Objective: To determine the expression of α subunits and different isoforms of Na⁺,K⁺–adenosine triphosphatase (ATPase) in human corneal endothelial cells (HCECs).

Methods: Immunoblot and RNA analysis of Na⁺,K⁺-ATPase α subunit expression were performed in preparations from HCECs that had been immortalized by transformation with simian virus 40. Na⁺,K⁺-ATPase activity was determined by constructing dose-response curves for the ouabain inhibition of Na⁺,K⁺-ATPase activity in human corneal endothelial cells.

Results: Both messenger RNA analysis and immunoblot studies indicated that HCECs express ATPase catalytic α₁ and α₃, but not α₂ and α₄, subunits. A limited amount of α₃ subunit was expressed in HCECs compared with the α₁ subunit. Biochemical analyses of Na⁺,K⁺-ATPase activity revealed 2 independently active Na⁺,K⁺-ATPase isoenzymes, a low-affinity site with a kinetic parameter for ouabain inhibition constant (Kᵢ) in the micromolar range and a high-affinity site with a constant Kᵢ in the nanomolar range. These 2 sites may be associated with α₁ and α₃ isoforms, respectively, expressed in HCECs.

Conclusions: Human corneal endothelial cells express α₁ and α₃ isoforms of Na⁺,K⁺-ATPase, and both polypeptides are catalytically competent in these cells. Defining the components of Na⁺,K⁺-ATPase in HCECs is an important step toward elucidating the mechanisms that regulate corneal endothelial ionic pump function as well as the pathogenesis of corneal diseases associated with corneal edema.

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THE CORNEAL endothelium is essential for the maintenance of normal corneal hydration, thickness, and transparency.¹ ² Corneal transparency is achieved by precise arrangement of the collagen fibers within the corneal stroma and depends to a great extent on the maintenance of a relatively low level of stromal hydration. The corneal stroma tends to imbibe water owing to the charge characteristics of the proteoglycans present within the structure. The continual movement of water into the stroma would lead to stromal swelling and loss of transparency if mechanisms were not present to remove fluid from the stroma. The endothelium has been shown to be responsible for maintaining stromal hydration by the activity of ionic pumps. A variety of studies suggest that metabolic energy is needed to maintain normal corneal thickness,³ ⁴ and this energy requirement has been shown to be associated with the activity of Na⁺,K⁺–adenosine triphosphatase (ATPase) pumps located in the basolateral membrane of the corneal endothelial cells at an intensity of 1.5 × 10⁶ pump sites per cell.⁵ ⁷ These ATPase pumps are believed to function by creating a net ionic flux from the intracellular to the extracellular milieu. The osmotic gradient produced then causes water to be drawn passively from the stroma to the aqueous humor.⁵ ¹⁰

The Na⁺,K⁺-ATPase pump is a plasma membrane enzyme that mediates the ATP-dependent exchange of Na⁺ and K⁺ across the plasma membrane, establishing the low internal Na⁺ and high internal K⁺ concentrations characteristic of most eukaryotic cells. By using the energy from the hydrolysis of 1 molecule of ATP, the enzyme transports 3 Na⁺ ions out in exchange for 2 K⁺ ions that are taken into the cell.¹¹ ¹² The Na⁺ gradient that the Na⁺,K⁺-ATPase pump generates provides the energy that fuels the cotransport of water, other ions, sugar, and amino acids across the cell membrane.¹³ ¹⁴ In epithelial cells, the asymmetrical distribution of Na⁺,K⁺-ATPase on the plasma membrane is essential to drive the vectorial transport of fluid and salt to either the capillary or luminal side of the cells. In this manner, the enzyme is important in controlling the reabsorption of Na⁺ and water in the kidney.¹⁵ and the compo-
position of transcellular fluids, such as cerebrospinal fluid in the central nervous system, aqueous humor of the eye, and endolymph in the inner ear.

Structurally, Na⁺,K⁺-ATPase is an oligomer, resulting from the association of distinct molecular forms of 2 major polypeptides, the α and β subunits. The α subunit is a multispanning membrane protein with a molecular mass of approximately 112 000 Da and is responsible for the catalytic and transport properties of the enzyme. The β subunit is a polypeptide that crosses the membrane once and, depending on the degree of glycosylation in different tissues, has a molecular weight between 40 000 and 60 000 Da. The β subunit is essential for the normal activity of Na⁺,K⁺-ATPase, and it influences the K⁺/Na⁺ affinities of the enzyme. In addition, in vertebrate cells the β subunit acts as a chaperone, stabilizing the correct folding of the α polypeptide to facilitate its delivery to the plasma membrane.

There are different genes encoding distinct molecular forms of the α and β polypeptides. Three structural variants of the α polypeptide, known as α1, α2, and α3, were originally cloned from mammals. More recently, a fourth α isoform (α4) has been cloned from a rat testis library. For the β subunit, 3 isoforms (β1, β2, and β3) have been identified in vertebrates. Both the α and β isoforms are differentially expressed depending on the tissue. In the adult rat, the α1 and β1 subunits are expressed in nearly every tissue; α2 is present in the brain, skeletal muscle, heart, and adipocytes; and α3 and β2 are restricted to the nervous system. Also, the expression of the isoforms varies in relation to cell differentiation and tissue maturation. Interestingly, the association of different α and β pairs results in Na⁺,K⁺-ATPase isozymes that have distinct cation, substrate, and ouabain affinities. These results suggest that Na⁺,K⁺-ATPase isoforms have unique physiological roles and that the precise regulation of their expression may be important in modulating the activity of the enzyme to meet the functional requirements of the various cell types and tissues.

In this study, we have characterized the expression of the messenger RNA (mRNA) and protein of the various Na⁺,K⁺-ATPase α isoforms in an established human corneal endothelial cell line. This cell line contains human corneal endothelial cells (HCECs) that have been immortalized by transformation with simian virus 40 (SV40), thereby providing a useful tool in characterizing and understanding endothelial functions in vitro. To our knowledge, this is the first report that HCECs are able to express the α1 and α3 isoforms of Na⁺,K⁺-ATPase and that both polypeptides are catalytically competent in these cells.

**METHODS**

**CELL CULTURE**

The SV40-transfected HCEC line was kindly provided by Steven Wilson, MD. Cells were grown at 37°C in a 5% carbon dioxide atmosphere in medium containing 10% fetal bovine serum and 5% calf serum. The cells were plated at a density of 3 to 6 × 10⁴ and allowed to grow until confluence before they were used.

**TISSUE AND CELL PREPARATIONS**

Membrane fractions were prepared from HCECs following the protocol described previously. Briefly, samples were homogenized on ice in 320 mM sucrose, 25 mM imidazole (pH 7.4), and 0.1 mM ethyleneglycoltetraacetic acid using a glass–glass homogenizer. The lysate was then centrifuged at 1000g for 10 minutes. The pellet was resuspended, homogenized again, and centrifuged at 1000g for 10 minutes. Both the supernatants of the first and second centrifugations were combined and centrifuged for an additional 30 minutes at 30 000g. The final pellet was resuspended in the original homogenization buffer. The same procedure was used to prepare membranes from rat brain and from SC-9 cells expressing the Na⁺,K⁺-ATPase α4 isoform.

**RNA BLOT ANALYSIS**

We isolated RNA from tissue and cells using RNAzol (Tel-Test, Inc, Friendswood, Tex), according to the supplier’s protocol. RNA content was quantified spectrophotometrically. Equal amounts of RNA (15 µg) were separated by electrophoresis in a formaldehyde gel and transferred overnight by capillary action to nylon cellulose (Micron Separations Inc, Westboro, Mass). The blots were ultraviolet cross-linked, prehybridized, and hybridized according to protocols previously described. Phosphorus 32 (32P)-labeled probes for the Na⁺,K⁺-ATPase α isoforms were made from N-terminal fragments of the corresponding complementary DNA (cDNA), using the Rediprime (Amersham, Little Chalfont, England) random primer labeling kit. The following cDNA segments for each isoform were used: For α1 and α3, a segment from the 5’ end to the AjIII restriction site was used. For α2, the probe was made with a 5’ end-Nhel segment. For α4, a region of the isoform between base pairs 1314 and 1592 was used. Blots were washed in 0.1 X SSC (15 mM sodium chloride and 0.15 mM sodium citrate; pH 7.0), containing 1% sodium dodecyl sulfate (SDS) at 65°C and exposed for autoradiography.

**POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNOBLOT ANALYSIS**

Proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Proteins were separated by SDS-PAGE, transferred to nitrocellulose (Hybond C+, Amersham) and immunoblotted as previously described. The Na⁺,K⁺-ATPase α1, α3, and α4 isoforms were identified with polyclonal antibodies raised against a synthetic peptide derived from the N-terminal region of each polypeptide. The α2 subunit was identified using the monoclonal antibody McB2 provided by Kathleen Sweadner, PhD (Massachusetts General Hospital, Boston). As secondary antibodies, horseradish peroxidase–conjugated antibodies were used. Detection was performed by chemiluminescence.

**BIOCHEMICAL ASSAYS**

Protein assays were performed using the biecinchonic acid–copper sulfate solution as described by the supplier (Pierce Chemical Co, Rockford, Ill) after lysis of the cells in 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate. Na⁺,K⁺-ATPase activity was assayed through determination of the initial rate of release of [32P]inosorganic phosphorus from γ[32P]-ATP as described previously. Membrane fractions from HCECs containing a total of 20 µg of protein were assayed. Na⁺,K⁺-ATPase activity was measured in a final volume of 0.25 mL of medium containing 120 mM sodium chloride, 30 mM potassium chloride, 3 mM magnesium chloride, 0.2 mM ethyleneglycoltetraacetic acid, 2.5 mM sodium azide, 30 mM Tris buffer–hydrochloric acid (pH 7.4), 3 mM cold ATP, 0.2 µCi (0.007...
activity was approximately 3 mmol of Pi released per millidrolysis in the absence and presence of 1mM ouabain. Specific ATPase activity was determined as the difference in ATP hydrolysis in the absence and presence of 2 populations of Na+,K+-ATPase isozymes with different affinities for ouabain.

Figure 1. RNA hybridization analysis of Na+,K+-adenosine triphosphatase (ATPase) α isoforms in human corneal endothelial cells (HCECs); 15 mg of total RNA from HCECs was loaded per lane. After transference to nylon membranes, RNA was hybridized with Na+,K+-ATPase isoform-specific complementary DNA probes for the indicated isoforms. Specificity of the probes is shown by their hybridization profile with rat kidney RNA, which contains only α1, rat brain DNA, which expresses the α1, α2, and α3 isoforms, and testes, which contain the α4 isoform.

Figure 2. Immunoblot analysis of Na+,K+-adenosine triphosphatase (ATPase) α isoforms in human corneal endothelial cells (HCECs). Proteins (30 mg total) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted as described in the “Methods” section. The Na+,K+-ATPase α1, α2, and α3 isoforms were identified with polyclonal antibodies raised against a synthetic peptide derived from the N-terminal region of each polypeptide. The α2 subunit was identified using the McB2 monoclonal antibody. Horseradish peroxidase–conjugated antibodies were used as secondary antibodies. Detection was performed by chemiluminescence.

MBq) of γ[32P]-ATP±different concentrations of ouabain. The reaction was started by the addition of ATP and incubated at 37°C for 30 minutes. The tubes were then placed on ice, and the reaction was terminated by the addition of 25 µL of 55% trichloroacetic acid. Released [32P]-P, was converted to phosphomolybdate and extracted with isobutanol. Radioactivity of 170 mL of the isobutanol-phosphomolybdate phase was measured by liquid scintillation counting. The hydrolysis of ATP in all assays was maintained in the linear range. Specific Na+,K+-ATPase activity was determined as the difference in ATP hydrolysis in the absence and presence of 1 mM ouabain. Specific activity was approximately 3 mmol of P released per milligram of protein per hour.

DATA ANALYSIS

Curve fitting of the experimental data was performed using a Marquardt least-squares nonlinear regression computer program (SigmaPlot; Jandel Scientific, San Rafael, Calif) and applying the equations described previously. The best fitting of the experimental points was obtained by assuming the existence of 2 populations of Na+,K+-ATPase isozymes with different affinities for ouabain.

EXPRESSION OF α SUBUNITS IN HCECS

To characterize the expression of the Na+,K+-ATPase α isoforms in HCECs, total cellular RNA was prepared from the cultured cells and evaluated by Northern blot analysis. [32P]-labeled probes were made to the cDNA of the various α isoforms of Na+,K+-ATPase of rat. Because of the high homology of the Na+,K+-ATPase isoforms across species, the probes can identify human isoforms with the same efficiency of those from the rodent. To ensure the specific identification of the α subunits, regions in the cDNA that show the highest divergence for each isoform were chosen as templates. In addition, hybridization with the probes at 55°C and stringent washing conditions were also applied. As shown in Figure 1, transcripts for the α1 and α3 subunits of Na+,K+-ATPase were detected in the HCECs, whereas no α2 or α4 subunits were expressed in these cells. As a control, total RNA from rat brain was included to identify the α1, α2, and α3 isoforms. For α4, RNA from rat testes, a source of this Na+,K+-ATPase isoform, was used.

EXPRESSION OF α GENE PRODUCTS IN HCECS

To identify the protein products of Na+,K+-ATPase α gene expression, crude membrane preparations (30 mg total) were isolated from the HCECs, separated by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis as described in the “Methods” section. The Na+,K+-ATPase α1, α3, and α4 isoforms were identified with polyclonal antibodies raised against a synthetic peptide derived from the N-terminal region of each polypeptide. The α2 subunit was identified using the McB2 monoclonal antibody. Horseradish peroxidase–conjugated antibodies were used as secondary antibodies. Detection was performed by chemiluminescence. As shown in Figure 2, isoform-specific Na+,K+-ATPase antibodies detected only the α1 and α3 polypeptides. This result is consistent with the mRNA analysis in that only α1 and α3 subunits are expressed in HCECs. Although...
the α1 isoform could be easily detected in the HCECs with the immunoblot analysis, it is of interest to note that 50 mg of total protein was necessary to detect the α3 polypeptide in the cells. This indicates that HCECs may express limited amounts of the α3 isoform compared with α1.

**OUABAIN-INHIBITABLE Na⁺,K⁺-ATPASE ACTIVITY IN HCECs**

An interesting functional difference among the Na⁺,K⁺-ATPase isoforms is their distinct affinity for cardiotonic steroids, such as ouabain. This property can be used to determine the composition and relative amount of Na⁺,K⁺-ATPase isoforms in a particular sample. Na⁺,K⁺-ATPase activity in the various preparations was assayed at various concentrations of ouabain to estimate the heterogeneity of Na⁺,K⁺-ATPase. The dose-response curves of the inhibition of Na⁺,K⁺-ATPase activity by ouabain in HCECs are depicted in Figure 3. These curves showed a biphasic shape that spanned at least 6 logarithmic units, suggesting that more than one Na⁺,K⁺-ATPase–inhibiting process was occurring. The kinetic parameters for the interaction of the enzymes with the inhibitor as well as their relative amounts are shown in the Table. These results strongly suggest that 2 independent sites exhibiting 2 different affinities for ouabain are present in HCECs. According to the parameters shown in the Table, the first enzyme component would be a low-affinity site with an inhibition constant (Kᵢ) in the micromolar range (7.7 ± 1.5 × 10⁻⁷). This agrees well with the Kᵢ reported for the human α1 polypeptide and most probably reflects the presence of this isoform in the corneal endothelial cells. The second enzyme component is a high-affinity site with a Kᵢ in the nanomolar range (1.1 ± 1.0 × 10⁻⁹). This enzyme component must represent the α3 isoform, the only other Na⁺,K⁺-ATPase α polypeptide expressed in the corneal cells. The contribution of low-affinity isoform (α1) is predominant (82.4% ± 1.5%) compared with that of high-affinity α3 isoform (17.5% ± 1.8%). This result is consistent with those of the immunoblot and RNA analyses in which HCECs were shown to express a limited amount of α3 isoform.

**COMMENT**

Na⁺,K⁺-ATPase is a membrane-embedded protein whose enzymatic function was shown to be determined to a large extent by the nature of constituents composing the membrane components. The question of which tissue- and isoform-specific ATPase pump is expressed in HCECs provides the first insight into the possible kinetic function and modulation of these pump activities. The Na⁺,K⁺-ATPase pump has been shown to be located in the basolateral membrane of the corneal endothelial cells at an intensity of 1.5 × 10⁶ pump sites per cell. It is believed that normal stromal hydration, which is critical for maintaining corneal thickness and transparency, was achieved by the dynamic balance of corneal endothelial cell barrier function and ATPase pump activity. The adjacent endothelial cells are joined by “leaky” intercellular tight junctions that limit the movement of large molecules across the endothelium through the paracellular shunt. However, ions and small molecules are able to penetrate the physical barrier provided by the tight junctions in the endothelium. The ATPase pump located in the endothelial cell layer is responsible for the fluid-coupled active transport of ions from the stroma to the aqueous humor, thus maintaining corneal hydration and transparency.

Reduction of ATPase pump activity and number has been shown to be responsible for the pathogenesis of corneal edema from chronic inflammation and herpetic stromal disease. In herpetic stromal disease it has been shown that endothelial barrier function was not altered significantly, whereas endothelial Na⁺,K⁺-ATPase activity was significantly reduced by 22% to 33% during periods of maximal corneal edema. Contact lens wear has also been shown to induce corneal swelling by inhibiting Na⁺,K⁺-ATPase pump activity via the synthesis of the
cytochrome P-450 arachidonic acid metabolites, particularly the production of 12(R)-hydroxyicosatetraenoic acid and 8(R)-hydroxyhexadecatrienoic acid. However, the exact cellular mechanism through which these agents act on Na⁺,K⁺-ATPase in the corneal endothelial cells is not clear. Previous studies have shown that in other tissues and species intracellular second messengers, such as different protein kinases and arachidonic acid, are able to differentially regulate the function of the Na⁺, K⁺-ATPase isozymes. One of the mechanisms by which these messengers affect ATPase activity is through direct phosphorylation of the α subunit. Normal function of Na⁺, K⁺-ATPase requires the association of 2 polypeptides, the α and β subunits. The α unit is a multispanning membrane protein that contains the binding sites for ATP, the cations, and the specific inhibitor, ouabain. The β subunit is a type II glycosylated polypeptide essential for normal ATPase activity. The β subunit appears to be involved in the occlusion of potassium and in the modulation of the Na⁺ and K⁺ affinity of the enzyme. The α subunit exists in several distinct isoforms that are regulated during development and in tissue distribution and is solely responsible for the kinetic property of Na⁺, K⁺-ATPase. The α 1 isoform of Na⁺, K⁺-ATPase is found in all cells and is present at high concentrations in epithelial tissue with transport functions. The α2 and α3 isoforms of Na⁺, K⁺-ATPase have a more limited distribution. The α2 isoform of Na⁺, K⁺-ATPase is the primary isoform of adult skeletal muscle, whereas the α3 isoform is located predominantly in neural tissue. This tight regulation of isoform distribution suggests that functional differences may also exist.

In the present study, we demonstrated for the first time that HCECs express ATPase catalytic α1 and α3 but not α2 and α4 subunits. These findings were confirmed with both mRNA analysis and immunoblot studies. Our results also indicated that limited amounts of α3 isoforms are expressed in HCECs compared with that of α1 isoforms. This result is of particular interest in that α3 isoform has been found predominantly in neural tissues and the corneal endothelium is embryologically derived from the neural crest. The expression of α3 isoform may indicate important functional implications for HCECs. In addition, biochemical analyses of Na⁺, K⁺-ATPase activity have indicated that there may be 2 independently active Na⁺, K⁺-ATPase isoenzymes, which may be associated with α1 and α3 isoforms, respectively, in HCECs.

Because differences in sensitivity to ouabain in various tissues have been correlated to the presence of different isoforms of Na⁺, K⁺-ATPase, our results suggest that 2 independent isoforms are present in HCECs. Nevertheless, because a single Na⁺, K⁺-ATPase isoform might be present in different conformations with various affinities for ouabain, it might not be sufficient to detect Na⁺, K⁺-ATPase isoforms based solely on the sensitivity of the isoform to ouabain. However, this explanation is not valid because the dose-response curve for the ouabain inhibition of Na⁺, K⁺-ATPase activity spanned at least 6 logarithmic units in our study. In addition, the K₁ values reported in our experiments correlate well with those previously reported for the α1 and α3 isoforms. Extensive evidence in other tissues has indicated a low- and high-affinity form of Na⁺, K⁺-ATPase, which correspond to α1 and α3 isoforms, respectively, of Na⁺, K⁺-ATPase. The kinetic properties of Na⁺, K⁺-ATPase have not been shown to be influenced by the β isoform in the tissue.

The presence of more than a single form of Na⁺, K⁺-ATPase is of interest because, among the different regulatory mechanisms of corneal transparency, Na⁺, K⁺-ATPase is believed to play a critical role in maintaining a low level of stromal hydration. A variety of corneal diseases such as herpetic keratitis and chronic inflammation have been shown to directly reduce Na⁺, K⁺-ATPase pump density and activity in HCECs. The expression of different isoenzymes of Na⁺, K⁺-ATPase may indicate unique physiological functions of these enzymes in the corneal endothelial cells. The determination of the possible functions of these different isoforms may provide first insights into the pathogenesis of a variety of corneal diseases associated with corneal edema. The modulation of the different kinetic activities of Na⁺, K⁺-ATPase in HCECs may have important future therapeutic implications.

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