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Intracellular pH regulation in cecal epithelial cells from the chick

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Intracellular pH (pH_i) regulation has been investigated in cells isolated from the proximal ceca of the chicken. pH_i was measured with the pH-sensitive dye, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein in nominally HCO_3^--free solutions. Under resting conditions the pH_i was 7.08. Removal of extracellular Na^+ decreased pH_i by approx. 0.24 pH units and the subsequent addition of Na^+ increased pH_i towards the control value. This Na^+-dependent pH_i recovery was inhibited by 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA). Following an intracellular acidification, by abrupt withdrawal of NH_4Cl, pH_i alkalinized in the nominally absence of Na^+. Rotenone, *N*-ethylmaleimide, *N*,*N*′-dicyclohexylcarbodiimide, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole, iodoacetic acid and SCH 28080 inhibited the Na^+-independent pH_i recovery rate by 82, 82, 67, 74, 77 and 50%, respectively. Bafilomycin A_1 was without effect. Na^+-independent cell alkalization was stimulated by external K^+. In the presence of *N*-ethylmaleimide addition of Na^+ induced a rapid pH_i recovery. The initial rate of this recovery exhibited first-order dependence on Na^+ concentration and it was inhibited by EIPA. The initial rate of Na^+-dependent cell alkalization increased with a Hill coefficient greater than one when pH_i was reduced from 7.2 to 6.2. The 'set point' for the exchanger is appox. 7.5. These studies demonstrate that in cecal epithelial cells exist at least two mechanisms for proton secretion: a Na^+-H^+ exchanger and a Na^+-independent proton transport system.

Introduction

In birds the cecum plays an important role in the absorption of salt and water [1,2] and in osmoregulation [3]. Other functions of bird cecum is partial digestion of protein [4], vitamin synthesis [5] and the production of volatile fatty acids [6]. Recent reports have shown that rectal epithelium transports sugar [7] and dipeptides [8] against a concentration gradient. Dipeptide uptake in cecal epithelial cells was dependent on the Na^+ gradient in an indirect manner, via the Na^+-H^+ antiporter [8].

We have investigated the presence of Na^+-H^+ exchange activity and its role in pH_i homeostasis in epithelial cells isolated from chicken ceca. We have found an additional Na^+-independent pH_i regulating system.

Materials and Methods

Solutions. Four salt solutions (pH 7.4) were employed. The standard solution contained 80 mM NaCl, 1 mM $CaCl_2$, 100 mM mannitol, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 20 mM Tris-HCl, 0.5 mM β-hydroxybutyrate, 10 mM fructose, 1 mM L-glutamine, and 1 mg/ml bovine serum albumin. β-Hydroxybutirate, fructose and L-glutamine are present as passively transported nutrients. NaCl was replaced isosmotically by choline chloride in the Na^+-free solution and by KCl in the high K^+ solution. K^+ was replaced isosmotically by choline chloride in the K^+-free solution.

Isolation of cecal epithelial cells. Cecal epithelial cells were isolated from Hubbard chickens, 4–6-week old, by hyaluronidase incubation as described in Ref. 8. Cell viability was assessed by determining the fraction of the cell population able to exclude 0.2% Trypan blue [9] and usually ranged from 60% to 75%.

Intracellular pH. Intracellular pH was measured fluorimetrically at 25°C with 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) as described in Ref. 10. The excitation wavelengths, 500 and 450 nm, were alternated automatically every 3 s and the ratio of the fluorescence (emission wavelength 530 nm) stored in a computer. Cell autofluorescence was less than 2% of the total signal and was not corrected. The initial pH_i rate after an experimental maneuver is defined as the change in pH_i that occurred during the first minute.

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Fluorescence ratios (500/450) were correlated with pH_i at the end of each recording session by permeabilizing the cell membranes with 70 μM digitonin, the solution was then titrated with acid or base and a calibration curve was constructed (Fig. 1A). In some experiments the nigericin null-point determination and the digitonin-based calibration were performed in the same cell batch (Fig. 1B). For these experiments the cells were suspended in 100 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. 1C 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 0.13 ± 0.008 ($n = 8$) pH units. Therefore a correction factor of 0.13 pH units was applied to the calibration utilizing digitonin.

Acid-loading. Cells were acidified by the ammonium chloride technique [11]. Unless otherwise stated BCECF-loaded cells were incubated at 25°C in NaCl-solution containing 30 mM NH_4Cl for 10 min. Cells were then centrifuged and washed quickly in NH_4^+- and Na^+-free buffer (choline substitution), before being suspended in the appropriate Na^+-free solution.

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

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

where V is cell volume (3.7 μl/mg protein) as determined previously [8], 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 negligibly small and β_t was taken to equal β_i.

Chemicals. Valinomycin, digitonin, *N,N'*-dicyclohexylcarbodiimide (DCCD), *N*-ethylmaleimide (NEM), iodoacetic acid, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), hyaluronidase, rotenone and all the salts used in the current study were obtained from Sigma, St. Louis, MO. BCECF-AM from Molecular Probes (Eugene, OR). 5-(*N*-Ethyl-*N*-isopropyl) amiloride (EIPA) was purchased from Merck, Sharp and Dohme. Bafilomycin A_1 was kindly provided by Dr. Altendorf. SCH 28080 was a gift from Schering-Plough, S.A. None of the chemicals used in the current work interfered with the BCECF fluorescence.

The BCECF-AM (1.45 mM), digitonin (14 mM), NBD-Cl (40 mM), DCCD (100 mM), NEM (100 mM), rotenone (4 mM), EIPA (4 mM), SCH 28080 (20 mM), Bafilomycin A_1 (2 mM) and valinomycinin (3.6 mM) were prepared in DMSO. They 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.

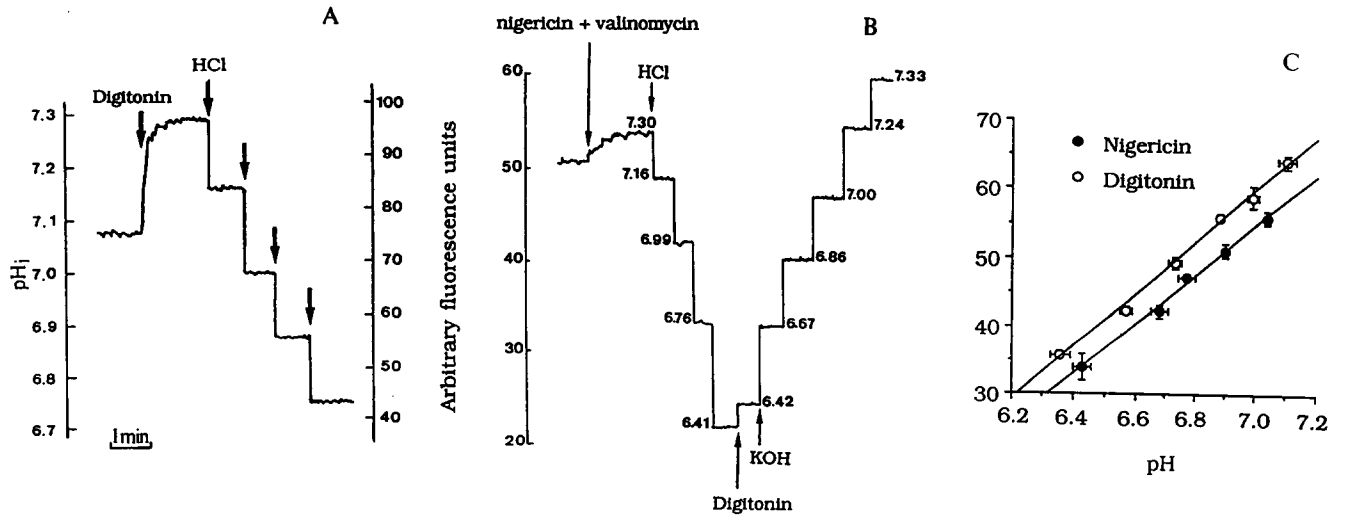

Fig. 1. pH_i calibration protocol. (A) Calibration of fluorescence utilizing digitonin. (B) Dye-loaded cells were suspended in KCl-buffer at the beginning of the trace. Nigericin and valinomycin was added as shown and medium pH was lowered by addition of HCl. Then the cells were lysed with 70 μM digitonin and titration with KOH was carried out in steps. The trace is representative of six experiments. (C) Plot of fluorescence intensities vs. pH for nigericin- and digitonin-treated cells.

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

Results and Discussion

Intracellular steady-state pH and intracellular buffering capacity of cecal epithelial cells

Steady-state pH_i in cecal epithelial cells measured in nominally bicarbonate-free solutions, at 25°C, was 7.08 ± 0.01 ($n = 47$).

Intracellular buffering power, β_i, was calculated from the value of pH_i reached by the addition of 20 mM NH_4Cl in cells exposed to an NH_4^+ pulse (0–40 mM of NH_4Cl to obtain different pH_i) as described in Materials and Methods. It was found that β_i increased (range: 50–58 mM/pH unit) as the pH_i decreased (range: 7.1–6.4). The mean value for β_i was 53.4 ± 2 mM/pH unit ($n = 19$). The inverse linear relationship between β_i and pH_i is given by the formula:

$$\beta_i = -20\ pH_i + 188\ (r = 0.96)$$

Na^+-H^+ exchanger and pH_i regulation

Na^+-H^+ exchange activity has been detected in virtually every type of cell in which it has been investigated. To test whether the cells isolated from chicken ceca possess a functional Na^+-H^+ antiporter at resting pH_i we studied the effects of Na^+ removal on pH_i. Cells incubated in Na^+-containing solution 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^+. When extracellular Na^+ was removed pH_i fell by approx. 0.24 units to a new pH_i value of 6.74 ± 0.02 (Fig. 2A). Readdition of Na^+ resulted in the recovery of pH_i. TEACl did not elicit pH_i recovery (Fig. 2B) suggesting that the effect of Na^+ was not due to changes in osmolarity or ionic strength of the suspending medium, but to a reversal of the transmembrane Na^+ gradient. Incubation with EIPA in Na^+-containing solution acidified the cells and Na^+-free conditions did not produce further acidification (Fig. 2C). Under these experimental conditions the readdition of Na^+ did not result in pH_i recovery. These results suggest that cecal epithelial cells posses a Na^+-H^+ exchanger operative under resting conditions.

The role of Na^+-H^+ antiporter in the pH_i recovery from an acid load was investigated in acidified cecal epithelial cells. Acid-loaded cells suspended in Na^+-containing solutions recovered towards the basal pH_i value with an initial rate of 0.31 ± 0.012 ($n = 10$) pH unit/min (Fig. 3). Regulatory cell alkalization was inhibited, but it was not abolished, by EIPA. In the presence of EIPA the initial recovery rate was 0.06 ± 0.003 ($n = 4$) pH unit/min.

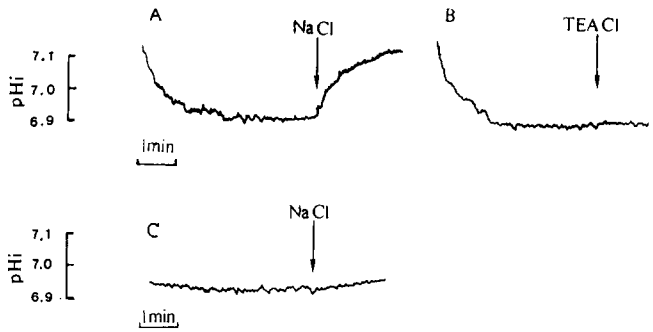

Fig. 2. Effect of Na^+ removal on resting pH_i in cecal epithelial cells. (A and B) At the beginning of each trace, dye-loaded cells incubated in standard solution were suspended in Na^+-free solutions (choline substitution). In (C) cells were incubated for 5 min in standard solution containing 50 μM EIPA and suspended in Na^+-free solutions containing 50 μM EIPA. At the times indicated by the arrows 80 mM NaCl or 80 mM TEACl were added to the cuvettes. Each trace is representative of nine independent experiments.

In Na^+-free solutions (choline substitution) the pH_i slowly but significantly increased (Fig. 3) with an initial rate of 0.06 ± 0.003 ($n = 18$) pH unit/min. After about 30 min pH_i reached a steady-state value of 6.68 ± 0.03 ($n = 4$). The subsequent addition of Na^+ brings pH_i back to control values.

The results suggest that in cecal epithelial cells pH_i recovery from an acid load is mediated by at least two mechanisms: the Na^+-H^+ antiporter, inhibited by EIPA, and a Na^+-independent pH_i regulatory process. The contribution of the Na^+-independent mechanism to the regulation of pH_i is much smaller (initial velocity was about 20% of total pH_i recovery) than that of the Na^+-H^+ antiporter.

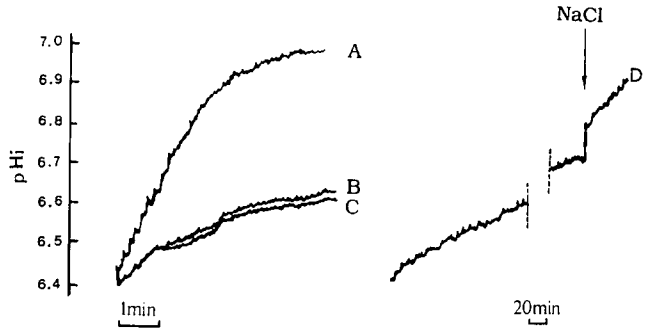

Fig. 3. pH_i recovery from acid load. Dye-loaded cells were acidified as described in Materials and Methods. At the beginning of the trace cells were suspended in NaCl-medium (A) or in Na^+-free medium (C and D). In (B) cells were incubated in standard solution containing 30 mM NH_4Cl and 50 μM EIPA for 10 min and suspended in NaCl-medium. At the time indicated by the arrow 80 mM NaCl was added. Each trace is representative of six independent experiments.

Kinetics of Na^+-H^+ antiporter

The kinetics of the Na^+-H^+ antiporter were determined in the presence of 1 mM NEM to avoid the interference of Na^+-independent mechanism (see below). Two protocols were used to initiate the Na^+-dependent pH_i recovery from acidified cells. First, various amounts of NaCl were added to medium containing acidified cells. Second, acidified cells were added to an isosmotic Na^+-containing medium (various amounts of NaCl isosmotically replacing choline chloride). In the latter case some experiments were repeated in the absence of NEM. The results were not significantly different indicating that NEM has no significant effect on Na^+-H^+ antiporter.

External Na^+ initiated prompt concentration-dependent pH_i recovery in both cases, and the Na^+-dependency of the rate of pH_i recovery was a saturable process (Fig. 4A). The relationship between the dpH_i/dt and external Na^+ concentration was different in the two protocols. Lineweaver-Burk plots of the data (Fig. 4B) led to calculate values of apparent Michaelis constant for Na^+ (K_m) and of the maximal velocity (V_{max}). V_{max} was significantly greater when NaCl isosmotically replaced cholineCl than with NaCl addition. Whether the difference between V_{max} were due to changes in medium osmolarity and/or cell volume or surface was beyond the scope of this work and will not be discussed further. The K_m for Na^+ is similar to that obtained by the same or different methods in other epithelial cell types [12–15].

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

In most cells the Na^+-H^+ antiporter is allosterically regulated by intracellular H^+. The antiporter is virtually silent near physiological pH_i, but becomes activated below a certain threshold pH_i or set-point [16].

The dependence of Na^+-H^+ exchange activity on pH_i was studied in cecal epithelial cells acidified by treatment with different concentrations of NH_4Cl. The initial rate of Na^+-dependent alkalization was measured in the presence of NEM. The relationship between dpH_i/dt or net H^+ efflux (nmol/mg per min) and pH_i did not follow simple Michaelis-Menten kinetics (Fig. 5A). The Lineweaver-Burk plot was used to calculate the apparent J_{max} and the data are presented in the form of a Hill plot (Fig. 5B). The Hill plot gives an interaction coefficient (n) of 1.46 and a $[H^+]_{0.5}$ of 190 nM. This indicates a positive cooperative mechanism for the influence of cytosolic protons on Na^+-H^+ antiporter, that is the antiporter is allosterically regulated by intracellular pH. In Fig. 5C all the individual values are plotted and fitted to a line by computer. The extrapolated pH_i value at which no measurable cell alkalization occurred, set-point, (approx. 7.5) is greater than the resting pH_i value (approx. 7.08). This indicates that the resting pH_i is maintained by continuous Na^+-H^+ exchange, as suggested by the effect of Na^+ removal on resting pH_i discussed above. Similar observations have been described for other epithelial cell types [17].

Na^+-independent pH_i recovery after an acid load

Na^+-independent cell alkalization was significantly decreased by rotenone and iodoacetic acid (Table I), inhibitors of ATP production, indicating a requirement for metabolic energy. The inhibitors were disolved in DMSO, which had no effect by itself.

NEM, DCCD, SCH 28080 and NBD-Cl significantly slowed down pH_i recovery, while bafilomycin A_1 had no effect (Table I). The first two agents inhibit all proton pumps and the last inhibits the vacuolar proton pump at the concentrations used in our experiments [18, 19]. Bafilomycin A_1, a macrolide antibiotic, differ-

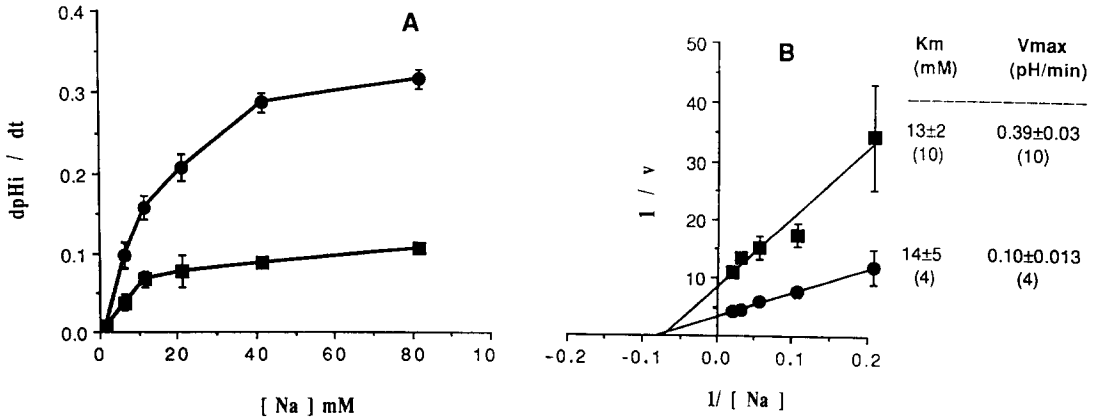

Fig. 4. Na^+-dependent changes of pH_i after an acid load. Cells were acidified as explained in Materials and Methods to a pH_i value of 6.62 ± 0.02. The experiments were carried out in the presence of 1 mM NEM. (A) The initial increment in intracellular pH vs. Na^+ concentration. •, NaCl isosmotically replacing choline chloride; ■, various amounts of NaCl were added to the cuvettes containing acidified cells. (B) Lineweaver-Burk plot of the data represented in (A). The line was calculated by linear regression analysis, (•) $r = 0.998$ and (■) 0.96.

entiates the three types of proton pumps, since it inhibits the vacuolar type very strongly, the E_1E_2 type moderately, and the F_0F_1 type not at all [18,19]. SCH 28080, an imidazopyridine, inhibits the gastric H^+/K^+-ATPase, a E_1E_2 type proton pump, by competing with K^+ [20]. A H^+-K^+-ATPase is also present in epithelial cells of colon [21,22] and kidney [23,24].

Effects of K^+ concentration on alkalization in the absence of added Na^+

Na^+-insensitive regulatory cell alkalization was dependent on extracellular K^+ concentration and not affected by Ba^{2+} (Table II). Since Ba^{2+} modifies membrane potential by inhibiting some types of K^+ channel [25–27], the results suggest that K^+ activated cell alkalization by itself and not indirectly by modifying the membrane potential. In addition the K^+-induced cell alkalization was prevented by SCH 28080.

Na^+-independent pH_i regulation and external pH

The results obtained with the putative proton-pump inhibitors suggest that the Na^+-independent proton transport system involved in pH_i regulation may be a proton-pump. However, since NEM reacts with sulfhydryl groups, DCCD with carboxyl groups and NBD-Cl with amino groups, they may alter cellular functions, including transport mechanisms, other than proton pumps [28–33].

SCH 28080 is believed to be much more specific and its effect indicates the presence of a H^+/K^+-ATPase in cecal epithelial cells. We failed to reveal the presence of a K^+-activated ATPase by measuring the P_i hydrolysed from ATP with the method of Del Castillo and Robinson [34].

Additional experiments throw doubts on the presence of an ATPase able to translocate H^+ against an

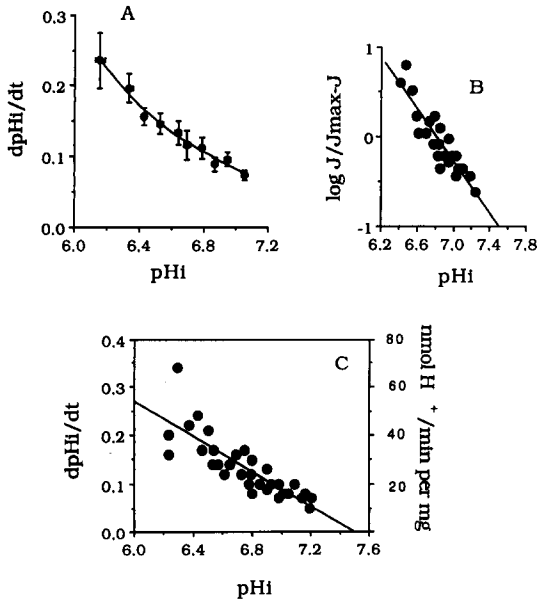

Fig. 5. Relationship between initial rate of pH_i recovery and pH_i. Dye-loaded cells were acidified as described in Materials and Methods except NH_4Cl concentration prepulse was 0, 15, 20, 25, 30 or 40 mM and incubation time ranged from 5 to 10 min to obtain different pH_i values. Acidified cells were added to cuvettes containing 80 mM NaCl and 1 mM NEM. (A) Means ± S.E. ($n = 5$) of rates of pH_i recovery or H^+ efflux against the means of pH_i at 0.1 pH unity intervals. (B) Hill plot of data in (A). The line was calculated by linear regression analysis, $y = 9.81-1.46x$, $r = 0.95$. J_{max} was calculated from the Lineweaver-Burk plot of the data. (C) All the individual data are plotted and adjusted to a line by computer.

TABLE I

Effect of various inhibitors on Na^+-independent pH_i recovery

Means ± S.E., between brackets the number of independent determinations. Initial rate of pH_i recovery from acid load to 6.4 ± 0.01, attained as indicated in Materials and Methods. The inhibitors were present for the time indicated in the table and throughout the entire experiment. Significant differences with the control: * $p < 0.001$; ** $p < 0.05$.

Inhibitor	Preincubation Time (min)	Rate of pHi recovery (pH/min)	% of control rate
None		0.060 ± 0.003 (18)	100
Rotenone (10 μM)	10	0.005 * ± 0.004 (11)	8
NBD-Cl (20 μM)	1	0.016 ** ± 0.02 (5)	26
Iodoacetic acid (500 μM)	30	0.014 * ± 0.008 (11)	23
NEM (500 μM)	10	0.005 * ± 0.006 (4)	8
DCCD (500 μM)	10	0.020 * ± 0.001 (5)	33
Bafilomycin (40 μM)	20	0.060 ± 0.006 (6)	100
SCH 28080 (100 μM)	10	0.030 * ± 0.007 (6)	50

TABLE II

Effect of external K^+ concentration on the rate of pH_i recovery in nominally absence of Na^+

Cells were acidified as described in Materials and Methods and transferred to cuvettes containing the indicated modifier. $[K^+]_o$ is the extracellular K^+ concentration. In some experiments 5 mM Ba^{2+} or 100 μM SCH 28080 were also present during the incubation with NH_4Cl. Data are mean values ± S.E., n is the number of independent determinations. * $p < 0.001$ compared with 6 mM K^+ concentration (first row).

$[K^+]_o$ (mM)	n	pH_i		Rate (min^{-1})
		initial	1 min	
6	23	6.65 ± 0.02	6.72 ± 0.02	0.066 ± 0.004
6 + Ba^{2+}	12	6.68 ± 0.04	6.75 ± 0.04	0.07 ± 0.007
6 + SCH	6	6.65 ± 0.04	6.68 ± 0.04	0.030 ± 0.007 *
100	14	6.57 ± 0.05	6.65 ± 0.04	0.110 ± 0.004 *
100 + SCH	6	6.54 ± 0.05	6.58 ± 0.04	0.035 ± 0.01 *
0	8	6.53 ± 0.05	6.56 ± 0.05	0.03 ± 0.005 *

electrochemical gradient. In buffer at pH 6.5 the cells, that were acid-loaded in a pH 7.4 buffer, did not recover from the acid load, but acidified further with an initial rate of 0.40 ± 0.001 ($n = 10$) pH unit/min. Addition of Na^+ resulted in pH_i recovery with an initial rate of 0.15 ± 0.001 ($n = 3$) pH unit/min. This rate of pH_i was lower than that obtained at external pH 7.4 (see above). This finding agrees with previous reports showing that H^+ competes with Na^+ for binding to the external transport site [35].

Alternatively, the Na^+-independent cell alkalization may be mediated by a passive proton conductance sensitive to various inhibitors. Recovery of opossum kidney cells from an acid load is mediated by a proton permeability insensitive to DCCD and NEM [36]. A similar mechanism does not seem to be responsible for our results: Ba^{2+} should modify Na^+-independent cell alkalization by depolarizing the cell membrane, but we find no Ba^{2+} effect. These observations together with the K^+ dependence of pH_i recovery in the absence of added Na^+ may be compatible with the presence of a K^+-H^+ exchanger.

In summary, the present study provides information on pH_i regulation in cecal epithelial cells. The Na^+-H^+ antiporter is involved in pH_i recovery from an acid load and participates in setting the resting pH_i. In addition, a Na^+-independent H^+-transport process is also involved in pH_i regulation. The nature of this latter mechanism requires further investigation.

Acknowledgements

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