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Na^+-H^+ exchange activity and cellular pH regulation in enterocytes isolated from chick small intestine

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Intracellular pH (pH_i) and Na^+/H^+ exchange activity have been examined in isolated chicken enterocytes using pH sensitive fluorescence dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), and in a nominally bicarbonate-free buffer. Under resting conditions the pH_i (7.18) was higher than that observed in the presence of the proton ionophore FCCP (6.98), indicating that $[H^+]$ is below the value predicted for electrochemical equilibrium across the plasma membrane, i.e., pH_i is regulated. Removal of extracellular Na^+ lowered pH_i by 0.28 units and subsequent addition of 80 mM Na^+ rapidly increased pH_i towards the control value. The acidification induced by Na^+-removal was prevented by 1 mM amiloride. After an intracellular acidification by exposure to 30 mM NH_4Cl during 5 min, the pH_i decreased from approx. 7.18 to approx. 6.86. Subsequent alkalinization of cells back to control pH_i was observed after addition of Na^+ or Li^+ but not TEA^+. Na^+-dependent recovery of pH_i after an acid-load was unaffected by valinomycin, and was 82% reduced by 1 mM amiloride. The inhibitory action of amiloride was abolished by 10 μM monensin. The initial rate of pH_i recovery from an acid-load following exposure to Na^+ exhibited simple saturation kinetics, with an apparent K_m of 12.5 mM Na^+ and maximum velocity of alkalinization of approx. 0.2 pH units · min^{-1}. The rate of pH_i recovery was inversely proportional to pH_i. The 'set point' for the exchanger is approx. 7.35. It is concluded that in chicken enterocytes the Na^+/H^+ exchange system is not quiescent at resting pH_i and, thus contributes to the maintenance of a steady-state pH_i at neutral or slightly alkaline levels.

Introduction

The plasma membranes of a wide variety of animal cells [1–4], including intestinal epithelial cells [5–13], contain a carrier-mediated transport system that brings about the transmembrane exchange of Na^+ and H^+. The Na^+/H^+ antiport appears to be involved in multiple cellular functions [1,2], such as regulation of intracellular pH (pH_i), control of cell volume, transepithelial transport of Na^+ and HCO_3^-, initiation of cell growth, and metabolic responses to hormones such as insulin. In addition, in the case of enterocytes, the brush-border membrane Na^+/H^+ antiport has been suggested to play an important role in maintenance of a microclimate pH gradient in the close vicinity of the cell surface, which seems to play a role in intestinal absorption, particularly for H^+-dependent transport systems such as transport of dipeptides [14,15].

Although there have been several studies demonstrating the presence of a Na^+/H^+ exchanger in chicken enterocytes [5,7,11], little is known about its regulation (i.e., control by pH_i) or its role in pH_i homeostasis in these cells.

The aim of the present work was to assess the ability of isolated chicken enterocytes to regulate pH_i and to determine the role of Na^+/H^+ exchanger in the regulation of pH_i. In addition some kinetic characteristics of the exchanger were studied.

Materials and Methods

Solutions. The following physiologic salt solutions were employed: (1) Standard solution (in mM): NaCl, 80; $CaCl_2$ 1; mannitol, 100; K_2HPO_4, 3; $MgCl_2$, 1; Tris-HCl (pH 7.4), 20; β-hydroxybutyrate, 0.5 and 1 mg/ml bovine serum albumin. β-Hydroxybutyrate is present as a passively transported nutrient.

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(2) Na^+-free solution: the same composition as the sodium medium except that the sodium chloride was replaced mole-per-mole with choline chloride.

(3) KCl solution contained (in mM): KCl, 150; $MgCl_2$, 1; $CaCl_2$, 1; Hepes, 20.

All solutions were adjusted to pH 7.4.

Isolation of enterocytes. 4–6-week-old Hubbard chickens were used. Cells were isolated following the method described by Kimmich [16]. Briefly, the animals were decapitated and a section of the mid intestine, about 25 cm long, was removed. The tissue was rinsed clean with ice-cold standard solution containing 0.1 mM DL-dithiothreitol (to reduce the mucus content of the preparation), everted, and opened longitudinally. Pieces 2–3 cm long were then transferred to standard solution containing 1 mg/ml hyaluronidase and incubated at 37 °C in a shaking water bath (70 cycles/min) for 20 min. After incubation, intestinal segments were transferred to hyaluronidase-free buffer where cells were released by gentle shaking with a plastic pipette tip. The cells were filtered through a cotton gauze and then through a 200 μm mesh nylon sieve before being washed twice with standard buffer by resuspension and centrifugation.

Cell viability was assessed by determining the fraction of the cell population able to exclude 0.2% trypan blue [17] and usually ranged from 60% to 75%.

Measurement of pH_i. pH_i was measured fluorimetrically using the pH-sensitive carboxyfluorescein derivate BCECF and all the experiments were carried out at 25 °C. The nonfluorescent and membrane-permeant acetoxymethyl ester of this compound (BCECF-AM) enters cells readily and cytosolic esterases cleave the ester bonds and form the anionic, non-permeant, fluorescent BCECF.

Isolated enterocytes were suspended in Ca^{2+}-free solution (cytocrit approx. 5%) and incubated with BCECF-AM (4 μM dissolved in DMSO), for 15 min, in a shaking water bath. Omission of Ca^{2+} minimized the activity of any carboxyesterase released from damaged cells [7]. After 15 min Ca^{2+} was restored to 1 mM and the incubation was continued for an additional 15 min. After loading, the cell suspension was diluted to half the original cell concentration with saline and centrifuged ($100 \times g$ for 5 min) to remove extracellular dye. Then the cell pellet was resuspended (cytocrit approx. 10%) in standard saline solution and kept at ice temperature until use. Immediately before each fluorimetric measurement, 100 μl of cells were rinsed twice with the appropriate solution by resuspension and centrifugation, the supernatant was aspirated and the cells resuspended in the same solution to a final volume of 2 ml. Fluorescence (excitation 500 nm, emission 530 nm, slitwidths 10 nm and 15 nm, respectively) was monitored in a thermostatically controlled acrylic cuvette in a Kontron SFM 25 spectrofluorimeter, equipped with a magnetic stirrer. The fluorescence signal was continuously displayed on a chart recorder. The initial pH_i change after an experimental maneuver is defined as the change in pH_i that occurred during the first minute.

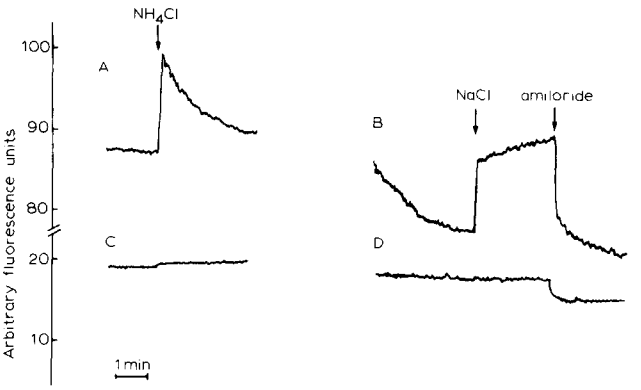

Fig. 1. Changes in fluorescence of BCECF at excitation wavelength of 500 nm or 450 nm in response to different modifiers. 200 μl of dye-loaded cells were rinsed twice with standard buffer by resuspension and centrifugation and the supernatant aspirated. Two cuvettes were prepared: in one cuvette fluorescence of BCECF was measured at 500 nm excitation (A, B) and in the other at 450 nm excitation (C, D). At the beginning of each trace the cells were suspended in Na^+-containing (A, C) or Na^+-free solution (B, D). Every one min the excitation wavelength was changed as well as the cuvette. At the times indicated by the arrows the following additions were made: 20 mM NH_4Cl, 80 NaCl and 1 mM amiloride. Each trace is representative of six experiments.

Cell autofluorescence was less than 2% of the total signal and no correction was applied for this component.

To avoid interferences due to dye leakage and photobleaching, pH_i should be calculated from the ratio of fluorescence intensity at 500 nm and 450 nm excitation. However, in parallel experiments we have observed that with exception of amiloride, the different experimental maneuvers caused very small changes in fluorescence intensity at 450 nm excitation. Amiloride decreased emission at both excitation wavelengths (see Fig. 1).

Calibration of fluorescence as a function of pH was made by the following protocol. At the end of each experimental procedure the pH_i and the extracellular pH (pH_o) were equilibrated by permeabilizing the cell membranes with 70 μM digitonin. The solution was then titrated with 10 μl of either 0.1 M HCl or 0.1 M KOH over the range of fluorescence values obtained during the experiment. By measuring the solution pH (WTW Microprocessor pH/ION meter, model PM 2,000 and electrode, model U402-M5) after each addition of acid or base, a calibration curve of fluorescence vs. pH_o was constructed for each individual experimental sample. A typical calibration of fluorescence intensity of BCECF vs. pH in digitonin-treated cells is shown in Fig. 2. Fig. 2 also shows that the fluorescence recorded under these conditions was not registered exclusively from a compartment which is inaccessible to rapid extracellular pH_o changes. Thus, addition of small

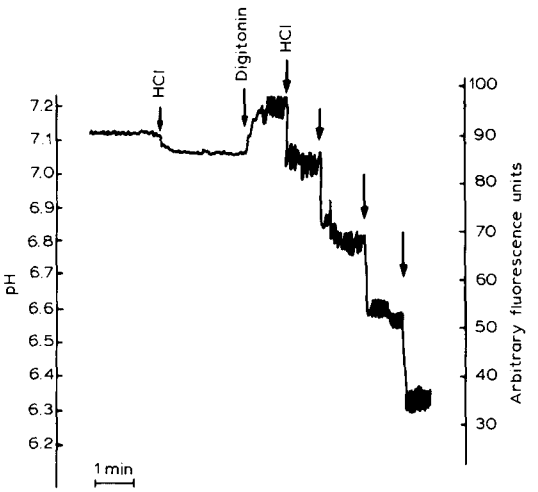

Fig. 2. pH_i calibration protocol. The rapid changes in fluorescence signal in response to changes in medium pH before addition of digitonin is assumed to be due exclusively to extracellular dye. The change in fluorescence intensity following addition of digitonin is assumed to represent total dye (intracellular plus extracellular).

aliquots of HCl caused instantaneous deflections in the signal suggesting that $10 \pm 0.1\%$ ($n = 4$ separate determinations) of the total dye was immediately accessible to the acid. This fraction (referred to as extracellular probe) of fluorescence could not be reduced by additional washing of the cells and probably represented binding of the dye to the cell surface or it was within very leaky cells in the population.

It has been reported [18–20] that the excitation spectrum of intracellular BCECF is red shifted compared with the spectrum of the extracellular dye. To assess whether in isolated chicken enterocytes the behavior of the dye inside the cell was similar to that in free solution, we performed four experiments in which we compared the excitation and emission spectra of intracellular and extracellular dye. We have observed that the excitation spectra (emission at 550 nm) of intracellular BCECF (peak at 502 nm) was red shifted about 5 nm compared with the extracellular dye (peak 497 nm). No differences were observed regarding the emission (peak at 530 nm, excitation at 500 nm) of intracellular and extracellular dye.

These observations indicate that the BCECF signal is best calibrated while the dye is inside the cells. Therefore, in some experiments the 'nigericin null-point determination' [21] and the digitonin-based calibration were performed in the same cell batch. For these experiments the cells were suspended in 150 mM potassium buffer, in the presence of the K^+/H^+ exchanging ionophore, nigericin (10 μM) and valinomycin (4 μM). Then the pH_o was titrated over the range of experimental fluorescence readings to obtain a fluorescence vs. pH calibration curve. Finally 70 μM digitonin was added to release the dye and another calibration curve was constructed. Fig. 3 shows that at any given value of pH_o the fluorescence signal from intracellular dye (KCl-nigericin-treated cells) was lower compared with extracellular dye (digitonin-treated cells). Consequently, the digitonin-derived pH_i values underestimated the actual pH_i values by about 0.15 pH units. Similar values have been reported for other cell types [9,18]. Therefore, a correction factor of 0.15 pH units was applied to the calibrations utilizing digitonin. As shown in Fig. 3, in both cases the ratio of fluorescence intensity was a linear function of pH at least between pH 6.6 and pH 7.4 ($r = 0.996$; $n = 10$, for both lines).

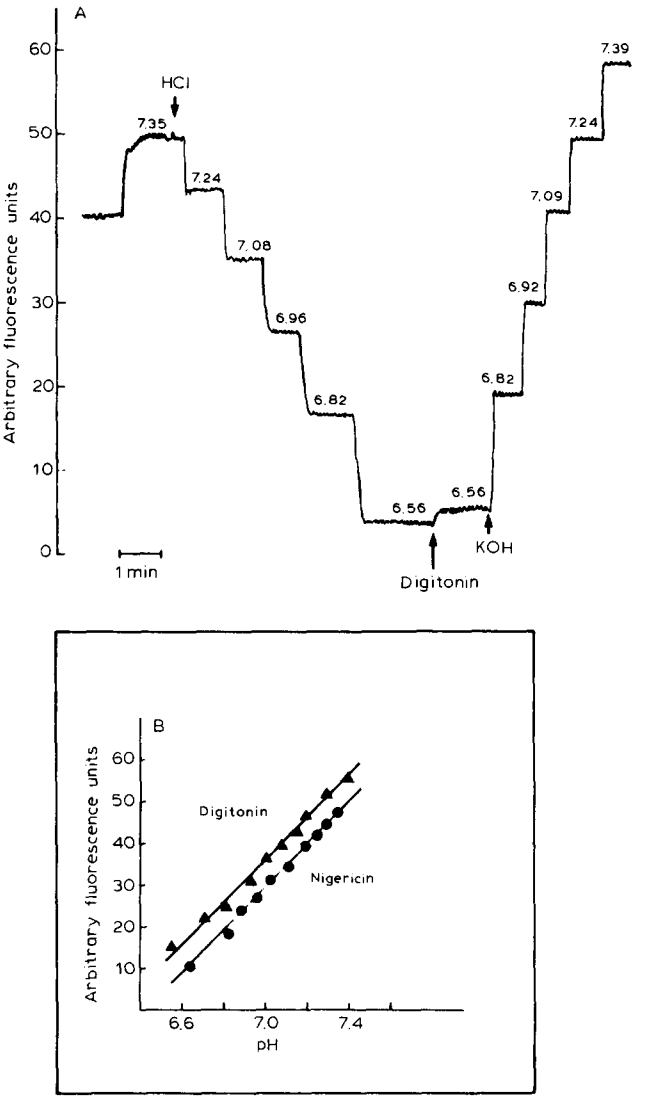

Fig. 3. Effects of addition of acid and base to nigericin- and digitonin-treated cells loaded with BCECF. (A) Dye-loaded cells were suspended in KCl-buffer at the beginning of the trace. Nigericin (10 μM) was added as shown and medium pH was lowered by addition of HCl. Then the cells were lysed with 70 μM digitonin and titration with KOH was carried out in steps. Trace is representative of six experiments. (B) Plot of fluorescence intensities vs. pH for nigericin- and digitonin-treated cells. Means of six experiments are shown. Standard errors of the means ranged from 0.3 to 0.5.

Dye leakage. The rate of BCECF leak out of the cells was quantified in six experiments by centrifuging (300 $\times g$ for 5 min) the suspension of dye-loaded cells at time intervals and measuring the fluorescence intensity in the supernatant. Over a period of 30 min, the leaked dye gave a signal that was less than 10% of the total signal (dye-loaded cells plus supernatant). Because the contribution of the dye leak to the total signal was small, this background was ignored.

Acid-loading. Cells were acidified by the ammonium chloride technique [22]. BCECF-loaded cells were incubated for 5 min at 25 °C in NaCl-solution containing 30 mM NH_4Cl. Cells were then centrifuged and washed quickly in NH_4^+- and Na^+-free buffer (choline substitution), before being resuspended in the appropriate solution.

Calculation of intracellular buffering power. The intrinsic buffering power (β_i) was measured using the NH_4^+ pulse as described by Roos and Boron [23]. The increase in pH_i after adding 20 mM NH_4Cl to control or ouabain-treated cells was used to calculate β_i. Cells were exposed to ouabain for 30 min.

Initial H^+ efflux rates (J_{H^+}) in nmol per min per mg protein were calculated according to the formula:

$$J_{H^+} = (dpH_i/dt)V\beta_t$$

where V is cell volume (3 μl/mg protein) as determined previously [24], dpH_i/dt was the rate of change of internal pH_i and β_t is the total intracellular buffering capacity (in mM/pH unit), i.e., the sum of the intrinsic intracellular buffering capacity β_i and the buffering capacity of intracellular HCO_3^- (β_{CO_2}). Since the current study was carried out in nominally HCO_3^--free solutions, β_{CO_2} was assumed to be negligible and β_t was taken to equal β_i.

Chemicals. Monensin, nigericin, valinomycin, digitonin and all the salts used in the current study were obtained from Sigma Chemical Co., St. Louis, MO. BCECF-AM from Molecular Probes (Eugene, OR), hyaluronidase and amiloride were purchased from Merck, Sharp and Dohme.

The BCECF-AM (1.45 mM), digitonin (14 mM), FCCP (2 mM) in DMSO, and monensin (4 mM), nigericin (2 mM), and valinomycin (1.4 mM) in ethanol, were stored for up to 30 days at −20 °C without loss in potency. Solvent concentration did not exceed 0.5% (v/v) and did not affect the fluorescence of BCECF.

Statistics. Results are expressed as mean ± S.E. Statistical significance was evaluated by the two-tailed Student's *t*-test for unpaired observations.

Results

Intracellular steady-state pH

BCECF-loaded cells equilibrated at 25 °C in bicarbonate-free buffer were first examined to measure the steady-state intracellular pH. Intracellular pH measurements in different preparations from different animals, were normally distributed around a mean value of 7.18 ± 0.005 (60 determinations).

To determine whether this value was more alkaline than would be expected if protons were distributed passively across the cell membrane, dye-loaded cells with a mean pH_i of approx. 7.18 were exposed to the proton ionophore FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone). Fig. 4 and Table I show that addition of 10 μM FCCP rapidly decreased pH_i by 0.20 pH_i units to a new steady-state pH_i value of approx. 6.98. This suggests that in the presence of a high proton conductance the pH_i shifts towards the electrochemical equilibrium. In the absence of FCCP, the K^+ ionophore, valinomycin, slightly decreased pH_i (0.03 pH_i units), and pH_i was further decreased following the addition of FCCP, indicating that the cells have enough H^+ permeability to respond quickly to changes in membrane voltage.

Intracellular buffering capacity (β_i) of chicken enterocytes

Intracellular buffering power (β_i) was evaluated from the pH_i changes produced by exposing the cells to a weak base [23]. Results of these experiments are displayed in Fig. 4 and Table I. As shown in Fig. 4, addition of 20 mM NH_4Cl led to a sudden intracellular alkalinization, followed by a decrease towards base line at an average rate of 0.05 ± 0.004 pH units/min. If the initial increase in pH_i due to the addition of NH_4Cl is used to calculate β_i, then β_i has a value of 65 ± 3 mM · pH unit^{-1} ($n = 21$). In ouabain-treated cells, β_i was 43 ± 3 mM · pH unit^{-1} ($n = 6$). β_i was also measured in cells acidified by the NH_4^+ pulse method [22], using a range (10–40 mM) of NH_4Cl concentrations to obtain different pH_i, and in the presence of ouabain. It was found that β_i increased (range 40–76 mM · pH

TABLE I

Effect of several modifiers on steady-state pH_i values and rate of pH_i change in cells incubated in standard saline solution

Data are the means ± S.E., n is the number of independent determinations. * $P < 0.001$, compared to control values (first column).

	n	pH_i		Rate (min^{-1})
		control	modifier	
FCCP (10 μM)	5	7.18 ± 0.02	6.98 ± 0.02 *	0.09 ± 0.01
Valinomycin (4 μM)	5	7.17 ± 0.02	7.14 ± 0.02	0.03 ± 0.01
[a] Valinomycin + FCCP	9	7.18 ± 0.02	6.92 ± 0.01 *	0.30 ± 0.01
[b] FCCP + valinomycin	9	7.18 ± 0.02	6.90 ± 0.01 *	0.30 ± 0.01
20 mM NH_4Cl	21	7.18 ± 0.02	7.45 ± 0.02 *	
Na^+-free buffer	8	7.18 ± 0.03	6.90 ± 0.02 *	0.13 ± 0.01

[a] Valinomycin was added first.
[b] FCCP was added first.

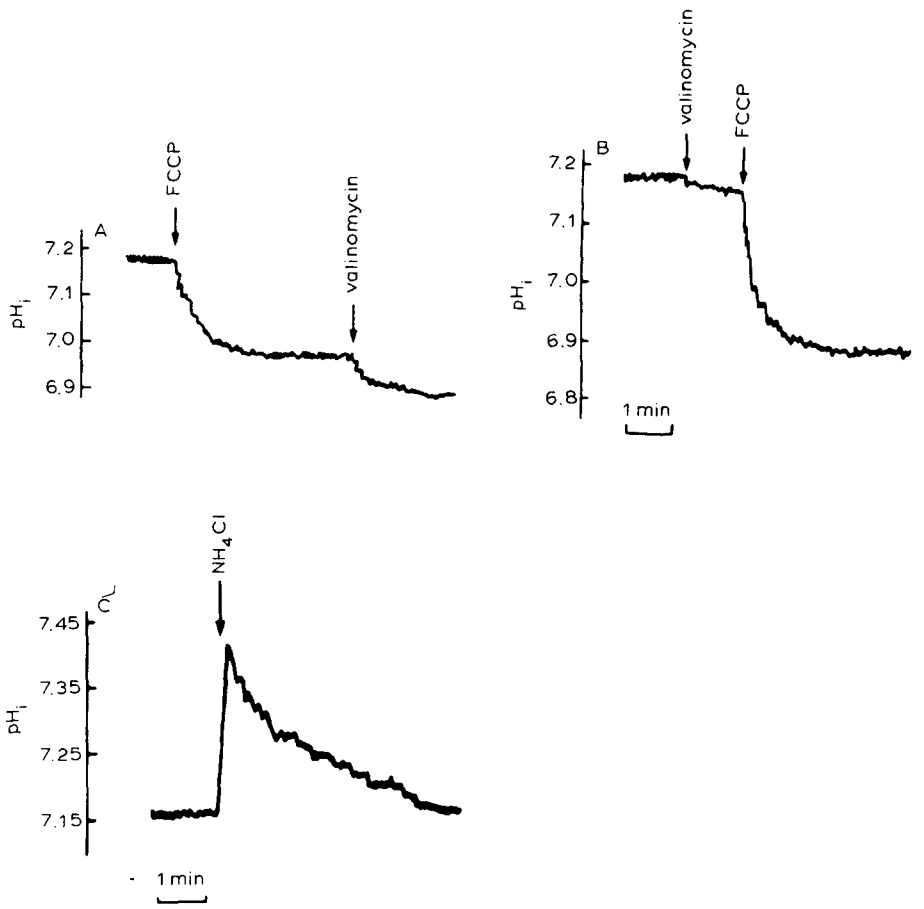

Fig. 4. Properties of resting pH_i of cells kept in standard solution. At the times indicated by the arrows the following additions were made to the cuvettes containing BCECF-loaded cells: 10 μM FCCP, 4 μM valinomycin (A, B), 20 mM NH_4Cl (C). Each trace is representative of 9 (A, B) or 21 experiments (C).

unit^{-1}) as the pH_i decreased (range: 7.22–6.55). The mean value for β_i was 59.6 ± 3 mM · pH unit^{-1} ($n = 22$). A weak ($r = 0.36$) inverse linear relationship between β_i and pH_i was observed

$$\beta_i = -20\,pH_i + 200$$

Na^+/H^+ exchanger activity and resting pH_i

To test whether the Na^+/H^+ antiport is normally functioning at the resting pH_i, we studied the effects of Na^+ removal on pH_i. In these experiments, dye-loaded cells incubated in NaCl containing buffer were suspended in Na^+-free medium (choline substitution) at the beginning of the fluorescence recording. This maneuver would reverse the ion gradient for Na^+ and an operational Na^+/H^+ exchanger in the membrane would produce a net influx of H^+. Fig. 5D and Table I show that removal of external Na^+ decreased pH_i by an average of 0.28 pH units, to a new steady-state value of approx. 6.90. Addition of 80 mM NaCl to the acidified cells led to a rapid alkalinization (see Fig. 5D and Table II).

Experiments with the electroneutral Na^+-H^+ ionophore monensin, clearly demonstrated that Na^+-removal reversed trans-membrane Na^+-gradient (Fig. 5 and Table II), since 10 μM monensin alkalinized cells incubated in Na^+-containing solution (Fig. 5B), but further acidified cells in Na^+-free solution (Fig. 5E). The acidification was promptly reversed when 80 mM NaCl was added back to the medium and the rate of pH_i recovery was faster in the presence than in the absence of monensin.

We also explored the effects of 1 mM amiloride, an inhibitor of Na^+-H^+ [25] on steady-state pH_i. We found that amiloride produced fluorescence quenching. As observed by Montrose and Murer [26], this quench was also seen in the absence of cells and precluded calculation of absolute intracellular pH. However, there was a change in the slope of the tracings (see Fig. 5C) which is not seen in the absence of cells, indicating that amiloride acidified the cells. Furthermore, amiloride prevented the

TABLE II

Effect of monensin and NaCl on steady-state pH_i and rate of pH_i changes in enterocytes suspended in Na^+-containing or Na^+-free buffer

Cells incubated in standard saline solution were suspended in Na^+-containing or Na^+-free buffer (choline substitution). Initial and final pH_i refers to the steady-state pH_i observed before and after the addition of the indicated modifiers (subsequent addition). Data are the means ± S.E., n is the number of independent determinations. * $P < 0.001$; ** $P < 0.005$; compared with initial pH_i (first column).

Suspending solution	Subsequent additions	n	pH_i		Rate (min^{-1})
			initial	final	
NaCl	Monensin (10 μM)	7	7.18 ± 0.02	7.28 ± 0.02 **	0.10 ± 0.01
Choline Cl	80 mM NaCl	8	6.88 ± 0.02	7.17 ± 0.02 *	0.17 ± 0.02
	Monensin	7	6.90 ± 0.01	6.80 ± 0.03 **	0.10 ± 0.02
	Monensin + NaCl	7	6.89 ± 0.02	7.22 ± 0.02 *	0.33 ± 0.01

cytosolic acidification induced by Na^+-free solutions (see Fig. 5F).

All these observations suggest that the Na^+/H^+ antiport is normally functioning at resting pH_i and operates reversibly depending on the Na^+ gradient.

Role of Na^+/H^+ exchange in pH_i recovery from an acid load

To further characterize the role of Na^+/H^+ antiport in pH_i homeostasis, enterocytes were acidified by the ammonium chloride prepulse method. Fig. 6A and Table III show that acid-loaded cells suspended in Na^+-containing solution recovered towards the steady-state pH_i value of approx. 7.18. Regulatory alkalinization was abolished if the acid-loaded cells were suspended in Na^+-free solution (choline substitution), but regulation was restored after addition of 80 mM NaCl or 80 mM LiCl to the medium (Figs. 6B and C). Since 80 mM TEACl did not elicit pH_i recovery (Fig. 6D), the effects of Na^+ and Li^+ were not due to changes in osmolality or ionic strength of the suspending medium.

We also investigated the effect of amiloride on pH_i regulation after an acid-load. The acid-loaded cells were suspended in Na^+-free medium plus 1 mM amiloride and incubated for 5 min before the fluorescence was

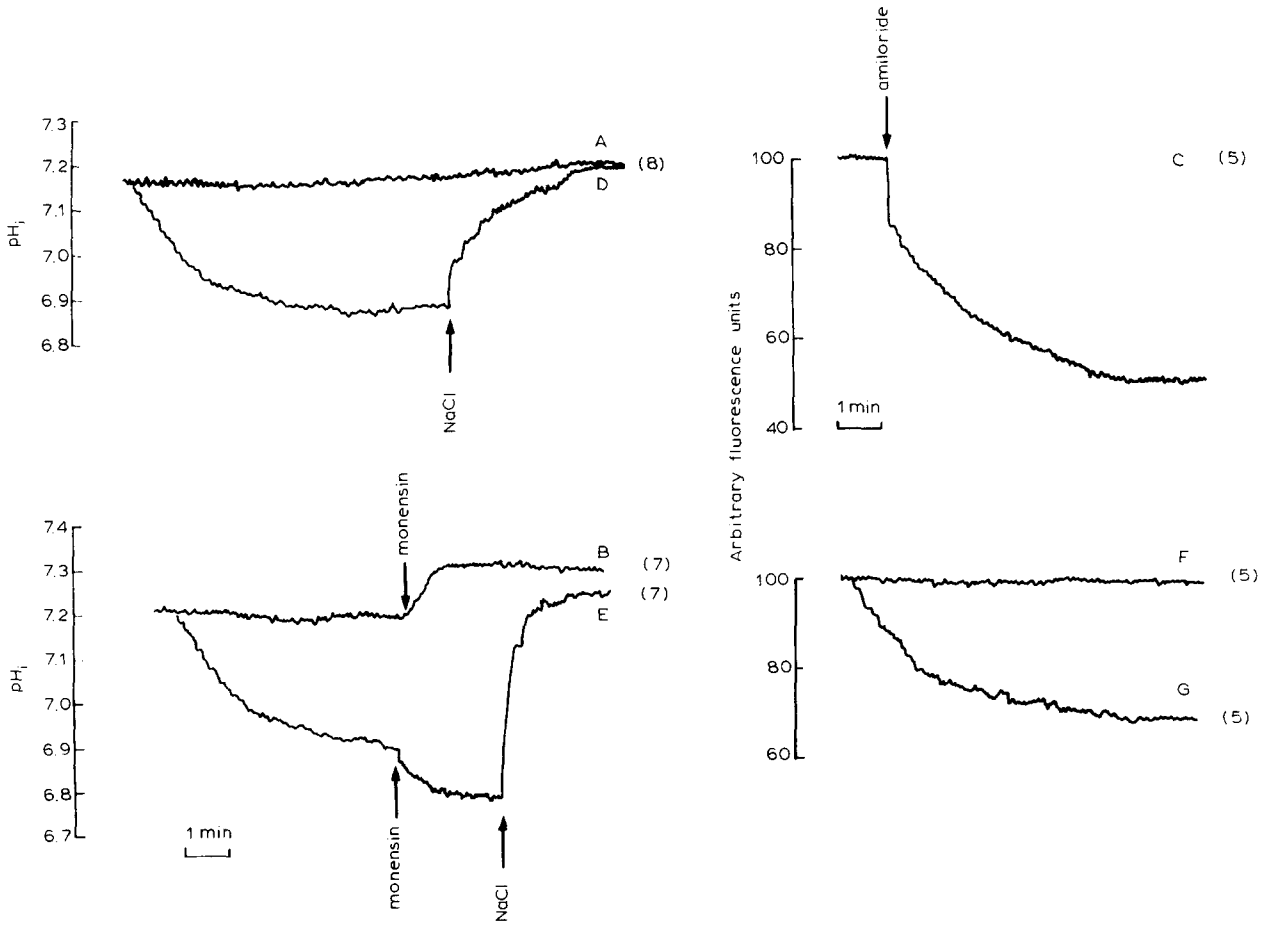

Fig. 5. Effects of Na^+ removal, amiloride and monensin on resting pH_i in isolated chicken enterocytes. At the beginning of each trace dye-loaded cells incubated in standard solution were suspended in Na^+-containing (A–C) or Na^+-free solution (D–G). At the times indicated by the arrows the following additions were made to the cuvettes: 80 mM Na^+, 10 μM monensin, 1 mM amiloride. In F amiloride-treated cells were suspended in Na^+-free buffer containing 1 mM amiloride. Each trace is representative of the number of similar experiments indicated between brackets.

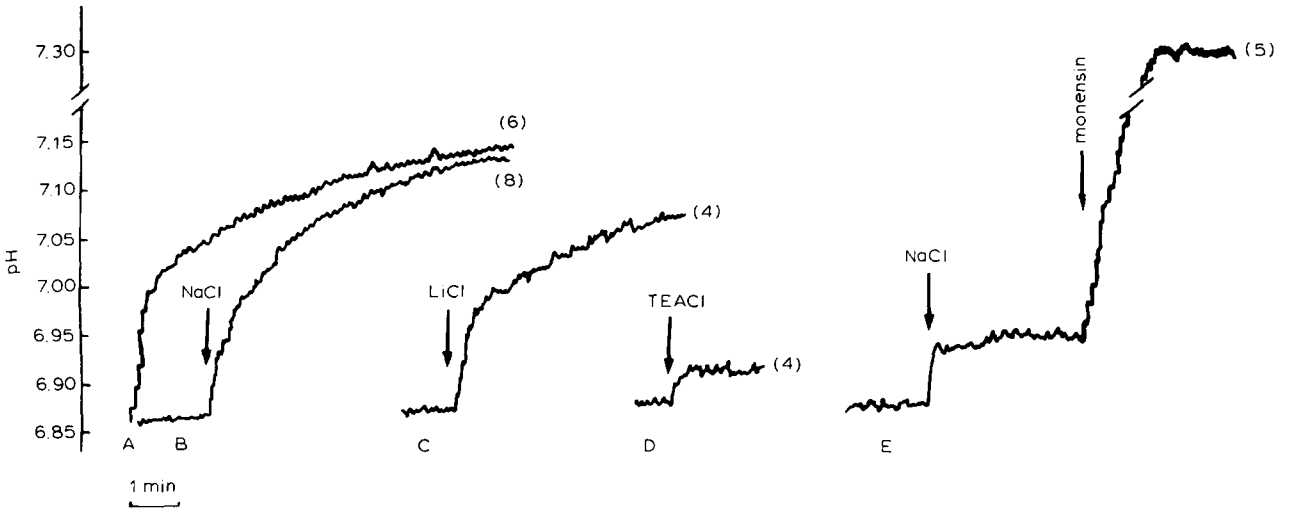

Fig. 6. pH_i recovery from acid load. Dye-loaded cells were incubated in NaCl-buffer containing 30 mM NH_4Cl for 5 min and washed in choline Cl-medium. In A, cells were suspended in NaCl-medium at the beginning of the trace. In B, C, and D cells were suspended in choline Cl-medium, and in E in choline Cl-medium containing 1 mM amiloride. At the times indicated by the arrows the following additions were made: 80 mM NaCl, 80 mM LiCl, 80 mM TEACl, 10 μM monensin. Each trace is representative of the number of similar experiments indicated between brackets.

recorded. Since, as mentioned above, amiloride causes a large fluorescence quenching, in each experiment the pH_i of acid-loaded, amiloride-treated cells was considered to be the mean pH_i value observed in acid-loaded cells suspended in Na^+-free buffer without amiloride, and the changes in pH_i induced by the modifiers were corrected accordingly. Fig. 6E and Table III show that the Na^+-depending regulation of pH_i was largely prevented (82% inhibition) by 1 mM amiloride and the amiloride block was overcome by 10 μM monensin.

To test if the pH_i-recovery mechanism is voltage sensitive, we explored the effect of valinomycin (4 μM). The rate of pH_i recovery following the addition of 80 mM NaCl was not modified by the presence of valinomycin.

These observations may suggest that the Na^+/H^+ exchanger plays an important role in pH_i recovery from an acid load and that the system is electroneutral.

Kinetics of Na^+/H^+ exchange

For these experiments cells were acidified as indicated above, by the ammonium chloride prepulse method. Acid-loaded cells were suspended in Na^+-free medium, and then Na^+ was added at increasing concentrations. The results of these experiments are displayed in Table IV. The pH_i achieved upon acid-loading was not significantly different among different cell batches and ranged from 6.94 to 6.96. Only one Na^+ concentration was tested in each cell batch. The relationship between extracellular Na^+ and the initial rate of alkalinization is depicted in Fig. 7. The increment in the rate of cytosolic alkalinization as the concentration

TABLE III

Steady-state intracellular pH values and rate of pH_i changes in cells acidified by NH_4Cl pulse

Data are the means ± S.E., n is the number of determinations. Initial and final pH_i refers to the pH_i values before and after addition of the indicated salt (80 mM), respectively. The concentration of amiloride was 1 mM and that of monensin 10 μM. * $P < 0.001$, compared with initial pH_i (first column).

Suspending solution	Subsequent additions	n	pH_i initial	pH_i final	Rate (min^{-1})
NaCl-buffer	none	6	6.85 ± 0.02	7.18 ± 0.02 *	0.15 ± 0.01
Choline-buffer	NaCl	8	6.85 ± 0.03	7.17 ± 0.03 *	0.15 ± 0.02
Choline-buffer	LiCl	4	6.86 ± 0.02	7.15 ± 0.04 *	0.11 ± 0.05
Choline-buffer	TEACl	4	6.87 ± 0.03	6.92 ± 0.04	0.05 ± 0.004
Choline-buffer + amiloride	NaCl	8	6.87 ± 0.05	6.92 ± 0.05	0.05 ± 0.009
Choline-buffer + amiloride + monensin	NaCl	5	6.86 ± 0.07	7.30 ± 0.04 *	0.45 ± 0.10

TABLE IV

Na^+-dependent changes of pH_i after acid load by NH_4^+ prepulse

$[Na^+]_o$ is the extracellular Na^+ concentration added to cuvettes containing acid-loaded cells. Initial and final pH_i are the pH_i values observed before and after Na^+ addition, respectively. Data are mean values ± S.E., n is the number of independent determinations.

$[Na^+]_o$ (mM)	n	pH_i initial	pH_i final	Rate (min^{-1})
5	6	6.94 ± 0.02	6.99 ± 0.02	0.05 ± 0.009
10	6	6.96 ± 0.02	7.05 ± 0.02	0.09 ± 0.006
20	6	6.96 ± 0.03	7.07 ± 0.03	0.11 ± 0.012
40	6	6.94 ± 0.02	7.10 ± 0.02	0.16 ± 0.005
60	6	6.96 ± 0.02	7.13 ± 0.03	0.17 ± 0.013
80	6	6.96 ± 0.03	7.16 ± 0.03	0.20 ± 0.016

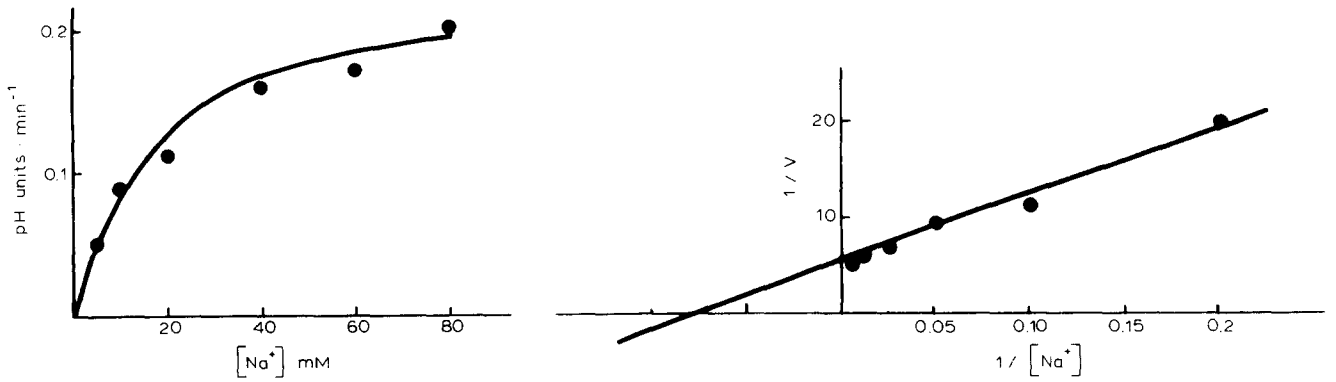

Fig. 7. Na^+-dependency of the rate of alkalinization after NH_4Cl acid load. Same conditions as in Fig. 6 except NaCl concentration varied between 0 and 80 mM. (A) The initial increment in intracellular pH on addition of different Na^+ concentrations is plotted vs. Na^+ concentration. Each point is the mean ± S.E. of six experiments. (B) Lineweaver-Burk plot of the data represented in A. Line was calculated by linear regression analysis, $r = 0.97$.

of external Na^+ increased showed simple saturation kinetics (Michaelis-Menten). The double-reciprocal plot of the data is shown in Fig. 7. The values fit a straight line giving an apparent transport constant, K_m, for Na^+ of 12.5 ± 1 mM and a maximal rate of alkalinization, V_{max}, of 0.2 ± 0.02 pH units · min^{-1}.

Relationship between pH_i and Na^+/H^+ exchanger activity

It has been reported [1,2] that, in a variety of cells, the activity of the Na^+/H^+ exchanger exhibits a threshold or set-point at or near physiological pH_i. Below this pH_i threshold the activity of the exchanger is increased.

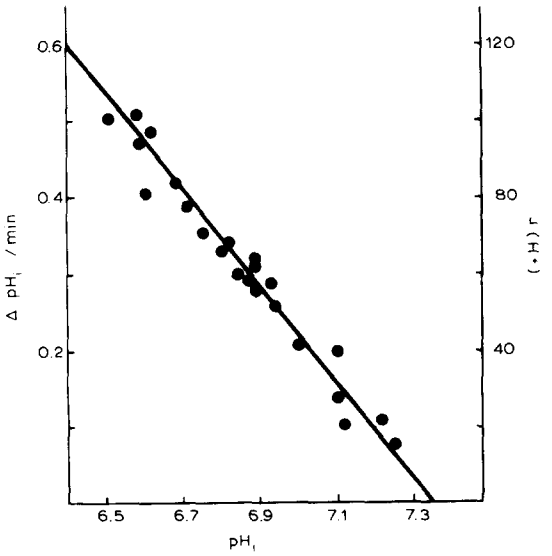

Fig. 8. Relationship between initial rate of pH_i recovery and initial pH_i. Same conditions as in Fig. 6 except NH_4Cl concentration prepulse was 0, 15, 20, 25, 30, or 40 mM. J_{H^+} (nmol H^+/min per mg) was calculated as described in Materials and Methods. Four different experiments were performed.

To study the dependence of Na^+/H^+ exchange activity on pH_i, dye-loaded cells were acidified by the ammonium prepulse method. Different concentrations of NH_4Cl (ranging from 10 to 40 mM) were used to obtain different pH_i. Cells were washed and resuspended in Na^+-free buffers and the rate of Na^+-dependent alkalinization was measured. The results are displayed in Fig. 8 and show that as the initial pH_i rose the initial rate of alkalinization slowed down, and that the data are compatible with an apparent linear relationship ($r = 0.98$, $n = 24$) between these two variables. Results are also expressed as changes in net H^+ efflux (J_{H^+}). J_{H^+} was calculated as described in Materials and Methods. β_i was calculated, for each pH_i, from the empirically derived formula given above. The extrapolated pH_i, at which no measurable alkalinization occured (set-point) was about 7.35. When a constant β_i value was used an inverse relationship ($r = 0.98$) between J_{H^+} and pH_i was again observed (not shown) and the pH_i at which J_{H^+} was estimated to equal zero was 7.38.

Discussion

Several reports have already demonstrated the presence of a Na^+/H^+ exchanger in intestinal epithelial cells [6,8–10,12,13], including chicken enterocytes [5,7,11]. In the present study the kinetics of Na^+/H^+ antiport and its role in pH_i homeostasis in chicken enterocytes have been evaluated by using a fluorimetric method.

The current work was conducted in the nominal absence of HCO_3^- because this species may mask Na^+-dependent pH_i changes by way of HCO_3^--dependent transport mechanisms [2] that may also be present.

The results show that cytoplasmic pH of chicken enterocytes is close to 7.0 when the cells are suspended in nominally HCO_3^--free solution. This value agrees

with available data of pH_i of most animal cells [23] including chicken [7,11], rabbit [12] and rat [8] enterocytes.

With exception of most red blood cells [2,27], the activity of H^+ in the cytosol of animal cells is lower than would be predicted based on extracellular pH and transmembrane potential (E_m). We are not aware of reports of E_m in avian enterocytes. Using TPP^+ (tetraphenylphosphonium) as a sensor of the membrane potential, we have observed that E_m of chicken enterocytes is around -48 mV (unpublished observation). Electrophysiological studies have shown that the E_m of mammalian enterocytes is around -35 mV [28,29]. If protons were passively distributed across the cell membrane, then with a pH_o of 7.4 and an E_m of -35 mV or -48 mV, the pH_i at equilibrium would be approx. 6.83 or 6.62, respectively. However, the resting pH_i measured was 7.18. Furthermore, pH_i observed in the presence of FCCP (6.98) or in Na^+-free buffer (6.90) was closer to the predicted equilibrium pH_i. All these observations indicate that chicken enteroctyes also maintain a cytosolic H^+ activity below electrochemical equilibrium and therefore, that pH_i is regulated.

The intracellular buffering capacity (β_i) of chicken enterocytes was evaluated by titration with NH_3 [23]. The results show that β_i of cells incubated in NaCl-buffer (pH_o 7.4) has a value of approx. 65 $mM \cdot pH^{-1}$, which is similar to that observed in rat enterocytes [8] and within the range of values measured for other tissues [23]. In the presence of ouabain β_i was 43 $mM \cdot pH^{-1}$. This would indicate that in control cells β_i was overestimated and that the initial rise in pH_i was not due solely to the entry of NH_3. It was also observed that β_i increases with decreasing pH_i. This finding has been previously reported in other cell types [23,30].

Na^+/H^+ exchanger and pH_i regulation

As indicated above chicken enterocytes exhibit a resting pH_i value which is above proton electrochemical equilibrium, and experiments with valinomycin showed that the membrane is slightly permeable to H^+ equivalents. These findings imply that in order to maintain pH_i in the physiological range an active or secondary active transport mechanism must be present to extrude H^+ equivalents against their electrochemical gradient. The following observations indicate that the Na^+/H^+ antiport may serve this function: (1) Na^+-removal under resting conditions led to an intracellular acidification, which recovered following readdition of Na^+. (2) Amiloride acidified the resting cells and prevented the acidification observed in Na^+-free conditions. (3) The steady-state pH_i observed in Na^+-free conditions (approx. 6.90) was close to the calculated pH_i at electrochemical equilibrium (approx. 6.83). That Na^+-H^+ is operative under resting conditions is not a general characteristic of animal cells. It has been observed in rat hepatocytes [30], frog skin epithelium [31] and cultured ciliary epithelium [32]. However, other studies [26,33,34] revealed no effect of amiloride or Na^+-removal on steady-state pH_i despite the presence of a Na^+/H^+ exchanger, and concluded that Na^+/H^+ antiport is quiescent under resting conditions.

The present study also provides evidence for a role of Na^+/H^+ antiport in pH_i recovery from an acid-load. This conclusion is drawn from the following observations: (1) After the cells were acid-loaded by the NH_4^+ prepulse method, pH_i did not recover in the absence of Na^+ in the external medium. (2) The acid-loaded cells regulated the pH_i back to control levels in the presence of Na^+ or Li^+, but not in the presence of TEA. (3) The Na^+-dependent realkalinization was largely inhibited by 1 mM amiloride. (4) The effect of amiloride was overcome by monensin.

As reported for other cell types [1,2] the Na^+/H^+ exchange system present in chicken enterocytes appears to be electroneutral because the rate of Na^+-dependent realkalinization was unaffected by valinomycin in the putative presence of a K^+ gradient.

We have also studied some of the kinetic characteristics of the Na^+/H^+ antiport. For this purpose we determined the initial rate of pH_i recovery from an acid-load at varying external Na^+ concentrations and at constant external pH_o of 7.4. The relationship between pH_i recovery and external Na^+ concentration appears to follow first-order kinetics with an apparent K_m for Na^+ of 12.5 ± 1 mM and a V_{max} of 0.2 ± 0.02 pH_i units $\cdot$ min^{-1}. The K_m for Na^+ is similar to that previously obtained by the same method in MDCK [20], other cultured cell lines [32] and rabbit cortical collecting tubule [35], and by a different approach by Montrose et al. [5] in chicken enterocytes.

The relationship between Na^+/H^+ exchange activity and pH_i is apparently linear. Data compatible with a linear relationship between these two variables have been also obtained in other cell types [26,30,36]. The observed relationship suggests that in chicken enterocytes the set-point of the Na^+/H^+ exchanger (no net flux) is a pH_i of approx. 7.35, i.e., above the observed resting pH_i (approx. 7.18). Similar behavior have been observed in other cell types [30,37]. Such behavior could be consistent with the existence of a second cytoplasmic H^+-binding site, which allosterically controls the activity of the antiporter [1].

In conclusion the present study provides evidence for the presence of an active Na^+/H^+ antiport in chicken enterocytes that appears to play a role in maintaining a resting pH_i more alkaline than would be expected if proton distribution were entirely on a passive basis. In addition the Na^+/H^+ antiport seems to act as both a pH_i sensor and as a mechanism for pH_i regulation in acid-loaded cells.

The pH_i may be also regulated by other mechanisms involving Cl^-/OH^-, or Cl^-/HCO_3^- or Na^+/HCO_3^- exchange systems. In the present study attention was focused on the role of Na^+/H^+ antiport. Further studies are needed to obtain a complete characterization of pH_i homeostasis in these cells.

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