Preincubation in acid medium increases Na/H antiporter activity in cultured renal proximal tubule cells

(cytoplasmic pH/cycloheximide/acidosis/fibroblast)

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ABSTRACT Chronic acidosis *in vivo* leads to an increase in proximal tubule Na/H antiporter activity that persists when the transporter is studied out of the acidotic environment. It is presently not clear whether a decrease in extracellular fluid pH alone is sufficient to elicit this adaptation. The present studies examined the effect of acid preincubation on Na/H antiporter activity in cultured proximal tubule cells. Antiporter activity was examined after a 2-day preincubation in control or acid medium, 1 hr after removal from the preincubation fluid. Na/H antiporter activity was assayed as the initial rate of Na-dependent alkalization after cell acidification in the absence of CO₂/HCO₃. Preincubation in low [HCO₃] acid medium or in high PCO₂ acid medium led to increases in amiloride-sensitive Na/H antiporter activity. This adaptation was inhibited by addition of cycloheximide to the preincubation medium. Preincubation of fibroblasts in low [HCO₃] acid medium did not lead to increased Na/H antiporter activity but rather caused a small inhibition. These studies demonstrate an adaptation in Na/H antiporter activity elicited by a low pH of the extracellular fluid, which is dependent on protein synthesis, and may be unique to certain H/HCO₃-transporting epithelia.

Chronic metabolic acidosis is associated with an increase in renal net acid excretion. One component of this response is an increase in the activities of the proximal tubule apical membrane Na/H antiporter and basolateral membrane Na/3HCO₃ symporter, the major proximal tubule H/HCO₃ transporter mechanisms (1–5). The transporter effects appear kinetically as V_{max} effects and persist when the transporter is studied outside the acidotic milieu.

Because these adaptations in transporter activity have only been elicited *in vivo*, it is difficult to determine the initiating extracellular signal. Changes in extracellular fluid pH may lead to changes in hormone levels, renal nerve activity, and renal hemodynamics, any of which could be responsible for increased transporter activity. In addition, it is difficult to study *in vivo* the intracellular signals that effect these adaptations. The purpose of the present studies was to examine whether decreases in extracellular fluid pH directly cause an adaptation in the Na/H antiporter of cultured proximal tubule cells that persists when the cells are removed from the acid milieu.

METHODS AND MATERIALS

Cell Culture. Renal proximal tubule cells were isolated from the kidneys of young male rabbits and placed in primary culture using an approach similar to that described (6–9). Male New Zealand rabbits (4–7 weeks old) were sacrificed and kidneys were aseptically removed. Cortex was trimmed from kidneys and sliced with a Stadie–Riggs tissue slicer.

Cortical sections were incubated with 0.1% collagenase for 40 min and then washed. The tissue was centrifuged on a Percoll gradient [50% (vol/vol) Percoll in a Beckman JA-20 rotor and J2-21-M centrifuge] at 20,000 × *g* for 30 min. Tubules aspirated from fraction F4 (9) were resuspended and centrifuged to wash out the Percoll. The suspension was then diluted by culture medium to achieve a concentration of 3000 tubules per ml, inoculated onto glass coverslips in 35-mm culture dishes (Corning), and incubated at 37°C in a 95% air/5% CO₂ atmosphere. Culture medium consisted of a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium supplemented with insulin (5 µg/ml), 50 nM hydrocortisone, transferrin (35 µg/ml), 29 nM sodium selenite, 20 µM ethanolamine, 1.5 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). Fetal bovine serum was added to 3% (vol/vol) to the culture medium for the first 3 days to allow better cell attachment. After achieving confluence (8–12 days), cells were rendered quiescent by removal of insulin and hydrocortisone for 48 hr prior to and during preincubation (10).

Cultured fibroblasts were derived from human foreskin and used in passages 5–7 (11). Fibroblasts were grown on glass coverslips in DMEM supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum. Fibroblasts were incubated with a reduced concentration of fetal bovine serum (1%) for 24 hr prior to and during preincubation.

Measurement of Intracellular pH and Na/H Antiporter Activity. Continuous measurement of cytoplasmic pH (pH_i) was accomplished using the intracellularly trapped pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5-(and -6)carboxyfluorescein (BCECF acid). Cells were loaded with the acetoxymethyl ester of BCECF (10 µM) for 40 min at 37°C. After washing, the coverslip was placed in a plastic cuvette in a computer-controlled spectrofluorometer (SLM 8000C) at a 30° angle to the excitation beam. pH_i was estimated from the ratio of fluorescence with excitation at wavelengths of 500 nm and 450 nm with emission at 530 nm (12). Slit widths were 4 nm. Background fluorescence was measured before dye loading and subtracted from fluorescence intensity at each excitation wavelength. Background fluorescence was less than 0.1% of the total fluorescence at both wavelengths. Calibration of the BCECF excitation ratio was accomplished using the nigericin technique as described (12). A linear response was obtained from pH 6.3 to pH 7.6. Cells were continuously superfused at 20 ml/min by solutions at 37°C.

Na/H antiporter activity was assayed as the initial rate of Na-dependent pH_i increase after an acid-load by NH₃/NH₄⁺

Abbreviations: pH_i, cytoplasmic pH; BCECF, 2',7'-bis(2-carboxyethyl)-5-(and -6)carboxyfluorescein; NS, not significant.

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prepulse in the absence of CO_2/HCO_3 (13). For this assay, Hepes-buffered standard solution contained 138 mM Na, 4.5 mM K, 1.1 mM Ca, 1.5 mM Mg, 147.7 mM Cl, and 15 mM Hepes. In Na-free solutions, Na was replaced with choline. In Na-free $\text{NH}_3/\text{NH}_4^+$ solutions, 20 mM NH_4^+ replaced 20 mM choline. All solutions were adjusted to pH 7.4 with *N*-methyl-D-glucammonium hydroxide at 37°C. Cells were first perfused with the Na-containing solution, and baseline pH_i was measured. Cells were then perfused with a Na-free solution containing 20 mM NH_4^+ for 5 min. Upon removal of NH_4^+ , pH_i decreased and reached a stable plateau. Subsequent addition of Na caused a rapid pH_i recovery that was inhibited by amiloride and due to the Na/H antiporter. The initial rate of this Na-dependent pH_i change (dpH_i/dt) was calculated by drawing a tangent to the initial deflection (over 20 sec).

The pH_i decrease caused by the removal of NH_4^+ (ΔpH_i) was used to calculate the cell buffer capacity (β), using the formula:

$$\beta = \frac{[\text{NH}_4^+]_i}{\Delta\text{pH}_i},$$

where $[\text{NH}_4^+]_i$ was calculated from the pH_i just prior to $\text{NH}_3/\text{NH}_4^+$ removal, the extracellular $[\text{NH}_4^+]$, and the extracellular pH (14).

For the measurement of pH_i during preincubation, cells were bathed in CO_2/HCO_3 -containing solutions similar to the preincubation culture media but without phenol red and vitamins.

All chemicals were obtained from Sigma unless otherwise noted as follows: collagenase (type I) from Boehringer; Percoll from Pharmacia; penicillin and streptomycin from Whittaker (M.A. Bioproducts); culture media from GIBCO; and BCECF from Molecular Probes.

Statistics. Data are reported as mean $\pm$ SEM. Statistical significance was assessed using the unpaired Student's *t* test. In all studies, control and experimental cells were from similar culture preparations and were assayed on the same day.

RESULTS

Preincubation in Acid Medium (Low $[\text{HCO}_3]$) Increases Na/H Antiporter Activity. Na/H antiporter activity was compared in cells that had been preincubated either in control or acid medium for 2 days. Acid medium for the first set of studies was prepared by titrating the control medium with sufficient HCl to decrease the concentration of HCO_3 by 50%. Medium pH was decreased by 0.32 pH unit (pH 7.41 vs. 7.09). Cells incubated in acid medium had a detectably lower pH_i than control cells at 4 hr ($\Delta\text{pH}_i = -0.29 \pm 0.06$; $P < 0.005$) and 8 hr ($\Delta\text{pH}_i = -0.15 \pm 0.04$; $P < 0.02$) of incubation, which returned toward control levels by 48 hr of incubation [$\Delta\text{pH}_i = -0.06 \pm 0.01$; not significant (NS)]. Na/H antiporter activity was assayed after cells had been removed from their respective preincubation medium for approximately 1 hr.

Fig. 1 shows a typical experiment. Addition of $\text{NH}_3/\text{NH}_4^+$ led to a cell alkalinization, which was followed by a cell acidification upon removal of $\text{NH}_3/\text{NH}_4^+$. Na/H antiporter activity, assessed as the initial rate of Na-dependent pH_i increase, was increased in acid-preincubated cells. These results are summarized in Fig. 2. Na/H antiporter activity was increased by 28% in cells preincubated in acid medium (0.36 ± 0.01 vs. 0.46 ± 0.03 pH unit/min, $P < 0.001$). Amiloride at 1 mM inhibited >85% of Na/H antiporter activity in these cells. In addition, when amiloride was present during the Na-induced cell alkalinization, the effect

FIG. 1. Effect of preincubation in low $[\text{HCO}_3]$ acid medium on Na/H antiporter activity: typical tracing. Cells were initially perfused with a Na-containing solution. At 1 min, Na was removed from the perfusate and 20 mM NH_4Cl was added, leading to a rapid cell alkalinization followed by a slow cell acidification. At 6 min, NH_4Cl was removed from the perfusate leading to a rapid cell acidification. When pH_i stabilized, Na was added leading to a rapid cell alkalinization that is mediated by the Na/H antiporter. As can be seen in the tracing, the rate of increase in pH_i was faster in cells preincubated in acid medium (---) than in cells preincubated in control medium (—). All solutions were free of exogenous CO_2/HCO_3 .

of preincubation in acid medium was eliminated (0.06 ± 0.01 vs. 0.05 ± 0.01 pH unit/min, control vs. acid; NS). Baseline pH_i measured in the absence of CO_2/HCO_3 , just prior to $\text{NH}_3/\text{NH}_4^+$ addition, was similar in the two groups (pH 7.46 ± 0.02 vs. 7.48 ± 0.02 , control vs. acid; NS). An increase in the initial rate of change in pH_i upon Na addition could be due to increased Na/H antiporter activity or decreased cell buffer capacity. Buffer capacities were similar in the two groups (21.2 ± 1.4 vs. 21.2 ± 1.1 mM/pH unit, control vs. acid; NS).

Preincubation in Acid Medium (High PCO_2) Increases Na/H Antiporter Activity. *In vivo* chronic respiratory acidosis has

FIG. 2. Effect of preincubation in low $[\text{HCO}_3]$ acid medium on Na/H antiporter activity in proximal tubule cells. Results are expressed as the initial rate of change in pH_i (dpH_i/dt) in response to Na addition. Open bars, rates in the absence of amiloride; solid bars, rates in the presence of 1 mM amiloride, added at the time of $\text{NH}_3/\text{NH}_4^+$ removal. (— amiloride: control, $n = 26$; acid, $n = 26$. + amiloride: control, $n = 7$; acid, $n = 7$.)

also been found by some investigators to cause an increase in Na/H antiporter activity (15–17). In the next series of studies, we performed experiments similar to those described above, with the exception that acid medium was identical to control medium, and acid cells were placed in a 10% CO₂/90% air incubator while control cells were placed in a 5% CO₂/95% air incubator. Medium pH was decreased by 0.28 pH unit in the high PCO₂ medium (pH 7.41 vs. 7.13). Once again, pH_i in acid cells was decreased compared to control cells at 4 hr of incubation ($\Delta\text{pH}_i = -0.15 \pm 0.04$; $P < 0.02$) but returned to values similar to that of control cells by 48 hr of incubation ($\Delta\text{pH}_i = -0.08 \pm 0.02$; NS).

Na/H antiporter was assayed 1 hr after removal from preincubation medium. Baseline pH_i measured prior to NH₃/NH₄⁺ addition in the absence of CO₂/HCO₃⁻, was 7.41 ± 0.02 in control cells and 7.43 ± 0.02 in acid-preincubated cells (NS). Na/H antiporter activity after 2 days of preincubation was increased in the cells incubated in acid medium (0.55 ± 0.02 vs. 0.62 ± 0.02 pH unit/min; $P < 0.02$; Fig. 3). Once again, when the assay was performed in the presence of amiloride, there was no effect of preincubation in high PCO₂ on the rate of Na-induced cell alkalization (Fig. 3). Buffer capacities were similar in the two groups (23.8 ± 1.2 vs. 22.8 ± 0.9 mM/pH unit, control vs. acid; NS).

Role of Protein Synthesis in the Antiporter Stimulation. The above studies demonstrate an adaptation in transporter activity that persists after the transporter has been removed from the acid milieu. To examine the role of protein synthesis in this adaptation, cycloheximide (2 $\mu\text{g}/\text{ml}$) was added to both control and acid media during the 2-day preincubation. A similar concentration of cycloheximide inhibited more than 90% of protein synthesis in A6 cells during a 6-hr incubation (18). For these studies, low [HCO₃⁻] acid medium was used because it was associated with a greater increase in Na/H antiporter activity. In the presence of cycloheximide, acid preincubation failed to stimulate Na/H antiporter activity [0.37 ± 0.03 ($n = 11$) vs. 0.34 ± 0.03 ($n = 12$) pH unit/min, control vs. acid; NS]. Buffer capacity was similar in the two groups (14.6 ± 0.9 vs. 14.6 ± 0.7 mM/pH unit, control vs. acid; NS).

In additional studies we examined the effect of actinomycin D during preincubation. However, with actinomycin D (0.05 $\mu\text{g}/\text{ml}$), cells sloughed from the plates. In chicken kidney cells, lower concentrations of actinomycin D were found to be ineffective in preventing uridine incorporation into RNA (19).

Preincubation in Acid Medium Does Not Increase Na/H Antiporter Activity in Fibroblasts. While the above effects on Na/H antiporter could represent an adaptation related to regulation of transepithelial transport in the proximal tubule,

FIG. 3. Effect of preincubation in high PCO₂ acid medium on Na/H antiporter activity in proximal tubule cells. Data are presented as in Fig. 2. (– amiloride: control, $n = 22$; acid, $n = 22$. + amiloride: control, $n = 4$; acid, $n = 4$.)

FIG. 4. Effect of preincubation in low [HCO₃⁻] acid medium on Na/H antiporter activity in fibroblasts. Data are presented as in Fig. 2. Amiloride concentration was 50 μM . (– amiloride: control, $n = 18$; acid, $n = 19$. + amiloride: control, $n = 5$; acid, $n = 6$.)

it was also possible that the adaptation was merely related to defense of pH_i and would occur in any cell possessing an Na/H antiporter. To examine this question, we performed similar experiments in cultured fibroblasts. Human foreskin fibroblasts were preincubated either in control or acid (low [HCO₃⁻]) medium. After 48 hr of incubation, baseline pH_i measured in the absence of CO₂/HCO₃⁻, prior to NH₃/NH₄⁺ addition, was 6.98 ± 0.02 in control cells and 6.95 ± 0.02 in acid-preincubated cells (NS). As shown in Fig. 4, Na/H antiporter activity was not increased in acid-preincubated fibroblasts, and in fact there was a tendency for decreased Na/H antiporter activity (0.65 ± 0.04 vs. 0.54 ± 0.04 pH unit/min, control vs. acid; $P = 0.06$). Amiloride (50 μM) inhibited most of Na/H antiporter activity in these cells and eliminated the difference between control and acid-preincubated cells (Fig. 4). Buffer capacities were similar between control and acid-preincubated cells (13.5 ± 0.8 vs. 14.1 ± 0.9 mM/pH unit, respectively; NS).

DISCUSSION

Chronic metabolic acidosis, chronic respiratory acidosis, chronic potassium deficiency, and chronic increases in glomerular filtration rate are associated with an adaptive increase in the activities of the apical membrane Na/H antiporter and the basolateral membrane Na/3HCO₃ cotransporter, the major H/HCO₃ transporters mediating proximal tubular HCO₃⁻ absorption (1–5, 15–17, 20–25). The altered activity of these transporters can be demonstrated after they have been removed from the altered *in vivo* milieu, indicating a change in the intrinsic properties of the transporters. Because all of these adaptations have been elicited *in vivo*, it has been difficult to determine the specific extracellular and intracellular signals that affect the adaptations. The purpose of the present studies was to develop an *in vitro* model to further examine these adaptations.

In theory, metabolic acidosis could lead to changes in proximal tubular transporter activities through changes in extracellular pH or through changes in hormone levels, renal nerve activity, or renal hemodynamics. The present studies demonstrate that decreases in extracellular fluid pH can directly lead to increases in Na/H antiporter activity. Na/H antiporter activity was assayed as the Na-dependent increase in pH_i after an acid load. This assay was performed in the absence of CO₂/HCO₃⁻ to eliminate any contribution from the Na(HCO₃)₂/Cl exchanger or the Na/3HCO₃ cotransporter (26, 27). The observation that amiloride inhibited most of the Na-dependent pH_i recovery in proximal tubule cells and in fibroblasts confirms that this goal was achieved.

Na/H antiporter activity was increased in proximal tubule cells preincubated in either low $[\text{HCO}_3^-]$ acid medium or high PCO_2 acid medium, indicating that the pH of the extracellular fluid is the key determinant rather than the $[\text{HCO}_3^-]$ or PCO_2 *per se*. While the demonstration of an adaptation in this setting proves that the adaptation can occur in the absence of hormones, renal nerves, and altered hemodynamics, it is possible that an additional component of the *in vivo* adaptation is mediated by these changes. In addition, autocrine factors released from the proximal tubule cells may also play a role. Kinsella *et al.* (3) found that normal levels of glucocorticoids were required for the *in vivo* acidosis-induced Na/H antiporter adaptation. In the present study, glucocorticoids were not required, suggesting that the *in vivo* glucocorticoid dependence may have been hemodynamic (decreased glucocorticoid levels lead to low glomerular filtration rates).

A change in transporter activity that persists after the transporter has been removed from the altered medium can be due to either covalent modification of the transporter, endo- or exocytosis, or changes in synthesis or degradation of the transporter. The present studies found that cycloheximide prevented the alteration in Na/H antiporter activity, suggesting a role for altered protein synthesis. However, these studies do not establish that the protein synthesized is the Na/H antiporter. It is equally plausible that a protein is synthesized that regulates Na/H antiporter activity through phosphorylation.

This "memory" effect in proximal tubular Na/H antiporter activity resembles in many ways long-term memory in *Aplysia* sensory neurons, where memory is mediated by a protein synthesis- and transcription-dependent effect on K-channel activity (28–30). In *Aplysia* sensory neurons, this effect is mediated by activation of the adenylyl cyclase/cAMP system. In the present studies, acid preincubation was associated with an early decrease in cell pH that returned toward normal as incubation continued. The role of cell acidification is not presently clear. However, the finding of increased transporter activity in K deficiency, which is associated with decreased pH_i (31) and increased extracellular pH, suggests that a decreased pH_i is a key signal. It is not presently clear how a decreased pH_i leads to transporter activation.

Fibroblasts in culture did not increase Na/H antiporter activity in response to preincubation in acid medium. In these studies, we found that pH_i measured in the absence of $\text{CO}_2/\text{HCO}_3^-$ was significantly lower in fibroblasts as compared to proximal tubule cells (pH 6.98 vs. 7.41–7.46). In the absence of $\text{CO}_2/\text{HCO}_3^-$, the major transport mechanism responsible for maintaining pH_i is the Na/H antiporter. Thus, this cell pH difference could be due to differences in the Na/H antiporter, differences in the regulation of Na/H antiporter activity, or differences in the rate of acid addition. Although control Na/H antiporter activities were similar in fibroblasts and proximal tubule cells (corrected for differences in buffer capacity), measurements were made at lower pH_i values in fibroblasts. Based on differences in amiloride sensitivity, Haggerty *et al.* (32) postulated the existence of two types of Na/H antiporters. Differences in the characteristics of the Na/H antiporter could explain the failure of fibroblasts to exhibit the adaptation in Na/H antiporter activity. Alternatively, fibroblasts could be deficient in any of the steps that mediate the effect of acid preincubation on antiporter activity.

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