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A sensitive technique for the determination of anion exchange activities in brush-border membrane vesicles. Evidence for two exchangers with different affinities for HCO_3^- and SITS in rat intestinal epithelium

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A large percentage (up to 70%) of $^{36}Cl^-$ influx in brush-border membrane vesicles from rat small intestine under equilibrium exchange conditions was found to be mediated by SITS-inhibitable anion exchange. This Cl^-/anion exchange could be measured 10–15-times more sensitive by determining the uptake of trace amounts of $^{125}I^-$ driven by a large Cl^- gradient (in > out) generated by passing the vesicles through an anion-exchange column. Voltage clamping of the vesicle membrane with K^+ and valinomycin did not effect the chloride driven $^{125}I^-$ uptake, showing that the 'overshooting' I^- uptake was not mediated by an electrical diffusion potential, as might be generated by the Cl^- gradient in the presence of a chloride channel. The Cl^-/anion exchange was further characterized in brush-border membrane vesicles from both rat ileum and jejunum by studying the inhibitory action of various anions on the Cl^- driven I^- uptake. NO_3^-, Cl^-, SCN^- and formate at 2 mM could inhibit Cl^-/I^- exchange for more than 80%. The ileal brush-border membrane vesicles displayed a clear heterogeneity with respect to the inhibitory action of SO_4^{2-}, SITS and HCO_3^- on Cl^-/I^- exchange. Approximately 30% of the Cl^-/I^- exchange was insensitive to SO_4^{2-} and showed a relatively low sensitivity to SITS ($IC_{50} = 1$ mM) but could be inhibited for 80% by 2 mM HCO_3^-. Presumably this component represents Cl^-/OH^- or Cl^-/HCO_3^- exchange. The residual 70% showed a high sensitivity to SO_4^{2-} ($IC_{50} = 0.5$ mM) and SITS ($IC_{50} = 2.5$ μM) but was less sensitive to HCO_3^-. This part of the exchange activity showed inhibition characteristics very similar to the Cl^-/I^- exchange in the jejunal vesicles. The latter process was also inhibited for 80% by 2 mM oxalate. As discussed in this paper both exchangers may be involved in the electroneutral transport of NaCl across the apical membrane of the small intestinal villus cell.

Introduction

The active transport of sodium and chloride across the epithelium of the small intestine can be modulated by intracellular second messengers like cAMP, cGMP and Ca^{2+} ions [1–4]. According to

Abbreviations: DIDS, 4,4′-diisothiocyanotostilbene-2,2′-disulfonic acid; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate.

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the model proposed by Field [5] the major cyclic nucleotide- and Ca^{2+}-sensitive ion transporters are (i) an electroneutral Na^+/Cl^- cotransport system, located in the apical membrane of the mature villus cell and (ii) an electrogenic Cl^- channel, presumably enriched in the intestinal crypt cell. The molecular nature of the transporters and of the signal transduction mechanisms involved is largely unknown.

Phosphorylation studies with isolated brush borders and brush-border membrane vesicles derived from intestinal villus cells provided evidence for the cophosphorylation of a 25 kDa proteolipid by an endogenous cAMP-dependent protein kinase and a unique isoenzyme of cGMP-dependent protein kinase [3,4,6]. A Ca^{2+}/calmodulin- and a Ca^{2+}/phospholipid-dependent protein kinase and specific substrate proteins for these kinases were also detected in brush-border membranes [3,4].

Transport studies with brush-border membrane vesicles from intestinal villus cells so far demonstrated the existence of (i) non-saturable electrogenic pathways for Na^+ and Cl^- [7–9] and (ii) an electroneutral Na^+/H^+ [10,11] and a Cl^-/OH^- or Cl^-/HCO_3^- exchanger [7,12] presumably coupled by circular proton movements, but failed to demonstrate unambiguously the presence of a true Na^+/Cl^- cotransporter [8,13]. Both exchangers are potential targets for second messengers, inhibiting salt transport across villus epithelium.

Prior to a detailed study of second messenger regulation of anion exchange at the level of isolated brush-border membrane vesicles, we first developed a simple and sensitive method to characterize Cl^-/anion exchange activity in apical membrane vesicles from rat ileum and jejunum. The method exploits a large concentration gradient of Cl^- across the membrane (in $\gg$ out) to energize an overshoot of $^{125}I^-$, mediated through a Cl^-/anion exchanger.

The results provided evidence for the existence of two different types of Cl^-/anion exchangers, a SITS- and SO_4^{2-}-sensitive exchanger found in both jejunum and ileum, and a HCO_3^--sensitive exchanger present only in the ileum. Both exchangers may be involved in electroneutral NaCl transport.

Materials and Methods

Materials

$H^{36}Cl$ (0.45 mCi/mmol) and $Na^{125}I$ (2000 Ci/mmol) were obtained from Amersham. Valinomycin was purchased from Boehringer and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate (SITS) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) from Sigma. The Cl^- channel blockers 130 B (5-nitro-2-[3-phenylpropylamino]benzoic acid) and 51 B (3′,5-dichlorodiphenylamine-2-carboxylic acid) were kindly donated by Dr. R. Greger, University of Freiburg. All other chemicals were analytical grade.

Methods

Preparation of vesicles. Each batch of brush-border membrane vesicles originated from 30 cm long segments of ileum or jejunum or from a 90 cm combined segment from small intestine freshly obtained from three or four adult male Wistar rats, weighing 300–350 g. Brush-border membrane vesicles were generated from isolated villus cells by a freeze-thawing technique and purified by differential Mg^{2+} precipitation and a washing step as described previously ('Mg-brush border membrane vesicles') [14]. Alternatively brush-border membrane vesicles were prepared from isolated brush borders [9] by treatment with 0.52 M KSCN as described by Hopfer [15]. Vesicles isolated by this procedure are virtually devoid of cytoskeletal proteins [9,15]. The vesicles were finally resuspended in buffer A (300 mM mannitol, 20 mM Hepes-Tris) pH 7.0 or pH 8.2 and loaded with salts by preincubation for 1 h at 0 °C.

Transport studies. The uptake of $^{36}Cl^-$ was measured by mixing 50 µl of a vesicle suspension in buffer A (pH 7.0) containing 25 mM KCl or potassium gluconate (3–5 mg protein/ml) with 250 µl of buffer A (pH 7.0) containing 25 mM $K^{36}Cl$ (3 µCi). With various time intervals 50 µl samples were loaded on minicolumns (0.6 ml of packed resin) of Dowex AG1-X8 anion exchanger (gluconate form, 50–100 mesh) and quickly eluted with 1 ml icecold buffer A. Radioactivity in the eluate was detected by liquid scintillation counting.

The Cl^--driven $^{36}Cl^-$ or $^{125}I^-$ uptake was measured by a modification of the procedure de-

scribed by Garty et al. [16] for the detection of cation channels in a heterogeneous population of vesicles. 100 μl of the brush-border membrane vesicle suspension in buffer A (pH 7.0 or 8.2) preloaded with 100 mM Cl^- (K^+ or choline) were applied to a similar minicolumn of Dowex anion exchanger (0.7 ml of packed resin) as used in the $^{36}Cl^-$ uptake studies, and the column was eluted with 450 μl of buffer A. The last 300 μl of the eluate which contained approx. 90% of the vesicles were collected and mixed with 10 μl $K^{36}Cl$ (0.68 μCi, 5 mM final concentration) or 25 μl of $Na^{125}I$ (0.5 μCi; 1 nM final concentration) one minute after the column loading. Exact timing was essential to avoid differences in intravesicular Cl^- concentration due to the rapid efflux of this ion following the generation of a steep chemical gradient. At various time points extravesicular $^{125}I^-$ or $^{36}Cl^-$ was removed by loading 50 μl of mixture on a second Dowex minicolumn and eluting this column with buffer A exactly as described for the $^{36}Cl^-$ uptake measurements.

In a typical experiment for the analysis of inhibition of the I^-/Cl^- exchanger, 300 μl vesicle suspension eluted from the first column was split into three aliquots (100 μl) and mixed with 25 μl potassium gluconate (final concentration 85 mM) additionally containing the inhibiting anion and $Na^{125}I$. Bicarbonate was added just before the start of the incubation from a 100 mM stock solution in freshly distilled water (pH 8.2). The ^{125}I uptake was terminated by column filtration 60 and 90 s after the mixing at which time points the $^{125}I^-$ uptake was at its maximum in the absence of inhibitors. Cl^-/I^- specific exchange was defined as the difference between the $^{125}I^-$ uptake in the presence or absence of a Cl^- gradient ($[Cl^-]_{out}$ = 100 mM).

Protein was measured by the method of Lowry et al. [17] with bovine serum albumin as a standard.

Results

The presence of 25 mM Cl^- at the interior of rat brush-border membrane vesicles obtained by differential Mg^{2+} precipitation caused a 2.5–3-fold stimulation of $^{36}Cl^-$ uptake at 15 s as compared to intravesicular gluconate (Fig. 1) or mannitol (not shown). SITS (4 mM), an inhibitor of anion exchange, completely abolished this stimulation (Fig. 1). This shows that intestinal brush-border membrane vesicles contain an active anion exchange mechanism, which can account for up to 70% of the $^{36}Cl^-$ uptake into the vesicles under equilibrium exchange conditions (Fig. 1, $t = 0.25$ min) and displays a low sensitivity to gluconate. To increase the sensitivity of the anion exchange assay, we tried to establish a large Cl^- gradient across the vesicle membrane ($[Cl^-]_{in} \gg [Cl^-]_{out}$) by passing the vesicles, preloaded with 100 mM Cl^-, through a Dowex anion exchange column in order to replace the extravesicular Cl^- by gluconate. In view of the low specific radioactivity of the $^{36}Cl^-$ isotope, however, this approach required the presence of millimolar concentrations of Cl^- in the extravesicular medium and therefore severely limited the magnitude of the Cl^- gradient needed to energize the overshooting uptake of $^{36}Cl^-$. The maximum overshoot seen in the presence of 5 mM external Cl^- was only 2–3-fold (Fig. 2); however, a 10–15-fold overshoot could be attained by replacing $^{36}Cl^-$ by nanomolar concentrations of $^{125}I^-$ (Fig. 3). This isotope is availa-

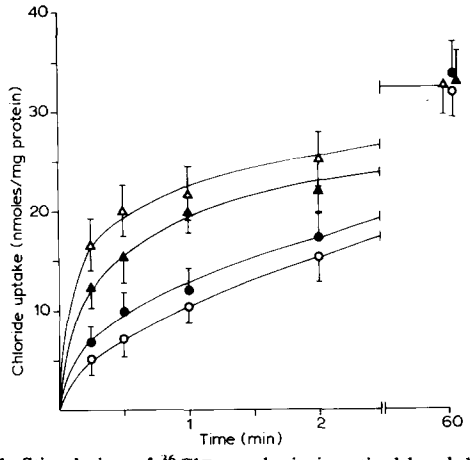

Fig. 1. Stimulation of $^{36}Cl^-$ uptake in intestinal brush-border membrane vesicles by internal Cl^-. Intestinal brush-border membrane vesicles in buffer A (pH 7.0) were preincubated for 60 min at 0 °C with valinomycin (10 μM) and 25 mM KI (▲), 25 mM potassium gluconate (●) or 25 mM KCl (○, △). $^{36}Cl^-$ (25 mM outside) uptake was measured as described in Materials and Methods at 25 °C as a function of time. ○, 4 mM SITS was present during both preincubation (15 min) and incubation. Vertical bars indicate S.E. ($n = 3$).

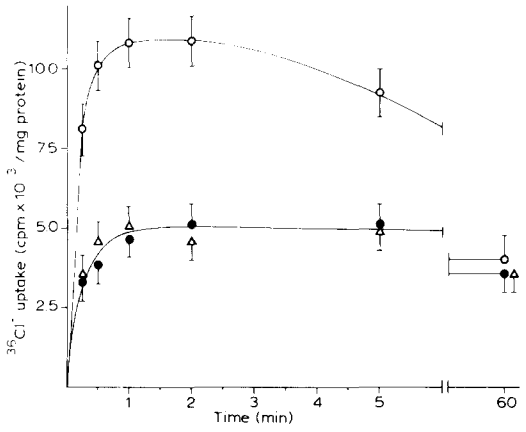

Fig. 2. Cl^--driven $^{36}Cl^-$ uptake in brush-border membrane vesicles plotted as a function of time. Effect of external Cl^- and I^-. Intestinal brush-border membrane vesicles in buffer A (pH 7.0) were preincubated for 1 h at 0 °C with 100 mM KCl. After replacement of the extravesicular Cl^- by gluconate on a Dowex AG-1 anion exchange column, $^{36}Cl^-$ (5 mM; 0.45 mCi/mmol) and, where indicated, additional 10 mM Cl^- or 10 mM I^- were added. $^{36}Cl^-$ uptake was measured at 25 °C as a function of time (see Materials and Methods). $^{36}Cl^-$ uptake was expressed in cpm/mg protein to compare the inhibition by 10 mM non-radioactive Cl^- with inhibition by 10 mM I^-. ○, no addition; △, +10 mM additional extravesicular Cl^-; ●, +10 mM extravesicular I^-. Vertical bars indicate S.E. ($n = 3$).

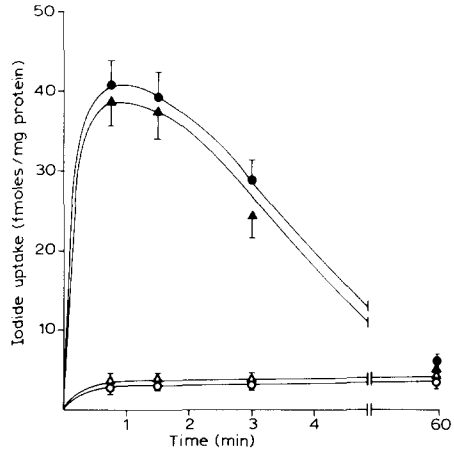

Fig. 3. Cl^--driven $^{125}I^-$ uptake in brush-border membrane vesicles plotted as a function of time. Intestinal brush-border membrane vesicles in buffer A (pH 7.0) were preincubated for 1 h at 0 °C with 75 mM choline Cl and 25 mM KCl. After replacement of the extravesicular Cl^- by gluconate on a Dowex AG-1 anion exchange column, $^{125}I^-$ (1 nM) uptake was measured at 25 °C as a function of time (see Materials and Methods). ●, no addition; ▲, +valinomycin (10 μM) present during preincubation; △+SITS (4 mM) present in both preincubation and incubation; ○, no Cl^- gradient present. Vertical bars indicate S.E. ($n = 3$).

ble at a much higher specific radioactivity and displays a high affinity for most anion exchangers studied [12,18]. As shown in Fig. 3 the overshoot gradually declined after 2 min presumably as a result of the collapse of the Cl^- gradient due to Cl^- efflux. Assuming that the half-filling time of the vesicles in the presence of 100 mM external KCl (i.e. 2–3 min) as determined by stopped-flow spectrometry of osmotic swelling [9] is nearly equal to the half-time of Cl^- depletion from vesicles preloaded with 100 mM KCl, Cl^--driven $^{125}I^-$ uptake is likely to become exceeded by $^{125}I^-$ exit downhill its chemical gradient within a few minutes following $^{125}I^-$ addition. If however the vesicles were preloaded with gluconate instead of Cl^-, uptake at 1 min and equilibrium uptake of $^{125}I^-$ were low (generally less than 10% of the overshoot observed in Cl^--loaded vesicles; results not shown), indicating that the overshoot could not be simply explained by excessive binding of $^{125}I^-$ to the exterior of the vesicle membrane in the absence of competing Cl^- and subsequent replacement of the bound isotope by Cl^- which is gradually released from the Cl^--loaded vesicles into the extravesicular medium.

The observation that voltage clamping of the vesicles with K^+ and valinomycin had no effect on the I^- uptake in the presence of a Cl^- gradient (Fig. 3) argues against electrogenic coupling of I^- and Cl^- transport by a membrane potential as might be generated by a large Cl^-/K^+ conductance ratio of the vesicle membrane (cf. Ref. 16). The stimulation of I^- uptake by a Cl^- gradient must therefore represent an electroneutral Cl^-/I^- exchange, which is in agreement with the inhibitory effect of 4 mM SITS.

In order to determine whether Cl^-/Cl^- and Cl^-/I^- exchange are catalyzed by the same transport system, we subsequently compared $^{36}Cl^-$ uptake in Cl^- versus I^- loaded vesicles (Fig. 1) as well as the efficacy of extravesicular I^- versus Cl^- to inhibit $Cl^-/^{36}Cl^-$ exchange (Fig. 2). In both type of experiments Cl^- and I^- appeared almost equally potent, indicating that the Cl^-/Cl^- exchanger could account for the major part of the I^-/Cl^- exchange activity in the vesicle membrane.

The I^-/Cl^- exchange process present in brush-border membrane vesicles was characterized further by measuring Cl^- driven $^{125}I^-$ uptake in the presence of various anions in the extravesicular medium. Because the I^- uptake was only linear during the first seconds we instead determined the percentage of inhibition of Cl^--driven I^- uptake at the time points when I^- uptake was maximal (between 1 and 2 min). Apparently the $^{125}I^-$ influx by the I^-/Cl^- exchanger is then balanced by the $^{125}I^-$ efflux.

The maximal overshoot (10–15-fold) in the absence of competing anions on the outside of the vesicles was considerably lower than would be expected if the Cl^- gradient across the vesicle membrane was exploited efficiently to build up a $^{125}I^-$ gradient in the opposite direction. Therefore it seems likely that accumulated $^{125}I^-$ is rapidly lost from the vesicle interior through a leak pathway different from halide/halide exchange. This is also conceivable considering the high concentration of Cl^- inside the vesicles which inhibits the back flux of $^{125}I^-$ by the exchanger. In analogy we also assume that in the presence of low concentrations of external anions (i.e. < 1% of the internal Cl^- concentration) or in the presence of anion species which bind to the exchanger but are not or only slowly transported, the exit pathway for I^- is not influenced by the presence of these external anions. Under such conditions the $^{125}I^-$ uptake between 1 and 2 min can be taken as an indicator of the activity of the Cl^-/I^- exchanger in the vesicle membrane.

If we additionally assume a competitive interaction between $^{125}I^-$ and the second anion for the extravesicular binding site on the Cl^-/anion exchanger, inhibition of the $^{125}I^-$ uptake by the non-radioactive anion can be described by the following equation: $IC_{50} = (1 + (S/K_m))K_i$ [19] in which IC_{50} is the concentration of anion causing 50% inhibition. S represents the concentration of external $^{125}I^-$, K_m is the Michaelis-Menten constant for carrier-mediated I^- uptake, and K_i is the inhibitory constant for the competing anion. Since $S \ll K_m$ (as evidenced by the lack of effect of addition of 100 nM unlabelled I^- on $^{125}I^-$ uptake; not shown), the IC_{50} approaches the K_i under our conditions. Therefore the IC_{50} can be used as an indicator for the affinity of the inhibiting anion for the external site of the exchanger. However, a complication may arise if the competing anion is transported itself by the Cl^-/anion exchanger and accelerates Cl^- efflux, provoking an earlier collapse of the Cl^- gradient which energizes overshooting $^{125}I^-$ uptake. In case of a transportable anion, possessing a low affinity for the exchanger and requiring high external concentrations for determination of the inhibition constant, this additional effect on the driving force for overshooting $^{125}I^-$ uptake is expected to result in a slight underestimation of the K_i for the competing anion when calculated from the IC_{50} by the forementioned equation.

In order to stabilize the HCO_3^- concentration during the assay all studies involving inhibitors were performed at pH 8.2. $^{125}I^-$ uptake in the absence of competing anions was not significantly different at pH 7.0 and pH 8.2 (compare Figs. 3 and 7). In Fig. 4 it is shown that extravesicular chloride inhibits the Cl^-/I^- exchange in a concentration-dependent manner ($IC_{50} \approx 0.5$ mM). Data on inhibition by other anions measured separately in jejunal and ileal vesicles are summarized in Table I. Externally added SCN^-, NO_3^-, I^-, Cl^-, formate and oxalate could strongly inhibit

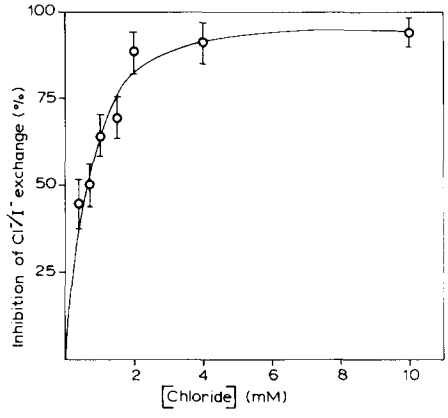

Fig. 4. Effect of external Cl^- on Cl^--driven $^{125}I^-$ uptake in intestinal brush-border membrane vesicles. Inhibition of $^{125}I^-$ uptake was measured in brush-border membrane vesicles from rat small intestine suspended in buffer A (pH 8.2) in the presence of a Cl^- gradient (100 mM KCl inside, 85 mM gluconate outside) at 25 °C between 60 and 90 s as described in Materials and Methods. KCl was mixed with the $^{125}I^-$ before the addition of the vesicles. Potassium gluconate was added at a concentration to establish a constant concentration of salt (85 mM) in the external medium. Vertical bars indicate S.E. ($n = 3$).

TABLE I

EFFECTS OF VARIOUS ANIONS ON Cl^--DRIVEN $^{125}I^-$ UPTAKE IN ILEAL AND JEJUNAL BRUSH-BORDER MEMBRANE VESICLES

$^{125}I^-$ uptake in brush-border membrane vesicles from rat ileum and jejunum suspended in buffer A (pH 8.2) was measured in the presence of a Cl^- gradient (100 mM KCl inside, 85 mM potassium gluconate outside) at 25 °C, between 60 and 90 s as described in Materials and Methods. Potassium salts of various anions were mixed with the $^{125}I^-$ to reach a final concentration of 2 mM. Data are means of triplicate experiments ± S.E. n.d., not determined.

Anion	% inhibition of Cl^-/I^- exchange	
	jejunal vesicles	ileal vesicles
Gluconate	0	0
Formate	77 ± 5	82 ± 5
Acetate	4 ± 15	25 ± 10
Oxalate	82 ± 5	n.d.
HPO_4^{2-}	18 ± 10	n.d.
SO_4^{2-}	78 ± 5	64 ± 6
HCO_3^-	25 ± 8	55 ± 7
NO_3^-	97 ± 3	95 ± 3
Cl^-	90 ± 4	91 ± 3
SCN^-	100 ± 3	100 ± 3
I^-	100 ± 3	100 ± 3
SITS	100 ± 3	92 ± 4

the I^-/Cl^- exchange at 2 mM concentrations while acetate and phosphate were much less inhibitory. Interestingly 2 mM of HCO_3^- caused a more than two times larger inhibition in ileal compared to jejunal brush-border membrane vesicles (55% versus 25%, respectively). SO_4^{2-} was more effective in inhibiting I^-/Cl^- exchange in vesicles from jejunum. The concentrations of HCO_3^- needed for half-maximal inhibition of the Cl^-/I^- exchange was 1.8 mM in vesicles from ileum but 6 mM in jejunal vesicles (Fig. 5), indicating a difference in the affinity of the Cl^-/anion exchanger for HCO_3^- between these two segments of the small intestine. In contrast, the dose dependency of the Cl^-/I^- exchange inhibition by SO_4^{2-} (Fig. 5) indicates that the difference in inhibition between vesicles from ileum and jejunum is due to a difference in the maximal percentage of inhibition but not to a difference in the IC_{50} for SO_4^{2-} (0.5–0.6 mM for both types of vesicles). Approximately 30% of the Cl^-/I^- exchange in ileal brush-border membrane vesicles could not be inhibited by relatively large concentrations (20 mM) of SO_4^{2-}, raising the possibility that Cl^-/I^- exchange in ileal vesicles is performed by at least two different exchangers, one of which has a very low affinity for SO_4^{2-}. This possibility is corrobated by the biphasic character of the inhibition curve of Cl^-/I^- exchange by SITS in ileal, but not in jejunal vesicles (Fig. 6). It can be established from Fig. 6 that virtually all of the Cl^-/I^- exchange activity in jejunal and 70%

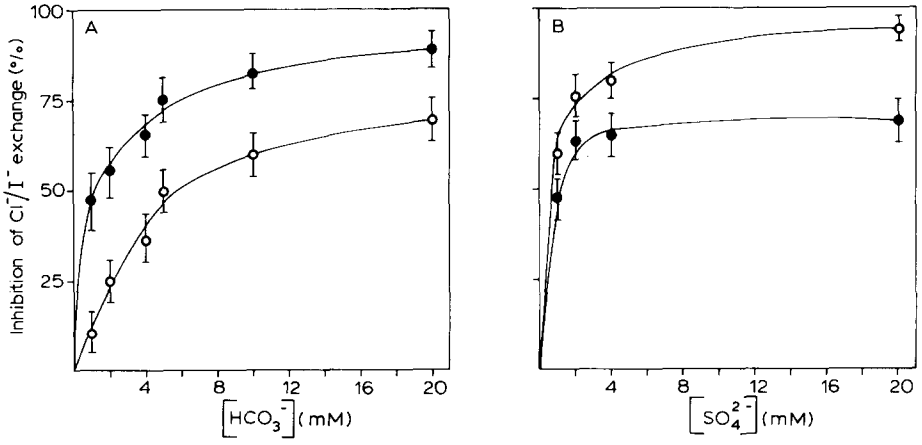

Fig. 5. Effects of SO_4^{2-} and HCO_3^- on Cl^--driven $^{125}I^-$ uptake in ileal and jejunal brush-border membrane vesicles. Inhibition of $^{125}I^-$ uptake was measured in brush-border membrane vesicles from ileum (●) or jejunum (○) in buffer A (pH 8.2) in the presence of a Cl^- gradient (100 mM KCl inside, 85 mM potassium gluconate/anion outside) at 25 °C between 60 and 90 s as described in Materials and Methods. $KHCO_3$ (A) or K_2SO_4 (B) were mixed with the $^{125}I^-$ before the addition of the vesicles to reach the final concentration as stated in the figures. The concentration of potassium gluconate was varied to keep the external salt concentration constant (85 mM). Vertical bars indicate S.E. ($n = 5$).

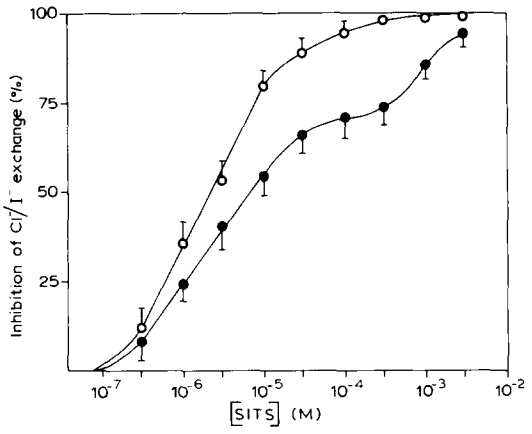

Fig. 6. Effect of SITS on Cl^--driven $^{125}I^-$ uptake in rat ileal and jejunal brush-border membrane vesicles. Inhibition of $^{125}I^-$ uptake was measured in brush-border membrane vesicles from rat ileum (●) or jejunum (○) in buffer A (pH 8.2) in the presence of a Cl^- gradient (100 mM KCl inside, 85 mM potassium gluconate outside) at 25 °C between 60 and 90 s as described in Materials and Methods. SITS was mixed with the $^{125}I^-$ before the addition of the vesicles to reach the final concentration stated in the figure. Vertical bars indicate S.E ($n = 3$).

of the exchange in ileal vesicles is extremely sensitive to SITS ($IC_{50} \approx 2.5$ μM), whereas the residual part of the Cl^-/I^- exchange in ileal vesicles is only half-maximally inhibited by 1 mM SITS.

The Cl^-/I^- exchange activity in jejunal vesicles appeared also extremely sensitive to DIDS ($IC_{50} \approx 1$ μM) but much less sensitive to the Cl^- channel blockers 51 B and 130 B ($IC_{50} \approx 50$ μM), potent inhibitors of epithelial Cl^- channels in the kidney [20] (results not shown).

Table II shows that the 30% of Cl^-/I^- exchange activity in ileal brush-border membrane vesicles which is not inhibited by low concentrations of SITS (0.1 mM) is also insensitive to SO_4^{2-} at a concentration (2 mM) which largely inhibits the SO_4^{2-}-sensitive part of the Cl^- driven I^- uptake. This indicates that the SO_4^{2-}- and the highly SITS-sensitive Cl^-/I^- exchangers in ileal vesicles are identical. The finding that 2 mM HCO_3^- in combination with either 0.1 mM SITS or 2 mM SO_4^{2-} can inhibit the Cl^-/I^- exchange in ileal vesicles up to 93% (Table II) additionally indicates that the component of Cl^-/I^- exchange, which is insensitive to relatively low concentrations of SITS or SO_4^{2-} has a high affinity for HCO_3^- ($IC_{50} \approx 0.5$ mM).

Fig. 7 shows that the activities of the Cl^-/I^- exchange per mg of protein in ileal and jejunal vesicles are only slightly different. It also shows

TABLE II

EFFECT OF SITS, SO_4^{2-} AND HCO_3^- ON THE Cl^--DRIVEN $^{125}I^-$ UPTAKE IN ILEAL BRUSH-BORDER MEMBRANE VESICLES

$^{125}I^-$ uptake in brush-border membrane vesicles from rat ileum suspended in buffer A (pH 8.2) was measured in the presence of a Cl^- gradient (100 mM KCl inside, 85 mM potassium gluconate outside) at 25 °C between 60 and 90 s as described in Materials and Methods. Potassium salts of the anions were mixed with the $^{125}I^-$ reach the final concentrations as stated in the table. Data are means of triplicate experiments ± S.E.

Anion	% inhibition of Cl^-/I^- exchange
2 mM SO_4^{2-}	65 ± 6
2 mM HCO_3^-	57 ± 6
0.1 mM SITS	70 ± 5
2 mM SO_4^{2-} + 2 mM HCO_3^-	90 ± 4
2 mM SO_4^{2-} + 0.1 mM SITS	71 ± 5
2 mM HCO_3^- + 0.1 mM SITS	93 ± 4

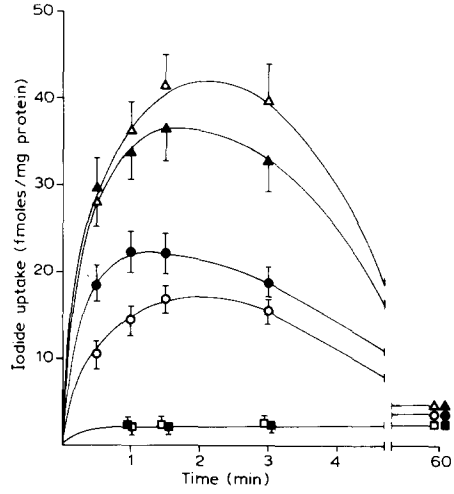

Fig. 7. $^{125}I^-$ uptake in ileal and jejunal brush-border membrane vesicles driven by an outwardly directed Cl^- or HCO_3^- gradient. Brush-border membrane vesicles from ileum (●, ▲, ■) or jejunum (○, △, □) were preincubated in buffer A (8.2) plus 100 mM KCl (△, ▲, □, ■) or 100 mM $KHCO_3$ (○, ●). After replacement of extravesicular anions by gluconate on Dowex AG-1 columns, (except □, ■), $^{125}I^-$ uptake was measured as described in Materials and Methods at 25 °C as a function of time. □, ■, no Cl^- gradient. Vertical bars indicate S.E. ($n = 3$).

that ileal and jejunal vesicles are both capable of HCO_3^-/I^- exchange when preloaded with $KHCO_3$ instead of KCl. However the activity of HCO_3^-/I^- exchange per mg protein is larger in ileal than in jejunal vesicles which is in line with the previous demonstration of a high affinity Cl^-/HCO_3^- exchange only in ileal vesicles.

Similar results as described above were obtained with brush-border membrane vesicles derived from brush-border caps by a thiocyanate treatment (not shown). These cytoskeleton-depleted vesicles could also be useful in future studies of the regulatory properties of Cl^-/anion exchange. In a previous study these KSCN-brush border membrane vesicles were successfully used to analyze a regulation of ion permeabilities by Ca^{2+} [9].

Discussion

Coupled NaCl transport in small intestine and particularly in ileum is presumably mediated by a Na^+/H^+ antiport in combination with Cl^-/anion exchange [7,12,13]. In the present study we obtained evidence for a highly active anion exchange process in brush-border membrane vesicles from rat small intestine, which was responsible for up to 70% of the $^{36}Cl^-$ uptake under equilibrium exchange conditions (25 mM Cl^- on both sides of the membrane).

By generating a large chemical gradient for Cl^- across the vesicle membrane and by the use of trace amounts of $^{125}I^-$ we could increase the sensitivity of the assay for the anion exchange activity 10–15-fold; $^{125}I^-$ may accumulate far above its chemical equilibrium in exchange for intravesicular Cl^-, without interfering with the magnitude of the gradient. Because most anion exchangers which transport Cl^- also have an affinity for I^- [12,18], this method seems generally applicable for detection of Cl^-/anion exchangers in isolated membrane vesicles.

As mentioned in the Results section this method could in theory also detect Cl^- channels in the vesicles in the presence of an impermeable cation (cf. also Ref. 16). A substantially contribution of Cl^- channel activity to Cl^--driven $^{125}I^-$ uptake in the intestinal brush-border membrane vesicles could however be ruled out by the insensitivity of overshooting $^{125}I^-$ uptake to (a) voltage clamping of the vesicles with K^+ and valinomycin strongly arguing against electrogenic coupling (cf. Ref. 16), and (b) micromolar concentrations of 51 B and 130 B acting as potent and selective inhibitors of Cl^- channels in other epithelial tissues [20]. It should be noted that the sensitivity of Cl^--driven $^{125}I^-$ uptake to micromolar concentrations of the stilbene derivatives SITS and DIDS can no longer be used as a diagnostic criterium for the operation of a band 3-like exchanger in view of the recent observation that epithelial Cl^- channels became also inhibited for about 80% by approx. 2 μM DIDS [21].

Extravesicular Cl^- inhibited the Cl^- driven I^- uptake in intestinal brush-border membrane vesicles in a concentration-dependent manner. The IC_{50} for Cl^- was approx. 0.5 mM. This is much lower than the K_m for the Cl^-/HCO_3^- exchanger (3.5 mM) or Cl^-/oxalate exchanger (4.0 mM) in rabbit ileum [12,22] and the K_i for the external binding site of the anion exchanger from red blood cells (4.3 mM in the presence of 100 mM Cl^- inside) [23]. It is not clear whether this discrepancy is caused by different properties of the exchangers or by methodological differences. Other anions like NO_3^-, SCN^-, I^- and SITS at relatively low concentration (2 mM) could also inhibit the Cl^-/I^- exchange for more than 90%, in both ileal and jejunal vesicles confirming a general affinity of Cl^-/anion exchangers for these ions [18]. In the inhibition studies with SO_4^{2-}, HCO_3^-, and SITS, evidence could be obtained for the existence of at least two different Cl^-/anion exchangers in brush-border membrane vesicles from rat ileum: (i) a Cl^-/anion exchanger with relatively high affinity for SO_4^{2-} ($IC_{50} \approx 0.5$ mM) and SITS ($IC_{50} \approx 2.5$ μM) and (ii) a Cl^-/anion exchanger with a high affinity for HCO_3^- ($IC_{50} \approx 0.5$ mM) but a low affinity for SITS ($IC_{50} \approx 1$ mM) and SO_4^{2-} ($IC_{50} > 20$ mM). Considering the similar affinities for SO_4^{2-} and SITS found for the first type of Cl^-/I^- exchanger in the ileum and the jejunal exchanger it seems likely that both exchangers are very similar or identical proteins. The second type found predominantly in ileum is presumably identical to the Cl^-/HCO_3^- or Cl^-/OH^- exchanger which was detected earlier by the use of pH or HCO_3^- gradients in a mixture

of ileal and jejunal brush-border membrane vesicles from rat [7] and ileal vesicles from rabbit [12]. The Cl^-/HCO_3^- exchanger from rabbit ileum was likewise insensitive to SO_4^{2-} and was only half-maximally inhibited by 1 mM SITS [12]. The absence of a Cl^-/HCO_3^- exchanger in jejunal vesicles is in agreement with the failure of Cassano et al. [24] and Gunther et al. [8] to detect Cl^-/OH^- exchange in brush-border membrane vesicles from rat and rabbit jejunum, respectively, and with the in vivo studies on salt transport in human ileum and jejunum by Turnberg who found evidence for Cl^-/HCO_3^- exchange in the ileum but not in the jejunum [25,26]. Because the SO_4^{2-}-sensitive Cl^-/anion exchange in jejunal vesicles can be largely inhibited by 2 mM oxalate, this anion exchange may be similar to the Cl^-/oxalate exchange described by Knickelbein et al. [22] in brush-border membrane vesicles from rabbit ileum, which could also be dissected kinetically from Cl^-/HCO_3^- exchange in the same preparation.

The role of the Cl^-/HCO_3^- exchanger in combination with a Na^+/H^+ antiporter in the neutral transport of NaCl in ileum is generally accepted. However, in our experiments the SO_4^{2-}- and SITS-sensitive but HCO_3^--insensitive anion exchanger accounts for the major part of the Cl^-/I^- exchange activity in ileal vesicles and for all exchange activity in jejunal vesicles. Moreover, Liedtke and Hopfer [7] have demonstrated SITS inhibition of NaCl uptake in rat jejunum where the Cl^-/HCO_3^- exchanger is apparently absent. Based on these results it seems likely that the HCO_3^--insensitive exchanger is also involved in transepithelial transport of Cl^-. We can think of three different mechanisms by which this type of exchanger may function in such a process:

(i) By catalyzing Cl^-/HCO_3^- exchange, as discussed by Turnberg [25]. Although the carrier has a low affinity for HCO_3^- it may accept HCO_3^- if present at high concentrations as demonstrated in the present study by the occurrence of HCO_3^-/I^- exchange in jejunal vesicles (Fig. 7).

(ii) By catalyzing Cl^-/formate exchange as proposed by Karmski et al. for the uptake of NaCl in the proximal tubule of the kidney [27]. Formate has a similar function as HCO_3^- because it may pass the membrane in the protonated form. The high affinity of the Cl^-/anion exchanger for this anion is evident from the 80% inhibition of Cl^-/I^- exchange in jejunal vesicles by 2 mM formate (Table I).

(iii) By catalyzing the exchange of Cl^- for another anion which can be subsequently exchanged for OH. In brush-border membrane vesicles from rabbit ileum for example Knickelbein et al. [22] found evidence for both an oxalate/OH or SO_4^{2-}/OH^- exchange and a Cl^-/oxalate exchange which was inhibited by SO_4^{2-}. Combination of these carriers may under the appropriate conditions result in a Cl^-/OH^- exchange. Because most anion exchangers have a broad substrate specificity it is not unlikely that a number of other anions in addition to oxalate and SO_4^{2-} can mediate the net uptake of Cl^- in a similar way.

In view of its simplicity and high sensitivity the technique for measuring Cl^-/anion exchange described in this paper may find general application in vitro studies of anion exchange mechanisms in plasma membrane vesicles and, in combination with an efficient procedure for the entrapment of regulatory factors inside brush-border membrane vesicles described recently [28,29], may be advantageously used to investigate a possible modulation of anion exchange activities by intracellular messengers (Ca^{2+}, cGMP, cAMP) in the apical membrane of mature enterocytes.

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