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Cl/HCO_3 Exchange in the Basolateral Membrane Domain of Rat Jejunal Enterocyte

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Summary. Basolateral membrane vesicles isolated from rat jejunal enterocyte and well purified from brush border contamination were tested to examine Cl and HCO_3 movements. Uptake experiments provided no evidence for a coupling between Na and HCO_3 fluxes; K-HCO_3 and K-Cl cotransports also could be excluded. Transport studies revealed the presence of a Cl/HCO_3 exchanger accepting other anions and inhibitable by the disulfonic stilbenes SITS and DIDS. We can exclude that the evidenced HCO_3-dependent Cl uptake is due to brush border contamination, since in jejunal brush border membranes this mechanism, if present, has a very low transport rate. Besides the Cl/HCO_3 antiporter, a Cl-conductive pathway seems to exist in jejunal basolateral membranes.

Key Words rat jejunum · basolateral membrane vesicles · Cl/HCO_3 exchange · Na-HCO_3 cotransport · disulfonic stilbenes

Introduction

Both bicarbonate and chloride are absorbed in the jejunal tract of the intestine [31]. The Na/H exchange mechanism [42] localized in the brush border membrane [26], coupled with CO_2 diffusion from the lumen and its subsequent hydration by means of carbonic anhydrase [8], can account for HCO_3 entrance into the cell. On the contrary, the ileum and proximal colon secrete HCO_3 [32]; since a Cl/HCO_3 exchange mechanism is evident in the apical membrane of these intestinal tracts [21, 24, 41], the functionally coupled Na/H and Cl/HCO_3 antiporters can promote NaCl entrance from the lumen into the enterocyte. Moreover, these mechanisms are involved in intracellular pH and volume homeostasis [19].

In the basolateral membrane domain of various epithelial tissues a number of anion transport systems have been described dealing with bicarbonate and chloride movements: Cl/HCO_3 exchange [3, 13, 16], Na-coupled Cl/HCO_3 exchange [3, 14, 16, 33, 37], electrogenic Na-$(HCO_3)_3$ cotransport [2, 5, 9, 12, 16, 17, 37, 44], passive rheogenic bicarbonate transfer [4, 6] and Cl conductance [20, 35]. There is evidence that in several cell types anions cross basolateral membranes by means of multiple pathways [13, 28, 33].

Despite the importance of HCO_3 and Cl absorption in the physiology of the jejunum, the molecular mechanisms underlying their transport across the basolateral membrane are obscure. In the present study HCO_3 and Cl movements were investigated using basolateral membrane vesicles; evidence is presented that the process which results in HCO_3 absorption involves a serosally located anion exchange process.

Materials and Methods

Basolateral Membrane Isolation

Two male albino rats (Wistar strain, Charles River Italiana) weighing 250–300 g (about two months age), fed a rodent laboratory chow and tap water, were used for each experiment. Jejunal enterocytes were collected by scraping off the mucosal layer and diluted in 250 mM sucrose, 0.2 mM phenylmethanesulphonylfluoride (PMSF), 0.01% (vol/vol) ethanol, 10 mM HEPES/Tris buffer, pH 7.5. Basolateral plasma membranes were isolated and purified exactly as described previously [29]. Briefly, basolateral membranes collected by self-orienting Percoll-gradient centrifugation (Kontron, Centrikon mod. T 2070 ultracentrifuge; Haake-Buchler, Auto Densi-Flow IIC apparatus), were suspended in the appropriate buffer (*see* single experiment). Five mM $CaCl_2$, which aggregates preferentially all membranes except brush border, was added. Collected pellets (basolateral membrane fraction) were washed and used for analysis and for uptake experiments. To ensure that the intravesicular space was loaded with the appropriate buffer, the collected pellets were then incubated in the same buffer at room temperature for at least 90 min (gassed with the appropriate CO_2 tension when $NaHCO_3$ was present), and used after that for Cl uptake by the rapid micro-filtration technique.

To control the purity of the basolateral membrane fraction, as a rule total protein, γ-glutamyltransferase (γ-GT, a marker enzyme for brush border membrane) and (Na,K)-ATPase (a marker enzyme for basolateral membrane) were determined as published [29].

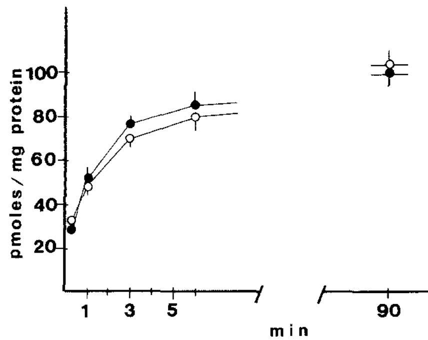

Fig. 1. 0.1 mM Na uptake into basolateral membrane vesicles. 50 μl vesicles obtained in 74 mM sorbitol and pre-equilibrated with 25 μM valinomycin were incubated in 200 μl of either 14 mM methylglucamine-HCO_3 and 54 mM sorbitol (filled circles) or 74 mM sorbitol (open circles). All solutions contained 20 mM HEPES/Tris buffer at pH 8.2, 100 mM K-gluconate, 0.2 mM PMSF, and 0.01% (vol/vol) ethanol. In addition, the incubating solution contained trace amounts of ^{22}Na and cold NaCl to a final concentration of 0.1 mM. *Ordinate:* Na uptake, mean values ± SE (= vertical bars, absent if less than symbol height). *Abscissa:* incubation time.

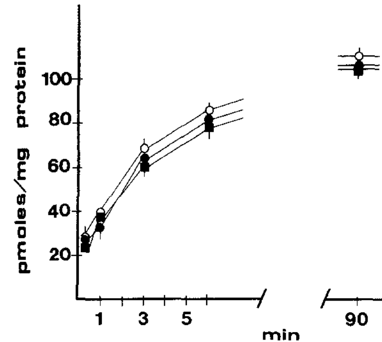

Fig. 2. 0.1 mM Rb uptake into basolateral membrane vesicles. 50 μl vesicles obtained in 70 mM sorbitol were incubated in 200 μl of either 14 mM methylglucamine-HCO_3 and 50 mM sorbitol (filled circles), or 14 mM methylglucamine-Cl and 50 mM sorbitol (filled squares), or in 70 mM sorbitol (open circles). All solutions contained 20 mM HEPES/Tris buffer at pH 8.2, 100 mM $NaNO_3$, 0.2 mM PMSF and 0.01% (vol/vol) ethanol. In addition the incubating solution contained trace amounts of ^{86}Rb and cold RbCl to a final concentration of 0.1 mM. *Ordinate:* Rb uptake, mean values ± SE (= vertical bars, absent if less than symbol height). *Abscissa:* incubation time.

Brush Border Membrane Isolation

Brush border membrane vesicles were isolated from rat jejunum enterocytes by the Ca^{2+} precipitation method first described by Schmitz et al. [38]. Mucosal scrapings, homogenized in hypotonic solution (50 mM sorbitol and 2 mM Tris Cl, pH 7.1) and incubated with 10 mM $CaCl_2$ for 15 min at 0°C, were centrifuged at 3,000 × *g* for 15 min. The supernatant was centrifuged at 27,000 × *g* for 30 min. The pellet was resuspended in the appropriate buffer and centrifuged at 43,000 × *g* for 20 min. The final pellet was resuspended again in the above solution.

Uptake Experiments

Sodium, rubidium and chloride uptakes were carried out. A volume of basolateral membrane suspension (2–4 mg protein/ml), equilibrated with 0.2 mM EGTA, was mixed at 28°C with the proper incubation solution. The composition of the resuspension buffers and incubation media are given in the legends of the figures. Samples were taken at selected times and diluted with 0.8 ml ice-cold reaction-stopping solution (139 mM K gluconate, 0.2 mM PMSF, 0.01% (vol/vol) ethanol, 20 mM HEPES/Tris buffer at either pH 7.5 or 8.2, according to the experiment), filtered on wetted cellulose nitrate filters (0.45 μm pore size) and immediately rinsed with 5 ml of the stop solution. When Rb uptake was tested, 139 mM K gluconate was substituted with 140 mM NaCl in the stop solution. The radioactivity of the filters was counted by liquid scintillation spectrometry (Tri-Carb, Packard, mod. 300). All experiments were performed in voltage-clamp conditions, except in the case reported in Fig. 4, where a diffusion potential was superimposed. The solutions used were prefiltered through 0.22 μm pore size filters. Individual uptake experiments in triplicate, representative of more than three repetitions with qualitatively identical results, are presented throughout the paper. Since uptake equilibria values differ for different membrane preparations, the effects of all the tested substances were always checked with a single basolateral membrane preparation. Details of experiments are reported in the legends of the figures.

Results

From the (Na,K)-ATPase and γ-GT determinations, we calculated that the basolateral membranes were enriched about 12 times over the initial homogenate, while brush border was reduced to one-half. The sidedness of our basolateral membrane preparation was determined with latency of (Na,K)-ATPase and reported in a previous work [39]; after activation of (Na,K)-ATPase with the detergent sodium dodecylsulfate (SDS) we calculated that the ratio of unsealed to right-side-out to inside-out vesicles is 2:2:1.

The first set of studies was designed to test for the presence of the transport system that achieves the greater part of basolateral HCO_3 efflux in mammalian kidney, namely the sodium-bicarbonate cotransport [2, 5, 12, 16, 17, 37, 44]. As shown in Fig. 1, sodium influx rate is not affected by an inwardly directed bicarbonate gradient, evidencing the absence of the Na-$(HCO_3)_3$ cotransport in jejunal basolateral membranes. Sodium binding on membrane surface was previously assayed [40], and its value, in the presence of 100 mM potassium, is small when compared with transport.

To examine the possible existence of a basolateral potassium-bicarbonate cotransport, as suggested by Lucas [25], we performed uptake experiments using ^{86}Rb as a tracer for potassium. Although Rb might not perfectly substitute for K, this cation can be replaced by Rb in most transport systems and ^{86}Rb is commonly used as a tracer for K fluxes. There was no significant binding of ^{86}Rb to the membrane vesicles under the incubation conditions used (not reported data). As depicted in Fig. 2,

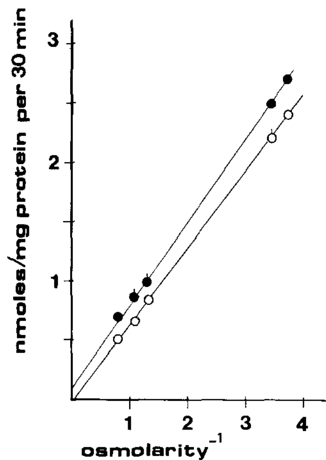

Fig. 3. Effects of extravesicular osmolarity (abscissa, 1/osmolarity values) on the uptake of 2 mM Cl after 30 min equilibration. Mean values ± SE (= vertical bars, absent if less than symbol height) are reported in nmol/mg protein (ordinate). 150 μl basolateral membrane vesicles (1–2 mg protein/ml) pre-equilibrated with 25 μM valinomycin were incubated for 30 min with 300 μl of incubation medium. Vesicles were obtained in 70 mM sucrose, 20 mM HEPES/Tris buffer at pH 7.5 (filled circles) or pH 8.2 (open circles), and the incubation medium contained 20 mM HEPES/Tris buffer at pH 7.5 (filled circles) or pH 8.2 (open circles), 2 mM ^{36}Cl and 70, 100, 820, 1070 or 1570 mM sucrose. All solutions contained 100 mM K-gluconate, 0.2 mM PMSF, 0.11% (vol/vol) ethanol. In both experiments, after 30 min incubation, 100 μl samples were processed as described above.

the lack of effect of bicarbonate on Rb flux excludes the presence of a K-HCO_3 cotransport in the basolateral membrane of rat enterocyte; moreover, from the same figure, it is evident also that Cl is not capable of sustaining an uphill movement of Rb, thus excluding the existence of a K-Cl symport.

As a subsequent approach, the uptake of Cl was evaluated under different experimental conditions. Figure 3 shows that 2 mM Cl uptake at 30 min is inversely proportional to the osmolarity of the incubation medium. By extrapolating Cl uptake to infinite osmolarity, it is evident that binding of chloride on vesicle surface is absent both at pH 7.5 and at pH 8.2 and Cl is transported into an osmotically sensitive intravesicular space.

In Fig. 4 the effect of both positive and negative superimposed membrane potentials on 2 mM Cl uptake is presented: since K gradients plus valinomycin affect Cl movement to a great extent, data strongly suggest the existence of a conductive pathway for chloride in basolateral membranes. Cl conductance is not affected by 1 mM SITS and 0.1 mM DIDS (not reported data).

The effect of different monovalent anions (Na salts) in transstimulating Cl uptake is summarized in Fig. 5. Intravesicular bicarbonate, chloride, bromide, nitrate and thiocyanate cause an increase of Cl uptake to a level that is transiently higher than

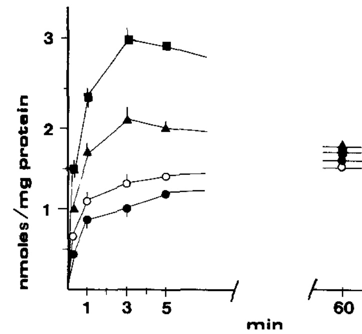

Fig. 4. Effect of membrane potential on 2 mM Cl uptake. 22 μl basolateral membrane vesicles obtained in 250 mM sorbitol and 1.14 mM K_2SO_4 were incubated in 420 μl of either 114 mM K_2SO_4 (filled squares) or 11.4 mM K_2SO_4 and 227 mM sorbitol (filled triangles), or 1.14 mM K_2SO_4 and 250 mM sorbitol (open circles), or 0.114 mM K_2SO_4 and 253 mM sorbitol (filled circles). All solutions contained 20 mM HEPES/Tris buffer at pH 8.2, 0.2 mM PMSF, 0.01% (vol/vol) ethanol. Vesicles were pre-incubated with 25 μM valinomycin. *Ordinate:* Cl uptake, mean values ± SE (= vertical bars, absent if less than symbol height). *Abscissa:* incubation time.

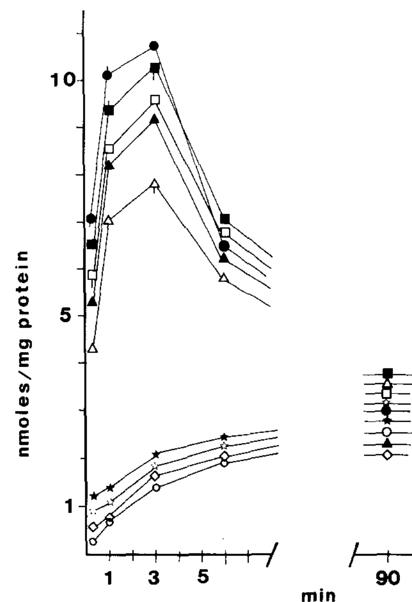

Fig. 5. 2 mM Cl uptake into vesicles preloaded with different anions at pH 8.2. 22 μl basolateral membrane vesicles obtained either in 100 mM $NaHCO_3$ (filled circles) or in 100 mM NaCl (filled squares) or in 100 mM NaBr (open squares) or in 100 mM $NaNO_3$ (filled triangles) or in 100 mM NaSCN (open triangles) or in 100 mM Na lactate (filled stars) or in 100 mM $NaNO_2$ (open stars) or in 100 mM Na-acetate (open rhombus) or in 196 mM sorbitol (open circles) were incubated in 420 μl of 2 mM ^{36}Cl and 196 mM sorbitol. All solutions contained 20 mM HEPES/Tris buffer at pH 8.2, 30 mM K-gluconate, 0.2 mM PMSF and 0.01% (vol/vol) ethanol. Vesicles were pre-incubated with 25 μM valinomycin. *Ordinate:* Cl uptake, mean values ± SE (= vertical bars, absent if less than symbol height). *Abscissa:* incubation time.

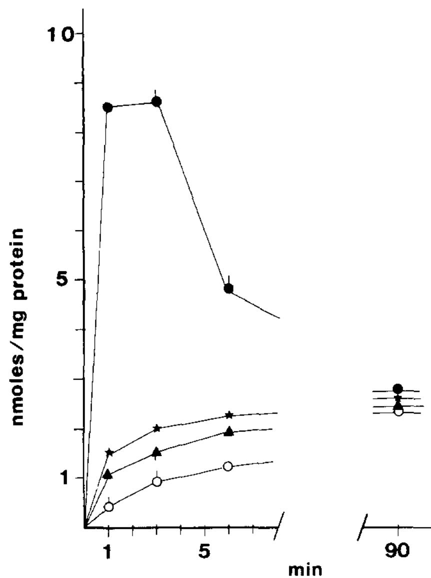

Fig. 6. Effect of SITS and DIDS on 2 mM Cl uptake. 22 μl basolateral membrane vesicles obtained either in 100 mM $NaHCO_3$ (filled symbols) or in 196 mM sorbitol (open circles) were incubated in 420 μl of 2 mM ^{36}Cl and 196 mM sorbitol (circles), added with either 1 mM SITS (filled triangles) or 0.1 mM DIDS (filled stars). All solutions contained 20 mM HEPES/KOH buffer at pH 8.2, 30 mM K-gluconate, 0.2 mM PMSF and 0.01% (vol/vol) ethanol. Vesicles were pre-incubated with 25 μM valinomycin. *Ordinate:* Cl uptake, mean values ± SE (= vertical bars, absent if less than symbol height). *Abscissa:* incubation time.

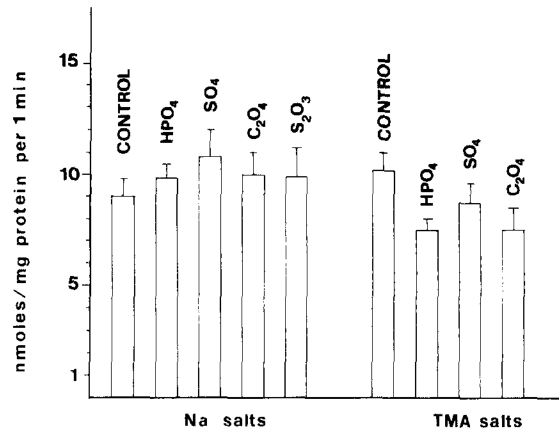

Fig. 7. Effect of bivalent anions on 2 mM Cl uptake after 1 min incubation. 22 μl basolateral membrane vesicles obtained in 100 mM $NaHCO_3$ and pre-incubated with 25 μM valinomycin were incubated in 420 μl of 2 mM ^{36}Cl and either 196 mM sorbitol or 88 mM Na or TMA salts. $S_2O_3^{2-}$ was tested only as Na salt since TMA salt cannot be tested. All solutions contained 20 mM HEPES/Tris buffer at pH 8.2, 30 mM K-gluconate, 0.2 mM PMSF and 0.01% (vol/vol) ethanol. *Ordinate:* Cl uptake, mean values ± SE (= vertical bars).

equilibrium value; on the contrary, lactate, nitrite and acetate are ineffective. This experiment, performed by increasing K concentration inside = outside to 100 mM, gave similar results. Thus the evidence suggests that a Cl/HCO_3 exchanger with broad specificity is present at the basolateral membrane. These results were obtained at pH 8.2; at pH 7.5 the maximal activation was achieved by intravesicular chloride instead of bicarbonate; the behavior of the other anions tested was not affected by pH variation (not reported data).

In Fig. 6 the effects of 1 mM SITS and 0.1 mM DIDS on Cl uptake are reported. In these experiments HEPES/KOH instead of HEPES/Tris was used, due to possible interactions between disulfonic stilbenes and Tris. Data of Fig. 6 give evidence that the transstimulating effect of HCO_3 on Cl uptake is drastically inhibited by both SITS and DIDS.

The effect of bivalent anions on Cl uptake in vesicles preloaded with 100 mM HCO_3 was tested in *cis*-inhibition experiments, and results are summarized in Fig. 7. Anions were employed as either Na salts or TMA salts; surprisingly, some anions, when present as Na salts, seem to stimulate Cl uptake. As this does not occur with TMA salts, it is likely that the effect is due to the presence of Na in the extravesicular medium.

The presence of Cl/HCO_3 exchanger in the apical membrane of various intestinal tracts [21, 24, 32, 41] could suggest that HCO_3-stimulated Cl transport evidenced in our preparation is actually occurring via the brush border membranes contaminating the basolateral membrane fraction. However, to our knowledge, there are no references concerning a possible Cl/HCO_3 antiport in jejunal brush border. Therefore, to get more insight on this subject we performed experiments using jejunal brush border membrane vesicles to test for the presence of such a mechanism. Results, depicted in Fig. 8, suggest that HCO_3 can transstimulate Cl uptake only to a very small extent.

Discussion

We can exclude any contribution of brush border transport systems in our results, since the marker enzyme analysis shows that the basolateral membrane preparation is very well purified; moreover, in a previous work [30], our basolateral membrane vesicles were tested for their ability to accumulate D-glucose in the presence of an inward Na gradient. The lack of effect is in agreement with the absence of brush border contamination. For this reason sucrose, which is a poorly permeating substance, could be used to increase osmolarity.

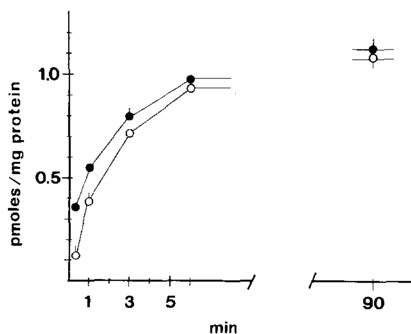

Fig. 8. 2 mM Cl uptake into brush border membrane vesicles. 22 μl brush border membrane vesicles obtained either in 100 mM $NaHCO_3$ (filled circles) or in 196 mM sorbitol (open circles) were incubated in 420 μl of 2 mM ^{36}Cl and 196 mM sorbitol. All solutions contained 20 mM HEPES/Tris buffer at pH 8.2, 30 mM K-gluconate, 0.2 mM PMSF and 0.01% (vol/vol) ethanol. Vesicles were pre-incubated with 25 μM valinomycin. *Ordinate:* Cl uptake, mean values ± SE (= vertical bars, absent if less than symbol height). *Abscissa:* incubation time.

In view of the presence of many similar transport mechanisms in renal and small intestinal basolateral membranes [27], we focused our attention on the Na-$(HCO_3)_3$ cotransport, which is the major basolateral membrane pathway for bicarbonate in the proximal tubule [2, 5, 12, 16, 17, 37, 44]. In our experimental conditions membrane potential was shunted by valinomycin and equal potassium concentrations in internal and external solutions; in order to maintain the imposed HCO_3 concentration, experiments were performed at pH 8.2. Data of Fig. 1 do not support the hypothesized mechanism, in agreement with previous studies carried out in this laboratory [11] and with more recent results presented by Hagenbuch et al. [17]. These Authors suggested the possibility that a K-HCO_3 cotransport could be involved in intestinal basolateral HCO_3 exit, as proposed by Lucas [25] on the basis of electrophysiological measurements. Once again the hypothesis was rejected, as shown by experimental results of Fig. 2.

In basolateral membranes of proximal tubule an electrically neutral K-Cl cotransport was evidenced [10, 36]; this transport system has been proposed as the mechanism for chloride exit from cells having a low basolateral conductance [34]. In the present study we found no evidence for Cl gradient-stimulated K influx, as reported for renal basolateral membrane vesicles [13]; thus a different mode for Cl movement must be taken into account. To shed more light on the process of chloride flux across the jejunal basolateral membrane, ^{36}Cl uptake experiments were carried out. Since both at pH 7.5 and 8.2 membrane surface should be negatively charged, the absence of Cl binding (*see* Fig. 3) is not amazing. We focused our attention on this point because a small binding of chloride was found in basolateral membrane vesicles from lobster hepatopancreas [1] and from rat lacrimal gland [23].

Results of Fig. 4 point out that chloride can cross the basolateral membrane through a conductive pathway. We cannot exclude that this conductance might be a consequence of the separating procedure; as a matter of fact, a magnesium-induced Cl conductive uptake was observed in renal cortical basolateral membrane vesicles [10]; however, a Cl conductance was also found in nonisolated basolateral membranes from other epithelia [15, 20, 43].

Data reported in Fig. 5 were obtained using vesicles preloaded with different monovalent anions; evidence is given that intravesicular HCO_3 is able to energize the overshooting uptake of Cl, suggesting the presence of a Cl/HCO_3 exchanger in basolateral membranes. Experiments were performed under voltage-clamped conditions, thus excluding electrodiffusional coupling. As 30 mM K could be insufficient to maintain shunting of the potential generated by 100 mM anion diffusion out of the vesicles, the experiment was also performed by increasing the voltage clamping of the vesicles with 100 mM K inside = outside; the transstimulation of Cl uptake was not reduced. Furthermore, the strong inhibition of Cl uptake exerted by the stilbene derivatives SITS and DIDS (Fig. 6) is consistent with the presence of a transporter operating a coupling between Cl and HCO_3 movements; as a matter of fact, Cl/HCO_3 antiporters that have been characterized in other cell types are sensitive to inhibition by SITS and DIDS [18]. The ineffectiveness of SITS and DIDS on conductive Cl uptake validates the presence of an actual anion exchange.

The data given in Fig. 5 show that preloading of vesicles with chloride, bromide, nitrate and thiocyanate can stimulate Cl uptake, whereas there is no significant effect of lactate, nitrite and acetate. These observations would suggest that Cl shares a transport system with HCO_3 and the first group of anions, but not with the second one. Similar anion selectivity was reported for exchangers present in other membranes [1, 20, 23]. The experiment described in Fig. 5 was also carried out at pH 7.5; in this case the maximal stimulation of Cl uptake was achieved by intravesicular chloride. It is likely that lowering pH value results in a reduction of intravesicular HCO_3 concentration, leading to a lowered driving force for Cl uptake.

It should be noted that jejunal basolateral membranes contain a Na/H exchanger [30, 40] that could be activated by the outwardly directed Na gradient present in our experimental conditions; this could

give rise to an intravesicular acidification and to a subsequent decrease of HCO_3 concentration. Therefore, the effects depicted in Fig. 5 could be underestimated. The activity of the Na/H antiport could also account for the results reported in Fig. 7. In this experimental protocol the Na gradient is inwardly directed when bivalent anions are present as Na salts; in this case the activation of the Na/H exchanger would cause an intravesicular alkalinization, resulting in a more stable HCO_3 concentration. Thus, the inhibitory effect of anions could be masked by the enhanced driving force for Cl uptake. In addition, Cl/HCO_3 exchange could be dependent on the presence of sodium and might be coupled to it, as reported for a number of similar antiporters [3, 14, 16, 33, 37].

The degree of inhibition by bivalent anions can be evaluated only in the presence of TMA salts; due to the instability of $H_2S_2O_3$ we could not perform experiments with $TMA_2S_2O_3$. As illustrated in Fig. 7, the ability of bivalent anions to inhibit Cl/HCO_3 exchange is very low considering the high concentrations employed.

Results of Fig. 8 support the conclusion that the HCO_3 transport system demonstrated is indeed basolateral, since the small HCO_3 effect on Cl uptake, evidenced in jejunal brush border membrane preparation, could be due either to a Cl/HCO_3 exchanger having a very low transport rate or to a basolateral membrane contamination. Also, Cassano et al. [7], using a different experimental approach, failed to provide evidence for the presence of Cl/HCO_3 antiport in rat jejunal brush border membrane vesicles.

The present results provide a possible mechanism by which bicarbonate absorbed across the apical membrane can be transported out of the cell across the basolateral membrane. Our conclusions are in agreement with recent results reported for the S_3 segment of renal proximal tubule, which in contrast to segments S_1 and S_2 does not contain the $Na(HCO_3)_3$ cotransporter [22]. In this model, chloride would enter the cell across both the apical and the basolateral membrane; the conductive pathway could account for chloride exit at the basolateral pole of jejunal enterocyte. Cl/HCO_3 antiport, together with Na/H antiport previously evidenced in jejunal basolateral membrane, can contribute to cell pH and volume regulation [19].

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