Corneal Endothelial Junctions and the Effect of Ouabain

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Paired rabbit corneas were perfused in vitro for endothelial permeability (Pac) determination with glutathione bicarbonate Ringer’s solution (GBR) and GBR plus ouabain (10⁻⁴ M). Results indicated no difference in Pac between the two groups (3.39 vs 3.67, respectively) despite significantly greater stromal swelling in the group perfused with ouabain. Freeze-fracture microscopy of similarly perfused corneas revealed intact tight junctional complexes in both groups, although the tight junctional complex of perfused corneas appeared less organized than that of freshly enucleated, nonperfused controls. Gap junctions were abundant as observed in freeze-fracture replicas of GBR-perfused endothelium, and appeared to be decreased or absent in ouabain-perfused endothelium. These results indicate that corneal endothelial tight junctions are unaffected by perfusion with ouabain, whereas gap junctions appear to be lost. The permeability and freeze-fracture data reaffirms the importance of tight junctions as permeability barriers and indicates that gap junctions are not of primary importance for maintenance or control of the corneal endothelial barrier. Invest Ophthalmol Vis Sci 31:933-941, 1990

Materials and Methods

Both physiologic and anatomic methods were employed to examine the status of the junctional complex of the rabbit corneal endothelium. For the physiologic study, paired rabbit corneas (n = 6) were perfused in the in vitro specular microscope for permeability determination, with carboxyfluorescein as the permeability tracer. A modification of the technique described by Araie⁵ was used as described previously.⁶ The experimental corneal endothelium was perfused with 10⁻⁴ M ouabain in an adenosine-free glutathione bicarbonate Ringer’s (GBR) solution (bubbled to pH 7.4 with 95% air/5% carbon dioxide prior to addition of the ouabain), while the control corneal endothelium was perfused with GBR (also bubbled to pH 7.4). The corneal epithelium was removed by scraping, and the anterior corneal surface was covered with silicone oil during the perfusion. Stromal thickness was measured by focusing the light slit of the specular microscope from the denuded epithelial surface to the endothelium, and recording the distance with a digital micrometer. After stabilization of the control corneal thickness, the silicone oil was removed by scraping, and then by swabbing with surgical sponges, and 3 ml 5(6)-carboxyfluorescein (0.26 mM) was applied to the anterior stromal surface for 30 sec. The dye was removed with a pipette and surgical sponges, and silicone oil was reapplied. The perfusion was continued for 30 min, during which time outflow perfusate was collected in a preweighed test tube. The mass of carboxyfluorescein in the perfusate (Mₚ) was determined fluorometrically. After termination of the perfusion, corneas were placed in

The primary permeability barrier in the corneal endothelial junctional complex is the macula oclu dens junction, which forms a “leaky” tight junction. This leaky junctional complex provides a relatively low electric resistance across the corneal endothelium (73 ± 6 ohms·cm²) as compared to occluding epithelium such as that in the rabbit corneal epithelium (1.6–9.1 Kohm·cm²).¹² Gap junctions, whose primary function is intercellular communication, may also act as permeability barriers due to their location in the lateral intercellular space. Ouabain, a cardiac glycoside which inhibits Na/K ATPase, has been shown to cause significant corneal swelling in the rabbit and human during corneal endothelial perfusion at 10⁻⁴ M concentration.³⁴ The purpose of this study was to determine the status of the junctional complex in the corneal endothelium after in vitro perfusion of the cornea with ouabain.

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Supported in part by National Eye Institute grants T32 EY-07016, RO1 EY-00933, and F32 EY-06206 and by funds from VAMC grant and Baptist Memorial Hospital Research Foundation, Memphis, Tennessee.

Submitted for publication: June 23, 1989; accepted September 28, 1989.

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BSS (Alcon Laboratories, Fort Worth, TX) for a minimum of 48 hr to allow for carboxyfluorescein elution and measurement of the stromal dye mass ($M_s$). The cornea–aqueous transfer coefficient ($k_{ca}$) and endothelial permeability ($P_{ac}$) were calculated as:

$$k_{ca} = \frac{\ln (M_p + M_s) - \ln M_s)}{t} \quad \text{(Ref. 6)}$$

$$P_{ac} = k_{ca} \times R_{ca} \times q \quad \text{(Ref. 5)}$$

where $t$ is the time following dye application (30 min); $R_{ca}$ is the steady state distribution ratio (1.07), and $q$ is the average stromal thickness from the three final readings of the perfusion. Swelling rates were calculated by plotting the change in thickness of the stroma over time and fitting the curve with a first order linear regression. The slope of the regression was taken to be the swelling rate (in micrometers per hour). Results of the experiments were analyzed with a paired t-test, comparing data obtained from the ouabain-perfused corneas to those of their paired controls.

For the anatomic analysis, six pairs of rabbit corneas were perfused as above for freeze-fracture analysis of the endothelial junctions, with no addition of dye to the cornea. Perfusion time averaged 2 hr. Immediately after cessation of the perfusion, corneas were fixed in a solution containing 0.1 M sodium cacodylate buffer, 2% paraformaldehyde, 2% glutaraldehyde, and 0.5% acrolein at pH 7.4–7.6. Corneas were immersed in the fixative for 2 hr at room temperature and were placed in 0.1 M Na-cacodylate buffer.

Freeze-fracture was carried out according to the method of McLaughlin et al. Corneas were hand-cut into 2-mm squares and glycerinated for 2 hr in 23% glycerol (v/v) to aid in cryoprotection. The squares were mounted endothelium side up on gold discs; frozen in liquid-nitrogen-cooled Freon 22; and stored in liquid nitrogen. The tissue was fractured at $-116\,^\circ\text{C}$; etched for 2 min; and replicated in a Balzers 400-T freeze-fracture apparatus fitted with electron beam guns for platinum and carbon shadowing and a quartz crystal monitor for standardizing replica thickness. The replicas were cleaned in Purex and mounted on coated grids for examination in the transmission electron microscope.

All aspects of this study were carried out according to the ARVO Resolution on the Use of Animals in Research.

**Results**

The permeability data obtained after endothelial perfusions with $10^{-4}$ M ouabain versus control (GBR alone) are listed in Table 1. Swelling curves (Fig. 1) reveal stromal swelling resulting from endothelial Na/K ATPase inhibition by ouabain (25 $\mu$m/hr), as compared to deswelling in the GBR-perfused control cornea ($-14 \mu$m/hr). Swelling rates were significantly different ($P < 0.01$, t-test). Despite this difference in stromal swelling, the ratio of perfusate to total dye concentration ($M_p/M_t$), an index of the amount of dye diffusing across the endothelium per total volume of dye, was greater in the control corneas than in corneas perfused with ouabain (0.235 vs 0.210 respectively, $P < 0.05$, paired t-test). This ratio data indicates, on a gross level, that more dye was lost across the control endothelial monolayer than across the ouabain-perfused monolayer. This is illustrated by the significantly lower $M_p$ following perfusion with ouabain as compared to control (Table 1). However, no significant difference in $P_{ac}$ values, the more sensitive indicator of endothelial barrier integrity, was observed between the two groups.

Freeze-fracture analysis did not reveal any dramatic changes in tight junctional complexes of either ouabain- or GBR-perfused corneas (Figs. 2–4), when compared to fresh, nonperfused corneal endothelium (Fig. 5, 6). In nonperfused corneas, tight junctions were composed of E-face strands and complimentary P-face ridges (Fig. 5), similar to that seen in perfused corneas (Figs. 2–4). Gap junctional aggregates were often interposed between tight junctional ridges (Fig. 3) and in the vicinity of tight junctional strands (Fig. 5) in both control and nonperfused endothelium. Gap junctional aggregates were present also on the lateral membranes of both control (Fig. 3) and fresh, nonperfused endothelium (Fig. 6). In both control and ouabain-perfused endothelium, there appeared to be small tight junctional areas along the apicolateral membrane borders. There also appeared to be a reduction of gap junctional specializations in the ouabain-perfused endothelium (Fig. 4) as compared to control (Fig. 3) and to fresh, nonperfused endo-

**Table 1. Permeability data: GBR versus ouabain**

<table>
<thead>
<tr>
<th></th>
<th>GBR</th>
<th>$10^{-4}$ M ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Swelling rate*</td>
<td>$14.28 \pm 3.00$</td>
<td>$25.02 \pm 2.31^+$</td>
</tr>
<tr>
<td>$M_p$ (µg)</td>
<td>$0.755 \pm 0.086$</td>
<td>$0.781 \pm 0.151$</td>
</tr>
<tr>
<td>$M_s$ (µg)</td>
<td>$0.229 \pm 0.032$</td>
<td>$0.202 \pm 0.020^+$</td>
</tr>
<tr>
<td>Ratio $M_p/M_s$</td>
<td>$0.235 \pm 0.046$</td>
<td>$0.210 \pm 0.043^+$</td>
</tr>
<tr>
<td>$P_{ac}$ ($10^6$ cm/min)</td>
<td>$3.39 \pm 0.23$</td>
<td>$3.67 \pm 0.24$</td>
</tr>
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Values are given in mean ± SD.

- $M_s$ is the stromal mass of dye; $M_p$ is the mass of dye in the perfusate; $M_t = M_s + M_p$; $P_{ac}$ is the corneal endothelial permeability.
- *Swelling rate as calculated from slope of fitted linear regression.
- Significantly different from control ($P < 0.001$) by slope comparison and t-test.
- Significantly different from control ($P < 0.05$) by paired t-test.
The results of this study show that the permeability of the rabbit corneal endothelium to 5(6)-carboxyfluorescein is not altered after in vitro perfusion with ouabain. The finding that no change is occurring in the permeability barrier of these cells is compatible with freeze-fracture analysis of the junctional complexes of corneal endothelial cells perfused with ouabain. These cells showed no apparent change in tight junctional complexes as compared to GBR-perfused controls. An interesting observation was the almost total loss of gap junctional regions in ouabain-perfused endothelia.

The difference in swelling rates of 39 μm/hr between control (−14 μm/hr) and ouabain-perfused endothelia (25 μm/hr) in the current study is similar to the values of 37-52 μm/hr reported previously.3,4 Given the results of the current study, the swelling appears to be entirely attributable to the actions of ouabain on the endothelial ion transport mechanism, namely Na/K ATPase inhibition.

A possible source of error in the permeability experiments would be the restriction of carboxyfluorescein loss from the stroma across the endothelium of ouabain-treated corneas by the net flow of fluid entering the stroma during the swelling period. During Na/K ATPase inhibition with ouabain, the swelling pressure of the stromal tissue is the primary variable leading to net fluid flow into the stroma. If this fluid flow was restricting dye movement out of the cornea across the endothelial junctional complexes, permeability values would be correlated to the swelling pressure. In fact, no correlation was found when comparing individual permeability values with their average final thickness values (μ), which reflect the stromal swelling pressure (r = 0.03, n = 6). The average final thickness values ranged from 107-128% of the mean control value (361.9 μm), which correspond to a range of swelling values of approximately 70-40 g/cm².8

In agreement with the current findings is the study by Trenberth and Mishima, who showed no change in the permeability of radiolabeled sucrose and urea across rabbit corneal endothelium following perfusion of the endothelium as high as 10⁻³ M ouabain.3 It was also shown that water fluxes across the en-
Fig. 3. Freeze-fracture micrographs of control endothelial cell junctions. (A) Tight junctional ridges are present on the protoplasmic (P) membrane leaflet of adjacent cells. Gap junctional particle aggregates are present on the P-face membrane leaflet interspersed between tight junctional strands (arrowheads). (B) Lateral endothelial membranes demonstrating large gap junctional particle aggregates (arrows) and pits (arrowheads) (A, B, bar = 0.1 μm).

Fig. 4. Freeze-fracture of ouabain-perfused endothelial cell junctions. (A) As in the control endothelial junctions, tight junctional particle strands (arrows) are present on the external membrane leaflet (E), and complementary tight junctional ridges (arrowheads) are present on the protoplasmic (P) membrane leaflet of adjacent cells. No gap junctional particles were observed between tight junction strands (bar = 0.1 μm). (B) Gap junctional aggregates are absent from the lateral P-face membranes of ouabain-perfused corneas (bar = 0.1 μm).
Fig. 5. Freeze-fracture micrographs of junctional complexes from fresh, non-perfused corneal endothelium. (A) Tight junctional particle ridges are present on the protoplasmic (P) membrane leaflet. (B) Complementary tight junctional strands are present on the external membrane leaflet (E) of adjacent cells. Gap junctional particles are present and interspersed between tight junctional strands (arrowheads). P, protoplasmic membrane leaflet (bar = 0.1 μm).
Fig. 6. Lateral endothelial cell membranes from fresh nonperfused corneal endothelium, demonstrating gap junctional aggregates of particle and pits (arrowheads) of various sizes and shapes. E, external membrane leaflet; P, protoplasmic membrane leaflet (bar = 0.1 μm).

The endothelium did not change following ouabain infusion. These results are consistent with the findings of Fischbarg, who reported no change in corneal endothelial electrical resistance following introduction of ouabain to the bathing solution. Melki and colleagues recently showed significant lanthanum penetration through endothelial tight junctions in ouabain-treated corneas, where no penetration was seen in control endothelium. Their results, however, are not consistent with previously findings, where lanthanum was shown to penetrate normal corneal endothelium.

Previous studies using TEM have shown that the apical tight junctions of ouabain-treated corneal endothelial cells appear to be intact. However, TEM is very limited in its resolution of both gap and tight junctional complexes and is not capable of recognizing junctional strands. The only technique currently available to examine the details of junctional complexes is freeze-fracture microscopy. Because the question of the effects of ouabain on the corneal endothelial junctional complex could be more completely analyzed both physiologically and anatomically, it was decided that along with \( P_e \) measurements, freeze-fracture microscopy would be employed. Freeze-fracture of endothelial cells from ouabain-perfused corneas revealed minimal change in tight junctional morphology and an apparent reduction in the number of gap junctions as compared to control. Gap junctional loss following ouabain treatment has also been reported in cardiac Purkinje cells and ventricular muscle cells as determined by cellular electrical uncoupling. These authors postulated that inhibition of the Na/Ca exchanger via Na/K ATPase inhibition led to an increase in internal calcium concentration, which was responsible for the loss of gap junctional communication. This seems unlikely in the case of the corneal endothelium, where there is no evidence of a Na/Ca exchanger. The loss of gap junctions in the corneal endothelium after ouabain treatment may be related to the ouabain-induced physical stress occurring within the intercellular space, leading to intercellular vacuolization as observed in Figure 7. The loss of gap junctions may also be related directly to the actions of ouabain on the membrane, as has been proposed by Ledbetter and Lubin. These authors noted that Na/K ATPase has been immunohistochemically localized to the basolateral membrane adjacent to gap junctional regions, and suggested that inhibition by ouabain may physically disrupt the junctions or create localized ionic changes which may disrupt the junctions.

The fact that there was no change in endothelial permeability despite the loss of gap junctions reveals that in the corneal endothelium, gap junctions are not of primary importance for the maintenance or operation of the corneal endothelial barrier. It should be noted that this does not rule out a possible role for gap junctions as selectivity filters, although gap junctions almost certainly function primarily as pathways for intercellular communication.

The current study also has demonstrated that perfused corneas appear to have tight junctional complexes that are smaller and less interdigitated than those of corneas fixed in the fresh, intact eye. Such observations illustrate that even when using the
Fig. 7. Transmission electron micrographs (unstained sections) of corneas perfused with GBR and GBR plus ouabain (10^{-4} M). (A) Transmission electron micrograph of GBR-perfused cornea (bar = 0.4 μm). (B) Transmission electron micrograph of ouabain-perfused cornea. The control endothelium appears to be an intact monolayer containing cuboidal-shaped cells with interdigitating lateral cell borders and intact intercellular junctions. The ouabain-perfused endothelium appears to be disrupted along the basal borders of the monolayer, where large holes are present in the cytoplasm. The basolateral membranes appear to be intact, whereas there is some disruption of the apicolateral membrane regions, where large and small vacuoles are present between cell borders (bar = 0.4 μm).
widely accepted in vitro specular microscopic perfusion technique, abnormal structural alterations to the endothelium may occur.

**Key words:** cornea, endothelial permeability, intercellular junctions, ouabain, freeze-fracture

**Acknowledgment**

The authors wish to thank Ms. Lou G. Boykins for her expertise in providing freeze-fracture replicas.

**References**