NaK-ATPase Pump Sites in Cultured Bovine Corneal Endothelium of Varying Cell Density at Confluence

Kenneth M. Crawford,* Stephen A. Ernst,* Roger F. Meyer,† and Donald K. MacCallum*

Purpose. The driving force for ion and water flow necessary for efficient deturgescence of the corneal stroma resides in the ouabain-sensitive sodium (Na) pump of corneal endothelial cells. Using a cell culture model of corneal endothelial cell hypertrophy, the authors examined the expression of Na pumps at the cell surface to see how this central element of the endothelial pump changed as corneal endothelial cell density decreased to a level associated with corneal decompensation in vivo.

Methods. 3H-ouabain binding to NaK-ATPase at saturating conditions was used to quantitate the number of pump sites on cultured bovine corneal endothelial cells as the confluent density decreased from ~2750 cells/mm² to ~275 cells/mm².

Results. The mean number of Na pump sites per cell at confluence (1.92 ± 0.07 × 10⁶) did not change as the cell density decreased 2.7-fold from 2763 cells/mm² to 1000 cells/mm². However, pump site expression doubled to ~4 × 10⁵ sites/cell as the cell density decreased from 1000 cells/mm² to 275 cells/mm². Despite the incremental increase in Na pump site expression that occurred as the cells hypertrophied below a density of 1000/mm² to achieve confluence, this increase was insufficient to prevent a decrease in Na pump site density of the intact monolayer, expressed as pump sites/mm².

Conclusion. The confluent cell density of cultured bovine corneal endothelial cells can be varied from that found in the normal native cornea to that associated with corneal decompensation. In confluent cultures with cell densities ranging from 2750 cells/mm² to 1000 cells/mm², the number of pump sites per cell remains relatively unchanged. Below cell densities of 1000 cells/mm², the number of pump sites per cell progressively increases. The increased Na pump site abundance in markedly hypertrophied endothelial cells cannot adequately compensate for the progressive reduction in the number of transporting cells per unit area within the intact monolayer. Even when considered with the decrease in the size of the paracellular ion conductive pathway that is a consequence of progressive endothelial hypertrophy, the overall pumping capacity of the intact endothelial monolayer declines. Invest Ophthalmol Vis Sci. 1995;36:1317-1326.

The corneal endothelium is a monolayer of neural crest-derived cells that lines the posterior surface of the cornea.1 When functioning properly, the endothelium regulates, with some contribution by the stratified squamous epithelium of the anterior cornea, the state of corneal stromal hydration and corneal clarity.2-4 When the endothelial monolayer is disturbed by either a disease process or a mechanical injury, the dynamic equilibrium of fluid penetration into the stroma and the removal of ions and water from the stroma to the aqueous by an energy-driven endothelial pump becomes unbalanced. As a consequence, the corneal stroma swells, the collagen lamellae become disorganized, impairing visual acuity, and the corneal endothelium loses its integrity, resulting in recurrent epithelial erosions, stromal scarring, and eventual blindness.5

Corneal transparency depends on an endothelial membrane pump that compensates for passive stromal hydration by coupling water flow to net transendothelial ionic flux to the aqueous, resulting in the maintenance of corneal deturgescence. Although the mecha-
nistic details remain to be completely defined, it is clear that the underlying driving force for transendothelial ion- and solute-linked water transport ultimately resides in the ouabain-sensitive sodium (Na) pump situated in the basolateral corneal endothelial cell membrane.6-11 The critical role of the Na pump in stromal deturgescence is demonstrated by ouabain-induced stromal swelling, a phenomenon that occurs without compromise of endothelial barrier function as judged by unaltered permeability to nonelectrolytes and fluorescent dyes and maintenance of normal junctional architecture.14-15

When corneal endothelial cells are lost in certain species, including humans, the remaining cells hypertrophy, thereby reducing the number of cells that comprise the endothelial monolayer.16 In this study, we investigated a cell culture model in which the number of bovine corneal endothelial cells present in confluent cultures can be varied (that is, the cell density varied). Subsequently, we determined the relationship between cell size and the number of Na pump sites present on the cell surface, demonstrated by the specific binding of the glycoside ouabain to NaK-ATPase.

The experiments were undertaken to learn how this critical element of the corneal endothelial pump changes as the cells exhibit compensatory hypertrophy in a manner analogous to that which occurs after corneal endothelial cell loss from disease or trauma in human and animal corneas.16-10

MATERIALS AND METHODS

Materials

Culture Media and Salt Solutions. Earle's balanced salt solution (EBSS), Dulbecco's modified medium (DMEM) with 4.5 g glucose/l, and Eagle's minimal essential medium (EMEM) were obtained from Gibco (Grand Island, NY). Iron-supplemented bovine calf serum (BCS) was purchased from HyClone (Logan, UT), and crystalline trypsin was purchased from CalBiochem (San Diego, CA). [1H]-ouabain (15 to 30 Ci/mmol) was obtained from New England Nuclear (Boston, MA), and liquid scintillation cocktail (Bio-Safe II) was obtained from Research Products International (Mount Prospect, IL). Dispase (grade II) was purchased from Boehringer Mannheim (Indianapolis, IN), ouabain octahydrate and all other chemicals were purchased from Sigma Chemical (St. Louis, MO). Human recombinant epidermal growth factor (hrEGF) was a gift from Chiron IntraOptics (Irvine, CA).

Cell Culture

Bovine corneal endothelial cell (BCEC) cultures were prepared with modifications as described by MacCallum et al. 20 Briefly, bovine eyes were obtained from a local abattoir, and the corneas were excised with an attached scleral ring and placed, endothelium side up, in a concave plastic cup. The endothelial surface was rinsed and subsequently incubated at 37°C for 1/2 hours in a sterile solution of 20 mM HEPES buffered EBSS, pH 7.4, containing 0.25% crude dispase. Endothelial cells were dislodged from Descemet's membrane by gentle scraping using a spatula with a tapered silicone surgical rubber tip. Dislodged cells were aspirated from the eyes with a pipette and added to 5 ml of DMEM containing 10% BCS and 50 μg/ml gentamicin sulfate (subsequently referred to as “standard medium”). The cells from a single eye were gently centrifuged (600g, 2 minutes), resuspended in 5 ml of standard medium supplemented with 10 ng/ml hrEGF, and incubated in 25-cm² flask at 37°C in 95% air–5% CO₂. Cultures were fed three times a week and were typically confluent in 5 to 6 days. The addition of hrEGF is not required for BCEC growth, but it was added to amplify rapidly the growth of cells from a single eye in primary culture. Confluent endothelial cultures (~2.2 - 2.5 X 10⁶ cells/25 cm² flask) were subcultivated using a 5- to 8-minute incubation in Ca²⁺, Mg²⁺-free EBSS, pH 7.4, containing 0.6 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% crystalline trypsin and subcultured.

Cells were grown in standard medium except as noted in Results, where either 10 ng/ml hrEGF or EMEM with 5% BCS (referred to as “basal medium”) were used to adjust the number of cells in a confluent monolayer. Confluence was defined as the condition in which the culture surface was completely covered by BCEC, and only rare mitotic figures were observed. Generally, experiments were conducted on cultures 2 days after they had reached confluence. Except where indicated, all cultures were fed with either standard or basal media 24 hours before ligand-binding studies. The data presented in this article were obtained from cells in the first three passages (primary culture = 0 passage.)

Cultures were routinely observed with an inverted phase-contrast microscope. For photomicrography, cultures were grown on glass coverslips, fixed in 70% methanol for 15 minutes, mounted in glycerol–phosphate-buffered saline (9:1), and photographed with a conventional phase-contrast microscope. Cell counts were made by counting the nuclei in three 1600-μm² microscopic fields for each of the cell densities illustrated and averaging the cell count.

Pump Site Determination

Equilibrium binding of [1H]-ouabain was determined in confluent bovine corneal endothelial cell cultures grown in 12 well plates (3.8 cm²/well) at 37°C. To determine saturation binding conditions, cultures were rinsed twice in potassium-free HEPES-buffered
Kreb's Ringer Henseleit solution (K⁺-free KRH), which contained in mmol/l: 149 NaCl, 1.7 CaCl₂, 1 MgCl₂, 1 NaHPO₄, 6 NaHCO₃, 5 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. The cultures were incubated for 120 minutes with concentrations of [³H]-ouabain ranging from 1 nM to 100 nM. Preliminary experiments demonstrated that 75% of equilibrium binding was achieved in 30 minutes, 94% was achieved in 60 minutes, and equilibrium was reached by 90 minutes. Duplicate incubations of [³H]-ouabain were carried out in which the medium also contained 0.5 mM unlabeled ouabain. At each concentration, specific binding was calculated from the difference between total counts and that measured in the presence of 0.5 mM unlabeled ouabain. Incubation with excess unlabeled ouabain suppressed [³H]-ouabain binding by 93% to 98% (see Results and Fig. 1). Immediately after incubation, cultures were rinsed three times with ice-cold K⁺-free KRH followed by additional 5-minute and 10-minute washes to remove unbound label. The ouabain bound to the cells after incubation (~24% of total added counts) was firmly bound to the Na pump as an additional six washes over 25 minutes reduced binding by only an additional 0.5%. Cells were then solubilized overnight in a mixture of 1% sodium dodecyl sulfate and 1 N NaOH. The solubilized cells were aspirated, and the culture well was rinsed with water to retrieve all counts. The samples were neutralized with 1 N HCl and counted by liquid scintillation spectroscopy.

**Determination of Cell Number**

The number of cells in a culture well grown and handled under conditions identical to wells used in radioligand-binding experiments was determined with an automated cell counter (Coulter Electronics, Hialeah, FL). Briefly, at the end of 2-hour incubation in K⁺-free KRH and subsequent washes, cultures were rinsed with Ca²⁺, Mg²⁺-free EBSS + 0.6 mM EDTA followed by a 5- to 5-minute incubation in Ca²⁺, Mg²⁺-free EBSS containing 0.6 mM EDTA and 0.05% crystalline trypsin. The detached BCEC were added to 0.5 ml serum to quench the action of trypsin, centrifuged (600g, 2 minutes), resuspended in an isotonic diluent, and counted.

**Statistical Analysis**

Data obtained under identical experimental conditions were pooled; means were calculated and are presented in Results with standard error (SE). Analysis of variance, regression analysis, and t-tests were applied where appropriate. Results of statistical tests were considered significant at \( P < 0.05 \).

**Construction of a Planar Paracellular Pathway Model**

A simple, two-dimensional geometric model was constructed to approximate the linear extent of the para-
cellular pathway in confluent cultures of different final cell densities. (The model actually describes the entrance to the paracellular pathway rather than its total volume, which is estimated to be 10 times endothelial cell thickness over a wide range of cell densities and cell shapes). A plot was constructed from the calculated area and perimeter of regular hexagons, a shape representative of normal, native corneal endothelium, whose sides were increased in increments of 1 μm. The area of an individual cell in 1 mm² of a monolayer at various cell densities was calculated, and its perimeter was obtained from the area-perimeter relationship of the regular hexagons (see Discussion and Fig. 6). To verify this relationship, the paracellular pathway was estimated from a model of irregular hexagons that more accurately reflected the actual arrangement of cultured corneal endothelial cells, drawn to scale at varying cell densities. The perimeter of 69 cells was measured, and the paracellular pathway was calculated as half the total perimeter. The results derived from the irregular hexagon model were virtually identical to the paracellular pathway calculations using the geometric relationships of the regular hexagons (see Discussion and Fig. 6).

RESULTS

Binding Kinetics

Preliminary studies were carried out to characterize the kinetics of ouabain binding to BCEC cultures at standard confluent densities of ~1200 BCEC/mm². The saturation binding curve for [³H]-ouabain to BCEC is illustrated in Figure 1. Saturation binding was achieved at [ouabain] = 20 nM. To ensure saturation of binding sites, 50 nM [³H]-ouabain was used for all subsequent binding determinations. A Scatchard plot (inset) of specific binding (r = 0.98) revealed a single population of binding sites with a maximal binding level and equilibrium dissociation constant (Kd) of 3.7 fmol/mm² and 6.7 nM, respectively.

Model of Corneal Endothelial Cell Hypertrophy

To achieve confluent BCEC cultures of different densities for ouabain binding studies, subcultures were initiated with a variable number of cells essentially as described. In general, the greater the number of cells subcultivated, the greater the resultant cell density at confluence. In practice, we varied both the number of cells used to initiate subcultures and the growth medium. To achieve routinely a cell density approaching the cell count in native bovine corneas, ~2500 cells/mm² ~0.5 X 10⁶ cells were subcultured in DMEM + 10% BCS + 10 ng/ml hrEGF for one medium change and then in standard medium alone. To achieve all other cell densities, ~275 ~2000 cells/mm², the number of cells used to initiate the subculture, was progressively reduced (essentially 1:2 to 1:64 subcultivation ratios) and grown in either standard medium (DMEM + 10% BCS) or basal medium (MEM + 5% BCS). The use of basal medium (MEM + 5% BCS) was necessary to achieve confluent cultures of low density (<500 cells/mm²) with all cells approximately the same size; the use of standard medium in similar circumstances resulted in confluent cultures composed of groups of cells exhibiting disparate sizes (not shown).

Representative phase-contrast photomicrographs of cultures with density similar to that of native bovine cornea (2221 cells/mm²), intermediate cell density (958 cells/mm²), and density exhibiting significant hypertrophy (325 cells/mm²) are shown in Figure 2. As cells enlarged, they became less regularly shaped when compared to cells at higher densities.

Effect of Cell Density on Na Pump Expression

Confluent BCEC cultures with density ranging from 2763 cells/mm² to 275 cells/mm² were used for [³H]-ouabain studies. When cell proliferation was stimulated with 10 ng/ml hrEGF and removed at least 48 hours before determination of ouabain binding, the resultant cell density at confluence was 2396 ± 112 cells/mm² (n = 11), with each cell containing 1.54 ± 0.03 X 10⁶ pump sites per cell (n = 8). When subcultivation ratios alone were adjusted, a wide range of final cell densities at confluence resulted. For example, a 1:2 split resulted in 1104 cells/mm², whereas 1:8 and 1:64 splits resulted in 811 cells/mm² and 308 cells/mm², respectively (Fig. 3). At these confluent cell densities, [³H]-ouabain binding revealed 1.71 ± 0.03, 2.10 ± 0.14, and 2.82 ± 0.05 X 10⁶ pump sites/cell, respectively (Fig. 3). Analysis of variance (P = 0.0008) revealed a significant effect of using progressively fewer cells to initiate subcultures on the number of ouabain-binding sites per cell in the resultant stable, confluent cultures.

Figure 4 shows the number of pump sites per cell plotted as a function of cell density at confluence for the experiments conducted in this study. As confluent cell density decreased from normal in vivo values, ~2700 cells/mm² to 1000 cells/mm², the number of pump sites per cell was independent of cell density (r = -0.25, P = 0.36). In this range, 2700 → 1000 cells/
Cultured BCE Sodium Pumps

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mm², BCEC exhibited a mean number of 1.92 ± 0.07 X 10¹ pump sites/cell even though the cell density decreased 2.7-fold. However, as confluent cell density decreased below 1000 cells/mm² to a minimum of 275 cells/mm², the number of pump sites per cell increased significantly from 1.92 x 10⁶ to 4 x 10⁶/cell (r = -0.77, P < 0.001).

Two features of the culture system, time and the use of epidermal growth factor (EGF), were further examined to see whether either feature could bias results. Confluence was usually achieved within 9 days of subcultivation for cell densities >500 cells/mm². However, to rule out any effect of time on pump site density, we determined pump site density at confluence (day 9) as well as 3 and 6 days after confluence (days 12 and 15) in one experiment. The differences between the number of pump sites per cell or the number of pump sites per mm² did not differ significantly between confluence and 3 and 6 days after confluence (Table 1).

To rule out an acute effect of EGF on Na pump site density, we added EGF 24 hours before conducting ouabain-binding experiments to half a set of matched cultures. The EGF group had a cell density of 1865 ± 11 (n = 6) and a pump site density of 2.01 x 10⁶ ± 0.03 sites/cell (n = 8), whereas the control group had a cell density of 2208 x 10⁶ ± 34 (n = 6) and a pump site density of 2.05 x 10⁶ ± 0.03 sites/cell (n = 6). The differences between the number of pump sites per cell in the untreated and treated groups was not significant (P = 0.32). Additionally, analysis of the data presented in Figure 4 on the pump sites per cell in cultures having a cell density of >1000 cells/mm², a group comprised of both untreated and EGF treated cultures, also ruled out any special effect EGF might have had on pump site density; in this study, pump site density varied only in relation to confluent cell density.

When the total number of pump sites within the entire endothelial monolayer (expressed as pump sites/mm²) was plotted, a linear decrease (r = 0.94, P < 0.001) in pump site density was observed as the cell density fell from 2700 cells/mm² to 275 cells/mm². The relationship between the number of ouabain-binding sites per unit of surface area and the cell density is illustrated in Figure 5 (for 2700 cells/mm², 4.65 x 10⁶ binding sites/mm²; for 1000 cells/mm², 2.04 x 10⁶ sites/mm²; for 275 cells/mm², 0.93 x 10⁶ binding sites/mm²). Thus, although hypertrophied bovine corneal endothelial cells do exhibit
FIGURE 3. The relationship of initiating cultures with progressively fewer cells (increasing "split ratios"), final cell number in a confluent culture, and the change in Na pump sites per cell is illustrated (each bar represents the mean ± SEM for eight 3.8 cm² wells.) As bovine corneal endothelial cells hypertrophy in culture, there is a progressive increase in the number of pump sites per cell.

increased numbers of pump sites per cell in the range of 1000 to 275 cells/mm², this increase did not prevent an overall decline in the number of pump sites within the endothelial monolayer as the cell density decreased.

FIGURE 4. The number of Na pump sites per cell in confluent bovine corneal endothelial cell cultures of varying cell density is illustrated for 43 different experiments. The number of pump sites per cell does not vary statistically in cultures with a final cell density of 1000 to 2700 cells/mm² (the line illustrated is the regression line for cell densities of 1000 to 2700 cells/mm²; r = -0.25, P = 0.36). As cell density declines from 1000 cells/mm² to 275 cells/mm², there is a significant increase in the number of pump sites per cell (r = -0.77, P < 0.001).

FIGURE 5. The number of pump sites for the entire endothelial monolayer, expressed as pump sites per mm², is compared with the cell density in confluent bovine corneal endothelial cell (BCEC) cultures. A linear decrease (r = 0.94, P < 0.001) in the number of pump sites per mm² is observed as the cell number in confluent cultures declines. Even though there is a progressive increase in the number of pump sites per cell as BCEC continue to hypertrophy below 1000 cells/mm² (Fig. 4), that increase is not sufficient to prevent a sustained loss of pump sites in the endothelial monolayer as a whole.

TABLE 1. Number of Cells, Pump Sites/Cell, and Pump Sites/mm² at Confluence and After Confluence

<table>
<thead>
<tr>
<th>Days in Culture</th>
<th>9</th>
<th>12</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁶ cells/well</td>
<td>1.90 ± 0.03</td>
<td>1.76 ± 0.03</td>
<td>1.85 ± 0.04</td>
</tr>
<tr>
<td>10⁶ pumps/cell</td>
<td>2.63 ± 0.06</td>
<td>2.78 ± 0.08</td>
<td>2.83 ± 0.06</td>
</tr>
<tr>
<td>10⁶ pumps/mm²</td>
<td>1.33 ± 0.03</td>
<td>1.29 ± 0.04</td>
<td>1.37 ± 0.03</td>
</tr>
</tbody>
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Each point is the mean ± SEM for eight 3.8 cm² wells. There is no statistical difference between the number of pump sites/cell (P = 0.17) and pump sites/mm² (P = 0.27) at the three time points indicated.

DISCUSSION

By varying the number of cultured bovine corneal endothelial cells used to initiate cultures as well as the culture conditions, a wide range of cell densities in confluent cultures can be achieved. Cell densities in this culture system ranged from those observed in the native cornea²,³,²³ to those associated with corneal decompensation.¹⁵ The ability to vary corneal endothelial density over a wide range, coupled with reports of altered corneal endothelial cell pump function associated with decreased cell density,¹⁶,¹⁷,²⁴,²⁵ prompted us to study how the Na pump density changed as a consequence of progressive cell hypertrophy. It is firmly established that the Na pump is the driving force required for vectorial ion and fluid transport to the
aqueous humor and is a central element in endothelial-mediated deturgescence of the corneal stroma. Additionally, the number of functional Na pump sites on the endothelial cell surface can be easily quantified by the specific binding of $^3$H-ouabain. The dissociation constant ($K_0$) for ouabain observed in this study ($K_0 = 6.7 \text{ nM}$) agrees well with the calculated $K_0$ for cultured bovine corneal endothelium in the studies by Savion and Farzame ($K_0 = 10 \text{ nM}$) and Geroski and Hadley ($K_0 = 25 \text{ nM}$). The mean number of Na pump sites per cell in cultures having a density greater than 1000 cells/mm$^2$ was $\sim 1.9 \times 10^6$ in this study, which is somewhat greater than that reported by Savion and Farzame ($0.8 \times 10^6$) or Geroski and Hadley ($1.4 \times 10^6$) for confluent BCEC cultures. In general, there is good agreement among these three studies of cultured bovine corneal endothelium given methodologic differences that exist between each study.

Na pump site density has not been measured in native bovine corneal endothelium. There is a report that total ATPase activity, as contrasted with ouabain-sensitive activity, is lower in cultured bovine corneal endothelial cells than in native cells. In contrast, however, cultured rabbit corneal endothelial cells have been reported to exhibit somewhat higher Na pump site density ($3.3 \times 10^6$) than native cells ($3.0 \times 10^6$) by ouabain-binding analysis. Regardless of the differences, if any, between Na pump site density in native versus cultured corneal endothelium, we think the ability to alter corneal endothelial cell density rapidly in a simple monolayer cell culture system provides a useful tool in which to study the relationship between cell density and the number of Na pumps on the cell surface, including any compensatory increase in pump sites per cell as cell density decreases.

A surprising finding in our study was the fact that the number of pump sites per cell ($\sim 2 \times 10^6$) did not change significantly from 1000 cells/mm$^2$ to 2700 cells/mm$^2$, a 2.7-fold increase in cell density. The first study to quantify the number of pump sites in corneal endothelium, Geroski and Edelhauser pointed out that corneal endothelial cells could theoretically accommodate many more cell membrane Na pump sites than were present on native rabbit corneal endothelium. Our study carries that observation further by suggesting that corneal endothelial cells are capable of regulating the number of Na pump sites on their surfaces, and they do so over a surprisingly wide range of cell densities. As discussed on the next page, where Na pump sites, cell density, and the size of the paracellular pathway are compared, it appears that a pump site density of $\sim 2 \times 10^6$ sites/cell is more than adequate to compensate for the geometric consequence of a larger paracellular pathway for passive ion movement that coincides with smaller cells and more cells per mm$^2$.

Cultured bovine corneal endothelial cells do exhibit an increasing number of Na pump sites per cell as they continue to hypertrophy below a density of 1000 cells/mm$^2$. We originally thought that this progressive increase in the number of Na pump sites per cell might actually compensate for the overall reduction in the number of cells within the monolayer. However, that hypothesis proved not to be true when the number of pump sites within the entire monolayer, expressed as pump sites per mm$^2$, was calculated. The number of pump sites declined in a generally linear fashion as the number of cells within the monolayer declined. When considered by itself, the increased number of pump sites per cell attending marked cellular hypertrophy cannot be regarded as "compensatory increase" because the pump site density of the monolayer as a whole (that is, the capacity of the endothelium to serve as a fluid pump) continues to decline as the cell number declines. Even when considered in relation to the decreased extent of the paracellular pathway in hypertrophied cells, discussed on the next page, the number of pump sites is reduced in confluent low-density cultures compared to cultures of higher cell density.

Solute accumulation on the basal or stromal side of corneal endothelium results in increased water uptake by osmotic equilibration. The so-called "fluid pumping" mechanism of the endothelium maintains a dynamic equilibrium by countering this leak by solute-coupled fluid transport to the aqueous driven by the Na pump. Previous studies indicated that the pathway for transendothelial water movement is likely to be by a cellular route, and recent studies suggest that CHIP28-type water channels, which are localized in apical and basolateral endothelial cell membranes, may account for as much 90% of endothelial cell osmotic permeability. It is also reasonable to speculate that solute movement from the stroma to the aqueous, ultimately driven by the sodium pump, results in water transport through the same cellular water channels. Within physiological limits, it would be expected that any increase in solute accumulation and, therefore, water uptake toward the stromal side would be compensated by increased solute-dependent fluid movement to the aqueous to maintain homeostasis. Corneal endothelium is likely to have relatively high paracellular or junctional ionic conductance, consistent with low transendothelial resistance measurements and discontinuous occluding junctions. Significant increases in the linear extent of this paracellular pathway, such as would occur as cell density increases, would be expected to increase passive ionic movement toward the stroma and, therefore, osmotic water flow, possibly through CHIP28-type water chan-

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nels. In this scenario, greater demands would be placed on the compensatory fluid pumping system. One possible mechanism for the latter could be an increase in the number of pumps, although increased pump activity by previously existing pumps would obviously be an additional mechanism.

To understand further the relationship among Na pump sites, cell density, and the "pumping capacity" of the intact endothelial cell monolayer as they relate to the area of junctional contact, we constructed a simple, two-dimensional model of corneal endothelial cell hypertrophy and measured the linear extent of the paracellular pathway at different cell densities (see Materials and Methods). What we measured corresponds to the "area of intercellular space facing the anterior chamber" calculated using a different method by Bourne and Brubaker (with which our method of calculation agrees closely) and not the volumetric extent of the paracellular pathway, the depth of which is reported to be approximately 10 times the endothelial cell height over a wide range of cell densities. The relationship between the linear (or apical) extent of the paracellular pathway (designated LP, "leak pathway") in a model of either regular or irregular hexagons representing the endothelial cell monolayer at various cell densities is shown in Figure 6. Both hexagonal models exhibit the same degree of reduction in the planar extent of the paracellular pathway as cells hypertrophy.

Despite the inherent limitations of such a simple geometric model, it is helpful in unifying the findings of the present study. For example, even though the number of Na pump sites per cell remains the same while the apical extent of the paracellular pathway (leak path [LP], Fig. 6) for ionic movement is enhanced as cell density increases above 1000 cell/mm², there is, nonetheless, an increase in the number of pump sites per mm² and pump sites per leak path as the cell density increases. Thus, simply increasing the number of cells within the monolayer without increasing the Na pump site density per cell increases the potential transport capacity of the monolayer (pump sites per mm²) and the number of pump sites associated with the linear extent of the paracellular pathway available for passive ion conductance (Fig. 7).

With regard to marked cell hypertrophy, the number of Na pump sites in our paracellular pathway model progressively decreases (Fig. 7) even though the number of pump sites per cell essentially doubles as the cells enlarge to a density of 275 cells/mm². The only degree of compensation that can be inferred by hypertrophied endothelial cells exhibiting progressively more pump sites per cell is that if they did not do so, the decrease in pumping capacity of the monolayer (pump sites per mm², pump sites per paracellular pathway) would be even greater than that observed in the present study. In fact, the increased number of pump sites present on markedly hypertrophied cells may be a simple reflection of maintaining cellular ionic homeostasis in the face of a greatly expanded plasma membrane.

Decreased pumping capacity, as well as decreased numbers of Na pump sites per unit length of cell mem-
brane, have been reported in damaged or diseased corneas that exhibit cell hypertrophy, suggesting that such endothelia might not have the ability to increase the number of Na pump sites on their surfaces to the extent of that observed in this study. Such studies provide ample warning about generalizing the Na pump site density–cell hypertrophy relationships observed in this study. Encouragingly, however, Na pump sites per cell calculated from a recent study of feline corneal endothelial cell repair by Bourne et al do fit with the Na pump site per cell–cell density relationship observed with the cultured bovine corneal endothelial system.

The changes in Na pump site density observed in this easily manipulated corneal endothelial culture system emphasize the usefulness of cultured bovine corneal endothelium in studying this important element of the corneal endothelial ion and water transporting system.

**Key Words**

Na,K-ATPase, Na pump, paracellular pathway, ouabain, bovine corneal endothelium, cell culture

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