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Basolateral Cl^-/HCO_3^- exchange in rat jejunum: evidence from $H^{14}CO_3^-$ uptake in membrane vesicles

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Bicarbonate transport across basolateral membrane vesicles from rat jejunal enterocyte was studied at 28°C and pH 8.2. These experimental conditions make possible the determination of [^{14}C]bicarbonate uptake. Inward gradients of Na^+, K^+, and Li^+ did not stimulate HCO_3^- uptake, suggesting that a cotransport mechanism with these cations does not occur. On the contrary a countertransport of bicarbonate driven by a Cl^- gradient was evidenced. The ability of other inorganic anions to exchange with HCO_3^- was examined and results indicate that Cl^- can be substituted by NO_3^-, Br^- and SCN^-. The Cl^--dependent HCO_3^- uptake was strongly inhibited by SITS and DIDS, whereas acetazolamide was ineffective: thus transfer of labelled CO_2 is eliminated as a possible mode of HCO_3^- permeation. HCO_3^- uptake was also affected by the presence of superimposed membrane potentials, suggesting that a HCO_3^- conductive pathway is present in the jejunal basolateral membrane. These results show that there are no fundamental differences between data obtained performing $H^{14}CO_3^-$ and $^{36}Cl^-$ (previously reported) uptake experiments.

Introduction

The presence of a Cl^-/HCO_3^- exchange mechanism in basolateral membranes (BLMs) isolated from rat jejunal enterocyte was recently documented [1]. Such an antiport could represent the transport system by which bicarbonate absorbed across the apical membrane [2–4] is translocated out of the cell at its basolateral pole. Evidence suggests that this Cl^-/HCO_3^- exchanger shares some features with anion antiporters identified in other membranes [5–8]. These results were obtained by means of $^{36}Cl^-$ uptake experiments; however, if a Cl^-/HCO_3^- antipert is present in BLM vesicles, it should be possible to demonstrate that not only an outwardly directed HCO_3^- gradient stimulates Cl^- uptake, but also that a Cl^- gradient is able to transstimulate HCO_3^- flux. Thus, the purpose of this study was to verify previous results by performing $H^{14}CO_3^-$ uptake experiments. However, since bicarbonate can be transformed into related forms (CO_2, CO_3^{2-}), special precautions (regarding temperature and pH of experiments) must be taken to keep constant HCO_3^- concentration and to avoid $^{14}CO_2$ loss into air.

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Results of this work give evidence that it is possible to measure directly bicarbonate fluxes across BLM vesicles under proper experimental conditions. Moreover, bicarbonate uptake data are in accordance with the presence of a Cl^-/HCO_3^- exchanger in BLMs of rat jejunal enterocyte and confirm the features previously reported, namely its sensitivity to stilbene derivatives and its selectivity to different anions.

Materials and Methods

Basolateral membrane isolation

Two male albino rats (Wistar strain, Charles River Italiana) weighing 250–300 g (about two months old), 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 phenylmethanesulphonyl fluoride (PMSF), 0.01% (v/v) ethanol, 10 mM Hepes-Tris buffer (pH 7.5). Basolateral plasma membranes were isolated and purified as described previously [9]. 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). 7 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 further incubated in the same buffer at room temperature for at least 90 min and used after that for $H^{14}CO_3^-$ 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 brusch-border membrane), Na/K-ATPase (a marker enzyme for basolateral membrane), KCN-resistant NADH oxidoreductase (a marker enzyme for endoplasmic reticulum) and cytochrome *c* oxidase (a marker enzyme for mitochondria) were determined as published [9].

Uptake experiments

Transport of 1 mM $H^{14}CO_3^-$ into BLM vesicles was measured at pH 8.2 and at 28°C by a rapid microfiltration technique. Experiments lasting not longer than 30 min were performed. Due to the low specific activity of sodium [^{14}C]bicarbonate (0.2–0.3 MBq/mmol, cristalline solid, NEN, Boston, MA, USA), the unlabelled $NaHCO_3$ was omitted from the incubation medium and the activity of the isotope was checked and arranged to have a final concentration of 1 mM. 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 (zero time). The composition of the resuspension

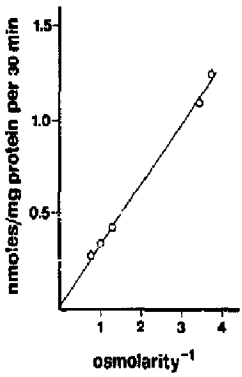

Fig. 1. Effect of extravesicular osmolarity (abscissa, 1/osmolarity values) on the uptake of 1 mM HCO_3^- after 30 min equilibration. Mean values±S.E. (vertical bars, absent if less than symbol height) are reported in nmoles/mg protein (ordinate). 150 μl BLM vesicles (1–2 mg protein/ml) obtained in 70 mM sucrose and pre-equilibrated with 25 μM valinomycin were incubated in 300 μl of incubation medium containing 1 mM $H^{14}CO_3^-$ and 70, 100, 820, 1070 or 1570 mM sucrose. All solutions contained 20 mM Hepes/Tris buffer (pH 8.2), 100 mM potassium gluconate, 0.2 mM PMSF, 0.01% (v/v) ethanol.

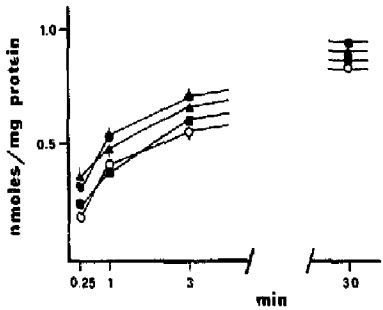

Fig. 2. Effect of monovalent cations on 1 mM HCO_3^- uptake into BLM vesicles. 20 μl vesicles obtained either in 100 mM KNO_3 and 22 mM sorbitol (filled circles and squares) or in 100 mM $NaNO_3$ and 22 mM sorbitol (filled triangles, open circles) were incubated in 380 μl of either 10 mM Na_2SO_4 and 100 mM KNO_3 (filled circles), or 10 mM K_2SO_4 and 100 mM $NaNO_3$ (filled triangles), or 10 mM Li_2SO_4 and 100 mM KNO_3 (filled squares), or 22 mM sorbitol and 100 mM $NaNO_3$ (open circles). All incubating solutions contained 1 mM $H^{14}CO_3^-$. All solutions contained 100 mM Hepes/Tris buffer (pH 8.2), 0.2 mM PMSF and 0.01% (v/v) ethanol. Ordinate: HCO_3^- uptake, mean values±S.E. (vertical bars, absent if less than symbol height). Abscissa: incubation time.

buffers and incubation media are given in the legends of the figures. Samples were taken up at selected times and diluted with 0.8 ml of ice-cold reaction stopping solution (isoosmotic potassium gluconate (136-139-140-157 mM according to the experiment), 0.2 mM PMSF, 0.01% (v/v) ethanol, 20 mM Hepes-Tris buffer (pH 8.2), filtered on wetted cellulose nitrate filters (0.45 μm pore size) and immediately rinsed with 5 ml of 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. 5 where a membrane potential was superimposed. To take into account the decrease of specific activity of labelled bicarbonate (always about 10% at the end of the 30-min experiment) samples of the reaction mixture were withdrawn at the same selected times and used as standards. The solutions used were pre-filtered 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

Both freshly prepared and frozen (−70°C) BLM preparations were used in this study. The purity of the

BLMs was checked by measuring various marker enzyme activities. The recovered fraction was enriched 10-fold compared to the homogenate in specific activity of Na/K-ATPase, whilst the enrichment factors of other marker enzymes were respectively 0.5 for γ-GT, 0.6 for KCN-resistant oxidoreductase, 0.3 for cytochrome *c* oxidase.

Fig. 1 shows that 1 mM HCO_3^- uptake at 30 min is inversely proportional to the osmolarity of the incubation medium. By extrapolating HCO_3^- uptake to infinite osmolarity, it is evident that bicarbonate does not bind on vesicle surface and is transported into an osmotically sensitive intravesicular space.

Electrogenic $Na/(HCO_3^-)_3$ cotransport is the predominant basolateral transport system of bicarbonate in proximal tubule [10–16]; recently it has been demonstrated that this mechanism involves a 1 : 1 : 1 cotransport of Na^+, CO_3^{2-} and HCO_3^- on distinct sites [17, 18]. To determine whether this process is present in rat jejunum, we performed the experiment whose results are depicted in Fig. 2. Bicarbonate uptake is not significantly affected by the presence of inward gradients of Na^+, K^+ or Li^+, suggesting that HCO_3^- movement does not occur by a cotransport mechanism with the tested monovalent cations.

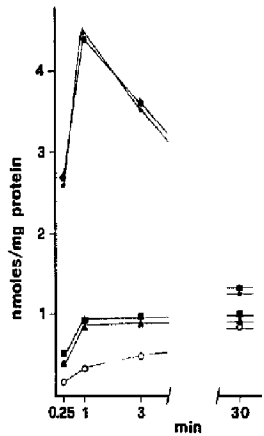

Fig. 4. Effect of acetazolamide, SITS and DIDS on 1 mM HCO_3^- uptake. 20 μl BLM vesicles obtained either in 100 mM NaCl (filled symbols) or in 196 mM sorbitol (open circles) were incubated in 380 μl of 196 mM sorbitol (filled and open circles) added with either 0.5 mM acetazolamide (filled stars), or 0.1 mM DIDS (filled squares), or 1 mM SITS (filled triangles). All incubating solutions contained 1 mM $H^{14}CO_3^-$. All solutions contained 20 mM Hepes/KOH buffer (pH 8.2), 100 mM potassium gluconate, 0.2 mM PMSF, 0.01% (v/v) ethanol. Vesicles were pre-incubated with 25 μM valinomycin. Ordinate: HCO_3^- uptake, mean values ± S.E. (vertical bars, absent if less than symbol height). Abscissa: incubation time.

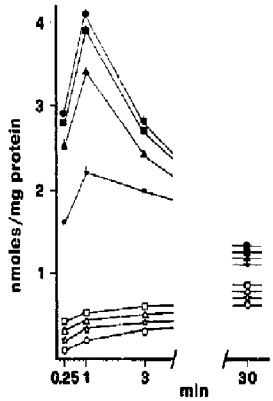

Fig. 3. 1 mM HCO_3^- uptake into vesicles preloaded with different anions. 20 μl BLM vesicles obtained either in 100 mM NaCl (filled circles), or in 100 mM $NaNO_3$ (filled squares), or in 100 mM NaBr (filled triangles), or in 100 mM NaSCN (filled stars), or in 100 mM $NaNO_2$ (open squares), or in 100 mM Na acetate (open triangles), or in 100 mM sodium lactate (open stars), or in 196 mM sorbitol (open circles) were incubated in 380 μl of 1 mM $H^{14}CO_3^-$ and 196 mM sorbitol. All solutions contained 20 mM Hepes/Tris buffer (pH 8.2), 100 mM potassium gluconate, 0.2 mM PMSF and 0.01% (v/v) ethanol. Slight pH variations, when present, were eliminated by titrating intravesicular solutions with small amounts of Hepes. Vesicles were preincubated with 25 μM valinomycin. Ordinate: HCO_3^- uptake, mean values ± S.E. (vertical bars, absent if less than symbol height). Abscissa: incubation time.

The subsequent experiments were designed to determine the effect of outwardly directed anion gradients on HCO_3^- uptake. As shown in Fig. 3, intravesicu-

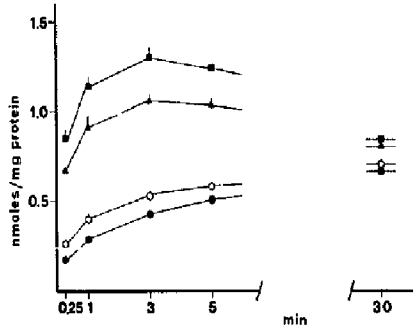

Fig. 5. Effect of membrane potential on 1 mM HCO_3^- uptake. 24 μl BLM vesicles obtained in 250 mM sorbitol and 1.14 mM K_2SO_4 were incubated in 456 μl of either 114 mM K_2SO_4 (filled squares), or 11.4 mM K_2SO_4 and 227 mM sorbitol (filled triangles), or 1.14 mM K_2SO_4 and 250 mM sorbitol (open circles), or 0.114 mM K_2SO_4 and 253 mM sorbitol (filled circles). All incubating solutions contained 1 mM $H^{14}CO_3^-$. All solutions contained 20 mM Hepes/Tris buffer (pH 8.2), 0.2 mM PMSF, 0.01% (v/v) ethanol. Vesicles were pre-incubated with 25 μl valinomycin. Ordinate: HCO_3^- uptake, mean values ± S.E. (vertical bars, absent if less than symbol height). Abscissa: incubation time.

lar Cl^-, NO_3^-, Br^- and SCN^- cause an overshoot phenomenon, indicative of a transient HCO_3^- accumulation above equilibrium distribution. On the contrary NO_2^-, acetate and lactate are ineffective. These findings support the hypothesis that an anion exchanger is present at the basolateral pole of jejunal enterocyte.

Directly coupled anion exchangers have been characterized in other cell types as being selectively inhibited by disulfonic stilbenes [5]. Fig 4 demonstrates that SITS and DIDS reduce drastically Cl^--dependent HCO_3^- uptake; in contrast acetazolamide, a known inhibitor of carbonic anhydrase, does not alter bicarbonate transport.

To determine the putative contribution of a conductance pathway to bicarbonate movement, the effect of a superimposed membrane potential on HCO_3^- uptake was evaluated. Results are reported in Fig. 5 and give evidence for the presence of a conductive pathway for bicarbonate. This conductance does not appear to be affected by the disulfonic stilbenes SITS and DIDS (not reported data).

Discussion

From the Henderson-Hasselbalch equation it is possible to calculate that at 28°C and pH 8.2, the CO_2 tension necessary to keep constant 1 mM bicarbonate concentration is 0.21 mmHg (= 28 Pa), i.e., 0.03% CO_2, which corresponds to the CO_2 per cent value in the air. This means that in our experimental conditions bicarbonate concentration keeps stable even if vessels are not gassed; furthermore only 0.9% of bicarbonate is present as either CO_2 or CO_3^{2-}. As a matter of fact studies dealing with HCO_3^- transport are generally carried out at pH ≥ 8 [19–21]. However, we must take into account that the specific activity of the labelled bicarbonate may be affected by the dehydration ($H^{14}CO_3^- \rightarrow {}^{14}CO_2$) and hydration ($CO_2 \rightarrow HCO_3^-$) reactions. From Magid and Turbeck data [22] we calculated the pseudo-first-order rate coefficient of dehydration ($\approx 1 \cdot 10^{-3}\ s^{-1}$) and hydration ($= 88 \cdot 10^{-3}\ s^{-1}$) reactions at 28°C and pH 8.2. The low rate of the former with respect to the latter, which moreover partly utilizes the $^{14}CO_2$ (vessels are kept closed during the incubation period) makes it possible to perform bicarbonate uptake experiments. Actually we determined experimentally that the decrease of bicarbonate specific activity after 30 min incubation is $10.8 \pm 0.6\%$ ($n = 62$). Only uncatalyzed reactions were considered since carbonic anhydrase activity is very low in rat jejunum [23–25]; furthermore, in rabbit ileum carbonic anhydrase seems to be mostly a cytosolic enzyme [26]. For safety's sake we performed bicarbonate uptake experiments in the presence of 0.5 mM acetazolamide without obtaining any detectable effect. These observations suggest that at alkaline pH values labelled HCO_3^- can be a useful tool to investigate bicarbonate transport systems; moreover it is less expensive than other radioisotopes normally used, such as $^{22}Na^+$ and $^{36}Cl^-$, and it emits low-energy radiations. It is worth underlining that pH 8.2 is far from physiological conditions and it would be of interest to investigate how a lower pH would alter experimental findings. However, experiments carried out at a more physiological pH (either by lowering HCO_3^- concentration or by increasing pCO_2) could give rise to criticism since the rate coefficients of hydration and dehydration reactions would be affected, causing a lowered specific activity of labelled HCO_3^-. Nevertheless $^{36}Cl^-$ uptake experiments provided evidence for a Cl^-/HCO_3^- exchanger both at pH 8.2 and 7.5, even if at the higher proton concentration its activity was reduced [1].

Purity of the membranes was assessed by various marker enzyme assays: the preparation showed minimal contamination of brush border, mitochondria and endoplasmic reticulum and BLMs were well purified compared with starting homogenate. It is worth while underlining that the low contamination of apical membranes leads us to exclude any contribution of brush-border transport systems in our results. Moreover, evidence was provided that in rat jejunal brush border Cl^-/HCO_3^- exchange, if present, has a very low transport rate [1,27].

Data of Fig. 2 confirm results previously reported [1,14] pointing out against the existence of a directly coupled Na^+ and HCO_3^- transport in jejunal BLMs. As indicated in the same figure we can also exclude a possible K^+ (or Li^+)/HCO_3^- cotransport, proposed by Lucas [28]. To avoid any influence of membrane potential on ion movements, equal intra- and extravesicular K^+ concentrations in the presence of valinomycin were used.

The mechanism by which HCO_3^- leaves the cell across the basolateral membrane could be represented by an anion exchanger exhibiting a broad specificity, as illustrated in Fig. 3. Electrodiffusional coupling is excluded since experiments were performed under voltage-clamp conditions. The anion selectivity resulting from this figure overlaps the one reported previously [1] and similar selectivities were demonstrated for exchangers present in other membranes [6–8]. From Figs. 3, 4 and 5 it seems that uptake data after 30 min incubation are not well equilibrated: however, experiments lasting longer than 30 min are not feasible due to HCO_3^- instability, nevertheless the results depicted are unambiguous.

Anion exchangers are characterized by their sensitivity to the stilbene derivatives, such as SITS and DIDS [5]. Therefore the strong inhibition depicted in Fig. 4 supports a direct coupling of HCO_3^- and Cl^- movements. The uneffectiveness of acetazolamide on Cl^--dependent HCO_3^- uptake argues against a CO_2

involvement in HCO_3^- transport; this point is further on strengthened by the agreement between the present data and the ones obtained by means of Cl^- uptakes [1].

The question then arises if, besides the exchange mechanism with chloride ions, HCO_3^- could exit the cell across the BLM by simple diffusion through conductive channels. Findings reported in Fig. 5 validate this hypotesis, even if we cannot exclude that HCO_3^- conductance is induced by the separation procedure. However, HCO_3^- (or equivalent ions) conductance was also found in non-isolated BLMs of other epithelia [13, 29–32] and a higher permeability for HCO_3^- than for Cl^-, Na^+ and K^+ was detected in the BLM of the proximal tubule [33]. The conductive pathway appears to exist separately from the Cl^-/HCO_3^- exchange, because SITS and DIDS, at the same concentrations that blunted Cl^--dependent HCO_3^- overshoot, have no effect on membrane potential-dependent HCO_3^- uptake.

In summary, results of this work estabilish that under proper experimental conditions it is possible to perform $H^{14}CO_3^-$ uptake experiments: as a matter of fact results reported in the present study overlap the ones obtained by means of $^{36}Cl^-$ uptake. These data suggest that the primary pathway for HCO_3^- exit across the BLM of jejunal enterocyte occurs via an exchange mechanism with Cl^- ions.

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