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Regulation of Cytoplasmic pH of Cultured Bovine Corneal Endothelial Cells in the Absence and Presence of Bicarbonate

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Summary. Intracellular pH (pH_i) in confluent monolayers of cultured bovine corneal endothelial cells was determined using the pH-dependent absorbance of intracellularly trapped 5(and 6)carboxy-4′,5′-dimethylfluorescein. Steady-state pH was 7.05 ± 0.1 in the nominal absence of bicarbonate, and 7.15 ± 0.1 in the presence of 28 mM HCO_3^-/5% CO_2. Following an acid load imposed by a NH_4Cl prepulse, pH_i was regulated in the absence of HCO_3^- by a Na^+-dependent process inhibitable to a large extent by 1 mM amiloride and 0.1 mM dimethylamiloride. In the presence of 28 mM HCO_3^-/5% CO_2, this regulation was still dependent on Na^+, but the inhibitory potency of amiloride was less. DIDS (1 mM) partially inhibited this regulation in the presence, but not in the absence of bicarbonate. With cells pretreated with DIDS, amiloride was as effective in inhibiting recovery from acid load as in the absence of HCO_3^-. The presence of intracellular Cl^- did not appreciably affect this recovery, which was still sensitive to DIDS in the absence of Cl^-. Removal of extracellular Na^+ led to a fall of pH_i, which was greatly attenuated in the absence of HCO_3^-. This acidification was largely reduced by 1 mM DIDS, but not by amiloride. Cl removal led to an intracellular alkalinization in the presence of HCO_3^-. The presence of a Cl^-/HCO_3^- exchanger was supported by demonstrating DIDS-sensitive $^{36}Cl^-$ uptake into confluent cell monolayers. Thus, bovine corneal endothelial cells express three processes involved in intracellular pH regulation: an amiloride-sensitive Na^+/H^+ antiport, a Na^+-HCO_3^- symport and a Cl^-/HCO_3^- exchange, the latter two being DIDS sensitive.

Key Words intracellular pH · sodium bicarbonate cotransport · Na^+/H^+ antiport · Cl^-/HCO_3^- exchange · amiloride · DIDS · cornea · endothelium · cell culture

Introduction

Since most biochemical processes are sensitive to changes in pH, the regulation of intracellular pH (pH_i) is expected to be important for almost every type of cell. Indeed, every animal cell examined so far expresses one or several plasma membrane transporters involved in pH_i regulation. In epithelial cells involved in transepithelial transport of acid equivalents, the situation is even more complex. In these cells, those transporters serve both housekeeping functions and mediate transepithelial transport [7].

The corneal endothelium, which covers the posterior surface of the cornea, is a tissue specialized in the transepithelial transport of bicarbonate and sodium. This transport is probably the driving force for pumping fluid out of the corneal stroma into the anterior eye chamber [15, 16, 20, 21, 31, 35], which is essential for the maintenance of corneal transparency.

In previous work, we have shown by intracellular microelectrode measurements [23, 25, 26] and $^{22}Na^+$-flux determinations [28], that cultured bovine corneal endothelial cells express an electrogenic sodium-bicarbonate symport which is inhibitable by disulfonic stilbenes (SITS and DIDS) and harmaline [24]. A similar symport had previously been identified in the basolateral membrane of the proximal tubule of the salamander kidney [9], and more recently in mammalian kidney cells of several different species [1, 2, 5, 17, 18, 22, 27, 29, 30, 42, 49] and in stomach oxyntic cells [14]. As suggested by studies of its kinetic properties in BSC-1 cells [30], this symport might actually be a $NaCO_3^-$-transporter (or a $NaCO_3^-$-HCO_3^- symport to account for a 3 : 1 stoichiometry). Also from $^{22}Na^+$ flux measurements, we postulated earlier the existence of a Na^+/H^+ antiport in the corneal endothelium [28], and electrophysiological data [26] yielded indirect hints at some type of Cl^-/HCO_3^- exchange in these cells.

In the present work, we use the pH-sensitive absorbance of 5(and 6)carboxy-4′,5′-dimethyl-

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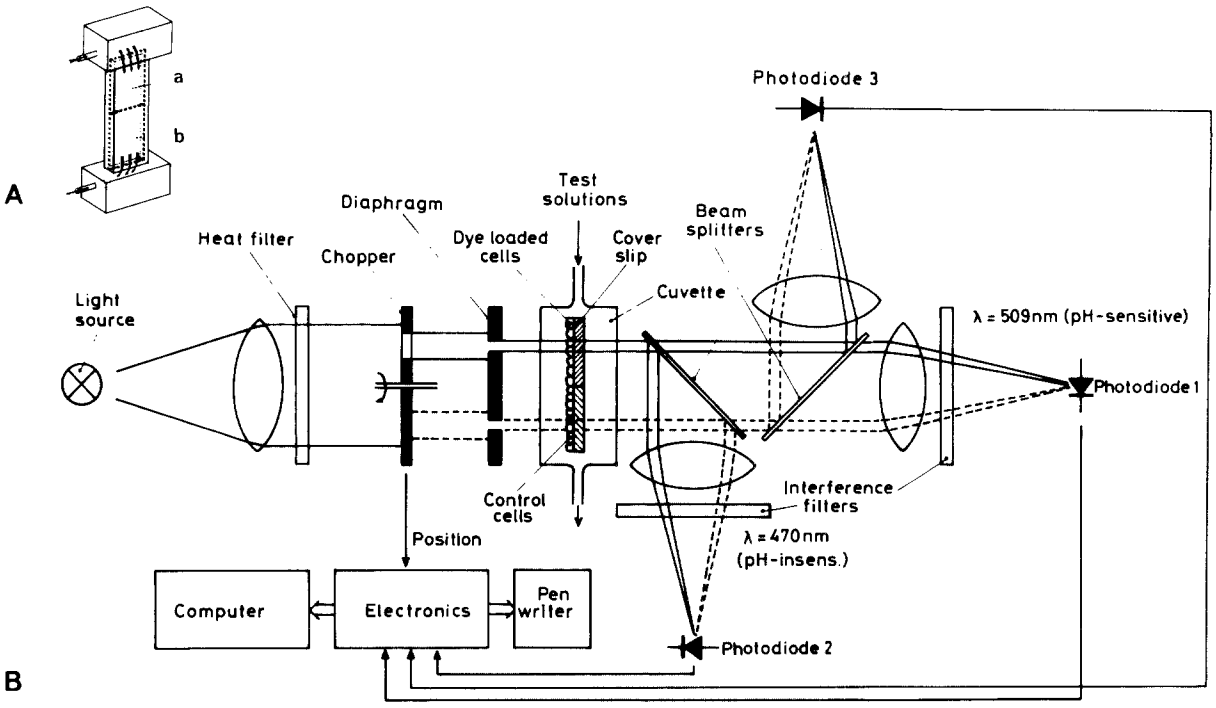

Fig. 1. Dual-beam dual-wavelength photometer designed for continuously measuring pH_i in cultured cell monolayers. (*A*) Detail of the cuvette; (*B*) schematic diagram of the setup

fluorescein trapped into confluent monolayers to investigate the regulation of intracellular pH in the bovine corneal endothelium. In addition to examining the roles of Na^+/H^+ antiport and Na^+-HCO_3^--($NaCO_3^-$) symport in this regulation, we provide evidence for a Cl^-/HCO_3^- exchanger in these cells both by determinations of pH_i and $^{36}Cl^-$ uptake and efflux measurements.

Materials and Methods

Materials

Cell culture media and supplements were purchased from Biochrom KG (Berlin, Federal Republic of Germany). 5(and 6)-carboxy-4′,5′-dimethylfluorescein-diacetate was obtained from Molecular Probes (Eugene, Oregon), 4,4′-diisothiocyanostilbene disulfonic acid (DIDS) from Fluka (Neu-Ulm, FRG), nigericin and digitonin from Sigma (Munich, FRG), and ouabain from E. Merck (Darmstadt, FRG). Amiloride was a general gift from MSD Sharp and Dohme (Munich), and dimethylamiloride was synthesized as described previously [13]. ^{36}Cl was obtained from New England Nuclear (Boston, Mass.).

Cells

Primary cultures of bovine corneal endothelial cells were established as described previously [25]. They were maintained at 37°C in DMEM (Dulbecco's modification of Earle's minimal essential medium) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 atmosphere. The cells were passaged at a split ratio of 1:3 using trypsin/EDTA (0.05/0.025%). For the pH experiments, cells were seeded on plastic cover slips in Leighton tubes (Costar, Cambridge, Mass.) and were used at least four days after having reached confluence. The cells used in the present study were from passage number 1 or 2. For the flux measurements, cells were seeded into 25 cm^2 tissue culture flasks. In these studies, cells were from passage number 3.

Measurement of Cytoplasmic pH

To determine intracellular pH, we used pH-sensitive absorbance changes of intracellular trapped 5(and 6)carboxy-4′,5′-dimethylfluorescein (CDMF). Due to a higher value of pK_a, this dye is better suited than carboxyfluorescein [45] to monitor changes of pH in the physiological range [43]. To load the dye into the cells, a plastic slip covered with a cell monolayer was cut into two equal parts. One part was dye loaded by a 10- to 20-min incubation at room temperature in saline [mM: 150 Na^+, 5 K^+, 1.7 Ca^{2+}, 1 Mg^{2+}, 160 Cl^-, 1 $H_2PO_4^-$, buffered with HEPES (10 mM) to pH 6.7] containing 50 μM CDMF-diacetate, which was added from a stock solution in DMSO (final concentration: 0.15% DMSO). The other part of the slip (control cells) was incubated under identical conditions except for the absence of CDMF-diacetate (but including DMSO). After incubation, the cells were rinsed with bicarbonate-free Ringer's (pH 7.4, sol.1) and were inserted on top of each other into a specially designed cuvette (Fig. 1*A*). This cuvette allows rapid fluid exchange (90% exchange in less than 2 sec) simultaneously on both sides of each cover slip (both sides may be covered with cells). The delay in fluid exchange in

front of the dye-loaded and unloaded cells, respectively, is also less than 2 sec. The cells are continuously superfused during the experiment (flow rate 70 ml/min), except for the calibration procedure described below. In the experiments described in this work, the temperature of the superfusing solution was 37°C, except when stated otherwise.

The cuvette is inserted into a specially designed dual-beam dual-wavelength photometer (Fig. 1*B*). The light of an incandescent lamp, operated on a constant current source, is focussed onto a diaphragm with two perforations after passing a heat filter. A chopper in front of the diaphragm (f = 70 Hz) illuminates the two perforations alternately. The size of the light spot was 2.7 mm^2, which means that the signal from roughly 5000 corneal endothelial cells was averaged during the measurement. The two chopped light beams pass through the control cells and dye-loaded cells in the cuvette, respectively, and are then split into two equal parts by a beam splitter. Both beams are focussed on two silicon photodiodes (1 and 2) (Hamamatsu type S1336-8BK, Hamamatsu, Japan) after passing interference filters (λ = 509 nm and λ = 473 nm, bandwidth 10 nm, Schott Glaswerke, type PIL1, Mainz, FRG). Before passing through the filter, part of the white light is focussed on photodiode 3 for light intensity reference. The placement of this photodiode behind the cells ensured that it measured only the light passing through the cells; thus, shifts in the focus of the lamp with time or diffraction effects during changes of solutions with different refraction indices could be conveniently corrected, while the error introduced by pH-sensitive absorption by the cells is small due to the broad range of wavelengths being measured. The absorbance spectrum of CDMF is maximally pH sensitive at about λ = 509 nm, whereas λ = 470 nm is an isosbestic point [12, 39]. Absorbance measurement at λ = 470 nm thus provides a means to determine the amount of dye trapped in the cells and to correct for dye leakage. The optical information is processed by analog electronics and then recorded on a pen writer or fed into a computer (Apple IIe, Cupertino, Calif.) by an analog-to-digital converter (sample rate: 2 per sec) and stored on a floppy disk. The availability of two channels (two beams) can be used in one of two ways: (a) since the test solutions superfusing the cells may absorb light at the measured wavelength, and since cells may change their absorbance independently of pH (and dye loading), the second channel may be used to measure the absorbance of control cells (and superfusate); this absorbance may then be automatically subtracted from the absorbance of dye-loaded cells (either by analog circuitry for on-line registration by a pen-writer or by the computer); this was the most frequent mode of operation used in the present study; (b) when the magnitude of these effects has been previously determined, both channels may be used to measure intracellular pH in two different cell populations simultaneously (both slips covered with dye-loaded cells); this has been exploited in some experiments to directly compare the regulation of pH_i in DIDS-pretreated and untreated cells.

Calibration of absorbance in terms of pH_i was done using the K^+/H^+-exchanger nigericin [45]. At the end of each experiment, the cuvette was filled with saline whose K^+ concentration approximates that of the intracellular compartment (mM: 140 KCl, 10 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, buffered with 10 HEPES, MOPS or Tris to the desired values of pH) and 10 μM of the ionophor (added from a stock solution in ethanol, final concentration of ethanol below 0.1%). This sets $pH_i = pH_o$, and pH_o may be varied to known values to obtain absorbance as a function of pH. Afterwards, the dye was released from the cells using 80 μM digitonin in order to determine null-point absorbance. The result from this calibration is given as an approximate pH_i scale in the Figures.

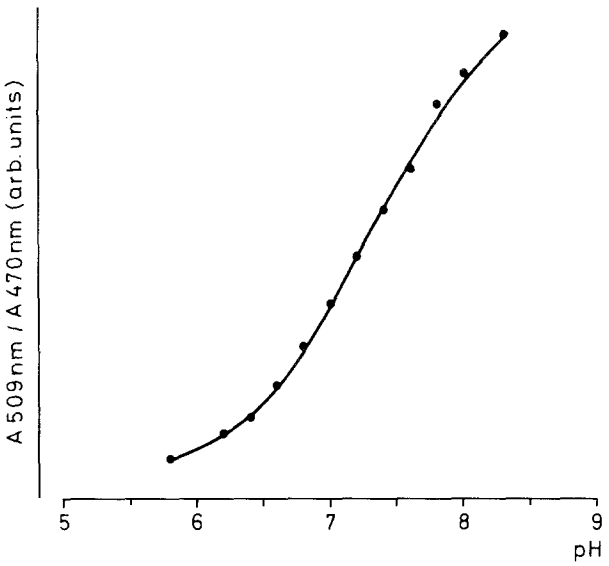

Fig. 2. Calibration curve for the determination of intracellular pH in bovine corneal endothelial cells using the absorbance of 5(and 6)carboxy-4′,5′-dimethylfluorescein. Dye-loading and calibration was done as described in Materials and Methods

An intracellular calibration curve for corneal endothelial cells is shown in Fig. 2.

Determination of ^{36}Cl Uptake and Efflux

^{36}Cl-uptake and -efflux measurements were performed essentially as described previously for $^{22}Na^+$ uptake [28]. Briefly, corneal endothelial cell monolayers grown in 25 cm^2 tissue culture flasks were preincubated at 37°C in solutions containing ions and inhibitors as described in the Figure legends. With bicarbonate Ringer's, the flasks were filled with 5% CO_2. This gassing was repeated at every solution exchange to keep pH_o constant throughout the experiment. In preliminary experiments (*data not shown*) we noted that preincubation of cells for 1 hr or more with 10^{-4} M ouabain increased steady-state accumulation of ^{36}Cl, possibly due to the dissipation of the plasma membrane voltage. Therefore, to increase the time during which ^{36}Cl uptake is approximately linear, cells were preincubated for 2 hr with saline containing 10^{-4} M ouabain. DIDS-pretreated cells were incubated at 37°C with saline containing 1 mM DIDS for at least 30 min. Uptake was determined by incubation with uptake saline containing 20 to 40 kBq/ml ^{36}Cl for the indicated periods of time either at 30 or at 37°C in a shaking bath. Uptake was terminated by three rapid washes with ice-cold stop solution (100 mM $MgCl_2$, 10 mM HEPES/Tris, pH 7.4). Cells were dissolved in 0.1 M NaOH and the resulting lysate was neutralized. Radioactivity was determined using Instant Scintillation Gel (Packard Instruments, Ill.) and a β-counter. Uptake was referred to surface area and to the number of cells, as described previously [28].

For ^{36}Cl-efflux measurements, cells were loaded at 37°C with ^{36}Cl for 90 min in sol. 1 containing about 30 kBq/ml ^{36}Cl, 10^{-4} M ouabain (and 1 mM DIDS for DIDS-pretreated cells). At the beginning of the efflux period, the radioactive saline was removed by aspiration and the flasks were rinsed three times (at 28°C) with isotope-free bicarbonate-Ringer's, and were incubated in this solution (at 28°C) for the indicated periods of time.

Table. Composition of solutions (concentrations given in mmol/liter)[a]

Sol. #	Na	K	NH_4	NMDG	chol	Ca	Mg	Cl	HPO_4	SO_4	HCO_3	acet	gluc	HEPES
1	151	5	—	—	—	1.7	1	159	1	1	—	—	—	10
2	—	5	—	151	—	1.7	1	159	1	1	—	—	—	10
3	131	5	20	—	—	1.7	1	159	1	1	—	—	—	10
4	151	5	—	—	—	1.7	1	131	1	1	28	—	—	—
5	—	5	—	123	28	1.7	1	131	1	1	28	—	—	—
6	131	5	20	—	—	1.7	1	131	1	1	28	—	—	—
7	151	5	—	—	—	6.0	1	—	1	67	28	—	—	—[b]
8	131	5	20	—	—	6.0	1	—	1	67	28	—	—	—[b]
9	151	5	—	—	—	6.0	1	—	1	1	28	—	131	—
10	151	5	—	—	—	1.7	1	81	1	1	28	50	—	—

[a] NMDG means N-methyl-D-glucamine, chol choline, acet acetate, and gluc gluconate, respectively. Every solution contained additionally 5 mM glucose. pH was 7.4 for all solutions, which was achieved by gassing with 5% CO_2 for the solutions containing bicarbonate.

[b] Osmolality was adjusted with mannitol.

This temperature was chosen to slow down the rate of efflux in order to be able to measure at convenient time points. Radioactivity remaining in the cells was determined as described above and expressed as percent of initial radioactivity prior to efflux measurements (determined immediately after loading the cells by rinsing the flasks 5 times with stop solution to thoroughly remove extracellular label).

Solutions

The composition of the solutions is given in the Table.

Results

Resting Intracellular pH

We measured resting intracellular pH by equilibrating the dye-loaded cell monolayer at 37°C for at least 15 min in the respective medium, while determining the associated absorbance of CDMF. Afterwards, each experiment was immediately individually calibrated using the nigericin method as described in Materials and Methods and extracellular solutions buffered to values of pH_o close to the expected values of pH_i. In nominally bicarbonate-free Ringer's buffered with HEPES to pH_o = 7.4 (sol. 1), intracellular pH averaged at pH_i = 7.05 ± 0.1. In bicarbonate Ringer's (sol. 4) gassed with 5% CO_2 to pH 7.4, mean pH_i was 7.15 ± 0.1. This difference was not statistically significant.

Regulation of pH_i Following an Acid Load

Regulation of intracellular pH is most easily observed by displacing pH_i and observing its subsequent recovery [38, 47]. To observe cellular acid extrusion mechanisms, we acidified the cell interior by using the NH_4Cl prepulse technique [10]. In short, extracellular addition of NH_4Cl leads to an intracellular alkalinization by nonionic diffusion of NH_3 across the plasma membrane and subsequent association with H^+. Conversely, an overshoot acidification is observed when NH_4Cl is removed after sufficient amounts of NH_4^+/NH_3 have accumulated in the cell due to influx of NH_4^+ during the plateau phase. In the experiment of Fig. 3, this has been done in the nominal absence of HCO_3^-. After acid loading, the cell regulates its pH towards more alkaline, physiological values of pH_i (recovery rate: 0.14 ± 0.01 (SEM) pH units/min at $pH_i \sim 6.4$ (n = 5)]. This regulation is dependent on Na^+, since extracellular removal of sodium leads to a block or even reversal of the alkalinization. A reversible, large, though not total block of this regulation is observed with 1 mM amiloride (Fig. 3*A*) (recovery rate: 0.03 ± 0.01 pH/min, n = 5), which is expected to block sodium/proton antiport by less than 90% at the high concentration of Na^+ used (151 mM) [4]. A similar inhibition is also observed with 0.1 mM dimethylamiloride, a more potent and specific inhibitor of Na^+/H^+ antiport [33, 48] (Fig. 3*B*). In other experiments (*data not shown*) we observed that lithium could partially substitute for sodium in acid extrusion. Taken together, these data strongly suggest that in nominally bicarbonate-free saline acid extrusion is predominantly performed by a Na^+/H^+ antiport.

When experiments similar to that of Fig. 3 were performed in the presence of bicarbonate (28 mM HCO_3^-, 5% CO_2, pH_o = 7.4), the acid extrusion could again be totally blocked by Na^+ removal (Fig. 4*A*). In the presence of HCO_3^-, the recovery rate (0.46 ± 0.08 pH/min, n = 5) was larger than in

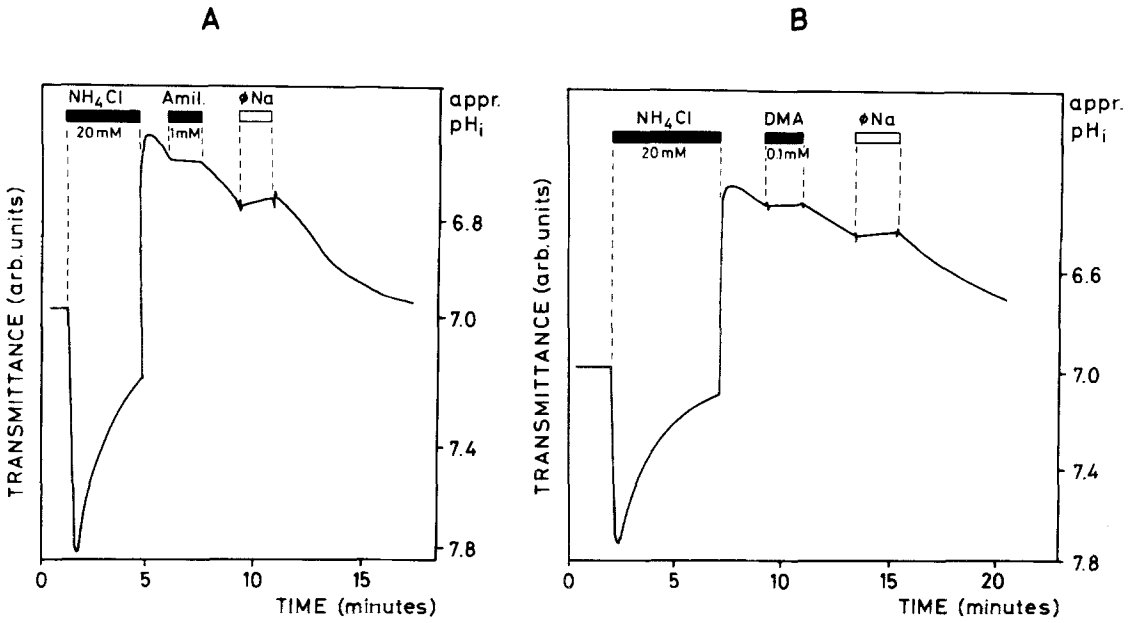

Fig. 3. Regulation of intracellular pH following an acid load in nominally HCO_3^--free saline. Acid loading was achieved by a prepulse of 20 mM NH_4Cl and the effect of Na^+ removal and 1 mM amiloride (*A*) and 0.1 mM dimethylamiloride (*B*) was explored. After the experiment, each measurement was individually calibrated using the nigericin method, resulting in the shown pH_i scale. Experiments as in (*A*) were performed 5 times, and as in (*B*) 3 times, with similar results. Used solutions: 1–3, with appropriate additions of inhibitors

nominally bicarbonate-free medium, even though the intracellular buffering power is increased.[1] This suggests the presence of a bicarbonate-dependent pH_i-regulating process. Indeed, in the presence of 1 mM amiloride the recovery rate was now 0.06 ± 0.03 pH/min (n = 3), which is larger than in the absence of HCO_3^-. In the experiment of Fig. 4(*B*), we explored the effect of 1 mM DIDS on this regulation. Though DIDS was ineffective in inhibiting acid extrusion in the absence of HCO_3^- (*data not shown*), it largely, though not totally, inhibited this regulation in the presence of HCO_3^- (recovery rate: 0.7 ± 0.01 pH/min, n = 6). Since DIDS is an aminoreactive reagent, its effect was only partially reversible after 4 min.

Thus, these experiments suggest that in bicarbonate Ringer's at least two processes may extrude acid equivalents, one being inhibitable by disulfonic stilbenes and the other by amiloride. If there is no third mechanism, application of both inhibitors should cause an inhibition as complete as the one observed with 1 mM amiloride in the nominal absence of HCO_3^-. Since DIDS, an aminoreactive compound, should not be applied together with amiloride (which contains primary NH_2 groups), we preincubated the cells for at least 30 min at 37°C with DIDS. This irreversibly blocks Na^+-HCO_3^- ($NaCO_3^-$) symport in cultured bovine corneal endothelium [28] and BSC-1 cells [29]. Indeed, with DIDS-pretreated cells (Fig. 4*C*), the effect of amiloride was larger than the one observed without DIDS pretreatment. The pH_i recovery rate (0.02 ± 0.01, n = 3) was somewhat less than the one observed with amiloride in the absence of HCO_3^-, which would be expected due to the increased buffering power in the presence of bicarbonate/CO_2. Thus, amiloride-sensitive Na^+/H^+ antiport and DIDS-sensitive mechanism(s) are the major processes performing acid extrusion in the presence of HCO_3^-. The DIDS-sensitive process(es) is (are) both Na^+- and HCO_3^--dependent. This is a characteristic feature of Na^+-HCO_3^--symport which we have previously identified in these cells [23, 28]. Another possible candidate is a DIDS-sensitive Na^+-HCO_3^-/Cl^--H^+-exchange mechanism, which is involved in pH_i regulation in several invertebrates [6, 47] and has been postulated to exist in A431 cells [40] and Chinese hamster lung fibroblasts [34] and possibly the basolateral membrane of the *Necturus* proximal

[1] Using the NH_3 technique, we measured the intracellular buffering power in the absence of HCO_3^- to be β_i = 14 ± 3 mM (SD, n = 5); this is a rather low value when compared with other tissues [38], but an even lower value has been described for other cultured cells [11]. Since under the present conditions (pH_i = 7.1, 5% CO_2) the additional buffering power introduced by the HCO_3^-/CO_2 system is about 29 mM [38], β should be higher by nearly a factor of 3 in the presence of HCO_3^-.

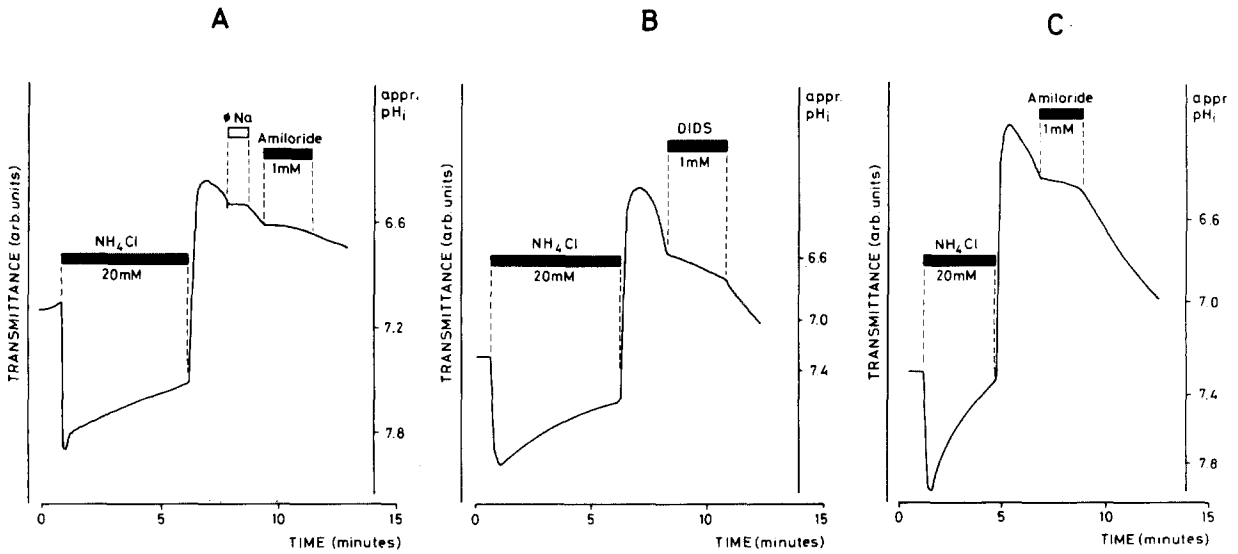

Fig. 4. Recovery from an acid load in the presence of 28 mM HCO_3^-/5% CO_2. Cells were acid loaded by a prepulse of 20 mM NH_4Cl, and the effect on ion substitution and inhibitors was tested. (*A*) Effect of extracellular Na^+ and 1 mM amiloride; (*B*) effect of 1 mM DIDS; (*C*) effect of 1 mM amiloride with DIDS-pretreated cells (pretreatment as described in Materials and Methods). Experiments as in (*A*) were performed 3 times, as in (*B*) 6 times, and as in (*C*) 3 times, with comparable results. Used solutions: 4, 5, 6

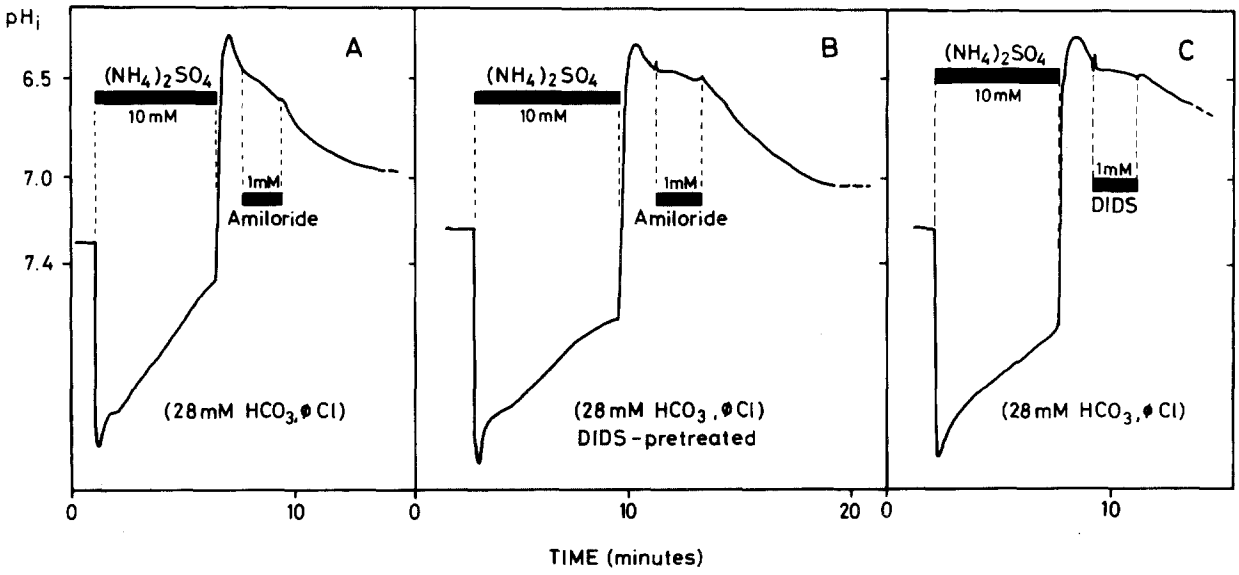

Fig. 5. Recovery from acid load in bicarbonate Ringer's in the absence of chloride. Cells were Cl depleted by a 1-hr incubation at 37°C in Cl-free saline (Cl substituted by SO_4), including the dye-loading period. The cells were then acid loaded using a $(NH_4)_2SO_4$ prepulse in the presence of 28 mM HCO_3^-/5% CO_2. The effect of 1 mM amiloride on acid extrusion was investigated using untreated (*A*) and DIDS-pretreated cells (*B*). In (*C*), the effect of 1 mM DIDS was explored under identical conditions. Experiments as in (*A*) and (*B*) were repeated 2 times, and as in (*C*) 10 times, with similar results. Used solutions: 7, 8, including appropriate concentrations of inhibitors

tubule [19]. Moreover, such a process is known to regulate pH_i after an acid load.

To examine this possibility, we performed similar experiments with Cl-depleted cells, since intracellular chloride would then be the substrate for exchange with extracellular Na^+ and HCO_3^- during recovery from acid load. Cells were Cl^- depleted by incubation in Cl^--free HCO_3^--Ringer's for 1 hr (including the dye-loading period), and the cells were acid loaded by a $(NH_4)_2SO_4$ prepulse in the absence of chloride (Fig. 5*A*). The effect of 1 mM amiloride was tested on the subsequent acid extrusion. With A431 cells (presumably expressing a Na^+-HCO_3^-/Cl^--H^+ exchanger) this led to a nearly complete

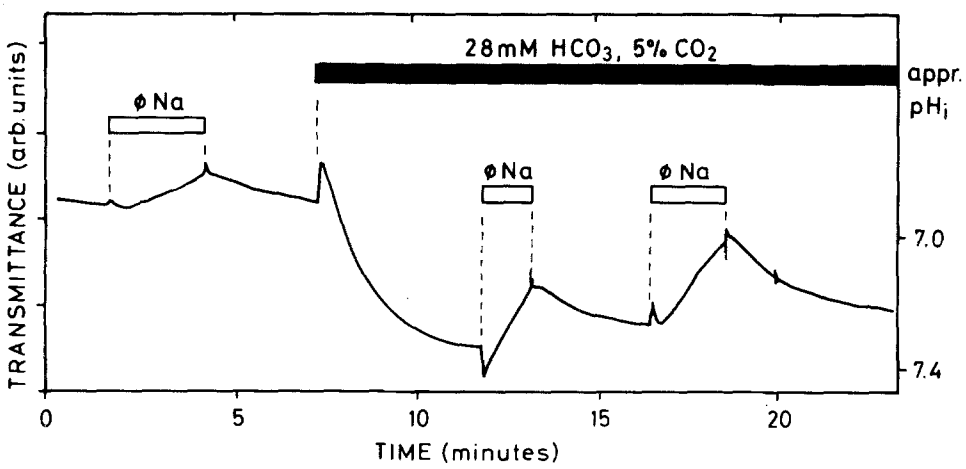

Fig. 6. Effect of sodium removal in the presence and absence of 28 mM HCO_3^-/5% CO_2. Note that the acidification caused by external Na^+ removal is much faster in the presence of HCO_3^-. The initial acidification observed with switching to bicarbonate/CO_2 saline is due to intracellular conversion of CO_2 (which enters the cell easily by diffusion) into carbonic acid. The initial alkalinization observed with the first Na^+ removal in the presence of bicarbonate is due to an insufficient equilibration of the sodium-free solution with CO_2 and disappeared when freshly equilibrated Ringer's had filled the tubings (second removal). In this type of experiment, pH_i seems to be significantly more alkaline in the presence of HCO_3^- than in its absence. However, this is probably due to an initial influx of $NaHCO_3$ after the solution exchange, since after sufficient equilibration (more than 15 min) this difference was much smaller and statistically not significant (7.15 ± 0.1 in the presence, versus 7.05 ± 0.1 in the absence of HCO_3^-). This type of experiment was repeated four times, with similar results. Used solutions: 1, 2, 4, 5

inhibition of pH_i regulation [40]. With the corneal endothelium, however, amiloride caused only a small inhibition, virtually ruling out such mechanism as the major DIDS-sensitive acid extrusion process. In another experiment, we repeated the same maneuver with DIDS-pretreated cells (Fig. 5*B*). As shown above in the presence of chloride (Fig. 4*C*), 1 mM amiloride now elicited a strong inhibition of acid extrusion.

The experiment shown in Fig. 5(*C*) is even more conclusive. Even in the nominal absence of Cl, 1 mM DIDS caused a significant inhibition of acid extrusion. Thus, a DIDS-sensitive, HCO_3^-- and Na^+-dependent, but Cl^--independent process plays a major role in recovery from intracellular acidification.

Effect of Na^+ Removal on pH_i Under Resting Conditions

Since this suggested that both Na^+/H^+ antiport and Na^+-HCO_3^--($NaCO_3^-$) symport played an important role in regulating pH_i in the acidic pH range, we subsequently tested whether these processes are equally operative at physiological values of pH_i. To this end, we removed extracellular sodium (replaced by NMDG and choline) both in the absence and in the presence of HCO_3^- (Fig. 6). With both processes being operative under these conditions, this should lead to a drop in pH_i in either case. Indeed, an acidification is observed both in the presence and in the absence of bicarbonate, the rate of acidification being, however, larger in the presence of bicarbonate. This strongly suggests that Na^+-HCO_3^--($NaCO_3^-$)-symport activity is significantly larger than the one of Na^+/H^+ antiport at physiological values of pH_i, especially since intracellular buffering power is expected to be larger in bicarbonate saline [46].[1] In the experiment shown in Fig. 7, we tested the effect of DIDS and amiloride on the effect of Na^+ removal in the presence of HCO_3^-. We exploited the dual-beam capability of the photometer to measure pH_i simultaneously in DIDS-pretreated (lower registration) and -untreated cells (upper registration). As expected from the previous experiment, DIDS pretreatment largely abolished the acidification caused by Na^+ removal in the presence of HCO_3^-. The minuscule effect of 1 mM amiloride on steady-state pH or on Na^+ removal-induced acidification again suggests a comparatively low activity of Na^+/H^+ antiport under these conditions.

An alternate explanation for the increased rate of acidification in the presence of HCO_3^- (Fig. 6) is the presence of a Na^+-independent acid-loading process dependent on the presence of bicarbonate. Furthermore, this process should be inhibited by DIDS (Fig. 7), and therefore could be the Cl^-/HCO_3^- exchanger described in the next paragraph. Though this mechanism is likely to contribute to the observed effects, the small effect of amiloride in Fig. 7 still suggests that $NaHCO_3^-$ symport is more

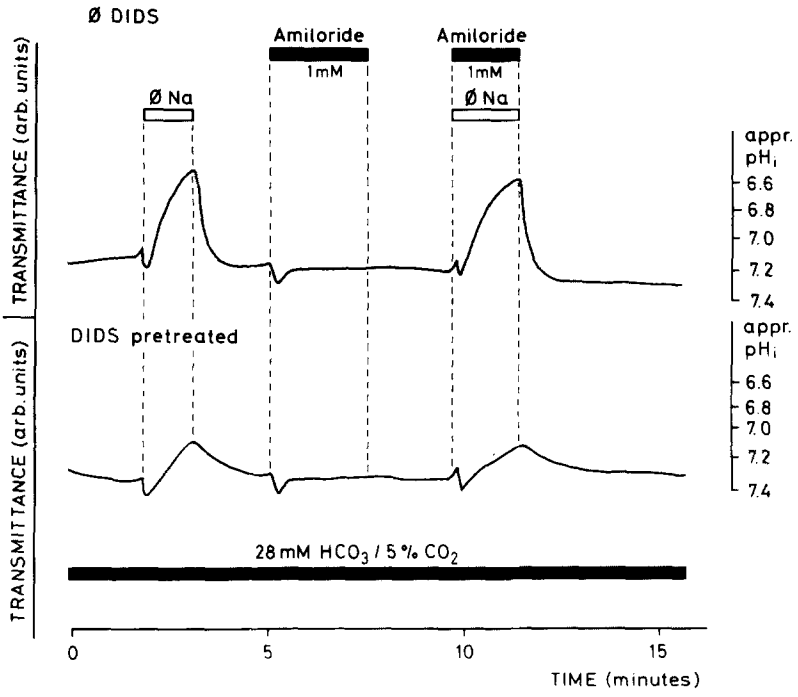

Fig. 7. Effect of DIDS and amiloride on Na^+ removal induced acidification in the presence of HCO_3^-. Cells treated with DIDS (lower trace) and not treated with DIDS (upper trace) were measured simultaneously exploiting the dual-beam capability of the photometer as described in Materials and Methods. Note that the acidification is largely reduced in DIDS-pretreated cells and that 1 mM amiloride has barely any effect on this acidification, nor on steady-state pH. Experiments of this type were performed 3 times, with similar results. Used solutions: 4, 5

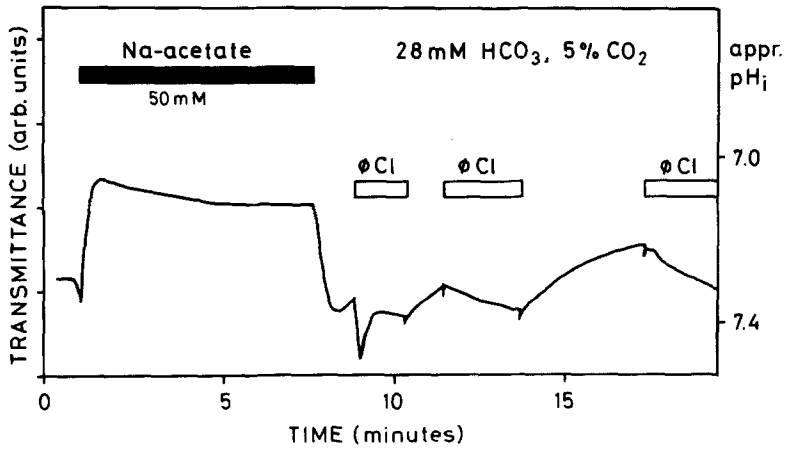

Fig. 8. Effect of Cl removal in bicarbonate saline after intracellular alkalinization induced by an acetate prepulse. Cl was substituted by gluconate, and Ca concentration was increased to 6 mM to account for the binding to gluconate. Four experiments of this type were conducted, with comparable results. Used solutions: 4, 9, 10

active at physiological values of pH_i than Na^+/H^+ antiport.

Effect of Cl^- Removal on Recovery from Base Loading

In mammalian cells the magnitudes of intra- and extracellular Cl^- and HCO_3^- concentrations are such that a simple Cl^-/HCO_3^- exchange can only mediate net intracellular acidification under physiological conditions. Therefore, we expected to detect such a transport activity most easily during recovery from intracellular alkalinization. In the experiment of Fig. 8, we slightly alkalinized the cytoplasm by a prepulse of 50 mM sodium acetate in the presence of HCO_3^-, and tested the effect of Cl^- removal on the subsequent recovery of pH_i. Cl^- removal led to an inhibition of this regulation or even to a net alkalinization. This is compatible with Cl^-/HCO_3^--exchange activity. Since the acetate prepulse did not produce a large alkalinization and since a comparable effect of Cl removal was still observed towards the end of the experiment, where pH_i had recovered, the Cl^-/HCO_3^--exchange activity does not seem to depend strongly on the value of pH_i.

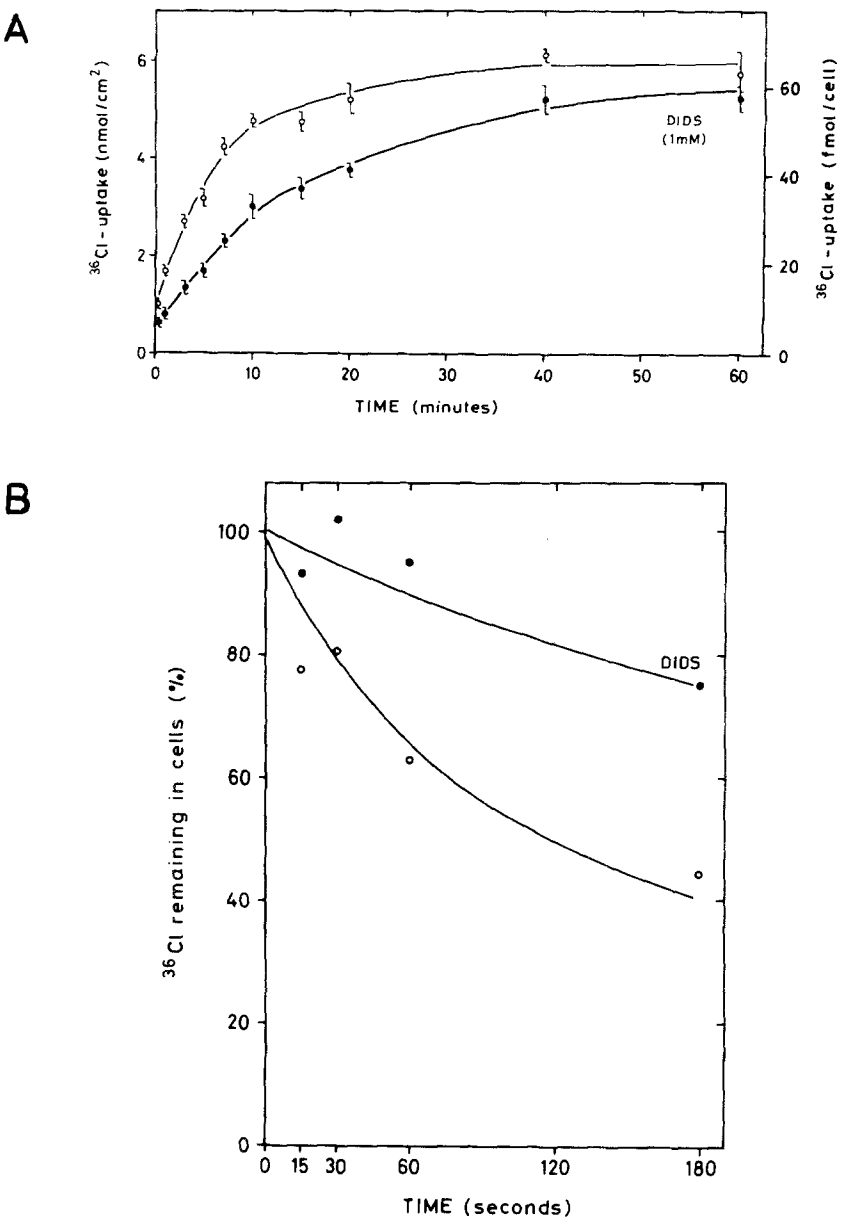

Fig. 9. Effect of 1 mM DIDS on $^{36}Cl^-$ uptake (*A*) and efflux (*B*) in HCO_3^--Ringer's. To measure under steady-state conditions, both preincubation- and uptake saline were identical (bicarbonate-Ringer's, solution 3) except for the presence of the isotopic tracer. Cells were preincubated for 2 hr in Ringer's containing 0.1 mM ouabain to approximate steady-state ion distribution. Uptake was measured with DIDS-pretreated (●) and -untreated cells (○). Each point represents the average of five determinations. Used solution: 3

^{36}Cl Uptake and Efflux is in Part DIDS Sensitive

To confirm the existence of a Cl^-/HCO_3^--exchange activity by an independent technique, we determined ^{36}Cl uptake into confluent monolayers of corneal endothelial cells.

If there is a typical Cl^-/HCO_3^- exchange, one would expect $^{36}Cl^-$ uptake to be inhibitable by disulfonic stilbenes. Indeed, in the experiment shown in Fig. 9(*A*), 1 mM DIDS reduced the rate of Cl^- uptake in the presence of bicarbonate by about 50%, while leaving steady-state accumulation of Cl unaffected. Similarly, ^{36}Cl efflux could be inhibited by DIDS (Fig. 9*B*).

Discussion

In this work, we have demonstrated by determinations of intracellular pH that cultured bovine corneal endothelial cells express at least three different processes involved in the transport of acid equivalents across the plasma membrane: a Na^+/H^+ antiport, a Na^+-HCO_3^- symport, and a Cl^-/HCO_3^- exchange. In the following, we will briefly summarize

the evidence for their existence and will discuss the role they play in intracellular pH regulation.

The Na^+/H^+ antiport [36] is clearly the most important process for recovery from acidic pH_i in the absence of HCO_3^-, since this recovery was totally Na^+ dependent and could be largely inhibited by 1 mM amiloride or 0.1 mM dimethylamiloride, a more specific and potent inhibitor of the antiport [33, 48]. While the Na^+/H^+ antiport was clearly active at acidic pH_i, it seemed to be quite inactive at resting values of pH_i. This is because (i) extracellular sodium removal (which should reverse the net transport direction of the exchanger) caused only a slight acidification in the absence of HCO_3^-, (ii) 1 mM amiloride had no significant effect on the rate of acidification observed with Na^+ removal in the presence of HCO_3^-, and (iii) because the application of 1 mM amiloride under steady-state conditions did not appreciably affect pH_i. Especially the latter point alone, however, is not totally conclusive, since e.g. in the salamander proximal tubule 2 mM amiloride even caused an alkalinization of the cell, which was explained by nonionic diffusion of the weak base amiloride [9]. On the other hand, our observations are in total agreement with data from other laboratories showing that the antiport is activated at acidic values of pH_i [3, 8].

The evidence for a Na^+-HCO_3^- symport (or $NaCO_3^-$ transport, HCO_3^--$NaCO_3^-$ symport [30]) requires more careful examination. While it is clear from our previous electrophysiological experiments [23, 25, 26], that bovine corneal endothelial cells express such an electrogenic symport, most of the data shown here (e.g. DIDS-sensitive and HCO_3^--dependent acidification upon Na^+ removal, the involvement of a process showing similar sensitivities in the recovery from acid load) may equally well be explained by a Na^+-HCO_3^-/Cl^--H^+ exchanger (or, equivalently, a $NaCO_3^-/Cl^-$ exchanger), which is known to be sensitive to DIDS and activated by intracellular acidification [6]. This possibility is especially intriguing since both intracellular pH measurements as well as $^{36}Cl^-$-uptake determinations showed evidence for a Cl^-/HCO_3^- exchange. To exclude this possibility, we have performed the experiment shown in Fig. 4 with Cl-depleted cells, demonstrating that the DIDS-sensitive, bicarbonate- and sodium-dependent process was not appreciably dependent on intracellular Cl^-. This conclusion obviously depends very much on the affinity of the putative transporter on intracellular Cl^- and on the degree of Cl^- depletion we have achieved by a 1-hr incubation in Cl^--free SO_4^{2-}-Ringer's. Bearing in mind the $^{36}Cl^-$-efflux experiment shown in Fig. 9(*B*), in which the cell lost more than 50% of internal Cl^- after 3 min even at 28°C, it is reasonable to assume that intracellular Cl^- should be depleted after 1 hr at 37°C in Cl-free medium to levels not allowing an operation of a Na^+-dependent Cl^-/HCO_3^- exchange. Thus, we conclude that the majority of Na^+-dependent, DIDS-sensitive bicarbonate flux is due to the electrogenic Na^+-HCO_3^--symport. This is also supported by preliminary experiments from our laboratory (C. Korbmacher, *unpublished observation*), showing that decreasing the plasma membrane voltage in the presence of HCO_3^- (by application of high extracellular K^+) leads to an alkalinization of the cell (*data not shown*). Since the symport carries net negative charge in the bicarbonate transport direction, this should indeed raise pH_i. In addition to this symport, the cells also express a Cl^-/HCO_3^- exchange. The missing effect of intracellular Cl depletion on recovery from acid load suggests that it is probably not dependent on Na^+. (A sodium-linked transporter should contribute to acid extrusion, while a sodium-independent exchanger can only acidify the cell.) However, we can not strictly exclude the presence of a Na^+-HCO_3^-/Cl^--H^+ exchanger in the corneal endothelium.

Since we have found earlier [24] that harmaline is an inhibitor of the sodium-bicarbonate symport, which has recently been confirmed for a similar transporter in rat kidney [18], we have also tested 1 mM harmaline on the recovery from acid loading (*data not shown*). However, probably due to its characteristics of a weak base [41], we observed a rapid alkalinization upon its addition during acid extrusion (T.J. Jentsch and I. Janicke, *unpublished observation*). Thus, this inhibitor cannot be used in pH_i experiments to block Na^+/H^+ antiport and Na^+-HCO_3^- symport simultaneously.

An interesting question is the role of these processes in determining steady-state intracellular pH, which might be expected to be different in the absence and presence of bicarbonate. Indeed, according to our model for transepithelial transport across the corneal endothelium [23], which is similar to schemes proposed for the renal proximal tubule [9], the Na^+-HCO_3^- symport should lead to a net efflux of bicarbonate, thus imposing a chronic acid load on the cell in the presence of HCO_3^-. Also a Cl^-/HCO_3^- antiport (not dependent on Na^+) can only lead to an acidification. However, the difference in pH_i under bicarbonate-free and bicarbonate-containing solutions was not statistically significant, and we rather observed a tendency towards more alkaline pH_i in the presence of HCO_3^-. Possible explanations for a more alkaline value of pH_i in the presence of HCO_3^- is the presence of a Na^+-dependent Cl^-/HCO_3^- exchange (which we did not detect in our measurements), or that sodium-bicarbonate symport has a

net inward transport direction in the corneal endothelium. The latter explanation would be compatible with the role of this process in the recovery from acid load we observed in Fig. 4. The net transport direction is closely related to the stoichiometry of bicarbonate-to-sodium coupling. A 2 : 1 stoichiometry would cause a net influx, and a 3 : 1 coupling an efflux at the values of pH_i observed in this study and the plasma membrane voltage of about −46 mV reported earlier [23, 25] (assuming intracellular Na^+ to be in the 10- to 20-mM range). Our previous electrical measurements would not allow us to choose between a 2 : 1 or 3 : 1 stoichiometry [23]. More recent measurements in kidney cells, however, yield hints at a 3 : 1 stoichiometry [44, 49]. On the other hand, however, we tentatively suggested that the Na^+-HCO_3^- ($NaCO_3^-$) symport of BSC-1 cells might mediate a net inward transport of sodium and bicarbonate [27]. Measurements of pH_i in BSC-1 [22] revealed no difference in steady-state pH in the absence or presence of HCO_3^-. However, this is not expected, since the large depolarization upon HCO_3^- removal in BSC-1 would have established the observed intracellular pH even by a passive distribution of H^+. In the corneal endothelium, however, the membrane voltage is largely given by the K^+ conductance [25]. Thus, hypothetically assuming a net inward transport by the symport, one would expect to see a lower value of pH_i in the absence of HCO_3^- in the corneal endothelium, but not in BSC-1. In that case, one would expect to see a similar acidification upon addition of 1 mM DIDS, which blocks the symport, but we were unable to detect a significant difference in steady-state pH_i between DIDS-treated and -untreated cells.

Of course, the presence of pH_i-regulating mechanisms does not necessarily imply a direct impact on steady-state pH_i. For instance, Na^+/H^+ antiport is activated by acidic pH_i, and two recent reports postulate a stimulation of Cl^-/HCO_3^- antiport by alkaline pH_i [32, 37]. In MDCK cells, which express both a Na^+/H^+ and a Cl^-/HCO_3^- antiport, no difference in steady-state pH_i could be detected between HCO_3^--free and -containing solutions at $pH_o = 7.4$. This is in contrast to a study of LLC-PK_1 cells, which express similar transporters, which were more acidic in the presence of HCO_3^- [11].

In conclusion, we have demonstrated the existence of three pH_i-regulating mechanisms in cultured bovine corneal endothelial cells: a Na^+/H^+ antiport, a Na^+-HCO_3^- symport (or $NaCO_3^-$ transporter, $NaCO_3^-$-HCO_3^- symport) and a Cl^-/HCO_3^- antiport. It is expected that these processes, in addition to regulating the internal milieu, also play an important role in transepithelial transport across this bicarbonate-secreting epithelium.

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