Quantitation of Na/K ATPase Pump Sites in the Rabbit Corneal Endothelium

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In these experiments, the binding of $^3$H·ouabain, a specific inhibitor of Na/K ATPase, was used to quantitate the density of Na/K ATPase pump sites in the rabbit corneal endothelium. The uptake of ouabain by the corneal endothelium shows two components: one that saturates at a ouabain concentration near $2 \times 10^{-7}$ M (specific binding), and one component that increases linearly with increasing glycoside concentration (nonspecific uptake). The nonspecific uptake can be accounted for by ouabain equilibrating with the extracellular space, which, estimated by inulin space, amounts to $13.0 \text{ nl/mm}^2$ of endothelium. The saturable component of endothelial ouabain uptake is displaced by K$^+$ ions, which is consistent with this fraction being bound to Na/K ATPase. Maximal endothelial ouabain binding was measured as $20.7 \text{ fmol/mm}^2$ of endothelium, which corresponds to $3.0 \times 10^6$ pump sites per cell. The density of Na/K ATPase pump sites in the rabbit corneal endothelium is comparable to densities reported for several transporting epithelia. These data are consistent with the known function of the endothelium in corneal deturgescence and corroborate the importance of Na/K ATPase in endothelial fluid transport. Invest Ophthalmol Vis Sci 25:1056–1060, 1984

Current evidence indicates that pump function of the corneal endothelium is mediated by the active transport of sodium and bicarbonate ions across the endothelium and into the aqueous humor. As in other transporting tissue, Na/K ATPase seems to be an essential component of this transport process. Since this enzyme first was measured in corneal endothelium by Bonting et al., considerable evidence has accumulated, which implicates it as a necessary component of endothelial fluid transport.

Brown and Hedbys and Langham and Kostelnik demonstrated that ouabain, a specific inhibitor of Na/K ATPase, prevented corneal temperature reversal of enucleated eyes. Ouabain also was shown to cause corneal edema when injected into the aqueous humor of rabbit eyes in vivo. Trenberth and Mishima have shown that isolated, ouabain-perfused, rabbit corneas swell in a dose-dependent manner due to pump-function inhibition. We recently have confirmed these findings in the human cornea as well.

The dose–response curve for ouabain-induced corneal swelling is strikingly similar to the ouabain inhibition curve for endothelial Na/K ATPase. Moreover, the effects of ouabain on both corneal deturgescence and Na/K ATPase are both biphasic with low concentrations of ouabain, enhancing enzyme activity and stimulating corneal deswelling as described by Anderson et al.

Electrophysiologic studies have further corroborated the importance of endothelial Na/K ATPase to pump function. Fischbarg has shown ouabain to inhibit the transendothelial potential difference, but not to affect the electrical resistance (barrier function) of the endothelium. Finally, the active sodium flux across the endothelium has recently been measured by Huff and Green and Lim. This net active sodium flux also is inhibited by ouabain.

Since it seems quite likely that endothelial pump function is linked to the activity of Na/K ATPase, these experiments were undertaken to further quantitate this enzyme in the rabbit corneal endothelium. In these studies the specific binding of $^3$H·ouabain by the corneal endothelium was investigated to determine the density of Na/K ATPase “pump sites” in this corneal layer.

Materials and Methods

Ouabain Binding Studies

$^3$H·ouabain (14.0–18.0 Ci/mmole) was obtained from New England Nuclear (Boston, MA). The ouabain was supplied in an ethanol-benzene solution which...
was evaporated to dryness under nitrogen, and the isotope was redissolved in deionized water. High performance liquid chromatography (HPLC) was used to determine the radiochemical purity of the \(^3\text{H}\) ouabain. A C18-µm Bondapak (10-µm particle diameter) column was eluted at a flow rate of 1.0 ml/min (1.0 ml fractions) using 84% H\(_2\)O:16% acetonitrile as the mobile phase. Ultraviolet absorbance was monitored at 220 nm. The \(^3\text{H}\) ouabain, which had a retention time of 7 min in this system, was found consistently to have a radiochemical purity greater than 95% (Fig. 1).

Eyes of 2-3-kg New Zealand albino rabbits were enucleated. The corneal epithelium was removed by scraping with a Gill knife, and the corneas were excised with a 1-2-mm scleral rim. Each deepithelialized cornea was incubated at 37°C in a shaking waterbath using 3.5 ml of a K\(^+\)-free bicarbonate Ringer's incubation solution of the following composition (g/liter): NaCl: 6.801; CaCl\(_2\)-2H\(_2\)O: 0.153; MgCl\(_2\) • 6H\(_2\)O, 0.158; NaH\(_2\)PO\(_4\):0.103; NaHCO\(_3\):2.453; and glucose: 0.903. In additional experiments designed to study the effects of K\(^+\) on ouabain binding, 16-mM KCl was substituted for an equivalent amount of NaCl. Carrier-free \(^3\text{H}\) ouabain was added to the incubation media in concentrations increasing to \(5 \times 10^{-7}\) M. Concentrations greater than \(5 \times 10^{-7}\) M were achieved by adding the appropriate amounts of cold ouabain to \(5 \times 10^{-7}\) M carrier-free \(^3\text{H}\) ouabain. To estimate extracellular space 1.0 µCi of \(^{14}\text{C}\) inulin (specific activity 3 mCi/g) was added to each of the initial 3.5-ml incubations. In a final group of experiments, \(10^{-4}\) M cold ouabain was added to the appropriate concentrations of \(^3\text{H}\) ouabain in order to distinguish specific from nonspecific binding.

Since initial incubations showed that steady-state ouabain uptake was achieved in 2.5-3 hr (Fig. 2), a 3-hr incubation period was chosen for subsequent studies. At the end of this incubation, the corneas were removed from the incubation medium, briefly drained, and rinsed three times (10 sec each) in ice-cold, ouabain-free, incubation medium. An 8-mm button of central cornea then was trephined out, and the endothelium plus Descemets was stripped away carefully as an intact sheet from the overlying stroma. Each cornea thus provided one sheet of endothelium and one sheet of stroma, each of which was dissolved in 1.0 ml of Protosol. The uptake of \(^3\text{H}\) and \(^{14}\text{C}\) was measured in each 8-mm diameter sheet using scintillation spectroscopy.

**Endothelial Cell Counts**

Corneas of 2–3-kg New Zealand albino rabbits were excised with a 2-mm scleral rim and placed into a balanced salt solution (BSS, Alcon)-filled chamber of a PRO eye bank microscope. Endothelial cells in five to ten 0.03 mm\(^2\) fields of each cornea were counted. Mean (±SEM) cell density was determined as 4126 ± 118 cells/mm\(^2\) (n = 10 corneas).

The use of animals in these experiments conforms to the ARVO Resolution on the Use of Animals in Research.

![Fig. 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933349/)

**Fig. 1.** High performance liquid chromatograph of \(^3\text{H}\) ouabain. A C18-µm Bondapak column was eluted at 1 ml/min using a mobile phase of 84% H\(_2\)O:16% acetonitrile. UV absorbance was monitored at 220 nm. Radiochemical purity of \(^3\text{H}\) ouabain was consistently greater than 95%.

![Fig. 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933349/)

**Fig. 2.** The time course of ouabain (\(10^{-8}\) M) and inulin uptake by the corneal endothelium in potassium-free medium. Values are the mean ± standard error. The number of measurements at each time is shown in parentheses.
Results

Endothelial ouabain (10^{-8} M) and inulin uptake as functions of time are shown in Figure 2. Steady-state ouabain uptake is achieved in 2.5 hr; therefore, a 3-hr incubation period was chosen for subsequent experiments. The uptake of inulin by the corneal endothelium is quite rapid with steady-state being reached within 1 hr. The inulin space of endothelium plus Descemet's membrane was determined to be 13.0 ± 0.4 nl/mm^2 of endothelium (±SEM, n = 42).

Figure 3 shows endothelial ouabain uptake as a function of glycoside concentration in potassium-free and 16-mM potassium media. Each point represents the mean (± standard error) of 5-10 determinations. Standard errors not shown lie within the boundaries of data point symbols.

Endothelial ouabain uptake consists of two components: one saturable at a glycoside concentration near 2 \times 10^{-7} M, while the other increases linearly with increasing concentration. Baker and Willis\(^{14}\) originally described this pattern of ouabain uptake and demonstrated that the saturable uptake component is associated with the specific binding of ouabain to Na/K ATPase, while the linear component reflects nonspecific uptake. Figure 3 also shows that potassium ions displace the saturable component but have no effect on the linear component. This is consistent with the known effects of potassium on reducing the affinity of Na/K ATPase for ouabain.\(^{15}\)

For comparison, Figure 4 shows stromal ouabain uptake at different glycoside concentrations. In this tissue layer, which consists primarily of extracellular space, ouabain uptake consists of only one component that increases linearly with ouabain concentration—nonspecific uptake. As expected, potassium has no effect on stromal uptake, and all of the glycoside taken up by this corneal layer can be accounted for by that entering the extracellular space, which was determined to be 981 ± 24 nl/mm^2 (n = 42). This inulin space is obtained from the stroma of a deepithelialized cornea following a 3-hr, in vitro incubation. The stroma is thus grossly swollen and the inulin space is large.

To determine the contribution of nonspecific binding to total endothelial ouabain uptake, uptake of \(^3\)H-ouabain was measured in the presence of a saturating concentration (10^{-4} M) of unlabelled glycoside. This data is shown in Figure 5. Mean (±SEM) values of ouabain uptake versus concentration are shown as is the calculated least squares line of best fit (r = 0.981). In the presence of 10^{-4} M cold ouabain, the uptake of tracer is a linear, nonsaturable function of concentration. Also depicted in Figure 5 is the calculated line for \(^3\)H-ouabain uptake into the inulin space, which is based on the measured inulin space of 13.0 nl/mm^2 endothelium. These data demonstrate that essentially all of the nonspecific uptake of ouabain is accounted for by that ouabain taken up by the extracellular space and that nonspecific binding (ie, nonsaturable uptake — inulin space uptake) is minimal in the corneal endothelium. Even at 5 \times 10^{-7} M, a concentration at which nonsaturable ouabain uptake accounts for 32% (7.6/23.4 to yield a value of 32%) of total uptake, nonspecific binding amounts to only 4.7% of total ouabain uptake.

Bound ouabain was determined by subtracting tracer uptake in the presence of 10^{-4} M unlabelled ouabain
Fig. 5. Nonsaturable endothelial ^3H^-ouabain uptake (solid line) measured in the presence of 10^-4 M unlabelled ouabain. Each point is the mean ± standard error of six determinations. Also shown is the inulin space ^3H^-ouabain uptake (broken line), which was calculated from the measured inulin space of 13.0 ± 0.4 nl/mm^2 of endothelium.

from total tracer uptake in the presence of carrier-free ^3H^-ouabain only. A double reciprocal plot was constructed and is shown in Figure 6. Each point represents the mean of 5–7 determinations. The least squares line of best fit was calculated (r = 0.999) to determine maximal binding and the apparent dissociation constant (K_D), which were determined to be 20.7 fmoles/mm^2 endothelium and 4.7 × 10^-8 M, respectively.

Discussion

The results of this study demonstrate that ouabain is bound specifically and with high affinity by the rabbit corneal endothelium. Since each Na/K ATPase "pump site" binds one molecule of ouabain,16 determinations of ouabain binding enable direct quantitation of Na/K ATPase pump sites. Maximal ouabain binding in the rabbit corneal endothelium is 20.7 fmoles/mm^2 of endothelium. This corresponds to 1.25 × 10^10 pump sites/mm^2. The mean endothelial cell density for the rabbits used in this study was measured as 4126 cells/mm^2 of endothelium. Therefore, each endothelial cell has 3.0 × 10^6 Na/K ATPase pump sites. Since endothelial Na/K ATPase has been localized histochemically to the lateral cell membrane,17–19 this membrane region must be capable of accommodating the pump sites measured in this study. Based on the measured cell density, the mean anterior (Descemet's surface) and posterior (aqueous surface) membrane surface areas for an endothelial cell are 242 μm^2 each. Assuming that the endothelial cell is a cylinder of 5 μm height, a minimal estimate of lateral membrane area can be calculated as 2.76 × 10^10 nm^2/cell. Since each Na/K ATPase "site" occupies a membrane area of 1000 nm^2,20 the lateral membrane area is capable of accommodating a minimum of 2.8 × 10^7 sites per cell—nearly 10 times the number measured. This calculation neglects the tortuosity of the lateral cell membrane and thus underestimates membrane area. In reality, many more pump sites could be accommodated in the lateral membrane than measured; therefore, the estimated density would appear reasonable.

For comparison to corneal endothelium, Table 1 presents Na/K ATPase pump site densities reported for several other tissues. The density of Na/K ATPase pump sites in the corneal endothelium is comparable with that reported for renal tubule and is of the same order of magnitude as that determined for choroid plexus. These comparisons suggest that, on a cellular basis, the transport capacity of the corneal endothelium is great and comparable to that of tissues known to have high rates of active transport. The data of this study thus provide a new perspective on the relative magnitude of endothelial transport capacity, which can

Table 1. Comparison of Na/K ATPase pump site densities and ouabain-ATPase dissociation constants reported for several other tissues to corneal endothelium

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Na/K sites per cell</th>
<th>K_D (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human erythrocyte21*</td>
<td>260</td>
<td>2.5 × 10^-7</td>
</tr>
<tr>
<td>Rabbit renal tubule22</td>
<td>4.0 × 10^6</td>
<td>5.1 × 10^-7</td>
</tr>
<tr>
<td>Cat ventricular muscle23</td>
<td>5.2 × 10^6</td>
<td>6.3 × 10^-5</td>
</tr>
<tr>
<td>Frog choroid plexus24</td>
<td>10.0 × 10^6</td>
<td>8.0 × 10^-7</td>
</tr>
<tr>
<td>Teleost chloride cell25</td>
<td>1.5 × 10^8</td>
<td>—</td>
</tr>
<tr>
<td>Rabbit corneal endothelium</td>
<td>3.0 × 10^6</td>
<td>4.7 × 10^-4</td>
</tr>
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* Reference number.
be difficult to appreciate because of the small mass of endothelium per cornea.

The high affinity of endothelial Na/K ATPase for ouabain as estimated by the apparent dissociation constant, $K_D$, suggests that endothelial Na/K ATPase should be quite sensitive to ouabain inhibition. This agrees well with the results of ouabain perfusion studies in both rabbit and human corneas, which have demonstrated the dose–response relationship of ouabain concentration to corneal swelling rate.

In these experiments, no stromal ouabain binding could be measured. This should not be interpreted as demonstrating the absence of Na/K ATPase in the stroma. Specific ouabain binding in this corneal layer, using the present techniques, is masked by a very high level of nonspecific uptake into the extracellular space which, in the rabbit cornea, comprises 90% of the stromal volume. Ouabain most certainly is bound by the stromal keratocytes. The amount bound, however, is immeasurable using the present method, and all stromal ouabain uptake is accounted for by that which equilibrates with the extracellular space.

Key words: cornea, corneal endothelium, ouabain, pump function, sodium-potassium ATPase

References


