Dye Coupling of Rabbit Ciliary Epithelial Cells In Vitro

Jeong Oh, Theodore Krupin, Li-Qi Tang, John Sveen, and Robert A. Lahlum

Purpose. To examine the in vitro properties of gap junctions on the isolated rabbit ciliary epithelium.

Methods. Intracellular potential was measured and lucifer yellow (5% in 1 M LiCl) was iontophoretically injected into a ciliary epithelial cell. Fixed tissue was examined with a laser confocal microscope.

Results. Dye spread was observed throughout both layers (horizontally and vertically) of the ciliary epithelium adjacent to the injected cell that was more intensely labeled. Dye reflux did not occur at the site of microimpalement. Microiontophoretic dye injection in a bathing solution with a high Ca²⁺ (10 mM) or an acidic pH (6.3) completely inhibited cell-cell dye coupling in the rabbit ciliary epithelial cells.


Aqueous humor is actively produced by the ciliary epithelium. This unique double epithelial layer consists of an inner nonpigmented epithelium in contact with an outer pigmented epithelial layer. The two layers are joined apex to apex. The basolateral membrane of the inner nonpigmented layer faces the posterior chamber aqueous humor, whereas the basolateral membrane of the outer pigmented layer faces the blood-side of the ciliary process. Using freeze fracture techniques, Raviola and Raviola have demonstrated by electron microscopy the presence of numerous gap junctions between the pigmented and nonpigmented ciliary epithelium, suggesting functional coupling of the two layers.

Microimpalement studies on rabbit preparations by Green et al² and Carré et al³ illustrate electrical coupling between the two ciliary epithelial layers. Moreover, iontophoretic intracellular injection of the fluorescent dye lucifer yellow (LY) shows spread of the dye between the two ciliary epithelial layers.² The present study uses laser confocal microscopy to define and study the regulation of gap junctions in the ciliary epithelium. In addition, we describe a modified perfusion chamber permitting immobilization of the ciliary processes without the need for an adhesive.

MATERIALS AND METHODS

Tissue Preparation

Adult, albino New Zealand rabbits weighing 5 to 6 kg each were sacrificed with intravenous (marginal ear vein) sodium pentobarbital (150 mg/kg) and bilateral thoracotomy.

Our experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyes were enucleated promptly and placed in modified Tyrode’s solution buffered with HEPES and bubbled with 95% O₂/5% CO₂. The solution was at room temperature and had a pH of 7.3 to 7.4 and an osmolality of 290 to 300 mOsm/l (Table 1). The enucleated globe was bisected 3 to 4 mm posterior to the limbus, and the anterior half was placed with the corneal surface down on a petri dish. Using a surgical microscope, the posterior lens capsule was opened, the lens was removed, and the zonules were incised.
TABLE 1. Solution Composition

<table>
<thead>
<tr>
<th>Solution</th>
<th>Control</th>
<th>Ca$^{2+}$ Free</th>
<th>10 mM Ca$^{2+}$</th>
<th>pH Study</th>
<th>Solutions</th>
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</thead>
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<tr>
<td>NaCl</td>
<td>110</td>
<td>110</td>
<td>100</td>
<td>110</td>
<td></td>
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<tr>
<td>HEPES acid</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>0</td>
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<td>KCl</td>
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<tr>
<td>MgCl$_2$</td>
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<tr>
<td>CaCl$_2$</td>
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<tr>
<td>NaHCO$_3$</td>
<td>30</td>
<td>30</td>
<td>30</td>
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<tr>
<td>KH$_2$PO$_4$</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
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<td>10</td>
<td>10</td>
<td>0</td>
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</tbody>
</table>

To study the effects of pH on dye coupling, solutions were prepared without HEPES buffer. After bubbling with 95% O$_2$/5% CO$_2$ for 15 minutes, the solutions were adjusted from pH 6.3 to pH 8.3 in 0.5 pH unit steps, with either 0.1 M HCl or 0.1 M NaOH. To maintain the adjusted pH, additional bubbling with CO$_2$ was not done. The pH of the solution was measured both before and after the experiment and showed fluctuations of less than 0.1 pH unit. All the solutions were at the room temperature and had an osmolality of 290 to 300 mOsm/l.

Membrane Potential Recording

Microelectrodes were fabricated from Omega-dot capillary tubing (1.5 mm outer, 0.86 mm inner diameter, World Precision Instruments, Sarasota, FL) using a horizontal Flaming/Brown puller (Model P-87, Sutter Instruments, Novata, CA). The microelectrode was filled with 4 M potassium acetate and connected to an Axoprobe-1A Amplifier (Axon Instruments, Foster City, CA) with a solid Ag-AgCl half cell. The reference electrode was another Ag-AgCl half cell that was in contact with the tissue bath. The microelectrodes had a resistance of 80 to 120 MΩ. The membrane potential (Vm) was monitored on an oscilloscope (Hameg Instruments, Frankfurt, Germany) and recorded on a chart recorder (Soltex Corporation, Sun Valley, CA).

The bridge was balanced during a brief current passage before cell impalement. The microelectrode was advanced into the tissue with a hydraulic micromanipulator (Narishige Scientific Instrument Lab, Tokyo, Japan) while being viewed under a microscope (Wild Leitz, Inc., Rockleigh, NJ). In some experiments, the electrode tip was advanced to the surface of the epithelium, as noted by a sudden hyperpolarization by −3 to −5 mV, and a brief oscillating current was passed to facilitate impalement. Successful impalements were judged by a rapid change in potential upon advance of the microelectrode; at least 5 minutes of continuous stable recording of the intracellular po-

![Diagram of iris-ciliary body mounting chamber](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933178/ on 10/19/2018)
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tential; maintenance of the membrane potential during iontophoretic injection; and sustained stability of the microelectrode resistance after removal from the cell.

A stable baseline Vm recording was obtained in control media for at least 5 minutes before the bathing media was switched to a solution with a different pH or Ca²⁺ concentration. The effect of the altered bathing solution on membrane potential was recorded during the next 15 minutes. Results are reported as the mean ± SEM. Statistical analysis was performed using the Student’s paired t-test.

Lucifer Yellow Fluorescent Dye Injection

The tip of the microelectrode was filled with 5% LY (Sigma Chemical Co., St. Louis, MO) solution and back-filled with 1 M LiCl. The mounted I-CB was immersed in oxygenated control or altered (pH or Ca²⁺) bathing solution for approximately 30 minutes before cell impalement. A stable Vm was obtained for approximately 5 minutes before the iontophoretic injection of LY, which was made by passing a hyperpolarizing current (−10 nA at 1 Hz) for 1 minute. Tissue was fixed in the chamber within 10 seconds after iontophoresis with 4% paraformaldehyde. The entire wedge of tissue was mounted on a slide in 2% DABCO (an antioxidant to prevent bleaching of the fluorescence) and covered with a plastic coverslip held in place with dental wax.

A laser scanning confocal microscope (Carl Zeiss, Thornwood, NJ) operated in the epifluorescence mode was used to analyze the extent of dye spread. LY was excited with the blue (476.5 nm) of an argon ion laser. A 50 μm laser spot was moved by analog electronics in either the horizontal (x-y) plane or along the optical axis (x-z plane) of the tissue. Z series of x-z images were recorded with intervals of 0.3 μm along the optical axis. Intensities detected by the photomultiplier as a function of the three-dimensional position of the light spot in the tissue were stored on a computer for further processing. The tissue sample was illuminated only while intensities were recorded.

In other experiments, the fixed tissue was cryosectioned at 5 μm thickness and examined under a fluorescent microscope (Carl Zeiss). Cryosections were also stained with hematoxylin-eosin to delineate the outer and inner epithelial layers.

RESULTS

Baseline Vm of the ciliary epithelial cells in control Tyrode’s solution was −65.3 ± 1.9 mV (N = 14). Iontophoresis caused slight hyperpolarization (−3 to −4 mV) in some experiments. However, in a majority of the cases, there was no shift in the membrane potential after the iontophoresis. When viewed under the confocal laser scanning microscope, extensive intracellular spread of the fluorescent LY was observed (Fig. 2A). The borders of the cells were clearly demarcated, and no leakage of LY into the stroma was seen. Fluorescent intensity decreased over an area of six to eight cells from the approximate region of the injected cell. The tissue was repetitively scanned along a straight line perpendicular to the surface with incremental 0.3 μm steps in the depth of focus (Z-sectioning). The computer assisted optical stacking (two-dimensional reconstruction) of the images showed approximately 18 μm vertical spread of the dye (Fig. 2B). This corresponded approximately to the combined thickness of both the inner and outer ciliary epithelial layers. Horizontal spread of the dye occurred within both epithelial layers (Fig. 2B).

A cryosection of the injected cell confirmed the presence of LY in neighboring cells of both epithelial layers (Fig. 3A). An adjacent section, stained with hematoxylin-eosin, showed the borders of the two epithelial layers (Fig. 3B). The cuboidal cells had a width and a height of approximately 8 to 9 μm.

Baseline Vm was not altered (N = 6, P > 0.7) when the bathing medium was changed to Ca²⁺ free solution. The confocal laser scanning image after iontophoretically injected LY demonstrated dye spread within both epithelial layers comparable to those observed in control (2.5 mM Ca²⁺) bathing solution (see Fig. 2).

Changing the bathing medium from control Tyrode’s solution to 10 mM Ca²⁺ Tyrode’s solution did not change the baseline Vm (N = 8, P > 0.8). However, confocal laser scanning after iontophoretic in-

FIGURE 2. Fluorescent confocal microscopy of rabbit ciliary epithelium after iontophoresis of lucifer yellow in control (2.5 mM Ca²⁺, pH 7.3) Tyrode’s solution. (A) the horizontal (x-y) surface plane. (B) The computer generated (two-dimensional reconstruction) Z series of x-y images obtained by scanning the tissue along the x-z plane (DZ is the total depth of tissue scanned).
jection of LY demonstrated restriction of the fluorescence to a single cell with an 8.5 μm depth of vertical dye distribution (Fig. 4A). Although the quantity of dye injected into a given cell varied, fluorescence was not detected in the extracellular space. In Figure 4, LY fluorescence was not observed with Z-sectioning in neighboring cells, either horizontally or vertically. However, in some 10 mM Ca²⁺ experiments, dye was seen in the epithelial cell vertically adjacent to the injected cell (photograph not presented). The vertical depth of dye distribution in this situation was 17 to 18 μm; however, there was no horizontal spread of dye.

Switching the bathing medium from pH 7.3 to pH 8.3 Tyrode’s solution (N = 5) significantly (P < 0.001) hyperpolarized baseline Vm by -4.8 ± 0.6 mV during the next 15 minutes. The intracellular spread of iontophoretically injected LY in pH 7.8 and 8.3 bathing medium was similar to that observed in control pH 7.3 medium (see Fig. 2). Dye was detected into the neighboring cells in both layers.

Acidification of the extracellular medium from pH 7.3 to pH 6.3 (N = 5) resulted in a significant (P < 0.0001) depolarization of baseline Vm (+5.0 ± 0.3 mV). Under this condition, intracellular injection of LY showed constraint of the fluorescence in a single cell within one epithelial layer (Z-sectioning), similar to that observed with high extracellular Ca²⁺ (see Fig. 4).

DISCUSSION

Our modified perfusion chamber securely immobilizes the iris-ciliary body tissue for intracellular recording at a high perfusion rate of 2.0 ml/min (chamber volume = 1.5 ml). Anchoring the tissue between two acrylic discs with central openings eliminates the need to immobilize the tissue with sutures² or an adhesive,³ which may affect integrity of the cells. This chamber can be further modified to continuously perfuse both sides of the tissue. Intracellular impalements result in stable recordings for at least 2 hours. Our baseline Vm in control Tyrode’s solution (~65.3 mV ± 1.9 mV) is similar to the values reported by Carré et al.⁸

Laser scanning confocal microscopy, in contrast to the light source used for conventional fluorescent microscopy, scans the tissue sample with a single spot of laser energy to produce an entire image.⁵ This technique improves fluorescent microscopy by illuminating only a small region of the specimen at any one
time. In addition, the region is viewed through an opening that masks the surrounding nonilluminated areas. By virtue of the small area of illumination and an aperture in the return light path, scattered and out-of-focus light resulting from the illumination is minimized. By incrementing the microscope’s focus distance, high-contrast optical sections are produced along the vertical x-z axis (Z-sectioning), which permits depth reconstruction and tracing the extent of fluorescent dye spread between the nonpigmented and pigmented epithelial layers of the ciliary process.

LY is widely used to study the presence of cell-cell coupling because the dye is readily permeable through gap junctions. Warner has demonstrated the specificity of LY for gap junctions. Binding the gap junction proteins with antibodies inhibits the transfer of LY between the cells of the amphibian embryo.

In the control state, gap junctions allow the rapid passage of LY from the injected cell to its neighboring cells within seconds (Fig. 2). The present experimental techniques permit only qualitative assessment of LY spread. The variable quantity of dye injected into the cell and the two-dimensional reconstruction limit a quantitative measurement. However, our results indicate that perturbations of extracellular Ca2+ (Fig. 4) or pH that presumably alter intracellular concentrations affect the conductance of LY through the gap junctions in the ciliary epithelium. Although the induced intracellular changes occurring after bath alterations would be smaller in magnitude, increased levels of intracellular Ca2+ and decreased intracellular pH close gap junctions in a variety of epithelia. Spray et al describe a more than 95% reduction in junction conductivity after a reduction in intracellular pH to 6.9. Vm is stable when the ciliary epithelial cells are impaled in a bathing solution with high Ca2+ or low pH, indicating the absence of transient permeability increases during impalement.

Gap junctions are ubiquitous in vertebrate epithelial cells. Gap junctions provide a pathway for intercellular communication, thereby allowing the cells to function as a syncytium. These specialized regions of cell-cell membrane contacts are composed of a hexameric configuration of identical protein subunits (connexins) that surround a central pore to form relatively large channels. The physiological role of gap junctions has not been established. However, these specialized channels, which exist in either the open or closed state, may function to allow the electrotonic spread of secretory potential between cells. The flux of molecules at least 12 Å in diameter and with molecular weight in excess of 1000 daltons occurs through these channels. Thus, gap junctions allow direct cell-cell transfer of ionic molecules, nutrients, and signaling molecules. These intercellular channels can also provide a pathway for cell-cell exchange of metabolic products. Cyclic AMP, the intracellular mediator of the response to adrenergic stimulation, passes between coupled heart cells through gap junctions.

Ciliary epithelial cells secrete aqueous humor by active ionic transport across the double-layer epithelium. Systemic acidosis has been shown to decrease intraocular pressure in rabbits by reducing the rate of aqueous humor production without altering outflow facility. Moreover, a portion of the intraocular pressure reduction with systemic carbonic anhydrase inhibitors may be related to an induced systemic acidosis. Our in vitro experiments show that acidification of the bathing solution and presumably intracellular pH causes depolarization and loss of cell-cell coupling in rabbit ciliary epithelium. High extracellular Ca2+ also closes gap junctions in the ciliary epithelium. Ca2+ functions as an intracellular second messenger and interacts with various ionic transport processes. The possibility of direct passage of cyclic AMP between ciliary epithelial cells may be important for the distribution and synchronization of sympathetic stimulation of ciliary epithelium. Both adrenergic agents and cyclic AMP have been shown to affect the transepithelial electrical measurements across the isolated rabbit iridociliary body. Halothane and other halogenated anesthetic drugs reduce the rate of aqueous humor formation and lower intraocular pressure in laboratory animals and affect electrical measurements across the isolated rabbit iridociliary body. Halothane is an effective gap junction blocker. The direct relationship between the inhibition of cell-cell coupling and the rate of aqueous humor formation requires further study.

Key Words

ciliary epithelium, intercellular potential, gap junctions, lucifer yellow, laser confocal microscopy, extracellular calcium, extracellular acidosis–alkalosis

References


