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Effect of Bicarbonate, pH, Methazolamide and Stilbenes on the Intracellular Potentials of Cultured Bovine Corneal Endothelial Cells

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Summary. Micropuncture of cultured bovine corneal endothelial cells led to registrations stable for hours. Intracellular potentials were mainly in the range of -40 to -55 mV, average 46.3 ± 0.6 mV (SEM). Changes of extracellular $[HCO_3^-]$ led to voltage transients, their amplitude depending logarithmically on $[HCO_3^-]$ with a mean slope of 37.3 ± 8.8 (SD) mV. After removal of bicarbonate/CO_2, a steady-state depolarization was seen. This steady-state depolarization, but not the voltage transients, could be reduced by 1 mM Ba^{++}. After removal of bicarbonate, the voltage response to changes of extracellular potassium was reduced. Alteration of pH_i induced by permeable buffers (butyrate, glycodiazine and ammonium) also resulted in voltage transients, internal acidification being correlated with a hyperpolarization, and internal alkalinization with a depolarization. Also changes of external pH caused voltage responses, alkalinization causing a hyperpolarization, acidification a depolarization. Methazolamide, an inhibitor of carbonic anhydrase, as well as stilbenes (SITS or DIDS) caused a reduction of the voltage response to HCO_3^- and pH. Their effects were additive. It is suggested that corneal endothelial cells possess one or two electrogenic transporters for HCO_3^- or related species, one of which is inhibitable by stilbenes.

Key Words corneal endothelium · cell culture · intracellular potential · bicarbonate · pH · stilbenes

Introduction

The corneal endothelium plays an important role in the maintainance of corneal hydration and transparency. It is a leaky epithelium which transports water, bicarbonate and sodium out of the corneal stroma into the aqueous humour of the anterior eye chamber.

In electrophysiological Ussing chamber-type experiments using (mainly rabbit) corneas denuded of their epithelium, a transepithelial voltage V_{te} of 0.5 to 1 mV, aqueous side negative, could be detected [6, 14, 16, 29, 41, 42]. Both V_{te}, the short-circuit current I_{sc} and water flow were dependent on the extracellular bicarbonate and sodium concentration and could be reduced by inhibitors of carbonic anhydrase [6, 14–16, 41, 42]. Carbonic anhydrase activity has been histochemically localized at the apical membrane [15]. Flux measurements with radioisotopes revealed a transepithelial transport of HCO_3^- [12, 15, 19, 23], which was dependent on sodium concentration and could be partly inhibited by inhibitors of carboanhydrase. Early work could not detect a transepithelial transport of sodium [15], but several recent papers [17, 18, 26, 27, 30] report a sodium transport similar in magnitude to the one of bicarbonate. Recent work also reports reduction of V_{te} and water transport by 1 mM amiloride and 1 mM SITS [25, 27].

The mentioned studies have been predominantly performed with rabbit corneas. Only three recent papers describe transepithelial electrophysiological measurements with human [16, 42] and bovine [41] corneal endothelium. In both cases a dependence of V_{te} and I_{sc} on the bicarbonate concentration similar to that of the rabbit could be shown. This indicates that the transport processes of these three species and probably other mammals are very similar.

The basic mechanisms involved in the transport of HCO_3^- are still obscure. The involvement of a Na^+/H^+-antiport [6, 25] as well as a HCO_3^--ATPase [32] were discussed. However, it seems that the latter enzyme is located only in the mitochondria [33, 39].

In order to clarify the underlying mechanisms, intracellular studies promised to be very fruitful. Until now, there are only a few published reports dealing with intracellular punctures of corneal endothelial cells. This is obviously due to the difficulties in obtaining stable impalements, since the cells are small and fragile. The study of Wiederholt and Koch [40] on corneal endothelium of rabbit and man reports potentials that were 'stable' for only a few seconds. Only very few recordings could be maintained stable for some minutes, thus making

a change of the external bathing solution during this period virtually impossible. With an improved mechanical support, Lim and Fischbarg [28] working with rabbit cornea could extend the period of stable puncture to several minutes with a maximum of 40 min.

Only one paper deals with the puncture of cultured corneal endothelial cells. Jumblatt [22] reported intracellular potentials of cultured rabbit corneal endothelial cells, but he could not extend the measuring period beyond some 10 sec.

In the aforementioned studies, the effects of high extracellular potassium, ouabain, metabolic inhibitors and temperature were investigated. In none of these studies, however, was a change of extracellular medium performed while a cell potential was recorded.

In the present study, we show that with cultured bovine corneal endothelial cells, which form polarized monolayers *in vitro,* it is possible to obtain intracellular recordings which are stable for hours. This has enabled us to perform many repetitive changes in extracellular medium during the puncture of a single cell for the first time. In this paper, we focus on the influence of bicarbonate and pH on the membrane potential difference.

Materials and Methods

Cell Cultures

Primary cultures of bovine corneal endothelial cells were established by a method similar to the one of MacCallum et al. [31]. Eyes from 3- to 4-years-old steers were obtained from a local abbattoir. They were removed immediately after death and transported to the laboratory in ice-cold Ringer's solution. Under clean, but unsterile conditions the corneas were incised with a razor blade at a distance of some millimeters from the limbus and removed with scissors. They were placed in a support, the endothelium facing upwards. A few drops of trypsin/EDTA-solution (0.05/0.02%) (Seromed, Munich, FRG) were given into the resulting cup. In order to prevent contamination with keratocytes of the corneal stroma, contact of the solution with the edge of the preparation was avoided. After approximately 2 hr of incubation at room temperature, the solution containing detached endothelial cells was aspirated with a sterile pipette after gently rubbing the surface of the endothelium. This suspension was given into a plastic culture flask (Falcon Plastics) and the culture medium [DMEM (Dulbecco's modification of minimal essential medium) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B; source of all components: Seromed, Munich, FRG] was added. The cultures were gassed with a 5% CO_2/air mixture and incubated at 37 °C. The medium was changed twice a week. When a sufficient number of colonies had grown, the cells were trypsinized (0.05/0.02% trypsin/EDTA solution) and allowed to grow in the same flask at an increased rate due to the removal of contact inhibition. After two to three weeks the cells formed a confluent monolayer covering totally the bottom of the culture flask. Then they were again trypsinized and passaged at a split ratio of 1:2 to 1:4. At this split ratio the cultures became confluent after a period of 3 to 5 days. For the electrophysiological experiments plastic tissue culture dishes (diameter 60 mm, Falcon Plastics) were used. The cell strains were propagated in tissue culture flasks (Falcon Plastics, 25 or 75 cm^2 growth area). Amphotericin B was no longer included in the culture medium for the subcultures.

While the group of Gospodarowicz stresses the importance of Fibroblast Growth Factor (FGF) for the maintenance of these cultures [10, 11], our experience is in line with MacCallum et al. [31], who avoid the additional expense of adding FGF while seeding the cells at a low split ratio without a negative effect on the cells.

For our investigations, only early subcultures (2nd to 5th passage) were used. Experiments were performed at least 4 days after the cell layer had reached confluency.

Solutions

The composition of the solutions is given in the Table. Solution 1, which will be named 'bicarbonate-rich solution,' is the control Ringer's. In the 'nominally bicarbonate-free' solution (solution 2) HCO_3^- was replaced by Cl^-. When solutions with different concentrations of bicarbonate were used (sol. 3 and 4), bicarbonate was always replaced by Cl^-. In the experiments with glycodiazine and butyrate (sol. 7 and 8), nominally bicarbonate-free solutions were used and 25 or 40 mM NaCl replaced by the sodium salts of glycodiazine or butyrate, respectively. In the experiments with NH_4Cl, 10 mM NH_4^+ replaced Na^+ (sol. 5 and 6). Solutions to which barium was added (sol. 11 and 12) were free of sulfate. The corresponding control solutions also contained no SO_4^{2-}.

The solutions containing 46 mM HCO_3^- were gassed with a 5% CO_2/air mixture and had a pH of 7.66. Sol. 3 and 4 were gassed both with air and 5% CO_2/air mixture. Sol. 3 (10 mM HCO_3^-) had a pH of 7.5 and 7.0, and sol. 4 (23 mM HCO_3^-) pH 7.9 and 7.4 when gassed with air or CO_2, respectively. The bicarbonate-free solutions (sol. 2, 6, 7, 8, 10, 12, 13) were gassed with air and were titrated with NaOH to pH 7.5.

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, St. Louis, Mo. Glycodiazine (Redul®) was a gift of Schering AG, Berlin, FRG, and methazolamide (Neptazane®) was generously provided by Dr. Stauder from Cyanamid/Lederle GmbH, Munich, FRG.

Experimental Setup

The experimental setup is shown in Fig. 1. For the experiments the tissue culture dishes (*a*) were placed into a plastic chamber (*b*) so that their bottoms were in contact with temperature-controlled water (*c*) which was heated by water flowing through the lower compartment (*d*) of the chamber. A Lucite flow chamber (*e*) was tightly pressed by springs (*not shown*) onto the bottom of the dish. It isolated a small channel (width: 1.5 mm, length: 30 mm) from the rest of the dish so that the cells growing in this area could be rapidly superfused with different solutions without appreciable leakage of fluid to the rest of the dish. The inlets (*f*) for six different test solutions were localized at one end of the channel. At the other end of the

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

Sol	Na^+	K^+	Mg^{++}	Ca^{++}	NH_4^+	Ba^{++}	Cl^-	$H_2PO_4^-$	SO_4^{--}	HCO_3^-	But^-	Gly^-	HEPES	Gluc[a]
1	151	5	0.9	1.7	–	–	112	1	0.9	46[b]	–	–	10	5
2	151	5	0.9	1.7	–	–	158	1	0.9	–	–	–	10	5
3	151	5	0.9	1.7	–	–	148	1	0.9	10	–	–	10	5
4	151	5	0.9	1.7	–	–	135	1	0.9	23	–	–	10	5
5	141	5	0.9	1.7	10	–	112	1	0.9	46	–	–	10	5
6	141	5	0.9	1.7	10	–	158	1	0.9	–	–	–	10	5
7	151	5	0.9	1.7	–	–	133	1	0.9	–	–	25	10	5
8	151	5	0.9	1.7	–	–	118	1	0.9	–	40	–	10	5
9	116	40	0.9	1.7	–	–	112	1	0.9	46	–	–	10	5
10	116	40	0.9	1.7	–	–	158	1	0.9	–	–	–	10	5
11	151	5	0.9	1.7	–	1	115	1	–	46	–	–	10	5
12	151	5	0.9	1.7	–	1	161	1	–	–	–	–	10	5
13	136	5	0.9	1.7	–	–	143	1	0.9	–	–	–	40	5

[a] But^- means butyrate, Gly^- means glycodiazine$^-$ and Gluc, glucose.
[b] This value has been chosen since the aqueous humour of various mammals contains a comparable concentration of HCO_3^- [42].

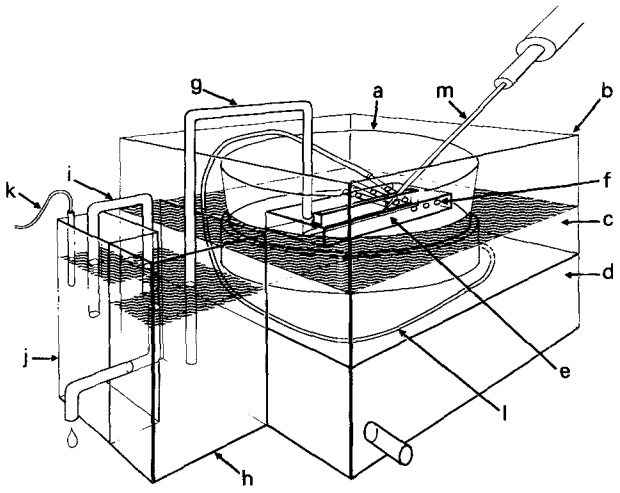

Fig. 1. Schematic diagram of the experimental setup for superfusion and recording of intracellular potentials of cultured cells. (*a*) tissue culture dish, (*b*) plastic chamber, (*c*) temperature-controlled water, (*d*) compartment for heated water, (*e*) flow chamber, (*f*) inlets for test solutions, (*g*) tubing for removal of test solution, (*h*) container with constant fluid level, (*i*) electrolyte-filled agar bridge, (*j*) compartment filled with 0.5 M KCl, (*k*) Ag/AgCl electrode, (*l*) tubing for the inlet of test solution (only one shown), (*m*) microelectrode connected to the probe of an amplifier

channel the solution was drained by a polyethylene tubing (*g*) which was immersed into a container (*h*) with a constant fluid level. By adjusting the height of this container, the level of fluid inside the channel of the perfusion chamber could be controlled. Generally the fluid level inside the container was located some 2 mm beneath the level of the cells. This resulted in a fluid level of approximately 1 mm inside the channel when it was perfused with the usual speed (22.5 ml/hr). In the latter container an agar bridge (*i*) provided electrical connection to a compartment (*j*) filled with 0.5 M KCl which was grounded with an Ag/AgCl electrode (*k*).

After gassing, the perfusion solutions were filled into glass syringes which were propulsed by a syringe infusor apparatus (Braun Melsungen, FRG). The syringes were connected to the flow chamber with gas-tight tubings (Tygon®, type R-3603, Norton Plastics, Ohio), which enclosed additional tubings leaky to gas (Silastic®, type 602–135, Dow Corning). The latter tubings were perfused with gas (air or CO_2/air mixture) to allow precise control of gas pressure in the Ringer's solution within a distance of 3 cm to the inlet into the flow chamber. Valves allowed each of the six solutions either to superfuse the cells or to flow into a bottle for waste fluid. Before reaching the flow chamber, the tubings (*l*) were led in three turns in the heated water of the chamber to allow the temperature to equilibrate. The temperature in the channel was adjusted to 32 ± 1 °C, since this is the temperature of mammalian corneal endothelium *in vivo* [7]. The flow of fluid was generally chosen to be 22.5 ml/hr. With this speed, the solution exchange next to the punctured cell as judged by its response to changes of K^+, was complete to 90% within 4 sec.

Measurement of Membrane Potential

Conventional Ling-Gerard microelectrodes were drawn by a horizontal puller (Narishige model PD-5) from filament borosilicate glass (OD 1.0, ID 0.58 mm, Hilgenberg, Malsfeld, FRG). When filled with 0.5 M KCl and immersed into control Ringer's solution, their resistance ranged from 70 to 140 MΩ. Microelectrodes with a tip potential of more than 5 mV were rejected. The electrodes were connected to the probe of an electrometer amplifier (WPI model M4-A, Hamden, CT) and fixed to a mechanical stepping device (David Kopf Instruments 607 W, Tujunga, Calif., or 'Heidelberg' Nanostepper, FRG) mounted on a micromanipulator (Leitz, FRG). The microelectrode (*m*, Fig. 1) was carefully lowered down in a flat angle to the plane of the culture dish inside the channel of the flow chamber until it reached its bottom. The electrode could then be advanced automatically by a purpose-built stepper control device ('cell finder'), until a cell as recognized by a negative potential was punctured. When the voltage broke down, the search for a new cell could be continued.

The voltage was read out on a digital voltmeter and its time course registered on a paper chart recorder (Rikadenki) together with a signal proportional to the number of steps the electrode was advanced. The punctures were performed on a vibration-damped table inside a Faraday cage for electrical shielding.

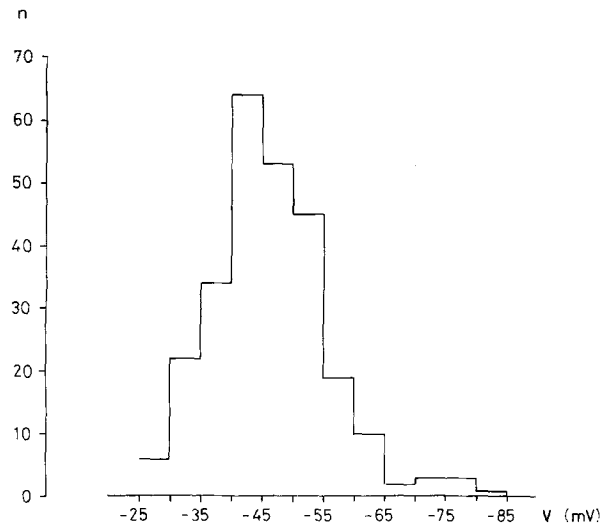

Fig. 2. Frequency distribution of the measured resting potentials V of 262 cells punctured in control Ringer's (sol. 1). Only potentials stable for at least 3 min were taken into account. The arithmetic mean amounts to -46.3 ± 9 mV (SD)

Results

I. Intracellular Potentials

Lowering of the electrode to the cell layer often led to a puncture of a cell. In most cases, however, the electrode had to be advanced further by the stepper. The results obtained by both types of impalement were indistinguishable. Puncture of a cell led to a rapid negative movement in the registered voltage, often followed by a further increase over several minutes until a stable value was reached. This was attributed to an improved sealing of the electrode in the membrane. Sometimes the potential reached a stable value immediately after puncture. The potentials obtained by these two patterns of puncture were equal within a few mV. Measurements with electrodes filled with 0.5 M NaCl instead of KCl gave similar results, indicating a negligible effect of potassium leakage out of the electrode on the membrane potential.

When the potential of a cell broke down, the electrode had to be advanced by about 20 μm to puncture a new cell. In most cases, the electrode could be advanced further without an appreciable change in resistance. About 5% of the attempts to puncture a cell led to potentials stable for more than one hour.

The frequency distribution of the measured intracellular potentials stable for more than 3 min is shown in Fig. 2. These punctures were performed in control Ringer's solution (sol. 1). The histogram displays a maximum in the range of -40 to -55 mV. A few cells had potentials of up to -80 mV. The arithmetic mean of the potentials of 262 punctured cells was -46.3 ± 9 mV ($\pm$ SD).

II. Effect of Bicarbonate and CO_2 on Intracellular Potential

(A) Removal of Bicarbonate/CO_2

The effect of bicarbonate on the membrane potential was explored by replacing the control Ringer's solution, which contained 46 mM HCO_3^- and was gassed with 5% CO_2, with a nominally bicarbonate-free solution, equilibrated with air. A representative experiment of this type is shown in Fig. 3. Removal of HCO_3^- led to a rapid fall in membrane potential of 20 to 30 mV. After reaching a minimum the potential recovered in a period of several minutes but did not regain its initial value. Instead, it remained lower by about 5 to 20 mV, as indicated by the arrow in Fig. 3. Often, as exemplified by the experiment of Fig. 7, it reached the steady state after passing through a maximum. Readdition of bicarbonate to the extracellular medium led to a rapid hyperpolarization which in some cases reached values of more than -90 mV. As shown in Fig. 3, the magnitude of this hyperpolarization increased with the time of superfusion with nominally bicarbonate-free solution. After this transient, the potential recovered to its original value, sometimes slowly oscillating over a period of about 10 min before reaching the steady state. With a slow rate of superfusion (about 1/4 of our usual rate), the amplitude and the slope of the voltage transients increased with the speed of solution exchange. This indicates that a fast build-up of HCO_3^- gradients is essential in the generation of these transients. The speed of superfusion generally employed in our experiments (90% exchange within 4 sec) was sufficient for the voltage transients to reach saturation of their amplitudes. Increasing the speed of superfusion further even by a factor of 4 did not result in a significant enhancement of the response.

To exclude the possibility that the observed effects were due to the increased concentration of Cl^- which had replaced HCO_3^-, rather than to the removal of HCO_3^-, in a few experiments SO_4^{2-} was used as a substitute for bicarbonate (the osmolarity being adjusted with mannitol). No difference in the reaction upon removal of HCO_3^- could be detected.

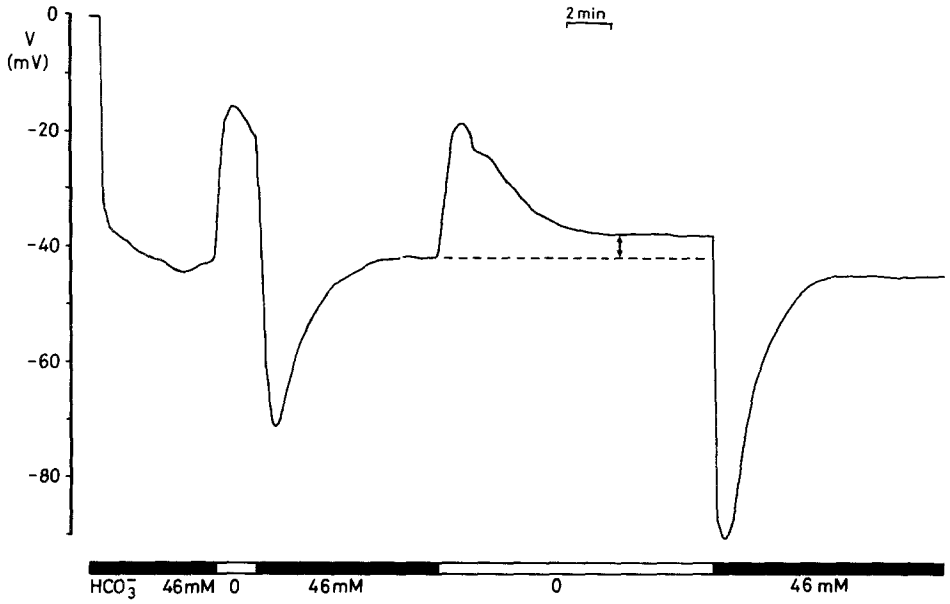

Fig. 3. Original registration demonstrating the effect of change of $[HCO_3^-]$ on the membrane potential V. The control Ringer's (sol. 1, containing 46 mM HCO_3^-, gassed with 5% CO_2, pH 7.66) was twice replaced by a nominally bicarbonate-free Ringer's (sol. 2, gassed with air, pH 7.5). Note (a) that the hyperpolarization after readdition of bicarbonate is greater after the second replacement, when the time of superfusion with 'bicarbonate-free' solution has been increased from 2 to 12 min, and (b), that after the transient depolarization the potential remains lower in 'bicarbonate-free' than in bicarbonate-rich solution. The difference is indicated by the arrow. A similar response to bicarbonate was seen in more than 30 experiments

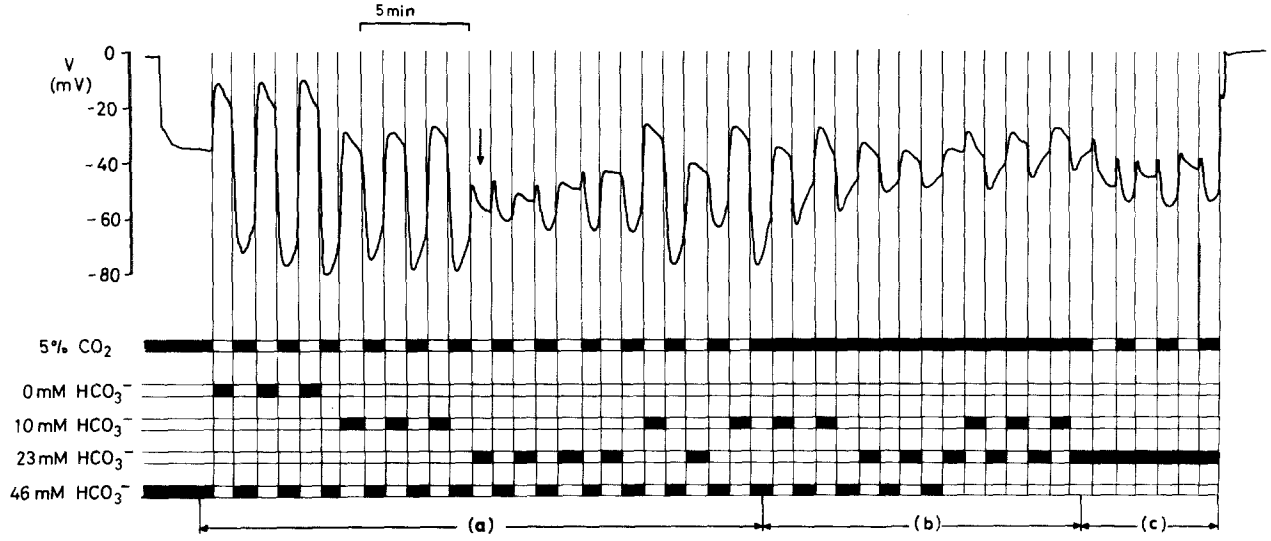

Fig. 4. Influence of varying concentrations of HCO_3^- and pCO_2 on the intracellular potential V. The perfusate was changed every minute (sol. 1, 2, 3 and 4). In part (*a*), $[HCO_3^-]$ is changed together with pCO_2; in part (*b*) this change occurs at constant pCO_2. In part (*c*), pCO_2 is changed at constant $[HCO_3^-]$. Eight such experiments were performed, all yielding similar results

(B) Differentiation of the Effects of HCO_3^- and pCO_2

In the type of experiment described above, the concentration of bicarbonate was changed together with pCO_2 to keep the extracellular pH within a reasonable range. Additional experiments were performed to elucidate the specific effects of the changes of bicarbonate, pCO_2 and pH.

Such an experiment is shown in Fig. 4, where the concentration of HCO_3^- was varied in four steps and pCO_2 in two, the solutions containing 10 and 23 mM HCO_3^- (sol. 3 and 4) being gassed both with 5% CO_2/air or with air.

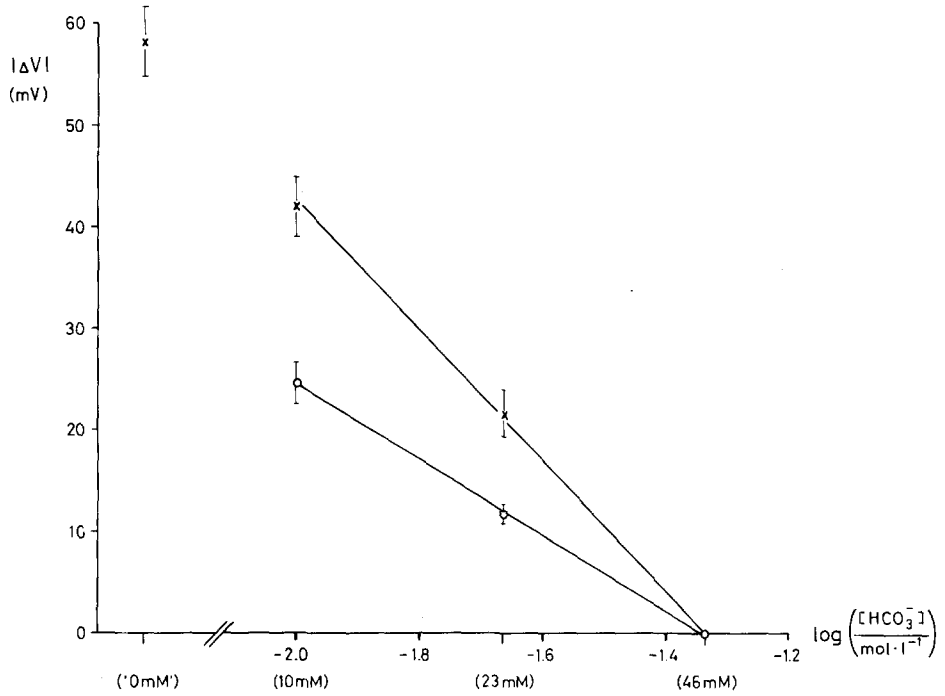

Fig. 5. Dependence of voltage transients on the magnitude of changes in $[HCO_3^-]$. As in the experiment of Fig. 4, the perfusate was changed periodically in intervals of 1 min from sol. 1 (46 mM HCO_3^-, 5% CO_2) to solutions containing less bicarbonate. The resulting voltage transients ΔV are shown as a function of log $[HCO_3^-]$ of the latter solutions. The arithmetic mean of the up- or downstroke of V following a change of $[HCO_3^-]_o$ has been evaluated. Changes of $[HCO_3^-]$ at constant pCO_2 (5%) are marked with circles (o), those effected to solutions gassed with air with crosses (×). Each point represents the arithmetic mean of 7 to 9 measurements on different cells, the bars indicating the standard error of the mean. The slopes of the dependences are 37.3 ± 3.1 and 63.5 ± 4.5 mV/decade ($\pm$SEM) for the experiments at constant and varying pCO_2, respectively. Change between sol. 1 and sol. 2 ('0 mM' HCO_3^-) resulted in these experiments in voltage transients of 57.9 ± 3.3 mV (SEM, $n=8$)

The experiment of Fig. 4 consists of three parts. At first, (*a*), $[HCO_3^-]$ was changed together with pCO_2. Then, (*b*), $[HCO_3^-]$ was changed at constant pCO_2, and in the last step, (*c*), pCO_2 was changed at constant $[HCO_3^-]$ (23 mM).

Both in part (*a*), where the solutions containing less than 46 mM HCO_3^- were equilibrated with air, and in part (*b*), where bicarbonate was changed at constant pCO_2, the amplitude of the voltage transients depended logarithmically on $[HCO_3^-]$. This is shown in Fig. 5. In part (*a*) the slope of the $V/\log[HCO_3^-]$ relationship was 63.5 ± 4.5 (SEM, $n=9$) mV/decade, whereas at constant pCO_2 (part (*b*)) it was only 37.3 ± 3.1 (SEM, $n=8$) mV/decade.

This difference in slopes indicates that in addition to bicarbonate, also changes in pCO_2 have an influence on the membrane potential. This was more explicitly investigated in part (*c*) of the experiment of Fig. 4, where pCO_2 was changed at nominally constant $[HCO_3^-]$ (23 mM). Also this maneuver induced transient changes of the membrane potential, which in this case had two phases. Increase of pCO_2 at first depolarized the cell and then led to a more prolonged hyperpolarization. A similar biphasic response was observed in part (*a*), when the medium was changed from 46 mM HCO_3^-/5% CO_2 to 23 mM HCO_3^-/air, as indicated by the arrow. This suggests that also this biphasic response, which was never seen with changes of $[HCO_3^-]$ at constant pCO_2, was due to the concomitant change in pCO_2. These biphasic responses were consistently observed in 8 experiments. The first phase always gradually disappeared after some minutes of repetitive solution exchange (as shown in Fig. 4, part (*a*), section following arrow). The mean response to a change of air to 5% CO_2 at $[HCO_3^-] =$ 23 mM was 13.3 ± 1.3 mV (SEM, $n=4$). A similar response was seen at 10 mM HCO_3^-.

III. Influence of Bicarbonate on Potassium Conductance

To test the involvement of K^+ conductance in the described voltage responses to bicarbonate, two types of experiments were performed. An example of the first one is shown in Fig. 6. Potassium conductance was checked by increasing extracellular potassium from 5 to 40 mM, which led to a depolar-

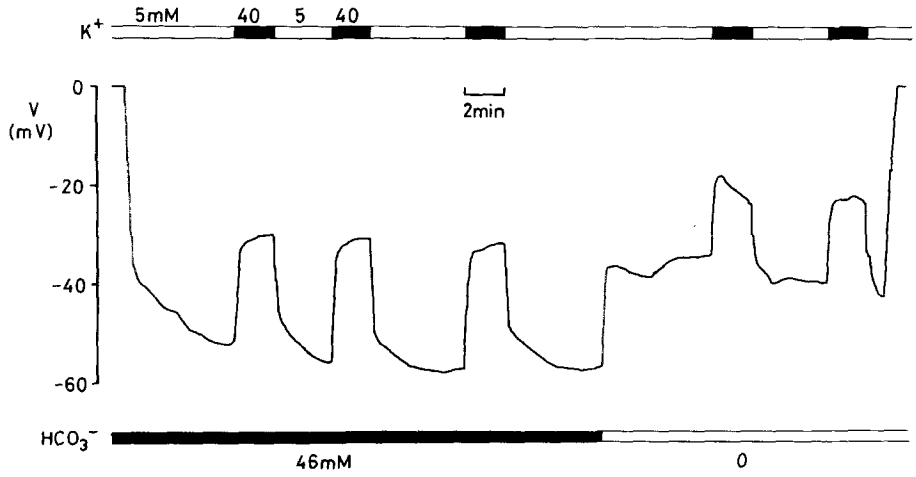

Fig. 6. Change of extracellular potassium concentration in the presence and absence of bicarbonate. Pulses of high potassium concentration (sol. 9 and 10) were applied in control Ringer's (sol. 1) and 'bicarbonate-free' Ringer's (sol. 2). Similar responses were seen in eight experiments

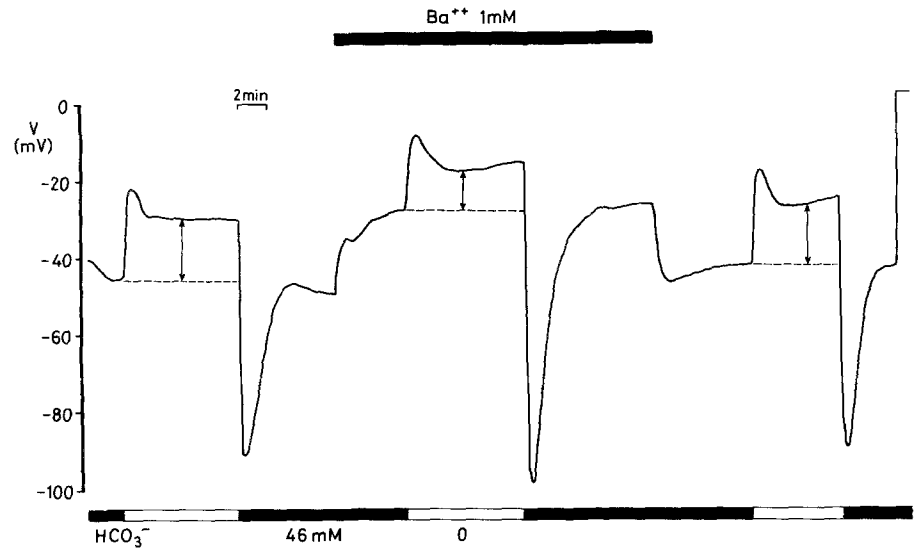

Fig. 7. Response of the membrane potential V to bicarbonate in the presence and absence of the K^+ channel blocker Ba^{++}. Ba^{++}, which depolarized the cell by nearly 20 mV, caused a reduction of the steady-state depolarization caused by the removal of bicarbonate. This is indicated by the arrows. Similar results were obtained in six experiments

ization. After removal of bicarbonate, which again led to a depolarization, this test was repeated. The changes in membrane potential induced by increased extracellular potassium were now about 30% smaller. The mean slope of the $V/\log[K^+]$ relationship decreased from 26.5 ± 4 (SD) mV/decade ($n=12$) in bicarbonate-rich solution to 18.8 ± 4 mV/decade ($n=8$) in nominally bicarbonate-free solution ($P<0.005$, t-test).

In the second type of experiment, potassium conductance was blocked by the application of 1 mM Ba^{++} and the reaction to removal of bicarbonate was compared to that in the absence of Ba^{++}. A typical experiment is shown in Fig. 7. The cell depolarized with the application of Ba^{++}, which is expected with a cell whose potential is largely determined by K^+ permeability. The effect of Ba^{++} was fully reversible. After its removal, there was an overshoot of V, suggesting an increased intracellular accumulation of K^+ during the application of the channel blocker by the Na^+/K^+-ATPase. In the presence of Ba^{++}, the steady-state depolarization after the removal of bicarbonate was reduced by about 40%, as indicated by the arrows. The mean depolarization after 6 min of bicarbonate removal was reduced from 16.4 ± 4 mV (SD) ($n=11$) to 9.2 ± 8 mV ($n=6$) in the presence of Ba^{++} ($P<0.05$, t-test). The voltage transients caused by removal and readdition of bicarbonate, however, were not suppressed. The hyperpolarization upon readdition of bicarbonate was even increased. This excludes changes in K^+ permeability as the sole factor responsible for the voltage changes induced by variation of $[HCO_3^-]$.

IV. Change of Intracellular pH

It may be suspected that the hyperpolarization induced by increasing pCO_2 (as demonstrated in Section II (B)) was due to an internal acidification

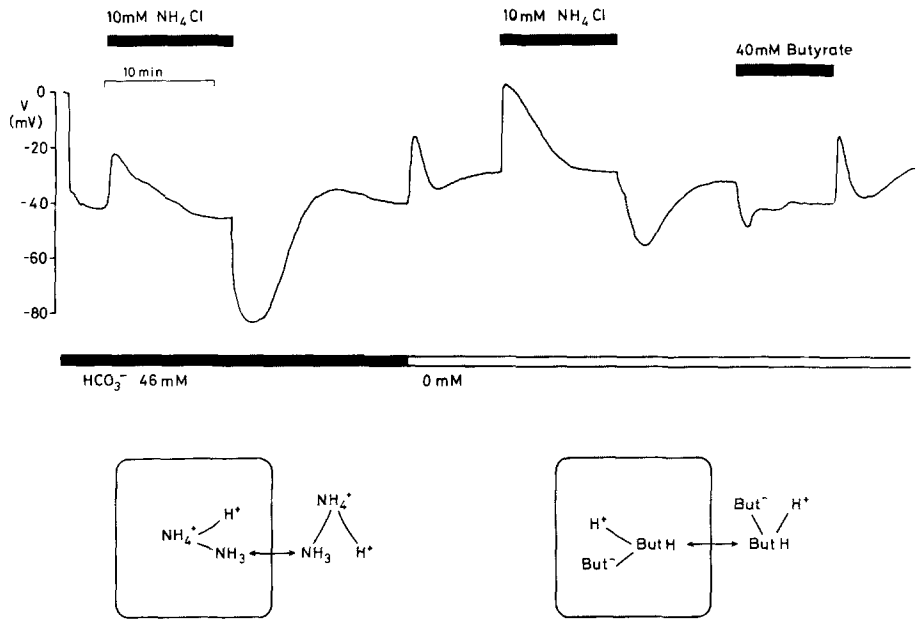

Fig. 8. Voltage response to changes of pH_i induced by external application of ammonium and butyrate (sol. 1, 2, 5, 6, 8). The mechanism of their effect on pH_i is shown in the lower diagrams. Similar effects of NH_4Cl were observed in 10 experiments and of butyrate in two experiments. The effect of NH_4Cl in bicarbonate-rich and bicarbonate-depleted Ringer's was compared in two experiments and results similar to the one shown were obtained

of the cell. To test this hypothesis, we changed intracellular pH (pH_i) by extracellular application of a variety of permeable weak acids and bases. It has been shown by several authors and with different systems that these maneuvers result in a change of pH_i (for a review *see* [34]). As shown in Fig. 8, these buffers can permeate the lipid bilayer of the cell membrane in their neutral form and then dissociate in the cytoplasma to form H^+ and A^- in the case of weak acids, or bind internally H^+ to form NH_4^+ in the case of the weak base ammonium. The former mechanism leads to internal acidification, the latter to internal alkalinization. A change of pH_i in the opposite direction is observed when these buffers are removed from the extracellular medium after they could partially equilibrate over the membrane.

In the experiment of Fig. 8, 10 mM NH_4Cl was at first added in bicarbonate-rich Ringer's. This should lead to an internal alkalinization. A concomitant transient depolarization was seen. After 12 min the extracellular ammonium was removed, which is expected to cause intracellular acidification. In this case, a transient hyperpolarization was observed. In other experiments we could show that the extent of this hyperpolarization was correlated with the period of incubation with NH_4Cl. This again is in line with an effect of pH_i, since it is known from work with pH-sensitive microelectrodes, that the extent of internal acidification depends on the time of preincubation with NH_4 Cl [5].

After removal of bicarbonate, which led to the already known depolarization, this maneuver was repeated. Again addition of ammonium caused a depolarization, its magnitude being even greater than in bicarbonate-rich Ringer's. Removal of NH_3 after 12 min again caused the cell to hyperpolarize, but to a lesser extent than in the presence of bicarbonate.[1]

In the next step, the weak acid butyrate (40 mM) was added extracellularly in nominally bicarbonate-free solution. This, which should lead to a cytoplasmatic acidification as in the case of removal of NH_4Cl, again hyperpolarized the cell. Its removal led to a depolarization very similar to those seen after removal of bicarbonate.

A possible effect of pH_i on V has also been checked by the application of 25 mM glycodiazine, a weak acid which in many bicarbonate-transporting epithelia such as the kidney [38] or the pancreas

[1] These altered amplitudes might be explained by a reduction of the cytoplasmatic buffer capacity due to the removal of HCO_3^-/CO_2. For example, in mammalian muscle the contribution of CO_2/HCO_3^- to the total intracellular buffering capacity has been estimated to be about 40% at $pCO_2 = 37$ mm Hg (5% CO_2) [1, 34, 37]. Thus, addition of ammonium in the absence of bicarbonate should lead to a more pronounced rise of pH_i and an increased voltage response. The reduced response after removal of NH_3 may be due to reduced intracellular accumulation of NH_3 and NH_4^+ in the bicarbonate-free system, since the concentration of the latter species should be reduced at the more alkaline pH_i which is expected in this case. Therefore, less NH_3 could leave the cell, resulting in a lesser degree of acidification and concomitant hyperpolarization.

On the other hand, in the nominal absence of bicarbonate there is still some HCO_3^- inside the cell, but very little outside. Then, in the presence of a HCO_3^- conductance, the depolarization caused by the application of NH_4^+ should be enhanced and the hyperpolarization reduced.

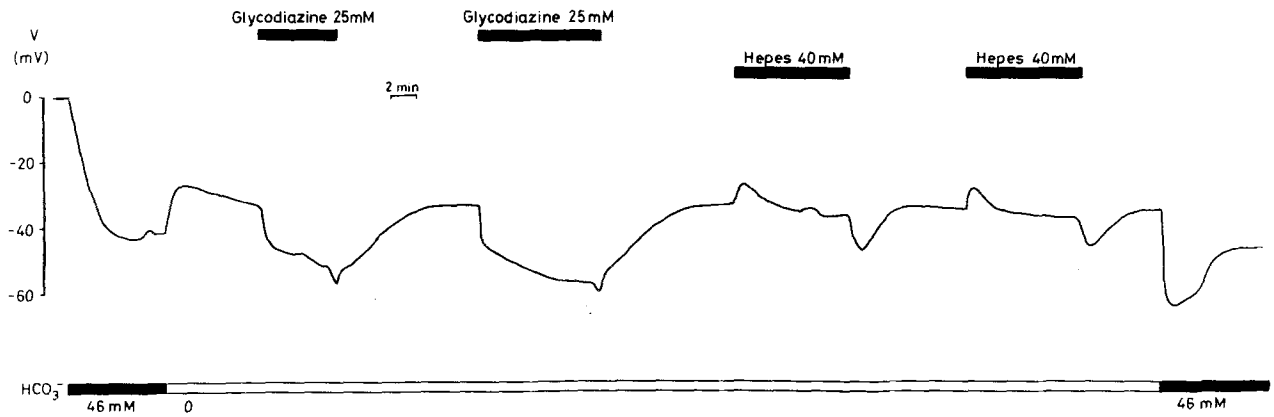

Fig. 9. Effect of 25 mM glycodiazine (sol. 7) and 40 mM HEPES (sol. 13) on membrane potential V in nominally bicarbonate-free solution. A similar effect of glycodiazine was seen in seven experiments. In three experiments, raising [HEPES] from 10 to 40 mM had no effect on V. In two experiments, a biphasic response similar to the one shown was observed

[35] could substitute for bicarbonate with respect to transport rates. Consistent with the extracellular addition of butyrate, it also caused hyperpolarization (Fig. 9). High concentration (40 mM) of the poorly permeable zwitterionic buffer HEPES, however, which does not easily penetrate into the cell, did not have such an effect. In some experiments, as shown in Fig. 9, HEPES produced a transient depolarization upon addition and a hyperpolarization upon removal. These changes were always smaller than those caused by butyrate or glycodiazine. This suggests that even with this buffer some penetration into the cell occurs. Either, (a), the protonated form of the buffer permeates; in this case, a net positive charge is transported with the molecule. Or, (b), it penetrates in the zwitterionic form, leading to a rise of pH_i. Both mechanisms could lead to the observed depolarization.

In summary, in the above described experiments maneuvers which should lead to a fall of pH_i consistently hyperpolarized the cells, whereas those expected to raise pH_i induced a depolarization, irrespective of the applied method. However, in these experiments pH_i is not the only parameter changing. For instance, V might also be directly influenced by the added ions, e.g. via conductive pathways for NH_4^+ or butyrate$^-$. Nevertheless, since we used three different methods to change pH_i, which all resulted in similar effects on V, it is tempting to speculate that there is an influence of pH_i on V. Whether this effect is due to conductive pathways for H^+, OH^- or HCO_3^- (which should accumulate in the cell under alkaline conditions), or to other mechanisms such as pH_i-sensitive conductances, cannot be concluded from these experiments.

V. Change of Extracellular pH

Extracellular pH (pH_o) was changed in nominally bicarbonate-free solutions which were buffered with HEPES. pH_o could be changed in six steps between pH 7.0 and 8.0. In order to explore the dependence of V on pH_o, the latter was altered in steps for periods of 1 min to avoid complications by adaptation of pH_i. An experiment of this type is shown in Fig. 10. Change of external pH led to voltage transients, the response increasing with the magnitude of change of pH_o. In many experiments no exact linear dependence of V on pH_o was obtained. The average voltage response of eight cells to a change of pH 7.0 to pH 8.0 in nominally bicarbonate-free solutions was 11.4 ± 3 mV (SD).

With changes of pH_o, external alkalinization was correlated with a hyperpolarization, and acidification with a depolarization. These voltage responses are thus opposite in sign to those associated with pH_i, probably elicited by the experimental procedures described in the previous section. This might mean — but this is by no means conclusive — that pH gradients across the plasma membrane (or gradients of HCO_3^- secondary to them) are involved in the generation of voltage changes observed with changes of pH.

VI. Effect of Inhibitors of Carboanhydrase and Anion Transport

To gain further insight in the mechanism of the voltage transients caused by changes of HCO_3^- and/or pH, two inhibitors were used.

Methazolamide, an inhibitor of carboanhydrase, led to a reduction of the response to changes

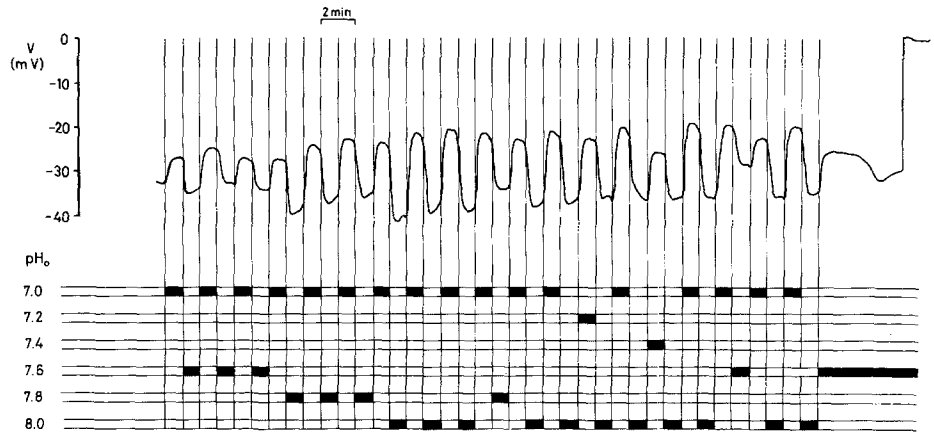

Fig. 10. Response of membrane potential difference to changes in extracellular pH in nominally bicarbonate-free solution (sol. 2). The effect of pH_o was explored in eight cells. The mean response was 11.4±3 mV/decade H^+ (±SD)

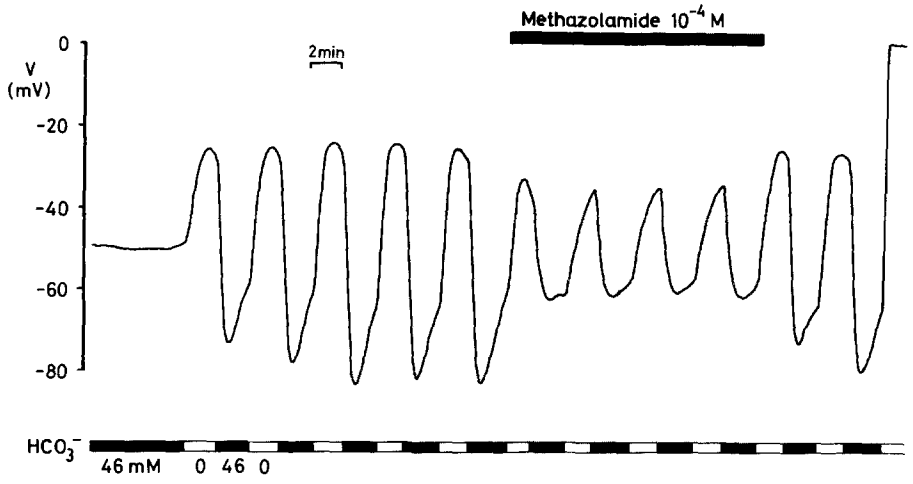

Fig. 11. Effect of methazolamide on the transients of V induced by changes of [HCO_3^-]. The perfusate was changed at intervals of 2 min between sol. 1 and sol. 2. During the time indicated by the bar, 10^{-4} M methazolamide was present in the perfusate. A similar reduction of the transients by methazolamide was observed in eight experiments

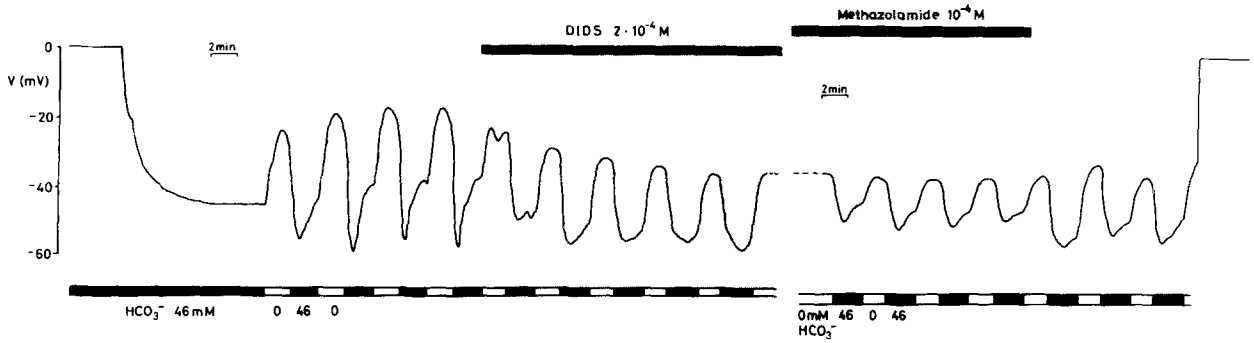

Fig. 12. Effect of DIDS and methazolamide on the voltage transients caused by bicarbonate (periodic change between sol 1 and 2). In the first part, a reduction of the response in the presence of DIDS ($2 \cdot 10^{-4}$ M) is shown. The second part is a continuation of the registration of the same cell. Between these two parts, 58 min had elapsed. The second part shows a further reduction of the response already attenuated by the preincubation with DIDS. Similar effects of DIDS and its congener SITS have been observed in four experiments

in HCO_3^-/pCO_2. This is demonstrated by the experiment of Fig. 11. As expected, the slopes of the transients were also reduced due to the reduced conversion of CO_2 to carbonic acid.

The stilbene derivates DIDS (or SITS) also caused a reduction of the response, but were unable to suppress it totally. This is shown in Fig. 12. The effect of DIDS (and in other experiments of SITS) was irreversible. At the same cell, which was now preincubated with DIDS, methazolamide led to a further reduction of the response. This suggests that stilbenes and inhibitors of carboanhydrase act on different processes.

The stilbenes seem to affect the hyperpolariza-

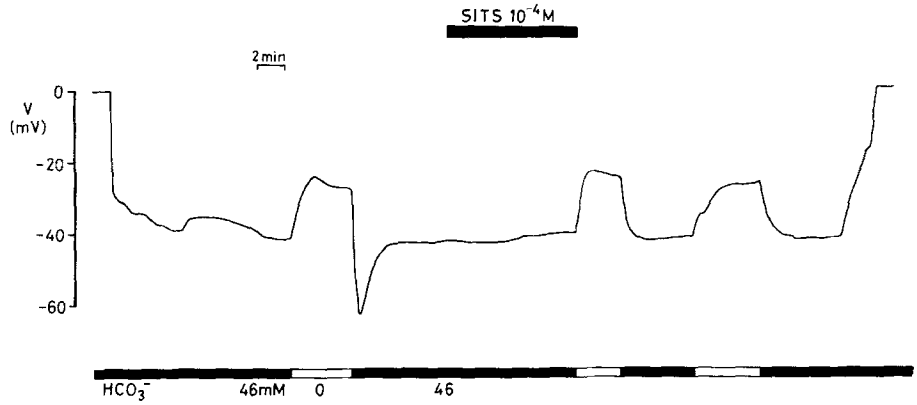

Fig. 13. Effect of SITS on the transient hyperpolarization caused by readdition of bicarbonate. The perfusate was changed between sol. 1 and 2 at longer time intervals than in the experiments of Figs. 11 and 12. Similar reduction of the hyperpolarization were seen in six experiments (in addition to the experiments stated in Fig. 11)

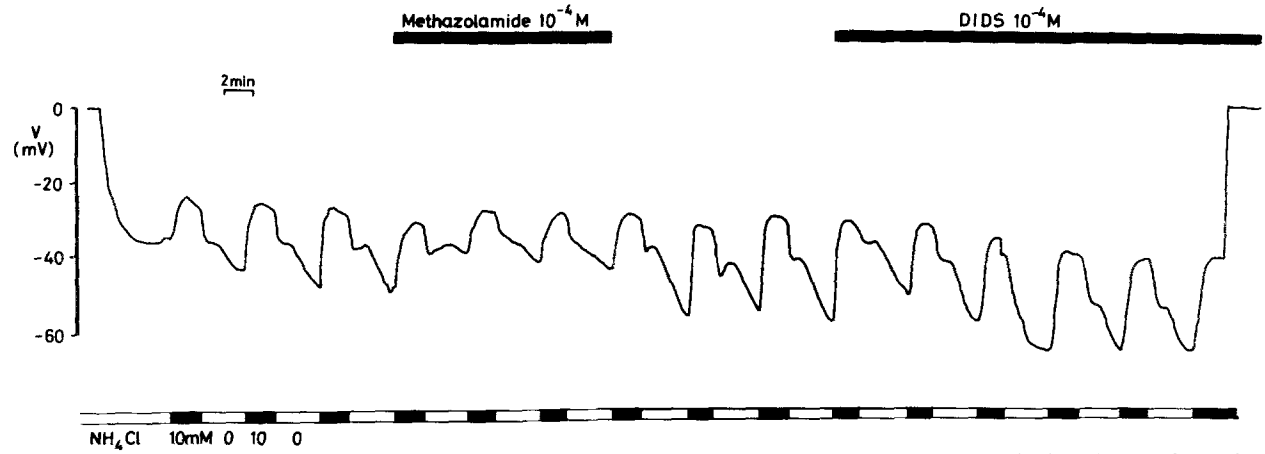

Fig. 14. Effect of methazolamide and DIDS on the voltage response elicited by ammonium in nominally bicarbonate-free solution (sol 2 and 6). Similar effects of methazolamide and DIDS were observed in three experiments

tion after readdition of bicarbonate more than the depolarization caused by its removal. This can be seen in Fig. 12 and is more clearly shown in Fig. 13, where bicarbonate removal was performed at greater time intervals and the different effects on de- and hyperpolarization become better visible. When bicarbonate was at first added to the HCO_3^--depleted cell, the already known transient hyperpolarization resulted. After treatment with 10^{-4} M SITS, which had no effect on the resting potential, this overshoot after readdition of bicarbonate was missing, while the depolarization was barely affected.

Neither inhibitor had any significant effect on the membrane potential under steady-state conditions. A combination of both, however, led to a slight depolarization of about 6 mV (5.9 ± 1.4 mV (SEM) ($n = 5$)).

These two inhibitors were also tested in nominally bicarbonate-free systems. An example is shown in Fig. 14. Voltage transients were produced by pH gradients generated by pulses of NH_4Cl. In contrast to the experiment shown in Fig. 8, the voltage response after removal of NH_4Cl had several phases. Both the patterns of Fig. 8 and Fig. 14 were seen in several experiments. Variable patterns of bi- and triphasic voltage responses to NH_4^+ have also been described by others [3]. In our case, we are inclined to attribute the rapid hyperpolarization after the removal of ammonium to an efflux of NH_4^+. The more prolonged hyperpolarization may be due to a rise of pH_i affecting V by a HCO_3^-/OH^- permeability or pH-sensitive conductances. This is supported by the fact that it was mainly this component which was affected by methazolamide. Even in this 'bicarbonate-free' Ringer's, methazolamide reduced the voltage response, though to a lesser extent than in bicarbonate-rich solution, whereas an effect of SITS was barely visible.

Discussion

In this work we have demonstrated that the intracellular potential of bovine corneal endothelial cells is sensitive to changes in bicarbonate, pCO_2

and pH, and that these effects can be reduced by stilbenes and inhibitors of carboanhydrase.

Whereas the puncture of corneal endothelial cells *in situ* has been proven to be very difficult, we were successful in obtaining stable potentials for periods of several hours using cells cultured on plastic.

In our opinion, there are three main reasons for this fact: (a) cells cultured on plastic are mechanically more stable than those punctured *in situ*. With our method of puncture, the electrode is advanced by gliding (with its shaft) on the surface of the plastic dish. This should lead to a mechanical fixation of the electrode relative to the cell. (b) Bovine corneal endothelial cells in culture are slightly bigger than those *in vivo* when cultured without high concentrations of fibroblast growth factor [10, 11], and (c) the same electrode can be advanced along the dish to puncture a great number of cells, whereas *in situ* the tips of the electrodes break easily when they impale Descemet's membrane or the stroma. Therefore, *in vitro* it is much easier to obtain a stable recording by repeated puncture.

As with all results obtained *in vitro*, the question of relevance of our data for the *in vivo* situation must be posed. In order to prevent alterations in the characteristics of the cells due to the conditions of culturing, we only used early subcultures for our study. Moreover, we used strains obtained from more than 10 different eyes, which all showed qualitatively the same reactions. Furthermore, bovine corneal endothelium (BCE) in culture seems to retain most of its *in vivo* characteristics and extrapolation to *in vivo* conditions seems to be less problematic than with other cultured cells [9]. Cultures of BCE have been thoroughly investigated by several groups as to morphology and biochemistry (e.g. *see* [11, 31]). They form contact-inhibited polarized monolayers, the cells being connected by junctional complexes [11] similar to those *in vivo*.

In our preparation, it is difficult if not impossible to decide whether the observed membrane processes are located in the apical or basolateral membrane. Since the cells form a polarized monolayer with a basement membrane secreted to the bottom of the dish [9, 11, 31], it will be primarily the apical surface which is superfused. With cells cultured on plastic, it is impossible to superfuse the basolateral membrane separately. This would only be feasible if the cells were grown on a permeable support such as a collagen film or a millipore filter. In those cases, however, we expect the puncture to be much more difficult.

On the other hand, corneal endothelium is leaky ($R = 20$ to $70\ \Omega\ cm^2$) [14, 29] and permeable even to molecules of high molecular weight [24]. Therefore it can be expected that the solutes also easily reach the basolateral membrane. Since we puncture the cells by advancing the electrode on the plastic surface, we puncture the cells from the lateral rather than the apical side. By disrupting the plasma membrane of the previously punctured cell, we presumably create an additional access for the solute to the lateral side of the punctured cell. Thus we assume that either the apical and/or the basolateral membrane could contribute to the observed phenomena.

Our method of cell puncture deserves a special comment. Though the electrode is advanced by gliding on the plastic surface, its very tip is not in contact with it. This is due to the conical shape of its shank. If the tip would actually glide on the plastic surface, no cell could be impaled due to the extracellular matrix secreted by the cell monolayer onto the dish which raises the cells above the plastic dish. Moreover, the tip would probably break. Thus, no piezoelectric effects (other than those possibly arising also with more traditional methods of puncture) should occur.

As to the values of the steady-state potentials of corneal endothelial cells, our results compare well with those obtained by other groups. For the rabbit cornea, Wiederholt and Koch [40] reported values of -30.6 mV, range 7 to 93 mV, Lim and Fischbarg [28] -45 ± 2 mV, and in a newer work [29] -61 ± 1 mV, and Jumblatt [22] for cultured cells -59.2 ± 1.8 mV. In most of these reports, a considerable scatter in the data is present, so that the mean values have to be considered with caution.

In the present study with cultured cells, the potentials average near -46 mV, the data scattering mainly between -40 and -55 mV, though in some cases also values of up to -80 mV have been observed. Within the same culture dish, the measured potentials were mostly equal within a few mV.

Since the corneal endothelium transports bicarbonate, we focused our work on the influence of bicarbonate, CO_2 and pH on the membrane potential. If one of these parameters has an influence on the potential V, then V will also be sensitive to changes in the two other parameters, since they are intimately linked. We have tried to answer the question which ionic species is directly involved in the change of membrane potential.

Since it is known from many systems [2, 34, 36] that potassium conductance is affected by pH or HCO_3^-, we checked this possibility by applying

K^+ pulses in the presence and absence of bicarbonate and by testing the response to HCO_3^- in the presence of the potassium channel blocker barium. Though the response to extracellular potassium was reduced in the absence of HCO_3^-/CO_2 and the steady-state depolarization caused by removal of HCO_3^-/CO_2 was reduced by Ba^{++}, this does not prove that a reduction of potassium conductance is responsible for the steady-state depolarization in the absence of bicarbonate. Indeed, in both experiments (Figs. 6 and 7) the baseline of V was appreciably changed either by removal of HCO_3^- or Ba^{++}, which might *per se* influence K conductivity.

On the other hand, the voltage transients caused by a change in $[HCO_3^-]_o$ could not be suppressed by Ba^{++}. This indicates that ionic species other than K^+ are involved in this effect. In Fig. 7 the transient depolarization caused by removal of HCO_3^- was slightly reduced, and the hyperpolarization due to addition of HCO_3^- increased in the presence of Ba^{++}. When K conductance is blocked, it is expected that the relative contribution of the conductances of other ions to the total membrane conductance increases. This should result in more pronounced voltage transients induced by bicarbonate in the presence of Ba^{++}, if these transients are caused by ions other than potassium. The slight reduction of the depolarization seen after removal of bicarbonate in the presence of Ba^{++} can be interpreted in terms of the reduction of the membrane potential by barium. This results in a reduced electrochemical gradient for the exit of negative species such as HCO_3^-, which may be responsible for the depolarization.

This leaves OH^-, H^+ and especially HCO_3^- as the most probable ions responsible for the voltage transients. We have demonstrated that transients of V depend linearly on $\log[HCO_3^-]$, thus suggesting a HCO_3^- conductance. At constant pCO_2, a slope of about 37 mV/decane HCO_3^- was obtained, which is compatible with such a process. When pCO_2 was lower in the solutions containing less bicarbonate, this slope (64 mV/decade) was slightly greater than the ideal slope predicted by the Nernst equation. This increased slope may be explained by the change in pCO_2. CO_2, as a small neutral molecule, will easily penetrate the plasma membrane [13]. In the cell, it will be converted by carbonic anhydrase to carbonic acid. This results in a lowering of pH_i, which was also shown experimentally in squid axons with pH-sensitive microelectrodes [5]. This lowering of pH_i could cause an influx of HCO_3^- or related species, which will result in a hyperpolarization. We have shown that this might be true also for changes of pH_i elicited by other methods (extracellular application of butyrate, glycodiazine and ammonium). The initial depolarization observed after increasing pCO_2 might be tentatively attributed to an initial efflux of HCO_3^-, which was rapidly generated from CO_2 by membrane-bound carbonic anhydrase. The reason for the gradual disappearance of this first phase, however, is not clear.

Since with most of the solutions changes in the experiment of Fig. 4 pH_o was changed together with $[HCO_3^-]$ or pCO_2, the influence of pH_o on the voltage response has also to be considered. This type of experiment provides evidence that pH_o is at least not the most important factor influencing V. This is because (1) the greatest voltage transients were obtained when the maximal change in $[HCO_3^-]$ was performed, and this was done with only a slight change of pH_o (7.66 to 7.5), and (2) that in these experiments the direction of voltage change was not correlated with the direction of change in pH_o. In part (*a*) and (*b*), the more alkaline solutions (containing more HCO_3^-) led to a hyperpolarization, whereas in part (*c*) the more alkaline solution (containing less CO_2) rather led to a depolarization.

Thus, this kind of experiment points to bicarbonate as the primary species determining the voltage transients. Changes in pCO_2 probably affect V indirectly by generating transmembranal gradients for pH and HCO_3^-.

On the other hand, also changes of pH in nominally bicarbonate-free solution caused voltage transients. This could be achieved by changing either extra- or intracellular pH. The latter effects, however, are more difficult to interpret because of simultaneous changes of other parameters, such as $[NH_4^+]$ or $[\text{butyrate}^-]$.

The response to pH in nominally bicarbonate-free systems, however, cannot be interpreted as an unequivocable evidence for a H^+ or OH^- conductance, since no effort has been made to exclude HCO_3^- totally. Even in nominally bicarbonate-free solutions equilibrated with air, it is expected that $[HCO_3^-]$ is more than a factor of 100 greater than $[H^+]$ or $[OH^-]$ [20]. There should also be a contribution of endogeneously produced CO_2. Moreover, methazolamide, an inhibitor of carbonic anhydrase, caused even in nominally HCO_3^--free solution a reduction of the voltage response induced by pH gradients, though not as pronounced as in bicarbonate-rich solution. This suggests an involvement of bicarbonate even in nominally bicarbonate-free solution.

Especially interesting is the effect of the stil-

benes SITS and DIDS. These are known inhibitors of anion transport in the red cell membrane and several epithelial cells. In our study, they reduced the response to bicarbonate by about 50%, being unable to suppress it totally even at concentrations of 5×10^{-4} M. Their effect was additive to that of methazolamide, indicating a different mode of action. Their effect was more pronounced in bicarbonate-rich than in nominally bicarbonate-free solutions, suggesting that the inhibited transport process needs bicarbonate at relatively high concentrations. Moreover, the stilbenes inhibited the hyperpolarization after addition of bicarbonate more than the depolarization caused by HCO_3^- removal. Thus it may be hypothesized that they inhibit a membrane process which under the experimental conditions mediates mainly bicarbonate influx, and that another process involved in electrogenic HCO_3^-/OH^- transport is operating in parallel. The latter process should not be inhibitable by stilbenes, explaining the residual voltage response unaffected by SITS or DIDS.

The process inhibited by stilbenes is not an electroneutral exchange mechanism as found in the red cell membrane and many epithelia. In this case it would rather be expected that application of stilbenes would cause an *increase* in the response, since now an electroneutral pathway for HCO_3^- would be blocked. Then most of the transport should occur via the (parallel) electrogenic channel which our experiments have shown to exist.

Both stilbenes and carbonic anhydrase inhibitors alone did not have a significant effect on steady-state membrane potential. A combination of both, however, led to a slight reduction of membrane potential by some mV. This suggests that in steady state the contribution of an electrogenic transport of HCO_3^- (or related species) to the membrane potential is only small, though bicarbonate is one of the main ions transported across the endothelium. This may be explained in two ways: (a) both the entry and the exit of HCO_3^- is electrogenic, both currents compensating each other, or (b) that with cultured cells the transport rates are rather small. This may be due to the growth of the cells on a plastic surface which is expected to reduce transcellular transport, since the availability of transportable ions to the basolateral membrane should be diminished and the net transepithelial transport abolished. The depolarization caused by a combination of both inhibitors points to a net electrogenic entry of HCO_3^- or related species into the cells or to a change of pH_i which affects pH-sensitive conductances. This is in contrast to the situation described for the proximal tubule of the kidney, another leaky epithelium involved in the transport of water, Na^+ and HCO_3^-. In that tissue exists an electrogenic exit for HCO_3^- at the basolateral membrane [4, 8] and the application of acetazolamide causes this membrane to hyperpolarize [8].

In conclusion, it is suggested, that one or possibly two electrogenic transport mechanism for HCO_3^- or related species exist in cultured bovine corneal endothelial cells, one of which is inhibitable by stilbenes.

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