΩ when bathed in Eagle’s minimum essential medium with Earl’s BSS containing 0.292 g/1 L-glutamine. Before the whole-cell configuration was achieved, the extracellular solution was changed to the standard extracellular solution containing (in mM) 80 Tris-HCl, 20 tetraethylammonium-Cl, 10 CsCl, 40 BaCl2, 1 MgCl2, 10 HEPES, and 10 glucose, which was designed to intensify calcium currents and block inward and outward potassium channels. Osmolarity was adjusted to ~310 mOsm. The pH of all solutions was adjusted to 7.5 with additional CsOH. All extracellular solutions, except for enzyme solutions and growth medium, contained 0.1 mg/ml of bovine serum albumin as a surfactant and 0.001% phenol red for easier control of pH. Experiments were performed within 5 hours of dissociation.

Currents were recorded by an amplifier (Axopatch 1B, Axon Instrument, Foster City, CA). The indifferent electrode was a Ag-AgCl wire connected to the chamber by an NaCl-agar bridge. The generation of holding and command voltages and storage and analysis of the data were performed by p-CLAMP (Axon Instrument) on a microcomputer. Before sampling at 2 ~ 10 kHz, data were low-pass filtered (Frequency Devices, Haverhill, MA; 902LPF 8 poles bessel) with an appropriate cutoff frequency.

Nonspecific leakage current, membrane capacitance, and series resistance were electrically compensated. Junction potentials were directly measured by 2 mM KCl filled electrodes, and the corrected values are given in this article.

Chemicals

BaCl2, CaCl, Tris-HCl, CoCl2, BSA, HEPES, nifedipine, magnesium adenosine triphosphate (MgATP), cyclic adenosine monophosphate (c-AMP), and leupeptin were purchased from Sigma; KCl, NaCl, MgCl2, CaCl2,
and D-glucose were purchased from Fisher Scientific (Pittsburgh, PA); tetraethylammonium-Cl was purchased from Eastman Kodak (Rochester, NY); Bay K 8644 was purchased from Research Biochemicals (Natick, MA); and bis-(o-aminophenoxy)-ethane-N,N',N'-tetraacetic acid was purchased from Molecular Probes (Eugene, OR).

RESULTS

Freshly Isolated Fetal Human Cells

Retinal pigment epithelial cells, isolated from human fetal eyes, were held at $V_h = -60$ mV in 40 mM barium solution, and positive step pulses to between $-50$ and 30 mV for either 200 or 400 msec were applied. An inward-going sustained current was observed (Fig. 1A; $n = 12$ out of 39). Figure 1B shows that the inward current reversibly disappeared in 4 mM cobalt solution. The current–voltage (I–V) relationship of the negative peak current, either in 40 mM barium solution or 4 mM cobalt solution, was normalized to the maximal current and averaged as shown in Figure 1C ($n = 3$). This inward-going sustained current activated with command pulses more positive than $-40$ mV from a $V_h = -60$, and the amplitude reached its peak at 10 mV (some cells peaked at -10 mV.)

In one cell, extracellular barium ions were replaced with equimolar (40 mM) calcium ions, and current amplitude decreased, as shown in Figure 2. From these features, it is likely that this current was carried through high-voltage-activated calcium channels.

Calcium channels are divided into several subtypes classified by pharmacologic sensitivity. For further characterization, we applied dihydropyridine compounds. The current was enhanced by $10^{-7}$ M Bay K 8644 and was blocked partially by $10^{-6}$ M nifedipine ($n = 4$) (Fig. 3A). Figure 3B shows that the peak of the I–V relationship shifted negatively with Bay K 8644 application, as is commonly observed.

Even when the $V_h$ was changed to $-100$ mV, current waveform did not change (Fig. 4A). Also, a transient component was not observed, and the waveforms were nearly identical to those obtained with $V_h = -60$ (Fig. 4B). From $V_h = -100$ mV, the current activated with command pulses more positive than $-30$ mV and reached a peak at $-10$ mV, as was observed in $V_h = -60$ mV, whereas total amplitude increased with more negative $V_h$. However, because negative shifts of the holding voltage caused the cell to deteriorate, we could not confirm these data in a sufficient number of cells. These findings suggested that the voltage-operated calcium channels in human fetal RPE cells are mainly dihydropyridine-sensitive high-voltage acti-
DHP-Sensitive Calcium Currents in Primate RPE Cells

Figure 4. Negative shifts of the holding voltage did not markedly change the waveforms in a freshly isolated human retinal pigment epithelial cell. (A) Holding voltage is -100 mV, and the cell was depolarized with step pulses between -90 and 30 mV in 20-mV steps. No marked change was observed when compared with the record from Vh -60 (B). (C) I-V relationship showed a small voltage-sensitivity, although no sign of other voltage-sensitive current was detectable. I-V = current-voltage.

Freshly Isolated Adult Monkey and Human RPE Cells

Retinal pigment epithelial cells were obtained from human adult postmortem eyes, and inward-going sustained current was observed when cells were depolarized to 10 mV from Vh -60 mV in 40 mM barium solution, whereas they showed less current at a command pulse of either -30 mV or 50 mV. The current reversibly disappeared in 4 mM cobalt. The barium current peaked at a command pulse of between 10 mV and 30 mV, and less current was carried in 40 mM calcium solution. The current was dihydropyridine (DHP) sensitive; 10^-7 M Bay K 8644 enhanced the current (n = 4). However, 10^-6 M nifedipine showed little (n = 2), or no effect (n = 2).

Because of the limited availability of RPE cells from adults, we could not further characterize their calcium channels. However, it is clear that adult primate RPE cells, both from human and monkey, possess high-voltage-activated calcium channels that are dihydropyridine sensitive.

Cultured Fetal and Adult Human RPE Cells

In a previous study from this laboratory, it was observed that cultured RPE cells from rat possess voltage-operated calcium channels that are identical to fresh RPE cells. Therefore, we tried to record voltage-operated calcium channels from cultured human RPE cells. Human RPE cells were cultured under several conditions. All the following cells failed to show voltage-operated calcium channels, similar or identical to the neuronal L type.
FIGURE 5. DHP-sensitive calcium current in adult human retinal pigment epithelial cells. (A) Step-pulse depolarization from -60 mV to 10 mV evoked an inward-going sustained current. This was not observed at the command pulse of -30 mV, and it disappeared at 50 mV. (B) In 4 mM of cobalt solution, the current reversibly disappeared, although current amplitude decreased, probably due to run down. (C) I–V relationship shows that the current peaked at a command step of 30 mV in 40 mM barium solution (closed circles), and the amplitude decreased in 40 mM calcium solution (open squares) or almost disappeared in 4 mM cobalt solution (open circles). (D) 10^{-7} M Bay K 8644 enhanced current amplitude. DHP = dihydropyridine; I–V = current–voltage.

age-operated calcium channels; cells from 30-day primary culture (with serum, from fetal eyes; n = 11), 16-day third passage culture (serum free, from fetal eyes; n = 10), 30-day third passage culture (with serum, from adult eyes; n = 15, and serum free, from fetal eyes; n = 9), 3-month primary culture (with serum, from fetal eyes; n = 2). Some of those cells were heavily pigmented.

DISCUSSION

We dissociated RPE cells from fetal and adult human and adult monkey eyes. Under the whole-cell configuration of the patch-clamp technique, fresh RPE cells from all these specimens exhibited calcium currents through DHP-sensitive voltage-operated calcium channels (VOCCs), whereas cultured human RPE cells did not show any sign of these currents.

VOCCs play important roles in excitable cells, and their presence in nonexcitable cells also has been reported. The functions of the channels are still obscure for many types of cells. In rabbit, epithelial cells of the ciliary body studied in vitro generated action potentials via barium currents through dihydropyridine-sensitive calcium channels, whereas RPE cells in vivo have not yet been reported to show spikes. The intracellular calcium concentration in cultured human RPE cells was observed to increase in response to muscarinic agents, vasopressin, or platelet-derived growth factor. It is likely that calcium is involved in the intracellular second messenger system of fresh RPE as well, and VOCCs may take part in such a system.

The calcium currents that we have described are relatively small when compared with other cells, and it was necessary to inhibit all other ionic currents and replace calcium with a high concentration of barium to observe them clearly. This may be due to a low density of channels, and it is questionable if the activation of VOCCs in RPE cells can elevate intracellular calcium concentration enough to activate any second messenger system. One possibility is that VOCCs are
highly localized and cause calcium hot spots, as is observed in neural growth cones. An intracellular calcium increase of as much as 1 μM did not alter the chloride conductance of rat RPE cells, although a large transient chloride conductance was triggered when 10 μM ionomycin, a calcium ionophore, was applied to a cell bathed in 2 mM calcium solution. This may represent additional indirect evidence that intracellular calcium ions work in a limited subcellular area where the local calcium concentration reaches extraordinarily high levels.

Calcium currents from freshly isolated primate RPE cells were similar to those from either freshly isolated or cultured rat RPE cells. This supports the notion that rat RPE cells may be a good model for human RPE cells as far as calcium currents are concerned. It is not clear why we did not record the same large transient chloride conductance of rat RPE cells, although a similar chloride conductance is present in fresh fetal human RPE cells (16 to 21 weeks gestation). Also, cultured human and monkey RPE cells exhibit a tetrodotoxin-sensitive sodium current, which is absent in fresh adult human and monkey. With regard to calcium channels, we have shown another possible difference between cultured cells (absence) and fresh cells (present) in human and monkey. This difference was not, however, present in rat, where cultured cells exhibit the same DHP-sensitive calcium current as fresh cells. Future experiments will be needed to determine why certain classes of channels appear (A-current and tetrodotoxin-sensitive sodium current) or disappear (DHP-sensitive calcium current) in cultured primate RPE.

Key Words
retina, retinal pigment epithelium, patch-clamp, voltage-operated calcium current, dihydropyridine

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References
18. Ueda Y, Steinberg RH. Chloride currents in freshly


