Nonpigmented Cells of the Rabbit
Ciliary Body Epithelium

Tissue Culture and Voltage-Gated Currents

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The aqueous humor of the eye is thought to be secreted by the epithelium of the ciliary body. This epithelium has been difficult to study, in part because of its complicated morphology. The authors attempted to circumvent this difficulty by growing the epithelial cells in tissue culture. A procedure is described for producing pure primary cultures of rabbit nonpigmented ciliary body epithelial cells. This procedure was used with whole-cell patch-clamp recording to characterize voltage-activated currents in the nonpigmented cells. These experiments show that most nonpigmented cells contain two kinds of currents: a rapidly activating and inactivating inward current, carried by Na+ and blocked by tetrodotoxin (TTX), and a more slowly activating and inactivating outward current, blocked by tetraethylammonium (TEA+), Ba2+, and 4-aminopyridine (4-AP) and presumably carried by K+. Both of these currents have been observed in freshly dissociated cells and in cultures up to 7 days old. The voltage-gated currents in ciliary body epithelial cells are remarkably similar to those of neurons and raise the possibility that these epithelial cells are capable of spike propagation. Invest Ophthalmol Vis Sci 32:1619-1629, 1991

The epithelium of the ciliary body lies at the inner margin of the eye between the retina and the iris and is thought to be responsible for secreting the aqueous humor.1 The physiology of this epithelium has been difficult to study, in part because of its complicated morphology. The epithelium lies on the surface of the highly invaginated ciliary processes and is difficult to mount in an Ussing chamber. Furthermore, there are two kinds of epithelial cells which occur in separate layers: the pigmented cells which face the stroma and the nonpigmented, which face the lumen of the posterior chamber. These two cell types have different populations of transport enzymes, receptors, and other proteins.2-4 The cells of these two layers are coupled electrically1 by an extensive network of gap junctions, both among the cells of each layer and from one layer to the other.5 This makes it difficult to interpret recordings from intact or semiintact epithelial preparations.7

To overcome these difficulties, it would be useful to isolate the two cell layers and grow each separately in tissue culture. The pigmented cells from mammals are easy to grow9-12 and can be identified by their dark-staining melanin granules. We have previously shown the feasibility of using such preparations for patch-clamp recording and characterized voltage-gated currents in the pigmented cells of adult rabbits.13

The nonpigmented cells are more difficult to culture. Methods for the isolation and growth in tissue culture of nonpigmented cells from human fetal and bovine eyes have been described,14-16 but it has not yet been possible to establish the identification of these cells firmly or to demonstrate their usefulness for physiologic investigations. We showed in the rabbit that it is possible to isolate the nonpigmented layer from the rest of the ciliary body17 and to identify the nonpigmented cells in culture with a monoclonal antibody against the transport enzyme H+-K+ adenosine triphosphatase (H+-K+ ATPase).6 However, we were also unsuccessful in our initial attempts at culturing these cells reliably enough to permit physiologic investigation.

We now describe methods that make possible the routine propagation of pure cultures of identified nonpigmented cells and show that it is possible to make patch-clamp recordings from these cells. In addition we use patch techniques to characterize two
types of voltage-gated membrane conductances. These experiments have given the surprising result that both freshly dissociated and cultured nonpigmented cells have tetrodotoxin (TTX)-sensitive, Na+-dependent inward currents. Since such currents are also present in the pigmented cells, our results raise the possibility that the syncytium of the ciliary body epithelium in mammals is capable of electrical activity.

Materials and Methods

Cell Culture

Nonpigmented ciliary body epithelial cells were isolated as a continuous layer free from pigmented cells and choroid by methods similar to those previously described. In brief, 2-, 7-, or 14-day-old pigmented rabbits (progeny of a New Zealand white female mated with an American blue male) were killed with a lethal injection of chloral hydrate and/or pentobarbital. This procedure was in accord with the ARVO Resolution for the Use of Animals in Research. The eyes were rapidly enucleated and hemicut posterior to the ora serrata. The ciliary body was dissected carefully from the remaining anterior segment by sectioning posterior to the attachment of the lens capsule and anterior to the ora serrata to exclude iris, iridial processes, and retina. The isolated ciliary bodies were incubated for 3–5 hr at 37°C in a 1:1 mixture of Nutrient Mixture F-10 (#320-1550; Gibco, Grand Island, NY) and Minimal Essential Media (#320-1095AG; Gibco), containing 3.46 mM CaCl2, 0.49 mM KCl, 0.49 mM MgCl2, 520 nM cholesterol, 5 μM O-phosphoethanolamine, 1 μM FeSO4, 3.3 μM ribose, 150 μM sodium pyruvate, 1.9 μM ATP, 5 μM ethanolamine, and 0.79 mg/ml bovine serum albumin. The resulting cell suspensions were counted in a hemocytometer, and 3 × 10^4 cells were plated into plastic petri dishes which had glass cover slips cemented (with paraffin) over a hole in the bottom. In most experiments, these petri dishes were prepared by first growing a confluent culture of pigmented ciliary body epithelial cells on them with tissue from albino rabbits. We then extracted the cells from these cultures with ammonium hydroxide, leaving behind a layer of extracellular matrix (ECM) on top of which the nonpigmented cells were plated. In some experiments, a different approach was used. The cells were plated onto 13-mm diameter cover slips precoated by the manufacturer with ECM (#TC1F13; Accurate Chemical, Westbury, NY). These cover slips were placed on top of the previously cemented glass cover slips in the dishes. The cultures were maintained in an incubator at 37°C in 5% CO2 in air.

Immunohistochemistry

**Antibody:** Nonpigmented cells were identified in culture with a monoclonal antibody generated against the H+-K+ ATPase from parietal cells of gastric mucosa. Hybridoma clone #12.18 was provided by Dr. Adam Smolka (Medical University of South Carolina, Charleston, SC). Monoclonal antibodies were raised in Balb/c mice and purified from ascites fluid with agarose-recombinant protein A chromatography. Anti-H+-K+ ATPase antibodies were assayed for protein content and stored at −20°C.

**Immunohistochemical reaction:** Cell cultures were fixed for 2 min in cold methanol (−20°C), rehydrated for 2 min in distilled water, and washed three times for 5 min in 0.1 M sodium phosphate buffer (pH 7.4) with 0.9% NaCl (PBS). All washes were done with agitation on an orbital shaker. Cultures were either processed immediately for immunohistochemistry or stored at 4°C.

After fixation, cell cultures were treated for 10 min with 0.003% saponin in PBS, washed for 5 min with PBS alone, and then treated for 10–30 min with 10% purified normal goat serum (NGS) and 1% normal mouse serum was used in place of the primary antibody. The cultures were incubated for 2 hr at 37°C and subsequently washed for 15–30 min in 3 X 10^4 cells were plated into plastic petri dishes which had glass cover slips cemented (with paraffin) over a hole in the bottom. In most experiments, these petri dishes were prepared by first growing a confluent culture of pigmented ciliary body epithelial cells on them with tissue from albino rabbits. We then extracted the cells from these cultures with ammonium hydroxide, leaving behind a layer of extracellular matrix (ECM) on top of which the nonpigmented cells were plated. In some experiments, a different approach was used. The cells were plated onto 13-mm diameter cover slips precoated by the manufacturer with ECM (#TC1F13; Accurate Chemical, Westbury, NY). These cover slips were placed on top of the previously cemented glass cover slips in the dishes. The cultures were maintained in an incubator at 37°C in 5% CO2 in air.

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PBS. They were then treated with 2% NGS for 15 min, and after removal of the NGS, the tissue was incubated for 1 hr at 37°C with 2% NGS containing goat anti-mouse immunoglobulin G conjugated to fluorescein isothiocyanate (FITC, #A10161; American Qualex) or tetramethylrhodamine isothiocyanate (TRITC, #A106RN; American Qualex). After this incubation, the cultures were washed in multiple changes of PBS for 1 hr, cover slipped with glycercol:PBS (9:1), and examined with Zeiss filter set #H485 (New York, NY) for FITC or #H546 for TRITC. Tissue sections prepared as previously described6 were run in parallel with the cultures as controls for the immunohistochemical reaction.

Histology: Cultures were fixed for 10 min with 2% glutaraldehyde in PBS and washed three times for 5 min each in PBS. Fixed cultures were stored at 4°C.

Electrophysiology

Electrophysiological recordings were made from isolated, nonpigmented ciliary body epithelial cells using standard patch-clamp techniques20 and an EPC-7 patch-clamp amplifier (Adams & List Associates, Great Neck, NY). The recordings were made from 2 hr to 7 days after dissociation of the intact ciliary epithelium. The cells used for the recordings came from a total of six dissociations of nonpigmented layers from 48 rabbits aged 2, 7, or 14 days (8 rabbits per dissociation).

During the experiments, the cells were superfused continually with HEPES- or bicarbonate-buffered saline at ambient temperature (20–23°C). Most of the recordings were made in HEPES-buffered solutions. When the bicarbonate-buffered saline was used, a mixture of 5% CO2 and 95% O2 gas was bubbled continuously into the perfusion reservoir to maintain the pH of the bathing solution at 7.4. No systematic differences were observed in the voltage-activated currents between cells in HEPES and bicarbonate buffer. The pH in the internal (pipette) solution was controlled by a solenoid-activated pinch valve (Neptune Research, Maplewood, NJ). It was impractical to use bicarbonate-buffered solutions with this system because of the difficulty of maintaining physiological pH in the U-tube. Therefore, HEPES-buffered external solutions were used for all experiments where drugs were applied. Tetrodotoxin (TTX) was obtained from Calbiochem (LaJolla, CA), tetraethylammonium (TEA+) chloride and tetramethylammonium (TMA+) chloride from Aldrich (Milwaukee, WI), and 4-aminopyridine (4-AP) from Sigma (St. Louis, MO).

Patch electrodes were pulled from either thick-walled borosilicate (internal diameter [ID], 0.86 mm; outer diameter [OD], 1.5 mm; Sutter, San Rafael, CA) or KG-33 (ID, 0.8 mm; OD, 1.6 mm; Garner, Claremont, CA) capillary tubing using a micropipetor-controlled puller (Model #P-80/PC; Sutter). Pipettes were used without coating or fire polishing. Electrode resistances ranged from 1–3 MΩ, and access resistances typically ranged from 5–20 MΩ. The series resistance compensation feature of the EPC-7 amplifier was used to cancel 50–70% of the voltage drop across the access resistance. No attempt was made to correct the recorded membrane potentials for the remaining voltage drop across the access resistance. Average tip potentials of the patch electrodes were measured as in Fenwick et al22 and were used to correct the recorded membrane potentials.

The data were recorded on videotape using a modified pulse-code modulator (Unitrade, Philadelphia, PA) after filtering with an eight-pole, low-pass Bessel filter (Model #902LPF; Frequency Devices, Haverhill, MA) at a cutoff frequency (Ff) of 500–1000 Hz. Data analysis was done off line with a personal computer (IBM AT, Armonk, NY) either with software developed by F. Bezanilla or with the PCLAMP program (Version 5.0; Axon Instruments, Burlingame, CA). Data were digitized at a sampling rate (Ff) at least four times greater than Fc.

Labeling of Cells With Lucifer Yellow

For further confirmation that cells from which recordings were made were nonpigmented ciliary body epithelial cells, we double labeled the cells with the intracellular dye Lucifer Yellow CH (#L1177; Molecular Probes, Eugene, OR) and with the H+-K+ ATPase antibody. Patch pipettes were filled with a KCl-based saline containing 1% Lucifer Yellow CH. The dye entered the cell by dialysis after the formation of a whole-cell recording. Voltage-activated currents were recorded from several cells in each culture dish using these pipettes. If the cell had voltage-activated inward and outward currents, the patch electrode was removed carefully, and the cell was left attached to the
bottom of the culture dish. If the cell did not have voltage-activated currents, the cell was destroyed, and its remains pulled off the bottom of the culture dish. After several cells were filled with the dye, the entire dish was fixed with methanol and stored at 4°C in the dark. The dish was then treated with the primary antibody to H⁺-K⁺ ATPase and secondary antibody coupled to rhodamine with the same procedure we used for identifying cultured cells.

Results

Culture of Nonpigmented Cells

We previously described a method for isolation of the ciliary epithelium which produces preparations that are highly enriched in nonpigmented cells. In the experiments described in this paper, the same isolation procedure was used, but the purity of our cultures was greater than previously reported, probably because of increased proficiency in the dissection of the epithelial cell layers. Cell cultures were screened systematically within 1-4 hr after plating, and most did not contain a single cell with pigment granules (out of approximately 30,000 cells in each dish). The few cultures which did contain pigmented cells were discarded.

Figure 1 illustrates the morphology of the cultured nonpigmented cells. One to 4 hr after plating (Fig. 1A), the cells had begun to attach to the substrate. Many cells exhibited microvilli, and some appeared slightly oblong. By 24 hr (Fig. 1B), many more cells had begun to flatten, and small colonies of cells could be observed. The cells continued to flatten and replicate over the next few days, forming larger groups of regularly arranged, polygonal cells (Fig. 1C). These

Fig. 1. Differential interference contrast photomicrographs of primary cultures of nonpigmented ciliary body epithelial cells fixed with glutaraldehyde at the times indicated. Cells were grown on ECM substrate prepared from albino ciliary body epithelial cultures. (A) At 3.5 hr after plating, nonpigmented cells were attached to the plate and showed microvilli. (B) After 21 hr in culture, many cells spread and small groups formed. (C) After 4 days in culture, many groups merged. (D) After 1 week in culture, confluent epithelial sheet formed. Magnification for (A) and (B) ×425 (1 cm = 23.3 μm); for (C) and (D) ×163 (1 cm = 61.4 μm).
Fig. 2. H⁺-K⁺ ATPase antibody staining of nonpigmented ciliary body epithelial cells as a function of time in culture. Cultures were fixed, incubated with HK12.18, and stained with FITC-conjugated goat anti-mouse IgG. Nonpigmented cells showed particulate staining over the entire cell at 3.5 hr (A), 21 hr (B), and 2 days (C) in culture. All cells on these plates showed fluorescence. By 4 days (D), fluorescence was present to a lesser extent in some cells. Fluorescence diminished at 1 (E) and 2 (F) weeks but was greater than the fluorescence of pigmented ciliary body cells or of nonpigmented cells treated with normal serum (not shown). Magnification for A-F, ×400 (1 cm = 25 μm).

Groups enlarged and reached confluence 1-2 weeks after plating (Fig. 1D). There was little difference between the cultures grown on ECM substrate prepared by us from previously cultured pigmented ciliary body epithelial cells or those grown on ECM substrate purchased commercially. It seemed that more cells attached initially to the commercial ECM and did so more consis-
Table 1. Frequency of occurrence of voltage-activated currents recorded from nonpigmented ciliary body epithelial cells as a function of length of time in culture

<table>
<thead>
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<th>Time in culture</th>
<th>No inward or outward</th>
<th>Inward, no outward</th>
<th>Outward, no inward</th>
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<td>0</td>
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<td>4</td>
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<td>47</td>
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</tbody>
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Identification of Cultured Cells

The monoclonal antibody HK.12.18 against the H⁺-K⁺ ATPase is a selective marker for the nonpigmented cells in vivo. Cultured nonpigmented cells also immunofluoresced when stained with this antibody, although the intensity of fluorescence diminished with time in culture. Cultures of pigmented ciliary body epithelial cells prepared separately did not exhibit fluorescence at any time.

Figure 2 illustrates the immunofluorescence observed from nonpigmented cell cultures which were fixed and then labeled with HK.12.18. Positive labeling was characterized by the appearance of particulate fluorescence, similar to that observed for cells in tissue sections. This labeling was observed for every cell without exception in cultures examined just after plating (Fig. 2A). A small, crescent-shaped region of intense fluorescence was often detected in these cells, similar to that seen along the apical membrane of nonpigmented cells in tissue sections after EGTA treatment (Cilluffo and Fain, unpublished). At 1 (Fig. 2B) or 2 days (Fig. 2C) after plating, bright particulate staining with the anti-H⁺-K⁺-ATPase antibody was still present in every cell in the dish, although the staining in the cells was now more uniform in distribution. By 4 days in culture (Fig. 2D), individual cells fluoresced with differing intensities. Cells in groups usually appeared dimmer than single cells, and rounder cells appeared brighter than flatter cells. Fluorescence continued to diminish as the cells reached confluence (Figs. 2E–F), although the entire cell sheet appeared brighter than either H⁺-K⁺-ATPase-stained pigmented cultures or control (normal serum-stained) nonpigmented cultures (data not shown).

Voltage-Activated Currents in Nonpigmented Cells

We used the whole-cell patch-clamp technique to record voltage-activated currents from cells having no apparent contacts with other cells. Currents were recorded in response to voltage pulses from a holding potential (V₀) of −70 to −90 mV. Most cells had both transient inward currents and sustained outward currents (Table 1). Voltage-activated currents were seen in freshly dissociated cells and in cells cultured for 1–7 days.

Figure 3 shows an example of voltage-activated currents in a freshly dissociated cell and also illus-

Fig. 3. Voltage-activated currents in a freshly dissociated nonpigmented ciliary body epithelial cell. V₀ = −75 mV. The external solution contained 128 mM NaCl, 4.3 mM KCl, 1.7 mM CaCl₂, 0.8 mM MgCl₂, 7 mM glucose, 10 mM sucrose, and 3 mM HEPES (pH to 7.4 with NaOH). The internal solution contained 112 mM KCl, 19 mM potassium aspartate, 4 mM MgCl₂, 10 mM K₂EGTA, 10 mM HEPES, and 3.5 mM KOH (pH = 7.2). (A) Currents were evoked by 100-msec pulses from −80 to 80 mV in 10-mV steps. Currents were filtered at a cutoff frequency (Fₘ) of 1 kHz with an eight-pole, low-pass Bessel filter and digitized at a sampling frequency (Fₛ) of 5.5 kHz. (B) Mean i-v curve for the last 55 msec of current during the voltage step (indicated by the line in A) as a function of pulse voltage. The straight line was a regression fit to the mean currents in this voltage range and had a slope of 3.8 GΩH. (C) Net voltage-activated currents. Currents produced by depolarizing and hyperpolarizing pulses of the same magnitude were digitally summed to remove leakage current. A rapidly inactivating inward current and a sustained outward current were visible. The outward current transient at the beginning of the trace and the inward current transient at the end of the trace were the result of incomplete cancellation of the capacitative transients to hyperpolarizing and depolarizing pulses. (D) Net transient inward current at a time base faster than in C. Fₛ = 11 kHz. Initial outward transient was due to an asymmetry in capacitative transients.
trates the methods used in several of the following figures. Figure 3A shows currents evoked by a series of 100-msec voltage pulses from -80 to +80 mV in 10-mV steps from a V_h of -75 mV. The mean current-voltage curve, estimated by averaging the current during the last 55 msec of the pulse (indicated by the line in Fig. 3A), is plotted in Figure 3B. The current-voltage curve was nearly ohmic for pulse voltages between -80 and +30 mV, with a slope of 3.8 GΩ. Therefore, the current produced by hyperpolarizing pulses between -80 and -10 mV was considered to be leakage current and was added digitally to the current produced by depolarizing pulses of the same magnitude to obtain the net voltage-activated currents (Figs. 3C-D). After using this procedure to remove the leakage current, we observed both voltage-activated inward currents (shown on a faster time scale in Fig. 3D) and outward currents.

In Table 1, we compare the frequency of voltage-activated currents in freshly dissociated cells (2-4 hr in culture) and in cells 1-3 days and 4-7 days in culture. Inward and outward currents were seen in all groups. The older cells showed a marginally significant increase (p < 0.05, by chi-square test) in the frequency of voltage-activated inward currents, although no significant change in the frequency of voltage-activated outward currents was observed. The amplitude of voltage-activated currents (both inward and outward) tended to become larger with time in culture, perhaps because the membrane area of the cells increased. We made no attempt to quantitate these changes.

Voltage-Dependent Inward Current

The voltage-dependent inward current was abolished by removal of extracellular Na⁺. This can be seen in Figure 4. For this cell, the currents evoked by hyperpolarizing pulses were again nearly ohmic and were added to the currents evoked by depolarizing pulses to produce net voltage-activated currents. Replacing external Na⁺ with TMA⁺ completely suppressed the transient inward current (n = 6). In four of six experiments (and for the cell in Fig. 4), Na⁺ replacement also produced a small decrease in the steady-state inward-going holding current. The effects on both the steady-state and voltage-activated currents were completely reversible on return to the Na⁺-containing Ringer's solution. There was no observable effect of Na⁺ replacement on the voltage-activated outward currents.

The Na⁺-dependent inward current appeared to be similar to that previously observed in the pigmented cells of the ciliary body, since it was inactivated at holding potentials more positive than -55 mV (n = 6, data not shown) and was largely blocked by 30 nM TTX (n = 6). Figure 5 shows the effect of 30 nM TTX on voltage-activated currents in a nonpigmented ciliary epithelial cell held at -75 mV. Leakage current was subtracted by the same procedure used in Figure 3. The TTX reversibly blocked most of the voltage-activated inward current but had no effect on the voltage-activated outward current. It also had no effect on the amplitude of the holding current.

Voltage-Activated Outward Currents

Outward currents evoked by positive-going voltage steps are shown in Figure 6 (control). Similar currents were observed in most of the cells we recorded (Table 1). Under the conditions of our experiments (10 mM EGTA in the internal solution), these currents are likely to be gated by membrane voltage per se, rather than by a change in cytoplasmic Ca²⁺ (ref. 23) or Na⁺ concentration. Our evidence for this, in addition to the high buffering capacity of the internal solution for Ca²⁺, is first, that the amplitude and wave form of the currents were unaffected by complete removal of either Ca²⁺ (n = 5, data not shown) or Na⁺ (Fig. 4).
Fig. 5. Block of the transient inward current by 30 nM TTX. Traces show net voltage-activated currents elicited by 100-msec depolarizing pulses from 0 to 80 mV in 10-mV steps from a holding potential of −81 mV. All records were from the same cell. Leakage current subtracted as in Figure 3 by digital summation of depolarizing and hyperpolarizing pulses. Initial brief inward current transients and subsequent brief outward transients were caused by incomplete cancellation of the capacitive transient. Fr = 11 KHz and Fc = 1 KHz. Control, internal and external solutions were as in Figure 3. Note rapidly inactivating inward current followed by outward current. 30 nM TTX. currents recorded in external solution containing 30 nM tetrodotoxin (TTX). TTX-containing solution was applied by microperfusion. Most of the inward current was blocked, although a small component was visible. Wash, voltage-activated currents from the same cell after washing out the TTX. Calibration for all three sets of traces is given below traces labeled TTX.

from the Ringer's solution. Second, there was no evidence under the conditions of our experiments for a component of outward current with a time course similar to the Na+-dependent inward current.

Voltage-activated outward currents in cells internally perfused with 10 mM EGTA were blocked by exposure to external solutions containing 20 mM TEA⁺ (n = 3), 1–3 mM 4-AP (n = 12), or 2–10 mM Ba²⁺ (n = 6). Figure 6 shows the effect of 20 mM external TEA⁺ on the net voltage-activated currents produced by a sequence of pulses identical to those used in Figures 3–5 from a Vh of −75 mV. Leakage current was subtracted by the same procedure used in Figure 3. The TEA⁺ reversibly blocked nearly all of the voltage-activated outward currents but had no consistent effect on the transient inward current.

Similar effects are shown for 4-AP and Ba²⁺ in Figure 7. For 11 cells, the peak outward current was decreased by 87 ± 9% (mean ± standard deviation) in 3 mM 4-AP and, for one cell, by 53% in 1 mM 4-AP. Exposure to 2 mM BaCl2 blocked peak outward current by 40% in one cell but had no effect in two others. At a higher concentration (10 mM), BaCl2 produced a nearly complete block in three cells, including the one in Figure 7B. Since external Ba²⁺, 4-AP, and TEA⁺ at millimolar concentrations selectively block voltage-activated K+ currents in a wide variety of preparations,28 it is likely that most if not all of the voltage-activated outward current seen in non-pigmented ciliary body epithelial cells under these recording conditions was due to voltage-activated K⁺ channels. However, the ion selectivity and kinetics of the outward currents were not studied in detail by us.

Double Labeling of Nonpigmented Epithelial Cells

The recordings in Figures 3–7 show that cells in our cultures contain TTX-sensitive, Na+-dependent inward currents which, with the exception of a previous report from our laboratory for pigmented cells from the ciliary body,13 have not previously been seen in mature, transporting epithelial cells. Although we believe our cultures to contain a pure population of nonpigmented ciliary body epithelial cells and have attempted to exclude any contaminating cell types, it seemed useful to provide a direct demonstration that the currents we recorded were in fact those of the non-pigmented cells.

To provide such a demonstration, voltage-gated currents were recorded from cells with pipettes filled with 1% Lucifer Yellow CH. Seven cells having such currents were fixed and labeled with antibody to H⁺-K⁺-ATPase. Figure 8 shows an example of a cell labeled in this fashion, which had currents typical of those illustrated in Figures 3–7. Above, we show the cell with differential interference contrast microscopy (Fig. 8A). Below, we show micrographs for this same cell using appropriate excitation and emission wavelengths to optimize the fluorescence of Lucifer Yellow (Fig. 8B) and the TRITC-conjugated secondary anti-
Fig. 7. Block of voltage-activated outward currents by 4-AP and Ba\(^{2+}\). Traces show net voltage-activated currents elicited by 100-msec (B) or 200-msec (A) depolarizing pulses from 0 to 90 mV in 10-mV steps. Leakage currents subtracted as in Figure 3. Initial brief inward current transients and subsequent brief outward transients were caused by incomplete cancellation of the capacitive transient. The control external solution contained 148 mM NaCl, 4.3 mM KC1, 1.7 mM CaCl\(_2\), 0.8 mM MgCl\(_2\), 7 mM glucose, 10 mM sucrose, and 3 mM HEPES (pH to 7.4 with NaOH). BaCl\(_2\) and 4-aminopyridine (4-AP) were added to the Ringer's solution (Ringer's). The internal solution contained 112 mM KCl, 19 mM potassium succinate, 4 mM MgCl\(_2\), 10 mM K\(_2\)EGTA, 10 mM HEPES, and 20 mM KOH (pH = 7.2). (A) 3 mM 4-AP. F\(_{s}\) = 2 KHz; F\(_{c}\) = 500 Hz. Holding potential was ~80 mV. Recordings show currents in control Ringer's, in Ringer's containing added 3 mM 4-AP, and in control Ringer's after removing the 4-AP (wash). (B) 10 mM Ba\(^{2+}\). F\(_{s}\) = 3.3 KHz; F\(_{c}\) = 700 Hz. Holding potential was ~87 mV. Recordings show currents in control Ringer's, in Ringer's containing added 10 mM BaCl\(_2\), and in control Ringer's after removing the BaCl\(_2\) (wash).

body bound to the H\(^+-K\(^+-ATPase antibody (Fig. 8C). Lucifer Yellow-stained cells did not fluoresce with the optics optimized for TRITC unless the cells were processed for H\(^+-K\(^+-ATPase immunoreactivity. Since the H\(^+-K\(^+-ATPase antibody is a selective marker for nonpigmented epithelial cells in ciliary body and is not known to label any other cells except for the parietal cells of gastric mucosa, we believe this experiment is a direct demonstration of voltage-gated currents in nonpigmented ciliary body epithelial cells.

Discussion

We showed that it is possible to produce pure cultures of nonpigmented ciliary body epithelial cells which are suitable for electrophysiological investigation. Our method was based on the isolation of sheets of nonpigmented cells from the ciliary body, followed by enzymatic dissociation and cell propagation in a complex culture medium on glass cover slips coated with ECM. The identity of the cells was verified with a selective marker, a monoclonal antibody to the transport enzyme H\(^+-K\(^+-ATPase. Although several previous attempts have been made to culture mammalian nonpigmented ciliary body cells, we believe we are the first to show that a pure population of identified nonpigmented cells in primary culture can be grown routinely to confluence. The specific aspects of our approach which we believe to have been responsible for our success were (1) the young age of our animals; (2) the use of an ECM as a substrate onto which the cells were plated; and (3) the composition of our culture medium.

We used whole-cell, patch-clamp recording to identify and characterize voltage-dependent conductances in the membranes of nonpigmented cells. This method does not permit us to discriminate conductances present on apical and basal membranes, nor is it possible to distinguish differences in the properties of cells in different parts of the ciliary body (eg, pars plana and pars plicata). Nevertheless, our recordings show that nonpigmented cells can contain two kinds of voltage-activated conductances: an inward current, carried by Na\(^+\) and blocked by TTX, and an outward current, blocked by TEA\(^+\), Ba\(^{2+}\), and 4-AP and presumably carried by K\(^+\). We do not know why some of the cells do not show both of these currents. It is possible that nonpigmented cells in different parts of the epithelium (pars plana and pars plicata) or tip and trough of ciliary processes have different electrical properties. It is also possible that the electrical properties of some cells were altered as a result of the dissociation procedure.

The voltage-gated currents we observed in the nonpigmented cells of the rabbit ciliary body epithelium are similar to those previously demonstrated for the...
Fig. 8. Double-labeled cell with voltage-activated, transient inward and outward currents. Cell was labeled with Lucifer yellow and H+-K+ ATPase antibody. Recording was made 4 days after dissociation of cells and preparation of cultures. Magnification for A, X709 (1 cm = 14.1 μm); and for B and C, X682 (1 cm = 14.7 μm). (A) Photomicrograph of the fixed cell using differential interference contrast (DIC). (B) Fluorescence micrograph of same cell labeled with Lucifer yellow. Dye was introduced into the cell from a patch pipette. Cell was viewed with Zeiss filter set #H485 for fluorescein isothiocyanate (FITC). (C) Fluorescence of same cell incubated with HK12.18 antibody, followed by goat anti-mouse IgG conjugated to TRITC. Viewed using Zeiss filter set #H546. Note the particulate appearance of the fluorescence, typical of positive labeling with this antibody.

pigmented cells. There is, however, one important difference. Some of the pigmented cells showed a large inward current activated by hyperpolarization (inward rectifier). Most nonpigmented cells, on the other hand, had nearly linear current-voltage curves for hyperpolarizing voltage steps (Fig. 3B) and showed at most a small degree of inward rectification. Inward currents activated by hyperpolarization, when they were observed, were always much smaller than those seen for the pigmented cells. The reason for this difference is unknown.

The inward-going Na+ current activated by depolarization appears to be generated by Na+ channels like those present in nerve and muscle, where they are responsible for the initiation of action potentials. These channels are unlike the Na+ channels which are more typically found in epithelia (such as collecting tubule and urinary bladder), which are only weakly voltage dependent and are blocked by amiloride rather than TTX. Our observations demonstrate that inward-going Na+ currents can be recorded from freshly dissociated cells (Fig. 3) and from cells which have been in culture for several days (Table 1). It is therefore likely that Na+ currents are characteristic of nonpigmented cells in vivo and are not an artifact of our culturing procedure.

These TTX-blockable, Na+-dependent inward currents have also been found in the pigmented cells of the ciliary body. The pigmented and nonpigmented cells are, to our knowledge, among the first examples of transporting epithelial cells with voltage-gated Na+ channels, together with the earlier report of Roberts and Stirling of Na+-dependent action potentials in tadpole skin. Since the pigmented and nonpigmented cells are electrically coupled by an extensive network of gap junctions, it is possible that action potentials could propagate across the syncytium of the ciliary body much as across the epithelium of some primitive invertebrates. In invertebrates, the purpose of action potential propagation is to synchronize secretion, contraction, or luminescence. It is as yet unclear what purpose Na+-dependent action potentials might serve in the epithelium of the ciliary body.

Key words: ciliary body, ciliary epithelium, tissue culture, Na+ currents, epithelia, glaucoma

References


