Characterization of Bicarbonate-Dependent Potassium Uptake in Cultured Corneal Endothelial Cells

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Bovine corneal endothelial (BCE) cells in culture demonstrated $^{86}$Rb$^+$ uptake which was mostly ouabain-sensitive with some (15 to 50%) ouabain-insensitive uptake that was dependent on the presence of bicarbonate in the incubation medium. Bovine smooth muscle (SM) cells demonstrated ouabain-sensitive $^{86}$Rb$^+$ uptake but the ouabain-insensitive $^{86}$Rb$^+$ uptake was not bicarbonate-dependent. Although omission of bicarbonate from the incubation buffer resulted in some reduction in the pH, this change was not responsible for the reduction in the ouabain-insensitive $^{86}$Rb$^+$ uptake. Furthermore, the removal of bicarbonate decreased the $^{86}$Rb$^+$ influx but not its efflux. This ouabain-insensitive and bicarbonate-dependent $^{86}$Rb$^+$ influx in BCE cells proceeded at a linear rate for at least 60 min and increased as a function of bicarbonate concentration such that almost maximal uptake was observed at a concentration of about 10 to 15 mM. Saturation of the bicarbonate-dependent $^{86}$Rb$^+$ pump in BCE cells occurred at a concentration of 2 mM Rb$^+$ in the incubation buffer, similar to the previously observed value for the Na$^+$, K$^+$-ATPase. Competition experiments with both unlabeled Rb$^+$ and K$^+$ demonstrated that likewise in the Na$^+$, K$^+$-ATPase the $^{86}$Rb$^+$ influx represented physiological influx of K$^+$. Furthermore, the energy requirements of the bicarbonate-dependent $^{86}$Rb$^+$ uptake were similar to those of the $^{86}$Rb$^+$ uptake via the Na$^+$, K$^+$-ATPase. The results described in this work demonstrated a novel bicarbonate-dependent K$^+$ pump in BCE cells and may contribute to the fluid pump activity in addition to the well established contribution of the Na$^+$, K$^+$-ATPase pump.

The corneal endothelium regulates corneal hydration and therefore the transparency of the cornea.\(^1\) The fluid transport from the stroma to the anterior chamber across the endothelium is an energy-requiring process.\(^2,3\) Cooling the cornea to 4°C triggers swelling, which is, however, reversible if the cornea is transferred to a moist chamber at 31°C.\(^4\) Further experiments measuring the in situ swelling of the cornea demonstrated inhibition of the endothelium water pump by ouabain\(^5\) and by carbonic anhydrase inhibitors.\(^6\) These results may indicate the involvement of the Na$^+$, K$^+$-ATPase and the carbonic anhydrase in the fluid pump activity.\(^7\) The movement of fluid has been shown to be dependent upon the sodium and bicarbonate concentration, with corneal swelling observed when the bicarbonate concentration is less than 10 mM.\(^6,8,9\) Both sodium and bicarbonate show a net flux in the stromal to aqueous direction.\(^10,11\) Each ion is dependent on the presence of the other for transport across the endothelium.\(^10,11\) Based on the information accumulated thus far, several models have been proposed to link the movement of the fluid and the various ions.\(^12-17\) However, none of the models can explain and correlate all the parameters involved in the system. In particular, the connection between the ouabain and the bicarbonate effects on corneal swelling is not clear.

Recently, we have studied the Na$^+$, K$^+$-ATPase pump in cultured bovine corneal endothelial (BCE) cells, evaluated the number of pump sites per cell and measured the rate of ouabain sensitive $^{86}$Rb$^+$-uptake in BCE cells.\(^18\) In the current work we extend our studies and demonstrate the presence of a novel bicarbonate-dependent and ouabain-insensitive K$^+$ uptake in BCE cells. The ability of both ouabain addition and bicarbonate removal to inhibit distinct fractions of the K$^+$ uptake in BCE cells may further indicate the dominant role of K$^+$ uptake in the fluid pump activity and may also explain the inhibitory effect of bicarbonate removal on the corneal endothelium fluid pump.
Materials and Methods

Materials

$^{86}$RbCl (1-8 mCi/mg rubidium) was obtained from Amersham Radiochemicals (Buckinghamshire, England). Ouabain and rubidium chloride were obtained from Sigma (St. Louis, MO). Sodium azide and sodium bicarbonate were obtained from Merck (Darmstadt, F.R. Germany). Dulbecco’s modified Eagle’s medium (DMEM H-16) and calf serum were purchased from Grand Island Biological Co. (Grand Island, NY). Fetal calf serum was obtained from Sera-Lab (Crawley Down, Sussex, UK). Penicillin, streptomycin, fungizone, glutamine and trypsin versene solution were obtained from Biological Industries (Beth Haemek, Israel). Gentamicin was purchased from Abic (Ramat Gan, Israel). Tissue culture dishes were purchased from Nunc (Roskilde, Denmark). Fibroblast growth factor was purified from bovine brain as previously described.

Cell Culture Conditions

Cultures of BCE cells were established from steer eyes as already described. Cultures were grown at 37°C in 10% CO$_2$ in DMEM H-16 supplemented with 5% calf serum, 5% fetal calf serum, glutamine (2 mM), streptomycin (100 µg/ml), penicillin (100 U/ml) and fungizone (0.25 µg/ml), as previously described. Fibroblast growth factor (100 ng/ml) was added every other day until the cells were nearly confluent. Stock cultures were grown in 10 cm tissue culture dishes and passaged every week with a split ratio of 1:10. Cultures for experiments were seeded at an initial density of 50,000 cells per 35 mm tissue culture dish and grown in culture for the time periods indicated in each experiment. Cultures from passages 2-6 were used for experiments. Primary cultures of bovine vascular smooth muscle (SM) cells were prepared from the bovine aortic arch as previously described. The cultures were grown as described above for BCE cells but without fibroblast growth factor and passages 6-12 were used for experiments.

$^{86}$Rb$^+$ Uptake Assay

$^{86}$Rb$^+$ uptake experiments were performed as previously described. BCE cultures on 35 mm dishes were washed three times with warm (37°C) Rb$^+$ uptake buffer of the following composition (mM): NaCl:116; Na$_2$PO$_4$-H$_2$O:1.0; NaHCO$_3$:26; CaCl$_2$:2H$_2$O:1.8; MgSO$_4$:7H$_2$O:0.8; glucose:25, and RbCl:5.0. Hepes buffer (pH 7.4) at a final concentration of 20 mM was added to the Rb$^+$ uptake buffer during all incubations in order to maintain physiological pH in experiments where sodium bicarbonate was omitted. Solutions containing less sodium bicarbonate were prepared by replacing it by equimolar amounts of sodium chloride. The pH of the various combinations of buffers was recorded at the end of the uptake experiments and bicarbonate-free buffer and complete Rb$^+$ uptake buffer had pH of 7.0 and 7.4, respectively. Preliminary experiments indicated that at this pH range neither the ouabain-sensitive nor the bicarbonate-sensitive $^{86}$Rb$^+$ uptake were affected. Dishes were preincubated for 60 min with 1 ml Rb$^+$ uptake buffer at 37°C in a 10% CO$_2$ atmosphere. Ouabain (0.1 mM) was added to some of the dishes for the last 20 min preincubation and was also present during the uptake period. At the end of the preincubation period $^{86}$RbCl (0.5 µCi/ml) was added and the incubation was continued for 30 min or as indicated in the Figures. Uptake was terminated by aspiration followed by ten rapid washes with ice-cold Dulbecco’s phosphate-buffered saline (PBS). Radioactivity was extracted with 1 ml NaOH (0.1 M) and mixed with 4 ml of Lumagel (Lumac, Landgraaf, The Netherlands) and counted in a Packard (Downers Grove, IL) Tri Carb liquid scintillation counter. Each experiment was repeated at least twice and experiments were performed in duplicates or tetraplicates.

Statistical Analysis

Results were analyzed by student t-test and only differences with $P < 0.001$ were considered as significant differences.

Results

Effects of Bicarbonate and Ouabain on $^{86}$Rb$^+$ Uptake by BCE and SM Cells in Culture

$^{86}$Rb$^+$ uptake by confluent BCE and SM cultures was measured in the presence or absence of both bicarbonate and 0.1 mM ouabain (Fig. 1). When incubated in Rb$^+$ uptake buffer, the two cell types demonstrated a ouabain-sensitive $^{86}$Rb$^+$ uptake of 80 to 110 nmol/10$^6$ cells/30 min. The ouabain-insensitive $^{86}$Rb$^+$ uptake in SM cells was 75 nmol/10$^6$ cells/30 min and in BCE cells it was slightly higher, at a level of 110 nmol/10$^6$ cells/30 min. An increase in the ouabain concentration up to 1 mM did not reduce the observed ouabain-insensitive $^{86}$Rb$^+$ uptake in BCE cells (data not shown). Exposure of BCE cultures to bicarbonate-free buffer significantly reduced the ouabain-insensitive $^{86}$Rb$^+$ uptake with minor non-significant change in the ouabain-sensitive $^{86}$Rb$^+$ uptake.

On the other hand, under this condition the ouabain-insensitive $^{86}$Rb$^+$ uptake in SM cells was not reduced. This experiment demonstrated a ouabain-
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In order to examine the possibility that the reduction in \(^{86}\text{Rb}^+\) uptake in the bicarbonate-free buffer was due to changes in the pH of the incubation medium, the pH at the beginning and at the end of the uptake period was measured. The pH of the \(\text{Rb}^+\) uptake buffer and the bicarbonate-free buffer was 7.4 and 7.0, respectively, and did not change during the incubation period. Titration of the bicarbonate-free buffer to pH 7.4 did not increase the amount of \(^{86}\text{Rb}^+\) uptake, and reducing the pH of the \(\text{Rb}^+\) uptake buffer to 7.0 did not decrease the amount of \(^{86}\text{Rb}^+\) uptake (Fig. 2).

Higher accumulation of \(^{86}\text{Rb}^+\) in BCE cultures exposed to \(^{86}\text{Rb}^+\) in the presence of bicarbonate could be due to either increase in the \(^{86}\text{Rb}^+\) influx or alternatively to decrease in the rate of \(^{86}\text{Rb}^+\) efflux from the cultures. In order to study this question, BCE cultures were prelabeled with \(^{86}\text{Rb}^+\) and then exposed to bicarbonate-containing or bicarbonate-free buffers and the rate of \(^{86}\text{Rb}^+\) efflux was measured (Fig. 3).

\(^{86}\text{Rb}^+\) efflux was higher in cultures which were exposed to bicarbonate-containing buffer than in those exposed to bicarbonate-free buffer. This result supported the conclusion that the higher accumulation of \(^{86}\text{Rb}^+\) in the presence of bicarbonate-containing buffer was probably due to higher \(^{86}\text{Rb}^+\) influx into the cells.

Ouabain-Insensitive \(^{86}\text{Rb}^+\) Uptake by BCE Cells as a Function of Bicarbonate Concentration and Time

The effect of bicarbonate concentration on ouabain-insensitive \(^{86}\text{Rb}^+\) uptake was studied (Fig. 4). The \(^{86}\text{Rb}^+\) uptake was increased as a function of the bicarbonate concentration, and at concentrations of 10 to 15 mM almost maximal uptake was observed with a slight increase at a concentration of 26 mM.

The rate of ouabain-insensitive \(^{86}\text{Rb}^+\) uptake in the...
presence or absence of bicarbonate was studied (Fig. 5). BCE cultures which were incubated in the presence of ouabain in a bicarbonate-containing buffer demonstrated a linear uptake of $^{86}$Rb$^+$ for 60 min. When BCE cells were exposed to ouabain in the absence of bicarbonate in the incubation buffer only minor $^{86}$Rb$^+$ uptake was observed. At the end of the 60 min uptake period the amount of $^{86}$Rb$^+$ in cultures exposed to bicarbonate-containing buffer was six times higher than in cultures exposed to bicarbonate-free buffer, and in the latter case the amount of $^{86}$Rb$^+$ associated with the cells probably represents nonspecific absorption of $^{86}$Rb$^+$ to the cultures.

Specificity of $^{86}$Rb$^+$ Uptake in BCE Cells

The ouabain-insensitive $^{86}$Rb$^+$ uptake by BCE cultures incubated in the presence or absence of bicarbonate was studied as a function of bicarbonate concentration (Fig. 6). The amount of ouabain-insensitive $^{86}$Rb$^+$ uptake in the presence of bicarbonate was measured as a function of $^{86}$Rb$^+$ concentration in the incubation medium (Fig. 6). The amount of ouabain-insensitive $^{86}$Rb$^+$ uptake in the presence of bicarbonate was determined as described under Materials and Methods. The experiment was performed in triplicates; the squares represent the mean and the circles the actual values of the triplicates.
Fig. 6. $^{86}\text{Rb}^+$ uptake in the presence or absence of bicarbonate by BCE cells as a function of $^{86}\text{Rb}^+$ concentration. Confluent BCE cultures were preincubated in $^{86}\text{Rb}^+$-free $^{86}\text{Rb}^+$ uptake buffer in the presence (+NaHCO$_3$) or absence (−NaHCO$_3$) of bicarbonate for 60 min. Ouabain (0.1 mM) was added for the last 20 min preincubation and then $^{86}\text{Rb}^+$ (0.1 μCi/μmol) was added in increasing concentrations, as indicated in the Figure. The amount of $^{86}\text{Rb}^+$ uptake was determined as described under Materials and Methods. The experiment was performed in duplicates; the squares represent the mean and the circles the actual values of the duplicates.

The ouabain sensitive Na$^+$, K$^+$-ATPase transport system was also shown to transport Rb$^+$ with an affinity similar to that of K$^+$. Indeed, increasing concentrations of both K$^+$ and Rb$^+$ were equally effective in competing on $^{86}\text{Rb}^+$ uptake in BCE cells (Fig. 7B). The ability of increasing concentrations of both K$^+$ and Rb$^+$ to compete on the $^{86}\text{Rb}^+$ uptake in bicarbonate-containing buffer in the presence of ouabain and 2 mM $^{86}\text{Rb}^+$ was studied (Fig. 7A). $^{86}\text{Rb}^+$ uptake by BCE cells was reduced in the presence of both Rb$^+$ and K$^+$ with half maximal competition at concentrations of about 1.5 and 2.5 mM, respectively (Fig. 7A). This result demonstrated close affinity of Rb$^+$ and K$^+$, thus indicating that the bicarbonate-sensitive $^{86}\text{Rb}^+$ uptake represented a bicarbonate-sensitive K$^+$ pump in BCE cells.

Energy Requirements of the Bicarbonate-Dependent $^{86}\text{Rb}^+$ Uptake

The bicarbonate-sensitive $^{86}\text{Rb}^+$ uptake could be a passive transport or an energy-dependent process, as previously described for the ouabain-sensitive $^{86}\text{Rb}^+$ uptake. In order to test whether the bicarbonate-dependent uptake was an energy-dependent process, the energy sources of the cells were blocked by the omission of glucose and the addition of sodium azide (5 mM) (Fig. 8B). The net ouabain-sensitive and bicarbonate-dependent $^{86}\text{Rb}^+$ uptakes were obtained by measuring the uptake in the presence and absence of ouabain or bicarbonate (Fig. 8). The omission of glucose and addition of azide resulted in a significant reduction (75%) in the $^{86}\text{Rb}^+$ uptake both via the...
Na\textsuperscript{+}, K\textsuperscript{+}-ATPase as well as via the bicarbonate-dependent uptake.

**Discussion**

Previous studies\textsuperscript{13} postulated that K\textsuperscript{+} flux in BCE cells occurs either via a K\textsuperscript{+} channel which is inhibitable by barium\textsuperscript{12} or via the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase pump which is inhibitable by ouabain.\textsuperscript{25} In the current study, we have demonstrated that cultured BCE cells possess both the well established ouabain-sensitive Na\textsuperscript{+}, K\textsuperscript{+}-ATPase pump and a novel bicarbonate-dependent K\textsuperscript{+} pump, which is not inhibitable by barium (data not shown). The bicarbonate-dependent \(^{86}\text{Rb}\textsuperscript{+}\) uptake was observed only in BCE cells, and although SM cells demonstrated an active \(^{86}\text{Rb}\textsuperscript{+}\) uptake via the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase, they did not demonstrate the bicarbonate-dependent uptake. This specificity of corneal endothelial cells may suggest that this process is involved in the fluid pump activity which is specific for corneal endothelium, although it is difficult to directly extrapolate from cultured cells experiments to the in vivo cornea. The bicarbonate-dependent K\textsuperscript{+} pump in BCE cells was not inhibited by ouabain even when added at the highest concentration of 1 mM, but was dependent on energy to the same extent as the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase pump. The bicarbonate-dependent pump demonstrated close affinity to Rb\textsuperscript{+} and K\textsuperscript{+}; therefore in this study we used \(^{86}\text{Rb}\textsuperscript{+}\) in order to monitor K\textsuperscript{+} uptake, as was routinely done in studies of the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase pump. Saturation of the bicarbonate-dependent pump occurred at a concentration of 2 mM \(^{86}\text{Rb}\textsuperscript{+}\), similar to the observed saturation in the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase pump.\textsuperscript{18} Maximal bicarbonate-dependent \(^{86}\text{Rb}\textsuperscript{+}\) uptake was observed at a bicarbonate concentration of about 10 to 15 mM in the incubation buffer, which is similar to the concentration of bicarbonate needed to allow maximal Na\textsuperscript{+} uptake via the bicarbonate-dependent Na\textsuperscript{+} uptake in BCE cells\textsuperscript{26} and also maximal corneal dehydration via the corneal endothelial fluid pump.\textsuperscript{9}

The transparency of the cornea depends critically upon its hydration\textsuperscript{1} and it has been shown that an endothelium pump is responsible for regulating the stromal hydration via an energy-requiring process. The fluid transport in corneal endothelium is inhibited in the presence of ouabain, indicating the role of the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase pump in this process.\textsuperscript{3} Furthermore, if bicarbonate is omitted from the perfusion solutions, swelling of the cornea occurs but at a rate slower than that induced by ouabain.\textsuperscript{2} The effect of bicarbonate on the fluid pump activity was suggested to be mediated through the involvement of a bicarbonate-sensitive ATPase located within the endothelial mitochondria.\textsuperscript{27,28} The results presented in this manuscript indicated that a fraction (15 to 50%) of the K\textsuperscript{+} influx to BCE cells was not inhibited by ouabain but was bicarbonate-dependent. Therefore, if one assumes that a full K\textsuperscript{+} influx rate is essential for the fluid transport, then inhibition of any fraction of the K\textsuperscript{+} influx would cause inhibition of the fluid pump activity, resulting in swelling of the cornea. The observation that ouabain inhibits a higher fraction of the K\textsuperscript{+} influx than the omission of bicarbonate from the incubation buffer and is also more effec-
tive in inducing swelling of the cornea than bicarbonate omission fits the hypothesis suggested above.

Although in all of the experiments the existence of a bicarbonate-dependent \( ^{86}\text{Rb}^+ \) uptake was clearly observed, this fraction represented between 15 to 50% of the total \( ^{86}\text{Rb}^+ \) uptake with actual values between 30 to 110 nmol/10^6 cells/30 min of \( ^{86}\text{Rb}^+ \) uptake. The reason for these variations was not clear and it was not correlated with the passage number of the cells used. It should be noted that since the cultures maintained their normal morphology as closely apposed contact inhibited confluent monolayers under the various buffers used, no correlation between \( ^{86}\text{Rb}^+ \) uptake and morphology of the cultures could be detected.

The effects of pH changes of the incubation buffer on the \( \text{Na}^+ \), \( \text{K}^-\) -ATPase pump activity in BCE and the \( \text{Mg}^{2+}\text{-ATPase} \) activity in rabbit corneal endothelium were demonstrated in previous studies. However, the results described above did not indicate any changes in the \( ^{86}\text{Rb}^+ \) uptake at the pH range used (between pH 7.0 to 7.4), and the bicarbonate-dependent \( ^{86}\text{Rb}^+ \) uptake could not be explained by the small change in the pH of the bicarbonate-free incubation buffer. However, our studies cannot exclude the hypothesis that the lack of bicarbonate in the incubation buffer induces changes in the intracellular pH which thereby inhibit the \( ^{86}\text{Rb}^+ \) uptake. Further studies should be done in order to test this mechanism.

In a recent publication Doughty and Maurice measured the active fluid flow across stroma-endothelium preparations from rabbit corneas and demonstrated an active corneal fluid pump which is not bicarbonate-sensitive. This result is in contrast to previous studies which measured the corneal deturgescence as a parameter for the fluid pump activity and claimed that the corneal endothelium fluid pump is bicarbonate-dependent. Doughty and Maurice explain that in their experimental model the corneas were exposed to a high volume of fresh solution on both sides of the preparations, such that the CO2 level remains constant, while in the previous experimental model the corneas were covered with a layer of silicone oil through which CO2 can easily pass and be lost and thereby cause an increase in pH. However, this hypothesis cannot explain our results because in our experiments the endothelial monolayers were exposed to fresh medium under controlled CO2 atmosphere. Therefore, the bicarbonate-dependent and ouabain-sensitive potassium uptake in corneal endothelial cells described above represents a novel K+ pump, but the role of this pump in corneal deturgescence is not yet clear.

Key words: K+ uptake, bicarbonate-dependent, corneal endothelium, fluid pump, Na+, K+-ATPase

References

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