Biochimica et Biophysica Acta, 1103 (1992) 45–50

BBAMEM 75457

Cl^-/base exchange and cellular pH regulation in enterocytes isolated from chick small intestine

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(Received 6 May 1991)
(Revised manuscript received 11 September 1991)

Key words: pH, intracellular (pH_i); Chloride–base exchange; Enterocyte

Intracellular pH (pH_i) and Cl^-/base exchange activity have been examined in isolated chicken enterocytes, both in the presence and absence of 25 mM HCO_3^-/5% CO_2. Intracellular pH was measured with BCECF, a pH-sensitive carboxyfluorescein derivative. Under resting conditions pH_i was 7.17 in Hepes and 7.12 in HCO_3^--buffered solutions. Cells became more alkaline upon withdrawal of Cl^-. Cells depleted of Cl^- acidified upon reinstatement of Cl^-. These changes were faster in the presence of HCO_3^- than in its absence. After an alkaline load (removal of HCO_3^- from the medium) pH_i decreases towards base line in the presence of Cl^-, but not in its absence. The Cl^--dependent pH_i changes were prevented by H_2DIDS and were unaffected by Na^+. The Cl^--induced recovery from an alkaline load exhibited simple saturation kinetics, with an apparent K_m of 12.5 mM Cl^- and maximum velocity of ≈ 0.20 pH units min^{-1}. The Cl^-/base exchange is functional under resting conditions, as shown by cell alkalinization on exposure to 0.5 mM H_2DIDS, both in the presence and in the absence of HCO_3^-. It is concluded that Cl^-/base exchange participates in setting the resting intracellular pH in isolated chicken enterocytes and helps recover from alkaline loads. The exchange operates both in the presence and in the absence of bicarbonate.

Introduction

The concentration of cytosolic H^+, like that of other cations, is finely regulated in all cells. Intracellular pH (pH_i) depends on intracellular buffers in the short term, and on effective transport of proton equivalents across the cell membrane on a longer term. In vertebrate cells pH_i is regulated by at least four membrane transport systems: an amiloride-sensitive Na^+/H^+ antiporter, a Na^+-dependent Cl^-/HCO_3^- exchanger, a Na^+-independent Cl^-/HCO_3^- exchanger, and a Na^+/HCO_3^- cotransporter (see Refs. 1–5 for reviews).

In the absence of CO_2 the Na^+/H^+ exchanger is the main pH_i regulatory mechanism in vertebrate cells [1–5] including enterocytes [6–13]. It is less clear to what extent HCO_3^--dependent transport systems participate in pH_i regulation in enterocytes. This is important because HCO_3^--CO_2 is the principal buffer in vivo.

The aim of the current work was to demonstrate the Cl^-/base exchange in chicken enterocytes and to determine its role in pH_i homeostasis.

Materials and Methods

Physiologic solutions. Physiologic solutions (Table I) were adjusted to pH 7.4 and equilibrated at room temperature with room air (Hepes-buffered solutions) or 95% O_2/5% CO_2 (HCO_3^--CO_2 buffered solutions). In experiments with gluconate as the substituted anion the Ca^{2+} concentration was increased to 6 mM.

Enterocytes. Enterocytes were isolated from Hubbard chickens, four-to-six-week old, by hyaluronidase incubation following the method of Kimmich [14] as described in Ref. 13. Cell viability was estimated as the fraction of the cell population able to exclude 0.2% Trypan blue [15] 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 [13]. The excitation wavelengths, 500 and 450 nm, were alternated

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TABLE I

Composition of solutions (mM)

All solutions contained 10 mM fructose, 1 mM L-glutamine, and 0.5 mM β-hydroxybutyrate.

	A	B	C	D	E	F	G	H
Mannitol	60	60	60	260	40	40	40	240
Na^+	100	100	0	0	100	100	0	0
Choline	0	0	100	0	0	0	100	0
Cl^-	102	0	102	0	102	0	102	0
Gluconate	0	106	0	0	0	106	0	0
K^+	6	6	6	6	6	6	6	6
Mg^{2+}	1	1	1	1	1	1	1	1
Ca^{2+}	1	6	1	6	1	6	1	6
SO_4^{2-}	0	1	0	1	3	4	3	4
$H_2PO_4^-$	0	0	0	0	3	3	3	3
HPO_4^-	3	3	3	3	0	0	0	0
Hepes	20	20	20	20	0	0	0	0
HCO_3^-	0	0	0	0	25	25	25	25

automatically every 3 s and the ratio of the fluorescence (530 nm) stored in a computer. 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 constructing a calibration curve. This procedure underestimates actual pH_i values by ≈ 0.15 pH units [13] and a correction of 0.15 pH units was applied to the results. Cell autofluorescence was less than 2% of the total signal and was not corrected. The initial pH_i change after an experimental maneuver is defined as the change in pH_i that occurred during the first minute.

The intracellular buffering power. The intracellular buffering power was determined as described by Roos and Boron [1]. The intrinsic buffering power (β_i) was calculated from the increase in pH_i after addition of 20 mM NH_4Cl to control cells. For cells incubated in HCO_3^- solutions the total buffering power, β_T is given [1] by

$$\beta_T = \beta_i + 2.3 \cdot [HCO_3^-],$$

Initial OH^- efflux rates (J_{OH^-}) are calculated as:

$$J_{OH^-} = (dpH_i/dt) \cdot V \cdot \beta_T$$

where V is the cell volume (3 μl/mg protein, as determined in Ref. 16) and dpH_i/dt is the rate of change of pH_i. Buffering power is measured in mM/pH unit and efflux rates in nmol per min per mg protein.

Chemicals. H_2DIDS (Molecular Probes, Eugene, OR, USA), was dissolved in dimethylsulfoxide (100 mmol/l) just before use. BCECF in the form of acetoxymethyl ester (Molecular Probes), dissolved in dimethylsulfoxide (1.45 mmol/l), and digitonin (Sigma, St. Louis, MO, USA), dissolved in distilled water (14 mmol/l), were stored for up to 30 days at −20°C without loss in potency. Hyaluronidase and other chemicals were from Sigma. The concentration of dimethylsulfoxide did not exceed 0.5% (v/v) and did not affect fluorescence.

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

Results

Resting pH_i and intracellular buffering power

The intracellular pH was 7.17 ± 0.01 (n = 20) in the nominal absence of HCO_3^- (solution A) and 7.12 ± 0.02 (n = 20) in HCO_3^--CO_2 buffer (solution E). Thus, at external pH 7.4, the steady-state pH_i was slightly more acidic in a HCO_3^--containing than in a HCO_3^--free solution (difference significant at the 5% level).

The cellular buffering power was evaluated from the response to 20 mM NH_4^+. NH_4^+ addition resulted in a much larger cell alkalinization in the absence of HCO_3^- than in its presence. The intracellular buffering power in bicarbonate-free solution was 57 ± 2 mM/pH unit (n = 8). In the presence of 25 mM HCO_3^-/5% CO_2 the total buffering power was 133 ± 11 mM/pH unit (n = 8).

Cl^-/HCO_3^- exchanger

To test for the existence of a Cl^-/HCO_3^- exchanger in the plasma membrane of chicken enterocytes the transmembrane Cl^- gradient was inverted by removal of extracellular Cl^-. The experiments were carried out either in the presence or the absence of HCO_3^- (Hepes buffer). Under these conditions and in the presence of an anion exchanger, the exit of Cl^- could be the driving force for the entry of base equivalents (HCO_3^- or OH^-). Cells were incubated for 15 min in the presence of Cl^- (solution E) and transferred to a buffer with Na^+ gluconate instead of NaCl (solution F); experiments in the absence of HCO_3^- used solutions A and B instead of solutions E and F, respectively. The cells became more alkaline following removal of Cl^- (Fig. 1A and Table II). This suggests that a gradient-driven efflux of Cl^- induced influx of base equivalents.

In another set of experiments cells were incubated without Cl^- (solutions F or B) for 15 min and then transferred to buffer with Cl^- (solutions E or A, respectively). Lack of Cl^- induced alkalinization and reinstatement of Cl^- reverted pH_i to normal values (Fig. 1B and Table II). These findings suggest that the influx of Cl^- now induced efflux of base equivalents.

The Cl^--dependent pH_i changes observed in the absence of bicarbonate were of the same direction but of lesser magnitude than in the presence of bicarbonate (Fig. 1 and Table II). This suggests that the ex-

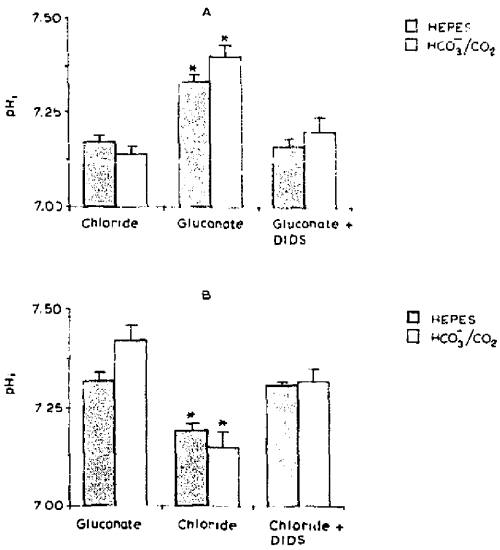

Fig. 1. Effect of external Cl^- on resting pH_i. Measurements were made 5 min after transfer to each solution. (A) Cells incubated in Cl^--containing solutions were transferred to either Cl^--containing solutions, or Cl^--free solutions (gluconate), or Cl^--free solutions plus 0.5 mM H_2DIDS. (B) Cells incubated in Cl^--free solutions were transferred to either Cl^--free solutions (gluconate) or Cl^--containing solutions with or without 0.5 mM H_2DIDS. Means and their standard errors in eight independent experiments. Significant differences with the first two columns: * $P < 0.001$.

changer responsible for the observed pH_i changes prefers HCO_3^- to other bases present in the external medium.

The Cl^--dependent pH_i changes were inhibited by H_2DIDS, an inhibitor of anion exchange in other cells [17] (Fig. 1 and Table II).

Two types of Cl^-/HCO_3^- exchangers have been described: Na^+-dependent and Na^+-independent anion exchangers [1–5]. In chicken enterocytes the pH_i response to external Cl^- was independent of Na^+. Repetition of the experiments in the absence of Na^+ (solu-

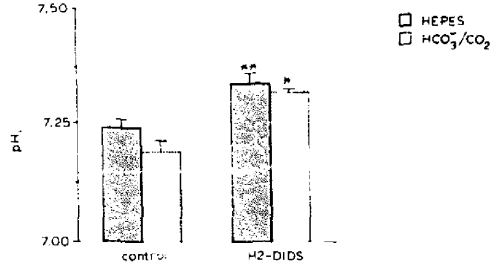

Fig. 2. Effect of H_2DIDS on resting pH_i. Dye-loaded cells were incubated for 15 min in the presence or in the absence of HCO_3^- and transferred to the same solution with or without 0.5 mM H_2DIDS. Means and their standard errors in eight independent experiments. Significant differences with the first two columns: * $P < 0.001$, ** $P < 0.005$.

tions C, D, G, and H) led to changes in pH_i identical to those found in the presence of Na^+ (Table II).

The results suggest that chicken enterocytes present a Na^+-independent Cl^-/base exchanger. Operation of this exchanger under steady-state conditions is implied, among other observations, by the significant increase in pH_i caused by H_2DIDS, both in the presence and in the absence of HCO_3^- (Fig. 2).

Cl^-/HCO_3^- exchanger and pH_i recovery from base loading

An alkaline load was imposed by incubation of the cells in 25 mM HCO_3^-/5% CO_2 (solution E) for 15 min and transfer to Hepes buffer nominally free of CO_2. This transfer resulted in an immediate alkalinization (Fig. 3 and Table II), attributed to the rapid efflux of CO_2, equivalent to a net proton extrusion. In the presence of Cl^- (transfer to solution A) this alkalinization was transient and pH_i decreased towards baseline. After 5 min, 87% of the initial pH_i increase had been recovered. This pH_i recovery was significantly smaller in the presence of H_2DIDS and in Cl^--free media (solution B instead of A).

Recovery from alkaline loads was independent of Na^+. Repetition of the experiments in the absence of

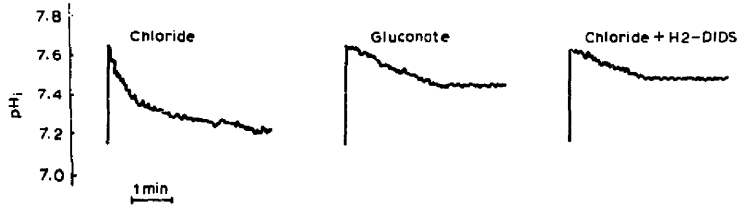

Fig. 3. Effect of external Cl^- and H_2DIDS on pH_i recovery from an alkaline load. Dye-loaded cells were incubated in the presence of HCO_3^- (solution E) with or without 0.5 mM H_2DIDS and transferred at the beginning of the trace to Hepes-buffered solution containing the indicated modifiers. The experiments were carried out in the presence of Na^+.

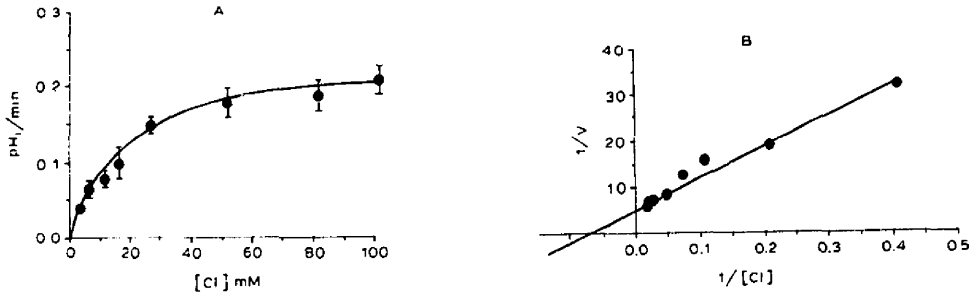

Fig. 4. Effect of external Cl^- on the rate of pH_i recovery from an alkaline load. Experiments as those in Fig. 3 were repeated in the presence of various concentrations of Cl^- (0 to 100 mM) in the Hepes-buffered solution. (A) pH_i change in the first min after the peak vs. Cl^- concentration. Means and their standard errors in 10 independent experiments. (B) Lineweaver-Burk plot of the data represented in A. Linear regression, $r = 0.99$.

TABLE II

Effect of external Cl^- on pH_i

Alkalinization and acidification are measured as the initial rates of change in pH_i observed upon removal and reinstatement of Cl^-, respectively. H_2DIDS was used at 0.5 mM. The net base flux, J_{OH}, is measured in nmol min^{-1} mg^{-1}. Means ± S.E. in eight independent experiments. Significant differences with the control: * $P < 0.001$.

Saline solution	Alkalinization on Cl^- removal		Acidification on Cl^- addition	
	pH units/min	J_{OH}	pH units/min	J_{OH}
HCO_3^-/CO_2 buffer				
Control	0.29 ± 0.03	116	0.27 ± 0.02	108
With H_2DIDS	0.06 ± 0.008 *	24	0.11 ± 0.01 *	44
Without Na^+	0.25 ± 0.02	100	0.23 ± 0.02	92
Hepes-buffer				
Control	0.16 ± 0.03	27	0.13 ± 0.03	22
With H_2DIDS	0.01 ± 0.002 *	2	0 *	0
Without Na^+	0.18 ± 0.02	22	0.16 ± 0.02	34

Na^+ (transfer from solution G to solution C) led to pH_i changes parallel to those found in the presence of Na^+ (Table III). Again pH_i recovery was significantly inhibited by H_2DIDS and by the absence of Cl^- (recovery in solution D instead of C).

These results suggest that a Na^+-independent, H_2DIDS-sensitive, Cl^-/base exchanger is responsible for most of the pH_i recovery from an alkaline load. We have no explanation for the residual acidification observed in the absence of Cl^- or in the presence of H_2DIDS.

Kinetics of Cl^-/base exchange

To estimate the kinetic parameters of pH_i recovery from an alkaline load, experiments such as those shown in Fig. 3 were repeated in the presence of various concentrations of external Cl^- (gluconate substitution). Because a residual acidification was observed in the

TABLE III

Effect of Cl^-, Na^+, and H_2DIDS (0.5 mM) on pH_i recovery from an alkaline load

Experimental conditions as for Fig. 3. Cl^--free solutions were made by gluconate substitution, and mannitol was used to replace NaCl. Means ± S.E. in n independent experiments. Significant differences with the control: * $P < 0.001$.

Recovery solution (Hepes)	n	pH_i			% recovery
		at peak	1 min after peak	5 min after peak	
With Na^+					
Chloride	5	7.65 ± 0.01	7.34 ± 0.01	7.20 ± 0.03	87
Gluconate	5	7.65 ± 0.02	7.53 ± 0.03 *	7.45 ± 0.02 *	38
Cl^- + H_2DIDS	7	7.65 ± 0.02	7.55 ± 0.01 *	7.50 ± 0.01 *	30
Without Na^+					
Chloride	10	7.57 ± 0.02	7.22 ± 0.03	7.19 ± 0.03	85
Mannitol	10	7.57 ± 0.04	7.50 ± 0.03 *	7.48 ± 0.01 *	20
Cl^- + H_2DIDS	5	7.57 ± 0.02	7.53 ± 0.02 *	7.45 ± 0.02 *	30

absence of Cl^-, the recovery rate with Cl^- was corrected for the recovery rate with gluconate. The initial acidification rate follows simple (Michaelis-Menten) saturation kinetics (Fig. 4). The rates were defined as the pH_i change in the first minute after the peak. Linear transformation of the data according to Lineweaver-Burk (Fig. 4) revealed an apparent K_m for external Cl^- of 12.5 mM and a V_{max} of 0.20 pH units/min.

Discussion

The resting pH_i of chicken enterocytes appears slightly more acidic in the presence of HCO_3^- than in its absence. All possible results have been reported for other cells: more acidic, like ours [18–22], more alkaline [23–28], and indistinguishable [29–33].

The total intracellular buffering power in HCO_3^--containing media is more than 2.3-times larger than the intrinsic one at external pH 7.4. Therefore, a given pH_i change represents more than 2.3 times the rate of transport of H^+ (or OH^- or HCO_3^-) in the presence of HCO_3^- than in its absence.

Chicken enterocytes posses a Na^+/H^+ exchanger operative under resting conditions and responsible for pH_i recovery from an acid load in the absence of HCO_3^- [13]. Now we present evidence for the existence in the same cells of a Cl^-/base exchanger that is functional at resting pH_i and involved in the recovery from an alkaline load. A recent report [23] has shown the involvement of a Cl^-/HCO_3^- exchanger in pH_i homeostasis in crypt and villus cells isolated from rabbit ileum.

The following observations are consistent with the presence of a Cl^-/base exchanger in chicken enterocytes and its operation under resting conditions: (i) increase of pH_i following removal of external Cl^-, (ii) return to resting pH_i values upon reinstatement of Cl^-, (iii) inhibition of these pH_i changes by H_2DIDS, (iv) Na^+-independence of these pH_i changes, and (v) cell alkalinization by H_2DIDS under resting conditions. Under physiological conditions the Cl^-/base exchanger would exchange intracellular HCO_3^- for extracellular Cl^-. The exchanger may also be responsible for the slight decrease in resting pH_i due to external HCO_3^-. Cl^-/base exchangers active at resting pH_i have been observed in rabbit ileal crypt and villus cells [23], rabbit S3 proximal tubules [34], gastric glands [35], Vero cells [20], IEC-6 cells [18] and LCC-PK cells [19].

The Cl^-/base exchanger in chicken enterocytes has no absolute requirement for exogenous HCO_3^-, since it works, at a slower rate, in Hepes-buffered solutions. This finding does not agree with previous observations that found no evidence for Cl^-/OH^- in chicken enterocytes [36]. The Cl^-/base exchangers of IEC-6 cells [18] and BSC-1 cells [29] require exogenous HCO_3^- to operate and do not work at all in Hepes-buffered solutions. Other Cl^-/base exchangers have a higher affinity for HCO_3^- than for OH^- [19,32,34,37–39]. An alternative explanation for our results is that metabolism produced sufficient endogenous CO_2/HCO_3^- to support a slow activity of a Cl^-/HCO_3^- exchanger without recourse to OH^- ions [40]. However, current experiments from our laboratory reveal the presence in chicken intestinal brush-border membrane vesicles of a Cl^-/base exchanger active in the absence of HCO_3^-.

Recovery from alkaline loads in chicken enterocytes required external Cl^-, was independent from Na^+, and was inhibited by H_2DIDS. These properties indicate that the process is mediated by the Cl^-/base exchanger operative under resting conditions. Cell acidification after an alkaline load as a function of external Cl^- concentration follows simple saturation kinetics; the affinity constant of the exchanger for external Cl^- is similar to that obtained with other epithelial cell types with radioisotope fluxes [37,41] or fluorescence methods [24,32].

Our results offer no cues on the cellular localization of the Cl^-/base exchanger. A Cl^-/HCO_3^- exchange has been repeteadly found in vesicles from brush-border membranes [42–44], but not in those from basolateral membranes [44] of the enterocyte. Our results are thus likely to refer to exchanges in the brush-border membrane.

Occurrence of both Cl^-/base and Na^+/H^+ exchanges in vesicles from the brush-border membrane of enterocytes allows absorption of NaCl across intestinal epithelia [42,43]. This does not preclude that the Cl^-/base exchanger intervenes in pH_i homeostasis in vivo, as it does in isolated cells.

Acknowledgement

The work was supported by a grant from the Spanish DGICYT No. PB89-0616.

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