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Cytosolic pH regulation in chicken enterocytes: Na^+-independent regulatory cell alkalinization

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Abstract

The mechanisms involved in intracellular pH (pH_i) recovery from an acid load have been investigated in enterocytes isolated from chicken. Following an intracellular acidification, by abrupt withdrawal of NH_4Cl, pH_i alkalinized in the nominally absence of Na^+ and bicarbonate. This Na^+- and bicarbonate-independent (NBI) regulatory cell alkalinization became negligible when the pH_i has reached a value of approx. 6.85. Addition of Na^+ induced a rapid pH_i recovery to control values. Rotenone, DCCD, vanadate, NBD-Cl, SCH 28080 and EIPA inhibited the NBI cell alkalinization, whereas bafilomycin A_1, ouabain and H_2-DIDS were without effect. Na^+-dependent pH_i recovery from an acid load was inhibited by EIPA and unaffected by SCH 28080 or DCCD. The rate of NBI cell alkalinization was a linear function of the electrochemical proton gradient. In high external K^+ buffer plus valinomycin the line goes through the origin. Gramicidin accelerated the rate of NBI cell alkalinization, whereas it was slightly reduced by low external potassium. The results demonstrate that in intestinal epithelial cells exist at least two mechanisms for proton secretion: a Na^+-H^+ exchanger and a Na^+- and bicarbonate-independent proton transport system. This latter mechanism appears to be a proton conductance pathway.

Keywords: pH_i; pH, cytosolic; Enterocyte; Sodium ion-independent alkalinization; Alkalinization

1. Introduction

Most animal cells, including epithelial cells, maintain a cytosolic pH (pH_i) more alkaline than expected from a passive proton distribution across the cell membrane, indicating that cells are able to regulate their pH_i [1–3]. Several mechanisms of pH_i regulation have been delineated over the past decade. Those studied include an amiloride-sensitive Na^+-H^+ exchanger; disulfonic stilbene-sensitive and bicarbonate-dependent acid/base transport systems and a variety of proton pumps differing in their inhibitor sensitivities, ion requirements and physiological function.

Chicken enterocytes possess a Na^+-H^+ and a Cl^-/base exchanger, operative under resting conditions and involved in pH_i recovery from deviations from its resting pH_i value [4,5].

In the current work we show that chicken enterocytes present a Na^+- and bicarbonate-independent mechanism involved in pH_i recovery from acid load.

2. Materials and methods

2.1. Solutions

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 Hepes-Tris, pH 7.4, and 1 mg/ml bovine serum albumin. NaCl was replaced isosmotically by choline chloride in the Na^+-free, 6 mM K^+ solution and by KCl and choline chloride in the 65 mM K^+ solution. The solutions were buffered with Hepes-Tris or Mes-Tris to different pH_o. 0.5 mM β-hydroxybutyrate, 10 mM fructose and 1 mM L-glutamine were present in all the solutions as passively transported nutrients. All solutions were equilibrated at room temperature with room air.

2.2. Enterocytes

Enterocytes were isolated from 4–6-week-old Hubbard chickens, by hyaluronidase incubation as described in [4].

2.3. Intracellular pH

pH_i was measured spectrofluorimetrically, using cell suspensions, at 25°C with 2′,7′-bis(carboxyethyl)-5,6-

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carboxyfluorescein (BCECF) as described in [5]. The initial pH_i rate after an experimental maneuver is defined as the change in pH_i that occurred during the first minute.

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 and constructed a calibration curve. This procedure underestimates the actual pH_i values by 0.15 pH units [4] and a correction of 0.15 pH units was applied to the results.

2.4. Acid-loading

Cells were acidified by the ammonium chloride technique [6]. BCECF-loaded cells were incubated at 25°C in Na^+-free, 6 mM K^+, pH 7.4 buffer containing 30 mM NH_4Cl for 10 min. Cells were then centrifuged and washed quickly in NH_4^+- and Na^+-free buffer before being suspended in the appropriated saline solution.

2.5. Membrane potential measurements

Electrical membrane potential (E_m) was measured using the lipophilic ion tetraphenylphosphonium (^{14}C-TPP$^+$) as described in [7]. Acid-loaded cells were incubated for 30 min in Na^+-free, 6 mM K^+ solutions buffered to different pH_o. Then ^{14}C-TPP$^+$ was added to a final concentration of 5 μM and uptake was measured for 15 min at 25°C. The E_m was calculated by applying the Nernst equation to the steady-state ^{14}C-TPP$^+$ distribution ratio:

$$E_m = (RT/zF) \ln \frac{[^{14}C - TPP^+]_i}{[^{14}C - TPP^+]_o}$$

where R, T, F and z have their usual meanings. The values for E_m are indicated in Table 1. In high potassium buffer plus valinomycin the cell membrane potential was not significantly different from zero.

2.6. Chemicals

Valinomycin, digitonin, vanadate, *N,N'*-dicyclohexylcarbodiimide (DCCD), 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), hyaluronidase, rotenone, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) and all the salts used in the current study were obtained from Sigma, Madrid, Spain. ^{14}C-TPP$^+$ was obtained from Amersham. BCECF-AM (2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester) and H_2-DIDS 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.

Stock solutions of BCECF-AM (1.45 mM) in the form of acetoxymethyl ester, NBD-Cl (4 mM), DCCD (100 mM), rotenone (4 mM), EIPA (4 mM), H_2-DIDS (100 mM), ouabain (400 mM), vanadate (20 mM), SCH-28080 (20 mM), bafilomycin A_1 (4 mM), digitonin (14 mM) and valinomycin (3.6 mM), FCCP (2 mM) were prepared in dimethylsulfoxide and stored for up to 30 days at −20°C without loss in potency. The concentration of dimethylsulfoxide (DMSO) did not exceed 0.5% (v/v) and it had no effect on fluorescence by itself.

Table 1
Electrical membrane potential (E_m)

Method	E_m (mV)		
pH_o:	6.5	7.4	8
FCCP	−16±2 * (13)	−30±3 (25)	−44±3 ** (8)
TPP^+	−37±4 (3)	−49±2 (3)	−55±3 (3)

Membrane potential was calculated from the TPP^+ distribution ratio and from the steady-state pH_i values obtained following the addition of 5 μM FCCP.

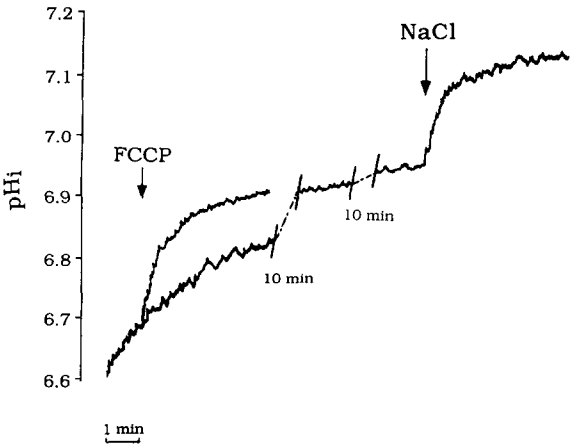

Fig. 1. pH_i recovery from acid load. Dye-loaded cells were acidified as indicated in Methods. At the beginning of the trace cells were suspended in Na^+-free, 6 mM K^+, pH 7.4 buffer. At the time indicated by the arrow 10 μM FCCP or 80 mM NaCl were added. The trace is representative of five independent experiments.

2.7. Statistics

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

3. Results

3.1. Na^+-independent pH_i recovery from an acid load

All the experiments described in this work were carried out in the nominal absence of bicarbonate.

Cells recovered from an acid load in Na^+-free saline solutions with a rate of 0.10 ± 0.002 pH units/min (Fig. 1). This Na^+- and bicarbonate-independent regulatory cell alkalinization becomes very slow when pH_i reaches a

Table 2
Effect of various reagents on the rate of Na^+-independent and Na^+-dependent regulatory cell alkalinization following an acid load

Reagent	Incubation (min)	Initial pH_i:	Rate (min^{-1}) $\times 10^{-2}$	
			6.62 ± 0.03 Na^+-independent	6.86 ± 0.02 Na^+-dependent
Control			10 ± 0.2 (73)	8 ± 0.3 (17)
20 μM Bafilomycin A_1	10		9 ± 0.4 (6)	
2 mM Ouabain	10		9 ± 0.5 (9)	
500 μM H_2-DIDS	20		9 ± 0.3 (10)	
20 μM EIPA	10 or 5		5 ± 1.0 [a] (7)	3 ± 0.3 [a] (9)
100 μM SCH 28080	5		4 ± 0.4 [a] (14)	8 ± 0.4 (10)
500 μM DCCD	5		2 ± 0.5 [a] (11)	8 ± 0.3 (15)
10 μM Rotenone	10		2 ± 0.2 [a] (14)	
100 μM Vanadate	10		2 ± 0.5 [a] (7)	
20 μM NBD-Cl	5		3 ± 0.4 [a] (8)	

Values are means ± S.E. Between brackets the number of independent determinations. Cells were acidified as described in Materials and methods. To measure the NBI cell alkalinization, acid loaded cells were placed in cuvettes containing Na^+-free, 6 mM K^+, pH 7.4 buffer and the indicated modifier. The rate of Na^+-dependent pH_i recovery from an acid load was measured following the addition of 20 mM NaCl to the acidified cells. The inhibitors were present for the time indicated in the table and throughout the entire experiment. In the case of the Na^+-dependent pH_i recovery the cells were incubated with EIPA for 5 min. A set of control studies were performed with vehicle. Significant differences with the control: [a] $P < 0.001$.

value around 6.85. Addition of 80 mM Na^+ brings pH_i back to control values. Throughout this paper this Na^+- and bicarbonate-independent regulatory cell alkalinization will be referred as NBI cell alkalinization.

3.2. Effect of several agents on the Na^+-independent and Na^+-dependent regulatory cell alkalinization

The NBI cell alkalinization was significantly inhibited by the Na^+-H^+ exchanger inhibitor EIPA and by several putative proton-pump inhibitors, such as rotenone, DCCD, NBD-Cl, vanadate and SCH 28080, whereas it was unaffected by bafilomycin A_1, ouabain or by the anion exchanger inhibitor H_2-DIDS (Table 2).

Na^+-dependent pH_i recovery from an acid load was significantly inhibited by EIPA, while DCCD and SCH 28080 had no effect (Table 2). In this set of experiments cells were acidified to a pH_i of about 6.88, at which the NBI mechanism is negligible.

3.3. Effect of extracellular pH on NBI regulatory cell alkalinization

An increase of pH_o from 7.4 to 8 increased the rate of NBI cell alkalinization (Fig. 2). In buffer at pH 6.5 the cells did not recover from the acid load, but acidified further. After approx. 30 min the pH_i reached a plateau. This pH_i value was not modified by the addition of the proton ionophore FCCP. This indicates that transmembrane proton distribution is at electrochemical equilibrium. The application of the Nernst equation:

$$E_m = (RT/zF) \ln([H]_o/[H]_i)$$

where R, T, F and z have their usual meanings, gives the values for E_m indicated in Table 1. The E_m values estimated from the distribution of protons at electrochemical equilibrium are lower than those measured using the equilibrium distribution of TPP^+. Since the cells were handled similarly, the differences in results could be due to the Donnan potential or to the methodology used to estimate the E_m. Using TPP^+ as a sensor of the E_m one must bear in mind that (i) TPP^+ accumulates within the cells as a function not only of E_m but also of the mitochondrial membrane potential [8] and (ii) a non-specific binding of TPP^+ to membrane constituents [9] and intracellular components may occur [10].

Addition of FCCP before reaching the plateau (see Fig. 1) greatly accelerated the rate of alkalinization but the final pH_i was not different from that attained by the NBI mechanism. This support the view that the NBI mechanism

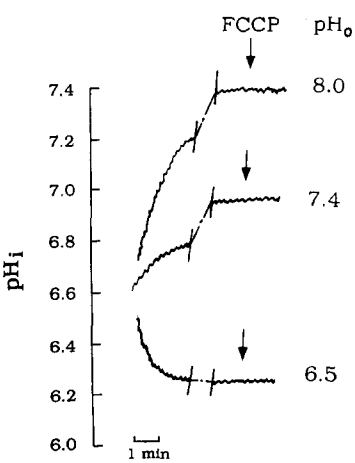

Fig. 2. Effect of pH_o on the rate of Na^+- independent regulatory cell alkalinization. Dye-loaded cells were acidified as indicated in Methods. At the beginning of the trace acid loaded cells were suspended in Na^+-free, 6 mM K^+ buffer with different pH_o. Fluorescence was recorded for 30 min. At the time indicated by the arrow 10 μM FCCP was added. Each trace is representative of five independent experiments.

Table 3
Effect of pH_o and $[K^+]_o$ on the rate of NBI cell alkalinization

$[K^+]_o$		Rate (min^{-1})$\times 10^{-2}$		
(mM)	pH_o:	7.4	8	6.5
6		7 ± 0.7	26 ± 2 [a]	-25 ± 3 [a]
65 + val		22 ± 3 [a,b]	41 ± 5 [a,c]	-16 ± 3 [a]

Data are mean values ± S.E. of five independent determinations. Acid loaded cells (pH_i 6.65 ± 0.001) were placed in Na^+-free solutions buffered to different pH_o and containing either 6 mM K^+ or 65 mM K^+ plus 18 μM valinomycin (val). [a] $P < 0.001$ compared with the values obtained at 6 mM K^+, pH 7.4. [b] $P < 0.001$, [c] $P < 0.01$ compared with values obtained at 6 mM K^+, (first row).

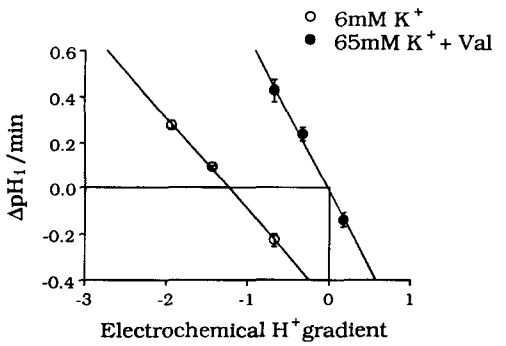

Fig. 3. Relationship between the rate of Na^+-independent regulatory cell alkalinization and electrochemical proton gradient. Electrochemical proton gradient has been calculated using the E_m values obtained from the TPP^+ distribution ratio. Rates of recovery are those shown in Table 3. Line was calculated by linear regression analysis, $r = 0.99$.

stops when there is not transmembrane electrochemical proton gradient.

3.4. Effect of external K^+ concentration and gramicidin on NBI regulatory cell alkalinization

High $[K^+]_o$ and valinomycin potentiated the effects of pH_o on the rate of NBI cell alkalinization at pH_o 7.4 or 8, and decreased the cell acidification observed at pH_o 6.5 (Table 3). The relationship between the rate of NBI cell alkalinization and the electrochemical proton gradient was linear (Fig. 3), both at 6 mM $[K^+]_o$ and at 65 mM $[K^+]_o$ plus valinomycin. In the absence of membrane potential (65 mM $[K^+]_o$ plus valinomycin) the net proton flux is zero when the transmembrane pH difference is zero.

At pH_o 7.4, the rate of NBI cell alkalinization was slightly decreased by the nominal absence of external K^+ (from 0.07 ± 0.006 to 0.05 ± 0.003, $n = 11$, $P < 0.01$). Addition of 1 μM gramicidin increased the rate of NBI cell alkalinization from 0.06 ± 0.007 to 0.14 ± 0.007 ($n = 3$).

4. Discussion

We have previously reported that in chicken enterocytes, and in the nominal absence of bicarbonate, Na^+-H^+ exchange was the mechanism responsible for pH_i recovery from an acid load [4]. The current results show that these cells also possess a Na^+- and bicarbonate-independent (NBI) mechanism that helps to recover pH_i from an acid load. The reason why the NBI cell alkalinization was not detected in the previous work was the initial pH_i value of the acidified cells, which was approx. 6.86. At this pH_i the NBI mechanism becomes negligible.

The inhibition of the rate of NBI cell alkalinization by the agents described in Table 2 was not due to a non-specific effects of the agents on cell function, as DCCD or SCH 28080 did not affect the rate of Na^+-dependent pH_i recovery from the acid load.

Na^+-independent pH_i recovery from an acid load has been reported in renal epithelial cells [11–14] and in lung macrophages [15]. Based on its sensitivity to several reagents it was concluded that the underlying mechanism was a H^+-translocating ATPase.

The pharmacological profile of inhibition may indicate that the NBI cell alkalinization reported in the current study is also mediated by a proton-ATPase. Interference with the supply of metabolic energy for transport by rotenone abolished the NBI cell alkalinization. It was also inhibited by DCCD, an inhibitor of all the proton-pump types so far described [16,17]; by vanadate, an inhibitor of E_1E_2-type proton pump [18]; by SCH 28080, inhibitor of the H^+/K^+-ATPase [19], and by NBD-Cl inhibitor of the vacuolar-type proton ATPases [16,17,20]. The NBI alkalinization was not affected by bafilomycin A_1, the most specific inhibitor for vacuolar proton pumps [16,17] or by ouabain, an inhibitor of Na^+, K^+ ATPase activity. In acidic media this pump becomes a H^+/K^+ antiporter and the H^+ transport is inhibited by ouabain [21].

The following observations argued against the involvement of a H^+-ATPase and suggest that the NBI regulatory cell alkalinization is a proton conductance pathway: (i) at pH_o 6.5 the cells did not recover from the acid load, but acidified further; (ii) the rate of NBI mechanism continues till transmembrane proton distribution reached electrochemical equilibrium; (iii) the rate of NBI cell alkalinization was a linear function of the initial electrochemical proton gradient and in the absence of transmembrane potential (high K^+ and valinomycin) the line goes through the origin; (iv) low $[K^+]_o$, a hyperpolarizing experimental condition [7], slightly decreased the rate of NBI cell alkalinization, and (v) gramicidin or high $[K^+]_o$ plus valinomycin, agents known to depolarize the cell membrane [22,23], accelerated the rate of pH_i recovery. This indicates that the NBI mechanism is sensitive to membrane voltage. Involvement of a proton permeability in pH_i regulation has been reported for opossum kidney cells [24].

The inhibition by rotenone, agent known to deplete cells from ATP, might indicate that the activity of the NBI mechanism requires a basal level of phosphorylation as reported for Na^+-H^+ exchanger [25,26].

In summary, under nominally bicarbonate-free condi-

tions, chicken enterocytes present a Na^+-independent H^+-transport process involved in pH_i recovery from an acid load. Our results offer no clues on the cellular localization of the NBI proton transport mechanism. Wilkes and Hirst [27] have recently reported the presence of an amiloride-sensitive proton-conductive pathway in rabbit duodenal and ileal brush-border membrane vesicles. Their results suggested that, at least in the absence of Na^+, the Na^+-H^+ exchanger may represent the pathway for proton permeation. The sensitivity of the NBI mechanism to EIPA described in the present work may also be consistent with the observation of Wilkes and Hirst. The physiological role of the mechanism is unknown since it only becomes apparent when the pH_i is below 6.8.

Acknowledgements

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