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\(^+\)), Ba\(^2+\), 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 electrically\(^5\) by an extensive network of gap junctions, both among the cells of each layer and from one layer to the other.\(^6\) 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 grow\(^9\)\(^-\)\(^12\) 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 body\(^17\) 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,13 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.17 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 hemic- sected 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 sterile 1:1 mixture of Nutrient Mixture F-10 (#320-1550; Gibco, Grand Island, NY) and Minimal Essential Medium (#320-1095AG; Gibco), containing 3.46 mM sterile 1:1 mixture of Nutrient Mixture F-10 (#320-1095AG; Gibco), containing 3.46 mM

epithelium in mammals is capable of electrical ac-

tion free of calcium and magnesium (CMF-BSS) of the following composition: 127 mM NaCl, 3.8 mM KCl, 1.08 mM Na3HPO4, 0.6 mM KH2PO4, 14 mM NaHCO3, 6.1 mM D-glucose, 0.31 mM D-gluconic acid (hemiacetate), 0.0035% phenol red. The pooled nonpigmented layers were then incubated in CMF-BSS containing 2 mg/ml collagenase/dispase. After this incubation, 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 culture17 with a monoclonal antibody generated against the H+-K+ ATPase from parietal cells of gastric mucosa.18 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—#V15SF; American Qualex, La Mirada, CA). The NGS was removed from the cultures and replaced with a solution of H+-K+ ATPase antibody at a concentration of 0.15-0.25 mg/ml in PBS. As controls for specificity, 10% NGS or 10% 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

1550; Gibco, Grand Island, NY) and Minimal Essential Media (#320-1095AG; Gibco), containing 3.46 mM ethylene-glycol-bis-n,n,n',n'-tetraacetic acid (EGTA). After this incubation, the nonpigmented layers were microdissected from the pigmented layers, and nonpigmented tissue from several eyes was then collected in a balanced salt solution with the following composition: 126 mM Na glutamate, 3.8 mM L-aspartic acid (mono K+ salt), 1.08 mM Na2HPO4, 0.6 mM KH2PO4, 14 mM NaHCO3, 6.1 mM D-glucose, 0.31 mM D-gluconic acid (hemiacetate), 0.0035% phenol red.

Cell suspensions were generated by washing the pooled nonpigmented layers in a balanced salt solution free of calcium and magnesium (CMF-BSS) of the following composition: 127 mM NaCl, 3.8 mM KCl, 1.08 mM Na3HPO4, 0.6 mM KH2PO4, 14 mM NaHCO3, 6.1 mM D-glucose, and 0.0005% phenol red. The pooled nonpigmented layers were then incubated in CMF-BSS containing 2 mg/ml collagenase/dispase (#1097 113; Boehringer Mannheim, Indianapolis, IN) for 45 min at 37°C. After this incubation, the tissue was triturated in a growth medium composed of NCTC 135 (#320-1350; Gibco) with 15% fetal bovine serum (#230-6140AG; Gibco), 3 mM L-glutamine, 50 μg/ml of gentamicin, 100 μg/ml of kanamycin, and the following additional additives: 0.49 μM adenine HCl, 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 X 104 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 them13 with tissue from albino rabbits. We then extracted the cells from these cultures with ammonium hydroxide,18 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.
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 glacial acetic acid (9:1), and examined with Zeiss filter set D or tetramethylrhodamine isothiocyanate (TRITC). Tissue sections prepared as previously described 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 techniques 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 always buffered to 7.2 with 5–10 mM HEPES. The ionic composition of the bathing and pipette solutions varied from one experiment to another. The dominant external cation was always Na+, and the dominant internal cation was always K+. The chloride was the dominant external anion in all the experiments and the dominant anion in the internal (pipette) solution in all but a few. All external solutions contained 1.7 mM Ca2+ and 0.8 mM Mg2+. Internal solutions contained no added Ca2+ and 0.5–10 mM EGTA. The precise ionic compositions of the internal and external solutions used for the experiments are given in the figure legends.

Drugs were applied to the cells with a gravity-fed, U-tube microperfusion system, whose outflow 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 micropipette 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 al 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 (F0) of 500–1000 Hz. Data analysis was done off line with personal computer software (IBM AT, Armonk, NY) either with software developed by F. Bezanilla or with the PCLAMP program (Version 5.0; Axon Instruments, Burlington, CA). Data were digitized at a sampling rate (F s) at least four times greater than F0.

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. We previously described a method for isolation of the ciliary epithelium which produces preparations that are highly enriched in nonpigmented cells.17 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 = 23.5 μm); for (C) and (D) ×163 (1 cm = 61.4 μm).](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933391/ on 10/17/2018)
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, X400 (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>
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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

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 (Vh) 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 illustrates 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. Fc = 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 Vh 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+ (Fig. 4). 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,13 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. This 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.24,25 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. 4. Effect of replacing [Na+]o with tetramethyl ammonium ion (TMA+) on the voltage-activated and steady-state holding current. Traces were the sums of responses to hyperpolarizing and depolarizing voltage pulses of equal magnitude. Pulse voltages were from 10 mV to 90 mV in 10-mV steps from a holding potential of -81 mV. Internal solution was same as in Figure 3. F1 = 11 kHz and F2 = 1 kHz. Brief transients at beginning of traces resulted from slight asymmetries in the capacitative transients. Control currents in control (Na+) external solution. External solution the same as in Figure 3, except that it contained 148 mM NaCl. TMA+ all of the NaCl in the external solution was replaced with TMACl. Note elimination of transient voltage-activated inward current. There was little or no effect on outward current. Note reduction in the inward current (by 3.7 pA in this cell) required to hold cell at holding potential (~81 mV), as shown by upward displacement of baseline current as compared with control. Wash, recovery of voltage-activated inward current and steady-state holding current was almost complete after returning cell to control saline. Calibration for all three sets of traces is shown beneath those labeled TMA.
of the voltage-activated outward current seen in nonpigmented 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,¹¹ 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 nonpigmented 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-

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**Fig. 5.** Block of the transient inward current by 30 nM TTX. Traces show net voltage-activated currents elicited by 100-mscc 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. F₁ = 11 KHz and Fc = 1 KHz. Control, internal and external solutions were as in Figure 3. Inward current 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.

**Fig. 6.** Block of the voltage-activated outward current by 20 mM external TEA⁺. Voltage-activated currents produced by the same pulse sequence used in Figure 5 from a holding potential of −75 mV. Leakage current subtracted as in Figure 3. Capacitive transients at the onset and offset of the pulses were reduced but not completely canceled by this procedure. F₁ = 5.5 KHz; Fc = 1 KHz. Control, external and internal solutions were as in Figure 3. In addition to the sustained outward current, a small voltage-activated inward current was visible after the capacitive transient at the beginning of the trace. 20 mM TEA⁺, voltage-activated currents in the same cell after substituting 20 mM external Na⁺. Wash, voltage-activated currents in the same cell a few minutes after washing out the TEA⁺.
Fig. 7. Block of voltage-activated outward currents by 4-AP and Ba²⁺. 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 KCl, 1.7 mM CaCl₂, 0.8 mM MgCl₂, 7 mM glucose, 10 mM sucrose, and 3 mM HEPES (pH 7.4 with NaOH). BaCl₂ and 4-aminopyridine (4-AP) were added to the Ringer’s solution (Ringer’s). The internal solution contained 112 mM KCl, 19 mM potassium aspartate, 4 mM MgCl₂, 10 mM K₂EGTA, 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 Ba²⁺. 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 BaCl₂, and in control Ringer’s after removing the BaCl₂ (wash).

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⁺, Ba²⁺, 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) 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 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.
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 \mu m); and for B and C, X682 (1 cm = 14.7 \mu m). (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


