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Kinetics of the chloride–anion exchanger of brush-border membrane vesicles isolated from chicken jejunum

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Abstract

The kinetic parameters of the apical Cl^-–anion exchanger of chicken jejunum have been evaluated using brush-border membrane vesicles. SITS inhibited pH gradient-driven Cl^- uptake in a dose-dependent manner with a IC_{50} of 700 μM. In the presence of a pH gradient (pH 7.7 inside, 5.5 outside), Cl^- uptake tends to saturate with increasing external Cl^- concentration. With 5 mM SITS the relationship between $^{36}Cl^-$ uptake and external Cl^- concentration was linear. SITS-insensitive Cl^- uptake may represent a diffusion component and has an apparent diffusion constant, K_d, of 0.068 ± 0.002 nmol/mg protein per 15 s per mM. Eadie-Hofstee analysis of the data obtained by subtracting the SITS-insensitive component from the total uptake indicates a single transport system with a K_m of 5 ± 0.9 mM. A 50 mM outwardly directed bicarbonate gradient increased the V_{max} from 3.63 ± 0.33 to 6.40 ± 0.54 nmol/mg protein per 15 s. The initial rate of H_2-DIDS-sensitive Cl^- uptake increased as the intravesicular pH increased, with a Hill coefficient greater than one. An intra- to extravesicular gradient of Cl^-, I^- and HCO_3^- stimulated Cl^- uptake in the absence of a pH gradient.

Key words: Brush-border membrane; Chloride–anion exchanger; Kinetics; (Chicken jejunum)

1. Introduction

The Na^+-independent Cl^-–anion exchanger is involved in the long-term control of intracellular pH (pH_i) [1–4]. The exchanger has been repeatedly found in the apical membrane of mammalian enterocytes [5] and recently in the basolateral membrane of rat jejunum [6]. Using a spectrofluorimetric method we presented evidence consistent with the presence of the exchanger in chicken enterocytes [7]. $^{36}Cl^-$ flux measurements in brush-border membrane vesicles revealed the presence of a Cl^-–anion exchanger in the apical membrane of chicken enterocytes [8].

In the current work we further characterize the apical Cl^-–anion exchanger of chicken jejunum by measuring the kinetics of the exchanger and its ionic selectivity.

2. Materials and methods

Brush-border membrane vesicles preparation. Brush-border membrane vesicles (BBMV) were obtained from the whole jejunum by double Mg^{2+} precipitation method as described by Cano et al. Purification of the BBMV preparation was assessed as previously described [8].

$^{36}Cl^-$ uptake studies. $^{36}Cl^-$ uptake was measured at 25°C by a rapid filtration technique as described by Cano et al. Briefly BBMV were left to stand at room temperature (approx. 25°C) for 10 min. Timed incubations at room temperature were initiated by adding 10 μl (400 μg of protein) of membrane vesicles suspension to 90 μl of uptake buffer. The composition of the intravesicular and uptake buffers is given in the figure legends. After designated periods of time, uptake was terminated by the addition of 2.5 ml of an ice-cold stop solution of the same composition as that of the intravesicular buffer. The samples were immediately filtered under vacuum through a 0.45 μm-pore size Millipore

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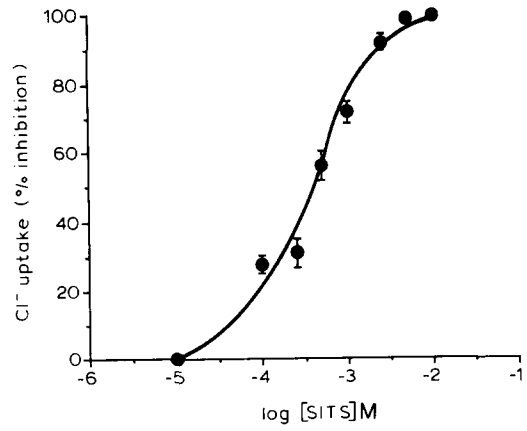

Fig. 1. Effect of SITS on chloride uptake. Uptake of Cl^- was measured during 15 s in the presence of a pH gradient. Vesicles contained 100 mM mannitol, 50 mM potassium gluconate, 70 mM Hepes-Tris (pH 7.7) and were preincubated for 15 min with SITS. Uptake buffer contained 100 mM mannitol, 50 mM potassium gluconate, 70 mM Mes-Tris (pH 5.5), 45 μM valinomycin, SITS and 5 mM $^{36}Cl^-$. Each point represents the mean value ±S.E. of triplicate assays using seven separate membrane vesicle preparations.

filter prewetted with the stop buffer. Filters were further washed twice with 5 ml of ice-cold stop solution, dissolved in 5 ml of Ready-Protein (Beckman) scintillation fluid and the radioactivity determined by liquid scintillation spectrometry. Nonspecific isotope binding to the filters was determined separately by adding stop solution to the vesicles before addition of uptake buffer, and subtracted from the total radioactivity of each sample.

The $^{36}Cl^-$ uptake measurements were carried out with the voltage across the membranes brought to zero by equal internal and external K^+ concentrations in the presence of valinomycin.

Materials. $H^{36}Cl^-$ (0.4 mCi/mmol, Amersham) was neutralized with Tris base before use. SITS (4-acetoamido-4'-isothiocyanostilbene-2-2'-disulphonic acid), valinomycin and all the salts used in the current study were obtained from Sigma (Madrid, Spain). The membrane filters (Millipore HAWP02500) were obtained from Millipore Products Division (Barcelona, Spain).

Statistical analysis. Individual experiments were carried out in triplicate. Results are presented as the mean and its standard error. In the figures vertical bars, that represent the S.E., are absent when they are less than symbol height. Comparison between different experimental groups was evaluated by the two-tailed Student's *t*-test.

3. Results and discussion

3.1. Effect of SITS on $^{36}Cl^-$ uptake

The stilbene-disulfonic acid derivative SITS inhibits Cl^-–anion exchange in several cell types and in chick jejunum [7,8]. Fig. 1 represents a log-dose response curve of SITS inhibition of $^{36}Cl^-$ uptake in the presence of a pH gradient (7.5 inside, 5.5 outside). Overall flux was inhibited by SITS with apparent half-maximal inhibition (IC_{50}) at 700 μM SITS under the conditions tested. The best fit curve and the calculation of the apparent IC_{50} were obtained using a nonlinear regression program to fit the data (ENZFITTER program).

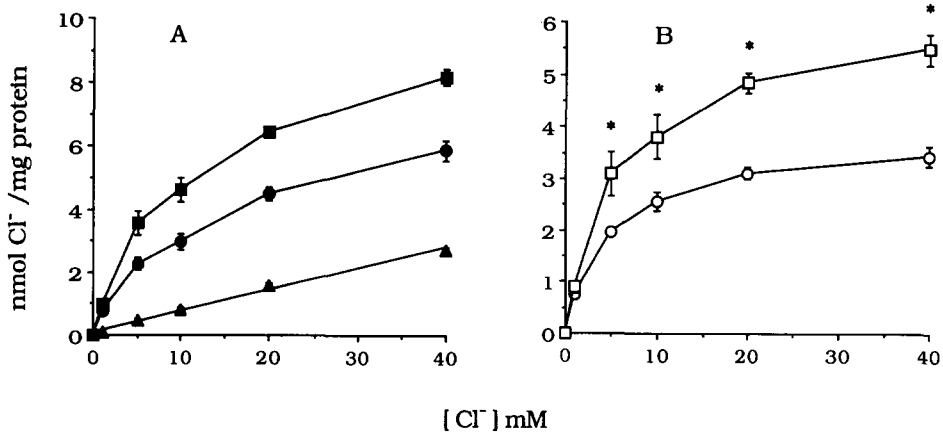

Fig. 2. Effect of increasing concentrations of external Cl^- on the initial rate (15 s) of Cl^- uptake. Vesicles were loaded with either 100 mM mannitol, 50 mM potassium gluconate, 70 mM Hepes-Tris (pH 7.7) (●) or 150 mM mannitol, 50 mM $KHCO_3^-$, 20 mM Hepes-Tris (pH 7.7) (■). Uptake buffer contained 100 mM mannitol, 70 mM Mes-Tris (pH 5.5), 45 μM valinomycin and the appropriated concentrations of gluconate and chloride to keep the potassium salt constant. The external chloride concentration ranged from 1 to 40 mM. When SITS was used, membranes were incubated for 15 min with SITS. (A) Total Cl^- uptake (■, ●) and uptake in the presence of 5 mM SITS (▲). (B) Difference between total uptake and that in the presence of SITS, with (□) or without (○) bicarbonate gradient. Each point represents the mean value ±S.E. of triplicate assays using five independent membrane vesicle preparations. * $P < 0.001$ as compared with data obtained in the absence of bicarbonate gradient.

Table 1
Kinetic parameters of chloride uptake into BBMV in the presence and in the absence of bicarbonate

	V_{max}	K_m	K_d
Without bicarbonate			
Calculated	3.20±0.42	3.94±0.80	0.077±0.004
Experimental	3.63±0.33	5.00±0.90	0.068±0.002
With bicarbonate			
Calculated	6.83±0.58 *	4.13±0.82	0.084±0.008
Experimental	6.40±0.54 *	5.26±0.80	0.068±0.002

Data are the means±S.E. of five independent determinations. Calculated kinetics parameters were determined with a computer program using an iterative non-linear regression procedure (ENZFITTER). Experimental: the diffusion component was evaluated by measuring chloride uptake in the presence of SITS and it was subtracted from the total uptake. V_{max} is expressed in nmol/mg protein per 15 s, K_d in nmol/mg protein per 15 s per mM and K_m is given as units of mM. * $P < 0.05$ as compared with data obtained in the absence of bicarbonate gradient.

The IC_{50} value for SITS inhibition is comparable to that reported for other epithelial cell types [9,10].

3.2. $^{36}Cl^-$ uptake vs. external Cl^- concentration

Uptake of $^{36}Cl^-$ was measured over the concentration range of 1 to 40 mM chloride in the presence of pH gradient and in the presence and absence of a bicarbonate gradient. $^{36}Cl^-$ uptake increased as Cl^- concentration increased (Fig. 2A). Although the curve shows an inflexion at low Cl^- concentration it becomes a straight line at higher concentrations, not yielding a saturation curve. The shape of this curve suggests the existence of at least two components in the uptake process: a saturation process upon which is superimposed a diffusion component. The data were subsequently fitted by computer (ENZFITTER program) to one transport system (Cl^-–anion exchanger) plus a nonsaturable (diffusion) term

$$v = (V_{max} \cdot S / K_m + S) + K_d \cdot S$$

where v is initial rate of uptake, S is the external chloride concentration, V_{max} is the maximal initial uptake rate, K_m is the Michaelis-Menten constant, and K_d is the apparent diffusion constant. The calculated apparent K_m, K_d and V_{max} for $^{36}Cl^-$ are given in Table 1. The results show that a 50 mM intra- to

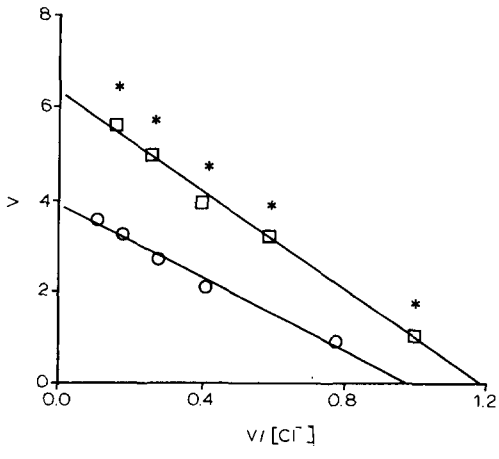

Fig. 3. Eadie-Hofstee plot of the data shown in Fig. 2B with (□) or without (○) bicarbonate gradient. V, nmol Cl^-/mg protein per 15 s. Kinetic parameters (i.e., the apparent K_m and V_{max}) of the Cl^-–anion exchanger were calculated using linear regression analysis and are summarized in Table 1. * $P < 0.001$ as compared with data obtained in the absence of bicarbonate gradient.

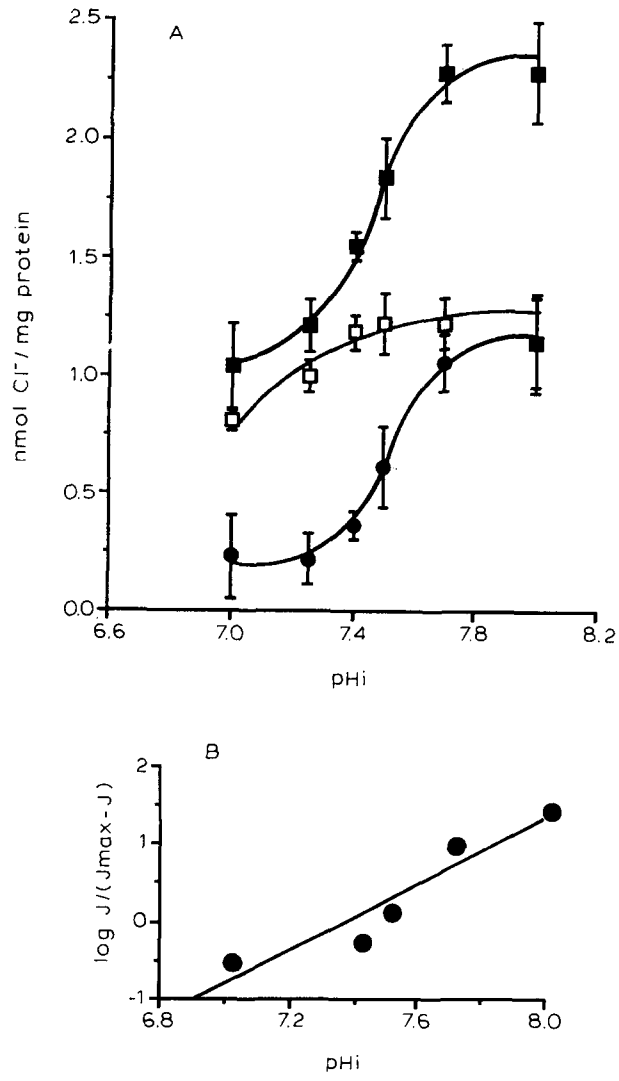

Fig. 4. Effect of intravesicular pH on Cl^- uptake. (A) Vesicles were loaded with 100 mM mannitol, 50 mM potassium gluconate and 70 mM Hepes buffered with Tris to indicated intravesicular pH. 15 s uptake of 5 mM $^{36}Cl^-$ was assayed in the presence of 100 mM mannitol, 50 mM potassium gluconate and 70 mM Hepes buffered with Tris to 6.5 (■) or 8 (□). In (●) chloride influx at pH_o 8 was subtracted from values at pH_o 6.5. Each point represents the mean value ±S.E. of triplicate assays using five independent membrane vesicle preparations. (B) Hill plot of data in A. The line was calculated by linear regression analysis, $y = -15.63 + 2.11x$, $r = 0.90$.

extravesicular bicarbonate gradient slightly increased the V_{max}.

The contribution of the simple diffusion component to the total uptake rate was also evaluated experimentally by measuring chloride uptake in the presence of a pH gradient and 5 mM SITS (Fig. 2A). With SITS the relationship between chloride uptake and external chloride concentration was linear and the data were used to calculate the apparent diffusion constant, K_d (Table 1). The K_d obtained for the SITS-independent component of Cl^- uptake was not significantly different from the calculated K_d (ENZFITTER program). The difference between total chloride uptake and that obtained in the presence of SITS gave the uptake mediated by the SITS-sensitive Cl^- uptake process (Fig. 2B). Kinetic analysis with an Eadie-Hofstee plot (Fig. 3) yielded a linear relationship, indicating that only one Cl^-–anion exchanger is present. The apparent transport constant, K_m, and maximal rate of transport for Cl^-, V_{max}, are given in Table 1. Again these values were not different from the calculated values. The apparent K_m for external Cl^- is similar to that reported for the Cl^-–anion exchanger of rabbit ileum [10], MDCK cells [11] and Vero cells [12] and lower than that reported for rat duodenum [9], rabbit ileum [13], rat colon [14], rat intestine [15] and rabbit collecting duct [16].

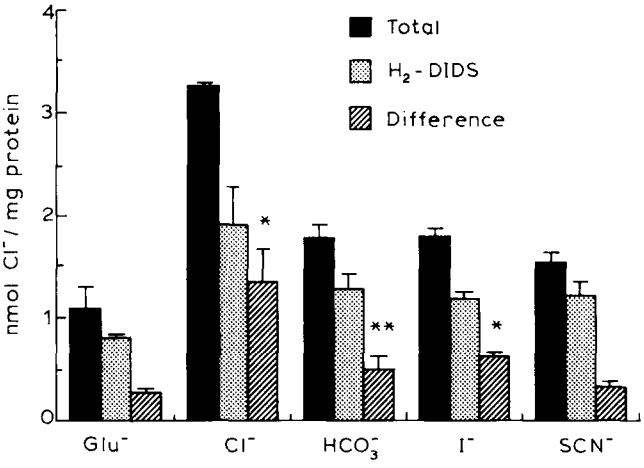

Fig. 5. Effect of intra- to extravesicular anion gradient on Cl^- uptake in the absence of pH gradient. The vesicles were loaded with a 7.7 buffer of the following composition: 100 mM mannitol, 50 mM potassium anion and 70 mM Hepes-Tris. Uptake buffer contained 100 mM mannitol, 50 mM potassium gluconate, 70 mM Hepes-Tris (pH 7.7), 45 μM valinomycin and 5 mM $^{36}Cl^-$. The concentration of H_2-DIDS was 1 mM and the vesicles were preincubated for 15 min with H_2-DIDS. The time of uptake was 15 s. Each point represents the mean value ± S.E. of triplicate assays using three independent membrane vesicle preparations. Difference: total uptake minus that in the presence of H_2-DIDS. * $P < 0.001$, ** $P < 0.05$ as compared to data obtained with gluconate gradient.

3.3. Cl^-–anion activity and intravesicular pH

In a variety of cell types the Na^+-independent Cl^-–anion exchanger functions at a low rate at resting pH_i, but becomes activated with increasing pH_i [11,12,17–22]. The sensitivity of the exchanger of chicken jejunum to pH was investigated by measuring chloride uptake into BBMV with the intravesicular pH preset to various values, and at two different external pH (pH_o): 6.5 and 8. Chloride uptake was stimulated by increasing internal pH from 7 to 8 at pH_o 6.5. The increase was almost completely inhibited at a pH_o of 8 (Fig. 4). The relationship between Cl^- uptake and pH_i did not follow simple Michaelis-Menten kinetics (Fig. 4A). The Hill plot gives an interaction coefficient (n) of 2.11 and a $[H^+]_{0.5}$ of 39 nM (Fig. 4B). This indicates that Cl^-–anion exchanger may be allosterically regulated by pH_i and that it may be involved in pH_i regulation.

3.4. Effect of trans-anions on chloride uptake

The apical Cl^-–anion exchanger of chicken jejunum was further characterized by investigating its anion selectivity (Fig. 5). Compared with the magnitude of chloride uptake measured under control conditions (gluconate), chloride uptake was significantly stimulated by outwardly directed gradients of several anions. Stimulation of chloride uptake by those anions were sensitive to inhibition by H_2-DIDS. The Cl^-–anion exchanger in chicken jejunal BBMV is capable of catalyzing Cl^-–Cl^-, Cl^-–OH^-, Cl^-–bicarbonate and Cl^-–I^- exchange. The H_2-DIDS-sensitive component of the anion-driven Cl^- uptake suggest a selectivity series of $Cl^- > I^- \geq HCO_3^- > SCN^-$ = gluconate.

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