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Cl^-/HCO_3^- exchanger is operative in isolated enterocytes from rat jejunum

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Abstract. Enterocytes isolated from rat jejunum were tested for the existence of a Cl^-/HCO_3^- exchange, previously evidenced in basolateral membrane vesicles but not in brush border. Cells were found to retain functional integrity and transport capabilities long enough to allow Cl^- fluxes to be measured. Both efflux and uptake experiments indicate that a Cl^-/HCO_3^- antiport, inhibited by 4,4′-diisothiocyanostilbene-2-2′-disulfonic acid (DIDS), is functional under resting conditions.

Key words. Rat jejunum; isolated enterocytes; Cl^-/HCO_3^- exchange.

In the plasma membranes of a wide variety of animal cells two kinds of Cl^-/HCO_3^- exchange have been identified. A Na^+-independent Cl^-/HCO_3^- antiport extrudes bases under physiological conditions[1–6], while a Na^+-linked Cl^-/HCO_3^- antiport utilizes the Na^+ gradient to bring $NaHCO_3$ into the cell, thereby extruding acid[1,5–9]. These two systems coexist in several cell types[1,4,5–9]. Recent studies performed with membrane vesicles[10–12] indicate that at the basolateral pole of rat jejunal enterocyte a Na^+-independent Cl/HCO_3^- exchanger could account for HCO_3^- efflux from the cell during HCO_3^- absorption. The presence of Na^+ positively affects the rate of anion exchanger, but neither HCO_3^- nor Cl^- gradients can drive the countertransport of Na^+. Based upon these observations a model was proposed which implies the existence of a Na^+-stimulated modifier site at the inner surface of the basolateral membrane[13].

To assess the effectiveness of the physiological model, an absolute requirement is to perform experiments on whole cells; the aim of the present work was to isolate intact and viable jejunal enterocytes in which the Cl^-/HCO_3^- exchanger was operative. Its sensitivity to 4,4′-diisothiocyanostilbene-2-2′-disulfonic acid (DIDS) and the effect of sodium removal will be considered.

Material and methods

Cell isolation. Two male albino rats (Wistar strain, Charles River italiana), weighing 200–300 g, fed with a standard rat chow and starved for 24 hours, were used. Under 2,2,2-tribromoethanol anaesthesia, a 20 cm length of jejunum was taken. The method of intestinal epithelial cell isolation used in this study was a modification of the method described by Weiser[14]. Briefly, after removal the length of intestine was rinsed thoroughly with oxygenated, ice-cold wash solution (154 mM NaCl, 1 mM 1.4-dithiothreitol (DTT)) and then filled with buffer A (96 mM NaCl, 1.5 mM KCl, 27 mM Na citrate, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml bovine serum albumin (BSA), 5.6 mM Na_2HPO_4/KH_2PO_4 pH 7.3) pre-oxygenated with 100% O_2 and warmed to 37 °C. The filled intestine was submerged in oxygenated buffer A and incubated for 10 min in a 37 °C water bath with shaking at 70 cycles/min. The luminal content was discarded and the procedure was repeated using buffer B (140 mM NaCl, 1.5 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 mg/ml BSA, 16 mM Na_2HPO_4/KH_2PO_4 pH 7.3). The intestine, submerged in buffer B, was shaken for 5 min in the 37 °C thermostated water bath. After that, the jejunal tract was gently palpated for 2 min to facilitate cell dispersion. The solution containing isolated cells were then drained from the jejunal loop, diluted in buffer C (137 mM NaCl, 5.2 mM KCl, 0.6 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.1 mM DTT, 0.2 mM PMSF, 10 mM glucose, 5 mM glutamine, 1 mg/ml BSA, 3 mM Na_2HPO_4/KH_2PO_4 pH 7.3) at 0–4 °C and filtered through a 250 μm pore size nylon mesh. The treatment with buffer B was repeated once again, reducing to 3 min the incubation time. All collected cells were filtered through a 100 μm pore size nylon mesh before being washed twice with buffer C by resuspension and centrifugation at 59 × g for 3 min at 4 °C. Isolated cells resuspended in the desired volume were held in polyethylene vessels, immersed in crushed ice until ready for use.

Colorant exclusion. Cell viability was assessed by determining the fraction of the cell population able to exclude 0.2% Trypan blue for up to 1 h after isolation.

Lactate dehydrogenase determination. The enzyme that leaked out of the cells during incubation time was determined in the supernatant of centrifuged (59 × g for 3 min) samples by means of an enzymatic determination

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Table. Test of the functional integrity of the isolated enterocytes. A) Lactate dehydrogenase released to the medium as a percentage of total lactate dehydrogenase activity measured after cell lysis by 0.5% Triton X–100. Total lactate dehydrogenase activity does not change during incubation time. B) Lactate production in the presence of 20 mM glucose. C) 3-O-MG accumulated by isolated enterocytes. Na^+-driven sugar uptake was measured by incubating cells in solution C without glucose in the presence and absence of 0.1 mM phlorizin. The incubation medium contained 0.1 mM 3-O-MG and trace amounts of ^3H-labelled sugar (0.5 μCi/ml).

		0 min	30 min	60 min	N
A) LDH (%)		–	11.33 ± 1.36	32.25 ± 0.64	6
		0–10 min	10–20 min	20–30 min	
B) Lactate production (μmoles/mg protein)		0.633 ± 0.061	0.667 ± 0.058	0.594 ± 0.066	6
			1 min	5 min	
C)3-O-MG uptake (cpm/mg protein)	137 mM Na		2289 ± 606	6212 ± 475	5
	137 mM Na + Phlorizin		1089 ± 135	2607 ± 678	5

kit (Boehringer, Germany). Total lactate dehydrogenase activity was measured after cell lysis by using 0.5% Triton X-100. Determinations were always performed at least in triplicate.

Lactate determination. Lactate produced by the isolated cell metabolism during incubation in the presence of 20 mM glucose was determined in the supernatant of centrifuged (59 × g for 3 min) samples using a commercially available enzymatic kit (Boehringer, Germany). Determinations were always performed at least in triplicate. Data are normalized to the amount of cellular protein.

Uptake studies. 3-O-Methyl glucose (3-O-MG) and Cl^- uptake experiments were performed. After isolation, cells were washed two times with the desired uptake medium (see figure legends) and resuspended at a final concentration of 2–3 mg cell protein/ml. Uptake experiments were performed at 37 °C and within 1 h after isolation. Under oxygenation 200 μl prewarmed cell suspension were mixed with 200 μl of the prewarmed incubation medium containing the appropriate solute and isotope tracer. At selected times the uptake was stopped by diluting the 400 μl cell suspension in 400 μl of ice-cold incubation medium without isotope tracer. Cells were immediately separated by centrifugation at 13,000 × g for 30 s through a 250 μl layer of the oil misture di-n-butyl phtalate: di-nonyl phtalate 3:2. Pellets were resuspended in 200 μl of water, stirred for 3 min and counted by liquid scintillation after protein precipitation with 10% trichloroacetic acid (TCA). Pellets were resuspended in 1 M NaOH and cellular proteins determined by the Coomassie blue method. All values are normalized to the amount of cellular proteins.

Cl^- efflux. To measure the release of $^{36}Cl^-$ from the isolated cells, a concentrated cell suspension (15–20 mg protein/ml) was pre-loaded by incubating in solution C at 37 °C for 10 min in the presence of $^{36}Cl^-$ (9.5 μCi/ml) under oxygenation. The rate of $^{36}Cl^-$ loss form the pre-loaded cells was then evaluated by hundred fold dilution of aliquots of this suspension with radioisotope-free buffer incubated at 37 °C. At selected times (0, 5 min) the efflux was terminated and samples taken for counting were processed as described in the uptake experiments.

Statistical analysis. Values reported in the text are means ± SE. Student's t-test was used for statistical analysis.

Results

At the end of the isolation procedure, cell viability was assessed by determining the fraction of the cell population able to exclude 0.2% Trypan blue. Viable cells were 73.33 ± 2.78% (number of experiments = 7). Viability decreased to 63.87 ± 4.21% (n = 8) after 30 min of incubation at 37 °C with agitation and oxygenation and was drastically reduced to 49.25 ± 5.28% (n = 8) after 60 min.

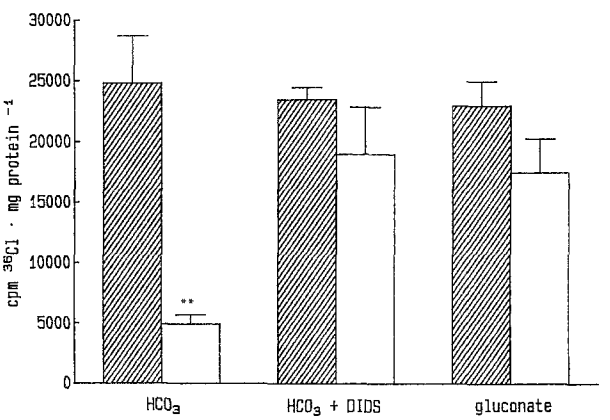

Figure 1. Cl^- efflux from preloaded ($^{36}Cl^-$) enterocytes. Cells preloaded with $^{36}Cl^-$ were incubated in solution C in which Cl^- was iso-osmotically substituted either with HCO_3^- (added or not with 0.1 mM DIDS) or with gluconate. $^{36}Cl^-$ content of the cells is reported after preloading (hatched columns) and after 5 min incubation in the Cl^--free solution (open columns). Significant difference from the filled column: $^{**}p < 0.001$.

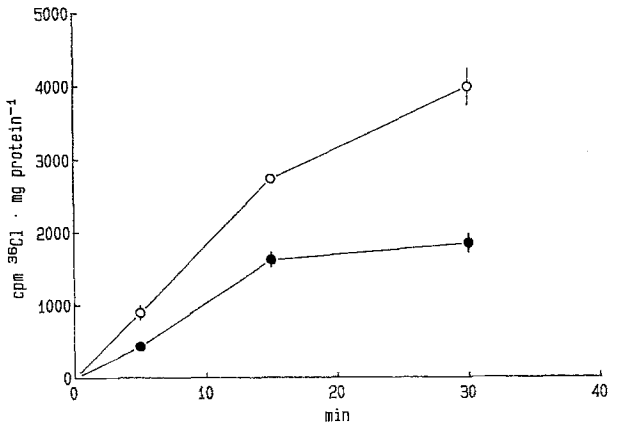

Figure 2. Time course of $^{36}Cl^-$ uptake into isolated enterocytes. Cells were incubated in solution C in the absence (open circles) and in the presence (filled circles) of 0.1 mM DIDS. The incubation medium contained trace amounts of $^{36}Cl^-$ (0.2 μCi/ml). The extracellular $^{36}Cl^-$ associated with the cell pellet was subtracted from each uptake value.

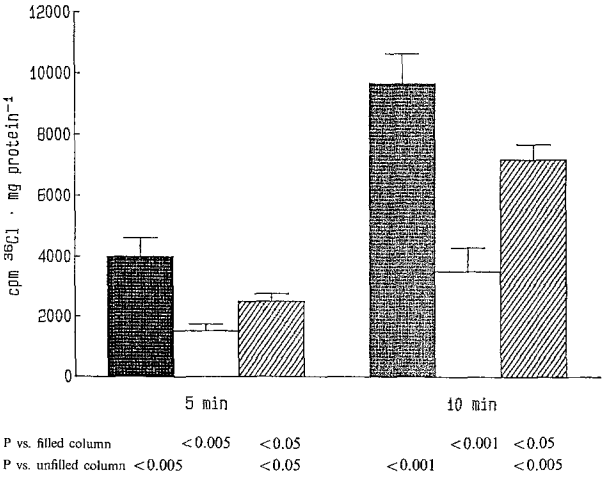

Figure 3. $^{36}Cl^-$ uptake into isolated enterocytes incubated in a Cl^--free buffer. Cells obtained either in solution C (filled and unfilled columns), or in solution C in which Na^+ was iso-osmotically substituted with TMA (dashed columns), were incubated in soluction C in which Cl^- was iso-osmotically substituted with gluconate. The incubation was performed either in the absence (filled columns) or in the presence (open columns) of 0.1 mM DIDS, or in solution C in which, besides Cl^- removal, Na^+ was iso-osmotically substituted with TMA (hatched columns). The incubation medium contained $^{36}Cl^-$ (0.2 μCi/ml). The extracellular $^{36}Cl^-$ associated with the cell pellet was subtracted from each uptake value. P values with respect to filled and open columns are reported beneath each column.

Table 1 shows the lactate dehydrogenase released to the medium as a percentage of the intracellular lactate dehydrogenase activity. Lactate dehydrogenase loss increased during incubation (30–60 min).

Subsequent experiments were performed to assess metabolic function of isolated jejunal cells with respect to time. Glycolytic activity was determined by measuring lactate formation from glucose. As shown in table 1, lactate production was linear for 30 min.

In separate experiments 3-O-MG uptake was evaluated in the presence and absence of 0.1 mM phlorizin, in order to evaluate whether cellular function was preserved. Results, shown in table 1, indicate that isolated enterocytes accumulate the sugar in a phlorizin-sensitive manner.

The presence of Cl^-/HCO_3^- exchanger was tested by means of both Cl^- efflux and Cl^- uptake experiments. To determine Cl^- efflux, cells were preloaded with $^{36}Cl^-$ at high activity in order to enhance the initial cpm value (fig. 1). Cells were then incubated for 5 min in the presence and absence of an inwardly directed bicarbonate gradient. The translocation of Cl^- mediated by the anion antiporter is operationally defined by its sensitivity to inhibition by DIDS[15]; as depicted in figure 1, efflux was significantly reduced by 0.1 mM DIDS.

Figure 2 illustrates the time course of $^{36}Cl^-$ influx into the isolated enterocytes in the absence and presence of DIDS. In the absence of DIDS the uptake follows an exponential time course; DIDS produces a drastic reduction in Cl^- uptake.

To establish the existence of a Cl^-/HCO_3^- exchanger in the plasma membrane of jejunal enterocytes, the transmembrane Cl^- gradient was inverted by removal of extracellular Cl^-. Cells were incubated in a Cl^--free buffer, osmotically balanced with gluconate and containing trace amounts of $^{36}Cl^-$. Under these conditions $^{36}Cl^-$ moves against its concentration gradient and is energetically driven by Cl^- efflux. As illustrated in figure 3, Cl^- accumulates in the cells and DIDS exerts a strong inhibition. If cells are Na^+-depleted and incubated in a Na^+-free solution, Cl^- uptake is significantly reduced, but is still higher than the value obtained in the presence of DIDS.

Discussion

The isolated cells were judged to be functionally intact because they satisfied a number of tests designed to ascertain three logically related aspects: 1) the plasma membrane integrity; 2) the ability of cells to perform biochemical functions; 3) the ability of cells to accumulate selected solutes from the external medium.

The microscopic appearance suggests that the cell surface membrane was relatively impermeable to Trypan blue for at least 30 min. Moreover, table 1 shows that no more than 13% of the total lactate dehydrogenase activity was lost into the suspension medium during the first 30 min of incubation under specified conditions; about 32% of the enzyme activity was found in the suspension medium after a 60-min incubation. These two independent tests suggest that the cell surface membrane of the isolated enterocytes remained quite stable for 30 min at 37 °C under standard conditions; therefore in all subsequent experiments incubation was restricted to 30 min or less.

A test of preserved metabolic function was then performed. Net lactate production from exogenous glucose proceeded linearly with incubation time for at least 30 min (table 1). The last data reported in table 1 concern 3-O-MG uptake studies. The sugar, which is not metabolizable, was used to examine the transport capability of the epithelial cell preparation. A rapid entry of 3-O-MG occurs under the experimental conditions imposed; influx is very sensitive to the addition of the sugar transported inhibitor phlorizin. Its action on the isolated cells is a significant clue that the transport capability of the cells has not been destroyed during the isolation procedure.

In summary, cells are found to retain their functional integrity and transport capabilities long enough to allow further investigation. The major experimental limitation of this system is the relatively short period of cell viability (about 30 min): however our data agree with those reported in the literature for rat enterocytes[16–19].

To test for the existence of a Cl^-/HCO_3^- exchanger in the plasma membrane of the isolated cells, Cl^- efflux was measured in the presence of 143 mM external HCO_3^-. Under this condition and if an anion exchanger is working, the exit of Cl^- could be driven by the entry of bicarbonate. The data shown in figure 1 give evidence that Cl^- efflux is transstimulated by HCO_3^- and inhibited by 0.1 mM DIDS. These observations indicate that the process could be mediated by the Cl^-/HCO_3^- exchanger, operative under resting conditions. Our results offer no clues on the cellular location of the Cl^-/HCO_3^- exchanger since both brush border and basolateral membrane are exposed and solute fluxes at all surfaces can contribute to observed transport rates. Nevertheless, previous work gave evidence that in rat jejunum the Cl^-/HCO_3^- antiport occurs in basolateral membranes and is absent in brush border[10].

Since in a previous study carried out with basolateral membrane vesicles we demonstrated that the Cl^-/HCO_3^- antiport can perform Cl^-/Cl^- self exchange[10], not only Cl^- efflux but also Cl^- uptake should be mediated by the DIDS-inhibitable anion transport mechanism. Figure 2 illustrates the time course of Cl^- uptake and the effect of DIDS.

To exclude the possibility that Cl^- moves through DIDS-sensitive channels, in subsequent experiments Cl^- gradient was inverted by removal of extracellular Cl^-. When cells are transferred from a medium containing 143.4 mM Cl^- to a Cl^--free buffer, osmotically balanced with gluconate and containing trace amounts of $^{36}Cl^-$, $^{36}Cl^-$ is accumulated in the cells by the antiport energetically driven by Cl^- efflux. Results, depicted in figure 3, show that in these experimental conditions Cl^- uptake is drastically reduced by DIDS. These data were obtained in the presence of physiological Na^+ concentrations. Studies previously performed with membrane vesicles[10–12] indicates that the jejunal basolateral Cl^-/HCO_3^- exchanger can work in the absence of sodium but is stimulated by its presence: therefore, to give a preliminary insight into the effectiveness of these findings in whole cells, we performed experiments with Na^+-depleted enterocytes incubated in the absence of sodium. These data are also presented in figure 3: it seems that the anion exchanger is also operative after Na^+ removal, even if to a lesser extent. This result is in agreement with previous studies[10–12].

In accordance with the findings in isolated basolateral membranes, jejunal enterocytes seem to possess Cl^-/HCO_3^- exchanger that is functional under resting condition. Under physiological conditions Cl^-/base antiporter would exchange intracellular bicarbonate with extracellular Cl^- and play a role in triggering cellular pH[4,7,8,13]. Having isolated jejunal enterocytes and demonstrated their viability and the presence of a Na^+-sensitive Cl^-/HCO_3^--exchange, our future studies will focus on the Na^+-dependence of the anion antiporter and on its role in the complex environment of the whole cell.

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