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Evidence for Coupled Transport of Bicarbonate and Sodium in Cultured Bovine Corneal Endothelial Cells

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Summary. Using intracellular microelectrode technique, the response of the voltage V across the plasma membrane of cultured bovine corneal endothelial cells to changes in sodium and bicarbonate concentrations was investigated. (1) The electrical response to changes in $[HCO_3^-]_o$ (depolarization upon lowering and hyperpolarization upon raising $[HCO_3^-]_o$) was dependent on sodium. Lithium could fairly well be substituted for sodium, whereas potassium or choline were much less effective. (2) Removal of external sodium caused a depolarization, while a readdition led to a hyperpolarization, which increased with time of preincubation in the sodium-depleted medium. (3) The response to changes in $[Na^+]_o$ was dependent on bicarbonate. In a nominally bicarbonate-free medium, its amplitude was decreased or even reversed in sign. (4) Application of SITS or DIDS (10^{-3} M) had a similar effect on the response to sodium as bicarbonate-depleted medium. (5) At $[Na^+]_o$ = 151 mM and $[HCO_3^-]_o$ = 46 mM, the transients of V depended, with 39.0 ± 9.0 (SD) mV/decade, on bicarbonate and, with 15.3 ± 5.8 (SD) mV/decade, on sodium. (6) After the preincubation of cells with lithium, replacement of Li by choline led to similar effects as the replacement of sodium by choline, though the response of V was smaller with Li. This response could be reduced or reversed by the removal of bicarbonate or by the application of SITS. (7) Amiloride (10^{-3} M) caused a reversible hyperpolarization of the steady-state potential by 8.5 ± 2.6 mV (SD). It did not affect the immediate response to changes in $[Na^+]_o$ or $[HCO_3^-]_o$, but reduced the speed of regaining the steady-state potential after a change in $[HCO_3^-]_o$. (8) Ouabain (10^{-4} M) caused a fast depolarization of −6.8 ± 1.1 (SD) mV, which was followed by a continuing slower depolarization. The effect was almost identical at 10^{-5} M. (9) It is suggested, that corneal endothelial cells possess a cotransport for sodium and bicarbonate, which transports net negative charge with these ions. It is inhibitable by stilbenes, but not directly affected by amiloride or ouabain. Lithium is a good substitute for sodium with respect to bicarbonate transport and is transported itself. In addition, the effect of amiloride provides indirect evidence for the existence of a Na^+/H^+-antiport. A model for the transepithelial transport of bicarbonate across the corneal endothelium is proposed.

Key Words corneal endothelium · cell culture · intracellular potential · pH regulation · bicarbonate · bicarbonate-sodium cotransport · lithium · stilbenes · amiloride · ouabain

Introduction

The corneal endothelium is a leaky epithelium which transports water out of the stroma of the cornea into the aqueous humour of the anterior eye chamber and thus maintains corneal transparency. During the last decade, transepithelial electrophysiological techniques and determinations of radio-isotope fluxes have been used to characterize this transport [11, 17, 19, 20, 22–25, 29, 31–33, 35–37]. These studies have shown that the transport of water is apparently coupled to a transport of bicarbonate and sodium. The transport rates of these ions are mutually dependent on each other. Ouabain has been shown to inhibit fluid transport as well as inhibitors of carboanhydrase, high doses of amiloride (1 mM) and SITS (1 mM). The above-mentioned work has been done with rabbit corneae, but recent papers on human [21, 53] and ox [52] corneae suggest that endothelial transport processes are very similar in different mammalian species.

The elementary membrane processes involved in the transendothelial transport are still obscure. Intracellular electrophysiological studies promised to improve our understanding considerably, but until recently only few data on intracellular voltages were available [28, 34, 35, 51] due to the difficulties in obtaining stable impalements of these small and fragile cells. In a recent work [26, 27], we have demonstrated that with cultured bovine corneal endothelial cells it is possible to obtain intracellular recordings which remain stable for hours. We have shown that changes of $[HCO_3^-]_o$, pH_i and pH_o lead to voltage transients which were in principle compatible to an electrodiffusive transport of bicarbonate. These voltage transients could be reduced by methazolamide, an inhibitor of carboanhydrase, and the stilbene derivates SITS and DIDS.

Table. Composition of solutions (concentration in mmol × liter^{-1})

Sol.[a]	Na^+	K^+	Li^+	$Chol^+$	Mg^{++}	Ca^{++}	Cl^-	$H_2PO_4^-$	SO_4^{--}	HCO_3^-
1	151	5	—	—	0.9	1.7	112	1	0.9	46
2	151	5	—	—	0.9	1.7	135	1	0.9	23
3	151	5	—	—	0.9	1.7	148	1	0.9	10
4	151	5	—	—	0.9	1.7	158	1	0.9	—
5	—	5	—	151	0.9	1.7	112	1	0.9	46
6	—	5	—	151	0.9	1.7	148	1	0.9	10
7	—	5	—	151	0.9	1.7	158	1	0.9	—
8	—	5	105	46	0.9	1.7	112	1	0.9	46
9	—	5	105	46	0.9	1.7	148	1	0.9	10
10	—	5	105	46	0.9	1.7	158	1	0.9	—
11	—	110	—	46	0.9	1.7	112	1	0.9	46
12	—	110	—	46	0.9	1.7	148	1	0.9	10
13	70	5	—	81	0.9	1.7	112	1	0.9	46
14	35	5	—	116	0.9	1.7	112	1	0.9	46
15	17	5	—	134	0.9	1.7	112	1	0.9	46
16	8	5	—	143	0.9	1.7	112	1	0.9	46

[a] All solutions contained additionally 5 mM glucose and 10 mM HEPES.

In this paper, we extend our previous observations and provide evidence for a coupled transport of sodium and bicarbonate (or related species) across the plasma membrane which is linked to a transport of net negative charge and is inhibitable by stilbenes.

Materials and Methods

Cell Cultures

Cultures of bovine corneal endothelial cells were established as described previously [27]. However, the cells were now trypsinized at 37°C for 10 to 20 min. The cultures were propagated at a low split ratio of 1:2 to 1:4 and fed twice a week with DMEM (Dulbecco's modification of minimal essential medium) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (source of all components: Biochrom KG, Berlin, FRG). For the experiments, only early (2nd to 4th) subcultures were used. Experiments were performed with cells cultured in polystyrene tissue culture dishes (Falcon Plastics). The cells were punctured at least 4 days after they had reached confluency.

Solutions

The composition of the solutions is given in the Table. In sodium-free solutions bicarbonate was added as choline bicarbonate. Thus all solutions in which sodium was replaced mainly by lithium or potassium were prepared to contain 46 mM choline. Solutions containing no bicarbonate were gassed with air and titrated to pH 7.5. Solutions containing bicarbonate were gassed with 5% CO_2/air mixture. pH was 7.66 ± 0.02 for solutions containing 46 mM HCO_3^-, 7.4 ± 0.02 for 23 mM and 7.0 ± 0.02 for 10 mM HCO_3^-.

Source of Chemicals

SITS (4-acetamido-4-isothiocyanostilbene-2,2-disulfonic acid) was obtained from SERVA, Heidelberg, FRG; DIDS (4,4-diisothiocyano-2,2-disulfonic acid stilbene) from SIGMA. Amiloride was obtained from Sharp and Dohme, Munich, FRG, and ouabain from Merck, Darmstadt, FRG.

Experimental Setup

The experimental setup was described in detail previously [27]. In short, a Lucite flow chamber was tightly pressed onto the bottom of a culture dish. It isolated a channel (length: 30 mm, width: 1.5 mm) which could be rapidly superfused with different solutions. The cells on the bottom of the channel were punctured with conventional microelectrodes (range of resistances: 60 to 140 MΩ) using a fast stepping device (Heidelberg Nanostepper, Science Trading, Frankfurt, FRG). Improvements on the situation, described in [27], included an increased number of inlets into the chamber (possible use of 8 instead of 6 test solutions) and an increased speed of fluid exchange (90% exchange within 3 sec, perfusion rate 45 ml/hr). The experiments were performed at $T = 32 \pm 1$°C, since this is the endothelial temperature *in vivo* [12].

Determination of Liquid Junction Potentials

Liquid junction potentials arising between different test solutions [30] were measured using the same experimental setup. Instead of microelectrodes, chlorided silver wires (diameter 1 mm) were lowered to the bottom of the channel. The voltage responses of three different electrodes to solution exchanges were recorded. The results were corrected for the response of the Ag/AgCl electrode to differences of chloride activities in the solutions, assuming a Nernstian behavior. Solution exchanges were performed from control Ringer's (sol. 1) to the test solutions indicated. This

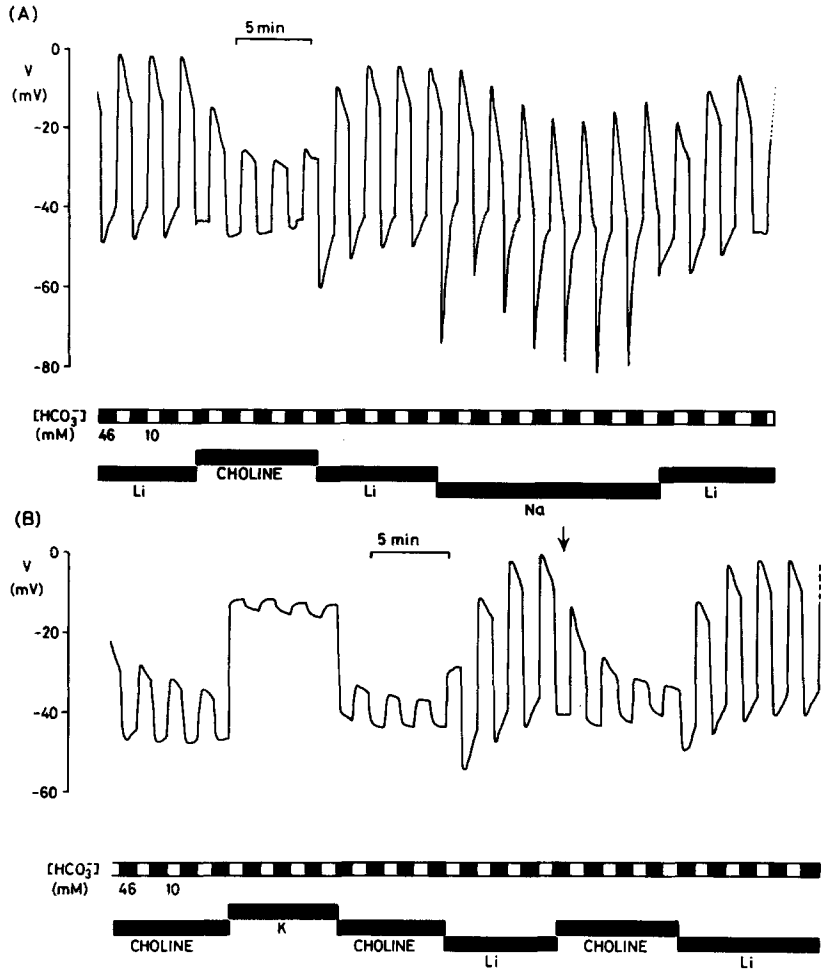

Fig. 1. (*A*) Original registration demonstrating the effect of different cations on the response of the voltage across the plasma membrane to changes of extracellular bicarbonate. $[HCO_3^-]_o$ was changed periodically from 10 to 46 mM at constant pCO_2 (5%), as indicated by the bars. The second registration (*B*) is from another cell of the same culture dish. The time elapsed between the two registrations is 10 min. (Used solutions: 1, 3, 5, 6, 8, 9, 11, 12.) (A similar reduction by replacement of Na by choline was seen when the solution was changed from 46 mM HCO_3^- to nominally bicarbonate-free solution (sol. 4) (4 cells, 2 different cell strains).)

resulted in the following liquid junction potentials (in mV ± SD): sol. 2: +1.3 ± 0.5, sol. 5: +2.4 ± 0.2, sol. 8: +5.5 ± 0.3, sol. 13: +1.7 ± 0.4, sol. 14: +2.3 ± 0.3, sol. 15: +2.6 ± 0.2, and sol. 16: +2.8 ± 0.2.

Results

Cation Requirement of Voltage Responses to Bicarbonate

We investigated the cation requirement of the electrical response to changes in bicarbonate by replacing extracellular sodium with other cations. This was done while measuring the voltage V across the cell membrane in response to changes in extracellular bicarbonate. In a typical experiment of this type, shown in Fig. 1, $[HCO_3^-]_o$ was periodically changed in intervals of 1 min between 46 and 10 mM at constant pCO_2 (5%). At first, part (*A*), this was done in a lithium Ringer's. As described earlier for sodium Ringer's [26, 27], removal of bicarbonate resulted in a depolarization and its readdition in a hyperpolarization. When extracellular lithium was replaced by choline, the voltage response of the cell to bicarbonate was greatly reduced, but not totally suppressed. In the next step, lithium was again substituted for choline, resulting again in an increase of the amplitude. Then, sodium was replaced for lithium. At first, when sodium was given together with high bicarbonate (46 mM), there was a great enhancement of the hyperpolarization. Later, the response to bicarbonate slowly reached a stable shape. Its total amplitude was greater than in the presence of lithium. While the shape of the voltage change in the presence of lithium was nearly symmetrical, with sodium its shape was asymmetric, i.e. addition of high concentration of bicarbonate led to a greater immediate voltage change than its removal (for more detail of the signal shape *see* inset of Fig. 10).

Part (*B*) was a registration of another cell of the same culture dish 10 min after the first registration. It demonstrated that potassium, as the main extracellular cation, led to a fast depolarization and to a reduction of the response to bicarbonate. This reduction was even greater than in the presence of

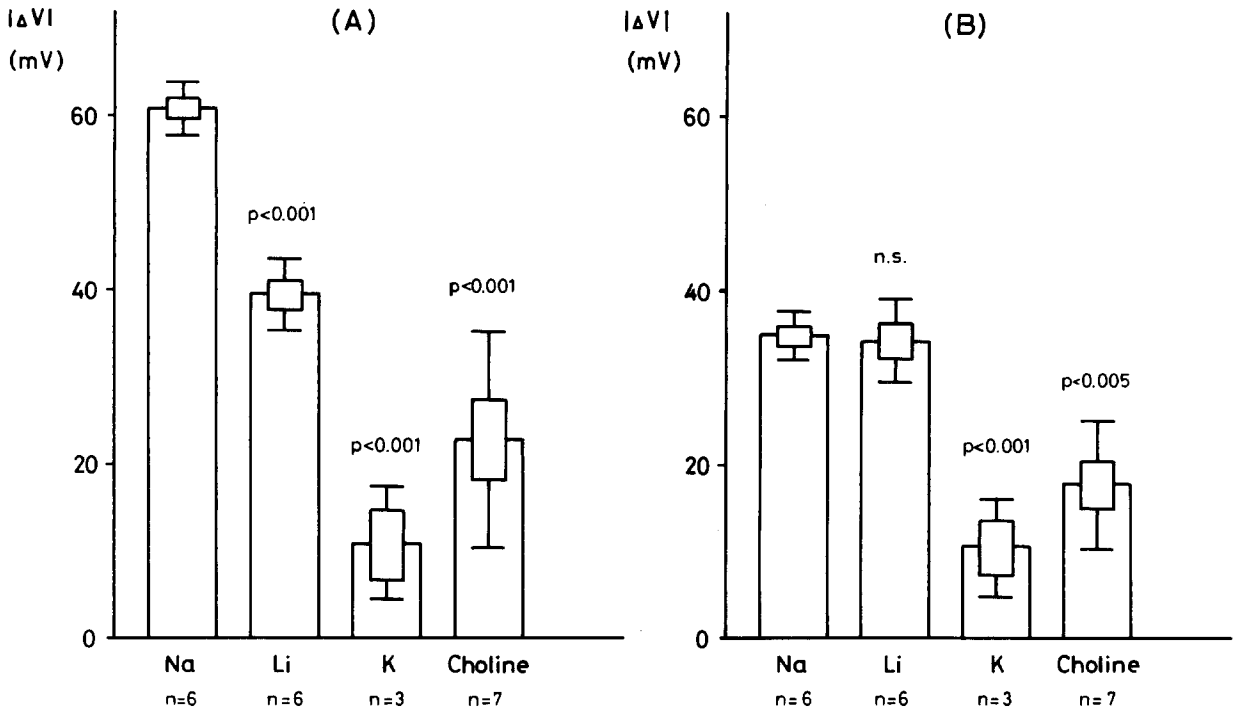

Fig. 2. Diagrams showing the voltage responses to changes in bicarbonate as a function of the main cation present. The data stem from experiments similar to the one shown in Fig. 1. Diagram (*A*) is a plot of the total (peak to peak) amplitude of the signal, whereas in part (*B*) the mean of the immediate up- or downstroke of the voltage following a change in $[HCO_3^-]$ has been evaluated. Error bars indicate SD and SEM (thick bars) and the number of cells is given under the columns. The statistical significance of the difference of the respective mean to the one for sodium using student's *t*-test is given above the columns. The levels of significance for the other pairs are: (*A*) Li-choline: $P < 0.005$; Li-K: $P < 0.001$; choline-K: $P < 0.1$ (n.s.). (*B*) Li-choline: $P < 0.05$; Li-K: $P < 0.001$; choline-K: $P < 0.2$ (n.s.)

choline. Lithium once again led reversibly to an enhancement of the reaction.

After a change of extracellular cations, the voltage responses to bicarbonate needed several minutes to reach a steady state of their amplitudes (e.g. *see* Fig. 1, part (*B*), section following the arrow, when choline was replaced for lithium). Since the time for an exchange of the extracellular medium was much shorter, this suggests that a slowly exchanging compartment—possibly the intracellular space—was involved in the full effect.

Figure 2 summarizes the results of several experiments on the cation dependence. In Fig. 2(*A*), the total (peak to peak) amplitude has been evaluated, whereas in 2(*B*) the arithmetic mean of the immediate up- and downstroke of V upon changes in $[HCO_3^-]_o$ was taken. Lithium could fairly well substitute sodium in enabling the voltage response to bicarbonate. Indeed, the immediate response to bicarbonate in the presence of Li (Fig. 2(*B*)) was not statistically different from the one in Na^+-Ringer's. Potassium and the larger organic cation choline were much less effective. In two experiments, other large organic cations (tetramethylammonium and bis(2-hydroxyethyl)dimethylammonium (BDA)) were substituted for sodium and gave results similar to that of choline. Thus, Na^+ is important for the electrical response to bicarbonate.

Voltage Response to Removal of Na^+

Removal and readdition of extracellular sodium led to changes of V very similar to those observed in the removal and readdition of extracellular bicarbonate [27]. This is shown in the experiment of Fig. 3, where extracellular sodium was three times replaced by choline. The time of exposure to sodium-free Ringer's was increased from 15 to 30 and to 60 sec. Removal of sodium in all cases led to a fast depolarization of nearly constant magnitude. With the used speed of perfusion, the maximal depolarization was reached in less than 10 sec. Afterwards, the membrane potential began to recover slowly. Re-introduction of sodium led to a fast transient hyperpolarization. Its magnitude increased with the time of preincubation in the sodium-depleted medium. After this hyperpolarization, the potential slowly oscillated before regaining its steady-state

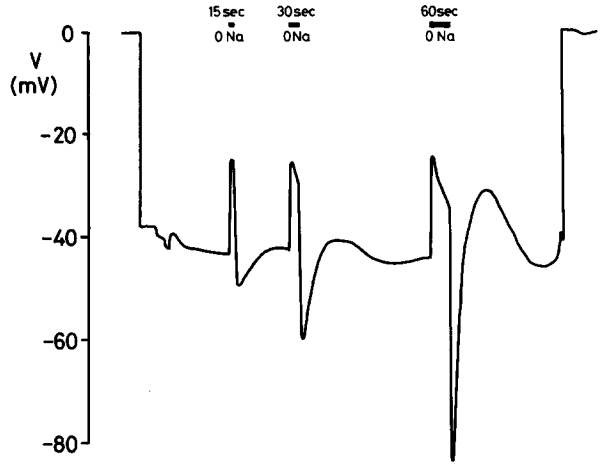

Fig. 3. Effect of sodium removal on the intracellular potential. In the intervals indicated by the bars, sodium was replaced by choline (change from sol. 1 to sol. 5). Note the increase in the hyperpolarization following readdition of sodium with increasing previous exposure to sodium-depleted medium (the time of exposure is given over the bars). During the recovery from the last exposure to sodium-depleted medium the impalement became unstable and the cell was eventually lost. A similar response to removal and readdition of sodium was seen in more than 50 cells of more than 8 different cell strains

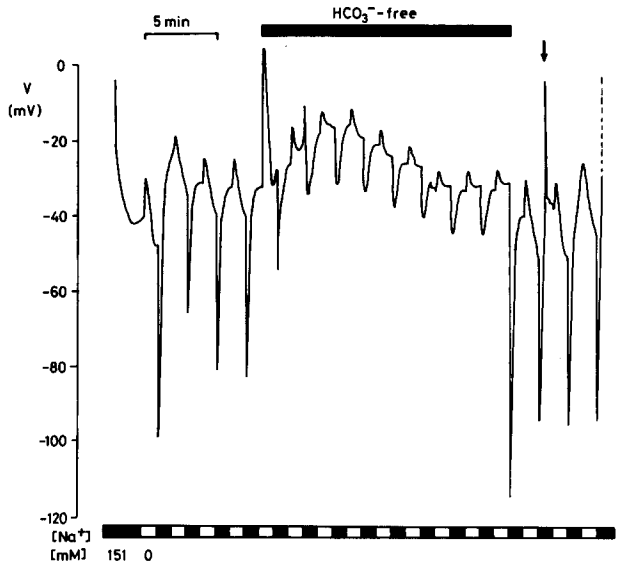

Fig. 4. Effect of bicarbonate on the voltage response to sodium. At the time indicated by the bar, the cell was perfused with nominally bicarbonate-free Ringer's, gassed with air (sol. 4 and 7). In contrast, the control Ringer's contains 46 mM bicarbonate and is gassed with 5% CO_2 (sol. 1 and 5). At the time indicated by the arrow, the impalement became unstable and a new cell was punctured by advancing the electrode. Similar sensitivities of the sodium response to bicarbonate were seen in more than 11 cells of 6 different strains

value. The magnitude of this hyperpolarization was quite variable among different cells, even with constant time of preincubation, while the voltage drop caused by removal of sodium was more constant. The hyperpolarization was greater with those cells whose potential quickly recovered or even hyperpolarized after the initial drop due to Na^+ removal (*compare* Figs. 3, 4, 5). Sometimes, when the cell was exposed to Na^+-free medium for periods of one minute or more, this hyperpolarization reached values of more than -100 mV.

This may suggest an association of the voltage responses to transmembranal sodium fluxes. When the cell is incubated in sodium-depleted medium, there will be a progressive decrease of $[Na^+]_i$. When sodium is finally re-added, the gradient for its influx should be increasing with the time of sodium depletion, resulting in an increased electrical signal associated with the increased influx. However, the voltage response is incompatible with a simple conductive pathway for sodium ions. In that case, changes of V opposite in sign are expected. Instead, the system behaves as if sodium flux is coupled to a flow of net negative charge.

Dependence on Bicarbonate

The depolarization on removal of Na^+ was dependent on bicarbonate. This is shown in Fig. 4, where at the same cell responses to sodium were explored in bicarbonate-rich Ringer's (46 mM HCO_3^-, gassed with 5% CO_2 (sol. 1 and 5)), and nominally bicarbonate-free Ringer's (gassed with air) (sol. 4 and 7). Removal of bicarbonate led to the known depolarization. In a bicarbonate-free medium, the amplitude of the response to Na^+ was drastically reduced. After readdition of bicarbonate, which at first, while given together with sodium, caused a large hyperpolarization, the original reaction to sodium removal was restored. In some experiments, the sign of the voltage response to sodium changed in a nominally bicarbonate-free solution. The removal of sodium then caused the membrane to hyperpolarize and its addition to depolarize.

Thus, voltage transients associated with changes in bicarbonate are dependent on sodium, and those associated with changes in sodium are dependent on bicarbonate. This might most easily be explained by a coupled transport process for bicarbonate and sodium.

Inhibition by Stilbenes

We have shown in an earlier work [26, 27] that the stilbene derivates SITS or DIDS reduced the voltage responses to changes in $[HCO_3^-]_o$. If the same

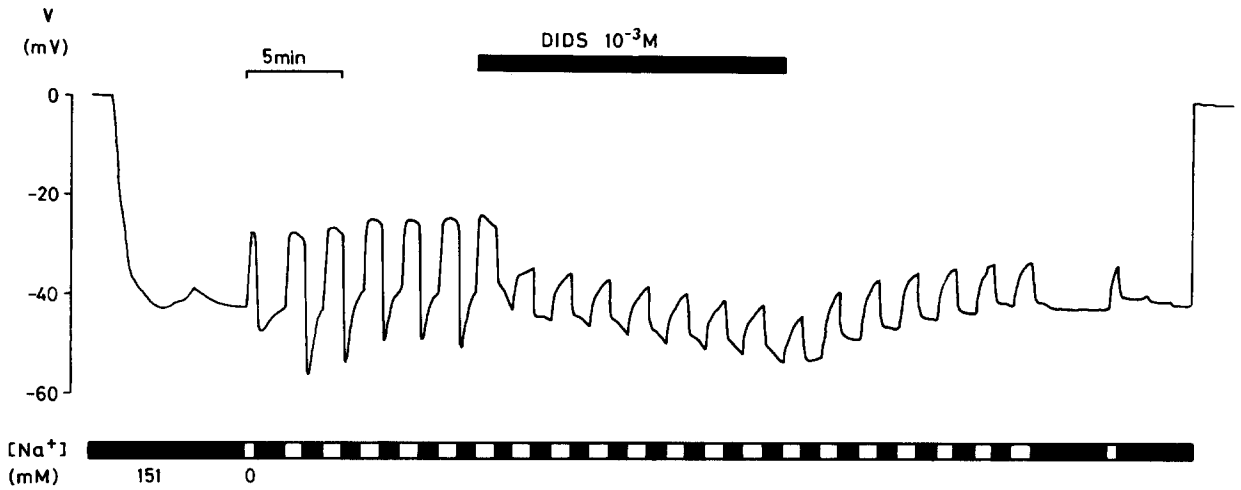

Fig. 5. Effect of DIDS on the response to sodium. Na^+ was periodically replaced by choline with 46 mM HCO_3^- being present (sol. 1 and 5). During the time indicated by the bar, DIDS (10^{-3} M) was applied. A similar reduction of the response to sodium by DIDS or SITS was seen in all 12 cells of 6 different strains examined

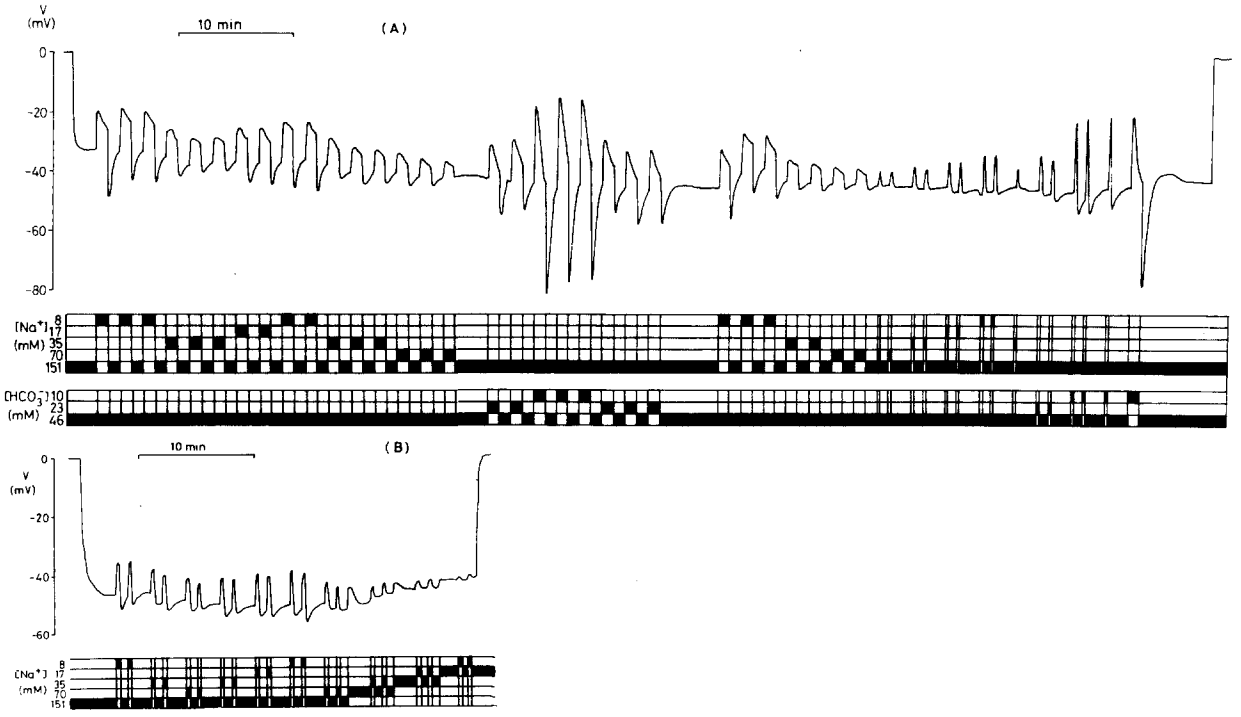

Fig. 6. Original registrations showing the quantitative dependence of voltage transients on the magnitude of step changes in $[Na^+]$ and $[HCO_3^-]$. (*A*) Comparison of the effects of sodium and bicarbonate at the same cell. At first, the solutions were switched in intervals of one minute to solutions containing less sodium or bicarbonate (sodium was replaced by choline and bicarbonate by Cl^-). In the last part, a change in the extracellular medium was performed in intervals of 10 sec, a sufficiently long time for the potential to pass its minimum. (*B*) Registration showing more explicitly the decline of the response to sodium with decreasing sodium concentrations. In the latter part of the experiment, step changes to lower $[Na^+]_o$ were performed from preincubation media containing less sodium. The number of cells explored is given in Fig. 7. (Used solutions: 1, 2, 3, 13, 14, 15, 16.)

process is shared by sodium, then stilbenes should also cause a reduction of the response to sodium.

As demonstrated in Fig. 5, this is indeed the case. Sodium was replaced by choline with 46 mM HCO_3^- being present. At first, this was done for 30 sec and later periodically for 60 sec, showing again the dependence of the hyperpolarization on the time of preincubation in sodium-free Ringer's. The appli-

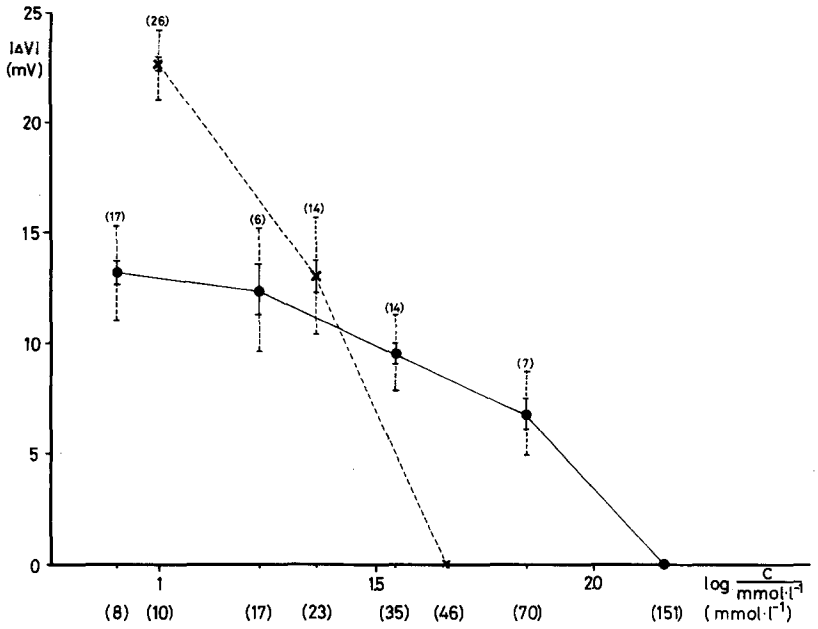

Fig. 7. Dependence of the voltage transients on the magnitude of changes in $[Na^+]_o$ (indicated by points) and $[HCO_3^-]_o$ (indicated by crosses). The concentration scale is logarithmic. Only the immediate downstroke of the potential after a change to a solution containing less Na^+ or HCO_3^- was evaluated. The solid bars indicate SEM, the thin bars SD. The number of cells investigated is given above each point. The calculated slopes are: 43.2 ± 8.9 (SD) mV/decade (n = 14) for bicarbonate taken from the step 46 to 23 mM, and 20.3 ± 5.7 (SD) mV/decade (n = 7) for sodium taken from the step 151 to 70 mM. When corrected for liquid junction potentials (*see* Materials and Methods), the calculated slopes are 15.3 mV/dec for sodium and 39.0 mV/dec for bicarbonate

cation of DIDS (10^{-3} M) (or in other experiments of its congener SITS) inhibited the fast depolarization upon removal of sodium and the hyperpolarization upon its readdition. After the removal of DIDS, the voltage response to sodium remained virtually constant, demonstrating the irreversibility of the action of DIDS. In some cells, as with nominally bicarbonate-free medium, a reversal of the sign of the voltage change was observed.

This suggests the existence of a common transporter for sodium and bicarbonate which is inhibitable by stilbenes and which transports net negative charge in the direction of sodium, as well as bicarbonate movement.

Dependencies of the Voltage Responses on the Magnitude of Changes in HCO_3^- and Na^+

A series of experiments was performed to obtain quantitative information about the magnitude of voltage transients associated with changes in $[Na^+]_o$ and $[HCO_3^-]_o$ in the same cell. An experiment of this kind is shown in Fig. 6(*A*). Bicarbonate concentration was changed in 3 steps between 46, 23 and 10 mM (at constant pCO_2 (5%)) with 151 mM Na^+ present, and sodium concentration in five steps (151, 70, 35, 17 and 8 mM) with 46 mM bicarbonate present (HCO_3^- was replaced by Cl^- and Na^+ by choline$^+$). The response to extracellular bicarbonate was more pronounced than that to sodium. Furthermore, the response to changes in $[Na^+]_o$ tended to saturate with increasing magnitudes of sodium concentration changes. This was observed in every single experiment.

In Fig. 6(*B*), this apparent saturation was further investigated. At first, as in Fig. 6(*A*), $[Na^+]_o$ was changed from 151 mM to lower values. In the second part of the experiment, step changes of $[Na^+]_o$ were performed to solutions containing about half the concentration of sodium while decreasing the sodium concentration of the preincubation medium progressively from 151 to 17 mM. The amplitudes of the responses were reduced with decreasing $[Na^+]_o$, though the relative magnitude of the change of $[Na^+]_o$ during these pulses remained nearly constant (change by a factor of about 2). Thus, the reason for the apparent saturation with larger steps of $[Na^+]_o$ may be a decrease of transport rates of the putative transporter with decreasing sodium concentrations. This cannot be attributed to the saturation of the transport site of a hypothetical carrier, since then a saturation of the voltage response should become apparent with high concentrations of Na^+. Rather it should reflect a limitation of the Na^+ gradient which drives the transport. Since intracellular sodium is the substrate for the process which produces the depolarization, with decreasing extracellular sodium, $[Na^+]_i$ probably becomes insufficient to drive the transport at the expected rate.

The results of these experiments are summarized in Fig. 7. The response to bicarbonate may be approximated by a straight line in the semilog diagram over the concentration range examined, whereas—as already noted for the experiment of Fig. 6—the slope for sodium decreases with decreasing $[Na^+]_o$. The calculated slopes are 43.2 ±

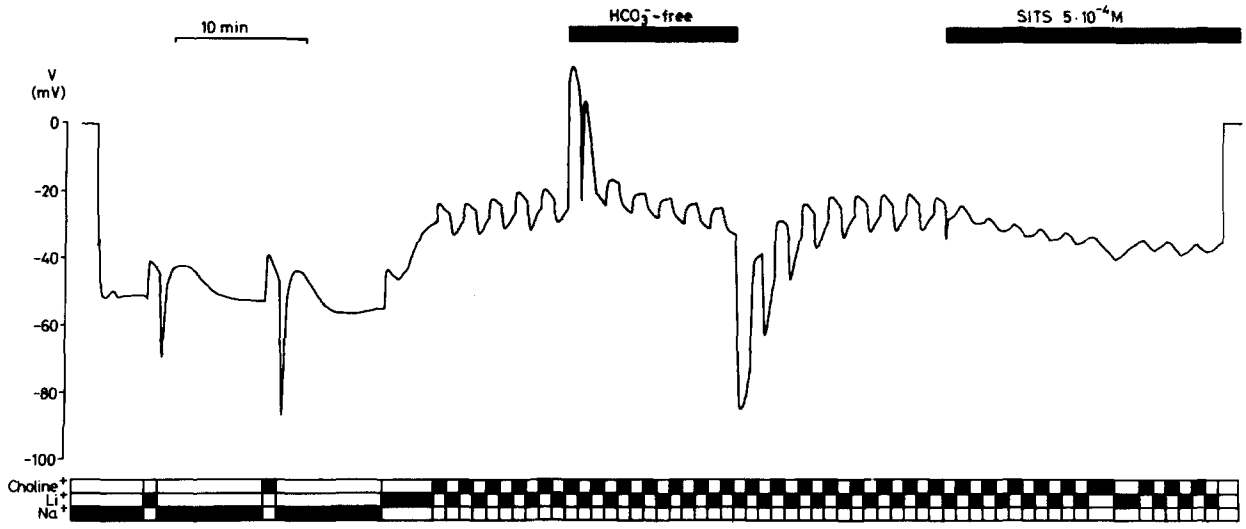

Fig. 8. Registration demonstrating the response of V to sodium, lithium and choline. Note that in bicarbonate-depleted Ringer's the response to lithium was reversed and that 5×10^{-4} M SITS reduced the response. A similar effect of replacement of Li by choline was seen in 4 cells. The influence of bicarbonate and SITS on this reaction was explored in 3 cells of 3 different strains and was similar to the one shown. (Used solutions: 1, 5, 7, 8, 10.)

8.9 (SD) mV/decade (n = 14) for bicarbonate (taken from the step 46 to 23 mM) and 20.3 ± 5.7 (SD) mV/dec for sodium (taken from the step 151 to 70 mM). When corrected for liquid junction potentials (*see* Materials and Methods), the calculated slopes are 39.0 mV/dec for bicarbonate and 15.3 mV/dec for sodium.

Does Lithium Share the Same Process?

The data on the cation dependence of the voltage response to bicarbonate suggest that the same process might also mediate a Li-HCO_3^- cotransport. If this is true, replacement of Li by choline should lead to a depolarization similar to the one observed with the correspondent replacement of sodium. More specifically, this reaction should be reduced in a bicarbonate-free Ringer's and in the presence of stilbenes.

These predictions were tested in another series of experiments. A typical registration is shown in Fig. 8. At first, extracellular sodium was replaced for one minute mainly by lithium (and 46 mM choline, sol. 8). This led to a depolarization with a subsequent hyperpolarization upon readdition of sodium. This reaction was similar to the response observed in the replacement of sodium by choline, which was performed at the same cell nine minutes later. However, in the latter case the amplitude of the reaction was greater by about 40%. (Correction for liquid junction potentials (2.4 mV for choline and 5.5 mV for Li) leads to an even greater difference in amplitudes.)

In the next step, lithium was substituted for sodium for a longer period of time. This led, at first, to a fast depolarization as observed earlier in the same cell. Later on, after passing through a maximum, the voltage continued to decline and reached a steady-state value only after several minutes. After this preincubation with lithium, Li^+ was periodically replaced by choline in intervals of one minute. In accordance with the hypothesis, this led to a depolarization similar to the one observed with replacement of Na^+ by choline. The amplitude was less than in the case of sodium, but since the baseline voltage was reduced with Li^+ as compared to Na^+, it is difficult to draw conclusions from this difference in amplitudes. When bicarbonate was removed, the cell depolarized and the response to replacement of Li^+ by choline$^+$ reversed in sign. The apparent hyperpolarization now seen with addition of choline Ringer's may be in part due to the liquid junction potentials and to a greater membrane permeability to Li^+ compared to choline$^+$. This was reversible after readdition of bicarbonate, which again resulted in a hyperpolarization. During the first minutes after readdition of bicarbonate, the response to Li was enhanced as compared to steady state. As the last step, SITS (5×10^{-4} M) was added. This caused a reversal of the response, with a very slow depolarization induced by the addition of lithium.

Thus, these experiments provide evidence that lithium may be transported by the same process which apparently mediates cotransport of sodium and bicarbonate.

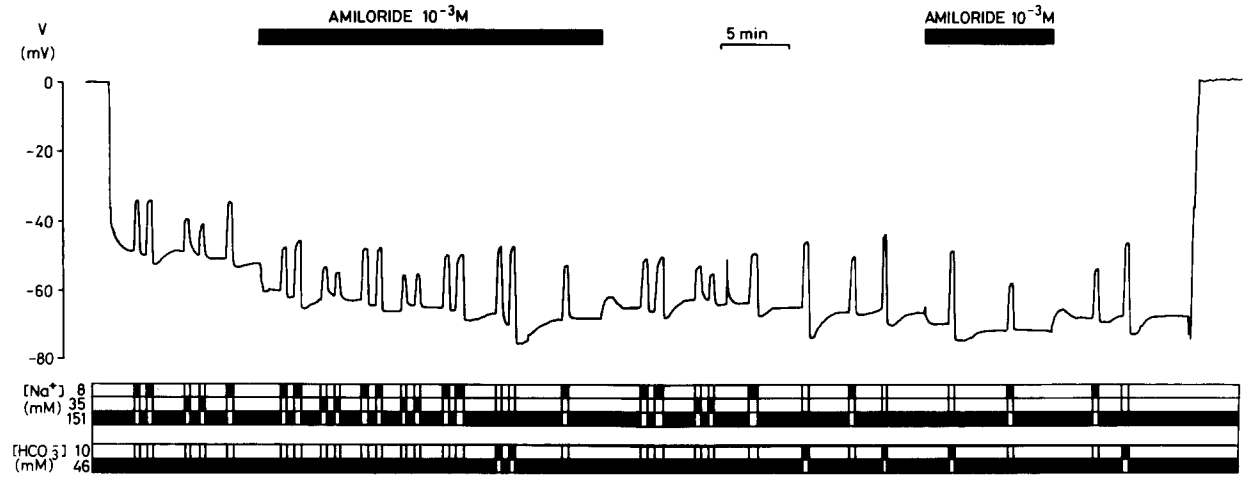

Fig. 9. Effect of amiloride (10^{-3} M) on the membrane potential and the voltage responses to changes in [Na^+] and [HCO_3^-]. The medium bathing the cells was replaced in short periods of 10 sec to solutions containing less sodium or bicarbonate. Note that there are no obvious differences of the reactions in the absence or presence of amiloride. Similar results were obtained with 5 other cells of 2 different strains. (Used solutions: 1, 3, 14, 16.)

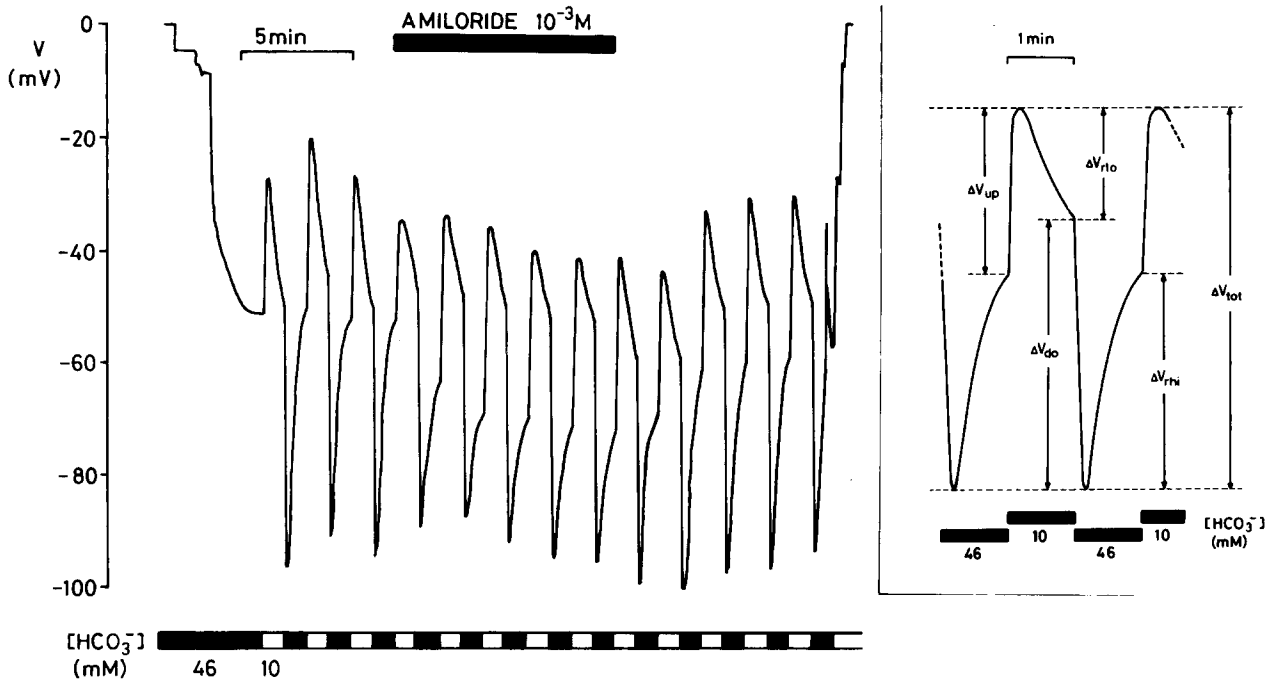

Fig. 10. Influence of amiloride (10^{-3} M) on the voltage response to bicarbonate. In contrast to the experiment of Fig. 9, the changes are performed in longer time intervals. [HCO_3^-] is changed in intervals of 1 min between 46 and 10 mM at constant pCO_2 (5%). The same experiment was performed in 4 cells, all yielding similar results. *Inset:* Definition of the different abbreviations used in the text. ΔV_{tot} = total amplitude, ΔV_{up} = immediate upstroke (depolarization) following removal of bicarbonate, ΔV_{do} = immediate downstroke (hyperpolarization), ΔV_{rhi} = restoring movement of V during the presence of a high concentration of bicarbonate, ΔV_{rlo} = restoring movement in the presence of low $[HCO_3^-]_o$. (Used solutions: 1 and 3.)

Effect of Amiloride

Amiloride (10^{-3} M) caused the membrane to hyperpolarize by 8.5 ± 2.6 mV (SD, n = 13). At 5 × 10^{-4} M, it hyperpolarized to a somewhat lesser extent (4.3 ± 1.5 mV, n = 8), whereas at 10^{-5} M virtually no effect was seen (ΔV = 0.8 ± 0.9 mV, n = 8).

In another series of experiments, we explored the influence of 10^{-3} M amiloride on the response to sodium and bicarbonate. A typical experiment is shown in Fig. 9. The external medium was switched to solutions containing 35 and 8 mM Na^+ or 10 mM HCO_3^- in intervals of 10 sec, a time sufficiently long for the voltage to pass its minimum. This was done

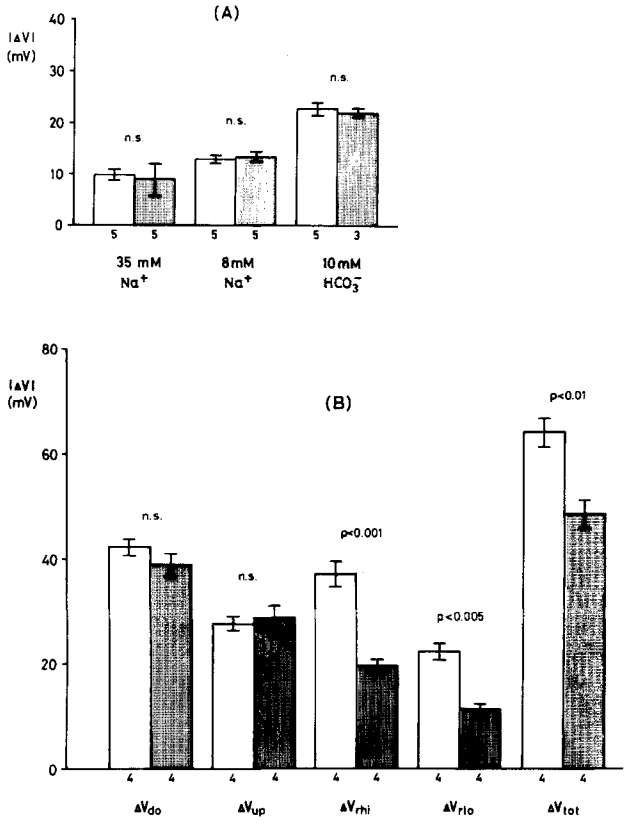

Fig. 11. Effect of amiloride on the voltage responses to sodium and bicarbonate. Hatched columns represent results in the presence of 10^{-3} M amiloride, whereas blank columns are control values. Bars indicate the standard error of the mean (SEM), and the number of cells investigated is given below the columns. Significance levels were calculated using student's *t*-test. (*A*) Responses to short pulses (10 sec) of low sodium (35 and 8 mM) and low bicarbonate (10 mM) (experiments as in Fig. 9). The immediate depolarization was evaluated (corresponding to ΔV_{up}). (*B*) Responses to periodic change of 10 to 46 mM HCO_3^- in intervals of one minute (as in Fig. 10). The response was divided into different segments according to the definitions given in the inset of Fig. 10 and explained in the text

in the absence and presence of 10^{-3} M amiloride. No obvious change in amplitudes can be seen in Fig. 9. The data from several similar experiments is compiled in Fig. 11(*A*). Amiloride had no significant effect on the voltage responses to sodium or bicarbonate.

However, when bicarbonate was changed periodically in intervals of 1 min instead of short pulses, amiloride (10^{-3} M) did have an influence on the reaction (Fig. 10). The overall amplitude of the reaction was reduced. This was not due to a change of the immediate voltage response to bicarbonate, but to a change in the slopes of the component of the signal which tends to restore the resting potential.

For a more quantitative evaluation, we divided the signal into different sections (inset of Fig. 10). We distinguished between the total amplitude ΔV_{tot}, the immediate upstroke ΔV_{up} or downstroke ΔV_{do} after a decrease or an increase of external bicarbonate, respectively, and the restoring movements ΔV_{rhi} and ΔV_{rlo} in the presence of high or low concentrations of bicarbonate. The result of this evaluation is given in Fig. 11(*B*). Amiloride did not significantly affect the immediate voltage change (which is in line with the experiment of Fig. 9 and Fig. 11(*A*)), but it influenced the restoring movement of the potential, which necessarily resulted in a changed total amplitude, ΔV_{tot}. Hence, amiloride probably did not exert a direct influence on the transporter, but did affect the response indirectly via different processes which are sensitive to amiloride.

Effect of Ouabain

Ouabain (10^{-4} M) caused a depolarization of the resting membrane potential of about 7 mV within seconds (Fig. 12(*A*)). This can be attributed to the abolition of the direct electrogenic contribution of the Na/K-ATPase to the membrane potential. Afterwards, the membrane potential decayed continuously over a period of more than half an hour, indicating the dissipation of the transmembranal potassium gradient responsible for most of the resting potential. The mean depolarization of four cells 30 sec after addition of 10^{-4} M ouabain was -6.8 ± 1.09 (SD) mV (resting potentials of these cells: $V = -45.03 \pm 8.75$ mV). At 10^{-5} M ouabain, the cells depolarized by -5.12 ± 0.87 mV ($n = 5$, mean resting potential -44.8 ± 4.2 mV). The response to bicarbonate was essentially unaffected by ouabain as is shown in the experiment of Fig. 12(*B*). No immediate effect on the voltage transients was observed. After a delay of several minutes, the voltage response to bicarbonate slightly increased and became more symmetrical, and only after more than 20 min, the response began to decrease, which might be a nonspecific or toxic effect of ouabain. Thus, the electrogenic cotransport of sodium and bicarbonate does not seem to be directly influenced by ouabain.

Discussion

Evidence for Coupled Transport of Sodium and Bicarbonate

In this paper, we have explored the voltage across the plasma membrane of cultured bovine corneal endothelial cells in response to changes of external bicarbonate and sodium in the presence of various

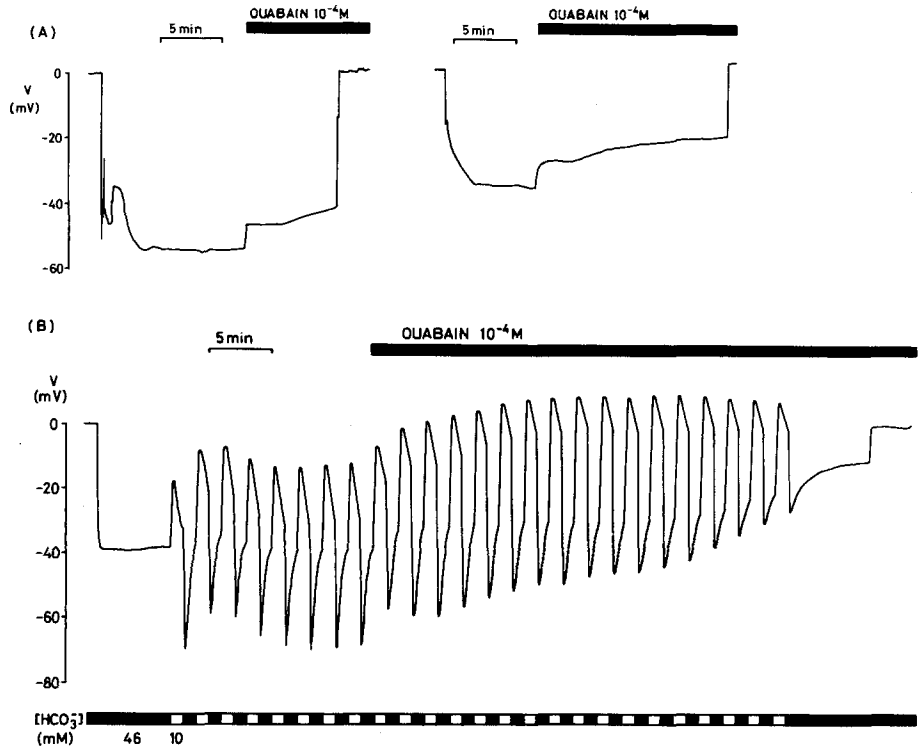

Fig. 12. Effect of ouabain. (*A*) Influence on the steady-state potential of two cells, demonstrating the fast initial drop of *V* due to the direct electrogenic component of the transport, and the following slower decay due to the dissipation of ion gradients. A similar effect of ouabain (10^{-4} M) was seen with 4 cells of 3 different strains, and with 10^{-5} M with 4 cells of 2 strains. (*B*) Effect of 10^{-4} M ouabain on the response to bicarbonate. Note that the response does slightly increase rather than decrease during the first 15 min and that it becomes more symmetrical. Similar results were obtained with 4 cells of 2 strains. (Used solutions: 1 and 3.)

cations and inhibitors. Though we did not measure transport rates and thus depend entirely on the interpretation of one parameter, i.e. *V*, we propose the existence of a tightly coupled transport for sodium and bicarbonate (or related species) in these cells. This transport system should move a net negative charge with the transported ions[1] and should be inhibitable by SITS or DIDS. Such a transport system has only recently been shown to exist in the basolateral membrane of the proximal tubule of the salamander kidney [5].

Such a model makes several predictions for the electrical response of the plasma membrane. The main predictions are:

(*i*) Removal of either bicarbonate or sodium should lead to a depolarization, the readdition of either ion to a hyperpolarization. The extent of this hyperpolarization should increase with the time of preincubation in a medium depleted of the respective ion, since this procedure increases the transmembranal gradient for the influx by depleting the intracellular ion concentration. For bicarbonate, this was shown to be true in [27] and in Fig. 6(*A*) (last part), and for sodium in Fig. 3.

(*ii*) The electrical signals associated with the bicarbonate concentration changes should depend on sodium and vice-versa. This was demonstrated in Fig. 1 for bicarbonate and in Fig. 4 for sodium.

(*iii*) Both the fluxes of bicarbonate and sodium as well as the associated electrical signals should be inhibitable by stilbenes (shown in [27] for bicarbonate and in Fig. 5 for sodium).

Additional support comes from the response to cations (Na or Li) after readdition of bicarbonate to HCO_3^--depleted cells. During some minutes, *V* is increased probably due to an influx of bicarbonate. When Na (or Li) is replaced by choline during this phase, this influx should be blocked. This leads to an enhanced response of *V* to cations as compared to cells equilibrated with bicarbonate. This has been observed for Na as well as for Li (Fig. 8).

Cation Dependence

We explored the dependence on cations by replacing sodium with lithium, potassium, choline and in some experiments with bis(2-hydroxyethyl)dimethylammonium (BDA) or tetramethylammonium (TMA). We have demonstrated that lithium can, to a certain extent, substitute for sodium by enabling the response of *V* to bicarbonate, while large or-

[1] Thus, this (probably passive) cotransport process, which is clearly associated with a change in membrane voltage, might be called "electrogenic" in the sense defined by S.G. Schultz et al. (S.G. Schultz, R.A. Frizzell, H.W. Nellans, 1974. Ion transport by mammalian small intestine. *Annu. Rev. Physiol.* **36**, p. 67).

ganic cations (choline, BDA, TMA) led to a pronounced reduction, though not complete inhibition, of the response. Potassium decreased the response to changes in $[HCO_3^-]_o$ even more than the larger organic cation choline. Although this difference was not statistically significant for the whole series, it was seen in every single cell examined. This may mean that factors other than the size of the cations might be important for their ability to facilitate the electrical response to bicarbonate. Such a factor might be V, which was greatly reduced by high extracellular potassium concentrations.

Moreover, the data suggest that Li does not only stimulate bicarbonate transport, but is probably also transported by the same process. This is because the substitution of lithium by choline led to similar responses as replacement of sodium by choline. This response had the same sensitivity to bicarbonate and stilbenes.

Furthermore, the immediate voltage response to bicarbonate in the presence of Li was not significantly different from that in Na-Ringer's. As will be discussed in more detail for the effect of amiloride, the immediate response rather than the total amplitude should reflect more directly the properties of the transporter. The faster return of V to baseline levels in the presence of sodium might be due to other processes which dissipate the imposed bicarbonate gradient. However, the Na^+/H^+-antiport, which will possibly participate in this return of V, is known to accept Li [41–43], but the influence of Li on its transport rates for the corneal endothelium is not exactly known.

It is interesting to note that besides the transport system described in this paper and the sodium-proton-antiport, also the coupled electroneutral $Na^+/H^+//HCO_3^-/Cl^-$ exchange mechanism in barnacle muscle fibers accepts lithium to some degree [7]. Potassium could not be substituted for sodium in the Na^+/H^+-antiport [41–43], nor could choline [41]. Also the stilbene-sensitive anion-exchanger of erythrocytes is known to transport sodium or lithium in the presence of bicarbonate [3, 9, 16]. In that case, the transport of the ion pairs $NaCO_3^-$ or $LiCO_3^-$ has been proposed.

Possible Stoichiometry of the Cotransport

At this point, no definite conclusions about the stoichiometry of this cotransport system are possible. We can only exclude a one-to-one stoichiometry (without involvement of other ions), since this would result in an electroneutral process. A coupling of two bicarbonate ions to one sodium ion, as has been suggested for the salamander system [5], could well describe the observed electrical phenomena.[2]

Effect of Amiloride

Amiloride (10^{-3} M) did not have an effect on the magnitude of the depolarizations caused by short pulses (10 sec) of low sodium or low bicarbonate. It did, however, influence the overall amplitude of the response to bicarbonate when the solution exchange was performed in greater time intervals. Nevertheless the immediate response to bicarbonate was unaffected in this case.

To explain these results, it is necessary to analyze the voltage response in more detail. If the voltage transients observed after a change in bicarbonate concentration are, to a first approximation, exclusively due to the cotransport system, then the immediate reactions of V to changes in bicarbonate reflect the electrical response of the cotransporter to the imposed transmembranal gradients, attenuated by parallel membrane conductances (e.g. for K^+). The return of the voltage to the steady-state value should be a consequence of the dissipation of the sodium and bicarbonate gradients across the plasma membrane. Hence, the speed of this restoring movement should not only reflect properties of the cotransport system (which simultaneously creates the electrical signal and dissipates the gradients), but of all other (inclusive electroneutral) processes which mediate a flux of either ion across the plasma membrane.

Such a dissipating process could be an electroneutral Na^+/H^+-antiport. Evidence for the existence of such a process in the corneal endothelium comes from the demonstration of an amiloride-sensitive proton influx into Li-loaded bovine corneal endothelial cells [42] and from amiloride inhibition of fluid transport and voltage across the rabbit corneal endothelium [31]. Blockade of this process by amiloride should indeed produce a slower return of V to baseline levels.

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[2] It is tempting to speculate that the ratio of the slopes $\partial V/\partial$ (log $[HCO_3^-]$) to $\partial V/\partial$ (log $[Na^+]$), which was between 2 and 3, reflects the stoichiometry of the cotransport. If the electrical response of a 2 : 1 cotransport of bicarbonate with sodium could be described by the simple relationship

$$V = \text{const} \times \ln([HCO_3^-]_o^2[Na^+]_o/([HCO_3^-]_i^2[Na^+]_i)),$$

then indeed the ratio of the slopes should be 2. However, this equation depends on the model assumed for the transporter. Moreover, in order to use this equation to determine the stoichiometry, $[Na^+]_i$ and $[HCO_3^-]_i$ must be known or must remain constant, which cannot be taken for granted in our experiments. Thus, no conclusion about the stoichiometry is possible.

Amiloride (10^{-3} M and 5×10^{-4} M) also induced a hyperpolarization of the membrane. When sodium was removed after the sodium-bicarbonate cotransport had been inhibited either by removal of bicarbonate or by the application of stilbenes, in some cases also a hyperpolarization was observed. This can most easily be explained by a sodium conductance which is inhibitable by amiloride. This effect is more difficult to reconcile with a Na^+/H^+-antiport. For that mechanism, both removal of sodium and inhibition by amiloride will result in an internal acidification of the cell. This could lead to an electrodiffusive efflux of protons or influx of OH^- or HCO_3^-, which would produce a (probably transient) hyperpolarization. A reduction of potassium conductance which is often associated with acidification [44] and which possibly is also operative in corneal endothelial cells [27] will produce just the opposite effect.

The high concentrations necessary to hyperpolarize the membrane ($\Delta V = 8.5$ mV at 10^{-3} M, $\Delta V = 4.3$ mV at 5×10^{-4} M and virtually no effect at 10^{-5} M) as well as the missing effect of amiloride on the depolarization caused by short exposure to low sodium (Fig. 11(*A*)) argue against the existence of a sizable sodium conductance in these cells. A minor conductive pathway for sodium ions, however, cannot be excluded.

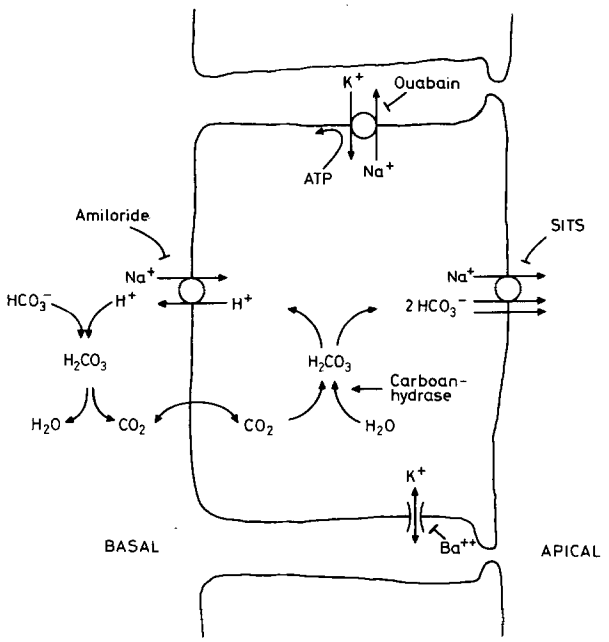

Fig. 13. Proposed model for the transport of sodium and bicarbonate across the bovine corneal endothelium. The stoichiometry of the HCO_3^--Na^+-cotransport is represented to be 2:1, though it is not yet definitely known. A thermodynamic analysis of a related 2:1 cotransport probably existant in the proximal tubule of the salamander kidney is given in [5]. The location of the transport processes is inferred from the transport direction of sodium and bicarbonate known from transepithelial studies

Effect of Ouabain

The electrogenic Na^+/K^+-ATPase makes an immediate contribution of about 7 mV to the resting potentials of corneal endothelial cells, which is comparable in magnitude to other epithelial cells, e.g. the proximal tubule of the rat kidney [13]. No direct effect on the voltage response due to the sodium-bicarbonate cotransport was observed. The slight increase in amplitude after a time lag of several minutes and the more symmetrical shape of the signal may be explained by a decrease in the sodium gradient across the plasma membrane. Normally, when external bicarbonate is varied in intervals of about one minute, the hyperpolarization (ΔV_{do}) correspondent to an influx of bicarbonate is greater than the depolarization (ΔV_{up}) (i.e. *see* inset of Fig. 10 and Fig. 11(*B*)). This might be attributed to the coupling to sodium, since this provides a driving force for bicarbonate entry. Therefore, in the absence of both a sodium gradient and a transmembranal voltage the response to bicarbonate should be symmetrical (if a symmetrical carrier is assumed). The slightly increased overall response to bicarbonate may be due to a stimulation of the exchange mechanism by intracellular sodium, and the final decrease to possible toxic effects of ouabain or effects secondary to intracellular concentration changes of other ions elicited by this inhibitor.

Interestingly, a similar symmetrical response was also seen when Li was the main extracellular cation. Li is only poorly transported by the Na^+/K^+-ATPase (for a review *see* [10]) and thus, after a sufficiently long time, intracellular levels of Li should be higher than those of Na. Therefore, it is not astonishing that with Li the response is similar to that in the sodium Ringer's with ouabain.

Model for Transendothelial Transport in the Cornea

When presenting a model for the transport of sodium and bicarbonate across the corneal endothelium, we must stress that our experiments are unable to distinguish between transport processes located in the apical or basolateral plasma membrane. As discussed previously [27], this limitation is inherent to the method of the puncture of cells cultured on plastic. Our suggestions for the localization of the processes are based on data concerning the transepithelial transport and are in analogy to schemes proposed for other epithelia [5].

The model we would like to propose is depicted in Fig. 13. We postulate the carrier for the coupled transport of sodium and bicarbonate, which has

been the main subject of the present paper, to be located at the apical cell membrane. This process is inhibitable by stilbenes and is linked to a transfer of negative charge. In Fig. 13, it is represented as a 2:1 cotransport of bicarbonate and sodium, though the exact stoichiometry is not clear at present. There might also be an involvement of chloride.

In addition, we assume a Na^+/H^+-antiport (inhibitable by amiloride) to be present at the basolateral side. Evidence for this carrier has been stated in the discussion of the effect of amiloride. Transport of bicarbonate by this sodium-proton-antiport involves diffusion of CO_2 across the cell membrane and conversion to carbonic acid by intracellular carbonic anhydrase. This enzyme has been shown to exist in these cells [20, 38, 39].

The energy for the transport by both carriers could be provided by the transmembranal sodium gradient which is generated by the Na^+/K^+-ATPase. Indeed, according to this model, both a transmembranal voltage, inside negative, or a sodium gradient with $[Na^+]_i < [Na^+]_o$ alone are sufficient to drive a transport of bicarbonate from the stroma to the aqueous humour. Thermodynamic calculations for the electrogenic HCO_3^--Na^+-cotransport in the salamander kidney [5] show that there should be a passive efflux of bicarbonate under control conditions. This is why we postulate these transport processes to be located as shown in Fig. 13. Furthermore, the electrical response to bicarbonate and sodium suggests that the putative sodium-bicarbonate-cotransport may operate in both directions. With a direct input of metabolic energy, the reaction should rather be biased in one direction. Thus, this cotransport system is probably passive.

While about 15% of the membrane potential are directly generated by the Na^+/K^+-ATPase (as indicated by the immediate effect of ouabain), the remaining 85% should be due to the potassium conductance which is inhibitable by barium [27]. Possibly there is also a conductance for sodium, which could explain the hyperpolarization caused by amiloride or the one sometimes seen with the removal of sodium in the absence of bicarbonate or the presence of stilbenes. Both procedures block the Na^+-HCO_3^--cotransport which normally would mask the effect of a sodium conductance. There is, however, no conclusive evidence for this conductance.

In addition to the Na^+-HCO_3^--cotransport system, there might also be a conductive pathway for HCO_3^-, which is not shown in the model. This permeability might explain the residual voltage response to bicarbonate after a blockade of the cotransport either by stilbenes [27] or in sodium-depleted Ringer's.

The proposed model can also explain most data on transendothelial transport. The coupling of sodium transport to bicarbonate [24] and vice-versa [17] can be explained both by the Na^+-HCO_3^--cotransport and the sodium-proton-antiport. The reduction of the transport by ouabain [11, 20, 37] should be due to the inhibition of Na^+/K^+-ATPase with subsequent dissipation of the transmembranal sodium gradient which drives both carriers. Inhibition of transport by amiloride [31] is explainable by the Na^+/H^+-antiport and the inhibition by stilbenes [31] by the Na^+-HCO_3^--cotransport. Inhibition of carboanhydrase also yielded a reduced transport [11, 20, 25].

The origin of the transepithelial voltage (1 mV aqueous (=apical) side negative) associated with transepithelial transport across the corneal endothelium is still an unsolved problem. Both direct electrogenic transport of anions as well as diffusion potentials across the leaky intercellular spaces have been discussed [11, 19, 32], though in the light of recent work [36] the latter possibility seems unlikely. The present results provide evidence for the existence of a coupled transport of sodium and bicarbonate linked to a transfer of negative charge. If this transport is located at the apical surface—and this is likely since the coupling to negative charge would rather favor an efflux of bicarbonate than an influx—then the electrical properties of this transport would have the correct sign to explain this voltage. Clearly, it will be premature to attribute the transepithelial voltage solely to this transport, since other ion fluxes whose relative magnitudes are not exactly known will contribute to the overall phenomenon.

Comparison with Other Systems Involved in Transport of HCO_3^- and/or H^+

In the present work we have shown evidence which suggests the existence of a tightly coupled, stilbene-inhibitable cotransport for sodium and bicarbonate, which transfers negative charge and is probably in series with a Na^+/H^+-antiport. A coupling of bicarbonate transport to sodium has been reported for a variety of tissues (for a review *see* [44]). These systems are believed to have important functions in the regulation of intracellular pH, or the transport of bicarbonate or chloride. A tight coupling of sodium, bicarbonate and chloride (and possibly H^+) has been described for the squid axon [6, 8], snail neurone [46, 47], and barnacle muscle [7, 45]. In crayfish neurones, a similar process is operative [40]. These transport processes are electroneutral and inhibitable by SITS. They mediate a cotransport of Na^+ and HCO_3^-, with Cl^- and possibly H^+ moving

in the opposite direction. There are, however, some differences between these systems. In the squid axon, intracellular ATP is required for acid extrusion [8] while the snail system is unaffected by metabolic inhibitors [46]. In barnacle muscle, the dependence on metabolic energy has not yet been examined. In mammalian tissue, however, no such coupling of bicarbonate to sodium fluxes has been reported. In sheep heart Purkinje fibers, a Cl^--HCO_3^--exchange system inhibitable by stilbenes has been identified [49] which does not depend on sodium [50]. In mouse soleus muscle, the parallel existence of Na^+/H^+-antiport and a Cl^-/HCO_3^- exchanger has been proposed [1, 2], the latter being inhibitable by stilbenes. In both systems, the exchange processes are apparently electroneutral.

Three recent papers [4, 5, 18] describe mechanisms of bicarbonate transport in the proximal tubule of amphibian kidneys. In the basolateral membrane of the salamander *Ambystoma* a transport system has been described [5] with electrical characteristics very similar to the ones found by ourselves. This process apparently couples the movement of one sodium ion to two bicarbonate ions and thus transports net negative charge in the direction of their movement. It is inhibitable by stilbenes and apparently independent on Cl^-. In the proximal tubule of *Necturus*, a tight coupling of sodium, bicarbonate and chloride movement inhibitable by SITS was reported [18]. As in the salamander kidney, there was an apparent cotransport of negative charge with sodium and bicarbonate and in opposite direction to chloride fluxes. Since this system depends on chloride, it is not identical to the salamander system, and a parallel operation of an electrogenic $Na^+/2HCO_3^-$ transporter with an electroneutral $Na^+/2HCO_3^-$-Cl^- exchanger has been taken into consideration by the authors [18]. However, they do not exclude the possibility that permeability changes for other ions cause the observed voltage changes.

In the mammalian proximal tubule, no coupling of basolateral bicarbonate transport to sodium has been described. In the rat, a conductive pathway for bicarbonate inhibitable by acetazolamide or stilbenes was reported [14, 15, 48].

It is interesting to note that bovine corneal endothelium thus bears several similarities to the proximal tubule of the kidney. Both are leaky epithelia involved in the transport of water, bicarbonate and sodium. In the proximal tubule, the exit of these ions occurs at the basolateral membrane, whereas in the corneal endothelium it is the apical surface. The coupling of a negative charge to the movement of sodium and bicarbonate ions provides an electrochemical gradient more favorable for the efflux of these ions than the electroneutral mechanism described for the invertebrate systems, where the main function of the coupled transport is probably regulation of pH_i, thus mediating mainly an influx of HCO_3^-.

Additionally, the coupling of negative charge to sodium should provide an economic means for transcellular transport of sodium in these sodium-transporting epithelia, which does no longer need to be extruded alone by the energy-consuming Na^+/K^+-ATPase.

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