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K^+-Conductance and Electrogenic Na^+/K^+ Transport of Cultured Bovine Pigmented Ciliary Epithelium

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Summary. Using intracellular microelectrode technique, we investigated the changes in membrane voltage (V) of cultured bovine pigmented ciliary epithelial cells induced by different extracellular solutions. (1) V in 213 cells under steady-state conditions averaged -46.1 ± 0.6 mV (SEM). (2) Increasing extracellular K^+ concentration ($[K^+]_o$) depolarized V. Addition of Ba^{2+} could diminish this response. (3) Depolarization on doubling $[K^+]_o$ was increased at higher $[K^+]_o$ (or low voltage). (4) Removing extracellular Ca^{2+} decreased V and reduced the V amplitude on increasing $[K^+]_o$. (5) V was pH sensitive. Extra- and intracellular acidification depolarized V; alkalinization induced a hyperpolarization. V responses to high $[K^+]_o$ were reduced at acidic extracellular pH. (6) Removing K_o^+ depolarized, K_o^+ readdition after K^+ depletion transiently hyperpolarized V. These responses were insensitive to Ba^{2+} but were abolished in the presence of ouabain or in Na^+-free medium. (7) Na^+ readdition after Na^+ depletion transiently hyperpolarized V. This reaction was markedly reduced in the presence of ouabain or in K^+-free solution but unchanged by Ba^{2+}. It is concluded that in cultured bovine pigmented ciliary epithelial cells K^+ conductance depends on Ca^{2+}, pH and $[K^+]_o$ (or voltage). An electrogenic Na^+/K^+-transport is present, which is stimulated during recovery from K^+ or Na^+ depletion. This transport is inhibited by ouabain and in K^+- or Na^+-free medium.

Key Words ciliary body · cell culture · intracellular potentials · K^+ conductance · Na^+/K^+-ATPase · ouabain

Introduction

The major function of the ciliary body is the formation of aqueous humor by ultrafiltration and active cellular electrolyte transport [4, 53]. In contrast to other transporting epithelia, which consist of only one cellular layer, the ciliary epithelium presents the unique situation of two different epithelial layers facing against each other: the pigmented epithelium (PE) at the stromal side and the nonpigmented epithelium (NPE) at the aqueous side. Both layers adjoin each other with their apical membranes.

Attempts to investigate the basic transport properties of this secreting epithelium have been made by mounting ciliary body preparations of different species [7, 11, 14, 25, 31, 35, 47, 52] in an Ussing-chamber. Because of technical problems only a few micropuncture studies have been performed [1, 8, 15, 21, 39, 51]. However, even in the same species the data are not always consistent and give no conclusive interpretation of localization, direction and amount of the electrolyte transport. There is no generally accepted model for the ion transport mechanisms involved in aqueous humor formation. One reason for the problems in these investigations is the double-layered anatomy of the ciliary epithelium. It is not easy to relate the obtained results to one of the two layers. Moreover, it is difficult, if not impossible, to perform fast solution changes at the stromal side. In *in situ* preparations there is a long diffusion distance across the connective tissue of the ciliary body. Therefore it is particularly difficult to investigate the PE *in situ*. For these reasons it is desirable to investigate PE and NPE separately. Cell culture provides this possibility. In this paper we describe the electrical membrane properties of bovine pigmented ciliary epithelial cells in culture using the micropuncture technique. We were able to show that K^+ conductance is pH sensitive and Ca^{2+} dependent, and we could characterize properties of electrogenic Na^+/K^+ transport.

Materials and Methods

Cell Cultures

Primary cultures of bovine pigmented ciliary epithelial cells were established by a method similar to the one previously described for human [34] and bovine [13] ciliary epithelia. Eyes from 2- to 4-year-old steers were obtained from the local slaughterhouse. They were enucleated immediately after death and transported to the laboratory, cooled on ice. All dissections were done under sterile conditions in a laminar flow hood. The posterior part of

the eyes was cut off and vitreous and lens were gently removed from the anterior part. The tips of the ciliary processes were dissected with small scissors and washed twice in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The tissue was digested using trypsin-EDTA solution (0.05/0.02%; Seromed, Munich, FRG) at 37°C. Every 30 min the processes were allowed to settle down. The supernatant containing trypsin solution and released cells was removed from the processes. Fresh trypsin was added to the processes at the bottom of the tube. The released single cells were spun down in a centrifuge and collected. After 2 to 3 hr the whole cellular suspension was filtered through a fine sterile gauze to remove clumped cells. The resulting single-cell suspension contained nonpigmented ciliary epithelial cells (NPE), pigmented ciliary epithelial cells (PE), blood cells and free pigment. These cells were incubated in a plastic cell culture flask (Nunc, Roskilde, Denmark) with Dulbecco's modification of minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B (all components from Seromed). The cultures were gassed with a 5% CO_2/95% air mixture and incubated at 37°C. The medium was changed twice a week. Amphotericin B was no longer included in the culture medium after the first medium exchange.

According to observations made by Kondo et al. [34], Coca-Prados and Kondo [13], and Ciluffo et al. [12] under these conditions only the PE were able to grow. NPE did not flatten or grow. To further confirm this observations, PE and NPE were separated by Percoll density gradient centrifugation [19]. The mixed suspension was layered on top of a Percoll (Seromed) discontinuous gradient (40%, 60%). NPE were found on top of the gradient. PE cells banded between 40 and 60% Percoll and at the bottom of the tube. The purified PE fraction contained less than 5% contamination by NPE, and the NPE fraction contained less than 5% PE contamination. Incubating the PE suspension in DMEM supplemented with 10% FCS resulted in a monolayer with the same morphology and the same electrophysiological properties (*data not shown*) compared with the mixed suspension. Incubating the NPE-containing fraction in the same medium, we observed according to [12, 13, 34] only a loose attachment of the cells to the substrate. The NPE cells never flattened or grew, and detached after a few days.

The PE cells formed a densely pigmented, epithelial-like confluent monolayer after 5 to 7 days in primary culture. Then they were passaged either for further propagation in tissue culture flasks or for electrophysiological experiments on tissue culture dishes. With a split ratio of 1:2 every week they could be propagated for 3 to 7 subpassages, becoming less pigmented with every splitting. Later the cells became very large and formed many vacuoles but did not divide any more. From the third passage on they were nearly unpigmented. Confluent monolayers in the first and second subculture were used for our investigations. Some experiments performed with primary cultures and later subpassages showed the same results.

Experimental Setup

The experimental setup has been described and discussed previously in detail [26]. In brief, a tissue culture dish was inserted in a temperature-controlled plastic container (37°C). A flow chamber was pressed on the bottom of a tissue-culture dish, isolating a small channel (width: 1.5 mm, length: 30 mm). This channel could rapidly be superfused by up to eight different test solutions with a 90% fluid exchange within 3 sec at a perfusion rate of 30 ml/hr. Solution exchanges at exact time intervals were performed using electromagnetic valves (Lucifer type 133A 54, Geneva, Switzerland) switched by a microcomputer (Commodore 2001-8C). The cells on the bottom of the flow channel were punctured with conventional microelectrodes filled with 0.5 M KCl solution (resistance in Ringer solution: 50-120 MΩ). They were advanced by a micro-stepping device (Heidelberg Nanostepper, Science Trading, Frankfurt, FRG) until a stable negative membrane voltage (V) was obtained. The microelectrode was connected to an electrometer amplifier (WPI model M4-A, Hamden, CT) and the time course of the voltage recorded on a chart recorder (Rikadenki KA-60, Tokyo, Japan).

Solutions and Source of Chemicals

The ionic composition of the test solutions is given in Table 1. Solution No. 1 will be called *control Ringer*. All HCO_3^--contain-

Table 1. Composition of solutions (concentrations given in mM)

n	Na^+	K^+	Ca^{2+}	Mg^{2+}	NH_4^+	$NMDG^+$	$Choline^+$	Cl^-	HCO_3^-	$H_2PO_4^-$	SO_4^{2-}	$acetate^-$	HEPES	Glucose
1	151	5	1.7	0.9	—	—	—	130.4	28	1	0.9	—	—	5
2	146	10	1.7	0.9	—	—	—	130.4	28	1	0.9	—	—	5
3	136	20	1.7	0.9	—	—	—	130.4	28	1	0.9	—	—	5
4	116	40	1.7	0.9	—	—	—	130.4	28	1	0.9	—	—	5
5	76	80	1.7	0.9	—	—	—	130.4	28	1	0.9	—	—	5
6	151	5	1.7	0.9	—	—	—	158.4	—	1	0.9	—	10	5
7	146	10	1.7	0.9	—	—	—	158.4	—	1	0.9	—	10	5
8	136	20	1.7	0.9	—	—	—	158.4	—	1	0.9	—	10	5
9	116	40	1.7	0.9	—	—	—	158.4	—	1	0.9	—	10	5
10	—	5	1.7	0.9	—	123	28	130.4	28	1	0.9	—	—	5
11	156	—	1.7	0.9	—	—	—	130.4	28	1	0.9	—	—	5
12	—	—	1.7	0.9	—	128	28	130.4	28	1	0.9	—	—	5
13	154.4		—	0.9	—	—	—	130.4	28	1	0.9	—	—	5
14	119.4	40	—	0.9	—	—	—	130.4	28	1	0.9	—	—	5
15	151	5	1.7	0.9	—	—	—	90.4	28	1	0.9	40	—	5
16	131	5	1.7	0.9	20	—	—	130.4	28	1	0.9	—	—	5

ing solutions were gassed with a 5% CO_2/95% air mixture, which led to a pH of 7.4. HCO_3^--free solutions were buffered with 10 mM HEPES to pH 7.4—if not indicated otherwise—and gassed with air. Solutions containing Ba^{2+} were SO_4^{2-}-free. In that case, also the control solutions did not contain SO_4^{2-}. Ouabain (*g*-strophantin) was obtained from Merck (Darmstadt, FRG).

Results

Membrane Potentials

Microelectrodes were advanced by a microstepper in 20-μm steps. Intracellular recordings were recognized by a steep negative movement in the registered voltage. The distribution of 216 intracellular potentials stable (±2 mV) for at least 5 min in control-Ringer solution (solution No. 1, Table 1) is depicted in Fig. 1. Impalements with a V less than −30 mV were thought to be leaky and excluded. Mean voltage was −46.1 ± 0.6 mV. There was a wide range from −31 to −69 mV, but within the same culture dish the potentials measured in different cells varied by only a few mV (<±3 mV).

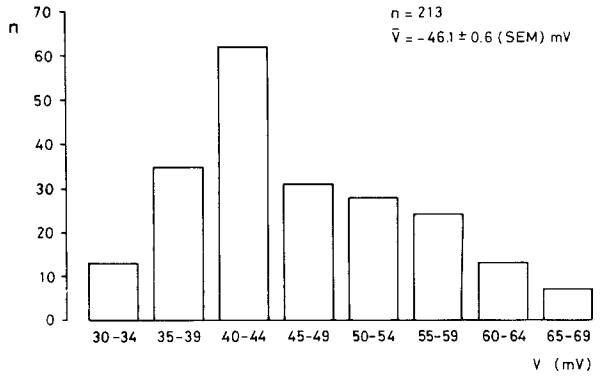

Fig. 1. Frequency distribution of resting potentials (V) of 213 cells punctured in control Ringer's (sol. 1). Only potentials stable for at least 5 min were taken into account. The mean amounts to −46.1 ± 0.6 mV (SEM)

K^+ Conductance

Increasing Extracellular K^+ Concentration

Changing the superfusing solution from 5 to 40 mM K^+ immediately depolarized the plasma membrane. Figure 2 shows the effect of Ba^{2+}, a known blocker of K^+ conductance. Ba^{2+} decreased V and reversibly reduced the amplitude of the voltage responses induced by increasing extracellular K^+. Figure 3A shows the effect of increasing the extracellular K^+ concentration ($[K^+]$) from 5 mM to 10, 20, 40 and 80 mM, respectively, on intracellular V. Figure 3*B* summarizes the results of nine experiments from different cells. Membrane voltage is plotted versus the logarithm of extracellular $[K^+]$. From a Nernstian behavior one would expect a linear relation. However, the slope of the curve increases by a factor of about five comparing the V response on doubling $[K^+]$ from 5 to 10 mM (+3.2 mV) with the response on doubling $[K^+]$ from 40 to 80 mM (+14.9 mV). To preclude that this flattening of the PD versus $[K^+]$ relation at lower $[K^+]$ is due to an inhibition of the electrogenic Na^+/K^+-ATPase by lowering $[K^+]$, we performed two experiments (similar to the one shown in Fig. 3*A*) in the absence and immediately after addition of 10^{-4} M ouabain. The mean values are depicted in Fig. 3*C*. The level of the curve in the presence of oubain is lower, but the flattening of the V versus $[K^+]$ relation is still present and the slope is not significantly different.

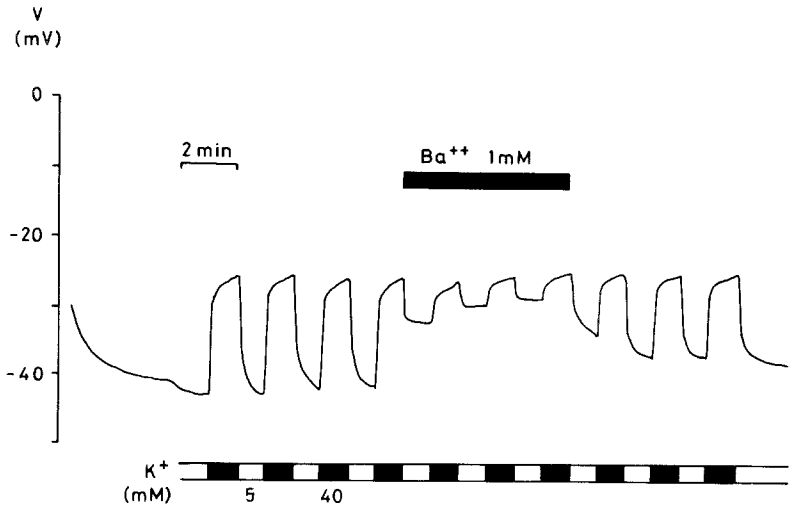

Fig. 2. Original recording demonstrating the blockade of K^+ conductance by Ba^{2+}. Extracellular $[K^+]$ was changed in 1-min intervals from 5 to 40 mM. The depolarization induced by 40 mM K^+ was markedly reduced when Ba^{2+} was added, as indicated by the bar. (Solutions: 1, 4 without SO_4^{2-})

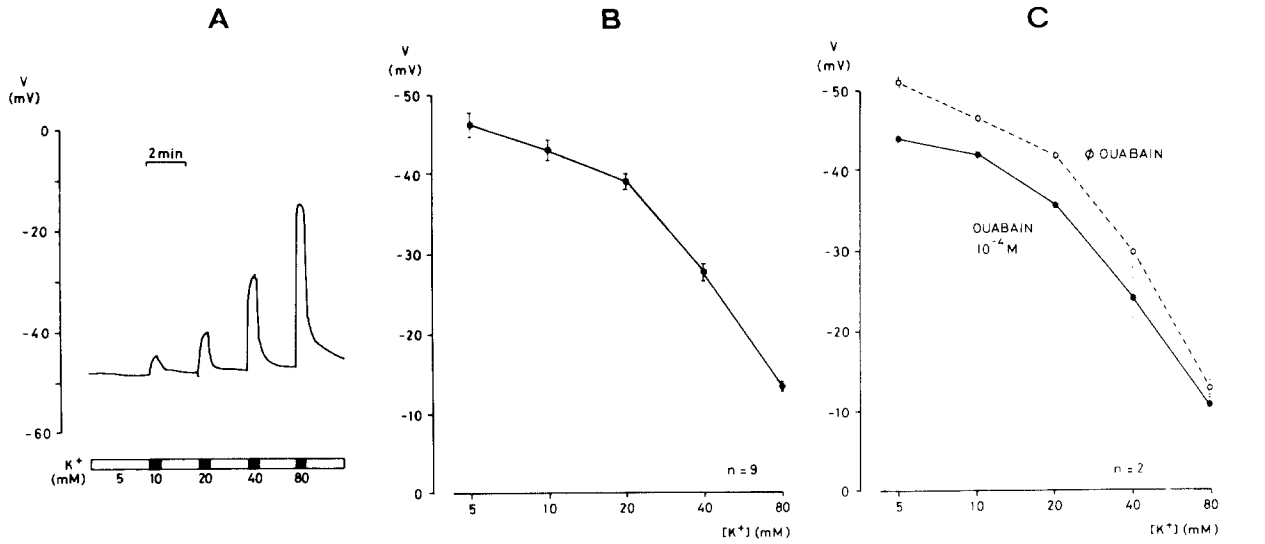

Fig. 3. (*A*) Influence of different K^+ concentrations on the membrane potential *V*. The superfusing solution was changed from 5 to 10, 20, 40 and 80 mM, respectively, for 30 sec each. (Solutions: 1, 2, 3, 4, 5.) (*B*) Mean values of the membrane voltage from nine experiments like the one shown in *A* are plotted as a function of the logarithm of K^+ concentration. Bars indicate SEM values. (*C*) Membrane voltage plotted versus log $[K^+]$. Experiments like those shown in *A* have been performed before and immediately after addition of 10^{-4} M ouabain. Mean values (±SEM) from two different cells are given

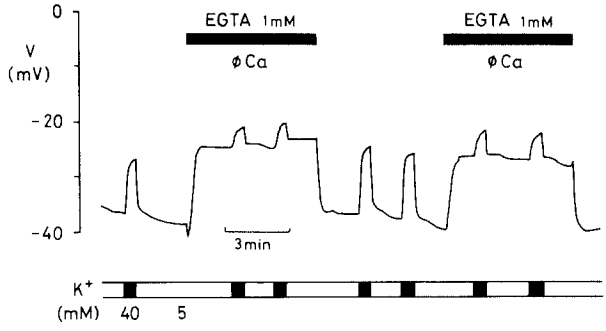

Fig. 4. Original recording of an experiment showing the effect of removing Ca^{2+} from the test solution and addition of 1 mM EGTA (as indicated by the bars) on membrane potential *V*. Extracellular $[K^+]$ was increased from 5 to 40 mM for 30 sec in the presence and in the absence of Ca^{2+}. (Solutions: 1, 4, 13, 14)

Changing Extracellular Ca^{2+} Concentration

Changing extracellular Ca^{2+} concentration $[Ca^{2+}]$ from 1 to 2 or 4 mM had no significant effect on *V* (*data not shown*). But in a Ca^{2+}-free solution in the presence of 1 mM EGTA the voltage reversibly depolarized by +13.3 ± 1.54 mV (n = 10). Figure 4 shows a typical recording. In Ca^{2+}-free medium (1 mM EGTA) *V* decreased and the depolarization induced by pulses with 40 mM K^+ were markedly reduced compared with the reactions in a Ca^{2+} containing solution. This effect was reversible. To test whether this depolarization was due to a direct effect of EGTA, we also performed some experi-

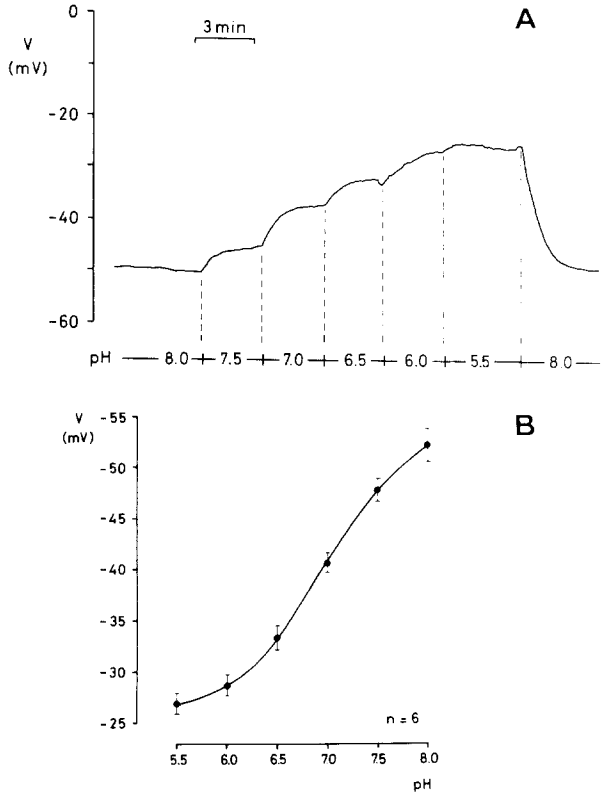

Fig. 5. (*A*) Effect of step changes of extracellular pH in HCO_3^--free solutions from 8.0 to 5.5 on membrane voltage *V*. (Solution 6 titrated to different pH values.) (*B*) Membrane voltage *V* plotted as a function of extracellular pH. Experiments like the one shown in *A* from six different cells are summarized. Bars indicate SEM

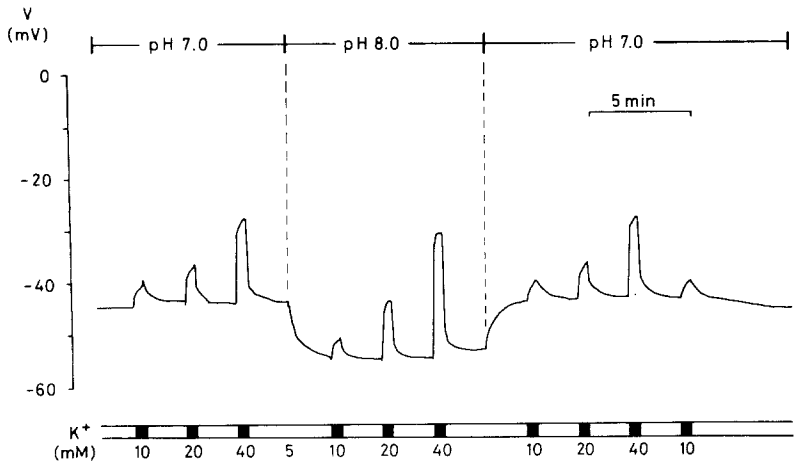

Fig. 6. Effect of extracellular pH on voltage (V) responses induced by increasing extracellular [K^+] from 5 to 10, 20, 40 mM, respectively. (Solutions: 6, 7, 8, 9 buffered with HEPES to pH 7.0 or 8.0, respectively)

ments in Ca^{2+}-free solutions without EGTA. Superfusing the cells with a nominally Ca^{2+}-free solution without adding EGTA also resulted in a significant depolarization.

Changing Extracellular pH

The effect of changing the extracellular pH (pH_o) was investigated in HCO_3^--free solutions buffered with 10 mM HEPES. Figure 5*A* shows an original recording demonstrating the potential responses on step changes in pH_o from 5.5 to 8.0. With increasing [H^+] the voltage depolarized. Figure 5*B* summarizes mean values from experiments with six different cells. There is a sigmoidal relation between pH_o and V. To explore whether this voltage change was due to an interaction of pH and K^+ conductance, we performed experiments as in Fig. 6. [K^+] was changed from 5 to 10, 20, and 40 mM in solutions buffered to pH 7.0 or 8.0, respectively. The reduction of the voltage response at pH 7.0 is obvious. In Fig. 7 voltage is plotted as a function of [K^+] at pH 7.0 and 8.0. At alkaline pH the slope of the curve is clearly steeper.

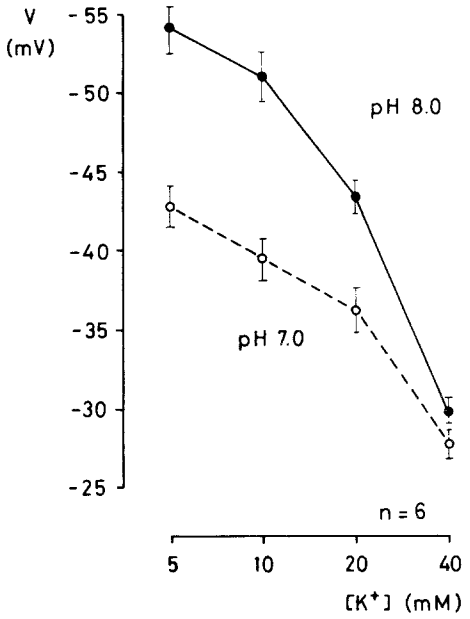

Fig. 7. Summary of experiments like the one shown in Fig. 6 from six different cells as a plot of membrane voltage (V) (mean ± SEM) versus logarithm of extracellular [K^+] at different extracellular pH values

Changing Intracellular pH

The intracellular pH (pH_i) was changed by exposing the cells to permeable weak acids or bases (for review, [45]). It has been well established that addition of the weak base NH_4^+ leads to an intracellular alkalinization and removal of NH_4^+ acidifies the cell interior. Addition and removal of the weak acid acetate has opposite effects. As shown in Fig. 8, maneuvers known to acidify intracellular pH (either removal of NH_4^+ or addition of acetate) depolarized the membrane. Addition of NH_4^+ or removal of acetate (both maneuvers are believed to increase pH_i) led to a hyperpolarization. This responses cannot be explained by a conductive pathway for NH_4^+ or acetate because the Nernst equation predicts reactions opposite in sign.

Electrogenic Na^+/K^+ Transport

Removing and Re-adding Extracellular K^+

Figure 9 shows a typical tracing of V responses induced by superfusing the monolayer with a nomi-

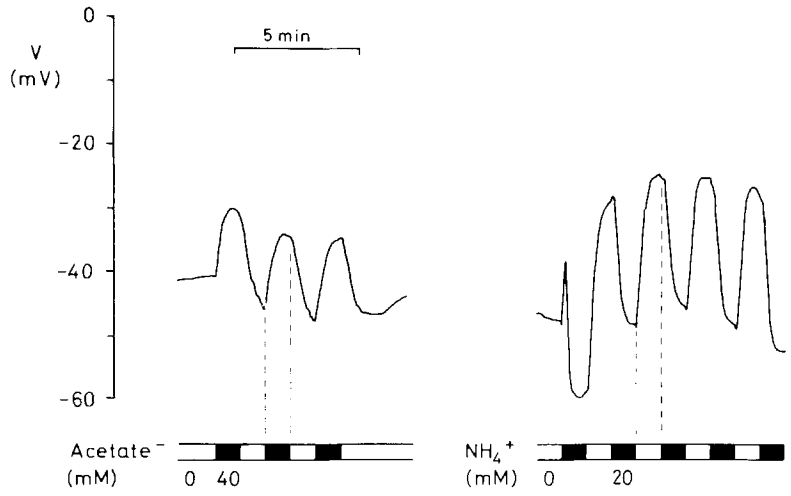

Fig. 8. Effect of changing intracellular pH on membrane voltage (V). Intracellular pH was acidified (as indicated by the broken lines) either by adding 40 mM acetate$^-$ or by removing 20 mM NH_4^+ (Solutions: 1, 15, 16)

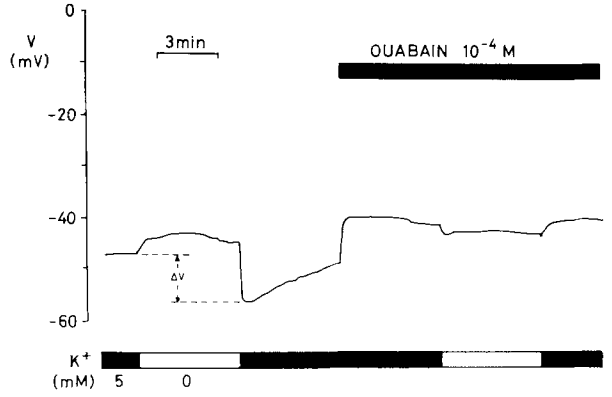

Fig. 9. Effect of changing extracellular K^+ concentration from 5 to 0 and vice versa before and in the presence of ouabain 10^{-4} M. (Solutions: 1, 11)

Table 2. Voltage response on removing extracellular K^+[a]

	Voltage response mean ± SEM (mV)	n
In control Ringer	+6.0 ± 0.7	14
In Ringer +2 mM Ba^{2+}	+7.0 ± 0.7	4
In Ringer + ouabain 10^{-4} M	−1.2 ± 0.5	5
In Na^+-free Ringer	−3.0 ± 0.4	6

[a] Determined 1 min after K^+ removal.

nally K^+-free solution. This maneuver resulted in a significant depolarization of +6.0 mV (±0.7 mV, n = 14). After the immediate decrease of PD on K^+ removal the voltage remained at a constant level. Cells depleted for some minutes (in Fig. 9 for 5 min) from K^+ responded with a marked hyperpolarization on K^+ readdition. V reached a maximum within 10 to 30 sec after K^+ addition. Then the voltage slowly decreased towards its initial value. In Fig. 10*A* the magnitude of this transient hyperpolarization is plotted versus the time of the preceding K^+ depletion. There is a clear dependence of the magnitude of the subsequent hyperpolarization on the time of K^+ depletion.

The observed V responses on K^+ removal and readdition cannot be explained by a K^+ conductance. The Nernst equation predicts a hyperpolarization on K^+ removal. In other epithelia [3, 9, 37, 49] including the ciliary body [21] such "anomalous" responses have been observed, too, and have been related to alterations in the pump activity of the Na^+/K^+-ATPase. In order to investigate whether the V responses on changes in $[K^+]$ from 5 to 0 mM (and vice versa) were also due to a change in the pump activity of the Na^+/K^+-ATPase, we performed the same experiments in the presence of ouabain. As shown in Fig. 9, immediately after ouabain addition (10^{-4} M) V depolarized by +8.9 mV (±0.7 mV, n = 15). This effect was complete in about 1 min and not easily reversible, if ouabain was present for longer than 5 min. For the next 30 min V depolarized by only a few more mV. Removing extracellular K^+ from ouabain-treated cells induced a voltage response opposite in sign to the V change in untreated cells (Fig. 9 and Table 2). In the presence of ouabain we observed a hyperpolarization (−1.2 ± 0.5 mV, n = 5) on K^+ removal. Moreover, the reaction on K^+ readdition after 5 min of K^+ depletion also changed. Instead of a hyperpolarization of −13.7 mV (±2.6 mV, n = 6) in untreated cells, we

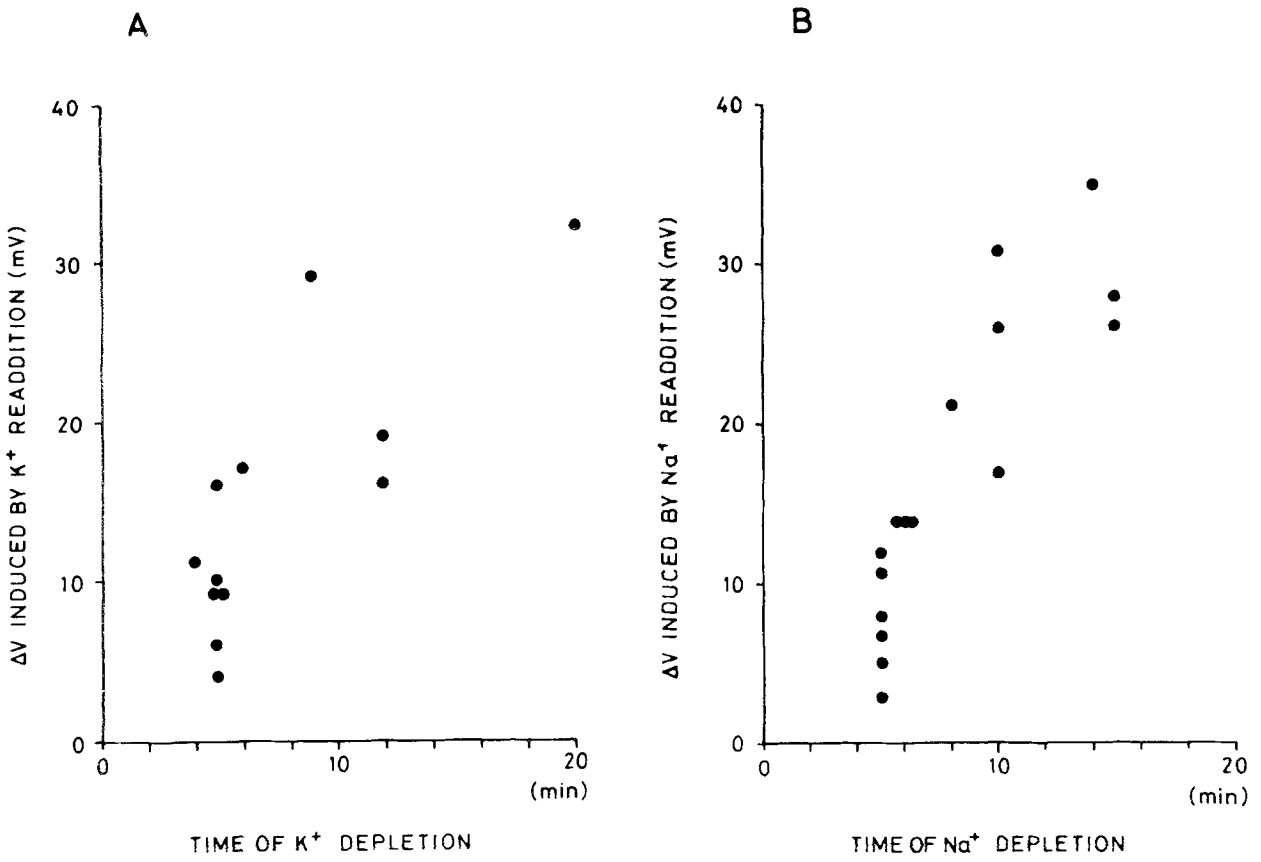

Fig. 10. (*A*) The magnitude of the transient hyperpolarization above steady-state level (ΔV in Fig. 9) on K^+ readdition is plotted versus the time of preceding K^+ depletion. (Correlation is significant: $r = 0.79$, $P < 0.01$.) (*B*) The magnitude of the transient hyperpolarization above steady-state niveau (ΔV in Fig. 12) induced by Na^+ readdition after Na^+ depletion is plotted versus the time of preceding Na^+ depletion. (Correlation is significant: $r = 0.65$, $P < 0.01$)

saw a depolarization of ±1.4 mV (± 0.6 mV, $n = 5$) in ouabain-treated cells.[1]

In order to investigate whether there is a coupling between Na^+ and K^+ transport, we compared the voltage changes on K^+ removal and readdition in sodium-containing and sodium-free medium. In experiments like those shown in Fig. 11*A*, $[K^+]$ was changed periodically from 5 to 0 mM in Na^+-containing and Na^+-free solution. In Na^+ Ringer we observed a depolarization, but in Na^+-free solution V hyperpolarized on K^+ removal (for summary and statistics *see* Table 2). The hyperpolarization on K^+ readdition seen in control Ringer (*see* Fig. 9) was totally abolished without Na^+ in the superfusing fluid (Fig. 11*B*). In Na^+-free medium V depolarized during recovery from K^+ depletion (+2.3 ± 0.4 mV, $n = 5$, *see* Fig. 11*B* and Table 3). Thus, the "anomalous" V response on K^+ removal seemed to be Na^+ dependent.

To exclude that the observed V responses on K^+ removal and readdition were due to an alteration of the K^+ conductance, we performed the same experiments in the presence of 2 mM Ba^{2+} (Fig. 14). Mean values are given in Table 2 and Table 3. It is obvious that the observed reaction could not be blocked by Ba^{2+}. The depolarization induced by K^+ removal was nearly unchanged and the hyperpolarization on K^+ readdition was not reduced (but slightly enlarged) in the presence of Ba^{2+}.

Thus, the V responses in control Ringer on changes of $[K^+]$ from 5 to 0 mM were opposite to the

[1] In the experiments shown in Figs. 9 and 12 the cells have been treated with ouabain for some minutes before extracellular electrolyte concentrations were changed. To exclude an indirect effect of ouabain on the observed V responses by changing intracellular electrolyte concentrations, we performed experiments in which ouabain was added simultaneously with the readdition of K^+ or Na^+, respectively. We observed the same reactions of V as shown in Figs. 9 and 12 (*data not shown*), indicating a direct action of ouabain.

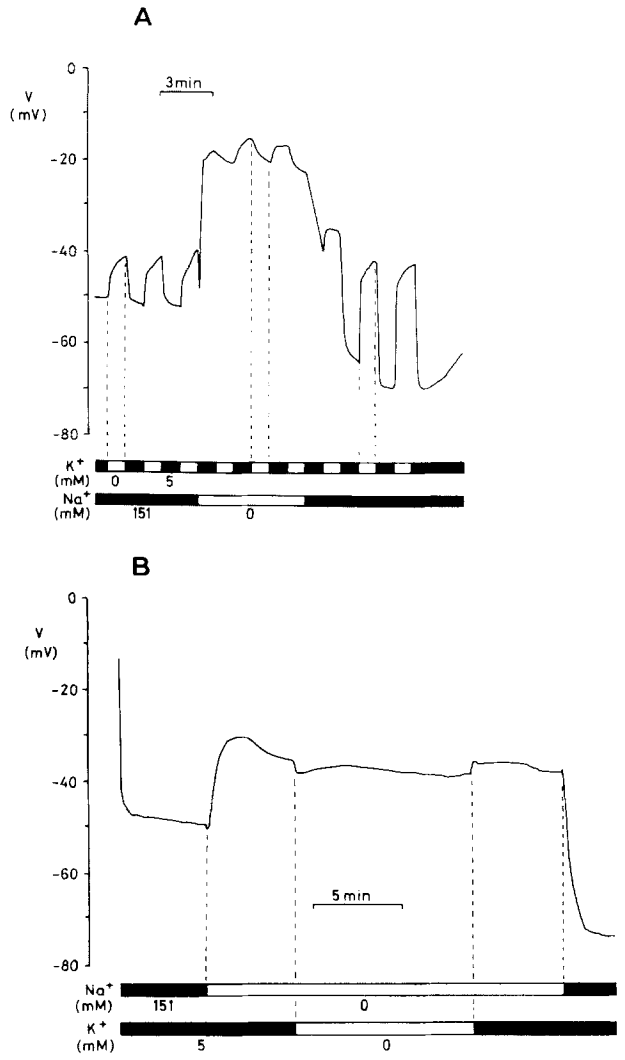

Fig. 11. (*A*) Extracellular [K^+] was changed periodically for 1 min from 5 to 0 mM in the presence and the absence of extracellular Na^+, and during recovery from Na^+ depletion. (Solutions: 1, 10, 11, 12.) (*B*) Effect of removing and readding extracellular K^+ in Na^+-free solution. (Solutions 1, 10, 12)

direction predicted by a K^+ conductance. These reactions could not be reduced by Ba^{2+}. On the other hand, in the presence of ouabain or in Na^+-free medium the *V* responses were in accordance with a Nernstian behavior.

Removing and Readding Extracellular Na^+

Replacing Na^+ in the superfusing solution by NMDG (solution No. 10, Table 1) markedly depolarized the cell membrane (as illustrated in Fig. 12). After a short period the voltage approached a new steady-state value at a lower level. The reaction on Na^+ readdition was similar to the one observed on K^+ readdition. Na^+-depleted cells responded with a transient hyperpolarization above the steady-state potential in control Ringer, when superfused again with Na^+-containing solution. A maximum was reached in about 2 min. The magnitude of this reaction was also dependent on the time of Na^+ depletion (Fig. 10*B*).

Table 3. Voltage response (total amplitude) on K^+ readdition after 5 min K^+ depletion[a]

	Voltage response mean ± SEM (mV)	*n*
In control Ringer	−13.7 ± 2.6	6
In Ringer +2 mM Ba^{2+}	−23.5 ± 1.3	4
In Ringer + ouabain 10^{-4} M	+1.4 ± 0.6	5
In Na^+-free Ringer	+2.3 ± 0.4	5

[a] Determined 10 to 40 sec after K^+ readdition.

In the preceding results we demonstrated an interaction of *V* changes induced by removing and readding K^+ with ouabain and Na^+. Therefore we tested the effect of ouabain on the *V* responses to Na^+ removal and readdition. As shown in Fig. 12, the initial decrease of *V* on Na^+ removal was still present in a solution containing ouabain. In the absence of ouabain the amplitude of the hyperpolarization on Na^+ readdition averaged −24.3 mV (±3.1 mV, $n = 6$) after 5 min of Na^+ depletion. However, the hyperpolarization during recovery from Na^+ depletion was reduced to −9.0 mV (±1.2 mV, $n = 3$, Table 4) in the presence of ouabain.

The K^+ dependence of the *V* responses induced by changing [Na^+] was tested in experiments like those shown in Fig. 13. Na^+ was removed in a K^+-free medium. As in control Ringer and in the presence of ouabain (*see* Fig. 12), the immediate depolarization induced by Na^+ removal was nearly unchanged. But the hyperpolarization on Na^+ readdition was suppressed in K^+-free conditions (*see also* Table 4).

Finally, we investigated the dependence of the observed *V* responses on the K^+ conductance. Na^+ was removed and readded in the presence of 2 mM Ba^{2+}, which blocks the K^+ conductance. Figure 14 shows such an experiment. The depolarization on Na^+ removal was slightly reduced in the presence of Ba^{2+}. However, the hyperpolarization induced by readdition of Na^+ was not markedly influenced by Ba^{2+} (*see also* Table 4).

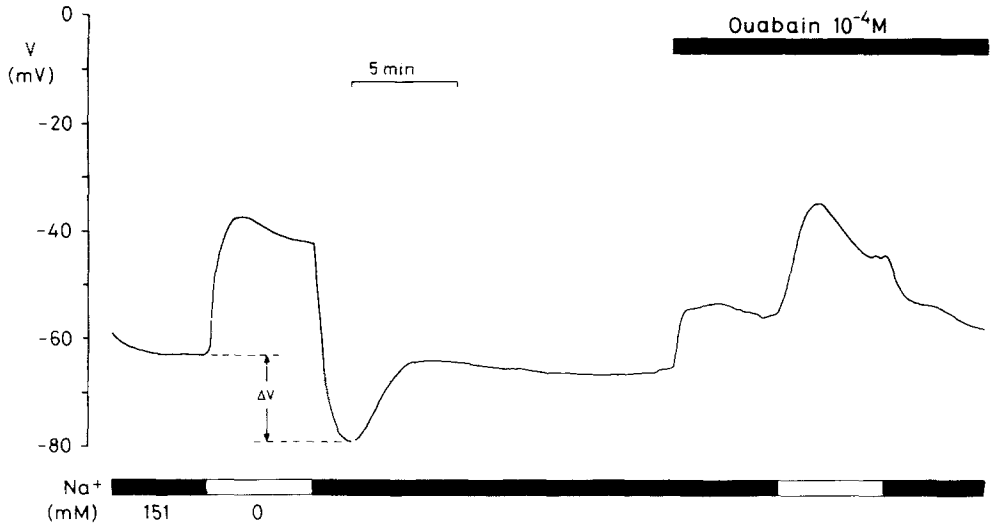

Fig. 12. Response of membrane voltage (V) on removing extracellular Na^+ from the superfusing solution and readdition of Na^+ after 5 min of Na^+ depletion before and in the presence of ouabain 10^{-4} M. (Solutions: 1, 10)

Table 4. Voltage response (total amplitude) on Na^+ readdition after 5 min Na^+ depletion[a]

	Voltage response mean ± SEM (mV)	n
In control Ringer	−24.3 ± 3.1	6
In Ringer +2 mM Ba^{2+}	−30.8 ± 2.9	5
In Ringer + ouabain 10^{-4} M	−9.0 ± 1.2	3
In K^+-free Ringer	−7.5 ± 2.5	2

[a] Determined 45 to 150 sec after Na^+ readdition.

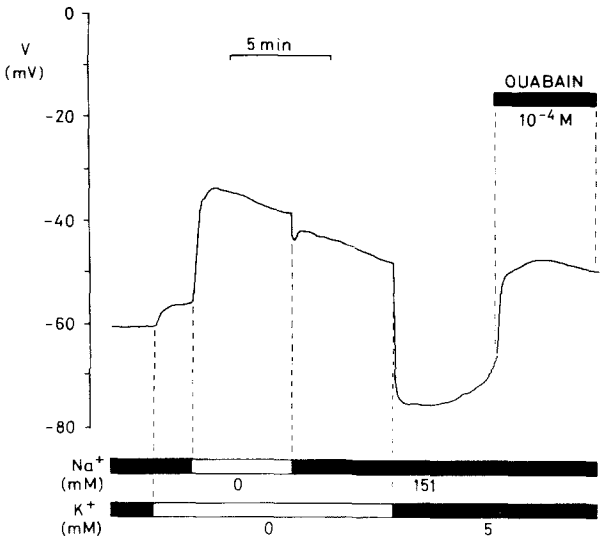

Fig. 13. Effect of removing and readding extracellular Na^+ in K^+-free solution. At the end of the recording ouabain 10^{-4} M is added during the recovery from Na^+ depletion. (Solutions: 1, 11, 12)

Thus, during the recovery after Na^+ depletion the membrane potential hyperpolarized. This reaction could be significantly diminished by ouabain or by omitting K^+ from the test solutions but not by application of Ba^{2+}.

Discussion

Membrane Potentials

This is the first report on electrophysiological properties of cultured ciliary epithelial cells. In the present study membrane potential averaged −46 mV. Previous micropuncture studies of this organ dealt with *in situ* preparations. In earlier studies with the rabbit ciliary body Berggren [1], Miller and Constant [39], and Cole et al. [15] reported membrane potentials of some −30 for NPE and of some −60 mV for PE. Green et al. [21] found both layers electrically coupled with an intracellular V of −65 mV. Candia et al. [8] measured −68 mV in PE and −69 mV in NPE, but in contrast to Green et al. [21] they postulated two compartments isolated electrically from each other. A recent paper on the shark

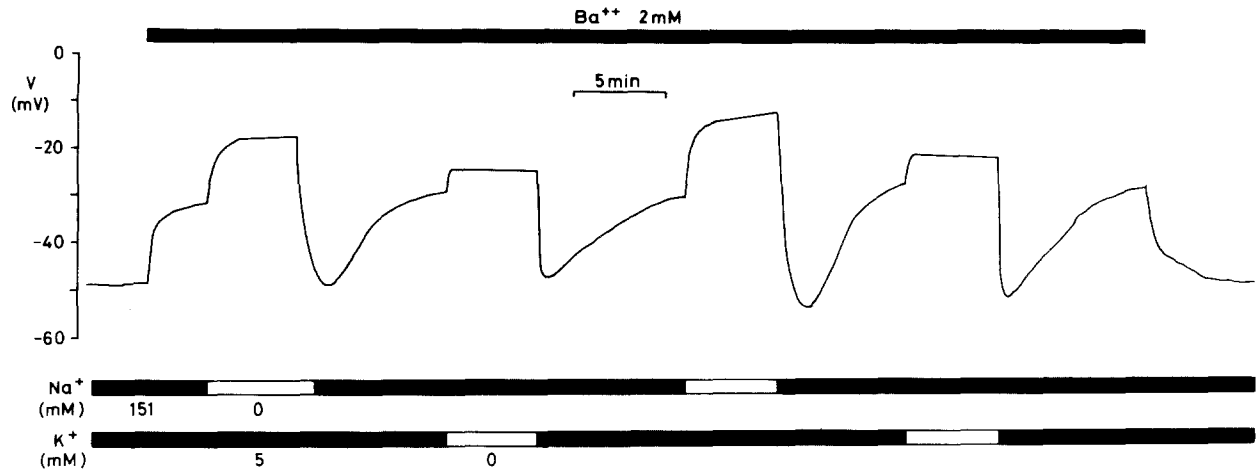

Fig. 14. Original recording of an experiment showing the effect of removing and readding of extracellular Na^+ or K^+, respectively, on membrane voltage (V) in the presence of 2 mM Ba^{2+}. (Solutions: 1, 10, 11 without SO_4^{2-})

ciliary body reports −53 mV for both layers, which are believed to be electrically coupled [51]. These differences reflect technical problems of micropuncture studies with *in situ* preparations. In this paper we demonstrate that cultured pigmented ciliary epithelial cells can be impaled with microelectrodes and that recordings stable for longer than one hour can be obtained. Moreover, we were able to perform fast solution exchanges. This method allows us to investigate electrical membrane properties. As discussed previously in detail [26, 27, 29], it does not allow a localization of the observed phenomena to the apical or basolateral membrane.

K^+ Conductance

Increasing extracellular $[K^+]$ depolarized the cell membrane. This reaction could be partially blocked by Ba^{2+}. These are typical characteristics of a K^+ conductance, which to a considerable extend contributes to the membrane potential in most vertebrate cells. The effect of increasing potassium and Ba^{2+} on V has been described in the isolated ciliary body [8, 21, 51].

The slope of the V versus log $[K^+]$ plot (Fig. 3*B*) gives an estimation for the relative K^+ conductance, often expressed as the potassium transference number (t_K). The relative K^+ conductance depends on the absolute magnitude of the K^+ conductance and additionally on the magnitude of other ionic conductances. Therefore, in the following discussion one should be aware that we determined only the relative K^+ conductance and not its absolute magnitude. We did not equivocally exclude that the observed phenomena were due to changes in the selectivity of the membrane to K^+.

In Fig. 3*B* it can be seen that the slope (and the relative K^+ conductance) is not constant over the investigated range from 5 to 80 mM K^+. The slope flattens at lower $[K^+]$, indicating a decrease of t_K at low $[K^+]$. In experiments with ouabain we could exclude that the deflection of V at lower $[K^+]$ was due to an inhibition of the electrogenic Na^+/K^+-ATPase by lowering its substrate. In some epithelia [37, 48] ouabain is known to markedly reduce the K^+ conductance. In our preparation there is no significant effect of ouabain on the relative K^+ conductance (Fig. 3*C*). In the presented data $[K^+]$ and voltage are closely related. With other methods evidence for a $[K^+]$ dependence [18, 32, 40] as well as for a voltage dependence [17, 36] of the K conductance has been found. With our method we are unable to differentiate between the effect of $[K^+]$ and V on K^+ conductance.

Removing Ca^{2+} from the superfusing solution depolarized the cell membrane. In other epithelia [6, 33, 43] a Ca^{2+}-dependent K^+ conductance has been described. Indeed, t_K in our experiments is reduced in Ca^{2+}-free medium. One could argue that this reduction in the relative K^+ conductance could be caused by a change in V induced by other mechanisms. However, this assumption leads to predictions contrary to those found experimentally, since depolarization has been shown to be associated with an increase in t_K. Thus, it is very likely that pigmented ciliary epithelial cells in culture express a Ca^{2+}-dependent K^+ conductance.

Another finding of this study is the fact that the intracellular potential was pH sensitive. Acidification caused a depolarization, alkalinization caused a hyperpolarization. This could be shown in the pH range between 5.5 to 8.0 (Fib. 5*B*). Similar pH-dependent voltage changes have been found in cul-

tured retinal pigment epithelial cells by Keller et al. [29]. The retinal pigment epithelium is embryologically closely related to the PE. For the discussion of the pH effect, it is obvious that manipulation of the extracellular pH results in a variety of primary and secondary changes (intracellular pH, $[HCO_3^-]$, pCO_2). There are three different mechanisms which could explain this pH effect: (i) Change in the conductance for other ions (K^+, Na^+, Cl^-), (ii) conductance for a pH-related ion (H^+, OH^-, HCO_3^-), (iii) change in the activity of electrogenic transport systems. (i) Indeed, the relative K^+ conductance is diminished at an acidic pH (Figs. 6 and 7). The argument that V could have been changed by another mechanism and depolarization induced the reduction of t_K can be rejected as discussed for the Ca^{2+} effect. A pH-sensitive K^+ conductance has been described for many other epithelia [2, 16, 41], including the retinal pigment epithelium [29]. At higher K^+ concentrations the pH effect is less marked. We assume that high $[K^+]$ (or low voltage) opens K^+ channels, which are less pH sensitive at high $[K^+]$. The possibility of two different K^+ channels could be taken into consideration.

(ii) A conductance for one or more of the ions mentioned is a possible explanation for the observed voltage changes. Since concentrations of H^+ and OH^- are several orders of magnitude below the K^+ concentration, conductance changes of ions such as H^+ and OH^- could only play a minor role in the observed effects. The pH experiments have been performed in nominally HCO_3^--free medium. Nevertheless, there will be a small HCO_3^- concentration due to endogenous CO_2 production. Furthermore, the possible existence of an unstirred layer may provide a considerable extracellular $[HCO_3^-]$ [5]. But a conductance for H^+, OH^- or HCO_3^- predicts a hyperpolarization on intracellular acidification and a depolarization on intercellular alkalinization. These predictions are opposite to the observed data (Fig. 8). Thus, a conductance for a pH-related ion as an explanation for the observed V responses induced by pH_o changes could be ruled out.

(iii) Depolarization due to inhibition of the electrogenic Na^+/K^+-ATPase by acidic pH should have no larger effect on V than the inhibition by ouabain. Ouabain depolarized V by only 8.9 mV. Acidification from pH 7.5 to 5.5 is accompanied by a 20-mV depolarization. This effect is too large to be explained by an inhibition of the electrogenic Na^+/K^+ pump.

Finally, maneuvers known to change intracellular pH also induced voltage responses in parallel to extracellular pH alterations. Although not further investigated, it is likely that these effects of pH_i on V may be due to modifications of K^+ conductance (as shown in [29] for the retinal pigment epithelium).

Na^+/K^+ Transport

The presented results clearly demonstrate a dependence of V on Na^+ and K^+ transport processes. Ouabain is a specific inhibitor of the Na^+/K^+-ATPase (for review [50]). The pump has been shown to extrude sodium ions for potassium ions entering the cell in a stoichiometry of about 3 to 2 [28]. Thus, the pump is electrogenic and contributes to the membrane potential. The immediate depolarization after ouabain application is likely to represent the inhibition of this electrogenic component. A significant accompanying reduction in potassium conductance by ouabain—described in some epithelia [48]—has not been observed in our preparation (Fig. 3C). The further slow depolarization probably represents the dissipation of the potassium (and sodium) gradient and the decrease of the equilibrium potential for K^+ (E_k). A similar biphasic response to ouabain has been reported [24, 27].

When extracellular $[K^+]$ is lowered, E_K becomes more negative. Assuming a Nernstian behavior, we should expect a hyperpolarization upon reduction of $[K^+]$. However, we observed a depolarization on K^+ removal from the bath solution and a hyperpolarization on K^+ readdition. Similar hyperpolarizing responses during recovery from K^+ depletion have been reported [3, 9, 21, 49]. There are two possible explanations for this phenomenon. First, a change of ion conductances. We could show that t_K depends on K^+ concentration. A decrease of $[K^+]$ to 0 should further diminish K^+ conductance [38]. It is possible that this effect is more important than the effect of E_k. The transient hyperpolarization on K^+ readdition could be explained by an overshoot activation of K^+ channels. But this assumption leads to predictions not in agreement with our results. If changes of V on K^+ removal and readdition were due to a change in K^+ conductance, they should be blockable by Ba^{2+}. This could not be found in our experiments[2]. Moreover, in potassium-

[2] Our data show a considerable increase of the hyperpolarization induced by K^+ readdition in the presence of Ba^{2+} compared with the amplitude in control conditions. This enlarged amplitude could be due to an experimental error. However, there is a reasonable explanation. Increasing extracellular K^+ depolarizes E_K. But this effect is less important than the hyperpolarization due to the activation of electrogenic Na^+ extrusion (as discussed below). Ba^{2+} is known to block the K^+ conductance and should reduce a depolarizing component due to E_K. Moreover, Ba^{2+} is expected to increase the cell membrane resistance (R). With a constant pump current (I) of the Na^+/K^+-ATPase, V (given by $V = I \cdot R$) should increase with increasing resistance. Thus, both lines of thought lead to the prediction that the hyperpolarization on K^+ readdition should be enlarged in the presence of Ba^{2+}.

free medium intracellular [K^+] is expected to drop [3] and E_k should decrease. This line of consideration argues against a change in K^+ conductance as the main cause for the observed phenomenon. In addition, ouabain could block these responses. (Although ouabain is known to interact indirectly with the K^+ conductance [24, 37, 48], it could be shown for our preparation that the reduction of the K^+ conductance plays no significant role. Therefore, the action of ouabain can be related to an inhibition of the Na^+/K^+-ATPase.) In the presence of ouabain the direction of the observed V responses is in accordance with a Nernstian behavior. This observation favors the second hypothesis, which will be discussed in the following section.

Extracellular K^+ is a substrate for the Na^+/K^+-ATPase [44]. Removing the substrate (extracellular K^+) will inhibit the Na^+/K^+ pump [46, 50] and thus abolish its hyperpolarizing effect. The longer the Na^+/K^+-ATPase is inactivated by potassium depletion, the higher intracellular Na^+ ($[Na^+]_i$) will rise [10, 30]. Addition of extracellular K^+ will immediately reactivate the pump. Intracellular Na^+ is known to regulate the activity of the Na^+/K^+-ATPase [44]. Because Na_i^+ concentration has been rising, the Na^+/K^+-ATPase will be more stimulated than under control conditions, until $[Na^+]_i$ has regained its normal low value. Therefore, the transient hyperpolarization on K^+ readdition very likely represents electrogenic Na^+ extrusion by the Na^+/K^+-ATPase. Further evidence could be presented by adding ouabain during hyperpolarization after K^+ readdition (Fig. 13), which resulted in a depolarization twice the amplitude than the depolarization induced by ouabain under control conditions.

This hypothesis is based on the assumption that Na_i^+ plays a major role in the observed V changes on K^+ readdition. To further confirm our theory, we tested the Na^+ dependence of the hyperpolarization on K^+ readdition by performing this maneuver in Na^+-free medium. These experimental conditions should prevent the rise in intracellular Na^+. Indeed, the hyperpolarization was abolished, confirming our assumptions. However, in Na^+-free medium other responses have also changed. As in the presence of ouabain, K^+ removal hyperpolarized V in Na^+-free Ringer. If our assumptions are correct, this effect should be due to an inhibition of the Na^+/K^+-ATPase by removing extracellular Na^+. Extracellular Na^+ *per se* is not necessary for the function of the Na^+/K^+-ATPase [50]. But it is very likely that removing extracellular Na^+ will reduce intracellular [Na^+] [30] (which is a substrate for the pump) and thus will reduce the pump rate of the Na^+/K^+-ATPase [37, 46].

These arguments led us to further investigate the effect of Na^+ on the membrane voltage. Na^+ removal markedly reduced V. This reaction is only slightly influenced by ouabain or K^+-free medium and can therefore not fully be explained by an inhibition of the Na^+/K^+-ATPase. Other underlying mechanisms will be investigated in further experiments; in this paper the emphasis will be laid on the ouabain-sensitive phenomena. After some minutes in Na^+-free medium addition of ouabain did not result in a significant depolarization (*data not shown*). This observation confirms the assumption that the Na^+/K^+-ATPase is inhibited in Na^+-free solution. Na^+ readdition after some minutes of Na^+ depletion induced a transient hyperpolarization, which reached a maximum about 2 min after Na^+ readdition. This reaction was much slower than the hyperpolarization on K^+ readdition, which was complete in about 30 sec. The reaction on Na^+ readdition could be in part inhibited by ouabain and was markedly reduced in K^+-free medium. Both procedures are believed to inhibit the Na^+/K^+-ATPase. Therefore, we are tempted to speculate that the hyperpolarization on Na^+ readdition is due to an activation of the Na^+/K^+-ATPase. (A change in K^+ conductance as the main cause for the observed V responses could be excluded by the experiments in the presence of Ba^{2+}.) A direct effect of extracellular Na^+ is unlikely because the pump has been shown not to be dependent on extracellular Na^+ [50]. The slower time course of the hyperpolarization also favors an indirect effect of Na^+ readdition. As discussed above, an inhibition of the Na^+/K^+-ATPase in Na^+-free medium may be due to a decreased intracellular [Na^+]. Na^+ readdition should increase intracellular [Na^+] towards the concentration in steady-state conditions. This should lead to a parallel increase in Na^+/K^+-ATPase activity and should hyperpolarize V to its initial value. For explaining the transient hyperpolarization of V above control, we have to postulate either a transient increase of intracellular [Na^+] above steady-state values or an increasing sensitivity of the Na^+/K^+-ATPase to intracellular Na^+ during Na^+ depletion, perhaps due to ATP accumulation. During the hyperpolarization accompanying the recovery after Na^+ depletion (believed to represent enhanced Na^+/K^+-ATPase activity), potassium removal (which should block Na^+/K^+-ATPase) resulted in an enhanced depolarization (Fig. 11*A*). This observation further supports the hypothesis of an enhanced Na^+/K^+-ATPase activity during recovery from Na^+-depletion.

The obtained data can only be a first step in interpreting the transport mechanisms responsible

for aqueous humor formation. For example, the effect of pH and Ca^{2+} on K^+ conductance and membrane potential could play a role in the regulation of intraocular pressure. However, at present there are no conclusive data concerning the effect of pH and Ca^{2+} on aqueous humor dynamics. The importance of the Na^+/K^+-ATPase for aqueous humor formation could be demonstrated in vivo. Ouabain induced a reduction of aqueous humor formation [20] and abolished the transepithelial current [7, 31, 35, 42, 52] in vitro when applied to the stromal side, suggesting an effect on the Na^+/K^+-ATPase of PE.

In summary, our results led us to suggest that K^+ conductance in cultured bovine pigmented ciliary epithelial cells depends on Ca^{2+}, pH and $[K^+]$ (or voltage). An electrogenic Na^+/K^+-ATPase is present, being inhibited by ouabain, Na^+ removal or K^+ removal, and stimulated during recovery from Na^+ or K^+ depletion.

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