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

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Bovine corneal endothelial (BCE) cells in culture demonstrated specific 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 Rb+ uptake but the ouabain-insensitive 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 Rb+ uptake. Furthermore, the removal of bicarbonate decreased the Rb+ influx but not its efflux. This ouabain-insensitive and bicarbonate-dependent 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 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 Rb+ influx represented physiological influx of K+. Furthermore, the energy requirements of the bicarbonate-dependent Rb+ uptake were similar to those of the Rb+ pump via the Na+, K+-ATPase. The results described in this work demonstrated a novel bicarbonate-dependent K+ pump in addition to the well established contribution of the Na+, K+-ATPase pump. This novel pump activity in addition to the well established contribution of the Na+, K+-ATPase pump.

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The corneal endothelium regulates corneal hydration and therefore the transparency of the cornea. The fluid transport from the stroma to the anterior chamber across the endothelium is an energy-requiring process. Cooling the cornea to 4°C triggers swelling, which is, however, reversible if the cornea is transferred to a moist chamber at 31°C. Further experiments measuring the in situ swelling of the cornea demonstrated inhibition of the endothelium water pump by ouabain and by carbonic anhydrase inhibitors. These results may indicate the involvement of the Na+, K+-ATPase and the carbonic anhydrase in the fluid pump activity. 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. Both sodium and bicarbonate show a net flux in the stroma to aqueous direction. Each ion is dependent on the presence of the other for transport across the endothelium. Based on the information accumulated thus far, several models have been proposed to link the movement of the fluid and the various ions. 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 Rb+-uptake in BCE cells. 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

\(^{86}\text{RbCl}\) (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 veseone 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. 19

Cell Culture Conditions

 Cultures of BCE cells were established from steer eyes as already described. 20 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 \(\mu\)g/ml), penicillin (100 U/ml) and fungizone (0.25 \(\mu\)g/ml), as previously described. 21 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. 22 The cultures were grown as described above for BCE cells but without fibroblast growth factor and passages 6–12 were used for experiments.

\(^{86}\text{Rb}^+\) Uptake Assay

\(^{86}\text{Rb}^+\) uptake experiments were performed as previously described. 18,23 BCE cultures on 35 mm dishes were washed three times with warm (37°C) \(\text{Rb}^+\) uptake buffer of the following composition (mM): NaCl:116; Na\(_2\)HPO\(_4\)-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 \(\text{Rb}^+\) 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 \(\text{Rb}^+\) 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 \(^{86}\text{Rb}^+\) uptake were affected. Dishes were preincubated for 60 min with 1 ml \(\text{Rb}^+\) 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 \(^{86}\text{RbCl}\) (0.5 \(\mu\)Ci/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 the incubation 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 \(^{86}\text{Rb}^+\) Uptake by BCE and SM Cells in Culture

\(^{86}\text{Rb}^+\) 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 \(\text{Rb}^+\) uptake buffer, the two cell types demonstrated a ouabain-sensitive \(^{86}\text{Rb}^+\) uptake of 80 to 110 nmol/10\(^6\) cells/30 min. The ouabain-insensitive \(^{86}\text{Rb}^+\) 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 \(^{86}\text{Rb}^+\) uptake in BCE cells (data not shown). Exposure of BCE cultures to bicarbonate-free buffer significantly reduced the ouabain-insensitive \(^{86}\text{Rb}^+\) uptake with minor nonsignificant change in the ouabain-sensitive \(^{86}\text{Rb}^+\) uptake.

On the other hand, under this condition the ouabain-insensitive \(^{86}\text{Rb}^+\) uptake in SM cells was not reduced. This experiment demonstrated a ouabain-
Fig. 1. Effects of bicarbonate and ouabain on 86Rb+ uptake by BCE and SM cells. Confluent cultures of BCE (A) and SM (B) cells were preincubated for 60 min, either in a Rb+ uptake buffer (+) or in a NaHCO3-free Rb+ uptake buffer (−). Ouabain (+Ou; 0.1 mM) was added where indicated in the figure for the last 20 min of preincubation, then 86Rb+ was added and its uptake was measured as described under Materials and Methods. The experiment was performed in tetraplicates and the mean ± SD is presented.

Insensitive and bicarbonate-sensitive 86Rb+ uptake of about 100 nmoI/10^6 cells/30 min in BCE cells (Fig. 1).

Characterization of a Bicarbonate-Dependent 86Rb+ Pump in BCE Cells

In order to examine the possibility that the reduction in 86Rb+ 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 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 86Rb+ uptake, and reducing the pH of the Rb+ uptake buffer to 7.0 did not decrease the amount of 86Rb+ uptake (Fig. 2).

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

86Rb+ 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 86Rb+ in the presence of bicarbonate-containing buffer was probably due to higher 86Rb+ influx into the cells.

Ouabain-Insensitive 86Rb+ Uptake by BCE Cells as a Function of Bicarbonate Concentration and Time

The effect of bicarbonate concentration on ouabain-insensitive 86Rb+ uptake was studied (Fig. 4). The 86Rb+ 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 86Rb+ 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 $^{86}\text{Rb}^+$ for 60 min. When BCE cells were exposed to ouabain in the absence of bicarbonate in the incubation buffer only minor $^{86}\text{Rb}^+$ uptake was observed. At the end of the 60 min uptake period the amount of $^{86}\text{Rb}^+$ 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 $^{86}\text{Rb}^+$ associated with the cells probably represents nonspecific absorption of $^{86}\text{Rb}^+$ to the cultures.

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

The ouabain-insensitive $^{86}\text{Rb}^+$ uptake by BCE cultures incubated in the presence or absence of bicarbonate was studied as a function of $^{86}\text{Rb}^+$ concentration in the incubation medium (Fig. 6). The amount of ouabain-insensitive $^{86}\text{Rb}^+$ uptake in the presence of bicarbonate was determined as percent of $^{86}\text{Rb}^+$ concentration in the incubation medium. Confluent BCE cultures were preincubated for 60 min in a $^{86}\text{Rb}^+$ uptake buffer containing the indicated concentrations of bicarbonate. Ouabain (0.1 mM) was added for the last 20 min of preincubation and then $^{86}\text{Rb}^+$ was added and its uptake was measured 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.

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**Fig. 3.** Effect of bicarbonate on $^{86}\text{Rb}^+$ efflux from BCE cells. Confluent BCE cultures were loaded with $^{86}\text{Rb}^+$ during 48 hr incubation in a complete growth medium (1 ml/35 mm dish) containing $^{86}\text{Rb}^+$ (2 μCi), and during the last 20 min incubation ouabain (0.1 mM) was added. At the end of the loading period the cultures were washed ten times with PBS and exposed to fresh $^{86}\text{Rb}^+$ uptake buffer (1 ml) containing ouabain (0.1 mM) in the presence (+; □) or absence (--; ○) of NaHCO$_3$. At the time points indicated in the figure aliquots of 50 μl were taken to measure the amount of radioactive material released into the incubation medium. The amount of $^{86}\text{Rb}^+$ remaining in the cell layer at the end of the efflux period was determined as described for the uptake experiments. The total amount of radioactivity associated with the cells was calculated and determined as 100%. The experiment was performed in duplicates; the squares represent the mean and the circles the actual values of the duplicates.

**Fig. 4.** Ouabain-insensitive $^{86}\text{Rb}^+$ uptake by BCE cells as a function of bicarbonate concentration. Confluent BCE cultures were preincubated for 60 min in a $^{86}\text{Rb}^+$ uptake buffer containing the indicated concentrations of bicarbonate. Ouabain (0.1 mM) was added for the last 20 min of preincubation and then $^{86}\text{Rb}^+$ was added and its uptake was measured 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.

**Fig. 5.** Effect of bicarbonate on the rate of $^{86}\text{Rb}^+$ uptake by BCE cells. Confluent BCE cultures were preincubated for 60 min in a complete $^{86}\text{Rb}^+$ uptake buffer (+NaHCO$_3$) or in a bicarbonate-depleted buffer (−NaHCO$_3$). Ouabain (0.1 mM) was added for the last 20 min preincubation and then $^{86}\text{Rb}^+$ was added and the incubation continued for the time periods indicated in the Figure. The amount of $^{86}\text{Rb}^+$ uptake was determined as described under Materials and Methods. The experiment was performed in tetraplicates and the mean ± SD is presented.
Fig. 6. $^{86}$Rb$^+$ uptake in the presence or absence of bicarbonate by BCE cells as a function of $^{86}$Rb$^+$ concentration. Confluent BCE cultures were preincubated in Rb$^+$-free 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 $^{86}$Rb$^+$ (0.1 μCi/μmol) was added in increasing concentrations, as indicated in the Figure. The amount of $^{86}$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$^+$,24 and indeed, increasing concentrations of both K$^+$ and Rb$^+$ were equally effective in competing on $^{86}$Rb$^+$ uptake in BCE cells (Fig. 7B). The ability of increasing concentrations of both K$^+$ and Rb$^+$ to compete on the $^{86}$Rb$^+$ uptake in bicarbonate-containing buffer in the presence of ouabain and 2 mM $^{86}$Rb$^+$ was studied (Fig. 7A). $^{86}$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 $^{86}$Rb$^+$ uptake represented a bicarbonate-sensitive K$^+$ pump in BCE cells.

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

The bicarbonate-sensitive $^{86}$Rb$^+$ uptake could be a passive transport or an energy-dependent process, as previously described for the ouabain-sensitive $^{86}$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 $^{86}$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 $^{86}$Rb$^+$ uptake both via the

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**Fig. 7. Titration of nonradioactive K$^+$ and Rb$^+$ effects on ouabain-sensitive and bicarbonate-dependent $^{86}$Rb$^+$ uptake by BCE cells.** Confluent BCE cultures were preincubated in Rb$^+$-free Rb$^+$ uptake buffer in the presence (A) or absence (B) of bicarbonate for 60 min. Ouabain (0.1 mM) was added for the last 20 min preincubation only to dishes of group A and then $^{86}$Rb$^+$ (0.1 μCi/μmol) was added to the dishes to a final concentration of 2 mM together with the indicated concentrations of KCl or RbCl. The amount of $^{86}$Rb$^+$ uptake was determined as described under Materials and Methods.
Discussion

Previous studies\(^{13}\) postulated that K\(^+\) flux in BCE cells occurs either via a K\(^+\) channel which is inhibitible by barium\(^{12}\) or via the Na\(^+,\) K\(^+\)-ATPase pump which is inhibitible by ouabain.\(^{25}\) In the current study, we have demonstrated that cultured BCE cells possess both the well established ouabain-sensitive Na\(^+,\) K\(^+\)-ATPase pump and a novel bicarbonate-dependent K\(^+\) pump, which is not inhibitible by barium (data not shown). The bicarbonate-dependent \(^{86}\)Rb\(^+\) uptake was observed only in BCE cells, and although SM cells demonstrated an active \(^{86}\)Rb\(^+\) uptake via the Na\(^+,\) K\(^+\)-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 difficulf to directly extrapolute from cultured cells experiments to the in vivo cornea. The bicarbonate-dependent K\(^+\) pump in BCE cells was not inhibitible by ouabain even when added at the highest concentration of 1 mM, but was dependent on energy to the same extent as the Na\(^+,\) K\(^+\)-ATPase pump. The bicarbonate-dependent pump demonstrated close affinity to Rb\(^+\) and K\(^+\); therefore in this study we used \(^{86}\)Rb\(^+\) in order to monitor K\(^+\) uptake, as was routinely done in studies of the Na\(^+,\) K\(^+\)-ATPase pump. Saturation of the bicarbonate-dependent pump occurred at a concentration of 2 mM \(^{86}\)Rb\(^+\), similar to the observed saturation in the Na\(^+,\) K\(^+\)-ATPase pump.\(^{18}\) Maximal bicarbonate-dependent \(^{86}\)Rb\(^+\) 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\(^+\) uptake via the bicarbonate-dependent Na\(^+\) uptake in BCE cells\(^{26}\) and also maximal corneal dehydration via the corneal endothelial fluid pump.\(^{9}\)

The transparency of the cornea depends critically upon its hydration\(^1\) 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 inhibitible in the presence of ouabain, indicating the role of the Na\(^+,\) K\(^+\)-ATPase pump in this process.\(^5\) Furthermore, if bicarbonate is omitted from the perfusion solutions, swelling of the cornea occurs but at a rate slower than that induced by ouabain.\(^5\) 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.\(^{27,28}\) The results presented in this manuscript indicated that a fraction (15 to 50%) of the K\(^+\) influx to BCE cells was not inhibitible by ouabain but was bicarbonate-dependent. Therefore, if one assumes that a full K\(^+\) influx rate is essential for the fluid transport, then inhibition of any fraction of the K\(^+\) 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\(^+\) 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 $^{86}$Rb$^+$ uptake was clearly observed, this fraction represented between 15 to 50% of the total $^{86}$Rb$^+$ uptake with actual values between 30 to 110 nmol/10$^6$ cells/30 min of $^{86}$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 $^{86}$Rb$^+$ uptake and morphology of the cultures could be detected.

The effects of pH changes of the incubation buffer on the Na$^+$, K$^+$-ATPase pump activity in BCE and the Mg$^{2+}$-ATPase activity in rabbit corneal endothelium were demonstrated in previous studies. However, the results described above did not indicate any changes in the $^{86}$Rb$^+$ uptake at the pH range used (between pH 7.0 to 7.4), and the bicarbonate-dependent $^{86}$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 $^{86}$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 are exposed to a high volume of fresh solution on both sides of the preparations, such that the 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 fresh medium under controlled CO$_2$ atmosphere. Therefore, the bicarbonate-dependent and ouabain-insensitive 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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