The Influence of Ascorbic Acid on Active Sodium Transport in Cultured Rabbit Nonpigmented Ciliary Epithelium

Yining Hou, William M. Pierce, Jr., and Nicholas A. Delamere

PURPOSE. Cultured rabbit nonpigmented ciliary epithelium (NPE) transports ascorbic acid (ASC) inward through a sodium-dependent mechanism. This study was conducted to test whether Na-K transport is activated to export the additional sodium, which enters the cell in cotransport with ASC.

METHODS. Studies were conducted using a cell line derived from rabbit NPE. ASC uptake was measured using [14C]ascorbic acid. The ouabain-sensitive potassium (^86Rb) uptake rate was measured as an index of active Na-K transport. Cellular sodium was measured by atomic absorption spectrophotometry or SBFI fluorescence.

RESULTS. In the presence of 200 μM ASC, ouabain-sensitive potassium (^86Rb) uptake rate increased ~70%; lesser concentrations of ASC produced lesser increases. Phloridzin (100 μM) inhibited ASC uptake and inhibited the stimulatory effect of external ASC on ^86Rb uptake. Dehydroascorbic acid (DHA) did not increase ^86Rb uptake. Neither DHA nor ASC altered the Na,K-ATPase activity measured in isolated membrane material. External ASC appeared to stimulate active sodium transport through a mechanism involving an increase of cytoplasmic sodium. In the presence of 200 μM ASC, cellular sodium increased ~26%; studies with cells, sodium loaded by nigericin treatment, suggested that this sodium increase could account for the degree of ^86Rb uptake stimulation observed in ASC-treated cells. However, the cellular sodium increase could not be explained simply on the basis of sodium entry through the ASC transporter. An additional sodium-entry pathway seemed to be activated in cells that accumulated ASC. Dimethylamiloride (DMA) abolished both the cellular sodium increase and the ^86Rb uptake stimulation caused by ASC. DMA did not prevent ASC uptake.

CONCLUSIONS. ASC significantly stimulated active Na-K transport in cultured NPE. The mechanism appeared to involve activation of a DMA-sensitive sodium entry pathway, which caused cytoplasmic sodium concentration to increase. (Invest Ophthalmol Vis Sci. 1998;39:143-150)

Humans and many other mammals maintain a high concentration of ascorbic acid in the aqueous humor.1 In the rabbit, the aqueous humor concentration of ascorbic acid exceeds 1 mM compared with a blood concentration of 40 μM.2 This indicates there is an active transport mechanism that shifts ascorbic acid into the eye. Most likely, the ascorbic acid transport mechanism is localized in the ciliary epithelium bilayer, the site of aqueous humor formation. In keeping with this idea, the rabbit iris-ciliary body accumulates ascorbic acid to a level several times that of the extracellular fluid.

Pigmented ciliary epithelium (PE) is capable of sodium-dependent ascorbic acid uptake (Helbig et al.), and it is possible that ascorbate is accumulated from the blood into the PE from which it diffuses through gap junctions into the nonpigmented ciliary epithelium (NPE). Provided that a sufficiently high concentration of ascorbic acid is established within the NPE, it could move passively down a concentration gradient into the aqueous humor. However, plasma solutes have free access to the apical surface of the NPE, and it is possible that some ascorbic acid from the blood is accumulated directly by the NPE. Ascorbic acid accumulation has been observed in a cell line derived from rabbit NPE in which the dependence of uptake rate on the concentration of external sodium suggested a sodium-ascorbic acid cotransport mechanism with a stoichiometry of approximately two sodium molecules to one of ascorbic acid.5 Because ascorbic acid accumulation brings sodium into the NPE cell, it could increase the rate of active sodium-potassium transport. Indeed, Candia and his co-workers6 determined that ascorbic acid causes a significant increase in the blood-to-aqueous flux of sodium across the rabbit iris-ciliary body. This is important because active (Na,K-ATPase-mediated) cation transport may contribute directly or indirectly to the osmotically driven process of aqueous formation. Here we report that allowing NPE cells to accumulate ascorbic acid can lead to the rate of active sodium-potassium transport being increased by as much as ~70%. This stimulatory response was more complex than anticipated—the increase of

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Supported by USPS research grant EY06915, the Kentucky Lions Eye Foundation, and an unrestricted grant from Research to Prevent Blindness.

Submitted for publication December 12, 1996; revised May 9, 1997 and July 23, 1997; accepted September 19, 1997.

Proprietary interest category: N.

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sodium-potassium transport appeared to be the result of an elevation of cytoplasmic sodium concentration, which could not be explained simply on the basis of sodium entry through the ascorbic acid transporter. Cytoplasmic ascorbic acid appeared to stimulate a dimethylamiloride-sensitive pathway for sodium entry into the NPE.

**Materials and Methods**

**Cell Culture**

Experiments were conducted using a rabbit NPE cell line generously provided by Dr. Miguel Coca-Prados (Department of Ophthalmology, Yale University). This cell line has been used previously in studies of the ascorbic acid transport mechanism,\(^5\) Na,K-ATPase,\(^7\) the Na-K-2Cl cotransporter,\(^8\) and prostanoid receptors\(^6\). It was derived by transformation of a semi-confluent primary culture with wild-type simian virus 40. Cells were grown at 37°C in Dulbecco’s modified Eagle’s medium (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), under a humidified atmosphere of 5% CO2/95% air. Penicillin (100 U/ml) and streptomycin (100 µg/ml) were added to the growth medium as antibacterial agents. At least 12 hours before each experiment the medium was replaced with penicillin and streptomycin-free Dulbecco’s modified Eagle’s medium. Confluent monolayers of cells grown in 24-well plates were used for experiments involving \(^86\)Rb fluxes, intracellular sodium content measurement by atomic absorption spectrophotometry, and \(^14\)C ascorbic acid uptake. For fluorescent dye measurement of intracellular sodium concentration, cell monolayers were grown on mini Petri dishes.

**Ascorbic Acid Uptake**

Monolayers of cultured rabbit NPE cells were bathed in Krebs solution (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 2.5 mM CaCl2, 1 mM MgCl2, and 5.5 mM glucose, at pH 7.4) containing the protease inhibitors phenylmethylsulfonyl fluoride (40 µM) and aprotinin (0.01 U/ml) and then broken up by ultrasonication. The mixture was placed in a centrifuge at 6000g at 4°C for 10 minutes, and the resulting pellet was discarded. The supernatant was centrifuged again at 100,000g at 4°C for 60 minutes, to produce a pellet containing membrane material, which was resuspended in a small volume of buffer A. Protein content was measured by Bio-Rad assay. This membrane material was used to measure Na,K-ATPase activity, defined as the difference between ATP hydrolysis rates measured in the presence and absence of 1 mM ouabain. ATP hydrolysis was measured at 37°C in a buffer containing 40 mM histidine, 10 mM KCl, 100 mM NaCl, 3 mM MgCl2, 1 mM EDTA, and 4 µg/ml alamethicin. Ouabain (1 mM) was added to half the samples 10 minutes before initiation of ATP hydrolysis by the addition of 2.5 mM ATP. After a 30-minute ATP hydrolysis period, the reaction was stopped by adding ice-cold 50% trichloroacetic acid to each tube. Then the tubes were centrifuged at 3000 rpm at 4°C. The supernatant was transferred to a test tube, and a color reaction was generated by adding 4% FeSO4/1% ammonium molybdate in 1.32 N HCl. The concentration of inorganic phosphate was estimated by measuring absorbance at 750 nm. Na,K-ATPase activity was expressed as nanomoles of PO4 released per milligram of membrane protein per 10-minute period.

**Measurement of Cellular Sodium Content by Atomic Absorption Spectrophotometry**

Cell monolayers were washed for 5 minutes in ice-cold isotonic (100 mM) MgCl2 solution (pH adjusted to 7.4 with Tris base) and blotted dry. The cells were then lysed by adding 200 µl of 30% nitric acid to each well for 2 hours. Then 1.8 ml of deionized distilled water was added, and the sodium concentration was measured using an atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, CT) at a wavelength of 566.5 nm.

**Measurement of Intracellular Sodium Concentration Using SBFI**

SBFI (Molecular Probes, Eugene, OR) was used to measure intracellular sodium concentration. To load the cultured NPE cells, SBFI-AM was dissolved in 20% pluronic acid-dimethyl sulfoxide solution and added to the Dulbecco’s modified Eagle’s medium cell culture medium for 3 to 4 hours at a final concentration of 10 µM SBFI-AM and <0.5% pluronic acid-dimethyl sulfoxide. After this dye-loading period, the cells were washed with Krebs buffer. SBFI fluorescence was measured by using a microscope (Zeiss; Carl Zeiss, Thornwood, NY) equipped with a digital fluorescence imaging system (Attofluor; Atto Instruments, Rockville, MD). The cells were continuously superfused with Krebs solution on a heated microscope stage that maintained temperature close to 37°C. The emission wavelength was 520 nm. Alternating wavelengths of 340 nm and 380 nm were used for excitation. The ratios of fluorescence intensity at 340 nm and 380 nm were continu-
Ascorbic Acid Uptake

Ascorbic acid uptake was measured at an ascorbic acid concentration of 200 μM. For comparison, the ascorbic acid concentration in blood is 50 μM, whereas the concentration in aqueous humor is 1 mM.2 Uptake was inhibited by phloridzin, a recognized inhibitor of ascorbic acid uptake in adrenomedullary chromaffin cells11 and human ileum epithelium.12 In the presence of 100 μM phloridzin, the rate of ascorbic acid uptake was 1.24 ± 0.01 nmol/mg of protein per 60 minutes; this was significantly (P < 0.01) less than the control rate of 14.23 ± 0.53 (n = 6).

Influence of Ascorbic Acid on Active Sodium–Potassium Transport

Monolayers of cultured NPE cells were exposed to ascorbic acid for 60 minutes, and then the rate of potassium (86Rb) uptake was measured over a subsequent 10-minute period in the presence or absence of ouabain (1 mM). In control cells (no ascorbic acid), the ouabain-sensitive potassium uptake rate was 139.52 ± 0.54 nmol/mg of protein per 10 minutes compared with a significantly (P < 0.01) higher value of 236 ± 6.9 nmol/mg of protein per 10 minutes (n = 44) measured in the presence of 200 μM ascorbic acid. The increased rate of ouabain-sensitive potassium (86Rb) uptake depended on the ascorbic acid concentration. In the range of 0 μM to 200 μM, a higher concentration of ascorbic acid resulted in a higher rate of ouabain-sensitive potassium (86Rb) uptake (Fig. 1A).

Some cells were pretreated for 60 minutes with Krebs solution containing 200 μM ascorbic acid and 100 μM phloridzin before 86Rb was added with or without 1 mM ouabain for a further 10 minutes. Under these conditions, no detectable elevation of ouabain-sensitive potassium (86Rb) uptake rate was observed (Fig. 1B). Phloretin (100 μM), a different inhibitor of ascorbic acid uptake,4 also prevented the stimulation of ouabain-sensitive potassium (86Rb) uptake by ascorbic acid (Fig. 1B).

Ascorbic acid can be oxidized to dehydroascorbic acid, which can be transported inward through a sodium-indepen-

data analysis

The data were expressed as mean ± standard error (SE). Statistical significance was determined by using either the Newman–Keul test, after one-way ANOVA, or Student’s t-test. Differences were considered significant when the probability was <0.05.

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Ascorbic Acid Increased the Intracellular Sodium Concentration

Active Sodium—Potassium Transport Stimulation which was not significantly different from the control activity was measured in cultured NPE monolayers after the cells were exposed to 200 μM ascorbic acid. The sodium concentration was approximately 25% higher than the baseline sodium concentration (Fig. 2A). An increase of cytoplasmic sodium concentration was also observed when freshly isolated porcine NPE was exposed to 200 μM ascorbic acid (Fig. 2B). Using atomic absorption spectrometry, the sodium content was measured in cultured NPE monolayers after the cells were exposed to 200 μM ascorbic acid for 1 hour. In the ascorbic acid-treated cell monolayers, the intracellular sodium content was 9.7 ± 0.4 nmol/mg protein (n = 6), which is significantly (P < 0.05) higher than in the control group in which the sodium content was 7.6 ± 0.3 nmol/mg protein (Fig. 2C).

Phloridzin, an inhibitor of the ascorbic acid transporter, was used to test whether the observed sodium increase was linked to ascorbic acid uptake. When ascorbic acid uptake was inhibited by phloridzin (100 μM), the intracellular sodium did not increase in cells incubated in the presence of 200 μM ascorbic acid (Fig. 2C). Similarly, cells exposed to a different inhibitor of ascorbic acid uptake, phloretin (100 μM), showed no evidence of increased sodium content in the presence of 200 μM ascorbic acid. Neither phloridzin nor phloretin changed cellular sodium content when added in the absence of ascorbic acid.

Increased Cellular Sodium and the Magnitude of Active Sodium—Potassium Transport Stimulation

Monolayers of cultured NPE cells were exposed to 0.25 μM to 2.5 μM nigericin for 10 minutes; then either sodium content or ouabain-sensitive potassium (86Rb) uptake rate was measured (Fig. 3A). When ouabain-sensitive potassium (86Rb) uptake rate was plotted as a function of intracellular sodium content (Fig. 3B), a linear relationship was evident (r = 0.99). Note that, after a 10-minute exposure to 0.5 μM nigericin, the ouabain-sensitive potassium (86Rb) uptake rate was 235.66 ± 5.68 nmol of potassium/mg protein per 10 minutes (n = 6), whereas the cellular sodium content was increased by 26% compared with control cells (Fig. 3A). This result fits well with the results from cells exposed to 200 μM ascorbic acid for 60 minutes, in which a similar increase of cellular sodium content was associated with a similar increase of ouabain-sensitive potassium (86Rb) uptake rate.

**Na-K-2Cl Cotransporter Activity Measured in the Presence of Ascorbic Acid**

It can be calculated (see Discussion) that, in the presence of ascorbic acid, the rate of sodium entry into the cell exceeds the rate of entry predicted simply from inward cotransport with ascorbic acid. Because it is a major route for sodium entry, we tested whether ascorbic acid stimulates the Na-K-2Cl cotransporter. Bumetanide-sensitive potassium (86Rb) uptake was used as an index of Na-K-2Cl cotransporter activity. However, when cells were pretreated with 200 μM ascorbic acid for 60 minutes, the rate of bumetanide-sensitive potassium (86Rb) uptake measured over a subsequent 10-minute period was 51.8 ± 6.2 nmol of potassium/mg of protein per 10 minutes, which was not different from the control value of 57.4 ± 4.8 (n = 8).

**The Effect of Dimethylamiloride on the Ascorbic Acid-Induced Stimulation of Active Sodium—Potassium Transport**

Because sodium can enter the cell through the NaH exchanger, experiments were conducted to determine whether dimethylamiloride (DMA) prevents the increase of intracellular sodium content, which occurs in the presence of ascorbic acid. First, we tested whether DMA alters ascorbic acid uptake. There was no evidence for diminished ascorbic acid uptake in the presence of 10 μM DMA; in cells exposed to 10 μM DMA for 10 minutes before 200 μM ascorbic acid was added to the medium for another 60 minutes, the ascorbic acid uptake rate was 10.8 ± 0.1 nmol/mg protein per 60 minutes. This was similar to the uptake rate of 9.9 ± 0.1 nmol/mg protein per 60 minutes measured in a paired batch of control (no DMA) cells (n = 6). However, in the presence of 10 μM DMA, ascorbic acid (200 μM) failed to increase intracellular sodium. The sodium content in control monolayers that received ascorbic acid for 60 minutes in the presence of DMA (10 μM) was 7.4 ± 0.3 nmol/mg protein (n = 6). This was similar to the sodium content in control cells (no ascorbic acid) or in cells that received DMA alone (7.5 ± 0.3 nmol/mg protein and 6.7 ± 0.3 nmol/mg protein, respectively) (n = 6) (Fig. 4A).

Because dimethylamiloride appears to prevent the intracellular sodium increase that occurs in the presence of 200 mM ascorbic acid, we tested whether DMA prevents the ascorbic acid-induced elevation of ouabain-sensitive potassium (86Rb) uptake. Cell monolayers were first exposed to Krebs solution containing 10 μM DMA for 10 minutes. Then, 200 μM ascorbic acid was added for a further 60 minutes before measuring 86Rb uptake was measured in the presence or absence of 1 mM ouabain. The ascorbic acid-induced stimulation of ouabain-sensitive potassium (86Rb) uptake rate was abolished by 10 μM DMA (Fig. 4B).

**DISCUSSION**

In the presence of ascorbic acid, active sodium—potassium transport is stimulated in cultured rabbit NPE cells as judged by an increased rate of ouabain-sensitive potassium (86Rb) uptake.
At an ascorbic acid concentration of 200 μM, approximately four times greater than the normal concentration in plasma but 20% of the concentration in aqueous humor, there was an approximate 70% increase of active sodium-potassium transport rate. This response fits well with the observation that ascorbic acid delivered to the NPE side causes marked stimulation of active sodium transport across the isolated rabbit ciliary epithelium. Ascorbic acid has also been reported to increase sodium transport and chloride transport in the cornea and stimulate corneal amino acid transporters. However, there have also been reports that ascorbic acid has an inhibitory effect on Na,K-ATPase activity, which in some cases can be attributed to hydrogen peroxide generated by oxidation of the ascorbic acid. In the present study, we found that the stimulatory effect of ascorbic acid on ouabain-sensitive potassium (86Rb) uptake was abolished by phloridzin, an inhibitor that prevented the cells from accumulating ascorbic acid. We interpret this to signify that ascorbic acid must enter the cells to stimulate active sodium-potassium transport. There was no evidence to suggest a direct stimulatory influence by either ascorbic acid or dehydroascorbic acid on Na,K-ATPase activity. Instead, we conclude that the active sodium-potassium transport rate was stimulated as the result of an increase in cytoplasmic sodium concentration, which occurred in cells that accumulated ascorbic acid.

In an earlier study using this same cultured cell line derived from rabbit NPE, it was proposed that the transporter shifts both sodium and ascorbic acid inward with a stoichiometry of at least 2:1. If this is the case, then when there is a linear pattern of ascorbic uptake with a rate of approximately 0.2 nmol of ascorbic acid/mg of protein per minute in the presence of 200 μM ascorbic acid, there should be an additional 0.4 nmol of sodium/mg of protein per minute, which enters the cell through the sodium-ascorbic acid transporter. To cope with this additional sodium entry, the cell would simply need to stimulate active sodium export by 0.4 nmol of sodium/mg of protein per minute. However, we observed a much greater increase of active sodium-potassium transport—in the presence of 200 μM ascorbic acid, ouabain-sensitive potassium (86Rb) uptake was increased by 9.6 nmol of potassium/mg of protein per minute, and, with a stoichiometry of 3 Na⁺ to 2 K⁺ for Na,K-ATPase; this signifies that the active outward sodium transport rate was increased by 14.4 nmol of sodium/mg of protein per minute.

The rate of active sodium-potassium transport is dependent on the concentration of cytoplasmic sodium. Here we report that addition of 200 μM ascorbic acid to the bathing medium increases cytoplasmic sodium concentration by approximately 26%. Could such an increase in cellular sodium...
The influence of nigericin on sodium content and ouabain-sensitive potassium (86Rb) uptake rate measured in cultured rabbit nonpigmented ciliary epithelium. (A) Cell monolayers were incubated for 10 minutes in Krebs solution containing 0.25 μM to 25 μM nigericin then either sodium content was measured by atomic absorption spectrophotometric analysis (right panel) or 86RbCl (1 μCi/ml) with or without ouabain (1 mM) was added for an additional 10 minutes to measure ouabain-sensitive potassium (86Rb) uptake (left panel). The data are mean ± SE (n = 12). (*) A significant difference from control (no nigericin) (P < 0.05).

In B, the same data were plotted together with the ouabain-sensitive potassium (86Rb) uptake rate on the vertical axis and sodium content on the horizontal axis. Superimposed on these data from nigericin experiments is a single point calculated from mean data obtained from cell monolayers incubated for 60 minutes in the presence of 200 μM ascorbic acid before measurement of cellular sodium (n = 12) or ouabain-sensitive potassium (86Rb) uptake rate (n = 44).

concentration cause a doubling of active sodium-potassium transport rate? To address this question, cells were exposed to nigericin over a 0.25 μM to 2.5 μM concentration range; this elevated cellular sodium to varying degrees, enabling us to establish the relationship between cellular sodium content and ouabain-sensitive potassium (86Rb) uptake rate. Based on this relationship, the increased level of cellular sodium caused by the external 200 μM ascorbic acid would have been sufficient to cause the observed approximate 70% increase of active sodium-potassium transport rate.

Our results suggest that, in addition to shifting sodium inward by way of a cotransport mechanism, ascorbic acid may stimulate other pathways of sodium entry into cultured NPE cells. Together this elevates the cytoplasmic sodium concentration and thus stimulates active sodium-potassium transport. In some cell types ascorbic acid can influence calcium channels and in corneal epithelium, McGahan and Bentley have suggested ascorbic acid might upregulate sodium channels. Such a response could well be expected to increase sodium entry in the presence of ascorbic acid. Because some ion transport effects of ascorbic acid can be modulated by external chloride concentration, chloride channels or chloride-dependent cotransporters could also play a role in the responses. Candia and his co-workers speculated that ascorbic acid may stimulate Na-Cl cotransporter activity in rabbit NPE cells. However, we found no evidence for stimulation of inwardly directed bumetanide-sensitive potassium (86Rb) uptake through the Na-K-2Cl cotransporter in the presence of ascorbic acid.

The elevation of cellular sodium content in the presence of 200 μM ascorbic acid was abolished by 10 μM DMA. In the
DMA, the amount of sodium that enters the cell, simply by increasing cellular sodium concentration, which stimulates the DMA-sensitive pathway for sodium entry, and this finding suggests that cytoplasmic ascorbic acid instead stimulates sodium entry through epithelial sodium channels, which can also be inhibited by amiloride analogs. Ascorbic acid has a notoriously broad range of actions, and it is not possible for us to identify whether the DMA-sensitive, sodium-entry pathway is directly responsive to ascorbic acid or whether the response is linked to antioxidant properties of ascorbic acid or other cellular changes caused by ascorbic acid. In some cells, arachidonic acid metabolites modulate the Na-H exchanger activity, and the ascorbic acid effects could possibly be linked to the modulation of arachidonic acid metabolism caused by ascorbic acid.

In summary, our findings indicate that cytoplasmic sodium concentration is increased in NPE cells that accumulate ascorbic acid and this causes stimulation of active sodium-potassium transport. Ascorbic acid accumulation appears to stimulate a DMA-sensitive pathway for sodium entry. Although there could be significant differences between responses of the NPE in vivo and responses in a cultured NPE cell line, it should be noted that a similar cytoplasmic sodium increase caused by ascorbic acid was observed in the cultured NPE and in NPE isolated freshly from porcine eyes. In the intact ciliary epithelium bilayer, ascorbic acid may have more than one route of entry into the NPE. Some may be accumulated into the NPE directly through an ascorbic acid transporter possibly at the apical surface (blood side) of the cell. Some may be first accumulated from blood into the PE layer from which it passes to the NPE through gap junctions. Accumulation of cytoplasmic ascorbic acid in the NPE to a concentration greater than that in aqueous humor would permit diffusional passage of the solute across the basolateral surface into the interior of the eye. In view of the importance of the sodium pump for the mechanism of aqueous production, activation of sodium-potassium transport by a high concentration of cytoplasmic ascorbic acid in the NPE could possibly have an impact on fluid formation.

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