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Na^+-Dependent HCO_3^- Transport and Na^+/H^+ Exchange Regulate pH_i in Human Ciliary Muscle Cells

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Summary. We investigated intracellular pH (pH_i) regulation in cultured human ciliary muscle cells by means of the pH-sensitive absorbance of 5(and 6)-carboxy-4′,5′-dimethylfluorescein (CDMF). The steady-state pH_i was 7.09 ± 0.04 (n = 12) in CO_2/HCO_3^--buffered and 6.86 ± 0.03 (n = 12) in HEPES-buffered solution. Removal of extracellular sodium for 6 min acidified the cells by 1.11 ± 0.06 pH units (n = 12) in the presence of CO_2/HCO_3^- and by 0.91 ± 0.05 pH units (n = 8) in its absence. Readdition of external sodium resulted in a rapid pH_i recovery, which was almost completely amiloride-sensitive in the absence of CO_2/HCO_3^- but only slightly influenced by amiloride in its presence. Application of DIDS under steady-state conditions significantly acidified the ciliary muscle cells by 0.25 ± 0.02 (n = 4) in 6 min, while amiloride had no effect. The pH_i recovery after an intracellular acid load was completely dependent on extracellular sodium. In HEPES-buffered solution the pH_i recovery was almost completely mediated by Na^+/H^+ exchange, since it was blocked by amiloride (1 mmol/liter). In contrast, a marked amiloride-insensitive pH_i recovery was observed in CO_2/HCO_3^--buffered solution which was mediated by chloride-independent and chloride-dependent Na^+ HCO_3^- cotransport. This recovery, inhibited by DIDS (0.2 mmol/liter), was also observed if the cells were preincubated in chloride-free solution for 4 hr. Analysis of the sodium dependence of the pH_i recovery after NH_4Cl prepulse revealed V_{max} = 0.57 pH units/min, K_m = 39.7 mmol/liter extracellular sodium for the amiloride-sensitive component and V_{max} = 0.19 pH units/min, K_m = 14.3 mmol/liter extracellular sodium for the amiloride-insensitive component. We conclude that Na^+/H^+ exchange and chloride-independent and chloride-dependent Na^+-HCO_3^- cotransport are involved in the pH_i regulation of cultured human ciliary muscle cells.

Key Words ciliary muscle · intracellular pH · cell culture · sodium-bicarbonate cotransport · sodium/proton exchange

Introduction

The human ciliary muscle has smooth muscle properties (Ishikawa, 1962; Van der Zypen, 1967; Lütjen-Drecoll et al., 1988). Its contraction mediates accommodation and increases aqueous humor outflow facility, thus lowering the intraocular pressure. In an earlier study, we characterized a cell line from the human ciliary muscle (Korbmacher et al., 1990). Using this cell line, we described an electrogenic Na^+-HCO_3^- cotransport system in cultured human ciliary muscle cells by measuring the membrane voltage with intracellular microelectrodes (Stahl et al., 1992). Furthermore, we reported a hyperpolarization induced by intracellular acidification (Stahl et al., 1992). This hyperpolarization was dependent on both extracellular bicarbonate and sodium, suggesting involvement of an electrogenic Na^+-HCO_3^- cotransport in the pH_i regulation of human ciliary muscle cells. In this paper, we investigated the regulation of intracellular pH (pH_i) in human ciliary muscle cells, since in other smooth muscle preparations intracellular pH is known to influence intracellular calcium (Siskind et al., 1989; Danthuluri, Kim & Brock, 1990) and muscular tone (Ighoroje & Spurway, 1985; Smeda et al., 1987; Arheden, Arner & Hellstrand, 1989; Danthuluri & Deth, 1989). Intracellular pH measurements have not yet been performed in ciliary muscle. In other smooth muscle cells, Na^+/H^+ exchange has been shown to be the major acid-extruding mechanism in the absence of HCO_3^- (Weissberg et al., 1987; Kahn et al., 1990; Kikeri et al., 1990*b*). In the presence of HCO_3^-, two additional acid-extruding transporters have been described: first, a DIDS-sensitive, chloride-dependent Na^+-HCO_3^- cotransporter (Kahn et al., 1990; Kikeri et al., 1990*b*), and, second, a 5-(N-ethyl-N-isopropyl)amiloride-sensitive sodium- and bicarbonate-dependent mechanism, which is not inhibited by SITS (Neylon et al., 1990). Furthermore, DIDS-sensitive chloride/bicarbonate exchange is present in smooth muscle cells (Aickin & Brading, 1984; Aickin, 1986) and may serve as an alkali extruder (Korbmacher et al., 1988; Vigne et al., 1988; Kikeri et al., 1990*b*). This study provides evidence for a chloride-independent DIDS-sensitive Na^+-HCO_3^- cotransport which

is involved in the pH_i regulation of cultured human ciliary muscle cells. Chloride-independent Na^+-HCO_3^- cotransport has been shown to play an important role in pH_i regulation of various epithelial cells (Jentsch et al., 1986, 1988; Lopes et al., 1987; Townsley & Machen, 1989; Kikeri et al., 1990*a*; Muallem & Loessberg, 1990) and glia cells (Deitmer & Schlue, 1987). In guinea-pig ureter an amiloride-insensitive pH_i recovery after intracellular acidification has also been demonstrated, which was not measurably affected by chloride-free conditions (Aickin, 1988). However, in contrast to our results, an inhibiting effect of DIDS was not shown.

Materials and Methods

Cell Culture

Cells of an established cell line (H7CM), which was derived from the ciliary muscle of a one-day-old human infant (Korbmacher et al., 1990), were cultured in cell-culture flasks (80 cm^2). When cells had reached confluency, they were subcultured at a split ratio of 1 : 4 using Ca^{2+}-free and Mg^{2+}-free PBS containing 0.05% trypsin and 0.02% EDTA. Finally, the cells were grown to confluent monolayers on coverslips in Leighton tubes. Cells were maintained at 37°C in an atmosphere containing 95% air and 5% CO_2. The culture medium was Medium 199 supplemented with 10% FCS and 100 units/liter penicillin and 100 μg/ml streptomycin. Medium exchange was performed twice a week. Under these conditions, H7CM cells reached confluency within 7 days and were used for experiments 3–4 weeks after subculture. The H7CM cells in this study were from passages 5 to 15.

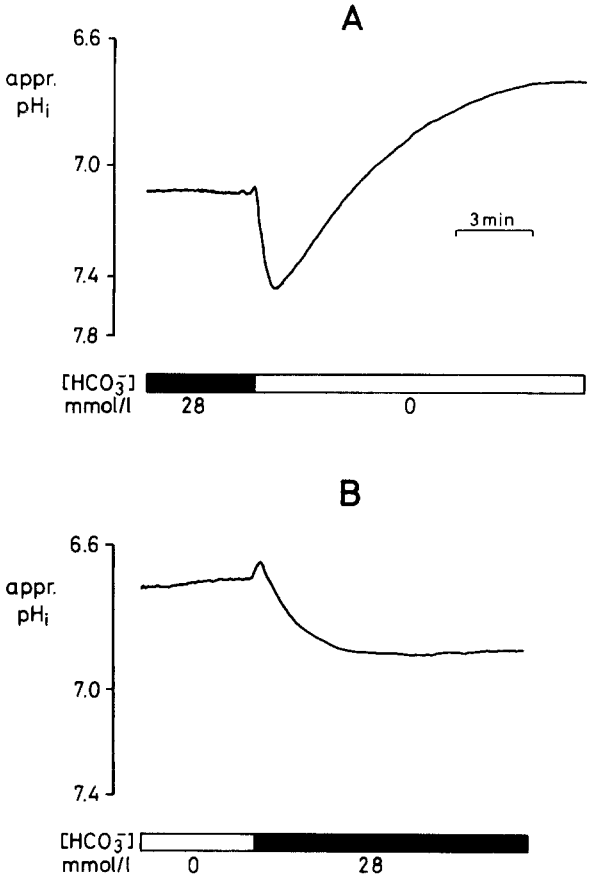

Fig. 1. Changes in pH_i induced by varying the extracellular solution from 5% CO_2/28 mmol/liter HCO_3^--buffered to HEPES-buffered solution (*A*) and vice versa (*B*). The extracellular pH was constant at 7.4. The steady-state pH_i was significantly more alkaline in the presence of CO_2/HCO_3^- (Table 1).

Measurement of Intracellular pH

Intracellular pH (pH_i) was measured, using the pH-sensitive dye 5(and 6)-carboxy-4′,5′-dimethylfluorescein (CDMF). The experimental setup has already been reported in detail (Jentsch et al., 1986). Briefly, the absorbance of CDMF is pH-sensitive at 509 nm but nearly pH-insensitive at 470 nm. Thus, pH can be estimated from the ratio of absorbance at 509 and 470 nm. For pH_i experiments, a cell covered coverslip was cut in half. One half (indicator cells) was incubated for 30 min with CDMF-diacetate (100 μM). The second half of the coverslip (control cells) was incubated in an identical solution without dye. Indicator and control cells were placed in a cuvette where they could be superfused with different test solutions. These test solutions were temperature regulated at 37°C and appropriately aerated with 5% CO_2 and 95% air. Transmittance was monitored continuously using a dual-beam dual-wavelength photometer. Data are presented as the ratio of transmittance at 509 and 470 nm, corrected for the non-pH_i-related absorbance variations in the control cells (arbitrary units). At the end of the recording, each experiment was calibrated using the nigericin method (Thomas et al., 1979), thus scales with approximate pH_i values (appr. pH_i) are added to the figures.

Solutions and Source of Chemicals

Standard bicarbonate solution contained the following ionic concentrations (in mmol/liter): 123 NaCl, 28 $NaHCO_3$, 4 KCl, 1.7 $CaCl_2$, 1 KH_2PO_4, 0.9 $MgSO_4$, and 5 glucose. Bicarbonate-containing solutions were aerated with 5% CO_2/95% air to yield a pH of 7.4. In bicarbonate-free solutions 28 mmol/liter $NaHCO_3$ were replaced by NaCl and the solutions were buffered with 10 mmol/liter N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) to pH 7.4. In solutions containing a lower sodium concentration, sodium was replaced by equimolar amounts of N-methyl-D-glucamine (NMDG). In solutions designed to change pH_i by nonionic diffusion, 20 mmol NaCl were replaced by 20 mmol NH_4Cl. For the experiments performed in chloride-free solutions, chloride was replaced by equimolar amounts of cyclamate. In solutions which contained 0.5 mmol/liter amiloride and 0.2 mmol/liter DIDS the substances were added from stock solutions in DMSO (10^{-1} mol/liter) as described previously (Muallem & Loessberg, 1990).

Cell culture media and supplements were purchased from Biochrom KG, Berlin; tissue culture flasks and dishes were from Nunc A/S, Roskilde, Denmark; Leighton tubes were supplied by Costar, Cambridge, MA. CDMF-diacetate was purchased from

Table 1. Variations in pH_i when the extracellular medium was changed from a CO_2/HCO_3^- to a HEPES-buffered solution (A) or vice versa (B)

A. 5% CO_2/28 HCO_3^- (pH_i)	0 CO_2/HCO_3^- (pH_i)	Transient alkalinization (ΔpH_i)
7.21 ± 0.03 (5)	6.90 ± 0.06 (5)	0.30 ± 0.07 (5)
B. 0 CO_2/HCO_3^- (pH_i)	**5% CO_2/28 HCO_3^- (pH_i)**	**Transient acidification (ΔpH_i)**
6.83 ± 0.04 (7)	7.02 ± 0.03 (7)	0.07 ± 0.02 (7)

The data were obtained from experiments like those shown in Fig. 1.

Molecular Probes, Eugene, OR. Nigericin, amiloride and 4,4'-diisothiocyanostilben-2,2'-disulfonic acid (DIDS) were obtained from Sigma Chemical, Deisenhofen, FRG.

Results

Steady-State pH_i in the Presence and Absence of CO_2/HCO_3^-

The change from 5% CO_2/28 HCO_3^- to a HEPES-buffered extracellular solution led to transient alkalinization followed by recovery to a significantly more acid pH_i value than in the presence of CO_2/HCO_3^- (Fig. 1*A* and Table 1*A*). The change from a bicarbonate-free to a bicarbonate-containing solution resulted in an initial transient acidification followed by recovery to a significantly more alkaline pH_i value than in the absence of CO_2/HCO_3^- (Fig. 1*B* and Table 1*B*). The steady-state pH_i calculated from the pooled data shown in Table 1*A* and *B* is 7.09 ± 0.04 (12) in the presence of CO_2/HCO_3^- and 6.86 ± 0.03 (12) in its absence.

Effects of Amiloride and DIDS on Steady-State pH_i

DIDS (1 mmol/liter) acidified the cells when applied under steady-state conditions in the presence of CO_2/HCO_3^- (Table 2). Amiloride (1 mmol/liter) had no significant effect on the steady-state pH_i either in the presence of CO_2/HCO_3^- or in its absence (Table 2).

Table 2. Acidification induced by amiloride (Ami) and DIDS

	ΔpH_i	n	$P <$
Ami (10^{-3} mol/liter) (0 HCO_3^-)	0.07 ± 0.04	4	NS
Ami (10^{-3} mol/liter) (+ HCO_3^-)	0.11 ± 0.03	3	NS
DIDS (10^{-3} mol/liter) (+ HCO_3^-)	0.25 ± 0.02	4	0.001
Ami (5×10^{-4} mol/liter) + DIDS (2×10^{-4} mol/liter) (+ HCO_3^-)	0.20 ± 0.03	4	0.05

pH_i Recovery after an Intracellular Acid Load

To investigate the mechanisms of pH_i regulation after intracellular acidification, we acidified the cells using the ammonium chloride prepulse technique (Boron & de Weer, 1976). 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 of 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 NH_4^+ influx during the plateau phase. This typical pH_i behavior is shown in Fig. 2. In HEPES-buffered saline, the pH_i recovery after intracellular acidification is almost completely blocked in the presence of 1 mmol/liter amiloride (Fig. 2*B*). In contrast, we observed a marked amiloride-insensitive component of the pH_i recovery in CO_2/HCO_3^--buffered solution (Fig. 2*A*). Statistical evaluation of our experiments revealed that, even in the presence of CO_2/HCO_3^-, an amiloride-sensitive process contributes significantly to the pH_i recovery after intracellular acidification (Fig. 3). Our data suggest that an amiloride-sensitive and amiloride-insensitive bicarbonate-dependent transport is operative in pH_i recovery. However, the spontaneous recovery rates did not significantly

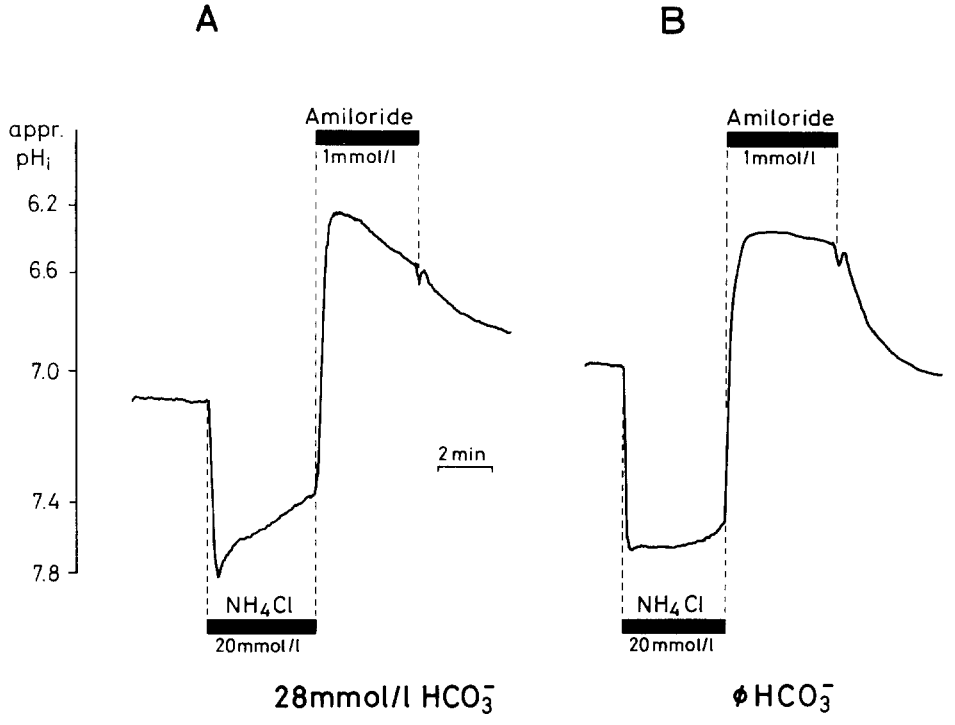

Fig. 2. The effect of amiloride (1 mmol/liter) on pH_i recovery was tested after an intracellular acid load by NH_4Cl prepulse. A marked amiloride-insensitive pH_i recovery was observed in the presence (*A*) but not in the absence (*B*) of CO_2/HCO_3^-.

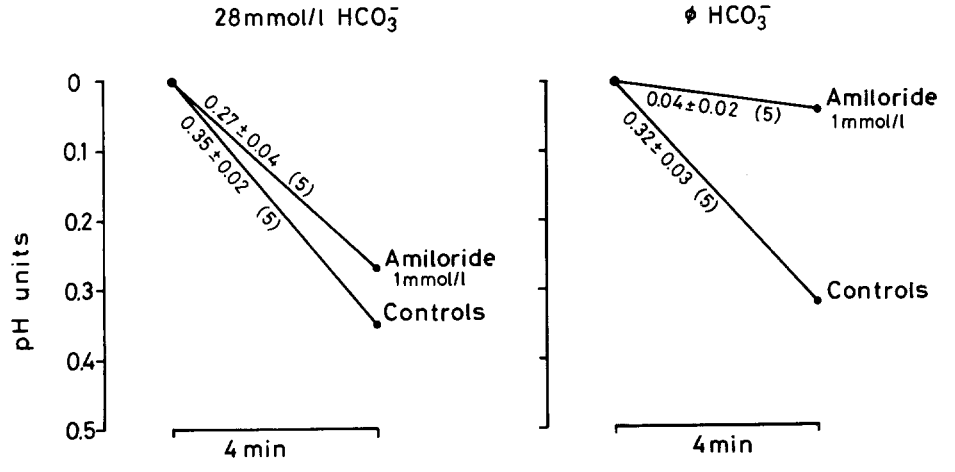

Fig. 3. Summary of 20 experiments similar to those shown in Fig. 2. The 4-min pH_i recovery rates from maximum acidification after a NH_4Cl prepulse are shown. Experiments were performed in the presence or absence of CO_2/HCO_3^- and in the presence or absence of 1 mmol/liter amiloride, respectively. The relative contribution of amiloride-insensitive processes to the pH_i recovery was larger in the presence of CO_2/HCO_3^- (approx. 70%) than in its absence (approx. 10%).

differ in the presence or absence of bicarbonate ("controls" in Fig. 3).

Amiloride-Sensitive pH_i Recovery

The amiloride-sensitive transport mechanism is most probably the Na^+/H^+ antiport. To further characterize this transporter in human ciliary muscle cells, we investigated the velocity of pH_i backregulation after an acid load in the presence of 0, 10, 25, 50 and 150 mmol/liter extracellular sodium. The experiments were carried out in the absence of CO_2/HCO_3^- to rule out the influence of bicarbonate transporters. A typical experiment is shown in Fig. 4. The velocity of pH_i recovery increases along with the extracellular sodium concentration, while virtu-

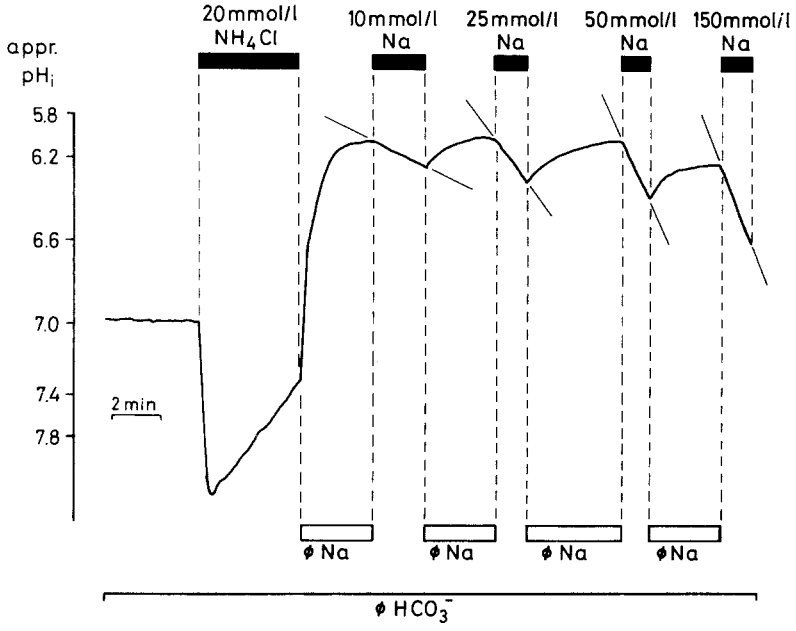

Fig. 4. Sodium-dependence of the pH_i recovery after an acid load. The experiments were performed in the absence of bicarbonate, thus the pH_i recovery can be almost completely attributed to Na^+/H^+ exchange. Cells were acid-loaded by means of NH_4Cl prepulse. Sodium was removed simultaneously with NH_4Cl. pH_i did not recover in the absence of sodium. The cells were subsequently exposed for short periods (indicated by the bars) to solutions containing increasing concentrations of sodium, followed each time by intervals of sodium removal. The slopes of pH_i recovery were used for kinetic analysis (Fig. 5).

ally no pH_i recovery can be detected in the absence of sodium. The initial rates of pH_i recovery for each sodium concentration were obtained from 9 individual experiments (Fig. 5*A*). Kinetic analysis by linear transformation of the data employing the method of Lineweaver-Burk revealed a V_{max} of 0.57 pH units/min with an apparent K_m = 40 mmol/liter for extracellular sodium (Fig. 5*B*).

Amiloride-Insensitive pH_i Recovery

This amiloride-insensitive and bicarbonate-dependent transport also requires the presence of extracellular sodium, since further pH_i recovery after NH_4Cl prepulse could not be observed in the presence of 1 mmol/liter amiloride when extracellular sodium was replaced by NMDG (*not shown*). The velocity of amiloride-insensitive pH_i backregulation increased along with the extracellular sodium concentration. Kinetic analysis of the amiloride-insensitive pH_i recovery by the method of Lineweaver-Burk revealed V_{max} = 0.19 pH units/min with an apparent K_m = 14 mmol/liter for external sodium (Fig. 6*A* and *B*). Data for kinetic analysis were obtained from five individual experiments similar to that shown in Fig. 4. In contrast to the experiments shown in Fig. 4, the recovery rates with various extracellular sodium concentrations were determined in the presence of amiloride (1 mmol/liter) and CO_2/HCO_3^-. Furthermore, the amiloride-insensitive pH_i recovery was inhibited by 0.2 mmol/liter DIDS, since the velocity of pH_i recovery was reversibly reduced when amiloride (0.5 mmol/liter) and DIDS (0.2 mmol/liter) were applied simultaneously (Fig. 7*A* and *B*). Our data suggest that the amiloride-insensitive process involved in the pH_i recovery after intracellular acidification is a DIDS-sensitive Na^+-HCO_3^- cotransport. To clarify whether this transport requires the presence of chloride, as would be expected for an electroneutral (Na^+-HCO_3^-)-(Cl^--H^+) transport we performed experiments similar to those shown in Figs. 2*A* and 3*A*. The experiments were performed in chloride-free solutions (chloride was replaced by cyclamate). Furthermore, the cells were preincubated for 1 hr in chloride-free solution before the experiment was initiated. These chloride-depleted cells, however, showed a similar amiloride-insensitive pH_i recovery after intracellular acidification, as shown in Fig. 8. A small amiloride-sensitive component was also detected. The velocity of amiloride-insensitive pH_i recovery did not significantly differ between chloride-depleted (Fig. 8) and chloride-containing cells (Fig. 3*A*). Even in ciliary muscle cells preincubated for 4 hr in chloride-free solution we could find an inhibiting effect of DIDS (1 mmol/liter) on the pH_i recovery after an acid load.

Removal of Extracellular Sodium

Replacement of extracellular sodium by NMDG led to intracellular acidification (Fig. 9) in both the presence and absence of CO_2/HCO_3^- (Fig. 9 and Table 3). The acidification induced by removal of extracellular sodium for 6 min was significantly greater in the

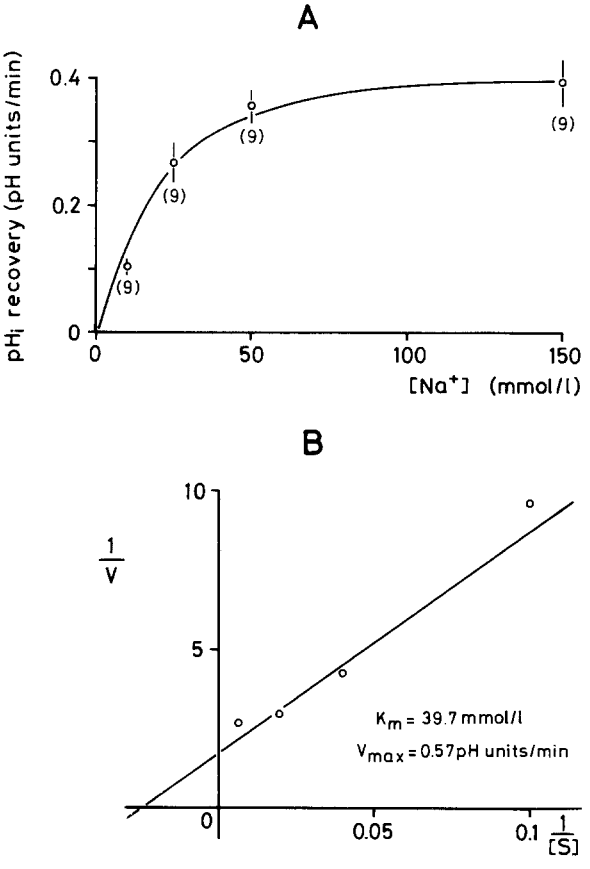

Fig. 5. Kinetic analysis of the sodium dependence of pH_i recovery in the absence of CO_2/HCO_3^-. Results were summarized for nine experiments similar to the one shown in Fig. 4. (*A*) The velocity of pH_i recovery (V) is plotted against the extracellular sodium concentration. (*B*) Linear transformation of the same data by Lineweaver-Burk plot.

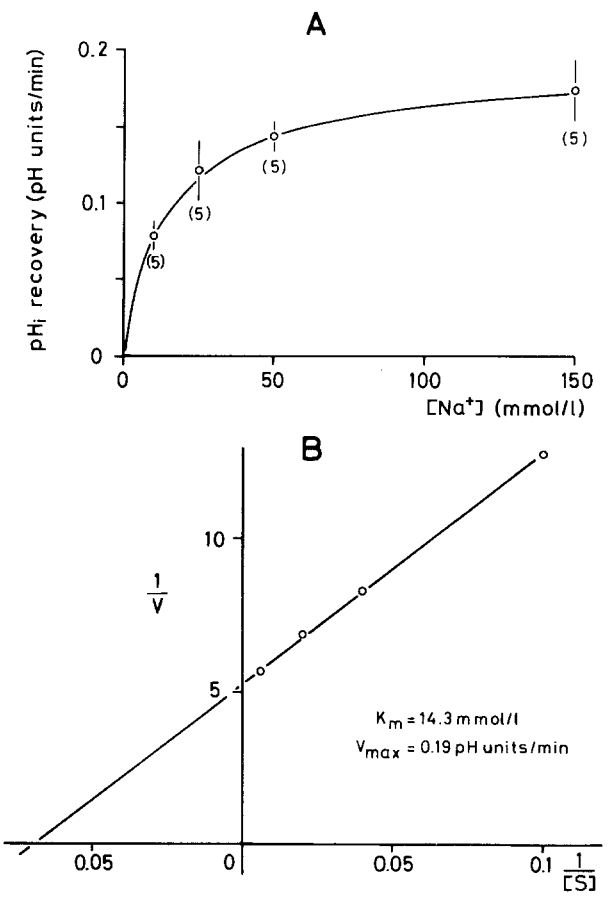

Fig. 6. Kinetic analysis of the sodium dependence of the amiloride-insensitive pH_i recovery in the presence of CO_2/HCO_3^-. Results were summarized for five experiments similar to the one shown in Fig. 4. The recovery rates were determined in the presence of CO_2/HCO_3^- and amiloride (1 mmol/liter). (*A*) The velocity of pH_i recovery (V) is plotted against the extracellular sodium concentration. (*B*) Linear transformation of the same data by a Lineweaver-Burk plot.

presence of CO_2/HCO_3^- than in its absence (Table 3). Unexpectedly, we could not find a difference in the acidification slope when we added either amiloride (1 mmol/liter) or DIDS (1 mmol/liter) or both substances (0.5 and 0.2 mmol/liter, respectively) in either the presence or absence of CO_2/HCO_3^- (*data not shown*). Readdition of sodium after its withdrawal for 6 min resulted in a rapid backregulation of pH_i. The initial pH_i recovery rate was higher than that observed after acidification by means of a NH_4Cl prepulse (*compare* controls in Figs. 3 and 10). When sodium was readded in the presence of amiloride (1 mmol/liter) we again observed an amiloride-insensitive pH_i recovery which was dependent on the presence of CO_2/HCO_3^- (Figs. 9*A* and 10). The slight inhibiting effect of amiloride in the presence of CO_2/HCO_3^- was not significant. Application of DIDS (1 mmol/liter) likewise had no significant inhibiting effect on the backregulation rate after removal of extracellular sodium for 6 min in either the presence or absence of CO_2/HCO_3^- (*not shown*). However, the rate of pH_i backregulation was significantly reduced by simultaneous application of amiloride (0.5 mmol/liter) and DIDS (0.2 mmol/liter). In the absence of CO_2/HCO_3^-, the pH_i recovery was almost completely blocked by 1 mmol/liter amiloride (Figs. 9*B* and 10). The chloride dependence of the pH_i backregulation after sodium removal was also tested. The amiloride-sensitive and amiloride-insensitive alkalinization was observed in the absence of extracellular chloride (Figure 11). Prior to the experiments, the cells were preincubated in chloride-free solution for 1 hr. We could still observe an amiloride-insensitive alkalinization when extracellular sodium was readded (Fig. 11). However, quantitative analysis revealed a significant decrease in the slope of amiloride-insensitive backregulation when

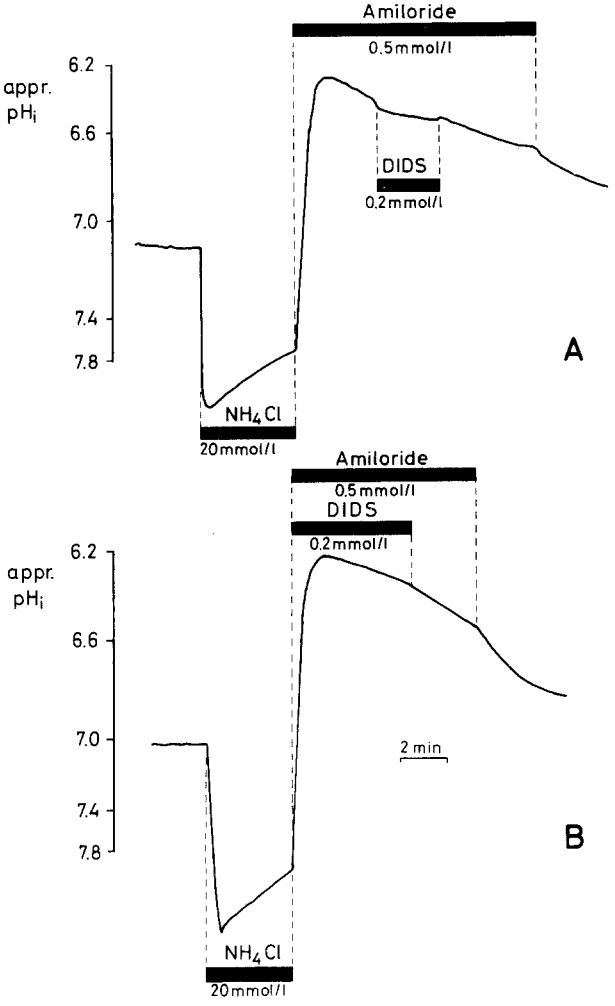

Fig. 7. The amiloride-insensitive pH_i recovery after an NH_4Cl prepulse is reversibly inhibited by DIDS. DIDS 0.2 mmol/liter was applied together with amiloride 0.5 mmol/liter either during the amiloride-insensitive pH_i recovery (*A*) or simultaneously with NH_4Cl removal (*B*).

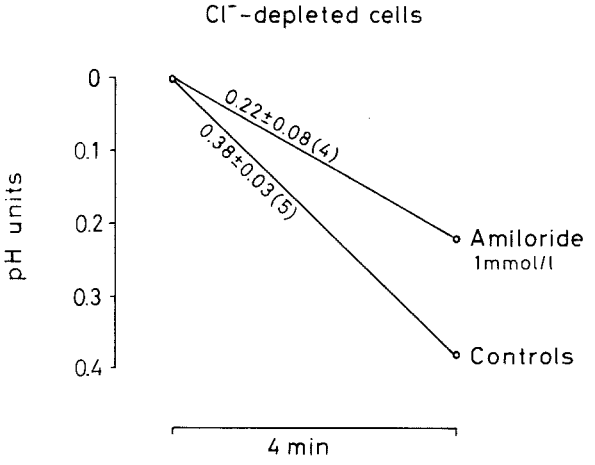

Fig. 8. The 4 min pH_i recovery rate after $(NH_4)_2SO_4$ prepulse in chloride-free, CO_2/HCO_3^- containing solution is shown in the presence and absence of amiloride, respectively. Prior to the experiment, cells were preincubated in chloride-free solution for at least 1 hr. The pH_i recovery was partly inhibited by amiloride. However, we could still observe an amiloride-insensitive pH_i recovery in chloride-depleted cells.

chloride-depleted were compared with chloride-containing cells (*compare* Figs. 10 and 12).

Discussion

Considering the negative membrane potential of 50–70 mV exhibited by our cultured human ciliary muscle cells (Korbmacher et al., 1990; Stahl et al., 1992), the intracellular steady-state pH is more alkaline than expected for passive H^+ distribution in either the presence or absence of CO_2/HCO_3^-. Thus acid extruding mechanisms must exist to overcome acid loading by passive fluxes of H^+, OH^-, HCO_3^-, by production of acid equivalents in the cell metabolism or by K^+/H^- exchange (Binder & Murer, 1986; Bonanno, 1991). At least one of these acid-extruding mechanisms in H7CM cells is bicarbonate dependent, since the steady-state pH_i of H7CM cells is more alkaline in CO_2/HCO_3^- containing extracellular medium than under CO_2/HCO_3^--free conditions. This difference in steady-state pH_i has already been observed for other smooth muscle cells (Aickin, 1986; Korbmacher et al., 1988; Kahn et al., 1990; Kikeri et al., 1990*b*; Putnam & Grubbs, 1990) and indicates the involvement of bicarbonate inward transporting processes in the pH_i regulation of these cells. This is also supported by the fact that DIDS *per se* had an acidifying effect when applied under steady-state conditions in the presence of CO_2/HCO_3^-. Such DIDS-induced steady-state acidification has already been described for smooth muscle cells (Aalkjaer & Cragoe, 1988; Korbmacher et al., 1988; Kahn et al., 1990; Putnam & Grubbs,1990). In the presence of CO_2/HCO_3^-, DIDS-sensitive transport mechanisms seem to contribute to a greater extent to the regulation of pH_i near the steady state than the amiloride-sensitive Na^+/H^+ exchanger, since application of amiloride did not significantly acidify the cells (Table 2) and simultaneous application of DIDS and amiloride did not induce a larger acidification than DIDS alone. Unexpectedly, amiloride also did not significantly acidify the cells, when it was applied in the absence of CO_2/HCO_3^-. Thus, even in the absence of CO_2/HCO_3^- the Na^+/H^+ exchanger seem not to contribute significantly to the maintenance of steady-state pH_i. Probably further mechanisms different from Na^+/H^+ ex-

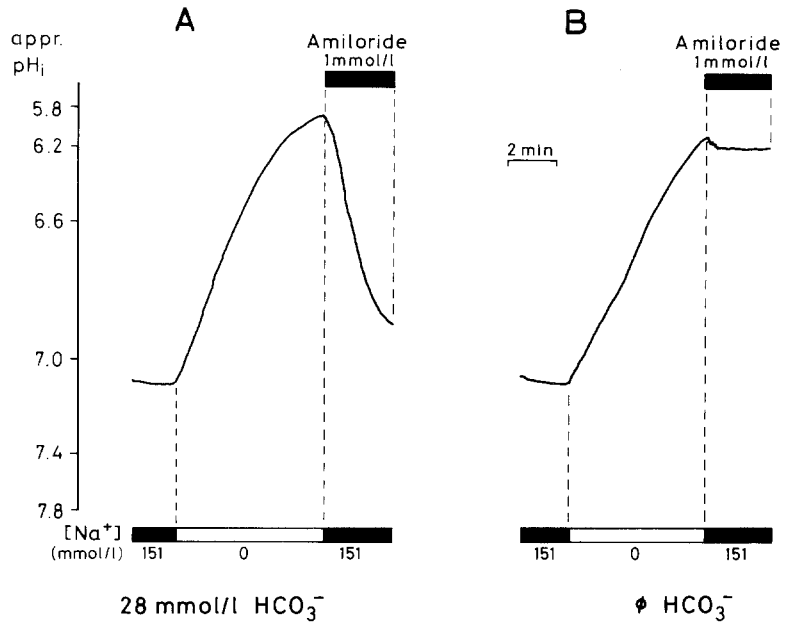

Fig. 9. The acidifying effect of extracellular sodium removal in either the presence (*A*) or absence (*B*) of CO_2/HCO_3^-. Extracellular sodium was replaced by NMDG. When sodium was restored in the presence of 1 mmol/liter amiloride, we observed a rapid pH_i recovery in CO_2/HCO_3^- containing solution (*A*). The pH_i recovery was largely attenuated in CO_2/HCO_3^--free solution (*B*).

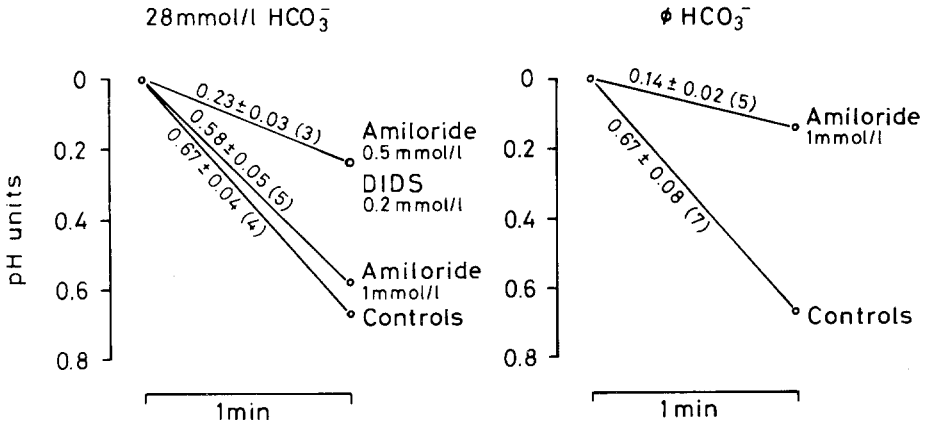

Fig. 10. The 1-min pH_i recovery rates induced by readding extracellular sodium after removal for 6 min are shown. Experiments were similar to those in Fig. 9. Drugs were added at the same time as extracellular sodium was restored. In the presence of CO_2/HCO_3^-, application of amiloride (1 mmol/liter) did not significantly influence the pH_i recovery. Simultaneous application of both 0.5 mmol/liter amiloride and 0.2 mmol/liter DIDS led to a markedly slower pH_i recovery than in the drug-free controls. In contrast, amiloride (1 mmol/liter) significantly reduced the pH_i recovery in the absence of CO_2/HCO_3^-.

change and Na^+-dependent HCO_3^- transport (i.e., H^+-ATPase) are involved in the maintenance of the steady-state pH_i.

Our further exploration of acid-extruding mechanisms after additional intracellular acidification by NH_4Cl-prepulse provides evidence for at least two sodium-dependent secondary active transporters. The first is an amiloride-sensitive Na^+/H^+ exchanger which almost completely mediates the backregulation after an acid load in the absence of CO_2/HCO_3^- (only 10% of the backregulation is amiloride-insensitive). Such a transporter has already been demonstrated in a large number of cell types, including smooth muscle cells (Owen, 1984; Owen, 1986; Weissberg et al., 1987; Korbmacher et al., 1988). The kinetic data obtained from our experiments are in good agreement with those reported for other smooth muscle cells (Berk et al., 1987; Weissberg et al., 1987; Korbmacher et al., 1988; Vallega et al., 1988; Vigne et al., 1988). In H7CM cells, Na^+/H^+ exchange also contributes to pH_i backregulation after an acid load in the presence of CO_2/HCO_3^-. However, more than 70% of the backregulation was insensitive to amiloride and thus must have been

Table 3. Variations in pH_i when extracellular sodium was removed.

	5% CO_2/28 HCO_3^-	0 CO_2/HCO_3^-
Control pH_i	7.03 ± 0.05 (8)	7.04 ± 0.02 (12)
0 Na^+ (6 min)	5.92 ± 0.05 (8)	6.13 ± 0.05 (12)
ΔpH_i	1.11 ± 0.06[a] (8)	0.91 ± 0.05 (12)

[a] The acidification induced by removal of extracellular sodium is significantly larger in the presence of CO_2/HCO_3^- than in its absence ($P < 0.05$). The data were obtained from experiments like those shown in Fig. 9.

mediated by another transport mechanism. Although the absolute values for backregulation do not differ significantly whether CO_2/HCO_3^- is present or not (probably due to the increased buffering power of CO_2/HCO_3^- containing cells), there is a difference in the relative contribution of the amiloride-sensitive Na^+/H^+ exchanger.

The second identified transport process is an amiloride-insensitive, DIDS-sensitive Na^+-HCO_3^- cotransport which is responsible for the marked amiloride-insensitive pH_i backregulation in the presence of CO_2/HCO_3^-. Kinetic analysis revealed that this transport is less potent in acid extruding than Na^+/H^+ exchange (V_{max} = 0.19 pH units/min for the amiloride-insensitive pH_i recovery and V_{max} = 0.57 pH units/min for the amiloride-sensitive pH_i recovery). However, comparison of the K_m values suggests that its affinity for sodium seems to be higher than that of Na^+/H^+ exchange.

The described amiloride-sensitive and amiloride-insensitive acid extruding mechanisms were also observed when the ciliary muscle cells were acidified by removal of extracellular sodium (Figs. 9 and 10). Readdition of sodium after its withdrawal for 6 min led to a rapid backregulation of pH_i. The initial pH_i recovery rates in the "controls" were much higher than those observed during the pH_i recovery after acidification by NH_4Cl prepulse (Figs. 3 and 10). This might be due to a higher degree of acidification being induced by extracellular sodium removal (Table 3) than by NH_4Cl prepulse (pH_i = 6.50 ± 0.04, n = 5 after NH_4Cl prepulse in the presence of CO_2/HCO_3^- and pH_i = 6.42 ± 0.06, n = 5 after NH_4Cl prepulse in the absence of CO_2/HCO_3^-). A higher inwardly directed sodium gradient achieved by sodium depletion when extracellular sodium was removed for 6 min might also contribute to this higher pH_i recovery rate. Not only a larger inwardly directed driving force for sodium but also an activation of the transporters by a low intracellular sodium concentration (similar to the H^+-induced activation) may contribute to the faster pH_i backregulation rate. Unexpectedly, amiloride and DIDS alone did not significantly reduce the pH_i recovery rates after sodium removal for 6 min (Fig. 10). Under such conditions (severe intracellular acidosis and intracellular sodium depletion) amiloride-sensitive and amiloride-insensitive transporters can thus replace each other in regulating pH_i and intracellular sodium concentration. However, simultaneous application of both inhibitors had a significant effect (Fig. 10). Although a bicarbonate-dependent acid extruding

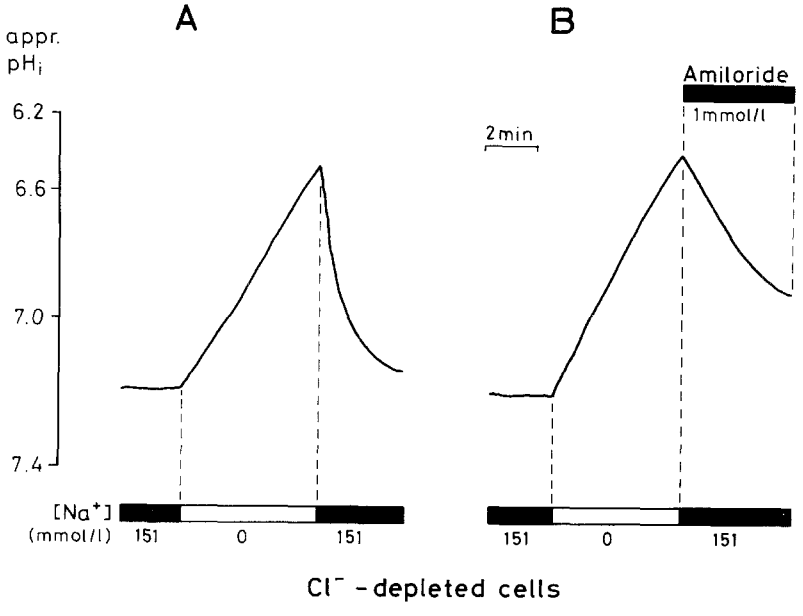

Fig. 11. The experiments were performed in CO_2/HCO_3^--buffered solution in the absence of extracellular chloride. Prior to the experiment, cells were preincubated in chloride-free solution for at least 1 hr. Even under these conditions, sodium removal induced an intracellular acidification which was reversed when sodium was restored (*A*). We could still observe an amiloride-insensitive pH_i recovery in chloride-depleted cells (*B*). Chloride was replaced by cyclamate.

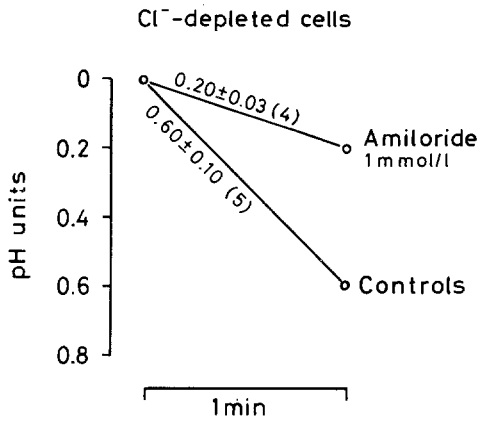

Fig. 12. Summary of nine experiments similar to those shown in Fig. 11*A* and *B*. The 1-min pH_i recovery induced by readdition of extracellular sodium after removal for 6 min was investigated in chloride-depleted cells (preincubation in chloride-free solution for at least 1 hr) in both the presence and absence of 1 mmol/liter amiloride. Amiloride inhibited the pH_i recovery. However, we still observed a chloride-independent, amiloride-insensitive recovery.

mechanism is operative besides the Na^+/H^+ exchanger in bicarbonate containing media, we did not observe a significant difference in the pH_i backregulation in the presence or absence of bicarbonate (Fig. 10). This can be explained by increased cytoplasmic buffering power in the presence of the CO_2/HCO_3^- buffer.

Other authors have attempted to distinguish between chloride-dependent and chloride-independent Na^+-HCO_3^- cotransport by preincubating the cells in chloride-free medium (Jentsch et al., 1988; Helbig et al., 1989; Townsley & Machen, 1989; Kahn et al., 1990; Kikeri et al., 1990*a*; Muallem & Loessberg, 1990). The preincubation times are between 10 min and 4 hr. The presence of chloride-independent Na^+-HCO_3^- cotransport was suggested when an amiloride-insensitive acid extrusion was observed after preincubation in chloride-free solution for 20 min (Muallem & Loessberg, 1990), 30 min (Townsley & Machen, 1989), 1 hr (Jentsch et al., 1988), 2 hr (Kahn et al., 1990) and 4 hr (Kikeri et al., 1990*a*). In our experiments we observed an amiloride-insensitive acid extrusion after chloride depletion for 1 hr. Furthermore, the backregulation after an acid load could still be inhibited by DIDS after preincubating cells in chloride-free medium for 4 hr. Thus we suggest that part of the amiloride-insensitive pH_i backregulation is mediated by chloride-independent Na^+-HCO_3^- transport. This is also supported by the results from Helbig et al. (1989), who could almost completely block the amiloride-insensitive pH_i backregulation in ciliary epithelial cells by removal of extracellular chloride for 15 min. Our data obtained from measurement of membrane voltage (Stahl et al., 1992) suggest that this transport is electrogenic, carrying one sodium ion together with two or more bicarbonate ions.

Quantitative comparison of the amiloride-insensitive recovery rates in chloride-containing (Figs. 3 and 10) and chloride-depleted cells (Figs. 8 and 12) revealed a slight (but not significant) decrease in chloride-depleted cells after NH_4Cl prepulse (Figs. 3 and 8). This decreased recovery rate was significant ($P < 0.05$) when the cells were acidified by removal of extracellular sodium (Figs. 10 and 12) instead of NH_4Cl prepulse. Thus, we conclude that both chloride-independent and chloride-dependent Na^+-HCO_3^- cotransport contribute to amiloride-insensitive acid extrusion in cultured human ciliary muscle cells.

In summary, our data provide evidence for at least three acid-extruding mechanisms in cultured human ciliary muscle cells. An amiloride-sensitive Na^+/H^+ exchanger and probably two different amiloride-insensitive, but DIDS-sensitive Na^+-HCO_3^- cotransporters.

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